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Enhancing sustainable crop production: biostimulants and biotechnological approaches in challenging climates

Edited by
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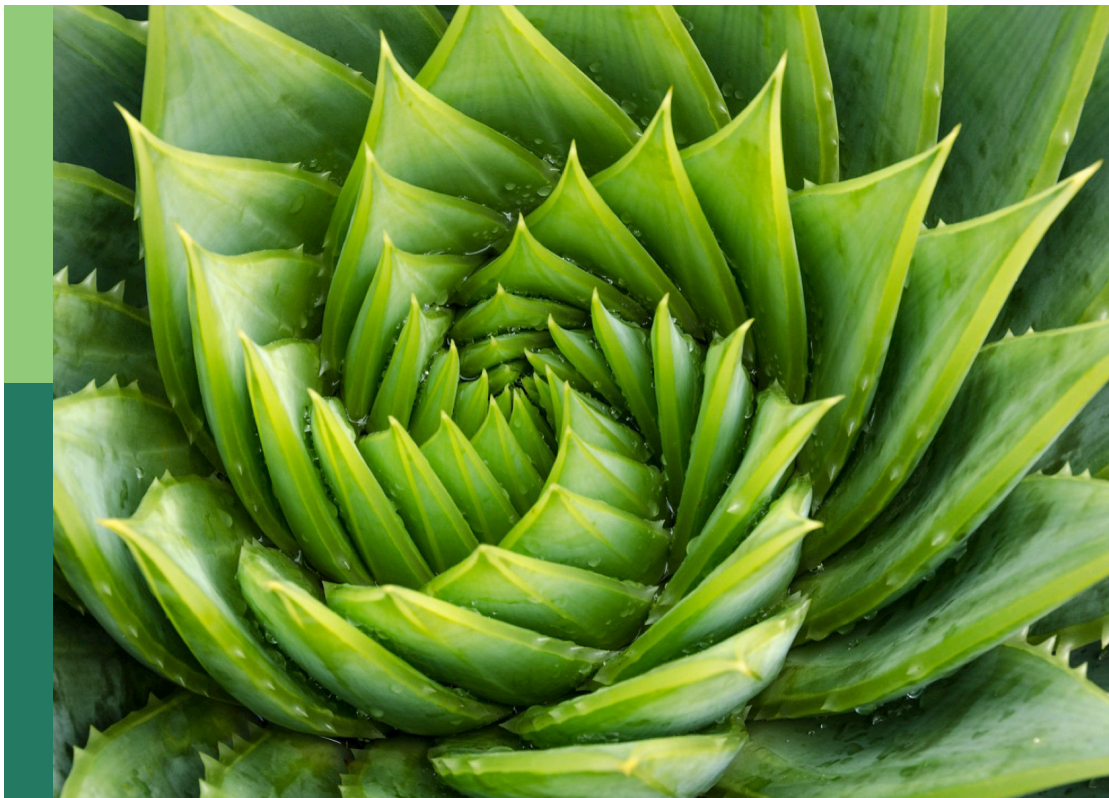

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Enhancing sustainable crop production: biostimulants and biotechnological approaches in challenging climates

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Editorial: Enhancing sustainable crop production: biostimulants and biotechnological approaches in challenging climates

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agricultural production systems, efficiency testing, environmental stress protection, green biotechnological strategies, microbial inoculants, non-microbial biostimulants

Editorial on the Research Topic
Enhancing sustainable crop production: biostimulants and biotechnological approaches in challenging climates

The implementation of biostimulants (BS; based on plant growth-promoting microorganisms or natural bioactive compounds) as plant strengtheners and other green biotechnological strategies are discussed as promising approaches to cope with increasing challenges for crop production related to climate change, limited availability of resources, and environmental protection. The principle effectiveness of these approaches has been frequently demonstrated, particularly in experiments conducted under controlled conditions, also contributing to a basic understanding of the underlying biological modes of action. However, the poor reproducibility of the expected benefits during field application remains a major challenge in bridging the gap between lab research and practical application, which is a major focus of this Research Topic.

As an initial overview, two review papers addressed various biotechnological approaches currently investigated in this context, including adaptive modification strategies for crops, modification of soil properties, and exploiting interactions with plant-beneficial microbes in different production systems (Melini et al.; Badiyal et al.).

The high variability of responses frequently observed under field conditions suggests a strong impact of environmental factors that can determine the beneficial functions of the respective adaptation strategies. This aspect is addressed by a multilevel approach, starting with three examples of investigations on the modes of action of various non-microbial BS to mitigate environmental stress under controlled conditions. The investigated stress responses comprised protective effects of the plant compound salvianolic acid on osmotic stress in maize and soybean (Kazerooni et al.) as well as mitigation of cold

stress and salinity by applications of seaweed extracts and protein hydrolysates in tomato (Borella et al.; Zhang et al.). Metabolomics, transcriptomics, and the analysis of various physiological stress indicators revealed an improved oxidative stress defense as a common mode of action, but also differential effects depending on the type of applied BS products.

At the next level, eight studies presented lab-to-field approaches to test the performance of microbial or non-microbial BS and management practices in different crops under variable environmental conditions. For better understanding of the critical factors interfering with the beneficial effects, it is more insightful to examine not only successful applications but also experiments that failed to produce the expected results. This last aspect was found in three experiments with applications of microbial consortia partially combined with micronutrients, seaweed extracts, and chitosan conducted with maize in Switzerland (Symanczik et al.) or winter rye (Behr et al.) and winter wheat (Gobel et al.) in Germany. In these cases, benefits were observed mainly in pot experiments under controlled conditions (Symanczik et al.; Gobel et al.) and during early growth in field trials (Behr et al.; Symanczik et al.), but did not fully translate into yield effects under field conditions. Conversely, microbial inoculants increased yield and resistance to biotic and abiotic stress factors in field experiments conducted with coffee and black pepper in Vietnam (Thanh Tam et al.), with tomato in Southern Italy (Cirillo et al.), and with maize, in combination with nano zinc fertilization, in Brazil (Jalal et al.). Fruit quality parameters of strawberries in Italy were improved by application of a protein hydrolysate and auxin-rich bacterial filtrates (Cardarelli et al.). Appropriate straw-returning to maize fields in Northern China decreased greenhouse gas emissions and improved the yield potential in maize (Wang et al.).

The third level consists of meta-analyses, which encompass a broad range of studies. This alternative approach offers large-scale insights into potential environmental factors influencing the performance of BS. Recently, various meta-studies have been conducted summarizing research achievements on the different groups of biostimulants (Schütz et al., 2018; Herrmann et al., 2022; Li et al., 2022). However, in a meta-analysis based on already published data, the interpretation of the results may be affected by the so-called "publication bias", as mainly positive results are usually considered for publication. Conversely, the present Research Topic provides a meta-analysis covering more than 140 pot and field experiments and 107 treatments with microbial and non-microbial BS applied as single products or as product combinations (Nkebiwe et al.). The data set derives from an EU-funded project (BIOFECTOR), investigating the performance of BS in European agriculture. It covers all data generated within the project over five years and is therefore not affected by a publication bias. Accordingly, the reported beneficial BS effects on plant performance with an average growth/yield increase of 9.3% in 945 observations (Nkebiwe et al.) were generally smaller than those reported by meta-studies based on published data (Schütz et al., 2018; Herrmann et al., 2022; Li et al., 2022).

A common outcome of all recently published meta-analyses is an apparent dependence of BS performance on various geo-climatic factors. Two meta-studies covering microbial (Schütz et al., 2018) and non-microbial BS (Li et al., 2022) suggested better performance of BS applications under arid and semiarid or subtropical/tropical climates as compared with more temperate climate conditions. Additionally, three meta-studies on microbial and non-microbial BS (Schütz et al., 2018; Li et al., 2022; Nkebiwe et al.) consistently showed a declining efficiency of BS applications with increasing soil organic matter. Both factors are closely correlated. Temperate climates often pose fewer challenges for crop production because they experience less extreme conditions in temperature, precipitation, soil pH, or salinity. Consequently, there is a reduced need for protective measures such as the application of biostimulants (BS). Moreover, soil organic carbon levels are frequently higher in temperate climates, often associated with higher levels of humic substances, higher fertility, better water-holding capacity, higher microbial activity and diversity, and a higher abundance of beneficial soil biota (Oldfield et al., 2019; Hoffland et al., 2020; Gerke, 2022). This may indicate a higher buffering capacity against the impact of environmental stress factors. In the respective soils, the effects of external BS applications may be at least partially replaced by higher levels of humic substances and native beneficial microbes with similar functions. Accordingly, also in this Research Topic, the absence of beneficial yield effects after BS application was restricted to field experiments conducted under temperate climate conditions in Germany (Behr et al.; Gobel et al.) and Switzerland (Symanczik et al.), while the remaining studies showing positive effects were performed under tropical, subtropical or Mediterranean climates (Thanh Tam et al.; Cirillo et al; Jalal et al.; Wang et al.).

For microbial inoculants, Symanczik et al. highlighted the importance of root colonization and rhizosphere competence for the establishment of beneficial effects, which was sufficient in controlled greenhouse studies during the early growth of maize but rapidly declined under field conditions. This is in line with the meta-analysis carried out by Nkebiwe et al., showing better field performance after BS application in crops maintained in a protected nursery before transplanting to the field compared with BS inoculation performed directly under field conditions.

Improved performance of microbial inoculants in combination with manure-based organic fertilizers in comparison with mineral fertilization was reported by Behr et al., similar to various previously published studies (Thonar et al., 2017; Mpanga et al., 2018; Bradáčová et al., 2019) and the meta-analysis by Nkebiwe et al. in this Research Topic. The application of organic fertilizers with easily available carbon sources might improve the carbon supply for fast-growing copiotrophic inoculants as well as indigenous plant growth-promoting microorganisms and support the establishment of a beneficial microbial community (Behr et al.). Furthermore, the high availability of N and P in manure-based fertilizers may serve as a starter fertilization for the host plant, facilitating root growth and the establishment of microbial inoculants in the rhizosphere (Bittman et al., 2006; Chekanai et al., 2018).

All the meta-studies cited here highlighted genotypic differences at the plant species level as key factors influencing BS) interactions with host plants. These differences may stem from variations in compatibility, as well as differences in growing conditions (Nkebiwe et al.), the severity and timing of imposed stress

conditions, and/or variability in stress tolerance of different plant varieties (Mahmood et al., 2022). Seven studies of this Research Topic used BS combinations (Behr et al.; Cirillo et al.; Gobel et al.; Jalal et al.; Mendes et al.; Symanczik et al.; Zhang et al.), frequently employed as a strategy to provide higher flexibility under variable environmental conditions (Nuti and Giovannetti, 2015; Sekar et al., 2016; Furlan et al., 2019). This was confirmed by the meta-study of Herrmann et al. (2022). However, the benefits of BS combinations were preferentially observed under stress conditions (Bradáčová et al., 2019; Nkebiwe et al.), and increased the propability of beneficial effets but not necessarily the absolute effect size (Bradáčová et al., 2019; Mamun et al., 2024).

Three studies pointed out the importance of interactions of microbial inoculants with native soil-microbial communities for the expression of beneficial BS effects in different crop species (Behr et al.; Cirillo et al.; Mendes et al.) as an aspect that deserves particular attention in future BS research, together with the impact on different genotypes inside a species. Finally, methodological difficulties related to the efficiency testing of BS-assisted strategies and green-biotechnological approaches were addressed by Mendes et al., Neuhoff et al., and Sun et al.

Collectively, the articles included in this Research Topic offer diverse examples for critical evaluation and characterization of conditions promoting the development of integrated plant production systems supported by environmentally friendly approaches based on BS applications and other green biotechnological strategies.

Author contributions

GN: Conceptualization, Writing – original draft, Writing – review & editing. FN: Writing – review & editing. MW: Writing – review & editing. VA: Writing – review & editing. RB: Writing – review & editing. CP: Writing – review & editing. MG-G: Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Salvianolic Acid Modulates Physiological Responses and Stress-Related Genes That Affect Osmotic Stress Tolerance in *Glycine max* and *Zea mays*

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Drought is a serious threat worldwide to soybean and maize production. This study was conducted to discern the impact of salvianolic acid treatment on osmotic-stressed soybean (*Glycine max* L.) and maize (*Zea mays* L.) seedlings from the perspective of physiochemical and molecular reactions. Examination of varied salvianolic acid concentrations (0, 0.1, 1, 5, 10, and 25 μM) on soybean and maize seedling growth confirmed that the 0.1 and 1 μM concentrations, respectively, showed an improvement in agronomic traits. Likewise, the investigation ascertained how salvianolic acid application could retrieve osmotic-stressed plants. Soybean and maize seedlings were irrigated with water or 25% PEG for 8 days. The results indicated that salvianolic acid application promoted the survival of the 39-day-old osmotic-stressed soybean and maize plants. The salvianolic acid-treated plants retained high photosynthetic pigments, protein, amino acid, fatty acid, sugar, and antioxidant contents, and demonstrated low hydrogen peroxide and lipid contents under osmotic stress conditions. Gene transcription pattern certified that salvianolic acid application led to an increased expression of *GmGOGAT*, *GmUBC2*, *ZmpsbA*, *ZmNAGK*, *ZmVPP1*, and *ZmSCE1d* genes, and a diminished expression of *GmMIPS2*, *GmSOG1*, *GmACS*, *GmCKX*, *ZmPIS*, and *ZmNAC48* genes. Together, our results indicate the utility of salvianolic acid to enhance the osmotic endurance of soybean and maize plants.

Keywords: hydrogen peroxide, lipid metabolism, fatty acid, amino acid, antioxidant enzymes, sugar, protein

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INTRODUCTION

Climate change has increased the prevalence of various abiotic stress conditions around the world (Pryor et al., 2014; Ogata et al., 2017). A vast range of stressful environmental stimuli, such as salinity, ultraviolet radiation, heat, flooding, drought, and heavy metals, pose a serious threat to plants (He et al., 2018; Keep et al., 2021; Liu et al., 2021, 2022; Sheteiwy et al., 2021c; Simioniuc et al., 2021). These stress factors are the major constraints for crop survival, accounting for a dramatic

reduction in crop yield globally (Lamers et al., 2020; Sheteiwy et al., 2021a). Drought is among the most prevalent abiotic stress condition that adversely influences plant growth, quality, and yield (Thirumalaikumar et al., 2018; Du et al., 2020b). Different factors, including water deficiency, high temperature, and low humidity, can be induced by drought conditions (Bartels et al., 2004; Abdoulaye et al., 2019). Drought stress can intrude various physiological processes, namely, plant photosynthesis, oxidative stress, enzyme activity, nucleic acids, proteins, membrane integrity, and cell metabolism (Valliyodan and Nguyen, 2006; Sheteiwy et al., 2021c), which could subsequently result in the prevention of plant growth.

Salvia miltiorrhiza Bunge (Lamiaceae), known as red sage, has been clinically applied in traditional Chinese medicine for more than 2,000 years. In recent years, it has been extensively approved as a health product in western countries (Ma et al., 2019). It is consumed as a drug for cardiovascular disorders, dysmenorrhea, angina pectoris, cancer, thrombosis, hepatitis, hepatocirrhosis, and neurasthenic insomnia (Li, 1998; Wang et al., 2017). Phytochemical studies have demonstrated that *S. miltiorrhiza* is composed of large amounts of compounds with strong anti-oxidative activity, including flavonoids, polyphenols, triterpenoids, lipophilic diterpenoids, and phenolic compounds (such as salvianolic acids) (Lu and Foo, 2002; Li et al., 2009). Salvianolic acids are the most water-soluble compounds in *S. miltiorrhiza,* and among them, salvianolic acid A and salvianolic acid constitute the most abundant compounds (Ma et al., 2019). It has been reported that salvianolic acids exhibited antioxidative properties and free radical scavenging activities in *in vitro* and *in vivo* conditions, and showed protective effects on cells exposed to detrimental agents (Zhao et al., 2008; Zhang et al., 2014).

Soybean (*Glycine max* L.) and maize (*Zea mays* L.) are the most substantial feed crops cultivated around the world. These crops contain beneficial metabolites and nutrients, which prevent cancer, kidney diseases, obesity, and diabetes (Messina, 2016; Mao et al., 2021; Poku et al., 2021). Soybean is considered as an ample source of oil and protein for humans. In addition to their consumption, soybean and maize are considered a future source of fuel and alternative for plastics, respectively (Candeia et al., 2007; Song et al., 2011; Wang et al., 2016). Despite these benefits, the growth and productivity of soybean and maize are substantially interfered by various abiotic stress factors (Deshmukh et al., 2014; Li et al., 2019; Mao et al., 2021). Amid the detrimental environmental factors usually faced by soybean and maize, drought is believed to be the harshest, since it influences all phases of plant development and subsequently reduces the final yield (Le et al., 2012; Yang et al., 2018). Thus, research on enhancing the growth and endurance of soybean and maize plants under drought stress is important to diminish the effect of water deficit and improve crop yield (Sheteiwy et al., 2021a,b).

Evaluations on the incorporation of climate alteration and crop yield models have anticipated greater loss in the production of major crops, including rice, soybean, wheat, and maize, which may have severe consequences for food safety (Waqas et al., 2019). We envisaged that salvianolic acid could promote osmotic stress survival in soybean and maize plants under destructive environmental situations. This work was conducted to examine the impact of the salvianolic acid application on attenuating osmotic stress in soybean and maize plants and determine its effect on plant development and production. We attempted to specify the appropriate salvianolic acid concentration that was efficient toward osmotic-stressed plants. In this study, physiochemical and molecular analyses were employed to perceive the mechanisms of salvianolic acid in soybean and maize plants under osmotic stress conditions. In particular, we show how exogenous salvianolic acid application influences the sugar content, amino acid content, fatty acid content, and transcription patterns of various genes. Our work presents a convincing demonstration of the positive effects of salvianolic acid on ameliorating the osmotic stress tolerance in soybean and maize plants.

MATERIALS AND METHODS

Determination of Proper Concentrations of Polyethylene Glycol

Soybean and maize seeds were rendered by the Agricultural Research and Extension Services (Gyeongsangbuk-do, South Korea) and Asia Seed Ltd. (Seoul, South Korea), respectively. Similar-sized seeds were selected and disinfected in 70% ethanol and 2.5% sodium hypochlorite and evaluated for vitality (Ke et al., 2018; Silva et al., 2020). Seeds were transferred onto pot trays (28 × 54 cm) filled with horticultural soil (Shinsung Mineral Co., Ltd., Chungcheongbuk-do, Korea), grown in a climate chamber (16/8 h day/night), and irrigated daily. The flow rate, humidity, and temperature in the chamber were maintained at 250 μmol m^{-2} s^{-1}, 65%, and 26 ± 2°C, respectively. Following emergence, one plant per pot (10 × 10 cm) received assorted treatments. Maize and soybean seedlings were either irrigated with distilled water (50 ml/pot), as control, or with polyethylene glycol 6,000 (PEG-6000, Merck-Schuchardt, Hohenbrunn, Germany) at −0.15, −0.3, −0.49, and −0.73 MPa concentrations (50 ml/pot). We used PEG-6000 to simulate drought stress conditions (PEG-generated osmotic stress). Each group comprised six replicates. After the completion of each treatment period (8DAT), plants were assayed for various agronomic characteristics. Finally, 25% polyethylene glycol 6,000 (PEG-6000; −0.73 MPa) was designated to be the right concentration for use in further experiments.

Determination of the Suitable Salvianolic Acid Concentration

Average-sized seeds were surface-sterilized (Ke et al., 2018; Silva et al., 2020) and grown in a climate chamber as described earlier. Next, maize and soybean seedlings were partitioned into two groups: (i) control, which received 50 ml/plant of distilled water, and (ii) osmotic stress treatment, irrigated with 25% PEG (50 ml/plant) and exposed to these treatments for 8 days. Later, the osmotic-stressed seedlings were treated with 50 ml per plant of 0, 0.1, 1, 5, 10, and 25 μM salvianolic acid (Sigma-Aldrich, St. Louis, Missouri, USA) daily for 8 days. The salvianolic acid

solution (25 μM, stock) was prepared by dissolving the solute in water, and then the stock solution was diluted in distilled water to obtain different concentrations. Each treatment contained six replicates. All the plant growth characteristics were measured after 8 days (8DAT). Subsequently, 0.1 and 1 μM salvianolic acid (SAL) concentrations were identified to be the appropriate concentrations for further experiments.

Physiochemical and Molecular Effects of Salvianolic Acid on Osmotic-Stressed Soybean and Maize Seedlings

Growth Condition and Treatments

The soybean and maize seedlings were maintained in a greenhouse at 43% relative humidity and 25/23°C day/night temperature. Three weeks after emergence, plantlets with similar maturity were used in this study. Plantlets (one seedling/pot, irrigated with 50 ml/pot) were irrigated with distilled water, 0.1 μM SAL, 1 μM SAL, or 25% PEG, according to Kazerooni et al. (2021) (**Table 1**). Each treatment consisted of six replicates. The maize and soybean seedlings were consistently irrigated with SAL, and leaves were collected 8 days after treatment. The maize and soybean leaves were promptly used or deep-frozen in liquid nitrogen before storage at −80°C.

TABLE 1 | Experimental work plan.

Symbol	Treatment
Soybean	
Cont	irrigated with sterile distilled water
SAL	irrigated with 0.1 μM SAL
PEG	irrigated with 25% PEG
PEG+SAL	irrigated with 25% PEG + 0.1 μM SAL
Maize	
Cont	irrigated with sterile distilled water
SAL	irrigated with 1 μM SAL
PEG	irrigated with 25% PEG
PEG+SAL	irrigated with 25% PEG + 1 μM SAL

Measurement of Physiological Characteristics and Chlorophyll Index

Diverse agronomic traits were measured to determine the impact of one-by-one treatment on the soybean and maize seedlings. These traits were recorded 8 days after treatment. A digital Vernier caliper and a ruler were used to measure the stem diameter and leaf area (leaf length/width). Plant height and root length were assessed with a tape meter. In primary osmotic stress and SAL screening test, a SPAD meter (SPAD-502, Konica Minolta, Tokyo, Japan) was employed to determine the chlorophyll concentration in leaves. The plants and roots were oven-dried at 60°C for 48 h to assess their dry weights (Valentovic et al., 2006).

Changes in Chlorophyll, Carotenoid, and Amino Acid Contents

The contents of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Total Chl), and carotenoid were determined according to Hosseini et al. (2017). Freshly harvested leaves (0.5 g) were immersed and homogenized in 80% acetone solution (20 ml). The absorbance of the extract was then recorded at the selected wavelength using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Waqas et al. (2015) proposed a method for determining the amino acid content. Powdered freeze-dried leaves (50 mg) were hydrolyzed with 1 ml of hydrochloric acid (6 N HCl, 24 h, 110°C), followed by evaporation and condensation under vacuum (80°C, 24 h). Then, hydrochloric acid (1 ml of 0.02 N HCl) was added to dissolve the condensed remnant. The extract was filtered (0.45-μm membrane) before loading into Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

Hydrogen Peroxide, Lipid Peroxidation, and Fatty Acid Analysis

The concentration of H_2O_2 was evaluated based on a modified method (Velikova et al., 2000) originally described by Kazerooni et al. (2021). The powdered leaf tissue (0.3 g) was homogenized in an ice bath with 0.1% trichloroacetic acid (TCA; 5 ml). The absorbance of the mixture was recorded at 390 nm using a spectrophotometer.

López-Serrano et al. (2019) method was applied to evaluate the lipid content in soybean and maize leaves. The mixture comprising 0.1% TCA (0.5 ml) extract was added to 0.5% TBA (1 ml; prepared in 20% TCA). The reaction was commenced by incubating the mixture at 95°C for 30 min and terminated by placing the mixture in an ice bath for 10 min. Then the mixture was immediately centrifuged at 12,000 rpm for 5 min. Lipid content was measured at wavelengths of 532 and 600 nm (Thermo Fisher Scientific, Waltham, MA, USA).

We used a previously published method (Poirier et al., 1999) to determine the fatty acid content in soybean and maize leaves. Gas chromatography–mass spectrometry analysis was conducted on an Agilent Model 7890A series (Agilent, Dover, DE, USA).

Protein and Sugar Content Quantification

The concentration of protein was assessed using the Brilliant Blue G-250 reagent with bovine serum albumin following Bradford's method (Bradford, 1976).

The soluble sugar content of leaves was quantified according to a former report (Du et al., 2020b). A ground sample (0.1 g) was extracted with 80% (v/v) ethanol (at 80°C for 30 min) and then centrifuged at 10,000 rpm for 10 min. The obtained remnant was extracted twice utilizing 80% ethanol. The collected supernatants were mixed, and 80% ethanol was added to reach a final volume of 5 ml. Then, the soluble sugar content was quantified at a wavelength of 620 nm using a spectrophotometer.

Measurement of Antioxidant Activities

The polyphenol oxidase (PPO) and peroxidase (POD) activities were assayed following the method described by Putter (1974). Catalase (CAT) and superoxide dismutase (SOD) activities were inspected according to de Azevedo Neto et al. (2005). Flavonoids, DPPH radical scavenging performance, and total polyphenols

were assessed according to Zheng and Wang (2001), Barka et al. (2006), Wang et al. (2009), and Kazerooni et al. (2021). The absorbance of the reaction mixture was characterized at preferred wavelengths using a spectrophotometer.

Estimation of Nutrient Content in Soybean and Maize Plants

Collected samples were freeze-dried and powdered to unravel the nutrient content of soybean and maize plants. The nutrient (potassium, K; phosphorus, P; and calcium, Ca) concentration in plants was determined using inductively coupled plasma mass spectrometry (Optima 7900DV, Perkin-Elmer, Akron, OH, USA). Treatments without osmotic stress or salvianolic acid were used to inspect the initial concentration of the selected elements.

RNA Extraction and Expression Analysis

Total RNA from leaves of soybean and maize at 8DAT was isolated using Trizol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNAs were generated from total RNA (1 μg) using BioFACT RT-Kit (BIOFACT, Daejeon, Korea) according to the manufacturer's conventional instructions. qRT-PCR was performed with an Illumina Ecosystem (Illumina, San Diego, CA, USA) to determine the relative transcript levels of the selected genes. Primer sequences used for qRT-PCR are given in **Supplementary Tables S1, S2**.

Statistical Analysis

The SAS statistical software (version 9.4, SAS Institute, Cary, NC, USA) was utilized to evaluate the data through analysis of variance (ANOVA). Substantial contradictions between treatments were clarified using Tukey's test at $p < 0.05$. All data are shown as six biological replicates. Graphs are drawn using Origin Pro (version 9.85, Origin Lab Corporation, Northampton, MA, USA).

RESULTS

The Growth Changes of Soybean and Maize Seedlings Under Varied Polyethylene Glycol Concentrations

Diverse plant growth characteristics were recorded in soybean and maize plants exposed to different concentrations of PEG (−0.15, −0.3, −0.49, and −0.73 MPa). In general, they exhibited a decline in plant growth parameters (**Supplementary Figures S1, S2** and **Supplementary Tables S3, S4**). Soybean and maize plants showed no evident changes when treated with the lowest concentration of PEG (−0.15 MPa) when compared to the control plants. However, treatment with maximum PEG concentration (-0.73 MPa) depicted considerable mitigation in the growth attributes of soybean and maize plants. For instance, attenuated plant height (58.50%), root length (50%), stem diameter (57.89%), leaf length (59.59%), leaf width (68.33%), chlorophyll content (51.22%), plant fresh weight (88.49%), plant dry weight (89.47%), root fresh weight (93.38%), root dry weight (100%), and leaf number (52.60%) ($p < 0.05$) were recorded in PEG-treated maize plants (-0.73 MPa) in contrast to control plants (**Supplementary Figure S2** and **Supplementary Table S4**). These data showed that PEG (−0.73 MPa) decreased the plant growth of maize and soybean noticeably.

Agronomic Traits of Stressed Soybean and Maize Seedlings Under Diverse Salvianolic Acid Concentrations

Mitigation in the varied plant growth parameters during osmotic stress is shown in **Supplementary Table S5**. On the other hand, when osmotic-stressed plants were treated with different concentrations of SAL (0, 0.1, 1, 5, 10, and 25 μM), they significantly displayed alleviation of osmotic stress (**Supplementary Figures S3, S4** and **Supplementary Table S5**). At 8DAT, osmotic-stressed maize plants treated with a minimum (0.1 μM) or a maximum concentration of SAL (25 μM) showed no significant fluctuation when compared to stressed soybean plants (**Supplementary Figure S4** and **Supplementary Table S5**). However, osmotic-stressed maize plants irrigated with SAL (1 μM concentration) began to display enhanced plant growth characteristics. By 8DAT, elevated plant height (69.23%), root length (53.57%), stem diameter (54.16%), leaf length (75%), leaf width (60.40%), chlorophyll content (36.49%), plant fresh weight (89.63%), plant dry weight (96.35%), root fresh weight (80.72%), root dry weight (75%), and leaf number (42.85%) ($p < 0.05$) were observed in SAL-treated stressed maize plants in contrast to osmotic-stressed plants alone (**Supplementary Figure S4** and **Supplementary Table S5**). In addition, stressed soybean plants irrigated with a minimum concentration of SAL (0.1 μM) showed marked improvement in their growth parameters compared to osmotic-stressed plants alone (**Supplemantary Figure S3** and **Supplementary Table S5**). These outcomes suggested that the osmotic stress was repressed in soybean and maize plants treated with 0.1 and 1 μM SAL, respectively.

Effect of Salvianolic Acid on Stressed Soybean and Maize Seedlings

Salvianolic Acid Improves Plant Growth Attributes Under Osmotic Stress

The impact of the salvianolic acid (SAL) on the growth of soybean and maize seedlings under osmotic stress conditions and without stress was investigated in pot trials. This detrimental abiotic stress factor negatively affected growth attributes (**Figures 1A–D**, **2A–T**) in unstressed and untreated soybean and maize plants. Conversely, these growth attributes were promoted in SAL-treated plants under stress. For instance, soybean plant height and root length were promoted by 59.18 and 41.33% in the osmotic stress treatments, respectively. Similarly, in SAL-treated soybean plants, plant fresh weight and root fresh weight were elevated by 54.54 and 47.65% in the osmotic stress treatment, respectively, when compared to the control plants (**Figures 2K,O**).

FIGURE 1 | Impact of salvianolic acid on soybean and maize plant growth and root under normal and stress conditions 8 days post-treatment **(A–D)**. Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the means ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey test).

Photosynthetic Pigments

Our results depicted that Chla, Chlb, and carotenoid contents were significantly higher in the SAL-treated stressed plants. Similarly, increased total chlorophyll content (TCC) was observed in SAL-treated stressed plants (**Figures 3A,B**). A decline in TCC was perceived in osmotic-stressed soybean

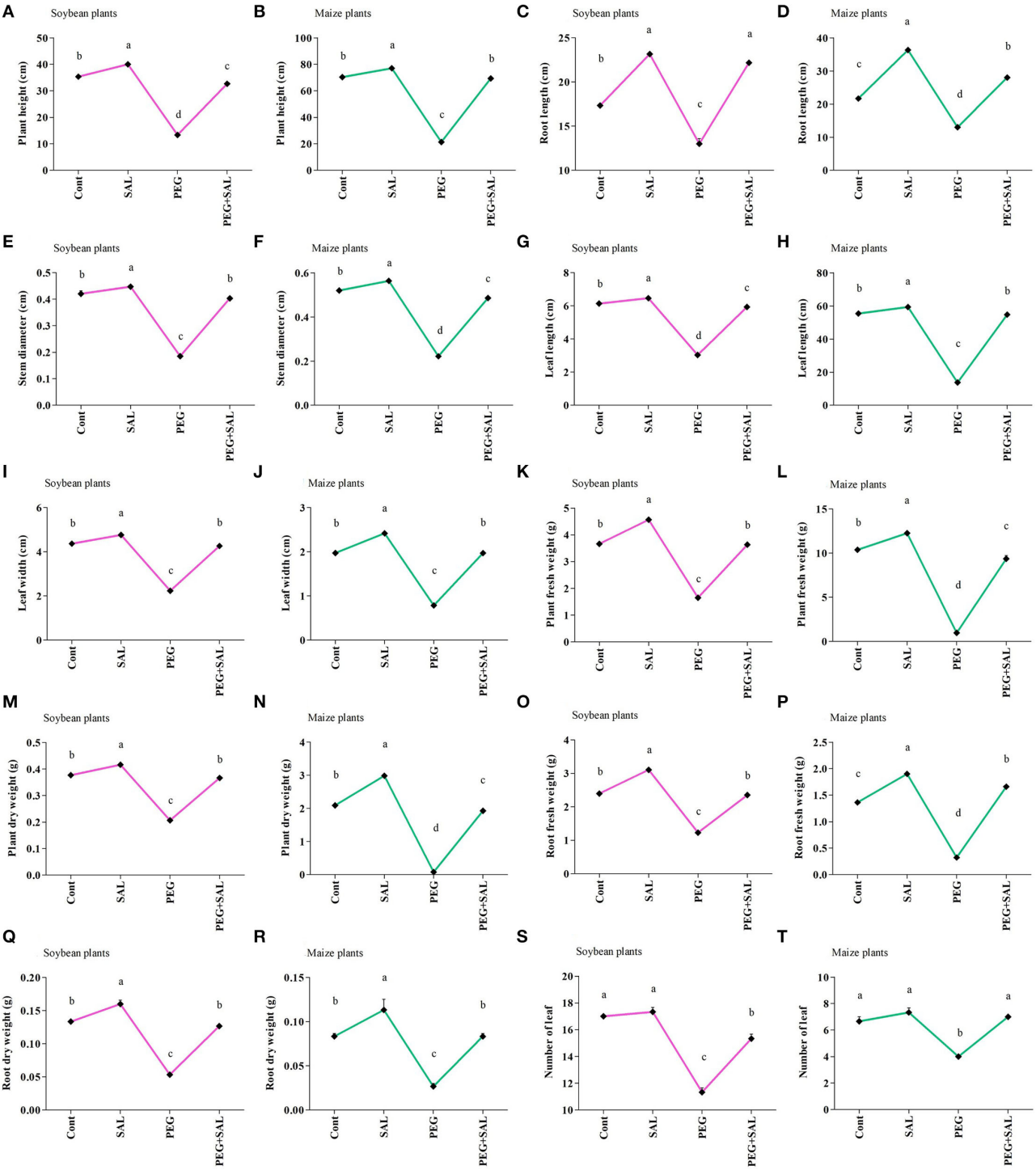

FIGURE 2 | Impact of salvianolic acid on soybean and maize plant growth parameters under normal and stress conditions 8 days post-treatment **(A–T)**. Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the mean ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey's test). Bars with different lowercase letters are significantly different from each other.

and maize plants (7.56 and 27.28%, correspondingly). On the other hand, SAL application contributed to an 8.02% (soybean) and 15.75% (maize) promotion in TCC under osmotic stress conditions when compared to the stressed control plants, and the difference was significant ($p < 0.05$; **Figures 3A,B**).

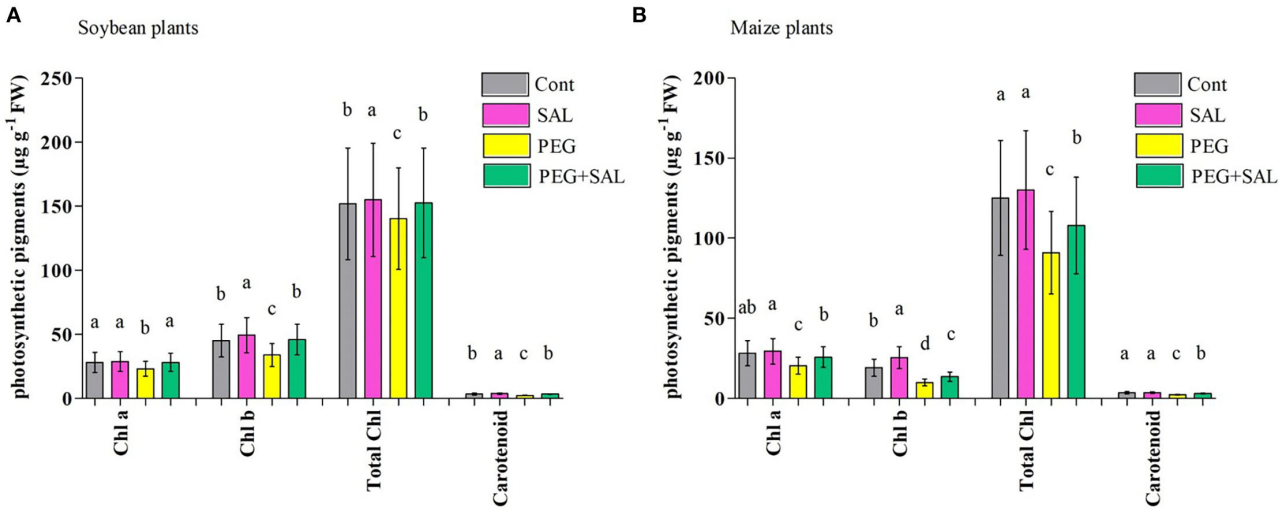

FIGURE 3 | Effect of salvianolic acid application on soybean and maize plant photosynthetic parameters "chlorophyll a, Chla; chlorophyll b, Chlb; total chlorophyll, total Chl; and carotenoid contents" **(A,B)**. Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the mean ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey's test). Bars with different lowercase letters are significantly different from each other.

Amino Acid Accumulation

Eighteen amino acids were identified in soybean and maize seedlings exposed to various treatments (**Figures 4A,B**). Osmotic stress caused a noticeable decrease in amino acid content in maize and soybean seedlings over 8 days. Proline content decreased by 26.91% (soybean) and 51.74% (maize) in osmotic-stressed plants, correspondingly. In contrast, plants treated with SAL had increased proline content (soybean, 32.66%; maize, 56.49%). Moreover, asparagine was the highest in osmotic-stressed plants 8 days after the application of SAL on the impaired plants, while alanine and tyrosine were the lowest in control and stressed soybean seedlings (**Figure 4A**).

Salvianolic Acid Regulates H_2O_2, MDA, and Fatty Acid Content

The H_2O_2 content was elevated by 41.83 and 28.76% in soybean and maize plants under osmotic stress, correspondingly (**Figure 5A**). Utmost mitigations of 19.89 and 26.77% in H_2O_2 content were recorded in SAL-treated soybean and maize plants under osmotic stress, correspondingly ($p < 0.05$).

As exhibited in **Figure 5B**, stress conditions elevated the generation of malondialdehyde (MDA) in the untreated soybean (35.81%) and maize (34.45%) plants. SAL treatment ameliorated MDA formation in osmotic-stressed plants by 30.46% (soybean) and 19.30% (maize) ($p < 0.05$).

The total fatty acid content in soybean and maize seedlings diminished in response to osmotic stress (soybean, 48.01% and maize, 49.87%) (**Figure 5C**). However, SAL-treated plants exhibited higher fatty acid content under stress and no stress conditions (soybean, 51.41% and maize, 57.32%).

Protein and Sugar Synthesis

We observed an increase in protein values in soybean (11.85%) and maize (17%) upon SAL application when compared to the untreated plants. However, the values were found to be diminished by 29.74% (soybean) and 29.82% (maize) under osmotic stress (**Figure 5D**). Under stress conditions, SAL treatment resulted in higher protein content (soybean, 34.35% and maize, 33.82%).

Stress resulted in a marked decline in the sugar content in soybean (33.43%) and maize (47.97%) plants (**Figure 5E**). SAL application increased the sugar content by 62.07% (soybean) and 55.05% (maize) under osmotic stress conditions (**Figure 5E**).

Antioxidant Assay

Stress resulted in a decrease in enzymatic and non-enzymatic antioxidant functions (SOD, CAT, DPPH, flavonoid, total polyphenol, POD, and PPO) in soybean and maize plants. SAL application distinctly upraised antioxidant activities under stress conditions (**Figures 6A–G**). For instance, DPPH and total polyphenol activity were higher in SAL-treated soybean (DPPH, 37.86% and total polyphenol, 9%) and maize (DPPH, 71.88% and total polyphenol, 26.46%) plants affected by osmotic stress in comparison to untreated stressed plants (**Figures 6A,B**).

Characterization of Nutrient Content in Plants

The nutrients like Ca, K, and P were inspected in soybean and maize plants to investigate the impact of the salvianolic acid on the nutrient value of soybean and maize plants and its recovery function (**Table 2**). In unstressed plants, a rise was observed in Ca, K, and P in plants treated with salvianolic acid compared to the control plants. Plant nutrients were modulated in SAL-treated stressed plants, which indicated enhancement in *K*- and *P*-values and a reduction in the Ca value under unfavorable conditions.

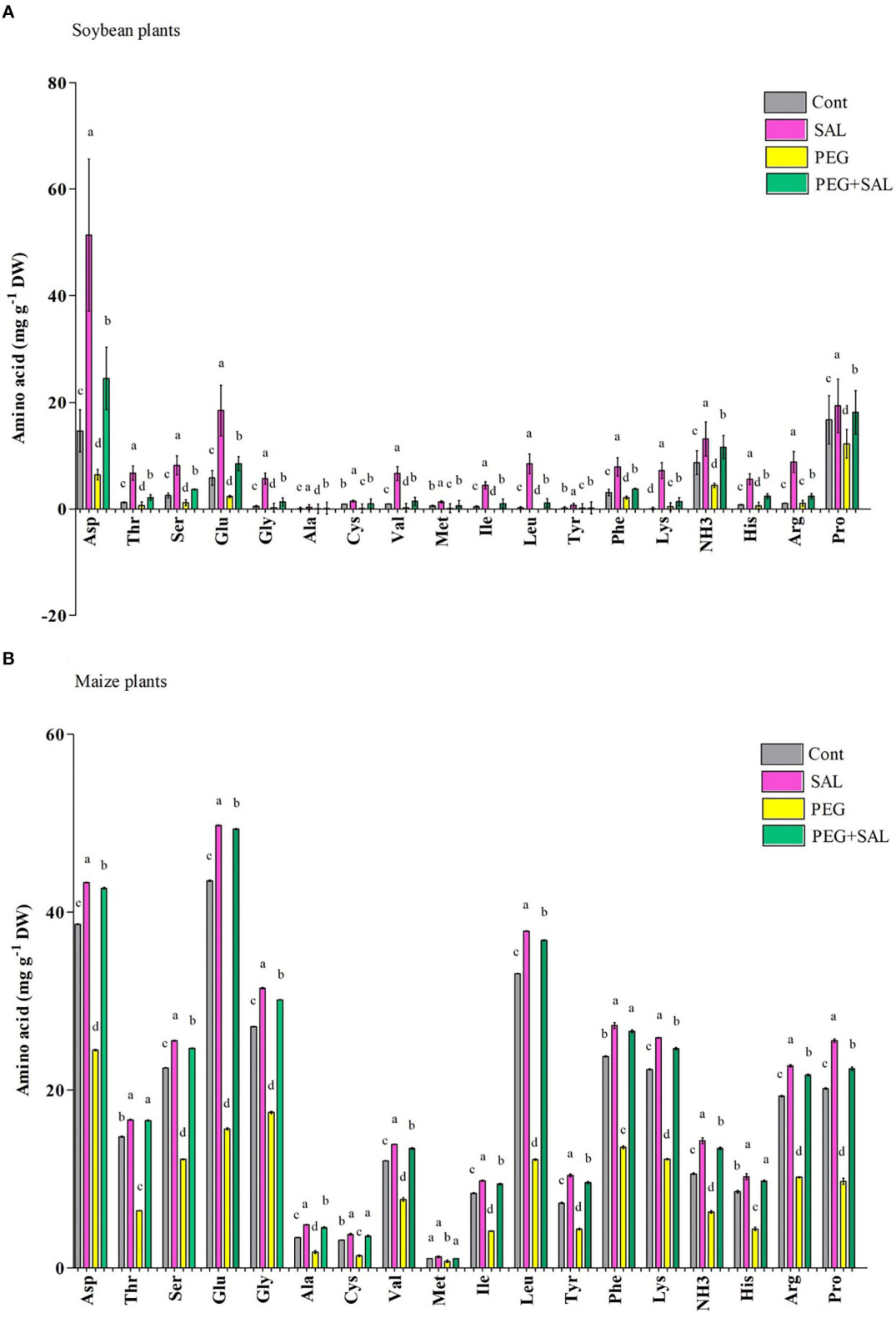

FIGURE 4 | Effect of salvianolic acid application on amino acid content in leaves of soybean and maize grown under normal and stress conditions 8 days post-treatment **(A,B)**. Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the mean ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey's test). Bars with different lowercase letters are significantly different from each other.

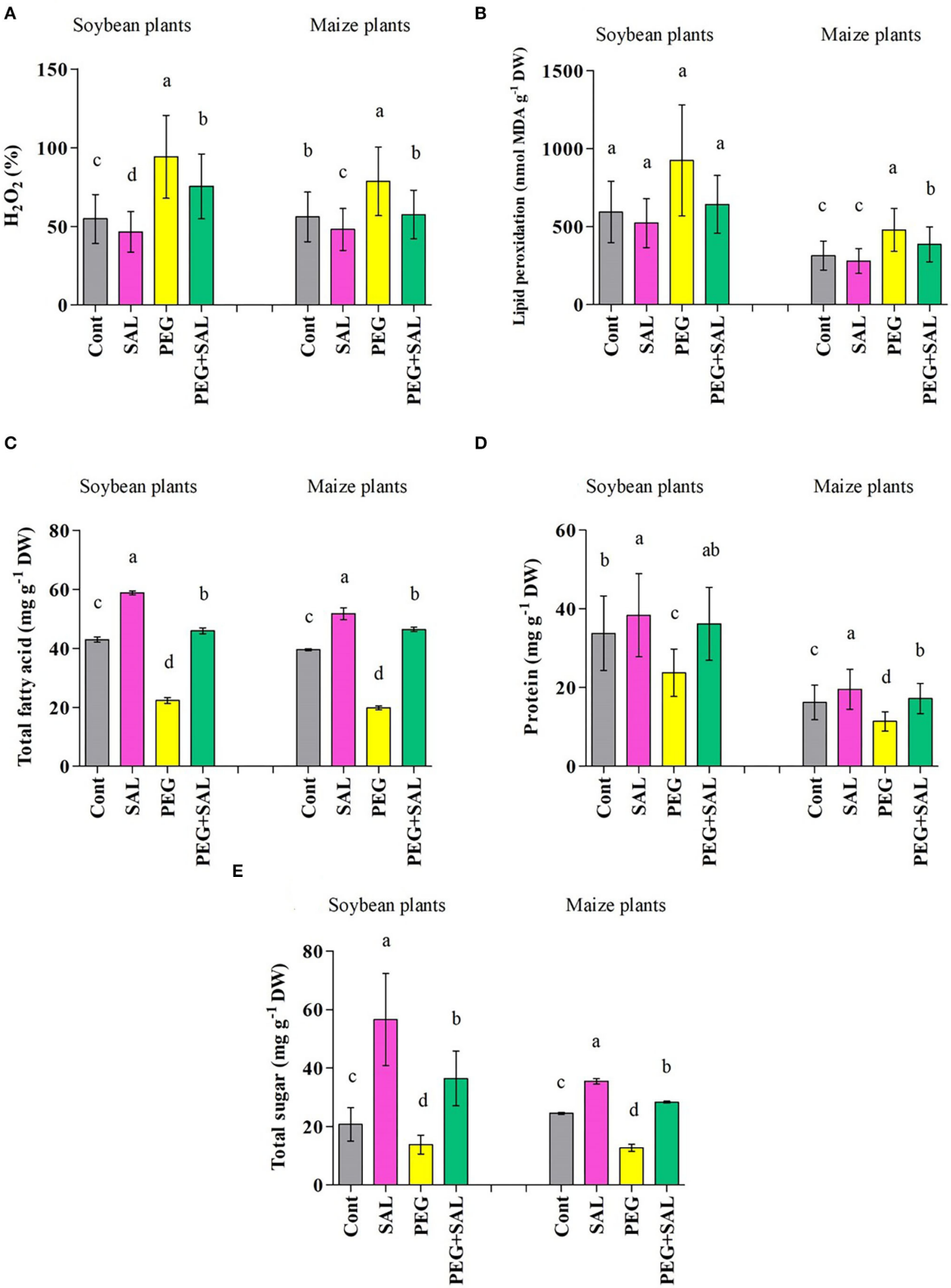

FIGURE 5 | (A) H_2O_2, **(B)** MDA, **(C)** total fatty acid, **(D)** protein, and **(E)** sugar content in leaves of soybean and maize grown under normal and stress conditions and treated with salvianolic acid for 8 days (8DAT). Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the mean ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey's test). Bars with different lowercase letters are significantly different from each other.

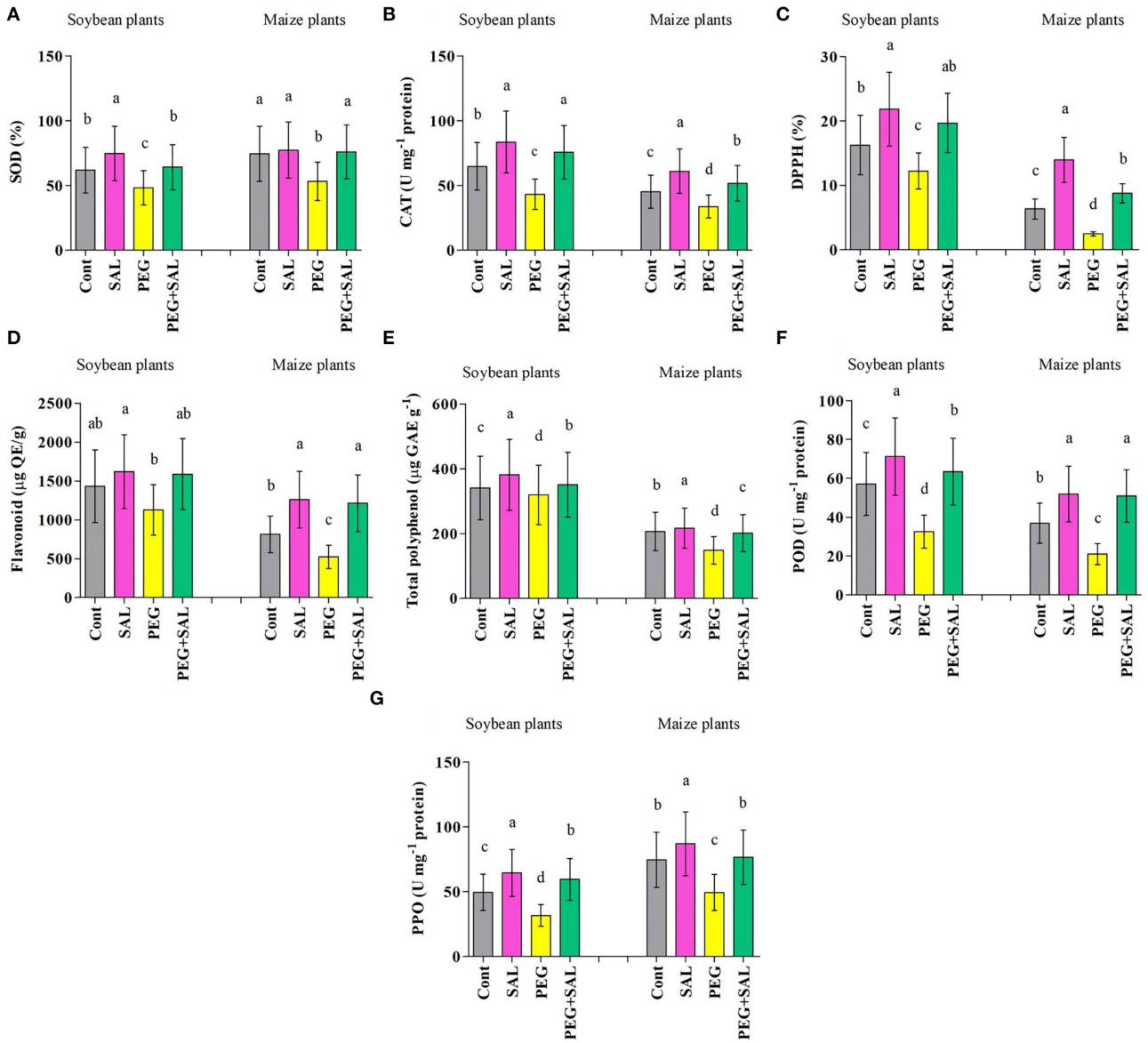

FIGURE 6 | Effect of salvianolic acid application on antioxidant content "SOD **(A)**; CAT **(B)**; DPPH **(C)**; Flavonoids **(D)**; Total polyphenol **(E)**; POD **(F)**; PPO **(G)**" of soybean and maize leaves grown under normal and stress conditions 8 days post-treatment. Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the mean ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey's test). Bars with different lowercase letters are significantly different from each other.

Salvianolic Acid Altered the Expression Pattern of Osmotic Stress-Responsive Genes in Soybean and Maize

Twelve genes were examined for their transcript patterns in soybean and maize plants under SAL application and abiotic stress conditions.

Association Analysis of Stress-Responsive Genes in Soybean

This study characterized the gene *GmMIPS2* in soybean, which showed contrasting responses under SAL application and abiotic stress conditions. An increase in *GmMIPS2* expression levels was found in osmotic-stressed plants. However, SAL treatment of osmotic-stressed plants decreased the expression by 50.94%, in contrast to that observed in the untreated plants (**Figure 7A**).

The stress diminished *GOGAT* gene expression in soybean plants, while SAL treatment elevated the expression of this gene (**Figure 7B**). For instance, the SAL application enhanced *GmGOGAT* gene expression by 81.90%, under osmotic stress.

The effect of osmotic stress and SAL application on *SOG1* was examined through the modification of the *SOG1* gene expression (*GmSOG1*) (**Figure 7C**). Differences were observed in

the expression of the *GmSOG1* gene in control and SAL-treated plants under stress conditions. Furthermore, improved *GmSOG1* expression was noticed in the stressed plants. SAL-treated plants revealed a decline in *GmSOG1* expression in contrast to untreated stressed plants (41.59% under stress conditions).

An increase in *GmACS* expression was discerned in osmotic-stressed plants (86.13%) in comparison to a decrease in SAL-treated stressed plants, which decreased by 57.37% (**Figure 7D**).

As illustrated in **Figure 7E**, osmotic-stressed plants exhibited an 87.04% reduction in *GmUBC2* expression when compared to the control plants. On the other hand, improved expression of *GmUBC2* was perceived in SAL-treated plants exposed to abiotic stress. SAL treatment increased *GmSAP16* expression by 82.34% under osmotic stress (**Figure 7E**).

Abiotic stress increased *GmCKX* expression by 83.74% (**Figure 7F**), but SAL treatment decreased *GmCKX* expression by 90.07%.

Association Analysis of Stress-Responsive Genes in Maize

The *ZmpsbA* transcript pattern in maize seedlings under abiotic stress and SAL treatment is exhibited in **Figure 7G**. A decline in *ZmpsbA* expression was discerned in osmotic-stressed plants by 63.42%. On the other hand, SAL treatment upraised the *ZmpsbA* expression in osmotic-stressed soybean plants by 74.52%.

As shown in **Figure 7H**, the abiotic stress reduced *ZmNAGK* expression in untreated stressed plants, but the expression increased by 79.36% in SAL-treated stressed plants (**Figure 7H**).

In the current experiment, we assessed the *ZmPIS* transcription pattern in maize plants. An increase in *ZmPIS* expression level was recorded in osmotic-stressed plants when compared to the expression in the unstressed plants. Although *ZmPIS* expression level increased in osmotic-stressed plants, the SAL implementation declined the expression by 64.06% (**Figure 7I**).

The effects of abiotic stress and SAL application on the expression of *ZmVPP1* were evaluated in maize seedlings through modification in the *VPP1* gene expression (*ZmVPP1*) (**Figure 7J**). During unstressed conditions, variations were shown in the *ZmVPP1* gene expression in control and SAL-treated plants; conversely, declined *ZmVPP1* expression was perceived in stressed plants. SAL-treated plants showed a rise (62.41%) in *ZmVPP1*expression.

The findings of the current study demonstrated a reduced *ZmSCE1d* expression in maize plants under osmotic stress conditions in contrast to the control plants. The association of maize plants with SAL noticeably enhanced the upregulation of *SCE1d*. A higher *ZmSCE1d* expression level (29.12%) was exhibited in SAL-treated stressed plants (**Figure 7K**).

We examined the expression pattern of *ZmNAC48* under normal and stress conditions. Enhanced *ZmNAC48* expression was discerned in maize seedlings affected by abiotic stress. The *ZmNAC48* expression level increased by 71.42% under osmotic stress (**Figure 7L**). In addition, SAL-treated maize plants depicted reduced *ZmNAC48* expression under stress conditions. Under SAL application, osmotic-stressed plants exhibited 39.28% lower *ZmNAC48* expression, compared to the untreated stressed plants.

TABLE 2 | Macronutrient accumulation in soybean and maize plants grown under stress and control conditions with or without salvianolic acid.

Sample name	Ca (μg/kg)	K (μg/kg)	P (μg/kg)
8DAT			
Soybean			
Cont	6.55 ± 0.2bc	41.18 ± 0.59c	5.26 ± 0.10c
SAL	7.27 ± 0.15a	49.94 ± 0.97b	6.79 ± 0.04a
PEG	6.77 ± 0.11ab	50.18 ± 0.09b	5.29 ± 0.09c
PEG+SAL	6.14 ± 0.03c	55.84 ± 0.92a	6.30 ± 0.20b
Maize			
Cont	8.31 ± 0.06bc	61.18 ± 0.59b	7.37 ± 0.16b
SAL	9.28 ± 0.14a	64.93 ± 4.02ab	8.40 ± 0.06a
PEG	8.61 ± 0.06b	65.18 ± 0.09ab	7.40 ± 0.04b
PEG+SAL	8.18 ± 0.01c	69.34 ± 0.58a	8.21 ± 0.11a

Values show the mean ± SE (n = 6). Data within the same column and different lowercase letters are significantly different at $p < 0.05$ (Tukey's test).

DISCUSSION

To ascertain the impacts of exogenously applied salvianolic acid (SAL) on osmotic stress, we examined whether the irrigation of soybean and maize seedlings with SAL would mitigate the symptoms of osmotic stress. SAL-treated seedlings (0.1 and 1 μM) seemed to be healthier, and we perceived that SAL, when applied to the soil, enhances plant growth and development. Additionally, it enhances osmotic stress tolerance and delays foliar wilting, which is evident from the improved growth attributes. Our data showed that treatment of unstressed and stressed plants with SAL results in increased height, root length, leaf area, chlorophyll and carotenoid values, K and P contents, etc. Additionally, the K and P content of osmotic-stressed plants treated with SAL was higher than that of untreated stressed plants. These functions are presumably achieved through amelioration processes associated with photosynthesis and other metabolisms.

Photosystem II consists of a multi-protein complex (D1 and D2 proteins) and performs a function in the oxygen-evolving photosynthetic organisms. The D1 protein is an essential constituent of oxygenic photosynthesis in plants. The *psbA* (encoding D1 protein) has a vital role in protecting photosystem II (PSII) from oxidative damage in plants (Nelson and Yocum, 2006; Mulo et al., 2012). Here, we found that SAL application improved *ZmpsbA* expression, which leads to enhanced D1 protein and confers osmotic stress endurance in maize plants. A study conducted by Huo et al. (2016) demonstrated that enhanced *ZmpsbA* expression, along with higher antioxidant enzyme activities, reduced hydrogen peroxide, malondialdehyde, and ion leakage during osmotic stress, suggesting the role of overexpressed D1 in removing immoderate ROS and boosting antioxidant capability.

Vacuolar H^+-pyrophosphatase has an important function in plant response to osmotic stress (Kriegel et al., 2015). The expression of its encoding gene (*VP1*) is prevalent in varied tissues (Gamboa et al., 2013; Yang et al., 2015), and

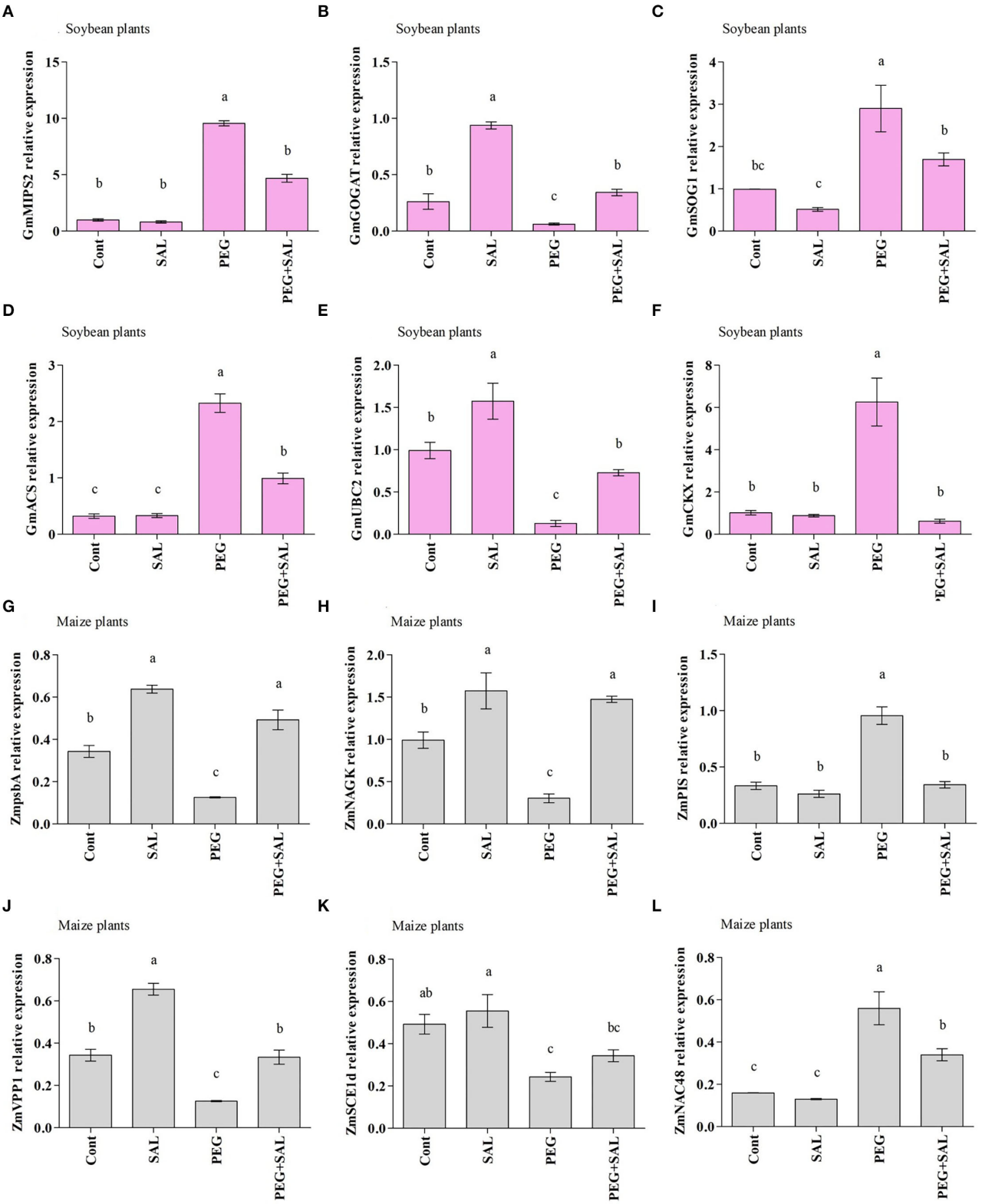

FIGURE 7 | Real-time expression analysis of *GmMIPS2* **(A)**, *GmGOGAT* **(B)**, *GmSOG1* **(C)**, *GmACS* **(D)**, *GmUBC2* **(E)**, *GmCKX* **(F)**, *ZmpsbA* **(G)**, *ZmNAGK* **(H)**, *ZmPIS* **(I)**, *ZmVPP1* **(J)**, *ZmSCE1d* **(K)**, and *ZmNAC48* **(L)** in leaves of soybean and maize plants grown under normal and stress conditions and treated with salvianolic acid for 8 days (8DAT). Treatments: Cont (control), SAL (0.1 μM salvianolic acid), SAL (1 μM salvianolic acid), PEG (25% polyethylene glycol), SAL (0.1 μM salvianolic acid) + PEG (25% polyethylene glycol), and SAL (1 μM salvianolic acid) + PEG (25% polyethylene glycol). Values show the mean ± SE ($n = 6$) and significant differences at $p < 0.05$ (Tukey's test). Bars with different lowercase letters are significantly different from each other.

its overexpression can improve crop tolerance to drought and salinity (Anjaneyulu et al., 2014; Schilling et al., 2014; Lv et al., 2015; Wang et al., 2016). In the present study, the expression level of *ZmVPP1* is rapidly upregulated in stressed maize plants upon SAL application, which proves the stress alleviation effect of SAL. Wang et al. (2016) reported that maize with increased *ZmVPP1* expression shows enhanced drought endurance. They believed that it is most probably due to improved photosynthetic efficiency and root development.

The plant-specific NAC gene family (NAC, ATAF, and CUC) encodes one of the biggest family of transcription factors and are extensively distributed in a wide range of plants (Olsen et al., 2005). Several NAC members have been practically distinguished in the developmental programs (Souer et al., 1996; Aida et al., 1997; Takada et al., 2001; Weir et al., 2004), growth hormone signaling (Xie et al., 2000; Fujita et al., 2004), defense (Collinge and Boller, 2001; Hegedus et al., 2003), and leaf senescence (Fraga et al., 2021). Various NAC genes participate in reactions to abiotic stresses, which include flood, water deficit, salinity, and cold (Fujita et al., 2004; Olsen et al., 2005; Hu et al., 2006; He et al., 2017; Gao et al., 2020, 2021; Mao et al., 2021). Enhanced expression of *ZmNAC48* was detected in maize plants subjected to osmotic stress. Mao et al. (2021) demonstrated that overexpressing *ZmNAC48* enhanced drought endurance, regulated ABA biosynthesis, reduced water loss, and improved stomatal closure. Therefore, we speculate that *ZmNAC48* expression is reduced in SAL-treated stressed plants due to the stress-relieving effect of SAL.

Phospholipids are one of the major structural components of membranes. They also function as signaling precursors or second messengers to modulate plant growth, development, and adaptation to environmental change (Xue et al., 2009). Phosphatidylinositol is a precursor of inositol-containing phospholipids in plant cells and phosphatidylinositol synthase (*ZmPIS*) is a main enzyme in the phospholipid pathway and accelerates the development of phosphatidylinositol (Liu et al., 2013). Here, we noticed overexpression of the *ZmPIS* in maize plants subjected to osmotic stress conditions. On the other hand, the transcript level of *ZmPIS* gene was altered in stressed maize plants under SAL treatment. Liu et al. (2013) reported that *ZmPIS* modulates the plant response to drought stress via modifying membrane lipid composition and enhancing ABA synthesis in maize. Thus, it could be presumed that *ZmPIS* is engaged in plant responses to osmotic stress in maize.

Phytohormones play essential roles in a broad range of physiological processes (Gerashchenkov and Rozhnova, 2013). Ethylene is a multifunctional plant hormone with varied functions, including germination, growth, cell elongation, fruit ripening, and senescence (Iqbal et al., 2017). Previous reports have confirmed the effect of abiotic stress on phytohormone performance, including ethylene (Habben et al., 2014; Riyazuddin et al., 2020). The findings of the current survey imply that osmotic stress enhances the expression level of ethylene-related gene (ACS). It has been proved that under water deficit conditions, ethylene caused leaf abscission and subsequently minimized water loss (Arraes et al., 2015). Our study shows that the application of SAL boosts soybean seedlings to confront stressful environments by diminishing the ethylene content.

Plants generally trigger a vast variety of defense mechanisms to improve endurance to water deficit conditions. Cytokinins (CKs) help to modulate plant development and conciliate plant endurance to water deficit stress. CK oxidases/dehydrogenases (CKXs) help to control CK metabolism. Growing evidence indicated that CKXs have an important role in diverse plant physiological and developmental alterations under stress (Pospíšilov et al., 2016; Hai et al., 2020). Our findings demonstrated that the expression of *CKX* was highly responsive to osmotic stress and was upregulated by dehydration. This was consistent with previous findings (Le et al., 2012). Moreover, we observed a reduction in *CKX* expression level in SAL-treated stressed plants. An enhancement in CK content was also demonstrated to promote leaf longevity and photosynthesis under drought stress, consequently improving water deficit tolerance without yield penalties (Rivero et al., 2007; Peleg et al., 2011). Our analysis of *CKX* has provided insights into CK metabolism in soybean under SAL application and osmotic stress conditions.

Ubiquitination is an essential kind of post-translational alteration of proteins observed in all eukaryotes. Ubiquitination modulates vital biological processes, including plant growth processes, photomorphogenesis, vascular differentiation, flower development, DNA repair, and biotic and abiotic stress factors (Dreher and Callis, 2007). The ubiquitin-conjugating enzyme, *UBC*, is a key enzyme that is involved in ubiquitination. A previous study demonstrated that *GmUBC2* is involved in the leaf development, oxidative stress responses, ion homeostasis modulation, osmolyte synthesis, and stress tolerance responses (Zhou et al., 2010; Zhiguo et al., 2015). Our findings have shown that *GmUBC2* is overexpressed in SAL-treated plants under stress conditions, which provides endurance to osmotic stress.

Reactive oxygen species (ROS) have a vital role in the adaptation mechanism of plants to unfavorable conditions (Huang et al., 2019). ROS at higher concentrations can cause oxidative damage, deteriorate membranes and proteins, disrupt metabolic activities, initiate programmed cell death, and debilitate enzymes (Choudhury et al., 2017). We detected higher H_2O_2 and MDA contents in stressed plants, which could be due to an imbalance in the rate of ROS production and removal (Huang et al., 2019). In contrast, SAL application evidently diminished the augmented H_2O_2 and MDA concentrations in osmotic-stressed plants toward the end of the inspection. Therefore, SAL might suppress the formation of ROS and thus inhibit oxidative-induced plasma membrane deterioration under abiotic stress (Sewelam et al., 2016; Castro et al., 2021).

Myo-inositol phosphate synthase (MIPS) is a central molecule needed for many processes, including cell metabolism, plant growth and development, and cell wall biogenesis (Loewus and Murthy, 2000; Meng et al., 2009). It has been demonstrated that myo-inositol is a key factor that ascertains whether oxidative stress activates or prevents defense responses during cell death provoked by hydrogen peroxide (Chaouch and Noctor, 2010). We found that the transcript levels of *GmMIPS2* increased in soybeans cultivated under osmotic stress conditions. A study

conducted by Ishibashi et al. (2011) showed that *GmMIPS2* is involved in drought stress signaling via ROS formation caused by drought stress.

As a crucial controlling process of post-translational alterations, Sumoylation have an important role in plants in developmental, hormonal, and environmental stress responses (Park and Yun, 2013; Wang et al., 2020). *ZmSCE1d*, a maize class-I SUMO conjugating enzyme, has been stated to take part in salt and drought tolerance activities (Wang et al., 2019). A previous study by Wang et al. (2020) reported that overexpression of *ZmSCE1d* enhanced SUMO conjugates and promoted drought endurance in plants. Taken together, our data showed that *ZmSCE1d* overexpression enhanced osmotic stress tolerance and antioxidant capability in maize plants under SAL treatment.

Land plants are exposed to hostile environments that suppress their growth and productivity. Therefore, they have evolved mechanisms to evade or endure adverse environmental conditions (He and Ding, 2020). It has been shown that environmental factors, including drought, salinity, and cold, cause alterations in the fatty acid content (Sui et al., 2018). Fatty acids control the concentration of ROS by specifically influencing the ROS-generating enzymes (Lim et al., 2017). The results displayed that osmotic stress affects fatty acid levels. The reduced fatty acid level was observed in soybean and maize seedlings under osmotic stress. Conversely, SAL-treated seedlings were less affected by this stress and exhibited a remarkably higher level of fatty acids. Singh et al. (2020) indicated that fatty acids boosted drought and salinity stress tolerance in soybean plants. Therefore, these data suggest that SAL utilization may induce the activation of phospholipases and phospholipid-derived molecules, which are engaged in plant protection mechanisms (Hou et al., 2016).

Plants have evolved intricate strategies to respond to stress via modifications at physiological and molecular levels (Qi et al., 2018). Accumulation of ROS in plants leads to DNA damage. One mechanism to combat oxidative damage and minimize immoderate ROS aggregation is via inner defensive mechanisms that entail antioxidant functions (Agarwal and Pandey, 2004; Gill and Tuteja, 2010). Besides the antioxidation pathway, plants have developed an effective system recognized as the DNA damage response (DDR) pathway (Baxter et al., 2013; Poku et al., 2021). A plant-specific transcription factor, the suppressor of gamma response 1 (*SOG1*) gene, has been identified as a major gene in plant response to DNA damage (Yoshiyama et al., 2009). The results of the present study revealed that the antioxidant function and *SOG1* expression level diminished in osmotic-stressed plants, while this function and expression pattern was enhanced upon SAL application in the stressed plants. A previous study implied that the expression of antioxidant enzymes could be triggered or hindered under abiotic stresses (Zhu et al., 2004). Moreover, it has been confirmed that SOG1 overexpression led to a higher survival rate and antioxidant accumulation in osmotic-stressed plants (Poku et al., 2021). This increase in antioxidant activity and SOG1 expression level suggests that SAL upraises the capability to scavenge excessive ROS, diminishes oxidative damage, and promotes osmotic stress endurance.

Deleterious environmental situations can severely alter sugar concentration in leaves. Sugar modulates various physiological functions, such as photosynthesis, osmotic homeostasis, and protein and lipid metabolism (Martínez-Noël and Tognetti, 2018). It has been confirmed that sugars may act as osmotically active molecules or protective agents upon membranes and enhance plant endurance under deleterious conditions (Sánchez et al., 1998; Sami et al., 2016). Our findings depicted an obvious aggregation of sugars in soybean and maize plants irrigated with SAL under normal and osmotic stress conditions. Saddhe et al. (2021) attested that sugar elevation also improves proline concentration under stress conditions. Considering the present study, the SAL treatment led to further sugar aggregation, which served as an osmoprotectant to modulate osmotic alterations, maintain membrane functions, and improve recovery from osmotic stress.

Previous studies have demonstrated that modulation of nitrogen metabolism is strictly linked to drought stress responses in plants (Zhong et al., 2018, 2019). Nitrogen is a fundamental element for crop growth, development, and yield and is involved in many physiological processes. Nitrogen in the form of NH_4^+ is changed to glutamate and glutamine via the glutamine synthetase, glutamate synthetase (GOGAT), and glutamate dehydrogenase pathways (Xu and Zhou, 2006). Considering that the augmentation of NH_4^+ in the process of nitrogen metabolism is harmful to plant cells (Nguyen et al., 2005), sustaining the activities of enzymes, such as GOGAT, in the nitrogen metabolism process is vital for plant growth (Du et al., 2020a). GOGAT activity is linked to drought stress response, and it is usually regarded as a metabolic indicator of drought tolerance (Nagy et al., 2013; Singh and Ghosh, 2013). In this experiment, osmotic stress conditions reduced the activity of GOGAT, which was in agreement with a previous study (Nagy et al., 2013). This reduced enzyme activity directly affects the efficiency of N uptake and utilization. On the other hand, SAL application enhanced GOGAT activity under osmotic stress conditions. This finding suggests that SAL assists in maintaining N metabolism and enhancing adaptation to osmotic stress.

Amino acids participate in the synthesis of numerous plant products that confer plant responses to adverse conditions (Batista-Silva et al., 2019). In this study, the amino acid content was elevated in soybean and maize plants subjected to osmotic stress conditions, which was in harmony with previous studies (Batista-Silva et al., 2019; Li et al., 2019; Trovato et al., 2021). This amino acid aggregation may be engaged in osmotic acclimatization, ROS scavenging, and protein maintenance (Wu et al., 2014). The SAL treatment recovered amino acid value in the stressed plants throughout the restoration time. A rise in proline concentration was discerned in stressed soybean and maize plants, which was consistent with the studies on varied plants (Khattab, 2007; Bassuony et al., 2008). Proline serves as a reactive oxygen species scavenger, stabilizes membrane and protein structure, minimizes cell damage, and enhances the tolerance of plants toward environmental changes (Teixeira et al., 2020). In addition, proline also functions as a nutritional repository that can be consumed throughout the revival stage to help plants withstand environmental

crises (Heinemann and Hildebrandt, 2021). In this conducted research, SAL treatment caused augmentation of amino acids, distinctly proline content, in stressed plants. This SAL-induced proline accumulation might be relevant to adaptive tactics that ameliorate osmotic acclimatization during osmotic stress.

Nitric oxide participates in numerous physiological processes in plants, such as growth, hormone responses, antioxidant activities, defense reactions, and abiotic stress responses (Peng et al., 2016; Hasanuzzaman et al., 2018; Su et al., 2018). It has been demonstrated that arginine (Arg) is a crucial precursor of NO (Winter et al., 2015). Since *N*-acetylglutamate kinase (NAGK) takes part in arginine biosynthesis, the increased osmotic stress endurance in the plants expressing *ZmNAGK* could be due to the NO augmentation through the arginine metabolism pathway (Huang et al., 2017). In this study, we observed enhanced expression of *ZmNAGK* in SAL-treated stressed plants. Furthermore, maize plants overexpressing *ZmNAGK* aggregated more arginine in response to SAL treatment under osmotic stress conditions. Liu et al. (2019) reported that enhanced expression of the *ZmNAGK* gene promoted drought endurance via greater water preservation, antioxidant defense capability, less oxidative damage, and aggregation of more arginine.

CONCLUSION

In summary, through this study, we have exhibited that salvianolic acid (SAL) can remarkably improve soybean and maize vitality and tolerance under osmotic stress conditions. The potential of SAL under abiotic caused stress modulated host growth by mitigating osmotic stress in maize and soybean plant. Furthermore, SAL application amended host biochemistry to lessen the drastic effects of osmotic stress. In this survey, osmotic stress restrained several genes, while SAL was able to confront the suppression impact of osmotic stress and reawakened varied suppressed genes. SAL also induced the expression of stress-related genes, particularly *GmGOGAT*, *GmUBC2*, *ZmpsbA*, *ZmNAGK*, *ZmVPP1*, and *ZmSCE1d*. Overall, our data provided confirmation for the evidence that salvianolic acid can noticeably strengthen resistance to osmotic stress and therefore can be a potential candidate to be utilized in agriculture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

EK conceived the project, designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. AA-S edited the manuscript. UR helped in data analysis. I-DK, S-MK, and I-JL provided the resources. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.904037/full#supplementary-material

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Nanozinc and plant growth-promoting bacteria improve biochemical and metabolic attributes of maize in tropical Cerrado

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Introduction: Plant growth-promoting bacteria (PGPBs) could be developed as a sustainable strategy to promote plant growth and yield to feed the ever-growing global population with nutritious food. Foliar application of nano-zinc oxide (ZnO) is an environmentally safe strategy that alleviates zinc (Zn) malnutrition by improving biochemical attributes and storage proteins of grain.

Methods: In this context, the current study aimed to investigate the combined effect of seed inoculation with PGPBs and foliar nano-ZnO application on the growth, biochemical attributes, nutrient metabolism, and yield of maize in the tropical savannah of Brazil. The treatments consisted of four PGPB inoculations [i.e., without inoculation, *Azospirillum brasilense* (*A. brasilense*), *Bacillus subtilis* (*B. subtilis*), *Pseudomonas fluorescens* (*P. fluorescens*), which was applied on the seeds] and two doses of Zn (i.e., 0 and 3 kg ha^{-1}, applied from nano-ZnO in two splits on the leaf).

Results: Inoculation of *B. subtilis* with foliar ZnO application increased shoot dry matter (7.3 and 9.8%) and grain yield (17.1 and 16.7%) in 2019-20 and 2020-2021 crop seasons respectively. Inoculation with A. brasilense increased 100-grains weight by 9.5% in both crop seasons. Shoot Zn accumulation was improved by 30 and 51% with inoculation of P. fluorescens in 2019-20 and 2020-2021 crop seasons. Whereas grain Zn accumulation was improved by 49 and 50.7% with inoculation of *B. subtilis* and P. fluorescens respectively. In addition, biochemical attributes (chlorophyll a, b and total, carotenoids, total soluble sugar and amino acids)

were improved with inoculation of *B. subtilis* along with foliar nano ZnO application as compared to other treatments. Co-application of P. fluorescens with foliar ZnO improved concentration of grains albumin (20 and 13%) and globulin (39 and 30%). Also, co-application of *B. subtilis* and foliar ZnO improved concentration of grains glutelin (8.8 and 8.7%) and prolamin (15 and 21%) in first and second seasons.

Discussion: Therefore, inoculation of *B. subtilis* and *P. fluorescens* with foliar nano-ZnO application is considered a sustainable and environmentally safe strategy for improving the biochemical, metabolic, nutritional, and productivity attributes of maize in tropical Savannah regions.

KEYWORDS

PGPB (plant growth-promoting bacteria), photosynthesis, plant growth, nutrient uptake, storage proteins, amino acids, zinc fertilization, grain yield

1 Introduction

Environmental disaster, food, and nutritional insecurities are the foremost devastating challenges to the agricultural sector. Malnutrition is a global dietary concern and one of the most a serious threats to agriculture crop production systems, affecting over half of the global population (Ramzan et al., 2020). Zinc (Zn) is one of the key dietary nutrients and its malnutrition has affected over one-third of agricultural soils because of the presence of excessive soil carbonates, oxides, silicates, and phosphates, as well as through extensive farming systems and practices (Masood et al., 2022). Zn is an essential micronutrient for the normal growth, development, and physiological activities of each living organism (Stanton et al., 2022). In addition, Zn is involved in numerous metabolic and biochemical functions of plants, such as protein and chlorophyll synthesis, lipid and carbohydrate metabolism, enzymatic activities and photosystems, pollen fertility, and energy production (Suganya et al., 2020; Zafar et al., 2022). Zn is responsible for the stabilization and catalyzation of ≈10% of human body proteins, and it presence helps in the mitigation of reactive oxygen species (ROS) through antioxidant metabolism and lipid peroxidation of cell membranes (Ojeda-Barrios et al., 2021; Li et al., 2022). Plants are the major source of Zn entrance into human body. Therefore, a quick and inexpensive alternative strategy is needed to improve Zn bioavailability in edible tissues and crop productivity to combat malnutrition and food security.

Nanotechnology is an ecofriendly alternative that increases targeted nutrient concentration and metabolism, as well as photosynthetic machinery of the chosen crop (Kapoor et al., 2022). Nano-fertilizer with zinc oxide (ZnO) is being recognized as an important and effective alternative for increasing growth and productivity by regulating primary photosynthetic activities and carbohydrate metabolism to satisfy the nutritional quality of plants (Singh et al., 2021; Jalal et al., 2022c). Nano-fertilizer reduces the use of synthetic fertilizers while increasing targeted nutrient availability for plant uptake and its intake by human in edible grains (Prasad et al., 2017). Foliar application of nano-fertilizer has been widely reported for enhancing plant nutrition and productivity, as it enters the cell membrane more effectively, contributing to the metabolism of proteins, sugars, and amino acids, and photosynthesis of plants to increase nutrient use efficiency and reduce environmental constraints (Weisany et al., 2021; Kandil et al., 2022). Foliar spray of ZnO is a more viable and prompt strategy than root/soil Zn application because of the large surface area and direct absorption through stomata and cuticles, and then translocation *via* the phloem into the chloroplast (Su et al., 2019; Zhu et al., 2020). The delivery of nano-Zn enhances plant growth, productivity, and Zn concentration in the edible tissues (Dimkpa et al., 2022). However, these benefits are still to be adapted at field scale because of the nature and size of particulates (Sudhakaran et al., 2020). Hence, the introduction of plant growth-promoting bacteria (PGPBs) in combination with nano-Zn fertilizer could be a better integrated alternative to improve agricultural productivity in a more sustainable and ecofriendly way to the environment.

PGPBs are applied *via* seeds, soil, and leaves to enhance efficiency of plant growth and manage abiotic stresses through root morphological alterations (Goswami and Suresh, 2020). Seed inoculation with PGPBs is a promising strategy to promote plant growth and development by facilitating nutrient use efficiency, modulating hormonal activities, and inhibiting pathogenic infestation (Di Benedetto et al., 2017; Kumar et al., 2019). In addition, PGPBs contribute to the synthesis of secondary metabolites, water absorption, nutrient [phosphorus

(P), Zn, and potassium (K)] solubilization, and tolerance to biotic and abiotic stresses (Hungria et al., 2018; Jalal et al., 2021; Lopes et al., 2021). The inoculants of the genus *Azospirillum* are being recognized in the biosynthesis of auxin synthesis, nutrient cycling and availability, and biological nitrogen (N) fixation by reducing N_2 into ammonia (NH_3) (Bhat et al., 2019; Carrillo-Flores et al., 2022; Galindo et al., 2022). *Bacillus subtilis* (*B. subtilis*) has the ability to promote plant growth through P solubilization, increase Zn use efficiency, bioremediation of heavy metals, and controlling phytopathogenic infestation, which in turn leads to increased root–shoot development and productivity (Lobo et al., 2019; dos Santos et al., 2021; Jalal et al., 2022a). In addition, *Pseudomonas fluorescens* (*P. fluorescens*) is considered to be one the most effective inoculants to synthesis antibiotics, metabolites, and volatile organic compounds to combat soil pathogens (David et al., 2018), improving Zn and P concentrations (Jalal et al., 2022b; Rosa et al., 2022), and also helping in N-fixing activities for sustainable crop production (Jing et al., 2020; Agbodjato et al., 2021).

PGPBs could increase Zn solubility and uptake through the production of organic and inorganic acids, and several chelators (Idayu et al., 2017; Khoshru et al., 2020). Green-synthesized ZnO increases morphological and biochemical attributes that lead to sustainable crop production systems (Natarajan et al., 2021). Zinc fertilization in combination with inoculation of *Azospirillum brasilense* (*A.brasilense*) increases Zn use efficiency and accumulation, and yield of cereal crops grown in tropical environments (Galindo et al., 2021). In addition, *B. subtilis* and *P. fluorescens* are being recognized as the most effective inoculants to solubilize Zn and P, and improve plant growth and development under different climatic conditions (Rosa et al., 2020; Ahmad et al., 2021; 2022b; Jalal et al., 2021; Jalal et al., 2022a).

Maize is recognized as the "queen of cereals" because of its extensive use and flexibility. It is the most frequently cultivated grain crop, serving as a major source of nutrition in many developing countries (Kumawat et al., 2020). Therefore, it is important to adapt new biotechnology, like the use of nano-fertilizers and PGPB inoculation, for improving physiochemical and yield traits of maize under changing environmental conditions. The literature is lacking data on the combined effects of PGPBs and nano-Zn on growth and development, and nutritional status of maize in the tropical savannah of Brazil. There exists a research gap on the effect of PGPBs and nano-Zn on primary metabolic and biochemical attributes, and yield of maize crop in the tropical savannah of Brazil. In this context, it was hypothesized that inoculation with PGPBs and foliar nano-Zn fertilization would be an interesting strategy to improve primary metabolic and biochemical attributes, and the yield of the maize crop. Therefore, the objective of the study was to evaluate the effect of inoculation with PGPBs in association with or without foliar nano-Zn application on the levels of chlorophyll a, b, and total chlorophyll, and concentrations of amino acids, sucrose, and total sugar in maize. In addition, we wanted to know the effect of PGPBs and foliar nano-Zn spray on the uptake of Zn in shoot and grains, and the grain yield of maize in the tropical savannah of Brazil.

2 Materials and methods

2.1 Description of experimental site

Two field experiments with maize were performed during the summer (October–March) of 2019–20 and 2020–1 cropping seasons at the Extension and Research Farm of School of Engineering, São Paulo State University (UNESP) at Selvíria, Mato Grosso do Sul, Brazil. The site is located at geographical coordinates of 20°22′ S latitude, 51°22′ W longitude, and an altitude of 335 m (Figure 1).

The soil is clayey oxisol defined as Rhodic Haplustox (Soil Survey Staff, 2014) and Red Latosol Dystrophic (Santos et al., 2018), with a granulometric characterization of 777, 98, 125 g kg^{-1} of sand, silt and clay at a soil depth of 0.00–0.25 m (Teixeira et al., 2017). The experimental site has a history of more than 30 years' cultivation with an annual cereal–legume crop rotation. In addition, the site was under a no-tillage system for the last 13 years while wheat was cultivated prior to the current maize experiments in both years.

The experimental region is characterized as Aw-Köppen with a rainy summer (an average rainfall of 1370 mm and 23.5°C), and is humid and tropical with a relative humidity of 70–80% (Alvares et al., 2013). Different climatic factors (e.g., rainfall, temperature, and light radiation) during the current experiments, in both cropping seasons, were carefully monitored (Figure 2).

2.2 Soil analysis

Twenty random soil samples were collected before the experiment started from a soil layer of 0.00–0.20 m in both cropping seasons. The collected samples were properly mixed to attain a composite sample, then air-dried, sieved (2 mm), and prepared for chemical characterization (Raij et al., 2001). The soil chemical characterization is summarized in Table 1.

2.3 Experimental design and treatments

The experiments were conducted in a randomized complete block design, with four replications in 4 × 2 factorial scheme. There were four types of seed inoculation with PGPBs (i.e., no inoculation, *A.brasilense, B. subtilis*, and *P. fluorescens*) and two foliar nano-ZnO applications (i.e., without or with 3 kg Zn ha^{-1}), applied at 50% tasseling and at the grain setting/filling stage of maize.

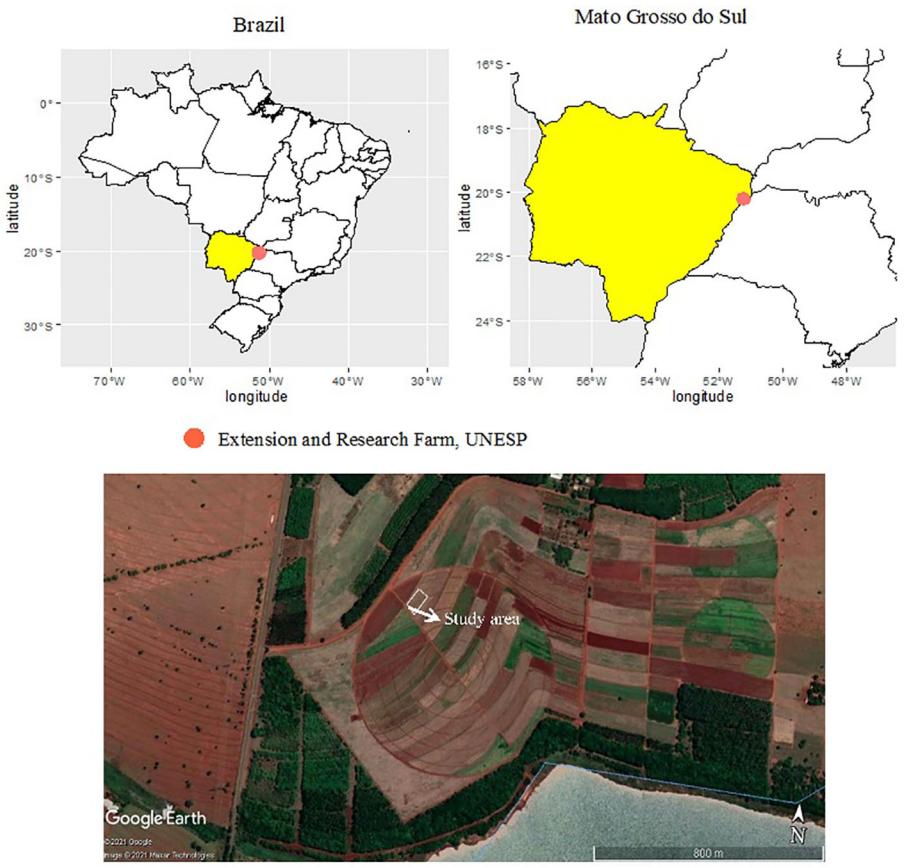

FIGURE 1
Geographical location of experimental site at Extension and Research Farm, UNESP–Ilha Solteira, at Selvíria, state of Mato Grosso do Sul, Brazil (20°22′ S, 51°22′ W, altitude of 335 m) during the 2019–20 and 2020–1 cropping seasons. The map was created using pacot, geobr, and ggplot within R software (R Core Team, 2015). Accessed on 27 February 2022. Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from the Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

The maize seeds were chemically treated with Standak Top™, a co-formulation of fungicide [arbendazim + thiram (45 g + 105 g of active ingredient (a.i.) 100 kg^{-1} seeds] and insecticide [imidacloprid + thiodicarb (45 g + 135 g of a.i. 100 kg^{-1} seeds)] 24 h prior to inoculation. Treating cereal seeds with Standak Top™ is a common practice in the Brazilian tropical savannah to prevent soil pathogen infection without any harmful effects on the bacterial inoculation (Munareto et al., 2018; Cardillo et al., 2019).

Seeds were manually inoculated by mixing seeds and the respective inoculant in a plastic bag 1 h before sowing. Inoculation with *A. brasilense* strains Ab-V5 (CNPSo 2083) and Ab-V6 (CNPSo 2084) was carried out at a dose of 200 ml of liquid inoculant per 24 kg of seeds with a guarantee of 2×10^8 CFU ml^{-1}, whereas *B. subtilis* strain (CCTB04) was inoculated with a guarantee of 1×10^8 CFU ml^{-1} and *P. fluorescens* strain (CCTB03) with a guarantee of 2×10^8 CFU ml^{-1}, at a liquid inoculant dose of 150 ml ha^{-1} per 24 kg of seeds. The inoculation was carried out by following the recommendation of the inoculant-providing company (Biotrop®, Curitiba, Brazil). These inoculants are commercially used in Brazil with strains of *A. brasilense* (AzoTotal™), *B. subtilis* (Vult™), and *P. fluorescens* (Audax™) used to promote growth and productivity. The gene sequencing of *A. brasilense* highlighted that strains Ab-V5 and Ab-V6 are carrying *fix* and *nif* genes, which promotes nutrient cycling and availability, biological N fixation, auxin production, and induces plant tolerance against biotic and abiotic stresses (Fukami et al., 2017; Fukami et al., 2018b; Galindo et al., 2021). *B. subtilis* is the first Gram-positive bacterium carrying non-ribosomal peptide synthetases and beta-glucanase that are resistant to phytopathogen attack and facilitate heavy metal accumulation, while *zntR* as Zn transporter induces plant growth promotion (Chaoprasid et al., 2015; Rekha et al., 2017; Muñoz-Moreno et al., 2018). *P. fluorescens* is considered an efficient biocontrol agent, with the synthesis of antibiotics and volatile organic compounds to deter soil pathogens, and helping in gluconic acid production, solubilization of nutrients, and biological N fixation (David et al., 2018; Jing et al., 2020).

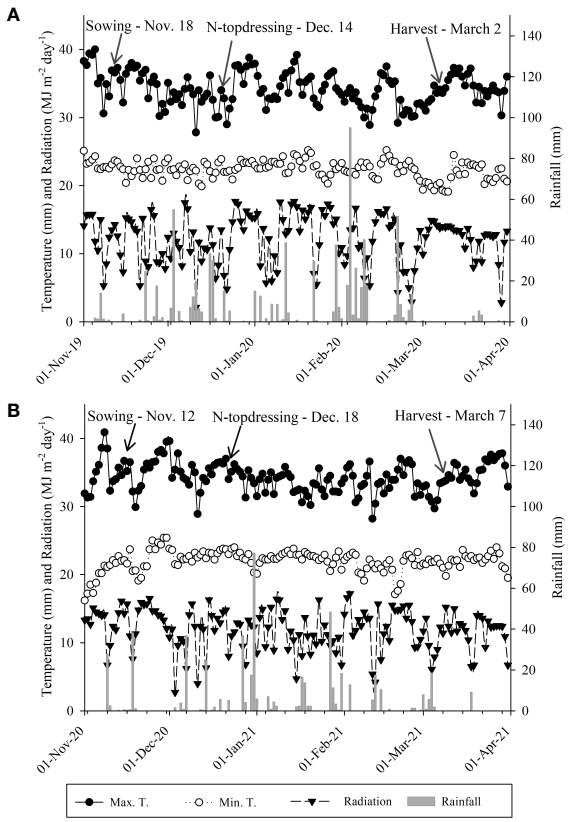

FIGURE 2
Rainfall, maximum and minimum temperatures, and light radiation during the experimental period at a weather station of the Extension and Research Farm of School of Engineering–UNESP, November to March 2019–20 **(A)** and 2020–21 **(B)**, respectively.

The foliar Zn application was performed from a liquid source of Zn (Nano R1 zinco™) that was obtained from Allplant® fertilizers industry, São Paulo, Brazil. The company is already registered with the Ministry of Agriculture, Brazil. Nano R1 zinc is a fluid suspension with 50% p/p Zn, 1000 g/l solubility, and 2.0 density, and has been successfully used in previous studies (Nakao et al., 2018; Jalal et al., 2022c). A total of 3 kg ha^{-1} of ZnO was applied in two splits, 50% Zn at V8/V10 and 50% at R1 stage of maize (Stewart et al., 2020). The application was performed through a manual sprayer pump with a 6.0-l water capacity (300 l/ha of volume application). The field was inspected soon after the foliar spray and no leaf damage was observed.

2.4 Field management

The field site was sprayed with glyphosate (Roundup™) + 2,4-D (1800 + 670 g ha^{-1} of a.i.) 15 days prior to the experiment being planted to control pre-emerged weeds. A simple maize hybrid FS500PWU-Forseed (registered with the National Technical Commission on Biosafety of Brazil under reference n°. 1596/2008 for tropical and sub-tropical regions) was planted on 18 November 2019 and 12 November 2020 in a no-tillage system at 3.3 seeds m^{-1}. All the treatments were uniformly fertilized with 350 kg ha^{-1} NPK (08 : 28 : 16, urea) on the basis of the soil analysis and expected yield. Seedlings emerged after 5 days of planting in both experimental years. Each experimental plot consisted of six 6-m-long maize rows with a 0.45m between rows. The total plot size was 16.2 m^2. The data were collected from four central rows with a useful area of 10.8 m^2. The post-emergence weeds were controlled by spraying herbicides atrazine and tembotrione (1000 + 84 g a.i. ha^{-1}) at the V3 growth stage of maize. N side dressing (120 kg ha^{-1}, applied in the form of ammonium sulphate; 21% N) at V6 growth stage (i.e., 30 and 31 days after emergence in the 2019–20 and 2020–1 maize cropping seasons, respectively) was applied to all treatments to uniformly distribute on the soil surface and was incorporated, by central pivot irrigation, on the same day. Irrigation was performed usinfg a central pivot sprinkler irrigation system at 14-mm water volume on a shift of 72 hours or as per crop requirement. The crop was manually harvested on 2 March 2020 and 7 March 2021.

TABLE 1 Pre-maize experiment soil analysis of composite sample in a soil layer (0–0.20 m) in the 2019–20 and 2020–1 cropping seasons.

Property	Unit	Status	
		2019–20	2020–1
pH ($CaCl_2$)	—	5.2	5.3
Organic matter	mg dm^{-3}	18	23
P (resin)	mg dm^{-3}	38	40
K	$mmol_c$ dm^{-3}	1.7	1.9
Ca	$mmol_c$ dm^{-3}	21	22
Mg	$mmol_c$ dm^{-3}	15	12
B (hot water)	mg dm^{-3}	0.14	0.39
Cu (DTPA)*	mg dm^{-3}	3.4	3.7
Fe (DTPA)*	mg dm^{-3}	25	28
Zn (DTPA)*	mg dm^{-3}	0.7	9.4
Mn (DTPA)*	mg dm^{-3}	38.1	37.3
S-SO_4	mg dm^{-3}	4.0	22
H+Al	$mmol_c$ dm^{-3}	34	31
CEC (pH7)*	$mmol_c$ dm^{-3}	75.7	66.9
V*	%	50	54

*CEC, cation exchange capacity; V, base saturation; DTPA, diethylenetriaminepentaacetic acid.

2.5 Assessments and evaluations

2.5.1 Growth and productivity attributes

Plant height was determined by measuring plant length from the surface of the ground to the upper apex of the tassel. The plants from four central lines were harvested, dried, and weighed with an analytical balance for the analysis of shoot dry matter. Ten random ears were collected at harvest to count number of rows and grains per ear. One-hundred-grain mass was measured with a precise scale at 13% humidity (wet basis). The ears from the central lines of each plot were manually harvested, threshed mechanically, and the grain weight was converted into kg ha^{-1} at 13% humidity to quantify yield.

2.5.2 Zinc nutrition and use efficiency

Zn accumulation in shoot and grains was estimated from the ratio of Zn concentration in shoot and grains, and shoot dry matter and grain yield, respectively. Shoot and grain Zn concentrations were determined by nitroperchloric digestion and quantified with atomic absorption spectrophotometry, following the protocols of Malavolta et al. (1997). Zinc use efficiency (ZnUE), *via* Eq. 1, and applied Zn recovery (AZnR), *via* Eq. 2, were calculated according to the methodology of Fageria et al. (2011):

$$ZnUE = \frac{GYF - GYC}{applied\ \ Zn\ \ dose} \quad (1)$$

$$AZnR = \frac{GSZnAF - GSZnAC}{applied\ \ Zn\ \ dose} \quad (2)$$

Where GYF is the grain yield with nano-Zn foliar fertilization, GYC is the grain yield in the control treatments, GSZnAF is the grain plus shoot Zn accumulation in nano-Zn-applied treatments, and GSZnAC is grain plus shoot Zn accumulation in control treatments.

2.5.3 Photosynthetic pigments

The photosynthetic pigments (chlorophyll a, b, and total, and carotenoid) were extracted and analyzed by the procedure of Lichtenthaler (1987). Fresh leaves were collected at flowering stage. The samples of 0.5 g were macerated in liquid nitrogen and 50 ml of 80% acetone, stored in the refrigerator and then centrifuged at 10,000 × *g* for 10 min. The absorbance of the acetone extracts were quantified at 663, 645, and 470 nm using a UV-160 A UV–vis spectrometer for chlorophyll a, total, and chlorophyll b and carotenoids concentrations, respectively.

2.5.4 Primary metabolism assay

2.5.4.1 Extraction for total soluble sugar and amino acids

Total soluble sugar (TSS) and amino acids were extracted from lyophilized leaves (≈0.5 g) in 10 ml of MCW solution (60% methanol, 25% chloroform, and 15% water) according to the procedure of Bileski and Turner (1966). The material solution was homogenized in a 15-ml polystyrene tube by vortexing, placed in a refrigerator for 48 h and centrifuged at 10,000 rpm for 10 min at 4°C. A 5-ml MCW extract supernatant was collected in a tube, and 1 ml of chloroform and 1.5 ml of distilled water added. After 24 h, the separation phase of aliquots from the hydrophilic portion was used for the determination of total soluble sugar and amino acid concentrations.

2.5.4.2 Determination of total soluble sugar

TSS in maize leaves was quantified according to the procedure of Dubois et al. (1956). A 20-µl MCW extract was mixed with 500 µl of 5% phenol (w/v) and 2 ml of concentrated H_2SO_4 in a glass tube. After homogenization in a vortex mixer, the tube was heated at 100°C for 10 min and then cooled down to room temperature. Afterward, the readings were performed at an absorbance of 490 nm in spectrophotometer (SP-220, bioespectro^{TM}). The standard sucrose curve was used to quantify total sugar concentration and was expressed in mg g^{-1} fresh weight (FW).

2.5.4.3 Determination of total amino acids

The protocols of Cocking and Yemm (1954) were used to quantify variation in total free amino acid concentration in maize leaves. An aliquot of 300 µl of MCW extract was mixed with 500 µl of 0.2 M sodium citrate, 200 µl of 5% ninhydrin in ethylene glycol, and 1 ml of 0.0002 M KCN solution in a glass tube. The content of the tubes was homogenized by vortexing and heated at 100°C for 20 min, and then cooled with tap water for ≈10 min. After cooling to room temperature, 1 ml of 60% ethanol was added to the glass tube and homogenized by vortexing. The readings were obtained at and absorbance of 570 nm using a spectrophotometer (SP-220, bioespectro^{TM}). The methionine standard curve was used to calculate free amino acid concentration and was expressed in mg g^{-1} FW.

2.5.4.4 Determination of storage proteins

The concentration of grain storage proteins (e.g., albumin, globulin, prolamin, and glutelin) was determined according to the protocols of Bradford (1976). Dried and ground grain samples of 0.25 g was extracted with 5 ml of deionized water in 15-ml falcon tubes. The material was homogenized by vortexing for 1 min and then centrifuged at 10,000 rmp for 20 min at 4°C. A 20-µl supernatant was extracted with 1 ml of Bradford's solution into 2-ml micro-tubes. The samples were homogenized, and read at an absorbance of 595 nm using a spectrophotometer (SP-220, bioespectro^{TM}) for the sequential extraction of albumin concentration. The same sample was used for the quantification of globulin by replacing water with 5 ml of 5% sodium chloride (NaCl) then replaced NaCl with 5 ml of 60% ethanol to determine prolamin concentration. Finally, the

glutelin fraction was quantified with 5 ml of 0.4% sodium hydroxide. Bovine serum albumin was used as a standard and was expressed in mg g^{-1} dry mass.

2.6 Statistical analysis

The entire dataset was tested for normality using the Shapiro–Wilk test and Levene's homoscedasticity test ($p < 0.05$), which showed that data were to be normally distributed ($W \geq 0.90$). The data were subjected to analysis of variance (*F*-test) where foliar nano-Zn spray, PGPB inoculations, and their interactions were considered fixed variables, and replication was considered a random variable in the model. When a main effect or interaction was observed as being significant by *F*-test ($p \leq 0.05$), then Tukey's test ($p \leq 0.05$) was used for mean comparison of nano-Zn spray and PGPB inoculation using R software (R Core Team, 2015).

A Pearson correlation analysis ($p \leq 0.05$) was conducted, and a heatmap was created using corrplot package of "color" and "cor.mtest" functions to calculate coefficients and evaluate the relationships between growth, yield, nutritional, biochemical, and metabolic attributes of maize using R software (R Core Team, 2015).

A principal component analysis (PCA) was used to evaluate maize growth, grain yield and components, and nutritional, biochemical, and metabolic attributes in both years of study. The PCA was performed using factoextra and FactoMineR packages in R software (R Core Team, 2015). The number of principal components (PCs) was selected based on eigenvalues. The biplot graphs represent PC1 on the *x*-axis and PC2 on the *y*-axis of the plot.

3 Results

3.1 Growth, yield components, and yield of maize

The current study addressed the impact of PGPBs and foliar nano-Zn application on the growth performance and nutrient metabolism of a maize crop in a tropical savannah region. Inoculation with PGPBs increased plant height in both years of study, whereas foliar nano-Zn and interaction of foliar nano-Zn and PGPBs did not influence plant height in the 2020–1 maize cropping season (Table 2). The interaction of foliar nano-Zn and PGPBs for plant height in the 2019–20 cropping season was significant (Figure 3A). Foliar nano-Zn at a dose of 3 kg ha^{-1}, along with inoculation of *A. brasilense*, *B. subtilis*, and *P. fluorescens* produced taller maize plants. All inoculation treatments were observed with taller plants under foliar nano-Zn application compared with the control treatments. There was no significant difference among inoculation treatments in the absence of foliar nano-Zn application (Figure 3A).

TABLE 2 Plant height, shoot dry matter, and number of rows per cob of maize as a function of plant growth-promoting bacteria inoculation, together with or without nano-zinc oxide spray in the 2019–20 and 2020–1 cropping seasons.

	Plant height		Shoot dry matter		Number of rows cob^{-1}	
	——— m ———		—— kg ha^{-1} ——		———	
Treatment	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1
Inoculation (I)						
Without	2.45	2.75 a	11526 b	11359	16.93 b	16.03 b
A. brasilense	2.64	2.74 ab	12157 a	12196	17.62 ab	16.91 ab
B. subtilis	2.65	2.64 b	12368 a	12478	17.81 a	16.80 ab
P. fluorescens	2.62	2.74 ab	12119 a	12623	17.25 ab	17.17 a
Foliar zinc (ZnF) spray (kg ha^{-1})						
0	2.45	2.69	11595 b	11521 b	17.22	16.40 a
3	2.73	2.74	12490 a	12807 a	17.59	17.05 b
F-test						
I	6.6*	4.2*	9.6**	2.0^{ns}	4.0*	3.8*
ZnF	54.6**	3.2^{ns}	59.3**	10.6**	3.7^{ns}	6.5*
I × ZnF	5.3*	2.0^{ns}	1.7^{ns}	0.13^{ns}	0.5^{ns}	0.24^{ns}
CV (%)	3.9	2.6	2.7	9.2	3.2	4.3

Means in the column followed by different letters are statistically different by Tukey's test, $p \leq 0.05$. ** and * significant at $p < 0.01$ and $p < 0.05$, respectively, while ^{ns} is non-significant by F-test.

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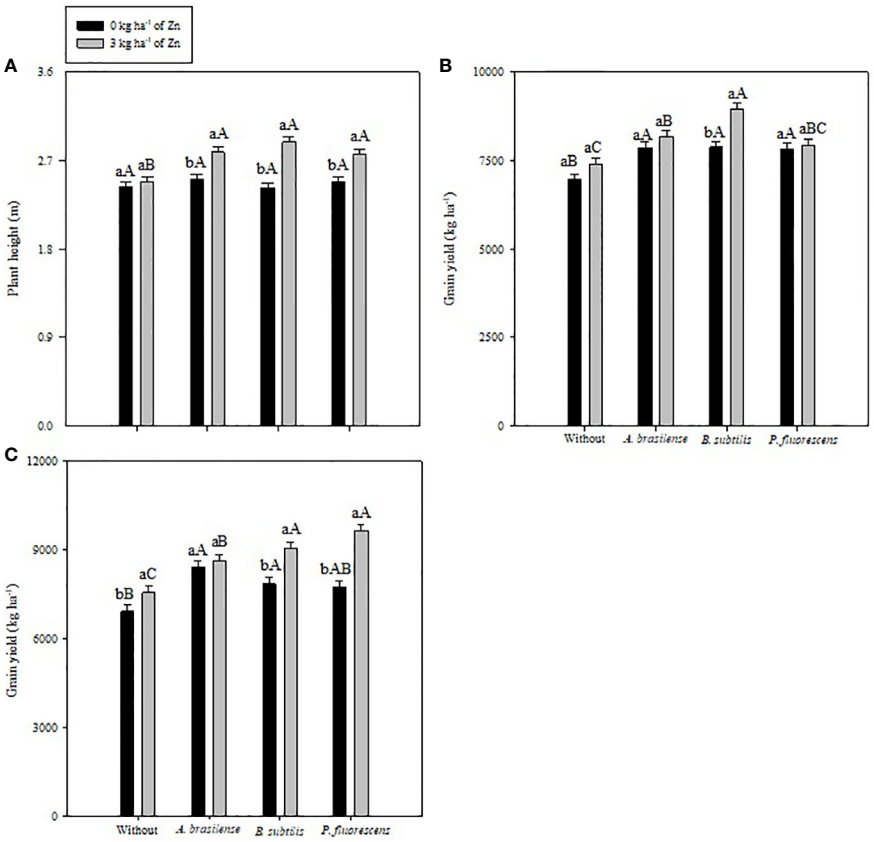

FIGURE 3
Plant height in 2020–1 **(A)**, and grain yield in 2019–20 **(B)** and 2020–1 **(C)** of common bean as a function of plant growth-promoting bacteria in combination with or without foliar nano-zinc oxide (ZnO) application. Without = control (no inoculation). The uppercase letters compare interactions of inoculations within each dose of foliar nano-ZnO application and lowercase letters are used to compare interactions of foliar zinc doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey's test ($p< 0.05$) for foliar ZnO doses and inoculations in the 2019–20 and 2020–1 cropping seasons. Error bars indicate standard error of the mean (n = 4 replications).

The interaction of PGPBs and nano-Zn foliar spray was not significant for shoot dry matter of maize. However, the effect of foliar nano-Zn spray was significant in both cropping seasons (Table 2). Nano-Zn foliar spray increased shoot dry matter of maize by 7.7% and 11.2% in the 2019–20 and 2020–1 cropping seasons, respectively. In addition, plants inoculated with *B. subtilis* were observed as having greater shoot dry matter (12,368 kg ha^{-1}), which was statistically at per with other inoculations, than the control treatment. There were no statistical differences among treatments regardless of inoculation in the 2020–1 cropping season (Table 2).

Inoculation with PGPBs and foliar nano-Zn application positively increased the number of rows per cob of maize (Table 2). The maximum number of rows cob^{-1} were observed in plants that had been inoculated with *B. subtilis* and *P. fluorescens* in the 2019–20 and 2020–1 cropping seasons, respectively. These results were statistically similar to other inoculation treatments in relation to the control. In addition, foliar nano-Zn application increased the number of rows cob^{-1} of maize in the second cropping season only. The interactions of inoculation with PGPBs and foliar nano-Zn application for number of rows cob^{-1} were not significant in both years of study (Table 2).

In addition, the number of grains cob^{-1} of maize was significantly influenced by inoculation with PGPBs and foliar nano-Zn application, whereas their interactions were not significant in the 2019–20 and 2020–1 cropping seasons (Table 3). The number of grains cob^{-1} was increased by 11.9% and 15% with the inoculation of *B. subtilis* and *P. fluorescens* in the first and second maize cropping seasons, respectively, in comparison to those maize crops without inoculation treatments. The foliar nano-Zn application increased the number of grains cob^{-1} by 10.4% and 16.6%, compared with the control (i.e., absence of foliar Zn spray).

The interaction of inoculation with PGPBs and foliar nano-Zn application was not significant for 100-grains weight of maize

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TABLE 3 Number of grains cob^{-1}, 100-grains weight, and grain yield as a function of plant growth-promoting bacteria inoculation, together with or without nano-zinc oxide spray in the 2019–20 and 2020–1 cropping seasons.

Treatment	Number of grains cob^{-1}		100 grains weight (g)		Grain yield (kg ha^{-1})	
	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1
Inoculation (I)						
Without	614 b	557 b	30.6 b	27.5 b	7172	7241
A. brasilense	675 a	623 a	33.5 a	30.1 a	8015	8521
B. subtilis	687 a	560 ab	32.7 ab	29.7 a	8405	8447
P. fluorescens	654 ab	642 a	31.8 ab	30.0 a	7866	8693
Foliar zinc (ZnF) spray (kg ha^{-1})						
0	625 b	559 b	31.3 b	28.5 b	7620	7730
3	690 a	652 a	32.9 a	30.2 a	8109	8720
F-test						
I	8.1**	11.3**	4.4*	10.5**	20.1**	19.9**
ZnF	32.7**	72.8**	7.9*	19.4**	18.1**	44.2**
I x ZnF	0.5^{ns}	1.8^{ns}	0.12^{ns}	1.9^{ns}	3.3*	6.1**
CV (%)	4.9	5.1	5.2	3.6	4.1	5.1

Means in the column followed by different letters are statistically different by Tukey's test, $p \leq 0.05$. ** and *—significant at $p< 0.01$ and $p< 0.05$, respectively, while ^{ns} is non-significant by F-test.

(Table 3). Inoculation with *A. brasilense* increased 100-grains weight of maize by 9.47% and 9.45% in the 2019–20 and 2020–1 cropping seasons, respectively, which was statistically similar to the inoculation of *B. subtilis* and *P. fluorescens* compared with the without inoculation treatments. Foliar application of nano-Zn at the dose of 3 kg ha^{-1} increased 100-grains weight by 5.1% and 5.9% in the 2019–20 and 2020–1 maize cropping seasons, respectively (Table 3).

The effect of inoculation with PGPBs and foliar nano-Zn application, and their interactions were significant for maize grain yield in the 2019–20 and 2020–1 growing seasons (Table 3). The treatment with inoculations of *B. subtilis* and *P. fluorescens* increased maize grain yield by 17.2% and 20.1% in the 2019–20 and 2020–1 cropping seasons, respectively, in relation to the without inoculation treatments. In addition, foliar-applied nano-Zn also increased grain yield of maize by 6.4% and 12.8% in comparison to the control treatments. In case of interactions, the treatments with inoculation of *B. subtilis* performed better with nano-Zn foliar spray in the first maize cropping season (Figure 3B). In addition, maize plants inoculated with *P. fluorescens* had a greater grain yield in the presence of nano-Zn foliar application, which was statistically at per with treatments inoculated with *B. subtilis* and foliar nano-Zn application in the 2020–1 cropping season (Figure 3C). In general, the treatments inoculated with PGPBs produced greater grain yields regardless of foliar nano-Zn application in both cropping seasons. The lowest grain yield of maize was noted in the control treatments in both cropping seasons (Figures 3B, C).

3.2 Shoot and grain zinc accumulation and use efficiencies

There was positive influence of the treatments on shoot Zn accumulation of maize; however, their interactions were not significant in the 2019–20 and 2020–1 cropping seasons (Table 4). Inoculation with *P. fluorescens* improved shoot Zn accumulation by 30% and 51% in first and second maize cropping seasons, respectively, compared with the without inoculation treatments. In addition, foliar nano-Zn application improved shoot Zn accumulation by 35% and 36% in first and second cropping seasons, respectively, in comparison with the control treatments.

Inoculation with PGPBs and foliar nano-Zn application had a positive influence on grain Zn accumulation of maize in the 2019–20 and 2020–1 cropping seasons (Table 4). The interactions of PGPBs and foliar nano-Zn application for grain Zn accumulation were also significant (Figure 4A). Inoculation with *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn application improved grain Zn accumulation by 49% and 51% in the first and second maize cropping seasons, respectively (Figures 4A, B). The treatments with inoculation of *P. fluorescens* and *A. brasilense* performed better regardless of

TABLE 4 Shoot zinc accumulation (SZnA) and grain zinc accumulation (GZnA), zinc use efficiency (ZnUE), and applied zinc recovery (AZnR) as a function of plant growth-promoting bacteria inoculation, together with or without nano-zinc oxide spray in the 2019–20 and 2020–1 cropping seasons.

	SZnA		GZnA		ZnUE		AZnR	
	——— g ha^{-1} ———				—– kg kg^{-1} —–		—— % ——	
Treatment	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1
Inoculation (I)								
Without	333 b	306 b	216	211	357 c	321 b	76	53 b
A. brasilense	330 b	379 ab	288	287	619 b	676 ab	95	92 ab
B. subtilis	379 ab	372 ab	322	287	876 a	816 a	147	111 ab
P. fluorescens	433 a	462 a	285	318	535 b	1016 a	136	160 a
Foliar zinc (ZnF) spray (kg ha^{-1})								
0	314 b	322 b	236	243	—	—	—	—
3	423 a	438 a	320	309	—	—	—	—
F-test								
I	3.7*	6.23**	28**	13.6**	78.3**	11.1**	3.9^{ns}	7.1*
ZnF	18.9**	20.7**	102**	28.0**	—	—	—	—
I x ZnF	0.5^{ns}	0.37^{ns}	10.8*	4.3*	—	—	—	—
CV (%)	19.2	19.0	8.5	12.7	8.2	24.9	30.2	32.2

Means in the column followed by different letters are statistically different by Tukey's test, $p \leq 0.05$. ** and *significant at $p< 0.01$ and $p< 0.05$, respectively, while ^{ns} is non-significant by F-test.

foliar nano-Zn application in both cropping seasons. In addition, the lowest grain Zn accumulation was observed in the treatments without inoculation of PGPBs and nano-Zn application in both maize cropping seasons (Figures 4A, B).

ZnUE and AZnR were increased in the treatments with inoculation of PGPBs and foliar nano-Zn application (Table 4). The treatments with inoculation of *B. subtilis* increased ZnUE by 145% in the 2019–20 cropping season. Interestingly, inoculation with *P. fluorescence* increased ZnUE by 216% in the second season, which was statistically at per with the treatments of inoculation with *B. subtilis* and *A. brasilense*, compared with the without inoculation treatment (Table 4). In addition, the treatments with inoculation of PGPBs positively influenced AZnR in the second maize cropping season only (Table 4). Inoculation with *P. fluorescence* was observed with a higher AZnR (160%), which was statistically similar to the treatment with inoculation of *B. subtilis* (111%) and *A. brasilense* (92%), than the without inoculation in the 2020–1 cropping season (Table 4).

3.3 Photosynthetic pigments

There was positive impact of PGPB inoculation and foliar nano-Zn spray on the photosynthetic pigments of maize leaves at the flowering stage (Table 5). The interaction and seed inoculation with PGPBs did not affect chlorophyll a content in the 2019–20 maize cropping season. However, the effect of treatments and their interaction for chlorophyll a content in maize leaves was significant in the 2020–1 cropping season (Table 5; Figure 5A). Inoculation with *B. subtilis* and foliar nano-Zn spray was observed with highest chlorophyll a content, compared with other inoculation and without inoculation treatments (Figure 5A). In addition, the treatments with inoculation of *P. fluorescence* were observed with a higher chlorophyll a content in the absence of foliar nano-Zn application than with other treatments (Figure 5A). The lowest leaf chlorophyll a content was observed in control treatments (Figure 5A). Foliar nano-Zn spray at the dose of 3 kg ha^{-1} increased chlorophyll a content by 5.7% and 6.7% in the first and second cropping seasons, respectively, in comparison to the without nano-Zn foliar spray (Table 5).

The interaction of PGPB inoculation and foliar nano-Zn spray for chlorophyll b was significant in the 2019–20 maize cropping season, whereas it was not significant in the 2020–1 maize cropping season (Table 5). Inoculation with *B. subtilis* in combination with foliar nano-Zn foliar spray was observed with highest chlorophyll b content, which was statistically similar to the inoculation of *A. brasilense* and foliar nano-Zn spray in the first maize cropping season (Figure 5B). The lowest chlorophyll b content was noted in the treatments without inoculation and foliar nano-Zn spray (Figure 5B). In addition, foliar nano-Zn spray did not influence leaf chlorophyll b content in the first season. Interestingly, foliar nano-Zn spray increased leaf

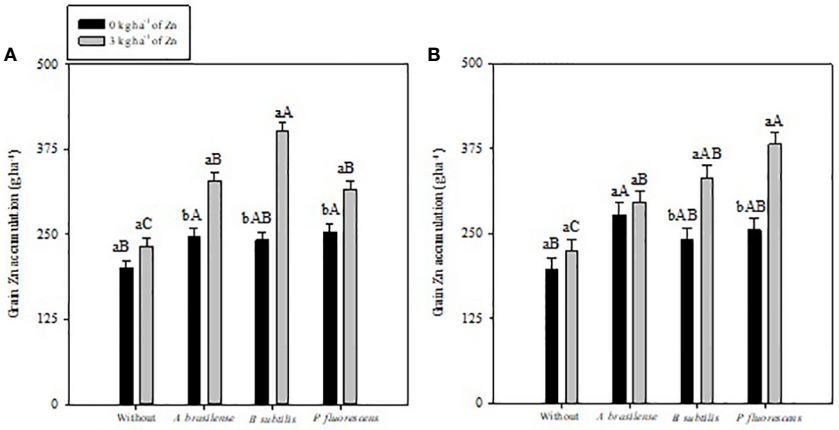

FIGURE 4
Maize grain zinc (Zn) accumulation in 2019–20 **(A)** and 2020–1 **(B)** as a function of plant growth-promoting bacteria with or without foliar zinc oxide (ZnO) application. Without = control (no inoculation). The uppercase letters compare interactions of inoculations within each dose of foliar nano-ZnO application and lowercase letters are used to compare interactions of foliar Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey's test ($p< 0.05$) for foliar ZnO doses and inoculations in the 2019–20 and 2020–1 cropping seasons. Error bars indicate standard error of the mean (n = 4 replications).

chlorophyll b content by 43% in the 2020–1 cropping season, compared with the control treatment (Table 5).

The interactions of PGPBs and foliar nano-Zn spray for total chlorophyll content were not significant in both cropping seasons studied (Table 5), although, leaf total chlorophyll content was positively influenced by the treatment effects. Seeds inoculation with *B. subtilis* increased total chlorophyll content by 15% and 16.8% in the 2019–20 and 2020–1 cropping seasons, respectively, which was statistically similar to the inoculation treatments with *P. fluorescens* and *A. brasilense*,

TABLE 5 Photosynthetic pigment of maize leaves as a function of plant growth-promoting bacteria inoculation, together with or without nano-zinc oxide spray, in the 2019–20 and 2020–1 cropping seasons.

	Chlorophyll a		Chlorophyll b		Total chlorophyll		Carotenoids	
	μg mL^{-1}							
Treatment	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1
Inoculation (I)								
Without	19.3	18.4	3.57	2.99 b	22.6 b	23.7 b	2.15 a	1.78 b
A. brasilense	20.4	19.7	4.82	4.47 a	24.8 ab	26.6 a	2.48 a	2.69 a
B. subtilis	20.1	21.6	4.97	4.8 a	26.0 a	27.7 a	2.61 a	3.25 a
P. fluorescens	19.9	20.3	5.10	3.98 ab	24.9 ab	26.2 a	2.68 a	2.85 a
Foliar zinc (ZnF) spray (kg ha^{-1})								
0	19.4 b	19.3	4.6	3.34 b	22.3 b	24.7 b	2.29 b	2.12 b
3	20.5 a	20.6	4.5	4.78 a	26.8 a	27.4 a	2.67 a	3.17 a
F-test								
I	2.3^{ns}	21.4**	8.4**	6.6**	3.8*	7.8**	1.7^{ns}	11.8**
ZnF	14.9*	21.4**	0.02^{ns}	22.3**	38.1**	19**	4.36*	33.6**
I x ZnF	0.3^{ns}	3.2*	3.9*	0.34^{ns}	0.31^{ns}	1.3^{ns}	0.47^{ns}	0.65^{ns}
CV (%)	4.1	4.1	14.7	21.2	8.5	6.6	20.9	19.4

Means in the column followed by different letters are statistically different by Tukey's test, $p \leq 0.05$. ** and * significant at $p< 0.01$ and $p< 0.05$, respectively, while ^{ns} is non-significant by F-test.

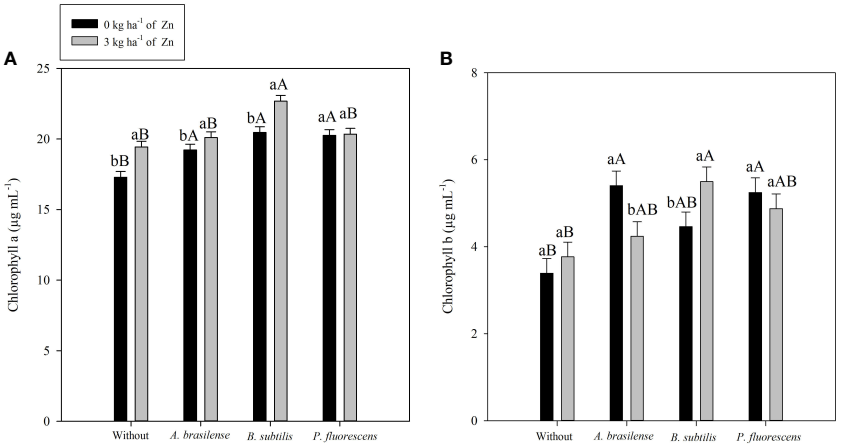

FIGURE 5
Concentrations of chlorophyll a in 2020–1 **(A)** and chlorophyll b in 2019–20 **(B)** of maize leaf as a function of plant growth-promoting bacteria with or without foliar zinc oxide (ZnO) application. Without = control (no inoculation). The uppercase letters compare interactions of inoculations within each dose of foliar nano-ZnO application and the lowercase letters are used to compare interactions of foliar zinc doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey's test ($p< 0.05$) for foliar ZnO doses and inoculations in the 2019–20 and 2020–1 cropping seasons. Error bars indicate standard error of the mean (n = 4 replications).

when compared with the without inoculation treatments (Table 5). In addition, foliar nano-Zn spray at the dose of 3 kg ha^{-1} increased total chlorophyll content of maize leaves by 20.2% and 10.9% in the first and second cropping seasons, respectively.

Leaf carotenoids content of maize was only significantly influenced by inoculation treatments in the 2020–1 cropping season, while foliar nano-Zn was observed to have a positive impact on carotenoid content in both cropping seasons (Table 5). Inoculation with *B. subtilis* increased leaf carotenoids content by 82.6%, which was statistically at per with other inoculations treatments during the second maize cropping season when compared with the without inoculation treatments (Table 5). In addition, the treatment with foliar nano-Zn spray increased leaf carotenoids content by 16.6% and 49.5% in the first and second cropping seasons, respectively, as compared with the control (Table 5).

3.4 Total soluble sugar, amino acids, and storage proteins

TSS content in maize leaves was significantly influenced by the inoculation treatments and the foliar nano-Zn spray in both cropping seasons (Table 6). Inoculation with *A. brasilense* increased TSS content in leaves by 33% and 40% in the 2019–20 and 2020–1 cropping seasons, respectively, when compared with the without inoculation treatments (Table 6). In addition, foliar nano-Zn spray increased TSS content by 35% and 56% in the first and second cropping seasons of maize, respectively, compared with the control. The interaction of inoculation and foliar nano-Zn spray was significant in the 2019–20 cropping season only (Table 6). The treatments with co-application of *A. brasilense* and foliar nano-Zn spray at a dose of 3 kg ha^{-1} were observed with the highest total soluble sugar content in maize leaves (Figure 6A). The treatments with foliar nano-Zn application and without PGPB inoculation were observed with the lowest TSS content in leaves of maize. However, treatments without foliar nano-Zn application and inoculation with *B. subtilis* were observed with higher TSS content, which was statistically at per with other inoculation treatments (Figure 6A).

The content of free amino acids in maize leaves was positively influenced by inoculation treatment and foliar nano-Zn spray in the 2019–20 and 2020–1 cropping seasons. The interaction was significant in only the second cropping season of maize (Table 6). Inoculation with *B. subtilis* increased amino acids content by 38.3% and 38.9% in the first and second cropping seasons, respectively, compared with the without inoculation treatment. Foliar nano-Zn spray also enhanced free amino acids content by 13.2% and 12.5% in the 2019–20 and 2020–1 maize cropping seasons, respectively, in comparison to the control. The interaction demonstrated that the treatments with foliar Zn spray at a dose of 3 kg ha^{-1} under inoculation of *B. subtilis* and *A. brasilense* increased the free amino acid content in maize leaves, compared with the without inoculation treatment (Figure 6B). Among PGPB inoculations, the treatments with *B. subtilis* were observed to have the higher amino acid content in the absence of foliar nano-Zn application. The lowest amino acid content was observed in the control treatments (Figure 6B).

Grain storage proteins of maize were significantly influenced by inoculation with PGPBs and nano-Zn foliar spray (Tables 6,

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TABLE 6 Total soluble sugar (TSS), free amino acids, and albumin concentration as a function of plant growth-promoting bacteria inoculation, together with or without nano-zinc oxide spray, in the 2019–20 and 2020–1 cropping seasons.

	TSS		Free amino acids		Albumin	
	mg g^{-1} DW					
Treatment	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1
Inoculation (I)						
Without	180	173 b	42.3 c	43.2	109 c	112
A. brasilense	240	242 a	51.2 b	54.3	119 bc	122
B. subtilis	235	224 ab	58.5 a	60.0	125 ab	122
P. fluorescens	210	204 ab	51.9 b	49.9	131 a	127
Foliar zinc (ZnF) spray (kg ha^{-1})						
0	184	165 b	47.8 b	48.8	116 b	117
3	249	257 a	54.1 a	54.9	126 a	124
F-test						
I	5.2*	4.15*	24.6**	25.5**	12.7**	12.8**
ZnF	29.1**	39.7**	22.6**	18.6**	12.8*	16.8**
I x ZnF	3.2*	1.8^{ns}	1.12^{ns}	3.12*	0.19^{ns}	3.1*
CV (%)	15.7	19.5	7.4	7.6	6.0	4.1

Means in the column followed by different letters are statistically different by Tukey's test, $p \leq 0.05$. ** and * significant at $p< 0.01$ and $p< 0.05$, respectively, while ^{ns} is non-significant by F-test.

7). Inoculation with *P. fluorescens* enhanced grain albumin concentration by 20.2% and 13.4% in the first and second cropping seasons, respectively, as compared with the without inoculation treatments (Table 6). Foliar nano-Zn spray also improved grains albumin concentration by 8.6% and 5.9% in the first and second cropping seasons, respectively, as compared with the control treatments. The interaction was significant in only the second cropping season, in which the highest grain albumin concentration was observed with the combined application of *P. fluorescens* inoculation and foliar nano-Zn spray, compared with the rest of the treatments (Figure 6C). All treatments with inoculation of PGPBs had improved grain albumin concentration regardless of the foliar nano-Zn application. The lowest albumin concentration was observed in the treatments without inoculation and nano-Zn application (Figure 6C).

The interactive effect of inoculation × foliar nano-Zn spray was significant for grain globulin concentration in both cropping seasons (Table 7, Figures 6D, E). The highest grain globulin concentration was observed with foliar nano-Zn fertilization under inoculation with *P. fluorescens*, which was statistically similar to the with inoculation of *B. subtilis* treatment in the 2019–20 (Figure 6D), and the with *B. subtilis* and *A. brasilense* in 2020–1 (Figure 6E) maize cropping seasons. The treatments with inoculation of PGPBs were observed to have higher globulin concentrations in the maize grains, even in the absence of foliar nano-Zn application, than the without inoculation treatments. The lowest globulin concentration in both studies was noted in the treatments without inoculation and nano-Zn application (Figures 6D, E).

The interaction of inoculations × foliar nano-Zn for glutelin concentration was not significant in both cropping seasons studied (Table 7). Grain glutelin concentration was improved by 15.3% and 8.8% with inoculation of *B. subtilis* in the first and second cropping seasons, respectively, which was statistically at per with other inoculation, when compared with the without inoculation treatments. Foliar nano-Zn spray improved grain glutelin concentration by 6.4% and 2.8% in the 2019–20 and 2020–1 cropping seasons in comparison to the control (Table 7).

Grain prolamin concentration of maize was not significantly influenced by inoculation and interaction of inoculation × nano-Zn spray in the 2019–20 cropping season, while the effect of only foliar nano-Zn spray was not significant in the 2020–1cropping season (Table 7). Foliar nano-Zn spray improved grain prolamin concentration of maize by 16.5% in the second cropping season. In the 2020–1 cropping season, inoculation with *B. subtilis* and *P. fluorescens* along with foliar nano-Zn spray were observed to have higher prolamin concentrations in the second cropping season (Figure 6F). In addition, inoculation with *A. brasilense* was observed with the highest grain prolamin concentration in the absence of foliar nano-Zn spray application. The treatments without inoculation were observed with the lowest grain prolamin concentration, regardless of the nano-Zn application (Figure 6F).

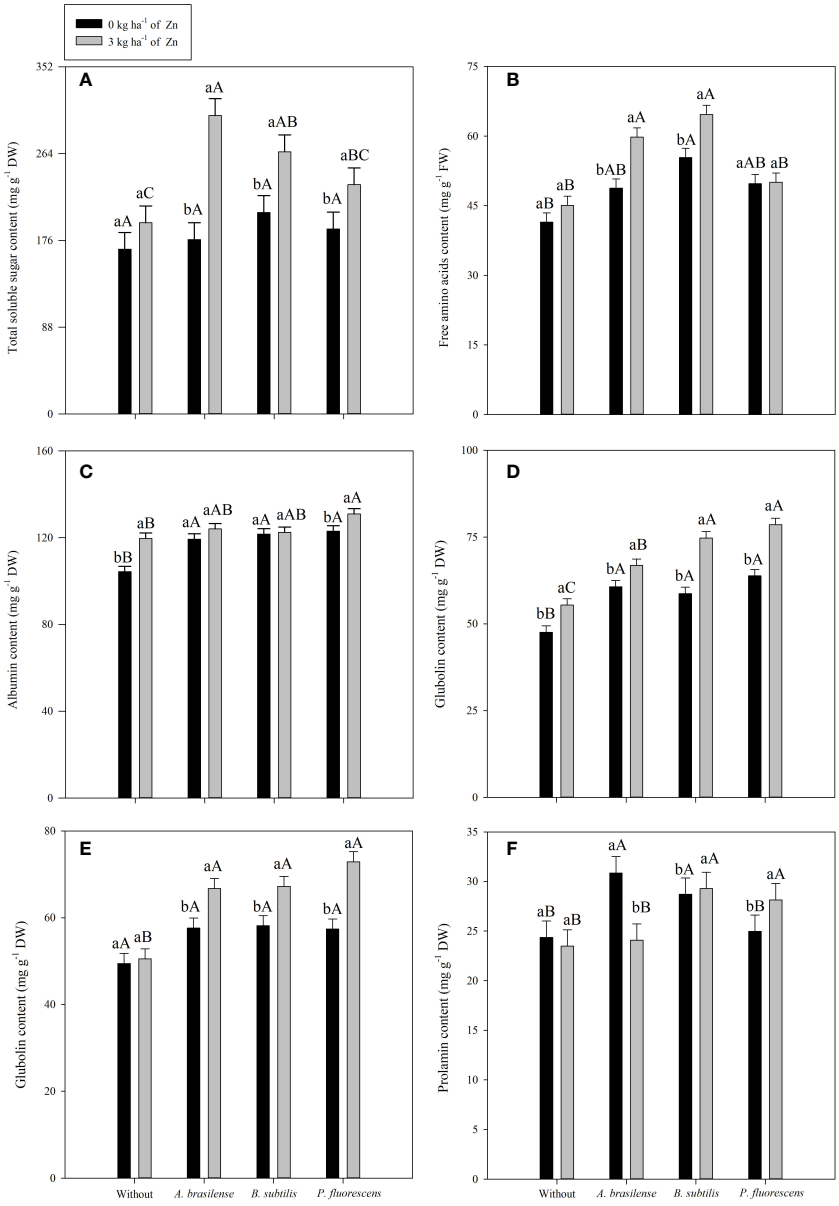

FIGURE 6
Concentrations of total soluble sugar in 2019–20 **(A)**, free amino acids in 2020–1 **(B)**, albumin in 2020–1 **(C)**, globulin in 2019 and 2020 (**D, E** respectively) and prolamin in 2020–1 **(F)** as a function of plant growth-promoting bacteria with or without foliar zinc oxide (ZnO) application. Without = control (no inoculation). The uppercase letters compare interactions of inoculations within each dose of foliar nano-ZnO application and the lowercase letters are used to compare interactions of foliar zinc doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not different from each other by Tukey's test ($p< 0.05$) for foliar ZnO doses and inoculations in the 2019–20 and 2020–1 cropping seasons. Error bars indicate standard error of the mean (n = 4 replications).

3.5 Pearson's correlation among evaluated attributes of maize

There were positive and significant correlations between Zn use efficiency and plant height, shoot dry matter, yield components, grain yield, grain Zn accumulation, chlorophyll a, amino acids, TSS, glutelin, and prolamin concentration of maize, regardless of the treatments applied in the 2019–20 cropping season (Figures 7A). A positive correlation was observed between Zn use efficiency and shoot and grain Zn accumulation, applied Zn recovery, shoot dry matter, grain yield, and photosynthetic and biochemical attributes of maize in the 2020–1 cropping season (Figure 7B).

TABLE 7 Globulin, glutelin and prolamin concentration of maize grains as a function of plant growth-promoting bacteria inoculation, together with or without nano-zinc oxide spray, in the 2019–20 and 2020–1 cropping seasons.

	Globulin		Glutelin		Prolamin	
	mg g^{-1} DW					
Treatment	2019–20	2020–1	2019–20	2020–1	2019–20	2020–1
Inoculation (I)						
Without	51	50	190 b	205 b	24.8 a	23.9
A. brasilense	64	62	215 a	222 a	26.5 a	27.5
B. subtilis	67	63	219 a	223 a	28.5 a	28.9
P. fluorescens	71	65	216 a	219 a	25.1 a	26.5
Foliar zinc (ZnF) spray (kg ha^{-1})						
0	58	56	203 b	214 b	24.2 b	26.2
3	69	64	216 a	220 a	28.2 a	27.2
F-test						
I	41.8**	17.3**	15.6**	14.2**	1.4^{ns}	3.3*
ZnF	73.4**	28.3**	16.2**	7.0*	8.5*	0.71^{ns}
I x ZnF	3.6*	3.27*	0.12^{ns}	0.52^{ns}	0.55^{ns}	3.3*
CV (%)	5.8	7.7	4.5	2.9	15	12.3

Means in the column followed by different letters are statistically different by Tukey's test, $p \leq 0.05$. ** and * significant at $p< 0.01$ and $p< 0.05$, respectively, while ^{ns} is non-significant by F-test.

In addition, there were positive and significant correlations between grain yield and Zn use efficiency, shoot dry matter, yield components, grain Zn accumulation, chlorophyll a, amino acids, total chlorophyll, and prolamin concentration of maize, regardless of the treatments applied in the 2019–20 crop season (Figures 7A). A positive correlation was observed between grain yield and all growth and yield components, as well as photosynthetic and biochemical attributes of maize in the 2020–1 crop season (Figure 7B).

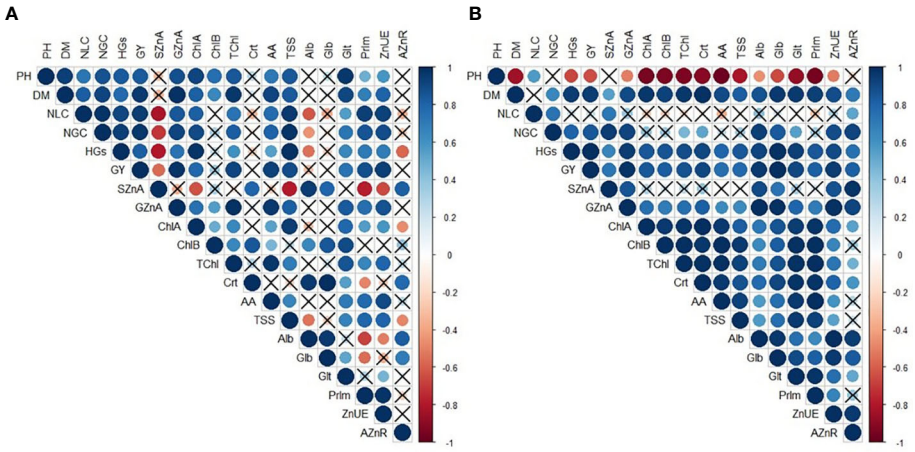

FIGURE 7
Heatmap color scale indicating Pearson's correlation among evaluated attributes of maize in response to plant growth-promoting bacteria and foliar nano-zinc oxide applications in the 2019–20 **(A)** and 2020–1 **(B)** cropping seasons. "X" indicates a non-significant relationship ($p \leq 0.05$). PH, plant height; DM, shoot dry matter; NLC, number of row per cob; NGC, number of grains per cob; HGs, 100-grains weight; GY, grain yield; SZnA, shoot Zn accumulation; GZnA, grain Zn accumulation; ChlA, chlorophyll a; ChlB, chlorophyll b; TChl , total chlorophyll; Crt, carotenoids; AA, free amino acids; TSS, total soluble sugar; Alb, albumin; Glb, globulin; Glt, glutelin; Prlm , prolamin; ZnUE, Zn use efficiency; and AZnR, applied Zn recovery.

3.6 Principal component analysis among evaluated attributes of maize

PCA was performed to investigate the changes in the yield, nutritional, and biochemical attributes of maize in the 2019–20 and 2020–1 cropping seasons (Figure 8). The eigenvalues of all eight principal components were greater than 1 and account for 100% of the data variation in both maize cropping seasons (Supplementary Tables 1, 2). The PC1 explained 79.6% and 70.6% of the data cumulative variation, while PC2 represented 88.9% and 82.7% in the 2019–20 and 2020–1 cropping seasons, respectively. The biplot graphs of PC1 and PC2 indicated that the group formed by inoculation with *A.brasilense, B. subtilis*, and *P. fluorescens* with foliar nano-Zn spray at a dose of 3 kg ha^{-1} obtained a positive correlation for all analyzed maize parameters in the first cropping season (Figures 8A, B). While analyzing the biplot graph of grouped PC1 and PC2 in the 2020–1 cropping season, all analyzed parameters showed a positive correlation with the group formed by inoculation with PGPBs, except plant height (Figures 8C, D).

4 Discussion

Plants' adaptation and responses to nutrients deficiency are being satisfied by ensuring minimal requirements and a carbon trade-off cost. Several strategies are being adapted to protect plants from the damages of harsh environmental conditions, especially in tropical rain-fed regions (Galindo et al., 2021). In this scenario, limited literature is available on the use of PGPBs in combination with foliar nano-ZnO on the growth and performance of maize. To address this incessant problem, the current study used Zn-improving PGPBs, such as *A. brasilense*, *B. subtilis*, and *P. fluorescens* in combination with a foliar nano-Zn application to assist biochemical attributes, primary metabolisms, and yield of maize crop in the tropical savannah.

The positive correlation between maize growth and yield, shoot–grain accumulation, photosynthetic pigments, and primary metabolism endorsed the hypothesis of the current study (Figure 7). Therefore, the current study is possible due to the synergetic effect of PGPBs with Zn enrichment and their role in different metabolic processes, nutrient use and acquisition, and synthesis of phyto-hormones (Mitter et al., 2017; Kudoyarova et al., 2019; Housh et al., 2021). In this context, the current study indicated that inoculation with *B. subtilis* and *P. fluorescence* along with foliar nano-Zn application produced taller plants (Figure 3A), greater shoot dry matter, higher number of rows, grains cobs^{-1}, heavier 100-grains weight (Tables 2, 3), and grain yield (Figures 3B, C) in two maize cropping seasons. It might be due to the role of PGPBs in nutrient solubilization and phytohormone production that stimulates nutrient availability and absorption through the

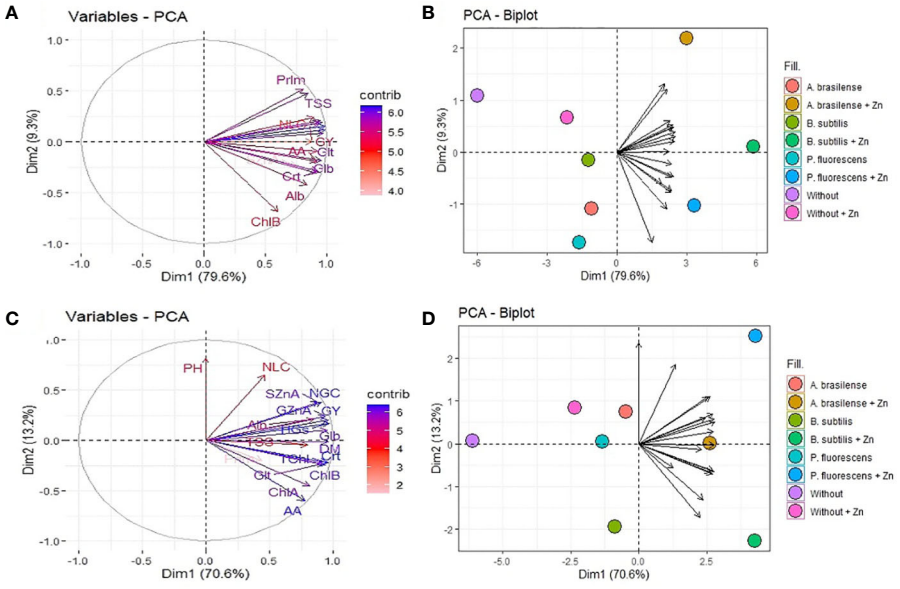

FIGURE 8
Loadings and biplot graphics of principal component analysis among the relationship of plant growth-promoting bacteria and foliar nano-zinc oxide applications for growth, nutritional, yield, and biochemical attributes of maize in the 2019–20 **(A, B)** and 2020–1 **(C, D)** cropping seasons. PH, plant height; DM, shoot dry matter; NLC, number of row per cob; NGC, number of grains per cob; HGs, 100-grains weight; GY, grain yield; SZnA, shoot Zn accumulation; GZnA, grain Zn accumulation; ChlA, chlorophyll a; ChlB, chlorophyll b; TChl, total chlorophyll; Crt, carotenoids; AA, free amino acids; TSS, total soluble sugar; Alb, albumin; Glb, globulin; Glt, glutelin; and Prlm, prolamin.

roots, as well the role Zn plays in cell multiplication and protein synthesis (Rossi et al., 2019; Swarnalakshmi et al., 2020). The individual or combined use of PGPBs with Zn could modulate phosphatase and invertase activities in soil as well as proline activities in plants (Tanveer et al., 2022), thus contributing to higher levels of plant growth and yield as an end product (Jalal et al., 2022a; Jalal et al., 2022b). It has also reported that inoculation with PGPBs promotes maize growth by regulating phytohormones and growth regulators, which could improve nutrient solubilization and nutrient uptake for better plant growth and production (Oleńska et al., 2020; Ribeiro et al., 2022). It has been reported that PGPBs are associated with greater root–shoot biomass and dry matter, which can lead to the promotion of vegetative growth at an early stage and a greater productivity at maturity (Sousa et al., 2021; Jalal et al., 2022b). In addition, foliar spray of nano-fertilizer improves growth and yield of maize by enhancing plant biochemical processes and resistance against ROS (Babaei et al., 2017). Zn regulates different biochemical attributes of plants through cell elongation and multiplication (as a result of auxin synthesis), thus leading to a greater biomass and productivity of cereal crops (Doolette et al., 2020; Jalal et al., 2022c). Despite of all this, the non-significant effect of foliar nano-Zn application for plant height and number of row cobs^{-1} in the first and second maize cropping season (Table 2) may be because plant nutrition with Zn was adequate, while these parameters are more influenced by genetic factors and tropical climatic conditions (Figure 2). In addition, low foliar Zn supply is another factor that can cause physiological and leaf anatomical alterations that can, consequently, affect nutrient penetration and accumulation, depending on the deficiency of target nutrient (Brian, 2008).

Results of the current study indicated that inoculation with PGPBs, such as *B. subtilis* and *P. fluorescens*, in combination with foliar nano-Zn application improved Zn accumulation in shoot tissue (Table 4) and grains (Figures 4A, B), as well as increased ZnUE and AZnR in maize crop cultivation (Table 4). It may be due to the positive interception of PGPBs in scavenging roots to produce growth-promoting hormones, and the increased water and nutrients uptake to shoot and grain tissues (Mumtaz et al., 2017). Previous studies indicated that applied inoculants interact with already existing microbes in the root rhizosphere, modifying root architecture, reducing phytic acid assimilation, and stimulating nutrient transportation to shoot and edible tissues (Singh and Prasanna, 2020). It has also reported that foliar Zn application could enhance translocation of Zn to shoot and edible tissues (Jalal et al., 2020; Jalal et al., 2022c) by developing coordination with amino acid (cysteine) and protein synthesis (Gupta et al., 2016). Foliar Zn application is an effective strategy to overcome the edaphic deficiency by improving bioavailability in edible tissue, leading to better biofortification (Mishra, 2022). In this context, the results of the present study are a progressive step to understanding the integrated use of PGPBs and foliar ZnO application for greater growth, yield, and biofortification of maize grains with higher Zn use efficiency (Table 4). It has been reported in another study that plant growth-promoting microbes are being identified as natural biofortifiers, synthesizing organic acids, acting as chelating agents, and producing siderophores that ultimately result in biofortification and higher crop yield in a sustainable manner (Ramesh et al., 2014; Upadhayay et al., 2022). Maize is one of the most important cereal crops for food and nutrition security, with high phytic acid and low Zn concentrations that may be the origin cause of malnutrition, especially in the regions under Zn deficiency like the tropical savannah of Brazil (Cakmak et al., 2010; Fageria et al., 2011). In this context, the present study exhibited that inoculation with *B. subtilis* and *P. fluorescens* along with foliar nano-Zn improved grain Zn accumulation (Figure 4) and Zn use efficiency in maize cultivation (Table 4). It has been reported that the inoculation with PGPBs in combination with foliar or soil Zn application contributes to the reduction of phytic acid, which consequently increases Zn concentration in the embryo, aleurone, endosperm, and whole grains of cereal crops (Rehman et al., 2018; Jalal et al., 2022b). In addition, foliar Zn spray considerably mobilized in the phloem compared with conventional soil Zn fertilization treatment, and the crop can deal with malnutrition because of its rapid remobilization and localization into the grains (Firdous et al., 2020; Rehman et al., 2021; Jalal et al., 2022c).

In the present study, the considerable increase in maize growth and Zn nutrition is due to the improvement of photosynthetic pigments (chlorophyll a and b, total chlorophyll, and carotenoids content) under inoculation with PGPBs and foliar nano-Zn application (Table 5). This increase in photosynthetic pigments might be due to the role of PGPBs in stabilizing the biochemical and physiological functions of plants, which can be attributable to stomatal conductance, transpiration, and intercellular gas exchange processes to increase photosynthetic rate of the plants (Pereira et al., 2020). The present results indicated that chlorophyll a, b, and total, and carotenoids concentrations were improved with the inoculation of *B. subtilis* and foliar Zn spray in both maize-cultivated seasons (Table 5, Figures 5A, B). It might be due to the critical role of PGPBs and Zn in the production of phytohormones, nitrogen fixation, and improving photosystem II efficiency (Seyed Sharifi et al., 2020). It has been reported that combined application of PGPBs and ZnO revealed itself as a promising technique to increase chlorophyll concentrations and performance of wheat (Azmat et al., 2022). The application of nano-Zn with PGPBs, including *Bacillus* and *Pseudomonas* sp., regulate defensive enzymes and intercellular homeostasis of plants to create optimal cellular conditions, which may lead to higher concentrations of photosynthetic pigments (Yasmin et al., 2020a). Batool et al. (2020) reported that inoculation with *B. subtilis* is an effective strategy that regulates chlorophyll a and b, and carotenoid content, as well as other biochemical process,

thus leading to sustainable growth and production of the plants under harsh environmental conditions. In addition, the non-significant effect of inoculation with PGPBs on leaf concentrations of chlorophyll a and carotenoids (Table 5) might be because the plant nutrition with Zn was adequate and tropical climatic conditions (Figure 2). Despite this, inoculation of PGPBs attribute to competition of already existing microbial consortium in rhizophere, which can ultimately affect nutrient transportation and growth performance of maize (Tang et al., 2020).

There was a remarkable increase in the concentration of TSS, free amino acids, and grain storage proteins (albumin, globulin, glutalin, and prolamin) of maize when treated with PGPBs and foliar nano-Zn spray (Tables 6, 7). It may be possible because of the role of foliar nano-Zn in upregulation of antioxidant systems and primary metabolites of the plant that contribute to enzyme activation and proteins synthesis (Ghani et al., 2022). Previous studies claimed that PGPBs regulate the production of photo-assimilates, interlinking the outcomes of foliar nano-Zn application with other physiological and biochemical functions that could ultimately improve primary metabolites in the leaves and storage proteins in grains of different crops (Batool et al., 2020; Azmat et al., 2022). The present results exhibited that combined application of PGPBs and foliar nano-Zn improved concentration of TSS (Figure 6A), free amino acids (Figure 6B), albumin (Figure 6C), globulin (Figures 6D, E), and prolamin (Figure 6F) in maize leaves and grains. The reason might be due to the rapid absorption and transportation of foliar nano-Zn with the involvement of several factors (i.e., thickness, density, and chemical composition of cuticle, trichomes, and stomata conductance), which are responsible for the operation of the entire plant machinery and, thus, improving metabolic and biochemical processes of the plants (Yumei et al., 2014; Xie et al., 2020). Zn fertilization increases grain reserve proteins because of its involvement in nitrate reductase activities and nitrogen assimilation pathways (Liu et al., 2015; Silva et al., 2021). Zn fertilization has also reported that co-application of Zn and PGPBs could modulate plant defensive system by improving photosynthetic pigments and primary metabolites, leading to better plant performance and yield (Tanveer et al., 2022). PGPBs induce multiple physiological functions by absorbing available nutrients through roots that may stimulate plant nutrition and primary metabolism in a sustainable manner (Yahaghi et al., 2019). The co-application of PGPBs and Zn improved TSS, amino acids, and protein content, leading to better performance and biofortification of maize (Upadhyay et al., 2021). Hence, inoculation with PGPBs and foliar nano-Zn application improved performance, primary metabolism, and the yield of maize. This strategy also proved to be a sustainable management practice for higher productivity and Zn biofortification of maize in tropical savannah conditions.

5 Conclusions

To satisfy the food and nutritious demands of an exponentially growing human population, use of PGPBs is one of the most sustainable and ecofriendly strategies that can increase nutrition, performance, productivity, and nutrient assimilation into the edible tissues of maize crop. In addition, foliar nano-Zn spray also proved to be a feasible and environmentally safe technique for improving Zn accumulation, growth, and biochemical attributes of maize. Therefore, it was verified from the current field findings that co-application of *B. subtilis* and nano-Zn at a dose of 3 kg Zn ha^{-1}, applied in two splits, increased plant height, shoot dry matter, yield components, and yield of maize in tropical savannah. Zn accumulation in shoot and grains, as well as Zn use efficiency and applied Zn recovery, were also improved with inoculation of *B. subtilis* and *P. fluorescens*, along with foliar nano-Zn application. Chlorophyll a, b and total, carotenoids, TSS, free amino acids in the leaves, and storage proteins (albumin, globulin, glutelin, and prolamin) in grains of maize were improved with inoculation of *B. subtilis* in combination with foliar nano-Zn application. Therefore, seed inoculation with *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn application is considered to be a highly effective and low-cost alternative strategy to improve Zn acquisition and Zn use efficiency, biochemical and primary metabolism, with higher productivity of maize in tropical savannahs. The present study gives an insight on the interaction of PGPBs and foliar nano-Zn application about various morphological and biochemical aspects of maize. Using this information, prospective research should aim to know the molecular and laboratory mechanisms (translocation, localization, loading, transporter proteins, etc.) behind the higher accumulation and improved biochemical and physiological attributes of maize to better understand the responses of PGPBs in different edaphoclimatic conditions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization, AJ and MT; methodology, AJ and CO; software, CO, GF, and FG; validation, AJ, FG; formal analysis, GF and AJ; investigation, AJ, GF and IG; resources; data curation, AJ, CO, AB, and PC; writing—original draft preparation, AJ; writing—review and editing, MT, EF, and FG;

visualization, BL, PC, and IG; supervision, MT; project administration, AJ and MT; and funding acquisition, AJ and MT. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1046642/full#supplementary-material

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The differential modulation of secondary metabolism induced by a protein hydrolysate and a seaweed extract in tomato plants under salinity

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Climate change and abiotic stress challenges in crops are threatening world food production. Among others, salinity affects the agricultural sector by significantly impacting yield losses. Plant biostimulants have received increasing attention in the agricultural industry due to their ability to improve health and resilience in crops. The main driving force of these products lies in their ability to modulate plant metabolic processes involved in the stress response. This study's purpose was to investigate the effect of two biostimulant products, including a protein hydrolysate (Clever HX®) and a seaweed extract with high amino acids content (Ascovip®), and their combination, on the metabolomics profile of tomato crops grown under salt stress (150 mM NaCl). Several stress indicators (leaf relative water content, membrane stability index, and photosynthesis activity) and leaf mineral composition after salinity stress exposure were assessed to evaluate stress mitigation, together with growth parameters (shoot and root biomasses). After that, an untargeted metabolomics approach was used to investigate the mechanism of action of the biostimulants and their link with the increased resilience to stress. The application of the biostimulants used reduced the detrimental effect of salinity. In saline conditions, protein hydrolysate improved shoot dry weight while seaweed extracts improved root dry weight. Regarding stress indicators, the application of the protein hydrolysate was found to alleviate the membrane damage caused by salinity stress compared to untreated plants. Surprisingly, photosynthetic activity significantly improved after treatment with seaweed extracts, suggesting a close correlation between root development, root water assimilation capacity and photosynthetic activity. Considering the metabolic reprogramming after plant biostimulants application, protein hydrolysates and their combination with seaweed extracts reported a distinctive metabolic profile modulation, mainly in secondary metabolite, lipids and fatty acids, and phytohormones biosynthetic pathways. However, treatment with seaweed extract reported a similar metabolic reprogramming trend compared to salinity stress. Our findings indicate a different mechanism of action modulated by protein hydrolysate and seaweed extract, suggesting stronger activity as a stress mitigator of protein hydrolysate in tomato crops under salinity stress.

KEYWORDS

biostimulants, metabolomics, plant stress, secondary metabolism, phytohormones

1 Introduction

Among the crop abiotic stresses that threaten world food security, soil salinity is considered a major challenge that affects the global agriculture sector, causing significant losses in production each year. Nowadays, about 20% of irrigated lands (1500 million hectares) are damaged by high salt content, and this percentage is estimated to grow to 50% (3750 million hectares) by the end of 2050 (Jamil et al., 2011; Chung et al., 2020; Kumar et al., 2020). The main reason for the increasing soil salinity accumulation lies in using saline water for irrigation, poor water management, high evaporation, and previous exposure to seawater. Soil salinity implies the presence of any salt at higher levels, including sodium ions (Na^+), chloride (Cl^-), sulfurates (SO_4^{2-}), nitrates (NO_3^-), borates (BO_3^{3-}), carbonates (CO_3^{2-}), bicarbonates (HCO_3^-), calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+), and ion (Fe^{2+}), which can exert adverse effects on plants (Bui, 2017). Indeed, salinity is well documented to affect the agronomical, physiological, and biochemical processes of plants (EL Sabagh et al., 2020; Khan et al., 2021). At the agronomical level, salinity stress causes a loss of both shoot and root biomass development and a reduction in crop productivity (EL Sabagh et al., 2020). While at the physiological level, it is able to cause ionic imbalances and ion toxicity through competition with several essential minerals such as K^+, Mg^{2+}, or Ca^{2+} ions, thus leading to chlorosis and necrosis of leaves (Almeida et al., 2017). Finally, salinity stress also induces biochemical changes in plants, including phytohormones modulation, ion uptake alteration, antioxidant enzyme activation, reactive oxygen species (ROS) accumulation, and photosynthetic pathway disruption and consequently compromising chlorophyll and carotenoids content and photosystem II (PSII) activity (Yoon et al., 2009; Chung et al., 2020).

Different strategies have been adopted to cope with this important issue, including traditional breeding, genetic engineering, and chemical fertilizer applications, which are not always feasible and sustainable for the ecosystem (Kumar et al., 2020). Thus, there is an urgent need to adopt and develop new innovative and eco-friendly strategies to manage this challenge in the agricultural systems. In the last decades, the use of plant biostimulants gained enormous importance as plant growth promoting products and plant defence elicitors, and is the most promising solution to cope with salinity stress (Rouphael et al., 2020). The latest European regulation on fertilizers, which includes biostimulants (REGULATION (EU) 2019/1009 OF, 2019), gave the latest definition of biostimulants: "Biostimulants are organic or inorganic products containing bioactive substances and/or microorganisms, which, when applied to the plant or rhizosphere, stimulate the growth and productivity of the plant by improving the absorption and assimilation efficiency of nutrients, tolerance to abiotic stresses and/or quality of the product regardless of their nutrient content".

Based on the definition above, there are two types of biostimulants: microbial biostimulants and non-microbial biostimulants. Focusing on non-microbial biostimulants, which are usually distinguished based on the starting material used to produce them, the most important ones are protein hydrolysate (PH) and seaweed extracts (SW). PHs are obtained by biological matrices after an optimised hydrolysis process that includes either chemical or enzymatic hydrolysis. PHs are mainly characterised by a mixture of amino acids, peptides, and proteins (Colla et al., 2015). Whereas SWs are produced by macroscopic marine algae belonging to different taxonomic groups, such as brown, red, and green algae (Khan et al., 2009). SWs are rich sources of nutrients, bioactive compounds, phytohormones, minerals, and organic matters, as well as characterised by complex polysaccharides such as laminarin, fucoidan, and alginates (Abraham et al., 2019; Flórez-Fernández et al., 2019). In the case of plants affected by salinity stress, the biostimulants were usually employed to overcome the osmotic and ionic stress by modulating both primary and secondary metabolism (Rouphael et al., 2020), and improving crops' growth, productivity, and tolerance to abiotic stresses (Rouphael et al., 2017; Paul et al., 2019a).

Despite the positive effect of biostimulants application in coping with various abiotic stresses, the crucial point highlighted by the latest European legislation on fertilizers includes the presence of scientific evidence to support the efficacy of a product registered as a biostimulant, including detailed clarification of their mechanism of action (REGULATION (EU) 2019/1009 OF, 2019). In recent years, the omics sciences (genomics, transcriptomics, proteomics and metabolomics) have gained a lot of notoriety in addressing this issue (Sahoo et al., 2020). Among these, metabolomics has contributed the most to understanding the mechanisms of action of plant biostimulants against abiotic stress (Paul et al., 2019a; Paul et al., 2019b; Nephali et al., 2020). Specifically, metabolomics is a qualitative and quantitative study of all metabolites involved in metabolic processes responsible for the maintenance of the normal function of an organism. In particular, the untargeted metabolomics approach has shown to have a high potential for unravelling the biochemical processes of plants affected by stresses and those regulated after biostimulant application (Burgess et al., 2014; Schrimpe-Rutledge et al., 2016; Rouphael et al., 2020).

This work aims to investigate and compare the potential salt stress mitigation effect of two biostimulant products such as PH (Clever HX®), SW (Ascovip®), and their combination PH + SW, applied by foliar spray on tomato plant (*Solanum lycopersicum* L.). For this purpose, agronomical, physiological, and biochemical parameters were assessed after salinity stress exposure and compared with the corresponding treatment with biostimulants' application. To this aim, an untargeted metabolomics analysis was used to comprehensively investigate the mechanism of action of the biostimulants and to unravel the processes underlying their stress tolerance mitigation. Considering that plant biostimulants can provide an extensive modulation of crop metabolism (Colla et al., 2015), and given the limited knowledge on the products used in this study, metabolomics has been chosen because of its hypothesis-free untargeted nature to better achieve the goal of the study.

2 Materials and methods

2.1 Plant material and growth conditions

Tomato plants (*Solanum lycopersicum* L., cv. Heinz 3402) were provided by a local nursery (Azienda F.lli Zermani, Piacenza, Italy)

and transplanted in single squared pots (12 cm x 12cm x 14 cm) at the four true leaves stage. After that, plants were grown for 18 days, starting from 21st of June 2021 to 8th of July 2021, under natural open field conditions at the experimental station of Università Cattolica del Sacro Cuore (Piacenza, Emilia-Romagna, Italy). The soil used for the experiment was a commercial Radicom universal potting soil contains a mix of fine white and brown peat (65%), green compost, Ecofibra®, and coir fibre (Vigorplant Italia srl, Fombio, LO, Italy). The specific characteristic of soil includes pH = 7.5, 0.4 ds/m electrical conductance, 180 kg/m^3 density, and 87% *v/v* total porosity.

2.2 Biostimulants

Two commercial biostimulants were supplied and produced by Clever Bioscience srl (Casanova Lonati PV, Italy). Clever HX® product is a protein hydrolysate characterized by 20% of peptides and 31% of free amino acids. Moreover, it contains 3.2% of organic nitrogen, 3.2% of potassium oxide, and 10% of organic carbon. The aminogram of the product (as %) is as follows: Ala (5.26), Arg (1.52), Asp (3.80), Cys (0.04), Glu (11.29), Gly (2.98), His (0.96), Ile + Leu (2.27), Lys (2.14), Met (0.9), Phe (1.42), Pro (4.23), Ser (2.19), Thr (2.24), Trp (0.34), Tyr (1.71), and Val (2.29). Ascovip® is a seaweed extract characterized by 15% free amino acids, 10% low molecular weight peptones, 10% organic carbon, 2% organic nitrogen, and 1% of betaines.

2.3 Experimental design

Tomato plants were randomly distributed into five experimental groups with eight biological replicates per group, amounting to 40 pots. The experimental groups were defined as follows: a control non-stressed (C), 150 mM of salinity stress (S), 150 mM salinity stress treated with Clever HX® 10% (*v/v*; PH), Ascovip® 10% (*v/v*; SW), and their combination 5% + 5% (*v/v*; PH + SW). The biostimulant treatments were performed three times: at 3 days after transplanting (DAT) referred to as Treatment 1 (T1), at 7 DAT referred to as Treatment 2 (T2), and at 14 DAT referred to as Treatment 3 (T3).

The 150 mM sodium chloride solution (Merck KGaA, Darmstadt, Germany), prepared using demineralized water, was applied by irrigation every day to induce salinity stress. The control pots were irrigated using tap water at the same volume (pH= 7.2, conductibility 572 µS/cm). Considering the biostimulants, 2 mL of each product, properly diluted in demineralized water to the concentration indicated above, was applied by foliar spray to each plant. At the end of the experiment, three out of eight replicates were destinated to morphological analysis, while the remaining five plants have been allocated for leaf membrane stability index (MSI), relative water content (RWC), metabolomics analysis, and minerals quantification analysis. Specifically, shoot and root of the three plants destinated for morphological measurements were collected to evaluate plant biomass. Shoot fresh weight (FW) was recorded immediately after sampling, while root samples were recorded after being washed under tap water and dried with paper. Shoots and roots were then dried for 48 hours at 80°C to measure dry weight (DW). Concerning the remaining five plants, one leaf of each plant per treatment was collected for RWC, while two leaves were collected for MSI. The remaining leaves were deep frozen in liquid nitrogen and immediately stored for metabolomics analysis and minerals quantification. For minerals quantification, leaves sample were dried for 48 hours at 80°C.

2.4 Leaf relative water content, membranes stability index, chlorophyll content, and photosynthetic activity

For the determination of RWC, one leaf of each of the five replicates was collected per treatment. The leaves were weighted (FW) and then incubated in 15 ml of ultrapure water for 24h at 4° C. After incubation, leaves were blotted and weighted to get the turgid weight (TW). Finally, leaf samples were oven-dried and newly weighted (DW). The RWC was finally determined using formula below indicated

$$\mathbf{RWC} = \frac{\mathbf{FW} - \mathbf{DW}}{\mathbf{TW} - \mathbf{DW}} \times 100$$

Leaf membrane stability index (MSI) was measured using two leaves from each of the five replicates for treatment. Tomato leaves were transferred in tubes with 5 ml of ultrapure water. They were heated at 30°C for 30 min and electrical conductivity was measured (C1). Samples were then heated in a bath at 100°C for 30 minutes, then cooled on ice, and the electric conductivity was measured again (C2). The MSI index was calculated using formula below indicated.

$$\mathbf{MSI} = \left(1 - \frac{\mathbf{C1}}{\mathbf{C2}}\right) \times 100$$

The effective photochemical quantum yield of photosystem II (Phi2), ratio of incoming light that is lost *via* non-regulated processes (PhiNO), non-photochemical quenching (PhiNPQ), and chlorophyll content were measured at 18 DAT by using a PhotosynQ instrument (PHOTOSYNQ INC., East Lansing, MI, USA).

2.5 Mineral and organic acid determination

The dried tomato leaves were extracted in ultrapure water in a 1:5 ratio using a shaking water bath at 80°C for 10 min. The samples were analysed after centrifugation at 10,000 *x g* for 10 min and filtration through a 0.20 µm cellulose cartridge. The quantification of anions (NO_3^-, PO_4^{3-}, SO_4^{2-}, and Cl^-) and cations (K^+, Ca^{2+}, Mg^{2+}, and Na^+), as well as organic acids (malate, oxalate, citrate, and isocitrate), were determined by using ion chromatography coupled to an electrical conductivity detector (ICS3000, Thermo Scientific™ Dionex™, Sunnyvale, CA, USA) as previously reported (Formisano et al., 2021). Specifically, cations were separated by isocratic chromatography through an IonPac® CS12A column (4 × 250 mm, Thermo Scientific™ Dionex™) with 25 mM methanesulfonic acid (Sigma Aldrich, Milan, Italy). Anions and organic acids were separated by a 5 mM–30 mM potassium hydroxide (KOH) gradient using an IonPac® AG11-HC IC column (4 × 50 mm; Thermo Scientific™ Dionex™) with 5 mM – 30 mM potassium hydroxide (KOH), setting the flow at 1.5 mL min^{-1}.

2.6 Metabolomics analysis

For each replicate per experimental group, leaf samples were ground in liquid nitrogen with mortar and pestle. Then an aliquot (1 g) was extracted in a methanolic solution (80% methanol + 0.1% formic acid) by homogenization process (Polytron PT 1200 E, Kinematica AG, Switzerland). The homogenised extracts were centrifuged at 5000 × g for 15 minutes and filtered (0.22 µm membrane) into vials ready to be analysed. The untargeted metabolomics analysis was carried out using ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS; Agilent Technologies, Santa Clara, CA, USA) as previously described (Pretali et al., 2016). The separation was performed by using an Agilent Poroshell 120 pentafluorophenyl (PFP) column (2.1 × 100 mm, 1.9 µm) and a binary mixture of water and acetonitrile acidified with 0.1% (*v/v*) formic acid as mobile phase (LC-MS grade, VWR, Milan, Italy). A linear gradient (5 to 95% of organic phase) and a 33 min run time were used for separation. The mass spectrometer worked in SCAN mode with a range of 100 to 1200 m/z, positive polarity and extended dynamic range mode, with a nominal mass resolution of 30,000 FWHM. The raw mass features acquired by the instrument were aligned and filtered, then annotated according to the 'find-by-formula' algorithm against the PlantCyc database (Hawkins et al., 2021) using the Agilent Profinder B.10.0 software (Agilent Technologies, Santa Clara, CA, USA). To this aim, only the features being putatively annotated within 75% of replications within at least one experimental group were retained, as previously described (Pretali et al., 2016). The annotation follows a LEVEL 2 of identification, referring to COSMOS standards in metabolomics (Salek et al., 2015).

2.7 Statistical analysis

The significance of the impact of the treatments on morphological and physiological parameters was investigated with a one-way analysis of the variance (ANOVA), followed by Tukey's HSD (honestly significant difference) (p-value< 0.05) by using the software PASW Statistics 26.0 (SPSS Inc., Chicago, IL, USA). The chemometrics analysis was carried out using Agilent Mass Profiler Professional B.15.1 (Agilent Technologies, Santa Clara, CA, USA) as previously described (Lucini et al., 2019). Differential compounds were identified by Volcano Plot analysis: the statistically significant compounds (p-value< 0.05, Bonferroni multiple testing correction) having a Fold Change value ≥ 2.5 were selected and used for pathway analysis using Omic Viewer Pathway Tool of PlantCyc (Caspi et al., 2013) (Stanford, CA, USA), to identify the biochemical processes most affected by treatments.

3 Results

3.1 Foliar biostimulants mitigate the negative effect of salinity stress in tomato plants at agronomical and physiological levels

The agronomical and physiological results of tomato plants treated with protein hydrolysate (PH), seaweed extract (SW), and their combination PH + SW, were assessed to investigate their potential salinity stress mitigation (Figures 1, 2).

For this purpose, at the end of the experimental period (18 DAT), different growing parameters were measured, including shoot and root fresh weight (FW; Figure 1A) and dry weight (Figure 1B), and shoot/root ratio (Figure 1C), together with representative images of the morphological differences (Figure 1D). Specifically, the FW and DW measurements pointed out, on average, a 57% biomass reduction (p< 0.05) under salinity stress compared to the control. The application of biostimulants results in increased stress tolerance and reduced biomass loss. In particular, the shoot biomass tended to increase in response to PH biostimulant application, while the root biomass for SW employment. This tendency was statistically significant (p< 0.05) compared to salinity-stressed plants. The combined application of PH and SW was found to be the worst in stress-mitigation potential at the morphological level, reporting no significant differences compared to salinity-stressed plants. Shoot/root ratio parameters highlighted the different mechanism of action driven by the two biostimulants. Indeed, PH application reported the highest and most significant values of shoot/root ratio compared to SW, indicating a preferentially shoot development driven by PH.

The photosynthetic performance was measured in terms of quantum yield of the photosystem II (Phi2), ratio of incoming light that is lost *via* non-regulated processes (PhiNO) and as non-photochemical quenching (PhiNPQ) (Figure 2A). In detail, salinity stress reduced the Phi2 and increased the PhiNPQ compared to the control. Interestingly, the decrease of photosystem II performance caused by salinity stress was reverted to the control condition after either PH or SW treatments. At the same time, the PH + SW application reported a distinctive behaviour, characterised by the lower performance of Phi2 and higher PhiNPQ values. Furthermore, PH and SW positively modulated the relative chlorophyll content in tomato leaves under salinity stress (Figure 2B).

Overall, salinity stress significantly increased the leaf relative water content (RWC; Figure 2C) and decreased membrane stability index (MSI; Figure 2D). However, the treatment with PH resulted in the only one capable of recovering MSI comparable to control plants.

3.2 Biostimulants differentially modulated tomato leaf mineral composition and organic acids accumulation

To gain insight into the mineral composition of leaves samples after biostimulant treatments, the main macronutrients, i.e., NO_3^-, PO_4^{3-}, K+, SO_4^{2-}, Ca^{2+}, Mg^{2+}, Na^+, and Cl^- have been quantified and reported in Table 1. Salinity stress modulated mineral accumulation in tomato leaves. In particular, NO_3^-, K^+, , and Ca^{2+} minerals were decreased under salinity stress, even though only $SO_4^{2-}$was statistically significant compared to control. Accordingly, tomato plants grown in the presence of salinity stress (150mM NaCl) showed an intensive and significant accumulation of Na^+ (7.27 g/kg DW) and Cl^- (85.59 g/kg DW) ions, compared to the control samples as 1.35 g/kg DW and 12.14 g/kg DW, respectively.

Plants treated with biostimulants reported a clear modulation of mineral compositions. Indeed, both foliar sprayed PH and SW generated an accumulation of K^+, SO_4^{2-} and Ca^{2+} at a level equal to

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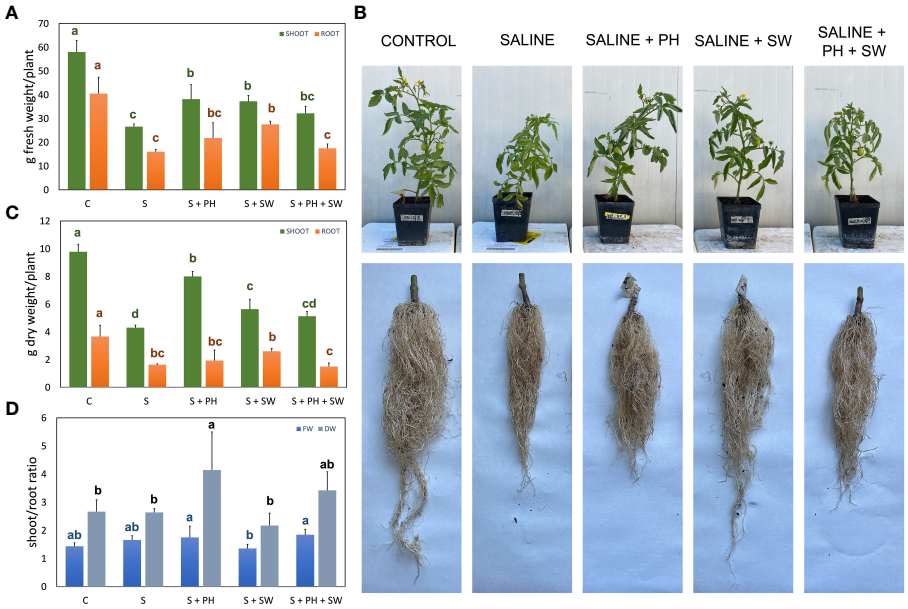

FIGURE 1
Morphological measurements of tomato shoot and root organ parts, affected by salinity stress (S) and treated with biostimulants i.e., protein hydrolysate (PH), seaweed extracts (SW), and their combination PH + SW, compared to the control non-stressed plants **(C)**. In the panels they show **(A)** fresh weight, **(B)** dry weight, and **(C)** the shoot/root ratio of fresh and dry weights for both tomato shoot and roots. In **(D)** shows the image of the aerial parts and roots after cleaning. Error bars indicate standard errors (n = 3). Different letters indicate significant differences according to Tukey's multiple-range test (p = 0.05).

or above compared to salinity stressed plants. The combined application of PH and SW produced a diverse mineral accumulation profile, suggesting a different stress response mechanism. Particularly, the PH + SW application generated a clear and significant accumulation of K^+. Moreover, a strong accumulation of Na^+ and Cl^- was observed after PH + SW treatment. Interestingly, the application of biostimulants, both alone and in combination, produced a relevant accumulation of Na^+, increasing by 2.44, 1.77, and 3.43 folds compared to that found in salinity stress only. This effect was not found in the case of Cl^- ion,

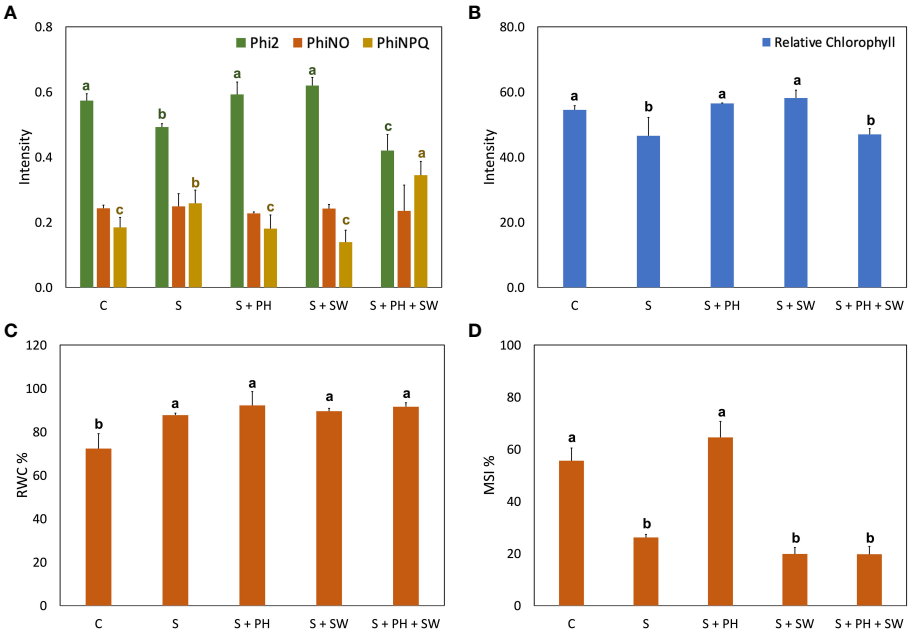

FIGURE 2
Physiological analysis of tomato leaves affected by salinity stress (S) and treated with biostimulants i.e., protein hydrolysate (PH), seaweed extracts (SW), and their combination PH + SW, compared to the control non-stressed plants **(C)**. In the panel shows **(A)** photosynthetic performance expressed as quantum yield of the photosystem II (Phi2), ratio of incoming light that is lost *via* non-regulated processes (PhiNO) and non-photochemical quenching (PhiNPQ). In **(B)** relative chlorophyll content. In **(C)** % of relative water content (RWC) in tomato leaves. In **(D)** % of membrane stability index. Error bars indicate standard errors (n = 3). Different letters indicate significant differences according to Tukey's multiple-range test (p = 0.05).

TABLE 1 Total minerals concentration of tomato leaves grown under salinity stress (S) and treated with biostimulants i.e., protein hydrolysate (PH), seaweed extracts (SW), and their combination PH + SW, compared to the control non-stressed plants (C).

	NO_3^-	PO_4^{3-}	K^+	SO_4^{2-}	Ca^{2+}	Mg^{2+}	Na^+	Cl^-
	g/Kg dry weight							
C	0.99 ± 0.77 ^{a}	3.33 ± 0.37	39.97 ± 1.19 ^{b,c}	6.55 ± 0.3 ^{a}	6.43 ± 1.72 ^{a,b}	5.57 ± 0.77	1.35 ± 0.51 ^{d}	12.14 ± 3.07 ^{c}
S	0.6 ± 0.09 ^{a,b}	3.14 ± 0.18	34.7 ± 2.71 ^{c}	4.8 ± 0.41 ^{b}	5.52 ± 0.7 ^{b}	6.45 ± 0.83	7.27 ± 13.29 ^{c}	85.59 ± 15.18 ^{a}
S + PH	0.29 ± 0.12 ^{b}	3.22 ± 0.35	43.35 ± 0.86 ^{a,b}	5.95 ± 0.56 ^{a}	7.42 ± 0.94 ^{a}	5.6 ± 0.67	17.8 ± 14.82 ^{b}	54.68 ± 6.98 ^{b}
S + SW	0.41 ± 0.14 ^{b}	3.07 ± 0.11	40.38 ± 8.6 ^{b,c}	5.9 ± 0.78 ^{a}	7.72 ± 0.88 ^{a}	5.45 ± 0.53	12.94 ± 15.87 ^{b}	51.07 ± 9.08 ^{b}
S + PH + SW	0.73 ± 0.29 ^{a,b}	3.21 ± 0.26	49.14 ± 5.86 ^{a}	4.6 ± 1.18 ^{b}	7.51 ± 0.56 ^{a}	5.45 ± 0.7	25.15 ± 13.84 ^{a}	74.92 ± 16.23 ^{a}

All data are expressed as the mean ± standard error, n = 5, g/Kg dry weight. Different letters within each column indicate significant differences according to Tukey's multiple-range test (p = 0.05).

which reported down accumulated under PH (54.68 g/kg DW) and SW (51.07 g/kg DW), and non-difference in the case of PH + SW treatment.

The effects of salinity stress on organic acids composition and content in tomato leaves were determined in terms of malate, oxalate, citrate and isocitrate quantification (Table 2). The malate and oxalate were reported a decreasing trend under salinity stress compared to the control, however, only malate variation was statistically significant. Instead, the behaviour of citrate and isocitrate showed an increasing trend, under salinity stress, although only citrate was statistically significant. The treatment with plant biostimulants modulated their accumulation considerably. Indeed, significant down-modulation of citrate and isocitrate was observed after PH and SW treatments on plants affected by salinity stress compared to the control.

3.3 Biostimulants application actively modulated essential metabolic pathways involved in stress response

The different metabolic responses induced by biostimulant application on tomato plants subjected to salinity stress were investigated using an untargeted metabolomics approach *via* UHPLC-ESI/QTOF-MS. The untargeted profiling allowed us to putatively annotate more than 3300 metabolites. Their comprehensive list is reported in the supplementary materials (Table S1), including compounds name, pathways, ontology classification, peaks abundances, retention time and masses of each metabolite.

The unsupervised hierarchical cluster analysis (HCA) was performed on the entire dataset to point out similarities/dissimilarities among treatments based on detected metabolites (Figure S1) and resulted in two main clusters. The first one showed clear metabolic profile similarities among plants treated with PH and the control, representing a unique subcluster, followed by PH + SW treatment (second subcluster). The second cluster was characterized by metabolites modulated by SW application and salinity stress. Although the application of SW reported distinctive morpho-physiological characteristics, the leaves' metabolic profiles resulted in no separation between stressed plants, suggesting a different mechanism of action involved.

The Volcano Plot analysis was used to investigate the mechanism of action of the biostimulants on tomato under salinity, combining ANOVA statistical analysis ($p< 0.05$) and fold change analysis (FC, threshold of 2.5). Overall, 609 differential metabolites were identified, and their complete list is provided in the Supplementary material (Supplementary Table S2). The graphical representation of the resulting biosynthetic pathways is reported in Figure 3, including biosynthetic pathways (Figure 3A), secondary metabolites biosynthesis (Figure 3B), lipids and fatty acids biosynthesis (Figure 3C), and hormones biosynthesis (Figure 3D), as well as the summary pathway table in Supplementary Table S3.

The main biosynthetic pathways modulated by salinity stress in tomato leaves, the biosynthesis of the secondary metabolites reported the highest down-modulation, followed by fatty acids and lipids and phytohormones biosynthesis. Instead, a clear accumulation was observed for amino acid biosynthesis (Figure 3A). Focusing on the pathways most affected by salinity stress, such as secondary

TABLE 2 Total organic acids concentration of tomato leaves grown under salinity stress (S) and treated with biostimulants i.e., protein hydrolysate (PH), seaweed extracts (SW), and their combination PH + SW, compared to the control non-stressed plants (C).

	Malate	Oxalate	Citrate	Isocitrate
	g/Kg dry weight			
C	28.24 ± 1.97 ^{a}	1.82 ± 0.37 ^{a}	10.94 ± 4.22 ^{b}	0.5 ± 0.22 ^{a,b}
S	20.92 ± 10.74 ^{b}	1.51 ± 0.17 ^{a,b}	16.46 ± 5.35 ^{a}	0.61 ± 0.2 ^{a}
S + HP	14.36 ± 1.05 ^{b}	1.26 ± 0.14 ^{b}	9.3 ± 2.46 ^{b}	0.38 ± 0.09 ^{b}
S + SW	17.15 ± 1.17 ^{b}	1.36 ± 0.15 ^{b}	9.35 ± 1.42 ^{b}	0.36 ± 0.1 ^{b}
S + HP + SW	15.18 ± 2.22 ^{b}	1.17 ± 0.28 ^{b}	12.24 ± 3.47 ^{a,b}	0.38 ± 0.19 ^{b}

All data are expressed as the mean ± standard error, n = 5, g/Kg dry weight. Different letters within each column indicate significant differences according to Tukey's multiple-range test (p = 0.05).

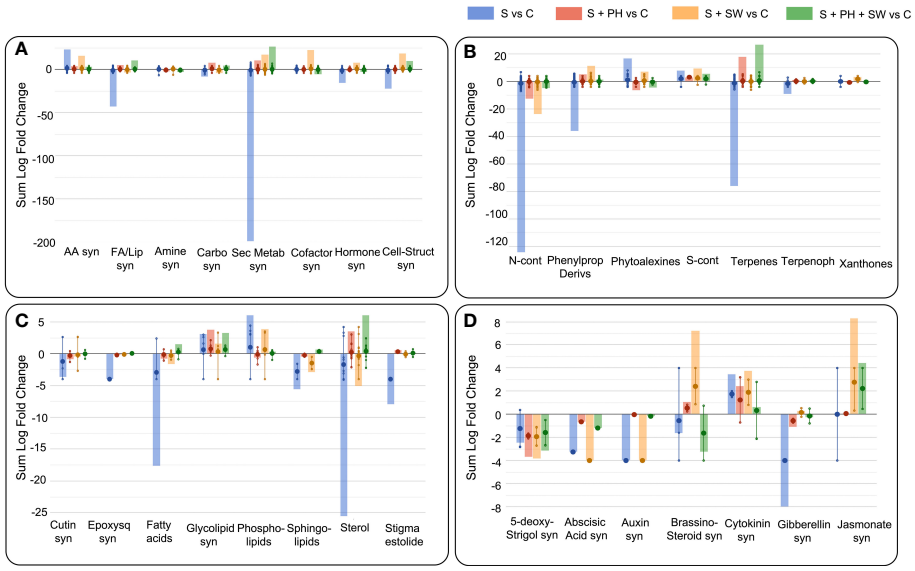

FIGURE 3
Pathway analysis of tomato leaves affected by salinity stress (S) and treated with biostimulants i.e., protein hydrolysate (PH), seaweed extracts (SW), and their combination PH + SW, compared to the control non-stressed plants **(C)**. The metabolites used to carry out the pathways analysis are those that have passed ANOVA (p-value< 0.05) and fold change (FC ≥ 2.5) analysis. Differential metabolites were interpreted in terms of biosynthesis pathways **(A)**, secondary metabolite **(B)**, lipids and fatty acids **(C)** and hormone biosynthesis (**D**). Error bars indicate the standard deviation of logFC values of all compounds belonging to the class. The larger dot indicates the median value, while the smaller dot indicates the actual value of each individual compound belonging to the class. The bars indicate the algebraic sum of the logFC values of the individual compounds belonging to the same class. Abbreviation = syn: biosynthesis; AA: amino acid; FA/Lip: fatty acid and lipid; Carbo: carbohydrate; Sec Metab: secondary metabolites; Cell-struct: cell-structure; N-cont: nitrogen-containing; Phenylprop: phenylpropanoids; S-cont: sulphur-containing; Terpenoph: terpenophenolics; Epoxysq: epoxysqualene.

metabolism, the down-modulated compounds belong to the macroscopic classes of nitrogen-containing, phenylpropanoids, terpenes, and terpenophenolics compounds. In contrast, classes of compounds belonging to phytoalexins and sulphur-containing, were up-modulated (Figure 3B). Considering fatty acids and lipids biosynthesis, the salinity stress negatively affected the biosynthetic pathways of sterols, fatty acids, sphingolipids, and cutin biosynthesis (Figure 3C).

Interestingly, foliar spray biostimulants application reported an effective modulation on those pathways damaged by salinity stress. Indeed, secondary metabolites following biostimulant application were up-modulated, and this trend was in common with fatty acids and lipids biosynthesis. Biostimulant treatments mitigated or even reverted both the biosynthesis of secondary metabolites such as nitrogen-containing, phenylpropanoids and terpenes compounds (Figure 3B), and the biosynthesis of fatty acids and lipids including sterols, fatty acids, and sphingolipids (Figure 3C).

Between the different biostimulants, in many cases, SW was found less effective in mitigating salinity stress-damaged pathways compared to PH and PH + SW. This was evident for secondary metabolites (nitrogen-containing, phytoalexins, and terpenes) and in lipids (fatty acids, phospholipids, sphingolipids, and sterols) biosynthesis. Indeed, PH reported a distinctive modulation pattern and their combination with SW. In particular, the combination of PH + SW showed an opposite modulation trend compared to stressed plants, suggesting a different stress response mechanism.

Phytohormones' biosynthetic pathways were also affected by salinity stress modulation, as well as the application of biostimulants (Figure 3D). Indeed, metabolites involved in the biosynthesis or degradation of 5-deoxy-stringol, abscisic acid (ABA), auxin, brassinosteroid, cytokinin, gibberellin, and jasmonate were reported to be highly modulated. Specifically, salinity stress firmly modulated gibberellin, cytokinin, auxin, ABA, and 5-deoxy-stringol biosynthesis. Instead, SW treatment strongly modulated brassinosteroid and jasmonate biosynthesis, comparted to PH treated one. The combination of PH + SW reported a distinctive modulation profile compared to their single components. However, given the low half-life of phytohormones, metabolites involved in both biosynthesis and degradation were considered to have a positive modulation.

4 Discussion

Salinity stress is a major environmental stress that affects crops' physiological, morphological, and biochemical processes, limiting their growth and productivity. According to literature, tomato plants grown under a high salt concentration (150 mM) were highly inhibited in the growing potential, reporting more than 50% of biomasses reduction, both considering FW and DW parameters. This effect was also observed by Tanveer et al. (2020). This imminent morphological effect was due to hyperosmolarity of the soil solution, which is translated into the plant system through two main phases: (1) a rapid osmotic stress response, which is independent of salt accumulation in the shoot, that causes plant growth inhibition (Almeida et al., 2017), and (2) plant adaptions to salinity stress by activation of different intracellular osmotic stress tolerance mechanism (Zhao et al., 2021). The resilience mechanism includes Na^+ or Cl^- exclusion, tissue tolerance, a modulation of signal molecules, stomatal closure and the maintenance of the water status

(Munns and Tester, 2008; Pardo, 2010). The application of biostimulants on tomato plants under salinity has reported a mitigating effect in terms of growth parameters, both on root and shoot organ parts. As known from the literature on salinity stress, in most cases Na^+ and not Cl^- is considered the ion that first reaches a toxic concentration in root of rice seedlings (Chi Lin and Huei Kao, 2001; Tsai et al., 2004). However, for several species, Cl^- is represented as the most toxic ion because Na^+ is usually withheld in roots and stems and only a small part reaches the leaves (Almeida et al., 2017). Conversely, Cl^- passes freely to the lamina, becoming the most significant toxic component under salt stress (Storey and Walker, 1998). According to our results, salinity stress significantly modulated Cl^- accumulation in saline-stressed plants (7 folds), whereas the employment of PH and SW reduced this value to about 4 folds. The combined PH + SW reported non-significant modulation in this Cl^- accumulation compared to stressed plants.

The localization of Na^+ on the roots and stems occurs mainly when the concentration of Na^+ in the soil solution increases and promotes in passive Na^+ transport by a family of Non-Selective Cation Channels (NSCCs family) within root cells. Na^+ taken up by the roots are then transported to shoots *via* xylem by bulk flow (Almeida et al., 2017). This process is driven by movement of water from the root through the plant to the surrounding atmosphere during transpiration. However, Na^+ is also the primary toxic ion as it interferes with the uptake of K^+ that is involved in the stomatal regulation (Tavakkoli et al., 2010). In this latter case, the water transpiration is limited by stomata closure through the action of K^+ to avoid water loss, thus leading to the segregation of Na^+ mainly in roots and stems compartment (Tavakkoli et al., 2010). Moreover, the Na^+ efflux process is considered to be an unfavourable action as it occurs through the action of Na^+/H^+ antiporters by the consumption of ATP and PPi (Zhao et al., 2021). Another mechanism that limits Na^+ accumulation in leaves is the Na^+ retrieval mechanism. Plants can reabsorb Na^+ from the xylem to the root cells as a mechanism to prevent large Na^+ accumulation in aboveground tissues (Maathuis et al., 2014). This finding was confirmand in *Arabidopsis*, where the disruption of HKT1 gene (Na^+ transporter) leads to hypersensitivity to salinity in mutant lines, with increased Na^+ in leaves. The knockout lines showed a higher amount of Na^+ in the shoots but a lower level of K^+ (Møller et al., 2009).

The application of biostimulants on tomato plants under salinity stress reported an increase of Na^+ accumulation in leaves, compared to the control. However, the beneficial effect of PH and SW biostimulants application were observed in terms of Phi2, PhiNPQ, and chlorophyll content, suggesting a different mechanism of action than compartmentalization and/or exclusion of toxic ions. In fact, plant responses to salt stress is also manifested by production of secondary signaling molecules such as ROS, contributing to the deleterious effects on plants (Mittova et al., 2004; Bai et al., 2018). Application of PH and SW biostimulants could enhance the biosynthesis of antioxidant agents to cope with salinity-driven oxidative stress and increase the amount of Na^+ ion tolerance. Indeed, the application of PH and SW clearly up-modulated powerful antioxidant compounds like phenolics and flavonoids, reported to have also a high antioxidant activity under salinity stress in *Salvia mirzayanii* (Valifard et al., 2014), *Carthamus tinctorius* L. (Golkar and Taghizadeh, 2018), *Phaseolus vulgaris* L. (Semida and Rady, 2014).

Salinity stress severely affects the photosynthesis apparatus caused by the reduction of plant water potential and chlorophyll biosynthesis. Giordano et al. (2021) reported the involvement of Cl^- in the biosynthesis of chlorophyll, which higher concentration interferes with its production. Interestingly, the negative correlation between leaf Cl^- content and chlorophyll production and photosynthetic performance in our plants, confirming the toxicity of Cl^- accumulation on the photosynthetic system of leaves. Accordingly, PH and SW showed the best ones in mitigating salinity stress in the photosynthesis activity and chlorophyll concentration.

Usually, the effect of salinity stress is superimposed on those caused by dehydration. Indeed, salinity stress is often associated with water deficit, as the salt dissolved in the soil reduces water availability and water uptake by the roots (Munns and Tester, 2008). RWC is a parameter that provides the degree of hydration of plant tissue and is considered a very important parameter for determining the maximum water contained in the plant Teulat et al. (1997). The tomato plants under salinity stress reported a value of RWC greater than those observed in the control plants. Although the RWC parameter has been reported by Suriya-arunruj et al. (2004) as an effective method to evaluate salt stress tolerance, where a high RWC value has been correlated with high salt stress tolerance, this observation was not in agreement with our data. However, the high RWC content in our salinity-stressed plants could be explained by the fact that water retention is a primary defense system adopted by plants under water shortage caused by osmotic effect (Zhu, 2002; Zhao et al., 2021). Indeed, sustained transpiration without sufficient water supply leads to loss of leaf turgor, decrease of photosynthetic activity, and growth capacity (Acosta-Motos et al., 2017). Similarly, no mitigating effect was reported after biostimulants application. Higher RWC values could be attributed to the leaf stomatal closure under salinity stress. In fact, in normal growth conditions (i.e., well-watered plants) leaf stomata are fully open during daylight periods, when light stimulates stomatal opening *via* blue light-specific and photosynthetic-active radiation-dependent pathways, to maximize the assimilation of CO_2 and ensure an optimal photosynthesis rate (Roelfsema and Hedrich, 2005). However, under stress conditions, stomata were closed to reduce water loss, with a consequent reduction in photosynthesis (Giménez et al., 2013; Giordano et al., 2021). Accordingly, salt-stressed tomato plants also reported a reduced value of Phi2 and increased value of PhiNPQ, as well as low chlorophyll content, compared to the control. Therefore, the high RWC value observed in the stressed samples is purely attributed to a saline stress response status activated by the tomato plant, resulting in the closure of the stomata and the following accumulation of water in the leaves. The stoma closure process is directly regulated by K^+ efflux through depolarisation-activated K^+ channels in guard cells (Demidchik, 2014). Due to the nature of the K^+ channels, it also requires the movement of counterions to balance the plasma membrane potential, such as Cl^-, PO_4^{3-}, NO^{3-}, citrate, and malate (Demidchik, 2014; Khan et al., 2020). Accordingly, salinity stress-affected tomato plants reported a low trend accumulation of K^+ and higher accumulation of potential counterions $Cl^-$and citrate in their leaves. However, biostimulants treated plants reported a different profile of mineral content in leaves, suggesting a different stress response mechanism, despite the similar RWC values reported.

Indeed, contrary to what was observed for the stressed samples, the application of PH reported an up-accumulation of K^+ and a milder decreased content of Cl^- and citrate, compared to stressed plants. In this sense, it is interesting the role of Ca^{2+}, which was observed to inhibit the efflux of K^+ under salinity stress (Davies and Newman, 2006). Accordingly, the Ca^{2+} level in plants treated with biostimulants were higher than those detected in salt-stressed leaves. Moreover, the beneficial effect of up-accumulated Ca^{2+} in treated plants is also correlated to the membrane stability index, as confirmed by several authors (Tuna et al., 2007; Khursheda et al., 2015; Tanveer et al., 2020). Specifically, salinity stress affected MSI of tomato plants severally, compared to non-stressed ones. However, the application of PH reported a clear mitigation capability on membrane integrity. This beneficial effect could be attributed to biostimulants' ability to increase Ca^{2+} coupled to enhancement of sterols biosynthesis, both involved in the regulation of membrane stability and permeability (Tuna et al., 2007; Guo et al., 2019).

The Ca^{2+} and K^+ could be attributed to the phytohormones regulation. Indeed, salt-stressed plants reported an overall down modulation of hormones biosynthesis, except for cytokinin and jasmonate. The application of PH and SW up-modulated the metabolites involved in the biosynthesis of brassinolide (BR), while SW and PH + SW application modulated those in the biosynthesis of jasmonic acid (JA), and finally all three biostimulants reported a modulatory capacity of gibberellin biosynthesis (GB). These phytohormones were reported to have a positive relationship to salinity tolerance response. The BR accumulation is directly related to the enhancement of plant antioxidant activity by implementing the activity of antioxidant enzymes and accumulating antioxidant compounds such as tocopherol, glutathione and polyphenols (El-Mashad and Mohamed, 2012). According to the literature, the application with SW reported an up modulation of BR and the following accumulation of antioxidant compounds, as well as the up modulation of phenylpropanoid biosynthesis pathways (Goñi et al., 2018; Deolu-Ajayi et al., 2022). Similarly, the up-regulation of JA is related to attenuating salinity stresses by accumulation of osmolytes to limit the uptake of Na^+ and Cl^- and non-enzymatic antioxidants such as flavonoids (Yastreb et al., 2016).

As we observed, Ca^{2+} ions mediate several mechanisms of the salinity stress response due to their flexibility in exhibiting different coordination numbers and forming complexes with proteins, membranes, and organic acids (Kudla et al., 2010). Indeed, the Ca^{2+} ion is the most important secondary messenger in plants. A potential alteration of the Ca^{2+} signalling cascade could regulate a wide range of physiological processes, including gene expression, protein activities, and biosynthetic pathways (*i.e.*, secondary metabolites, fatty acids and lipids, phytohormones, and other pathways) (Kudla et al., 2010; Roy et al., 2014). In regulating lipids biosynthesis, Ca^{2+} plays an essential role in processes that preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport and selectivity, and control ion exchange (Tuna et al., 2007). As well known, MSI is closely related to the composition of membrane lipids and strong changes in lipid composition induced by salinity stress could severally affect the membrane fluidity, permeability, and electrolyte leakage (Tuna et al., 2007; López-Pérez et al., 2009). In our samples, a strong down modulation of membrane lipid composition was observed in tomato plants affected by salt stress. The main changes involve the composition of sterols, sphingolipids, phospholipids, and fatty acids. Salinity stress strongly down-modulated the sterols and fatty acids synthesis, whereas biostimulants application reverted this negative effect. Particularly for phospholipids, we found a relevant up-accumulated under salinity stress and this observation was also supported by Salama and Mansour (2015). Interestingly, phospholipids were down-modulated after PH and slightly PH + SW applications. Concerning phytosterols, they were reported to be heavily down-modulated by salt stress. However, both PH and PH + SW applications resulted in an up-modulation of these lipids. Sterols are important structural components being constitutively down accumulated in plant membranes affected by salt stress (Guo et al., 2019). Salama and Mansour (2015) reported that maintaining a constant level of sterols in the membrane would be essential for plant salt tolerance. PH and PH + SW treatments stimulated the synthesis of planar sterols such as campesterol, brassicasterol, and 7-dehydrocholesterol, which are thought to regulate membrane fluidity and permeability in plant membranes by restricting the mobility of fatty acyl chains (Guo et al., 2019). The same result has been achieved for fatty acids composition, with salt stress strongly down modulated their biosynthesis despite a recovery could be observed after biostimulant application. Specifically, PH and PH + SW treated plants reported an improvement in saturated fatty acids *i.e.*, palmitoyl-CoA and lauroyl-CoA, which improve liquid-order phases that are directly related to membrane fluidity (Guo et al., 2019). The MSI reported from agronomical results were according to PH-treated tomato, but not in the case of PH + SW-treated plants. The reason could be due to the unsaturated fatty acid composition, which generates liquid-disordered phases and, consequently membrane instability (Guo et al., 2019). Unsaturated lipids were up accumulated under SW and PH + SW treatments, corroborating the morpho-physiological data reported from our results. In this case, we can state that the beneficial effect of PH treatment is strictly correlated with their ability to modify the composition of membrane lipids and change the ratio between saturated and unsaturated fatty acids. This effect is not evident in PH + SW treatment and is null in the case of SW application. The emerging evidence of the SW-driven mechanism of action may lie in the ability of SW to stimulate secondary metabolism, in particular in the production of phenylpropanoids and their antioxidant capacity (Deolu-Ajayi et al., 2022). Phenylpropanoids as well as nitrogen-containing and terpenes, were the secondary metabolites mostly affected by salinity stress in tomato plants. The biosynthesis of polyphenols is usually decreased in salt-stressed plants, as confirmed by several authors (Eryılmaz, 2006; Ben Dkhil and Denden, 2012; Yan et al., 2014; Wang et al., 2016; Bistgani et al., 2019). However, the biostimulants were able to up modulate their production, particularly under SW treatment. Plant phenolic compounds are involved in key metabolic and physiological processes, including growth and development, synthesis of photosynthetic pigment, and scavenging of harmful ROS (Golkar and Taghizadeh, 2018; Bistgani et al., 2019; Sharma et al., 2019). The scavenging of ROS is the most important beneficial property of polyphenols because the accumulation of salt-induced ROS is closely related to the activation of specific ROS-promoted signaling pathways, such as processes involved in the damage of photosynthetic systems, limitation of CO_2 fixation, as well as peroxidation and

destabilization of cellular membranes (Mittova et al., 2004). Moreover, generated ROS could also damage vital molecules such as nucleic acids, proteins, carbohydrates, and lipids (Sharma et al., 2019). Another mechanism of salinity stress-mitigation adopted by biostimulants was the modulation of N-containing compounds and terpenes. N-containing compounds are compounds characterized by a nitrogen group in their structure, such as glucosinolates, their hydrolysis products, and alkaloids. The relevant function of these classes of compounds in abiotic stress mitigation has been described by Del Carmen Martínez-Ballesta et al. (2013). The author also suggested a correlation between the increase of glucosinolates production and the synthesis of osmoprotective compounds e.g., proline (Matysik et al., 2002), glycine betaine (Mäkelä et al., 2000), and sugars (Saxena et al., 2013). Osmolytes are mainly involved in protecting the functions of cell structures and maintaining osmotic balance under salinity stress (Hare et al., 1999; Ghosh et al., 2021). As aforementioned, tomato plants treated with biostimulants showed an up-modulation of the precursors for the synthesis of proline (PH treatment) and betaine (SW treatment), as well as in the biosynthesis of carbohydrates (PH + SW treatment). Concerning the class of terpenes, we found a strong modulation of carotenoids and precursor for the biosynthesis of phytosterols, driven mainly by PH-based treatment, in accordance with the chlorophyll content and MSI results previously reported.

5 Conclusion

The search for sustainable approaches able to sustain crop productivity under unfavorable conditions has paved the way towards the use of plant biostimulants in agriculture. However, unraveling the mechanisms involved in biostimulants beneficial effects becomes of pivotal importance to identify tailored applications in line with the different agricultural scenarios worldwide. Our results evidenced a beneficial contribution of the tested biostimulants in terms of salinity stress mitigation. In agreement with previous reports on biostimulants, a broad metabolomic reprogramming could be observed following the application of biostimulants, with secondary metabolites, membrane lipids and phytosterols, as well as hormones showing the most extensive modulation.

Interestingly, distinct effects could be observed when the protein hydrolysate or the seaweed extract were applied, either alone or in combination, corroborating the differences observed at metabolomics level. On one side, this supports the need for understanding the mode of action, to properly design biostimulants-based agricultural solutions. On the other side, this opens the possibility towards an integrated strategy that uses complementary biostimulants, provided that synergistic effects are demonstrated. Starting from our results, a comprehensive evaluation that goes beyond plant science and includes also economic and environmental sustainability issues is recommended.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

LZ, GF, YR, SP, and LL: Conceptualization and project administration. LZ, GF, YR, SP, and LL: Methodology, validation, formal analysis, investigation, writing—original draft preparation. SDP and LL: Writing—review and editing. LZ: Software and data curation. GF, SP, YR and LL: Resources. LL: Visualization and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

GF was employed by company Clever Bioscience srl Casanova Lonati, PV, Italy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1072782/full#supplementary-material

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Two plant-associated *Bacillus velezensis* strains selected after genome analysis, metabolite profiling, and with proved biocontrol potential, were enhancing harvest yield of coffee and black pepper in large field trials

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Elimination of chemically synthesized pesticides, such as fungicides and nematicides, in agricultural products is a key to successful practice of the Vietnamese agriculture. We describe here the route for developing successful biostimulants based on members of the *Bacillus subtilis* species complex. A number of endospore-forming Gram-positive bacterial strains with antagonistic action against plant pathogens were isolated from Vietnamese crop plants. Based on their draft genome sequence, thirty of them were assigned to the *Bacillus subtilis* species complex. Most of them were assigned to the species *Bacillus velezensis*. Whole genome sequencing of strains BT2.4 and BP1.2A corroborated their close relatedness to *B. velezensis* FZB42, the model strain for Gram-positive plant growth-promoting bacteria. Genome mining revealed that at least 15 natural product biosynthesis gene clusters (BGCs) are well conserved in all *B. velezensis* strains. In total, 36 different BGCs were identified

in the genomes of the strains representing *B. velezensis, B. subtilis, Bacillus tequilensis*, and *Bacillus. altitudinis*. *In vitro* and *in vivo* assays demonstrated the potential of the *B. velezensis* strains to enhance plant growth and to suppress phytopathogenic fungi and nematodes. Due to their promising potential to stimulate plant growth and to support plant health, the *B. velezensis* strains TL7 and S1 were selected as starting material for the development of novel biostimulants, and biocontrol agents efficient in protecting the important Vietnamese crop plants black pepper and coffee against phytopathogens. The results of the large-scale field trials performed in the Central Highlands in Vietnam corroborated that TL7 and S1 are efficient in stimulating plant growth and protecting plant health in large-scale applications. It was shown that treatment with both bioformulations resulted in prevention of the pathogenic pressure exerted by nematodes, fungi, and oomycetes, and increased harvest yield in coffee, and pepper.

KEYWORDS

***Bacillus velezensis*, phylogenomics, biocontrol plant pathogens, microbial biostimulant, Vietnamese agriculture, black pepper, coffee trees, crop yield**

1 Introduction

Fruits from tropical tree crops, such as bananas, mangos, spices, coffee, and cacao, are widely traded and worldwide sought (Drenth and Guest, 2016). Coffee and black pepper are important crops in tropical, especially in Vietnamese agriculture. Coffee production was introduced to Vietnam by France around the year 1915. The cultivation of coffee is now a corner stone of the local economy. In 2022, the coffee area in Vietnam, which is mainly located in the Central Highland (Dak Lak province), amounted to 695.6 thousand hectares with the production of green coffee reaching 1,763.5 thousand tons per year (General Statistics Office – GSO, https://www.gso.gov.vn/en/). Robusta coffee (*Coffea canephora*) occupies most of the area (93%). The remaining part is used for cultivating the high-value Arabica coffee (*Coffea arabica*). The natural conditions in the Vietnamese mountain region, such as acidic soil, rain pattern and high elevation, provide a unique environment for Arabica coffee trees. Vietnamese coffee is exported to over 80 countries and territories. In 2018, Vietnam exported 1.9 million tons with an export turnover of 3.5 billion USD. This accounts for 14% of the market share and more than 10% of the global export value of green coffee, ranking second after Brazil (Rolshausen and Dzung, 2018).

Despite strong international competition, Vietnam made amazing progress in production of black pepper (*Piper nigrum*) fruits known as peppercorns, from nearly no-production in 1983 to the world number one today. In 2020, the area of pepper reached 131.8 thousand hectares, of which 112.9 thousand hectares were harvested with an output of 270.2 thousand tons. Pepper is grown mainly in the Southeast region and the Central Highlands. Vietnam's pepper output accounts for nearly 40% of this common daily spice worldwide (Vietnam Pepper Association (VPA), 2020).

However, in recent years harvest losses caused by pathogenic microorganisms, and root-knot forming nematodes have been steadily increasing (Tam et al., 2020; Jähne et al., 2023). A serious problem in Vietnamese agriculture is the occurrence of an aggressive wilt disease in coffee trees with symptoms as yellowing leaves, wilting foliage, and root rot (internally named ´YLRR´). The causative agent of the disease was identified as being the root-knot forming nematode *Meloidogyne* sp., often associated with the fungus *Fusarium oxysporum* (Nguyen et al., 2016). In addition, also the black pepper cultivation has been seriously affected by a number of diseases (Santos et al., 2023). Among them, the "quick wilt" or "quick death" disease caused by the root-knot nematode *Meloidogyne* spp, and the oomycete *Phytophthora palmivora* has a serious negative effect on black pepper growth and productivity (Nguyen et al., 2021). Widespread application of disease management strategies, based on chemically synthesized fungicides and nematicides, such as carbamate and organophosphorous, were found worldwide associated with increasing resistance of the target pathogens (Stukenbrock and Gurr, 2023). Toxic pollution of the environment reduces soil microbial diversity, lowers agro-product quality and food safety, and leads to increasing risks for farmers. Moreover, increasing resistance of the pathogens lowers the efficiency of chemically synthesized pesticides as a consequence of long-term application (Nicolopoulo-Stamati et al., 2016).

In order to favor a more sustainable agriculture in Vietnam, application of chemical fertilizers and pesticides were considered with growing concern by the government (Dao et al., 2015), and their substitution by environmentally friendly alternatives, such as the use of biologicals combined with "Good Agriculture Practices" (VietGAP), is a necessity for keeping the high-quality standard of Vietnamese agricultural products (Hoang, 2020).

Due to the problematic ecological as well as health hazardous properties of many of the chemically synthesized pesticides in use, new sustainable biological plant protection products are urgently needed. These environmentally friendly biocontrol agents should be specifically act against plant pathogens and effectively help to control common plant diseases without the environmentally harmful use of the chemically synthesized pesticides. We hypothesize, that plant growth promoting endophytic bacteria from the natural microbiome of healthy crops grown in pathogen-infested environments could help and serve as a basis for the development of improved biological pesticides.

This assumption was supported by a recent study in which we isolated endospore-forming Gram-positive bacteria with putative biocontrol function from healthy Vietnamese crop plants grown in fields infested with plant pathogens (Tam et al., 2020). Based on their draft genome sequences three main taxonomic groups able to control plant pathogens were distinguished: *Brevibacillus* spp. (Jähne et al., 2023), *Bacillus cereus* sensu lato, and 30 isolates assigned to the *B. subtilis* species complex (Fritze, 2004). Most of the latter group were representatives of *Bacillus velezensis* (Ruiz-García et al., 2005), a species which is known for its high potential to stimulate plant growth, and to suppress plant pathogens (Dunlap, 2019).

This study aims to perform a suitable approach for developing novel bioagents for disease control, which proved as being highly efficient in large field trials. Our approach includes the comprehensive analysis of (i) the genome sequences of the 30 isolates belonging to the *B. subtilis* species complex, (ii) their antagonistic metabolites, (iii) their action on selected plant pathogens, and (iv) their stimulatory effect on plant growth *in vitro* and *in vivo*. We show that bioformulations prepared with the most promising *B. velezensis* isolates, TL7 and S1, could be successfully applied in large field trials to protect black pepper and Arabica coffee plantations.

2 Materials and methods

2.1 Strain isolation, growth conditions and DNA isolation

Our strain isolation approach followed the procedure described by Wang and Liang (2014). The plant-associated bacteria were isolated from the rhizosphere soil and different organs of healthy crop plants, such as coffee, pepper, and others from fields located at Dak Nong, Dak Lac provinces, Vietnam, and infested with plant pathogens such as *Phytophthora palmivora*, *Fusarium oxysporum*, and the nematode *Meloidogyne* sp. (Tam et al., 2020). An overview about the isolation sources was given in Supplementary Table S1, and also reported previously (Tam et al., 2020). Routinely, leaf, stem and root of healthy plants were selected and taken to the laboratory for further processing. The plant organs were cut into 5 x 5 mm pieces, and washed twice with sterile water. Afterwards, the plant parts were dipped into 75% ethanol for one min, and then into 0.1% mercury dichloride ($HgCl_2$) for two min. Then, the cuts were three times washed with sterile water, and transferred into 10 mL sterile water. The suspension was grounded in a sterile and chilled mortar. After 30 min. incubation, 0.1 mL of the solution was transferred to Luria broth (LB) agar plates, and allowed to grow for 72 h at 28°C. Finally, single colonies were purified, transferred to 50 mL fresh LB medium, and cultured in a shaker at 28°C, and 180 rpm for 24 h. In addition, a similar procedure was used for strain isolation from soil adhering at the roots of healthy plants. Before further processing, the soil containing suspension was incubated for 10 min. at 80°C. The purified strains were maintained as glycerol stocks (20 %, w/v) at −80°C. Cultivation of the bacterial strains and DNA isolation have been previously described (Tam et al., 2020; Blumenscheit et al., 2022).

2.2 Genome sequencing, assembly and annotation

Short-read sequencing was conducted in LGC Genomics (Berlin, Germany) using Illumina HiSeqq in a paired 150 bp manner as described previously (Blumenscheit et al., 2022). Long read sequencing was done in house with the Oxford Nanopore MinION with the flowcell (R9.4.1) and prepared with the Ligation Sequencing Kit (SQK-LSK109). *De-novo* assemblies were generated by using the hybrid-assembler Unicycler (https://github.com/rrwick/Unicycler v0.4.8, (Chen et al., 2018). The quality of assemblies was assessed by determining the ratio of falsely trimmed protein by using Ideel (https://github.com/phiweger/ideel). Genome coverage of the obtained contigs was 50 x in average.

Automatic genome annotation was performed using the NCBI Genome Automatic Annotation Pipeline (PGAP6.2) for the general genome annotation provided by NCBI RefSeq. Functional annotation was done by using the COG- (Tatusov et al., 2000), and the KEGG database (Kanehisa et al., 2023). Prediction of core and pan genomes, and comparative analyses were performed with the EDGAR3.0 pipeline (Dieckmann et al., 2021). Genomic islands (GI) were predicted with the webserver IslandViewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/, Bertelli et al., 2017). Circular plots of genome and plasmid sequences were visualized with BioCircos (Cui et al., 2016).

Gene clusters for secondary metabolite synthesis were identified using the antiSMASH pipeline version 6 (Blin et al., 2021) under settings of all features, and BAGEL4 (van Heel et al., 2018). All biosynthetic gene clusters (BGCs) were investigated for their presence in the MIBiG repository (Kautsar et al., 2020).

2.3 Phylogeny and genome similarity assessment

The genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS) available at https://tygs.dsmz.de (Meier-Kolthoff and Göker, 2019). Information on nomenclature was provided by the List of Prokaryotic names with Standing in Nomenclature (LPSN, available at https://lpsn.dsmz.de) (Meier-Kolthoff et al., 2022). The genomes were compared with all type strain genomes available in the TYGS database *via* the MASH algorithm (Ondov et al., 2016), and the ten strains with the smallest MASH distances were chosen per user genome. Using the Genome

BLAST Distance Phylogeny approach (GBDP) the ten closest type strain genomes for each of the user genomes were calculated.

In silico DNA-DNA hybridization (dDDH) values were calculated in the TYGS platform using formula d_4, which is the sum of all identities found in the high score segment pairs (HSPs) divided by the total length of all HSPs. Pan-genome analysis was performed using the EDGAR software package (Dieckmann et al., 2021). ANIb values were obtained with the Jspecies WS online service (https://jspecies.ribohost.com/jspeciesws/#anib). Species cut off: 95% (ANIb), 70% (dDDH); subspecies cut off: 97% (ANIb), 79% (dDDH).

The EDGAR3.0 pipeline (Dieckmann et al., 2021) was used for elucidating taxonomic relationships based on genome sequences. High throughput ANI analysis (FastANI) was performed according to Jain et al. (2018). To construct a phylogenetic tree for a project, the core genes of these genomes were computed. In a following step, alignments of each core gene set are generated using MUSCLE. Then the alignments were concatenated to one huge alignment. This alignment is the input for the FastTree software (http://www.microbesonline.org/fasttree/) to generate approximately-maximum-likelihood phylogenetic trees. The values at the branches of FastTree trees are not bootstrapping values, but local support values computed by FastTree using the Shimodaira-Hasegawa test.

2.4 Mass-spectrometric detection of bioactive peptides

Cultivation of organisms, sample preparation and mass spectrometric detection of the bioactive compounds produced by the investigated endophytes were essentially performed as described in (Mülner et al., 2020). The strains were grown on Landy agar plates (Landy et al., 1948) after incubation for 24 h at 30°C. The cells were then transferred into 20 µL of water and mixed with 80 µL of trifluoroacetic acid (TFA, Sigma-Aldrich, Deisenhofen, Germany). The suspension was incubated for 30 min at room temperature. Then 10 µL were diluted 1:10 with double-distilled water to achieve a cell extract with a final concentration of 8% TFA. For mass-spectrometric detection of the peptides, a Bruker Autoflex Speed TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) was used with smart-beam laser technology using a 1 kHz frequency-tripled Nd-YAG laser. Two µL samples were mixed with 2 µL matrix solution (a saturated solution of a α-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% TFA), spotted on the target, air dried and measured. Mass spectra were taken by positive-ion detection in reflector mode. Monoisotopic masses were obtained with a resolution of 10.000.

2.5 Biocontrol activity against plant pathogens and plant growth promotion

Antifungal activity of the isolates was determined according to a method used by Soliman et al. (2022). Plugs (5 mm in diameter) with the pathogenic fungi were placed onto potato dextrose agar (PDA). Then, paper discs, saturated with the growing test bacteria ($O.D._{600\ nm}$ around 1.0), were added at a distance of 20 mm from the fungi. The cultures were incubated for six days at 27°C, and daily examined for colony diameter. Inhibition of fungal growth in vicinity of the bacteria was indicative for their antifungal activity. For the quantitative assay, two agar plugs (5 mm) containing either *Phytophthora palmivora* or *Fusarium oxysporum* were placed symmetrically onto potato dextrose agar (PDA). Then, a growing bacterial culture was streaked vertically between both plugs. The agar plates were incubated for seven days at 28°C. Then, the diameter of the fungal colonies was recorded. The experiments were repeated three times with ten replicates for each tested bacterial strain.

The bioassay of nematicidal activity was performed with *Caenorhabditis elegans* N2 (Carolina, U.S.A., https://www.carolina.com) fed with *Escherichia coli* OP50 cells. Culture and synchronization of the worms was performed as previously described (Lewis and Fleming, 1995). The L4 stage was used for two different bioassays performed as described previously (Liu et al., 2013). In the slow killing assay, around 40 L4 *C. elegans* roundworms were added to the nematode growth medium (NGM) agar plate containing the test bacteria. The mixture was incubated for 3-5 days at 25°C and daily inspected. In the liquid fast killing assay, the test bacteria were grown overnight under shaking (200 rpm) at 37°C in 3 mL liquid assay medium. 100µL of the bacterial culture were diluted with 500 µL M9 medium, and transferred into 12 well plates. Each well was seeded with 40 – 60 L4 stage N2 nematodes and the assay was performed at 25°C for 24 hrs. Mortalities of nematodes were defined as the ratio of dead nematodes to the total number of tested nematodes. All experiments were performed at least three times with each experiment comprising at least three replicates (N≥3).

An assay of plant growth was performed with wild type *Arabidopsis thaliana* (EDVOTEK, USA https://www.edvotek.com/) according to Budiharjo et al. (2014). The surface sterilized seeds were pre-germinated on Petri dishes containing half-strength Murashige-Skoog medium semi-solidified with 0.6% agar and incubated at 22°C under long day light conditions (16 h light/8 h dark) for seven days. Then, the roots of *Arabidopsis* seedlings were dipped into a diluted spore suspension of the test bacteria (10^5 CFU ml^{-1}) for five min. and five seedlings were transferred into a square Petri dish containing half-strength MS-medium solidified with 1% agar. The square Petri dishes were incubated in a growth chamber at 22°C at a daily photoperiod of 14 h. Fresh weight of the plants was measured 21 days after transplanting for estimating the ability of bacterial strains for growth promotion. Three replications per every variant including the control without bacterial treatment were performed.

The ´in planta´ assay using tomato plants infested with a natural isolate of *Meloidogyne* sp. was performed as following: The root-knot nematode *Meloidogyne* sp. was isolated from roots of infested pepper plants according to Hooper et al. (2005). Tomato plantlets were grown in pots containing natural soil, and exposed to the local subtropical climate conditions in the greenhouse of the

PPRI, Hanoi. Test bacteria and second stage juveniles (J2) nematodes were added to the pots two weeks after transplanting. Ten weeks after infesting with the nematodes the number of knots in tomato plants was counted (Bridge and Page, 1980).

In all cases, at least three replications were performed. The negative controls were performed as described for the test bacteria, but without treatment with the test bacteria. More details, such as number of repetitions, calculation of the results, and statistical analyses, are given in in the Supplementary Tables referred to in the Results section.

2.6 Greenhouse and field trials

2.6.1 Bioformulations manufactured from TL7 and S1

Bioformulations, named ENDOBICA1 and BIORHIZO1, were prepared from *B. velezensis* TL7 and *B. velezensis* S1, respectively. Bacterial cells were cultivated under shaking (220 rpm/min.) in LB supplemented with Mg^{++} ions for at least two days at 28°C. Further processing was performed, such as adjusting the concentration of the culture liquid to a titer of 3.5 x 10^{10} cells mL^{-1}, and adding of adjuvants and other additives for stabilization. Application of ENDOBICA1 plant took place as a water-diluted spray directed to the leaves and the stem. Diluted BIORHIZO1 was applied directly to the soil in vicinity of the plant roots.

2.6.2 Greenhouse

The "greenhouse" (location: PPRI, Hanoi), means here the so called "net"-house type, which is typical in tropical and subtropical regions. Here, the roof and the side walls are covered with nets not with glass panes. Plants grown in the net-house, therefore were protected from insects but directly exposed to natural subtropical climate conditions. Only watering was regularly performed.

All experiments were performed with completely randomized design with at least three independent repetitions.

Black pepper and coffee trees (variety Robusta) from the nursery were grown for two years in pots containing either 5kg (black pepper plants) or 10 kg (Robusta coffee trees) of the local Red River alluvial soil with a color ranging from bright brown to purple brown. In case of black pepper plants, 0.2 g mineral NPK (N 20: P 20: K 15) fertilizer, and 400 g earthworm excrements mixed with 100 g of rice husks were added to each pot. Before planting, the soil was sterilized by autoclaving at 120°C for 45 min. Two months after planting a second dose (0.1 g) of mineral NPK fertilizer was added to each pot. The same ingredients were added in proportional amount to the pots containing 10 kg soil and the planted Robusta coffee trees. 200 mL of the 5% liquid bioformulation corresponding to 7 x 10^{10} cfu/plant were poured to each pot. One month after this treatment, 2000 individuals of the pathogenic J2 *Meloidogyne* sp. nematode were added to each pot. Finally, two months after the treatment with the beneficial bioformulation, the plants were inoculated with either the pathogenic oomycete *Phytophthora palmivora* or the pathogenic fungus *Fusarium oxysporum* yielding a final concentration of 10^5 cfu g^{-1} soil, respectively. The negative controls were performed in the same way, but 200 mL tap water were used for treatment instead of the diluted bioformulations. Every treatment was carried out on ten plants. The results were recorded six months after the treatment with the bioformulations. The parameters for measuring the plant growth promoting effect on pathogen-infested coffee and black pepper plants were: height, plant canopy diameter, number of shoot branches, and the leaf chlorophyll content index (SPAD values, measured with the Chlorophyll Meter SPAD502Plus, Konica Minolta). The assays were performed as described by Nguyen et al. (2021).

2.6.3 Field trials

The field trials in which the bioformulations were used for the treatment of coffee trees and black pepper plants (Vinh Linh variety) were performed in large experimental plots with a size of one hectare at two different locations in Viet Nam. Each of the one-hectare plots including the control without the bioformulations was fertilized with a mixture of 5 tons humic fertilizer, and 50 kg mineral NPK fertilizer (N 20: P20: K15). Harvesting was performed after six months when the color of the fruits was changed to red. The harvest yield was determined by estimating the total weight of the dried coffee beans or the dried pepper fruits, respectively. For data collection obtained of an area of 10,000 m^2 per trial, five selected areas with 20 plants each were selected for each-one ha plot according to a fixed scheme developed by PPRI (Supplementary Figure S1).

The trials with the Arabica coffee trees were performed in the mountain region of Cau Dat, Xuan Truong, Da Lat, Lam Dong, Viet Nam (11°50'11.0"N 108°32'29.9"E) at an altitude of 1,500 -1,600 m above sea level. The basalt containing soil is red colored, and rich on clay minerals, well drained and fertile. The soil pH measured in H_2O is 5.5 to 6. The average temperature ranges from 18 to 21°C. The highest temperature is not exceeding 30°C, and the lowest temperature is not less than 5°C. Da Lat has two distinct seasons: the rainy season and the dry season. Dry season: from November to April, coinciding with the northeast monsoon season. The weather is generally warm, sunny, less cloudy, no rain, low temperature at night. Occasionally there will be rain in the afternoon, sometimes hail. The rainy season is from May to October, coinciding with the southwest monsoon season, often with heavy or prolonged rain. The average annual rainfall is 1562 mm. The humidity is 82%. For treatment of one ha with 6,250 Arabica coffee trees, 12.5 L of the bioformulation (3.5 x 10^{10} cfu mL^{-1}) were used, and diluted 1:1000 into water immediately before use. The control was performed with 2 L water per coffee tree instead treatment with the bioformulation.

The field trials with black pepper plants were performed under tropical monsoon climate conditions in the Chu Se, Gia Lai, Viet Nam (13°39'27.6"N 108°06'42.6"E) region at an altitude of 700 – 800 m above sea level. The area is flat or slightly sloping. The basalt containing soil is red colored, and rich on clay minerals, well drained and fertile. The soil pH measured in H_2O is 5.5 to 6. The climate is characterized by abundant humidity and high rainfall. The rainy season usually starts from May and ends in October. The dry season from November to April next year. The annual average temperature is 22 – 25°C. The average annual rainfall amounts to

2,200 - 2,500 mm. Gia Lai's climate and soil are very suitable for the development of many short- and long-term industrial crops. For treatment of one ha with 1,600 black pepper "pillars" (trellis) with one to three plants, each, 3.2 L of the bioformulation (3.5 x 10^{10} cfu mL^{-1}) were used, and diluted 1:1000 into water immediately before use (corresponding to 2 L per pillar).

2.7 Tests for estimation of plant pathogens

The number of zoospores of *P. palmivora* in soil was estimated six months after the treatment with the bioformulations by counting the number of zoospores able to "bait" and to decolorize rose petals (Drenth and Sendall, 2001). 100 g of dried root adhering soil were sieved through a 2-mm mesh, and suspended in 200 mL distilled water by stirring with a glass rod. After incubation overnight, 100 squared pieces of rose petals (1 mm x 1 mm, "traps") were added to the suspension. After one to two days, the decolorized petals were examined by light microscopy for the presence of zoospores, and the number of decolorized petals containing zoospores were counted.

The number of *F. oxysporum* var. *coffea* colonies g^{-1} soil were estimated six months after treatment with the bioformulation by plating of 10^{-4} diluted soil samples on PDA (potato dextrose agar) and CLA (green rice stem agar), and incubating at 28°C for one week according to Burgess et al. (2008). The *Fusarium oxysporum* f. *coffea* prototype occurring in the infested crop field soil, and in infested coffee plant roots, was previously isolated according to Burgess et al., 2008, and taxonomically assigned as representative of the *F. oxysporum* species by 18S rRNA sequencing. Interestingly, its 18S rRNA sequence was found identical with the sequence (OP010081.1) from *F. oxysporum* ZEHFO from Saudi-Arabia, reported as being associated with the wilt disease of Coffee arabica. Its pathogenicity according to Koch´s postulate was proven against coffee trees, which were damaged after treatment with the *F. oxysporum* isolates. Only colonies appearing white cottony and with the typical dark-purple undersurface pigment after growth in PDA, supplemented with streptomycin sulfate (1g/L) and neomycin sulfate (0.12 g/L), were counted. In addition, the morphological features typical for *F. oxysporum* (Burgess et al., 2008) were identified by light microscopy performed with selected colonies grown on carnation leaf or green rice stem agar.

The density of nematodes in soil was estimated six months after treatment with the bioformulations by counting the number of nematodes detected by light microscopy in a defined amount of soil (100 g) according to Hooper et al. (2005). The number of nematodes in plant roots was estimated according to Hooper et al. (2005). The washed and surface disinfected roots were separated from the stem at the soil line, and the number of nematodes in the cut root pieces were counted.

The disease severity index (%) for yellow leaf and root rot (YLRR) disease on coffee trees was calculated as described (Townsend and Heuberger, 1943) according to the formula:

$$Index\ \ of\ \ disease\ \ severity(\%) = \frac{\sum(\alpha \times b)}{N \times T} \times 100$$

where:

- ✓$\sum(axb)$: Sum of the product of the number of infected plants and its respective level of disease.
- ✓ T: The highest level of disease.
- ✓ N: Total number of investigated plants.

For YLRR disease on coffee plants, there are five levels of disease:

Level 0: plant is healthy or not infected.

Level 1: the percentage of yellowing leaves is ≤25%.

Level 2: the percentage of yellowing leaves is between 25% to 50%. The lateral roots and the main root are in part knotted or black rotted. Plant growth is impaired.

Level 3: the percentage of yellowing leaves is between 50% to 75%. Majority of the lateral roots and the main root are knotted or black rotted. Plant growth is heavily impaired.

Level 4: the percentage of yellowing leaves is ≥75%. Plants start to die.

The incidence rate of fast death disease (%) in black pepper plants is defined as:

$$Incidence(\%) = \frac{A}{B} x100$$

where:

A: the number of black pepper plant infected with fast death disease.

B: Number of all investigated black pepper plants

The greenhouse experiments for determining the number of plant pathogens in soil and severity and incidence of the YLRR and fast death disease were performed with ten plants and in three repetitions. The control plants were not treated with the bioformulation. Examples about calculating occurrence of plant pathogens, and severity and incidence of disease are given in Supplementary Tables S12, S13 for Robusta coffee and Supplementary Tables S16, S17 for black pepper. Also here, the greenhouse experiments were performed with ten plants and three repetitions. The details of the corresponding field trials were described in Supplementary Tables S14, S15 (Arabica coffee), and Supplementary Tables S18, S19 (black pepper).

2.8 Data analysis

Except large-scale field trials, the data obtained from biocontrol and plant growth promotion experiments were analyzed using one-factorial analysis of variance (ANOVA). Mean values were calculated from the results of the replicates (n≥3). The Fisher´s least significant difference (LSD) test was conducted as *post-hoc* test for estimating significant differences (p≤ 0.05) between the mean values.

The **formula** for the least significant difference is:

$$LSD_{A,B} = t_{0.05/2,DFW}\sqrt{MSW(1/n_A + 1/n_B)}$$

Where:

t = critical value from the t-distribution table

MSW = mean square within, obtained from the results of the ANOVA test

n = number of scores used to calculate the means.

Every experiment was conducted using a completely randomized design.

3 Results

3.1 Molecular taxonomy and comparative genome analysis of 30 isolates from Vietnamese crop plants representing the *B. subtilis* species complex

3.1.1 Molecular taxonomy revealed that the isolates belong to the *Bacillus subtilis* species complex

Since16S rRNA sequences are often not sufficient for species discrimination, we used the genome sequences for taxonomical assignment. The phylogenetic tree (Figure 1), containing the genomes of the 30 isolates from Vietnamese crop plants and of numerous type strains belonging to the *B. subtilis* species complex, was constructed with the Type (Strain) Genome Server TYGS (Meier-Kolthoff and Göker, 2019). The isolates were distributed in four different clusters, representing the species *B. velezensis*, *B. tequilensis*, *B. subtilis*, and *B. altitudinis*. Within the *B. velezensis* cluster, which contained 27 isolates, three subclusters can be distinguished. Eighteen isolates including TL7 and S1 formed a subcluster closely related with FZB42 (Borriss et al., 2011). Isolates MR2.1A and EG5.1A formed together with the type strain *B. velezensis* NRLL B-41580 (Ruiz-García et al., 2005) a second subcluster. A third branch consisted of five isolates. However, when compared with the type strain NRLL B-41580, their ANIb - and dDDH values were found above the species and subspecies cut off (Supplementary Table S1; Supplementary Figure S5), suggesting that they belong to one subspecies and no further discrimination according to their taxonomic level was necessary.

The isolate DL2.1, which was forming a species cluster together with *B. tequilensis* NCTC 13306 (Gatson et al., 2006), showed values below the subspecies cut off when compared with the type strain NCTCC 13306 (ANIb: 96.89%, dDDH:78.5%), suggesting that the isolate DL2.1 represented a novel subspecies.

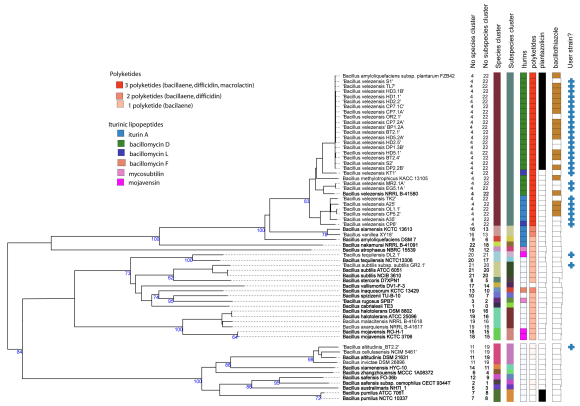

FIGURE 1
Phylogenetic tree inferred with FastMe 2.1.6.1 (Lefort et al., 2015) from GBDP distances calculated from whole genome sequences using the Type (Strain) Genome Server TYGS (https://tygs.dsmz.de). Analysis was performed using both, Maximum Likelihood and Maximum Parsimony, with 31 type strains (labelled by bold letters) and 30 genome sequences obtained from the *Bacillus* strains isolated from Vietnamese crop plants (labelled by the blue crosses at the right). The numbers above branches are GBDP pseudo-bootstrap support values > 60% from replications, with an average branch support of 85.1%. The first two colored columns to the right of each name refer to the genome-based species and subspecies clusters, as determined by dDDH cut-off of 70 and 79%, respectively. *B. tequilensis* DL2.1 represents a subspecies different from that of the type strain NCTC 13306. The clustering yielded 22 species clusters and the provided query strains were assigned to four of these. The third column refers to the iturinic lipopeptide, and the fourth column to the polyketide clusters detected in the genome sequences. The fifth column refers to the occurrence of plantazolicin, and the sixth column to the occurrence of bacillothizole gene clusters. The tree was rooted at the midpoint.

3.1.2 Pan genome analysis of the *Bacillus velezensis* isolates

The functional category analyses of the 27 *B. velezensis* isolates with *B. velezensis* FZB42 as reference revealed 110,824 COG functional categories distributed in core, dispensable, and single genes, A relatively high percentage of around 11% was dedicated to carbohydrate transport and metabolism. Nearly 5% were predicted to be involved in synthesis of secondary metabolites (Supplementary Figure S6). No differences between strains isolated from surface-sterilized plant material (roots, stems, and leaves), and strains isolated from the plant rhizosphere were detected (Supplementary Figure S7).

Singletons were defined as unique genes, not occurring in the other *B. velezensis* strains used for comparison. The majority of singletons were phage genes, and genes involved in synthesis of restriction-modification systems, ComX- pheromones, plasmid replication and mobilization, lantibiotics and other ribosomally synthesized and post-translationally modified peptides (RiPPs). The number of singletons detected in the isolates were obviously not dependent on their life style, and a direct comparison between the endophytic isolates, and the isolates obtained from the

rhizosphere yielded no genes specifically connected with the endophytic life style. The strain with the highest number of singletons (210) was *B. velezensis* TK2, a strain isolated from plant rhizosphere (Supplementary Table S7).

3.1.3 Whole genome analysis of *Bacillus velezensis* BP1.2A and BT2.4 revealed their close similarity with FZB42

In order to characterize the *B. velezensis* subcluster 1, sharing high similarity with FZB42 (Supplementary Table S1), in more detail, two randomly selected isolates, BP1.2A and BT2.4, were chosen for further analysis. Both strains were fully sequenced using a combined approach of two sequencing technologies which generated short paired-end reads obtained with Illumina HiSeq and long reads obtained with the Oxford nanopore MinION sequencing technology. The obtained sequences were then used for hybrid assembly (Blumenscheit et al., 2022). The data describing their general genomic features, summarized in Supplementary Table S2, have been already listed in a recent data paper (Blumenscheit et al., 2022), but were not comprehensively discussed until now.

Despite, that both strains were isolated from different sources (Supplementary Table S1), sequences of both strains were found closely related to *B. velezensis* FZB42, the model strain for Gram-positive, plant-beneficial bacteria, which has been isolated from a very remote area, an infested sugar beet field in Germany (Fan et al., 2017). The Venn diagram (Supplementary Figure S2) showed that the core genome of the three strains harbored a total of 3,633 genes (Supplementary Table S3). Only 75 genes of FZB42 were not detected in strains BP1.2A, and BT2.4. By contrast, 46 genes detected in BP1.2A and BT2.4 did not occur in FZB42. The pan genome formed by the three strains consisted of 3,757 genes (Supplementary Table S4). The chromosome of the isolate *B. velezensis* BP1.2A was used as reference for computing the core genome against *B. velezensis* BT2.4 and *B. velezensis* FZB42 (Figure 2). Genes probably involved in plant growth promotion, degradation of plant macromolecules (Supplementary Table S5), and synthesis of secondary metabolites, are indicated in the circular plot.

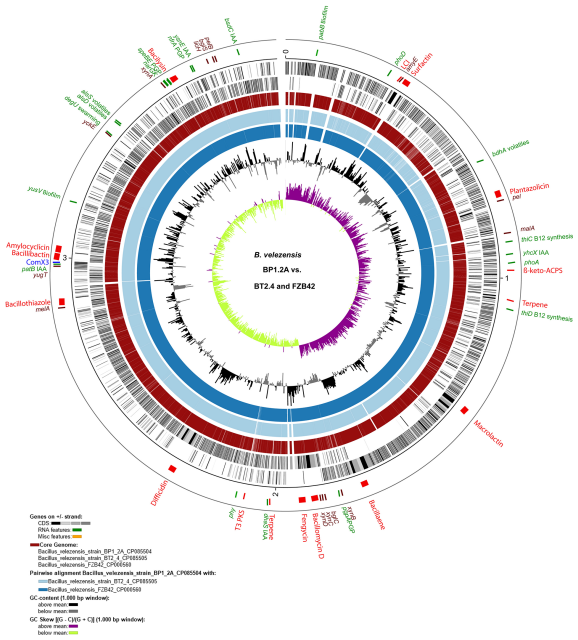

FIGURE 2
Circular plot of the BP1.2A genome (CP085504) compared with BT2.1.4 and FZB42. The plot was generated with BioCircos embedded within the EDGAR3.0 package (Dieckmann et al., 2021). The outer circle shows the position of the 17 BGCs (red), the genes and gene clusters involved in plant growth promotion (green), and of the enzymes degrading carbohydrate macromolecules (dark brown). Homologous regions representing the core genome formed by the three strains are shown in the fourth circle. Circles 5 and 6 represent the pairwise alignments with BT2.4 and FZB42, respectively. The inner circles 7 and 8 represent the GC-content, and the GC-skew [(G-C)/(G+C)] within 1,000 bp windows. Genes involved in plant growth promotion and degradation of plant macromolecules are listed in Supplementary Table S5.

3.1.4 Biosynthetic gene clusters encoding secondary metabolites

Genome mining using the software pipelines of antiSMASH and BAGEL4 was performed with the genomes of all the 30 isolates representing the *B. subtilis* species complex. Our survey yielded 17 gene clusters involved in biosynthesis of secondary metabolites in *B. velezensis*, which were found distributed within different parts of the genomes. Table 1 gives an overview about their position in the genomes of BP1.2A, BT2.4, and, for comparison, FZB42. Two of them, plantazolicin (Scholz et al., 2011), and the recently described bacillothiazole (Shen et al., 2022), were found to be not generally conserved throughout the species *B. velezensis*, but occurred sporadically in genomic islands, probably acquired by horizontal gene transfer (Figure 3). The gene cluster involved in biosynthesis of plantazolicin was detected in the genomic island of FZB42 covering region 732,136 – 736,434. The plantazolicin gene clusters, occurring in BP1.2A, and BT2.4, were detected in the corresponding regions (Supplementary Table S6). The bacillothiazole gene cluster occurred in all three strains in the genomic islands, and was covering the regions 2,864,692 – 2,888,497 (BT2.4), 2,865,076 – 2,882,593 (BP1.2A), and 2,868,284 – 2,887,865 (FZB42), respectively.

Fifteen "canonical" gene clusters were detected in the 27 *B. velezensis* isolates (Figure 4). They were responsible for non-ribosomal synthesis of the polyketides macrolactin, bacillaene, and difficidin, the lipopeptides surfactin, fengycin, and iturin-like compounds, the NRP-siderophore bacillibactin, and the dipeptide bacilysin. Biosynthesis of three different iturinic lipopeptides was predicted: The *B. velezensis* isolates, closely related to FZB42, harboured the gene cluster predicted to synthesize bacillomycin D (BGC0001090). Some of the more distantly related *B. velezensis* isolates harbored gene clusters predicted to be involved in the synthesis of iturin A (BGC0001098) and bacillomycin L, respectively (Supplementary Figure S8).

The gene clusters for ribosomal synthesis of RiPPs, such as LCS, and amylocyclicin were found in every *B. velezensis* isolate (Supplementary Figure S9). Well conserved in all *B. velezensis* isolates were also the clusters containing the genes for ß-keto-ACPS (PKS-like), and iterative type III polyketide synthesis, for synthesis of two different terpenes, and for synthesis of four

TABLE 1 Detection of 17 gene clusters involved in synthesis of secondary metabolites (BGCs) using antiSMASH (Blin et al., 2021) and BAGEL4 (van Heel et al., 2018) in the genomes of *B. velezensis* BP1.2A (CP085504), and *B.velezensis* BT2.4 (CP085505).

Region	BP1.2A (CP085504.1)	BT2.4 (CP085505.1)	FZB42 (CP000560.2)	Similarity MIBIG/NCBI	
LCI (antimicrobial peptide)	296,346 - 316,483	296,348 - 316,483	300,862 - 320,997	100%	Bacillus BAGEL4
Surfactin (NRP, lipopeptide)	318,208 - 383,067	318,208 - 383,067	322,723 - 387,582	95%	BGC0000433
Plantazolicin 91.1 RiPP : LAP	717,159 - 740,336	717,099 - 740,276	721,674 744,851	100%	BGC0000569 BAGEL4
ß-keto-ACPS PKS-like	935,682 - 976,926	935,298 - 976,542	940,739 - 981,983	100%	Bacillus
Terpene Squalene/phytoene	1,062,552 - 1,079,781	1,062,168 - 1,079,397	1,074,783 - 1,075,523	100%	Bacillus
Macrolactin H Polyketide	1,366,841 - 1,453,226	1,366,457 - 1,452,842	1,371,897 - 1,458,282	100%	BGC0000181
Bacillaene polyketide + NRP	1,676,755 - 1,777,357	1,676,371 - 1,776,973	1,681,811 - 1,782,413	100%	BGC0001089
Bacillomycin D NRP + polyketide	1,866,123 - 1,903,373	1,865,739 - 1,902,989	1,871,179 - 1,908,429	100%	BGC0001090
Fengycin NRP	1,907,878 - 1,963,948	1,918,319 - 1,963,564	1,923,759 - 1,969,004	100%	BGC0001095
Terpene Sporulene	2,010,880 - 2,032,763	2,010,496 - 2,032,379	2,024,219 - 2,026,102	100%	Bacillus
T3PKS type III-PKS	2,099,249 - 2,140,349	2,098,865 - 2,139,965	2,102,588 - 2,143,688	100%	Bacillus
Difficidin polyketide	2,269,142 2,362,931	2,268,758 - 2,362,547	2,344,012 - 2,286,309	100%	BGC0000176
Bacillothiazole NRP	2,851,295 - 2,900,808	2,850,911 - 2,906,712	2,873,990 - 2,884,225	100%	BGC0002641
ComX3 320.1 pheromone	2,994,084 - 2,994,275	2,999,980 - 3,000,171	2,997,539 - 2,997,712	100%	*B. velezensis* BAGEL4
Bacillibactin NRP siderophore	3,017,800 - 3,024,927,	3,023,696 - 3,030,823	3,021,021 - 3,033,995	100%	BGC0000309
Amylocyclicin RiPP head to tail cycl.	3,039,655 - 3,045,228	3,045,551 - 3,051,124	3,043,470 - 3,049,481	100%	BGC0000616 BAGEL4
Bacilysin other	3,574,134 3,615,552	3,580,030 - 3,621,448	3,593,882 - 3,599,780	100%	BGC0001184

For comparison FZB42 (CP000560.2) was also analyzed. Similarity to known metabolites listed in the MIBiG 3.0 repository (Kautsar et al., 2020) is indicated.

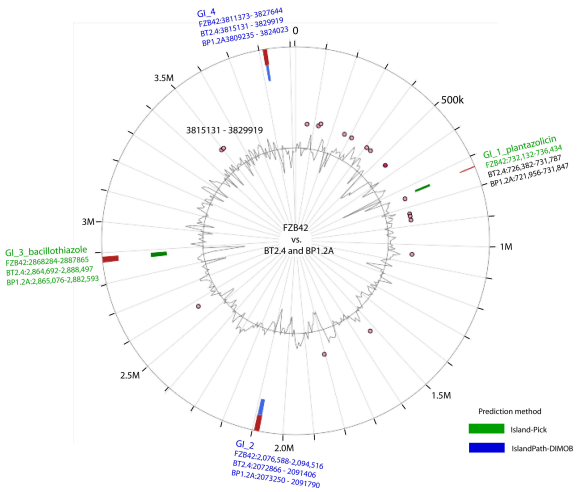

FIGURE 3
Occurrence of the genomic islands (GIs) in the chromosomes of *B. velezensis* BP1.2A, and *B. velezensis* BT2.4 using IslandViewer 4 (Bertelli et al., 2017). *B. velezensis* FZB42 is shown for comparison. Predicted GIs are shown as blocks colored according to the prediction method; Island Pick (green), Island-Path-DIMOB (blue), as well as the integrated results (dark red). The grey line within the inner circle shows deviations of the average GC-content. The genes present in the GIs are listed in Supplementary Table S6.

different types of the ComX pheromone (Supplementary Figure S10). As expected, the occurrence of the gene clusters involved biosynthesis of the RiPP plantazolicin, and the NRP-bacillothiazoles varied within the *B. velezensis* isolates. The BGC for plantazolicin synthesis was only detected within the closest relatives of FZB42, whilst the BGCs for bacillothiazole appeared more scattered in different isolates.

Six additional gene clusters, not occurring in FZB42, BT2.4, and BP2.1A, were identified in other *B. velezensis* isolates. Two uncharacterized gene clusters involved in non-ribosomal synthesis of NRP, and NRP-PK hybrids were detected in *B. velezensis* OL1, CP5.2, and MR2.1A (Supplementary Figure S11).

Two uncharacterized biosynthetic gene clusters were detected in *B. altitudinis* BT2.2 (Supplementary Figure S12): The NRP-independent siderophore cluster contained genes with similarity to schizokinen (BGC0002683), and a non-ribosomal peptide gene cluster harbouring genes similar to the genes present in BGC0000381 responsible for synthesis of the surfactant lichenicidin in *B. licheniformis*.

RiPPs of the sactipeptide type were found in most *B. velezensis* strains, but did not occur in FZB42, BP2.1A. and BT2.4 (Supplementary Figure S13). BGCs predicted to synthesize different types of lanthipeptides were detected in *B. subtilis* and *B. velezensis* (Supplementary Figure S14). The subtilomycin A

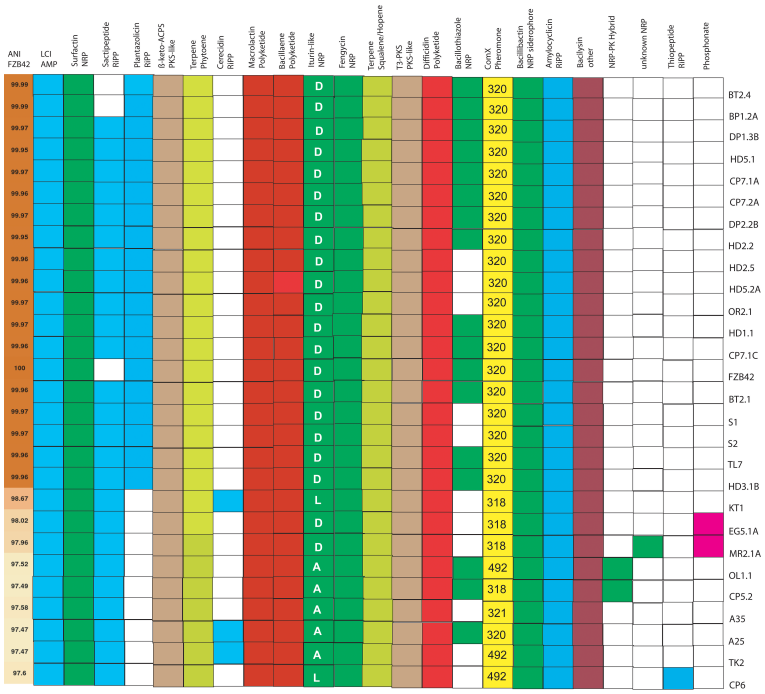

FIGURE 4
Occurrence of 23 biosynthetic gene clusters encoding secondary metabolites in 27 *B. velezensis* isolates obtained from Vietnamese crop plants. Representatives of cyclic iturinic lipopeptides were bacillomycin D (D), bacillomycin L (L), and iturin A (A). Gene clusters for synthesis of four different types of ComX pheromones (318, 320, 321, 492) were detected with BAGEL4. The first row shows the ANIb values of the *B. velezensis* genomes when compared with FZB42.

(lanthipeptide class I) gene cluster (BGC0000560) was detected in *B. subtilis* GR2. *B. velezensis* isolates harboured BGCs predicted to synthesize lanthipeptides representing class II (lichenicidin) and class IV (andalusin A). Other lanthipeptides, such as the thiopeptide thiocillin (*B. velezensis* CP6) were also detected (Supplementary Figure S15).

Known and hitherto unknown RiPPs were detected in *B. altitudinis* BT2.2. The head-to-tail cyclized pumilarin resembled amylocyclicin in *B. velezensis*. Another head-to-tail cyclized peptide (BhlA/UviB family) was similar to enterocin-48 from *Enterococcus lactis*. The leaderless class II bacteriocin aureocin A53 exhibited similarity to lacticin from *Lactococcus lactis* (Supplementary Figure S16). The BGCs responsible for synthesis of phosphonate were detected in *B. velezensis* EG5.1A, and MR2.1A (Supplementary Table S8).

3.2 Detection of bioactive peptides by MALDI-TOF mass spectrometry

The mass-spectrometric detection of bioactive peptides revealed that the *B. velezensis* isolates were able to synthesize the lipopeptides of the iturin family, either bacillomycin D or iturin A, the fengycins, and surfactin (Koumoutsi et al., 2004), the siderophore bacillibactin (Chen et al., 2009b), and plantazolicin (Scholz et al., 2011, Supplementary Table S8). Cyclic lipopeptides produced by FZB42, and other representatives of the *B. velezensis* species are known for their strong action against plant-pathogenic fungi (Gu et al., 2017), and to enhance plant defense responses such as induced systemic response (ISR, Chowdhury et al., 2015). Plantazolicin has been reported as being able to suppress plant-pathogenic nematodes (Liu et al., 2013). Most members of the *B. velezensis* subcluster 1 produced detectable amounts of surfactin, bacillomycin D, and plantazolicin. Figure 5 presents as a representative example of subcluster 1 the metabolic profile obtained from the isolate *B. velezensis* S1 grown on Landy agar, and further processed as described in Materials and Methods. Under these conditions, several bacillomycin D species containing lipopeptide chains of variable length, ranging from C14 to C16, were detected as the dominating compounds in surface extracts of S1.

The following bacillomycin D species were detected:

- C14-bacillomycin D: [M +H; Na;K]+ = 1031.7/1053.6/1069.6;
- C15-bacillomycin D: [M + H;Na;K]$^+$ = 1045.7/1067.6/1083.6;
- C16- bacillomycin D: [M +H; Na;K]$^+$ = 1059.7/1081.6/1097.7.

Surfactin was represented by:

- C14-surfactin: [M + Na : K]$^+$ = 1044.7/1060.7:
- C15-surfactin: [M + Na : K]$^+$ = 1058.7/1074.7.

The detected fengycins A and B were:

- C15-fengycin A: [M +H;Na;K]$^+$= 1449.9/1471.9/1487.9;
- C16-fengycin A: [M +H;Na;K]$^+$= 1463.9/1485.9/1501.9;
- C17-fengycin A: [M +H;Na;K]$^+$= 1477.9/1499.7/1515.9;

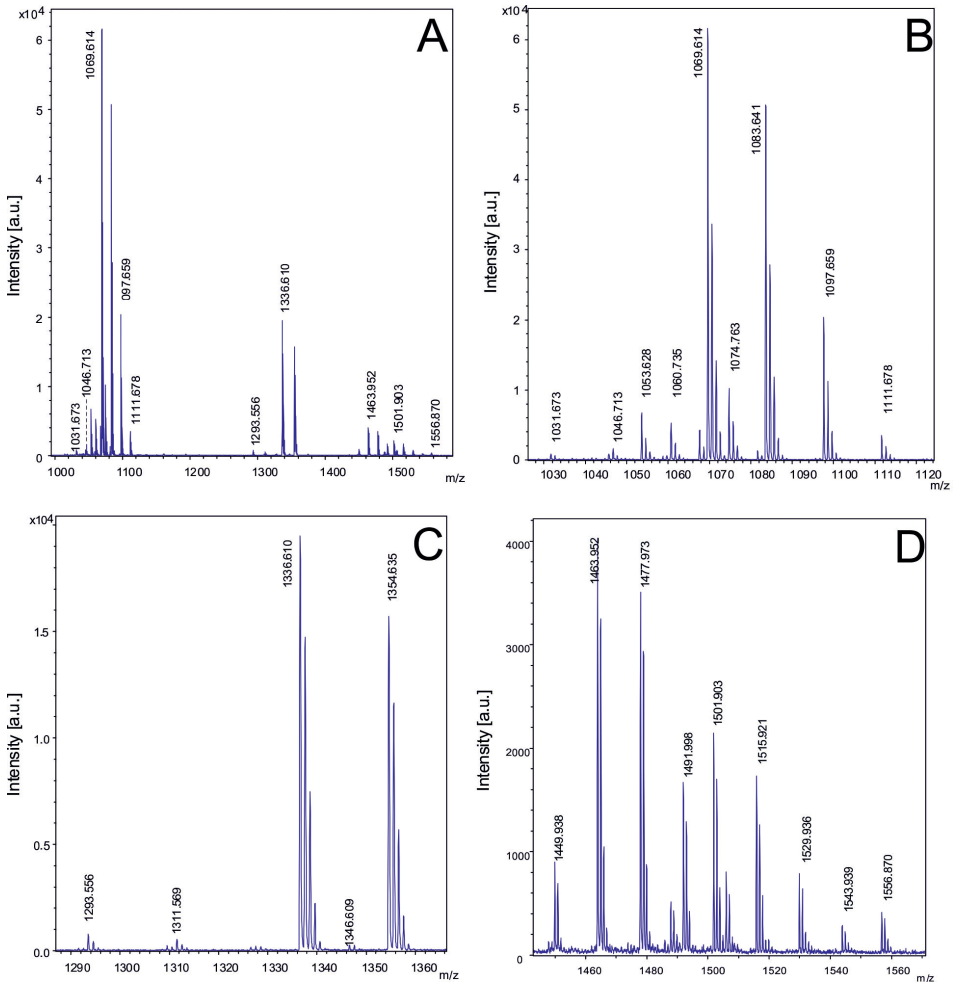

FIGURE 5
Detection of bioactive peptides produced by *B. velezensis* S1. **(A)** MALDI-TOF mass spectrum in the mass range from *m/z* = 1000 – 1500 of a cell-extract of strain S1 showing bacillomycin D species as the most prominent signals. Figures B, C, D show extended views of the signals visible in Figure 6A. **(B)**: Mass spectrum of C14-, C15- and C16 bacillomycin D, and C14 and C15-surfactin in the mass range from *m/z*=1000 to 1120. **(C)**: Mass spectrum of plantazolicin and its hydrated form with mass numbers of *m/z* = 1336.6 and 1354.6. **(D)**: Mass spectrum of the C14 - C17 fengycin species in the mass range *m/z* = 1400-1550. *B. velezensis* S1 was grown on Landy-agar for 48 (h) Cell material was picked from agar-plates, extracted with 50% ACN/0.1%% TFA, and processed as described under Materials and methods.

- C16-fengycin B: $[M + H,Na,K]^+$ = 1491.9/1513.9/1529.9.

Plantazolicin and its hydrated form were also detected:

- plantazolicin: $[M + H]^+$ = 1336.6/1354.6.

Corresponding results were obtained with the other representatives of subcluster 1 including isolate TL7. It was speculated that variations in the fatty acid chain-length of the lipopeptides affect their biological activity (Ramachandran et al., 2017).

3.3 *Bacillus velezensis* strains are highly efficient in suppressing plant pathogens and promoting plant growth

28 isolates, belonging to the *B. subtilis* species complex, were tested for their ability to promote plant growth in pot experiments performed with the model plant *Arabidopsis thaliana*. The *B. velezensis* isolates S1, S2, TL7, A35, and HD1.1 were found to stimulate plant growth based on biomass production by more than 25%. By contrast, the inoculation with the representatives of other species, such as *B. altitudinis* BT2.2, *B. subtilis* GR2.1, and *B. tequilensis* DL2.1 had no significant effect on growth of the *Arabidopsis* plantlets (Figure 6A; Supplementary Table S9).

Root-knot-forming nematodes of the genus *Melodogyne* are the main causative agents for the YLLR disease of coffee, and fast death disease of black pepper plants (Nguyen et al., 2016). A first screening for nematicidal activity was performed with the model nematode *Caenorhabditis elegans*, applying both the slow, and the fast killing-assay (see Materials and Methods). *B. velezensis* isolates TL7, S1, S2, and HD5.2A were found most efficient in killing *C. elegans* under *in vitro* conditions (Figure 6B; Supplementary Table S10).

In planta experiments performed with tomato plants infested with second stage juveniles of *Meloidogyne* sp., isolated from roots

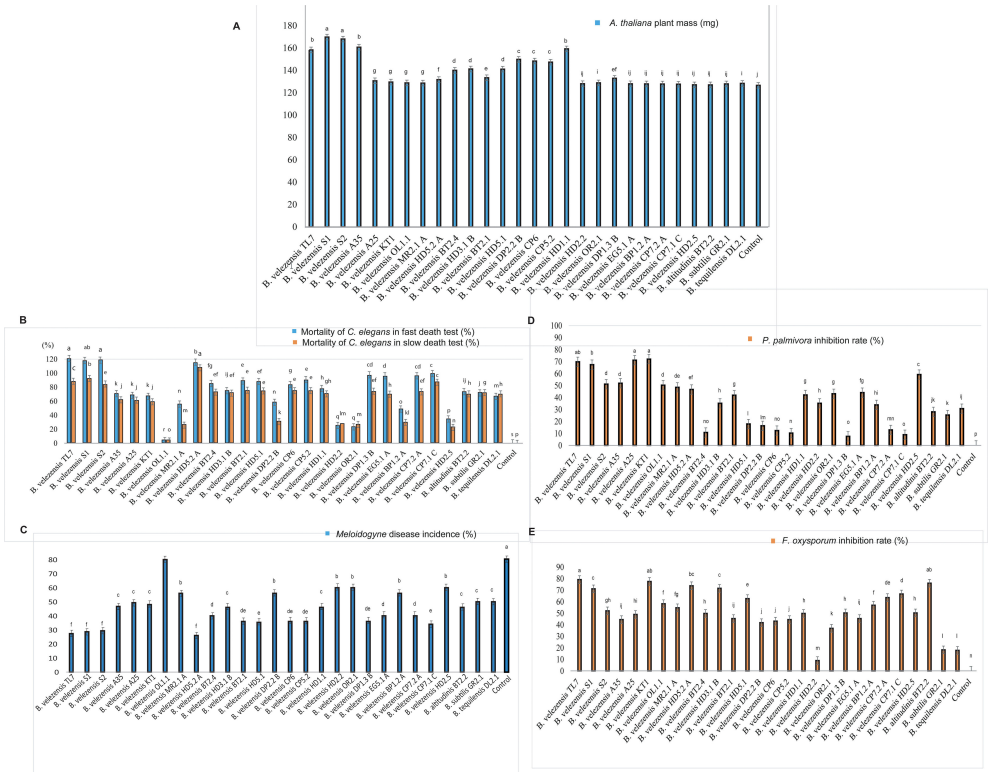

FIGURE 6
Strain evaluation for growth promotion, antagonistic and nematicidal activity. **(A)**: *Arabidopsis thaliana* growth promotion assay (Budiharjo et al., 2014). The columns in the diagram represent the fresh weight obtained after 21 days growth under controlled conditions in the growth chamber. **(B)**: Bioassay with *Caenorhabditis elegans*. Slow killing activity (slow death test) was determined on NGM plates, and fast killing activity (fast death test) in liquid medium. **(C)**: Determination of the biocontrol action of the *Bacillus* isolates on the root-knot nematode *Meloidogyne* sp. in greenhouse experiments. Tomato plants infested with *Meloidogyne* sp. were used for the test (counting of "knots" in tomato roots). The increase compared to the control without adding with the Bacillus isolate is shown. **(D)**: *In vitro* assay of antagonistic activity against *Phytophthora palmivora*. **(E)**: *In vitro* assay of antagonistic activity against *Fusarium oxysporum*. All diagrams showed the means of at least three replicates (n≥ 3). Negative controls were performed without treatment with the bacteria. Columns with superscripts with the same letter are not significantly different according to Fisher´s Least Significance Difference (LSD) Test ($p \leq 0.05$). The LSD values were indicated as bars above the columns.

of pepper plants, revealed that treatment with the isolates *B. velezensis* TL7, S1, S2, and HD5.2A were most efficient. They decreased the number of root-knots in the tomato plants infested with *Meloidogyne* sp. by around 65% (Figure 6C; Supplementary Table S11).

All isolates suppressed different plant pathogens known as the causative agents of important diseases in Vietnamese coffee and black pepper cultures, such as *Fusarium oxysporum*, and *Phytophthora palmivora*. *In vitro*-assays performed with all isolates revealed that the members of the *B. velezensis* species developed a strong antagonistic activity against the fungal pathogen *F. oxysporum*, and the oomycete *P. palmivora* Thereby, the *B. velezensis* isolates TL7, S1, and KT1 were found most efficient (Figures 6D, E; Supplementary Table S10).

In summary, the *B. velezensis* isolates TL7 and S1 were found very efficient in promoting growth of the model plant *A. thaliana*, and in suppressing the main causative agents of Vietnamese crop plant diseases *F. oxysporum* (Figure 7A), *P. palmivora* (Figure 7B), and *Meloidogyne* sp. (Figure 7C). Both strains were selected for the further investigations performed in greenhouse and field trials.

3.4 Greenhouse and field trials performed with the *Bacillus velezensis* isolates TL7 and S1 corroborated their efficiency in stimulating growth and harvest yield under pathogen pressure

Due to their high biocontrol and plant growth-promoting activity, bioformulations of the endophytic *B. velezensis* TL7, and the soil borne *B. velezensis* S1 were chosen for the treatment of the coffee and black pepper plants in greenhouse and large field trials.

3.4.1 Treatment with *Bacillus velezensis* S1 and TL7 reduced the pathogen pressure in coffee and black pepper plants

The effect of treatment with *B. velezensis* TL7 and *B. velezensis* S1 (7×10^{10} cfu/plant) on coffee trees infested with *Meloidogyne* sp., and *F. oxysporum* was investigated with Robusta coffee trees grown in greenhouse, and with Arabica coffee trees grown in one-ha mountain field plots. In the large field trials with 6,250 plants, 4×10^{14} cfu ha^{-1} of bioformulations were applied. The results obtained in greenhouse and field trials matched very well with

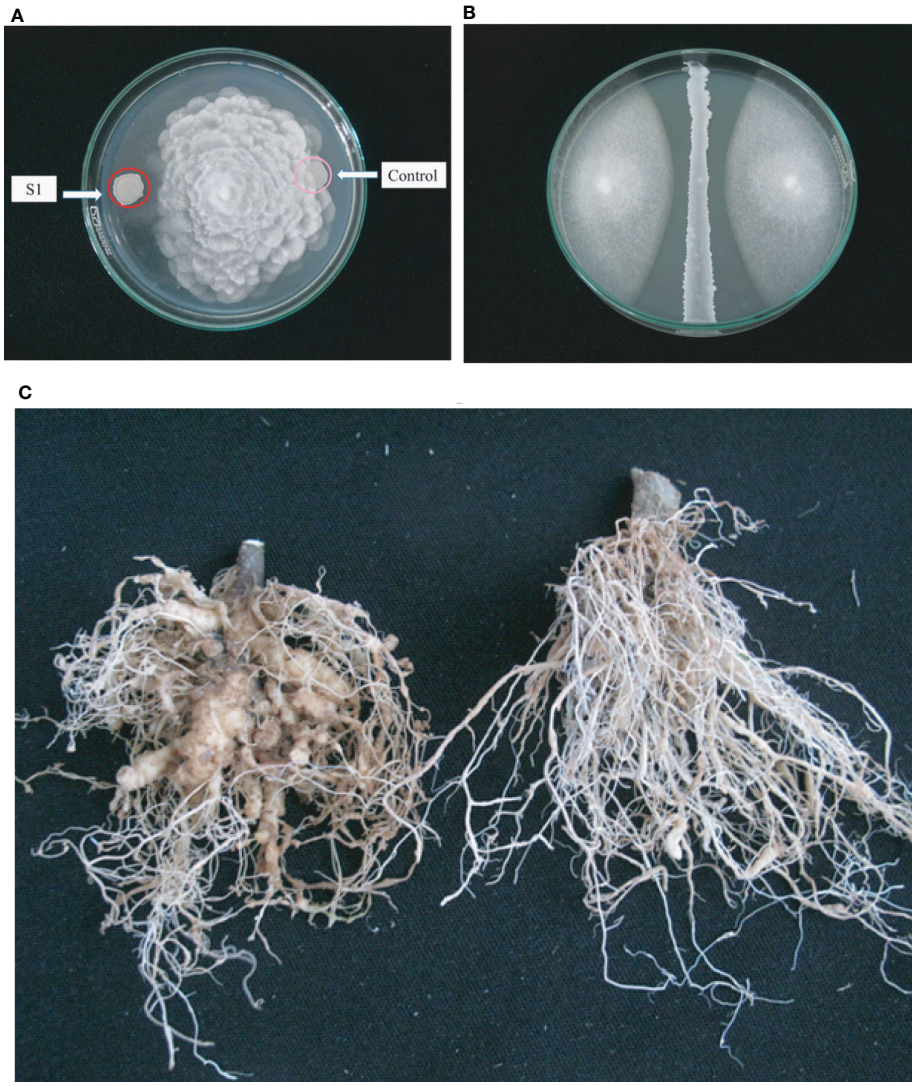

FIGURE 7
Antagonistic activity of *B. velezensis* S1 against fungal pathogens and nematodes. **(A)**: Qualitative assay of antagonistic activity of S1 (left side) exerted against the oomycete *P. palmivora* is indicated by growth inhibition of *P. palmivora* next to the bacterium. **(B)**: Semiquantitative assay of the inhibiting activity of S1 against *Fusarium oxysporum*. The diameter of the two fungal colonies growing to the left and to the right of the bacterial central line was found reduced compared to the control growing without bacteria. **(C)**: Demonstration of nematicidal activity. Formation of knots caused by *Meloidyne* sp. in tomato roots (left) is suppressed after treatment with *B. velezensis* S1 (right).

each other: By treatment with the S1 bioformulation the number of *F. oxysporum* and of *Meloidogyne* sp. in the rhizosphere soil was reduced by more than 60%, whilst treatment with TL7 had no significant effect. However, the number of root-knots in coffee trees infested with *Meloidogyne* sp. was found to be drastically reduced after treatment with *B. velezensis* S1 and *B. velezensis* TL7, as well. Interestingly, the prevention rate of root-knots was found slightly higher, when the plants were treated with the endophytic TL7 before infested with the nematodes (Figures 8A, C; Supplementary Tables S12, S14).

For calculation of the disease index the plant phenotype (percentage of yellow leaves, occurrence of black rot on roots), and the number of root-knot-forming nematodes - but not the number of *F. oxysporum* - in the rhizospheric soil was used. According to this definition, the index reduction observed in plants treated with *B. velezensis* TL7 was similar as in plants treated with *B. velezensis* S1, despite that the treatment with *B. velezensis* S1 had a much higher impact on the presence of *F. oxysporum* in soil than the treatment with *B. velezensis* TL7 (Figures 8B, D; Supplementary Tables S13, S15).

The effect of the treatment with the bioformulations obtained from TL7 and S1 on the black pepper plants (Vinh Linh variety) infested with *P. palmivora*, and *Meloidogyne* sp. was investigated in greenhouse and large field trials as well. Also here, the results obtained in greenhouse and field trials matched very well.

Treatment of pepper plants with *B. velezensis* S1 formulation resulted in a strong decrease (around 70%) of *P. palmivora* in the soil samples obtained in vicinity of the black pepper plant roots. The occurrence of *Meloidogyne* sp. in roots was found to be reduced in the same range (60% – 70%) after treatment with the *B. velezensis* formulations (Figures 8E, G; Supplementary Tables S16, S18). The incidence of black pepper plants infested with the fast

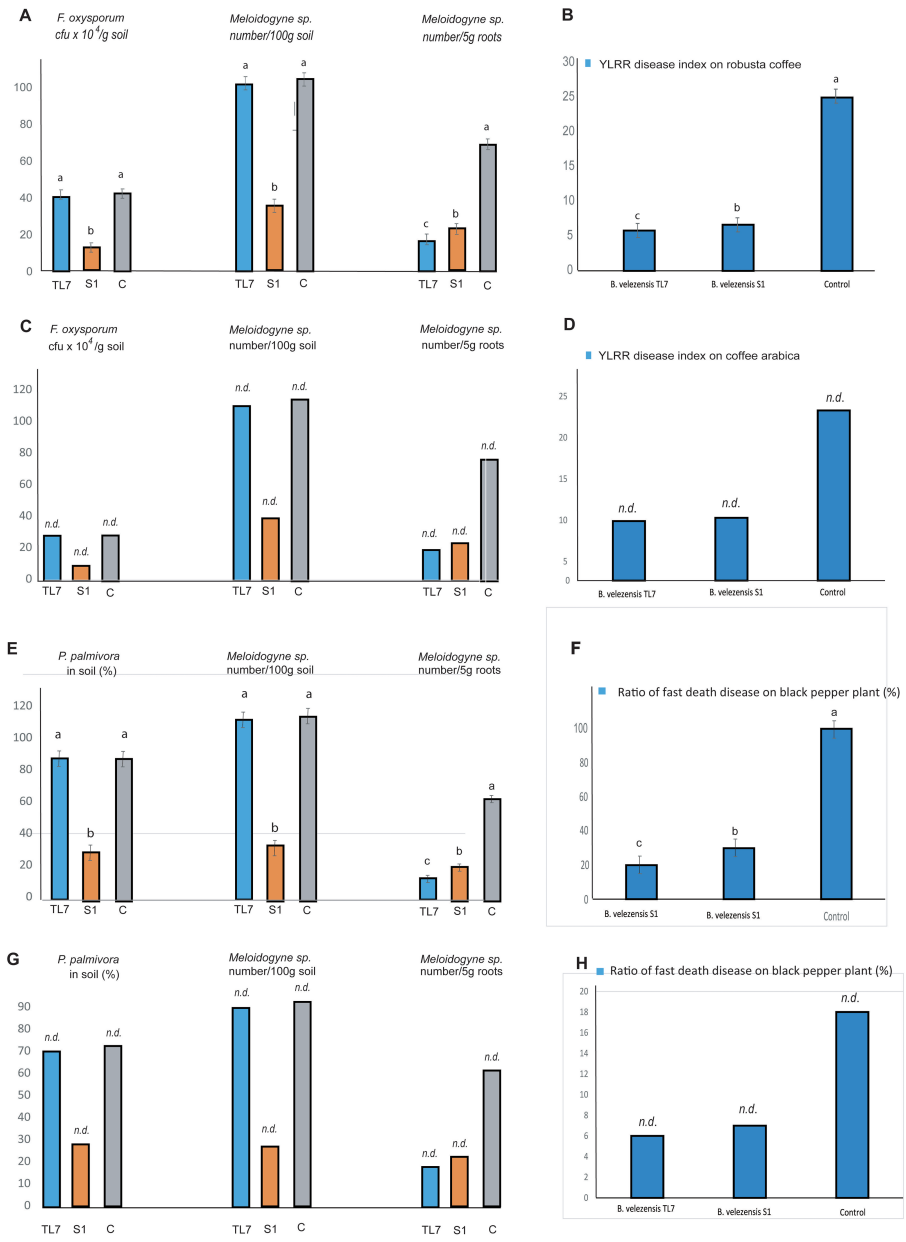

FIGURE 8
Effect of the treatment with *B. velezensis* TL7 and *B. velezensis* S1 on pathogens in coffee trees **(A–D)** and black pepper plants **(E–H)**. Samples were taken from the soil in vicinity of plant roots ("rhizospheric soil") and the roots directly. The presence of the oomycete *Phytophthora palmivora* in soil was estimated indirectly by using the rose flower decolorizing test as described in Materials and Methods. **(A)**: Effect on the number of *F. oxysporum* (cfu x 10^4/g soil) present in the rhizosphere and of *Meloidogyne* sp. (nematodes/5g root) present in the rizosphere and the roots of Robusta coffee trees (Vinh Linh variety) grown in greenhouse. The rate of reduction in comparison to the control without treatment is also presented. **(B)**: The YLRR disease index of Robusta coffee trees (Vinh Linh variety) grown in greenhouse, and treated with *B. velezensis* formulations was determined as described by Nguyen et al. (2016). **(C)**: Effect of inoculation with *B. velezensis* TL7 and S1 on the number of *F. oxysporum* (cfu x 10^4/g soil) and *Meloidogyne* sp. (nematodes/100g soil and 5g root, respectively) infesting Arabica coffee trees grown in one-ha plots located in the Cau Dat, Xuan Truong, Da Lat, Lam Dong-mountain region. Five randomly selected areas containing six coffee trees each (a total of 30 plants) were used for analysis of the three variants (TL7, S1, and control). **(D)**: The YLRR disease index of Arabica coffee trees grown in one-ha plots located in the Cau Dat, Xuan Truong, Da Lat, Lam Dong mountain-region, and treated with the *B. velezensis* formulations. Five randomly selected areas containing a total of 100 plants were used for analysis of the three variants (TL7, S1, and control). **(E)**: Effect on the occurrence of *P. palmivora*, and the number of *Meloidogyne* sp. (nematodes/100g soil and 5g root, respectively) in black pepper plants grown in the greenhouse. The rate of pathogen reduction in comparison to the control without treatment is also shown. **(F)**: The incidence of fast death disease in black pepper plants grown in green-house was calculated as the quotient of the number of plants with symptoms of the fast death disease and the total number of black pepper plants multiplied with 100. A strong decrease of infested plants after treatment with *B. velezensis* TL7 and *B. velezensis* S1 was registered. **(G)**: Effect on the occurrence of *P. palmivora* and the number of *Meloidogyne* sp. in black pepper plants grown in 1 ha field plots in Chu Se, Gia Lai, Viet Nam. **(H)**: The incidence of fast death disease in black pepper plants grown in 1 ha field plots in Chu Se, Gia Lai, Viet Nam was drastically reduced after treatment with *B. velezensis* TL7 and *B. velezensis* S1. The mean values from the greenhouse experiments (n≥3) were depicted as columns. The error bars indicate the values calculated for the least significant difference (LSD). Bars with superscripts with the same letter are not significantly different according to Fisher´s LSD Test ($p \leq 0.05$). The LSD values were indicated as bars at the top of the columns. Statistical analyses of the large-scale field trials (one ha plot per variant) were not performed (indicated on the bars by *n.d.*).

disease was lowered by 60-80% when treated with the *B. velezensis* bioformulations (Figures 8F, H; Supplementary Tables S17, S19).

3.4.2 Treatment with *Bacillus velezensis* S1 and TL7 enhanced growth and harvest yield in coffee and black pepper plants

The greenhouse experiments performed with Robusta coffee trees demonstrated a significant increase of plant growth parameters, such as height, diameter, and chlorophyll content of leaves (SPAD), when the coffee trees were treated with the bioformulations (Figures 9A, D). The plant height was found enhanced by 34% after treatment with TL7, and by 27% after treatment with S1. The plant canopy diameter was enlarged by 32% (TL7), or 20% (S1). After treatment with the bacterial bioformulations, the SPAD values were increased by around 15% (TL7), or 11% (S1), compared to the control without treatment (Supplementary Table S20).

Large field trials performed with Arabica coffee trees in the Central Viet Nam-mountain region (altitude 1,500 – 1,600 m above

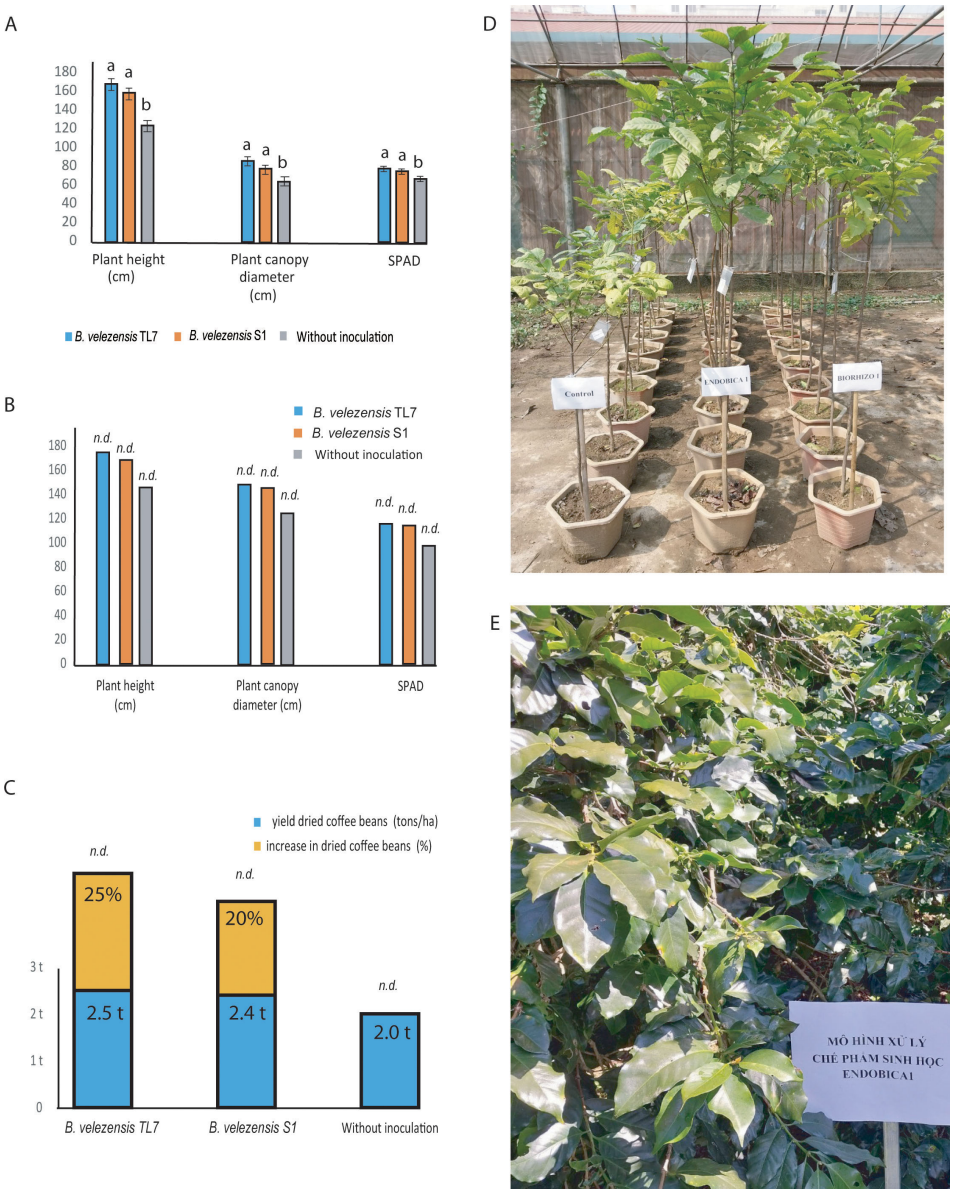

FIGURE 9
Growth promotion and enhancement of harvest yield of coffee trees by treatment with *B. velezensis* TL7 and *B. velezensis* S1 in greenhouse and large-scale field trials. **(A)**: Significant growth promotion of Robusta coffee trees inoculated with *B. velezensis* TL7, and *B. velezensis* S1grown in pots in the greenhouse. **(B)**: Growth stimulation of Arabica coffee trees grown in large field trials after inoculation with *B. velezensis* TL7, and *B. velezensis* S1 bioformulations. **(C)**: Increase of harvest yield in Arabica coffee trees grown under natural field conditions after treatment with *B. velezensis* TL7, and *B. velezensis* S1 bioformulations. **(D)**: Effect of treatment with *B. velezensis* TL7 (ENDOBICA1) and *B. velezensis* S1 (RHIZOBIO1) on Robusta coffee trees grown in greenhouse. **(E)**: Arabica coffee plantation plot (one ha) in Xuan Truong, Cau Dat, Da Lat, Lam Dong, Viet Nam (11°50'11.0"N 108°32'29.9"E). The plants were treated with a bioformulation prepared from *B. velezensis* TL7 (ENDOBICA1). Statistical analyses of the greenhouse experiments were performed as described in Figure 8. No statistical analysis was performed in large field trials (**B, C**). The bars without statistical analysis were labelled by *n.d.*

sea level) corroborated the results obtained in the greenhouse experiments. Treatment with the bioformulations manufactured from *B. velezensis* TL7, and *B. velezensis* S1 resulted in an increase of the plant size (height and diameter), and of the chlorophyll content of the leaves in the same range as obtained in the greenhouse experiments (Figures 9B, E; Supplementary Table S21). In addition to the growth promoting effect observed for coffee trees treated with the *B. velezensis* bioformulations, a strong increase in the harvest yield of the Arabica coffee trees treated with *B. velezensis* TL7 or S1 was registered. The harvest yield of Arabica coffee beans exceeds the yield of the control without treatment, and the average yield obtained for this coffee variety in this region by 20 – 25% (Figure 9C; Supplementary Table S22).

Likewise, as in coffee trees, the effect of the bioformulations on black pepper plants (variety Vinh Linh) was tested in greenhouse and field trials. Plant growth parameters, such as plant height, number of side-branches, and the chlorophyll content in leaves (SPAD) was found to be enhanced in greenhouse plants treated with the bacteria formulations (Figures 10A, D; Supplementary Table S23). The large field trials (one-ha plots per experimental variant) performed in the Chu Se, Gia Lai-mountain region (700 – 800 m above sea level) confirmed the results obtained in the greenhouse. All growth parameter were found enhanced in a similar range after treatment with *B. velezensis* TL7 and S1 (Figures 10B, E; Supplementary Table S24). Similar as in the coffee trees, the harvest yield on peppercorns was found to be enhanced after treatment with the bioformulations by more than 20% (Figure 10C; Supplementary Table S25).

4 Discussion

Due to the growing concern about utilization of chemically synthesized fertilizers and pesticides in agriculture, and the consumer demand for food security, their substitution by environmentally friendly biologicals, able to improve crop performance, is a pressing need (Stukenbrock and Gurr, 2023). However, partial or complete substitution of agrochemicals by biologicals, for example biocontrol agents and biostimulants, is a cost-intensive and challenging process. Many plant-beneficial microbes, which were found efficient under controlled laboratory and greenhouse conditions, failed or delivered inconsistent results under field conditions (Besset-Manzoni et al., 2019). We described here an approach for selecting suitable microbial candidates for developing efficient bioagents preferentially utilized in Vietnamese coffee, and black pepper production as integral part of the concept of Good Agriculture Practice (Dao et al., 2015). Our selection procedure based on the following steps (i) isolation of endospore forming bacteria from healthy plants grown in pathogen infested crop fields; (ii) *in vitro* test for their efficacy against the main local pathogens negatively affecting coffee and black pepper growth (nematodes, fungi, and oomycetes); (iii) genome analysis from 59 isolates for taxonomical assignment and predicting their potential for synthesizing secondary metabolites efficient against plant pathogens; (iv) in planta assays for determining the potential of 30 representatives of the *B. subtilis* species complex to suppress plant pathogens and to promote plant growth under pathogen pressure. Based on this strategy, two plant associated isolates, TL7 and S1, highly similar in their genome sequence with the biocontrol model strain *B. velezensis* FZB42, were selected and successfully applied in large field trials.

In our previous study (Tam et al., 2020) endospore-forming representatives of different genera and species were isolated from Vietnamese crop plants. Representatives of *Brevibacillus* spp. have a rich arsenal of bioactive peptides. They were found efficient in plant growth promotion and biocontrol in laboratory and greenhouse experiments (Jähne et al., 2023). However, due to their relatively slow growth rate under large scale conditions, manufacturing of bioformulations from Brevibacilli appears not economically appropriate. By contrast, many plant-associated representatives of the *B. subtilis* species complex (Fritze, 2004) were found suitable for large scale production of durable endospores and manufacturing bioagents under economically feasible conditions (Borriss, 2011). In this study we could show, that several *B. velezensis* isolates from Vietnamese crop plants were promising candidates for large-scale application in sustainable agriculture.

In recent years, it became increasingly evident that *B. velezensis* is by far the most important species for developing commercial biocontrol and growth-stimulating agents (Vallejo, 2023). However, for several reasons, this understanding has not been generally recognized. The studies performed by Borriss et al. (2011), and Dunlap (2019) demonstrated that most strains used for commercial agents were registered under inconsistent species names. For example, nine *B. velezensis* strains were registered as *B. subtilis* or *B. amyloliquefaciens*. Misclassification of commercial strains is mainly due to the insufficient species resolution of the members of the *B. subtilis* species complex, when solely based on phenotypic characteristics, and the highly conserved 16S rRNA sequence (Rooney et al., 2009). Despite that the majority of these strains have now been genome-sequenced, and their taxonomical boundaries can be corrected, the companies prefer to keep their "old" species names in order to avoid additional registration efforts and possible confusion of their customers. For example, *B. subtilis* GB03, registered as the biopesticide "Kodiak" by the EPA in 1992, is still referred as *B. subtilis*, despite its close relationship with *B. velezensis* FZB42 was clearly demonstrated (Choi et al., 2014). Comparative field trials performed with 13 strains representing different species of the *B. subtilis* species complex revealed that *B. velezensis* FZB42 exhibits the highest effect on growth and harvest yield of maize and potato plants, thereby surpassing *B. subtilis*, *B. atrophaeus*, and other species (Mülner et al., 2020).

B. velezensis, formerly designated as *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al., 2011), forms together with *B. amyloliquefaciens* (Priest et al., 1987) and *B. siamensis* (Sumpavapol et al., 2010) the "operational group *B. amyloliquefaciens*", whose genome similarities are slightly below the ANI cut off of 95-96% for species delineation (Fan et al., 2017). The three species are distinguished by their life style, and by the number of gene clusters involved in biosynthesis of important secondary metabolites (BGCs, Kautsar et al., 2020). The plant-associated *B. velezensis* FZB42 devotes nearly 10% of its genomic capacity to the synthesis of antimicrobial peptides, and polyketides

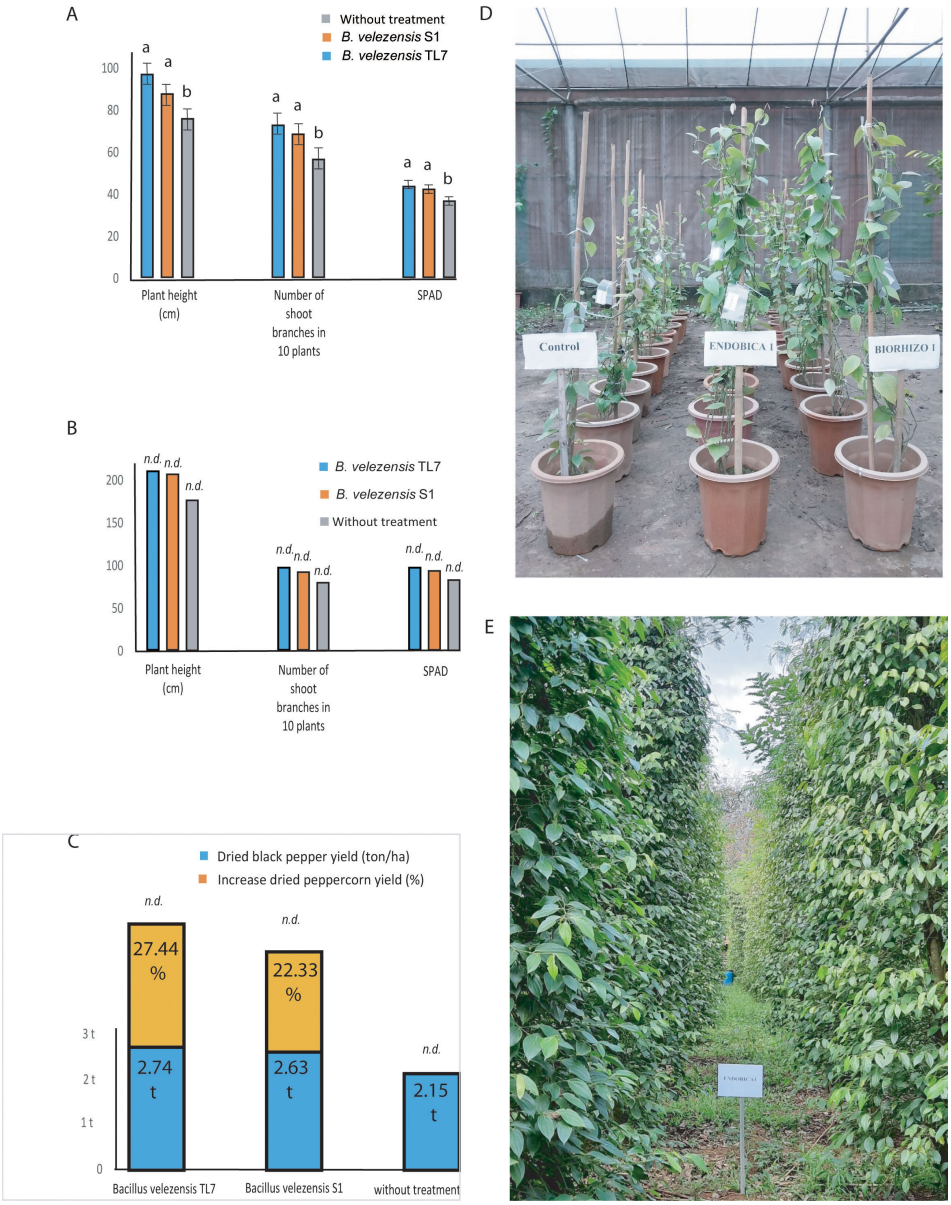

FIGURE 10
Growth promotion and enhancement of harvest yield of black pepper plants treated by *B. velezensis* TL7 and *B. velezensis* S1 in greenhouse and large-scale field trials. **(A)**: Significant growth promotion of the plants inoculated with *B. velezensis* TL7, and *B. velezensis* S1 grown in pots in the greenhouse. **(B)**: Growth stimulation of plants grown in large field trials (Chu Se, Gia Lai) after inoculation with *B. velezensis* TL7, and *B. velezensis* S1 bioformulations. **(C)**: Increase of harvest yield in black pepper plants grown under natural field conditions (one-ha plots, Chu Se, Gia Lai) after treatment with *B. velezensis* TL7, and *B. velezensis* S1 bioformulations. **(D)**: Effect of treatment with *B. velezensis* TL7 (ENDOBICA1) and *B. velezensis* S1 (RHIZOBIO1) on pepper plants grown in greenhouse. **(E)**: Black pepper plantation plot (1 ha), Chu Se, Gia Lai, Viet Nam (13°39'27.6"N 108° 06'42.6"E). The plants were treated with a bioformulation prepared from *B. velezensis* TL7 (ENDOBICA1). Statistical analyses of the greenhouse experiments were performed as described in Figure 8. No statistical analysis was performed in large field trials (10B, 10C). The bars without statistical analysis were labelled by *n.d.*

including bacillaene, macrolactin, and difficidin. It is able to promote plant growth, and to suppress plant pathogens (Chen et al., 2007). By contrast, the closely related soil bacterium *B. amyloliquefaciens* is unable to synthesize the polyketides macrolactin, and difficidin, and does not possess numerous hydrolases, present in *B. velezensis*, which are involved in degradation of plant cell material, such as the ß-1,4 endo-glucanases EglS, and BglC, or the xylanases XylA, and XynA (Rückert et al., 2011, Supplementary Table S26). *B. siamensis* strains were isolated mainly from plant food products. They do not harbor the giant gene cluster for macrolactin synthesis, but are able to synthesize the polyketides bacillaene and difficidin (Fan et al., 2017). During our screening procedure for plant-associated endospore-forming bacteria with potential to suppress plant pathogens, we could only detect representatives of *B. velezensis*, but not the other representatives of the *B. amyloliquefaciens*

operational group, suggesting that the latter species have no important role in the plant microbiome. For this reason, we focused our subsequent work mainly on the representatives of the *B. velezensis* isolates obtained from inside of different plant organs (endophytes), and from the plant rhizosphere (rhizobacteria). However, unique genes connected with the plant-associated life-style were not detected, when the singletons in the genomes of the 27 *B. velezensis* isolates were analyzed. Also functional category analysis revealed no apparent differences between the rhizosphere inhabitants, and the endophytes. We assume, that the ability of endophytes to cross plant barriers might be rather due to the expression level of genes enabling the bacteria to overcome plant stress responses, than to the presence of specific genes only occurring in endophytes.

Genome analysis revealed a striking similarity of the majority of the *B. velezensis* isolates with *B. velezensis* FZB42 (Figure 1), isolated from healthy plants growing within a pathogen-infested German sugar beet field (Krebs et al., 1998), suggesting that bacterial strains harboring closely related genomes can be isolated from very remote geographical regions. A core of 15 biosynthetic gene clusters (BGCs), encompassing clusters for non-ribosomal synthesis of lipopeptides (surfactin, fengycin, iturins), other peptides (bacillibactin, bacilysin), polyketides (macrolactin, bacillaene, difficidin, two unknown T1- and T3-polyketides), ribosomal synthesized peptides such as the LCI antimicrobial peptide, and amylocyclicin, two different terpenes, and the synthesis genes for four different types of the competence pheromone ComX, were detected (Figure 4). In total, 36 BGCs were detected in the genomes of the 30 representatives of the *B. subtilis* species complex investigated in this study (Supplementary Table S8).

Non-ribosomal synthesis of cyclic lipopeptides with antifungal action, such as iturins, and fengycins is common in different representatives the *B. subtilis* species complex (Dunlap et al., 2019). The *B. velezensis* isolates investigated in this study harbored BGCs for synthesis of different iturinic lipopeptides, such as bacillomycin D, iturin A, and bacillomycin L. *B. tequilensis* DL2.1 harbored a gene cluster predicted to encode the iturinic heptapeptide mojavensin. Together with fengycin, these compounds were detected directly in the isolates applying MALDI-TOF MS. We assume, that the antagonistic effect of the *B. velezensis* isolates against the fungal pathogen *F. oxysporum* was mainly due to the production of cyclic lipopeptides, especially bacillomycin D or other iturinic lipopeptides, whilst the suppression of *P. palmivora* might be due to bacilysin. There are many reports about the antagonistic action of cyclic lipopeptides against fungal pathogens including *F. oxysporum* (Koumoutsi et al., 2004; Chowdhury et al., 2015), one of the causative agents of the coffee YLRR disease. Non-ribosomal-synthesized polyketides (Chen et al., 2006), and the dipeptide bacilysin are known for their antibacterial action against the causative agent of fire blight disease on orchard trees, *Erwinia amylovora* (Chen et al., 2009a), and the rice pathogen, *Xanthomonas oryzae* (Wu et al., 2015). Recently, it was demonstrated that bacilysin from FZB42 antagonizes *Phytophthora sojae* and other representatives of the *Phytophthora* genus including *P. palmivora* (Han et al., 2021), one of the causative agents of black pepper diseases.

Besides their direct antagonistic effect on growth of plant pathogens, secondary non-ribosomally synthesized peptides can trigger the plant defense response known as induced systemic resistance (ISR). It has been early shown that purified surfactin, and to a minor extent fengycin, elicited the ISR-dependent plant immune resistance against fungal pathogens (Ongena et al., 2007). Mutants of FZB42, only able to produce surfactin, but no other antimicrobial peptides, were shown to induce systemic resistance against *Rhizoctonia solani* in lettuce plants (Chowdhury et al., 2015). Surfactin of *B. velezensis* was shown to be essential for colonization, biofilm formation on tomato root and leaf surfaces and subsequent protection (ISR) against *Botrytis cinerea* (Stoll et al., 2021).

Two gene clusters predicted to be involved in ribosomal synthesis of RiPPs were found conserved in all *B. velezensis* isolates. The head-to-tail-cyclized amylocyclicin, first described to occur in FZB42, was reported to display a high antagonistic activity against some related Gram-positive bacteria (Scholz et al., 2014). The antimicrobial peptide LCI have strong antibacterial activity against *Xanthomonas campestris*, and *Pseudomonas solanacearum* (Gong et al., 2011). One of the two terpene gene clusters detected in the *B. velezensis* isolates was encoding sporulene, discovered by Kontnik et al. (2008) in *B. subtilis*. The sporulene heptaprenyl lipids, synthesized by squalene-hopene cyclase, contributes to the resistance of spores to reactive oxygen species (Bosak et al., 2008). As in *B. subtilis*, the ability to uptake DNA (genetic competence) is controlled by an isoprenylated small peptide with variable amino acid sequence, the ComX pheromone. The signaling molecule ComX is synthesized as an inactive precursor, and is then cleaved and modified by ComQ before export to the extracellular environment, and its sensing by the ComP-ComA two-component system (Ansaldi et al., 2002). *B. velezensis* isolates possessed variable ComX precursor sequences. Four different variants were distinguished within the 27 isolates, which determine the specificity of the quorum-sensing system within the species (Figure 4; Supplementary Figure S8).

In addition to the 15 BGCs conserved in all *B. velezensis* isolates, eight were found sporadically distributed in some representatives of the species. The *nrs* gene cluster occurred in the genomes of most, but not all *B. velezensis* isolates. The product of the *nrs* gene cluster was unknown for long time, and has been recently identified as bacillothiazole. The compound is non-ribosomally synthesized, and modified by a discrete oxidase encoded by the *nrs* gene cluster (Shen et al., 2022). Most of the BGCs encoded ribosomally-synthesized and post-translationally modified peptides (RiPPs), such as plantazolicin (Scholz et al., 2011), and several classes of lanthipeptides. Plantazolicin was detected in the group of *B. velezensis* isolates, closely related to FZB42, including TL7 and S1 (Supplementary Figure S7). BGCs for synthesis and modification of class ii- and class iv-lanthipeptides were detected in a few *B. velezensis* isolates (Supplementary Figure S12), whilst the gene cluster for synthesis of a sactipeptide (sulfur-to-alpha carbon thioether cross-linked peptides) of unknown function was very common in most of the *B. velezensis* isolates, except BP1.2A, and BT2.4 (Figure 4). The gene cluster was similar to the uncharacterized sactipeptide gene cluster in *B. altitudinis* BT2.2,

and the subtilosin A gene clusters detected in *B. subtilis* GR2.1 and *B. tequilensis* DL2.1 (Supplementary Figure S11). A gene cluster encoding a representative of the thiocillin family RiPP (WP_109955211.1) was detected in *B. velezensis* CP6 (Supplementary Figure S13). Thiocillins have been shown to act as signaling molecules stimulating biofilm formation in *B. subtilis* (Bleich et al., 2015). The *B. altitudinis* BT2.2 genome proved as a rich source of BGCs encoding known and unknown RiPPs, such as the head-to-tail cyclized peptides pumilarin, and the BhlA/UviB peptide, which is resembling the enterocin AS-48 (BGC0000489), and the class ii bacteriocin aureocin A53, which is resembling lacticinQ/lacticin Z (Supplementary Figure S14). Whilst the importance of the non-ribosomal synthesized peptides as direct antagonists of bacterial and fungal pathogens, and as elicitors of plant ISR is without doubt, the function of most RiPPS is still less understood, and seems to be more complex. Besides their possible role in direct competition with the other members of the local microbiome, they might be important for governing the cell behavior, such as biofilm formation, regulating of cell density and genetic competence, and manyfold interactions with the environment.

Besides their suppressing effect on microbial pathogens, significant antagonistic actions of the *B. velezensis* isolates against root-knot nematodes were registered in laboratory and field trial experiments. This is especially important, because the root-knot nematode *Meloidogyne incognita* was identified as being the main causative agent of the coffee and black pepper diseases in Vietnam (Trinh et al., 2022). Different metabolites produced by *B. velezensis* were reported to be responsible for the antagonistic action exerted by this species against nematodes. By screening a random mutant library of FZB42 generated by the mariner transposon TnYLB-1, plantazolicin was identified as being involved in the antinematode effect of this bacterium (Liu et al., 2013). In addition, antinematode compounds recently identified in *B. velezensis* were thymine and the volatile compound hexahydropyrrolo [1,2-a] pyrazine-1,4-dione. The compounds were predicted to interact with acetylcholinesterase (Trinh et al., 2022). Volatiles of *B. atrophaeus* GBSC56, another member of the *B. subtilis* species complex, were shown to stimulate ISR in tomato against *M. incognita* (Ayaz et al., 2021). Nematicidal volatiles of GBSC56, FZB42, and *B. subtilis* SYST2 caused high killing rates due to production of reactive oxygen species (ROS) in the plant parasitic nematode *Aphelenchoides besseyi* (Ali et al., 2023).

Besides their impressive function in biocontrol of plant pathogens, promotion of plant growth was also observed, when the plants were treated with *B. velezensis* TL7 and S1. After an extensive genome analysis, a careful checking of their ability to produce antimicrobial metabolites, and their ability to enhance plant growth, and to suppress the most important local plant pathogens in laboratory scale, we choosed *B. velezensis* strains with different life style, the rhizobacterium S1, and the endophyte TL7, for greenhouse experiments, and large-scale field trials. Both isolates resembled in their genome sequence very much each other and FZB42 (ANI: ≥ 99.96%), but can be distinguished by the presence or absence of the bacillothiazole and the sactipeptide gene cluster (Figure 4). Proving efficacy in large field trials is the number one issue in the development line for better microbial agents having at least the same performance under field conditions as the chemicals they have to replace (Waltz, 2023).

Our greenhouse and field trial results obtained for two important Vietnamese crops, black pepper and coffee trees, demonstrated that treatment with the two selected *B. velezensis* strains, despite of their different plant-associated life-style, had a strong impact on growth and harvest yield. The harvest yields determined under natural farming conditions were found to be increased by more than 20% compared to the untreated control (Figures 9, 10). In a previous study an increase in harvest yield of 4.5% was obtained after a combined application of rhizosphere and endophytic bacteria in black pepper plants growing in selected farms in the Central Highlands of Vietnam (Nguyen et al., 2021). Simultaneously, we could show that growth promotion, and increase of harvest yield were closely connected with the ability of the *B. velezensis* strains TL7 and S1, to reduce the disease rate of the pathogen infested coffee trees, and the black pepper plants. Besides fungal and oomycetes pathogens, our main focus in the large field trials, and also in the greenhouse experiments, was directed on the occurrence of the root knot nematode *Meloidogyne* sp. It ruled out that treatment with the two *B. velezensis* strains resulted in different effects on the presence of the pathogens in the rhizosphere soil. Whilst the endophyte TL7 had virtually none effect on the number of nematodes, and the other pathogens present in the soil in vicinity of the plant roots, application of the rhizobacterium S1 reduced the number of pathogens by ≥ 60%. However, both, the TL7 endophyte, and the S1 rhizobacterium, were found very efficient in suppressing the root-knot nematodes inside of the roots, suggesting that both strains were similar efficient in decreasing the disease rates in black pepper, and coffee trees, as well. Bioformulations containing the endospores from selected *B. velezensis* isolates, applied within a holistic approach of Agriculture Good Manufacturing Practice, will contribute to further diminishing the use of harmful agrochemicals in coffee and black pepper plantations in Vietnam.

5 Conclusion

B. velezensis isolates from healthy Vietnamese crop plants grown in pathogen-infested environments have a high potential to enhance harvest yield of coffee trees, and black pepper plants, also under condition of high pathogen pressure mainly exerted by root-knot nematodes, plant parasitic fungi, and oomycetes. We could demonstrate that after a comprehensive genome analysis, and applying screening procedures for biocontrol and plant growth promotion, it was possible to select promising candidates for large-field trials. We could show that the bioformulations manufactured from durable endospores of the *B. velezensis* isolates TL7, and S1 were able to suppress plant pathogens, and to enhance growth and harvest yield of main Vietnamese crop plants under the conditions of large-field trials performed in their main cultivation regions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

LT: conceptualization, project administration, methodology, data curation, writing-original draft. JJ, PTL, LP, CB, AS, SH, JV, LN, PM: investigation, methodology, validation. JB: software. HT: methodology. MW and TH: formal analysis, validation, PM and TS: data curation. PH and NL: project administration. HT and LC: conceptualization, methodology. PL and TS: conceptualization, supervision, project administration. RB: conceptualization, project administration, writing-original draft, writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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A multi-attribute approach to evaluating the impact of biostimulants on crop performance

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An ever-growing collection of commercial biostimulants is becoming available in a wide variety of forms and compositions to improve crop performance. Given the intricate nature of deciphering the underlying mechanisms of commercial products, which typically comprise various biological components, it is crucial for research in this area to have robust tools to demonstrate their effectiveness in field trials. Here, we took a multi-attribute approach to evaluating the impact of biostimulants on crop performance. First, we assessed the impact of a biostimulant on the soil and rhizosphere microbiomes associated to crops in eight reference farms, including corn (3 farms), soybean (2), cotton (2) and sugarcane (1), in different biomes and production contexts in Brazil and Paraguay. Second, we modeled a set of integrated indicators to measure crop responses to biostimulant application, including five analytical themes as follows: i) crop development and production (9 indicators), ii) soil chemistry (9), iii) soil physics (5), iv) soil biology (6) and v) plant health (10). Amplicon 16S rRNA and ITS sequencing revealed that the use of the biostimulant consistently changes the structure of bacterial and fungal communities associated with the production system for all evaluated crops. In the rhizosphere samples, the most responsive bacterial taxa to biostimulant application were *Prevotella* in cotton; *Prauserella* and *Methylovirgula* in corn; and *Methylocapsa* in sugar cane. The most responsive fungal taxa to biostimulant use were *Arachnomyces* in soybean and cotton; and *Rhizophlyctis* in corn. The proposed integrated indicators yielded highly favorable positive impact indices (averaging at 0.80), indicating that biostimulant-treated fields correlate with better plant development and crop performance. Prominent indices were observed for indicators in four themes: soil biology (average index 0.84), crop production (0.81), soil physics (compaction reduction 0.81), and chemical fertility (0.75). The multi-attribute approach employed in this study offers an effective strategy for assessing the efficacy of biostimulant products across a wide range of crops and production systems.

KEYWORDS

impact assessment, multi-attribute indicators, rhizosphere microbiome, soil microbiome, sustainable agriculture

Introduction

As defined by Yakhin et al. (2017) a biostimulant is "a formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds." Considering the complexity to determine the underlying mechanisms of action of commercial products, which normally are constituted of diverse biological sources and obtained thru varied industrial processes, one important focus of the research in this field should be directed to proof the biostimulant efficacy (Yakhin et al., 2017). However, to determine the biostimulants technology efficacy more quantitative assessments on field trials are needed (Li et al., 2022).

The soil application of biostimulants is expected to impact not only plant performance, but also the soil/rhizosphere microbiomes associated with plants (Backer et al., 2018; Nuzzo et al., 2020). Soil and rhizosphere microbiomes function as extensions of the plant genome, playing a critical role on plant development and protection (Berendsen et al., 2012; Mendes et al., 2013). Microbial inoculants can modify the native soil community composition and structure, potentially altering soil functioning through changes in the soil microbiome (Mawarda et al., 2020). Microbiome modulation through microbial inoculants represents a sound strategy to promote plant development (Berg et al., 2021). Therefore, understanding the impact of biostimulants on microbial communities associated to crop field conditions is essential to assess their efficacy.

Biostimulants have been used in a wide variety of crops, in a whole range of cropping intensification levels, as well as in diverse agricultural production environments. Many studies have brought significant advances in the knowledge of soil biological functioning and the specific roles of different soil fertility attributes, characteristic to the varied types of soils, forms of management, and environmental contexts (Hungria et al., 2009; Lopes et al., 2013; Chamizo et al., 2018). Sets of biological indicators have also been devised to adequately focus on the role of biostimulants as a special kind of soil quality amendment (Mendes et al., 2015). However, in most instances these soil biology indicator sets apply to a partial assortment of variables, generally restricted to microbial activity, enzymatic functions, and soil organic matter composition (de Faria et al., 2021), lacking consideration on crucial aspects related to environmental, economic, and agronomic endpoints, essential for crop management decision-making.

More recent approaches address comprehensive soil health measures, relying on cutting-edge data analyses that include the use of microbiome machine learning for assessing soil health (Chang et al., 2017; Wilhelm et al., 2022). Nonetheless, such approaches may not suffice when a whole crop performance scenario is sought out, as to provide agricultural management recommendations in real farm settings (van Es and Karlen, 2019; Williams et al., 2020). In this sense, comprehensive indicator sets which aggregate crop performance (i.e., above and below-ground plant vigor, stand status, produce quality, productivity, and revenue), soil physicochemical and biological properties, and plant health markers are needed to properly assess the impacts of biostimulant technology and its role toward the sustainability of cropping systems (Doran, 2002). Given the diversity of formats, measurement units, and expression scales involved in such soil-biostimulant-crop performance impact assessment studies, in relation to the diversity of parameters analyzed, there is relative difficulty in aggregating, interpreting, and expressing the varied set of indicators in integrated indices, which would improve the understanding and communication of performance gains, thus favoring decision-making for adoption and expansion of the technology.

In this study, we first verified the impact of the use of a biostimulant on the microbiome associated to several crop systems, which served as a proxy for biostimulant's effectiveness. Then, to address the issue of variability and the absence of standardized indicators for biostimulants impact assessment studies, we proposed a multi-attribute system for integrating soil physicochemical, biological, crop performance and health indicators associated with biostimulant technology use. The proposed indicator system aims to favor the registration, interpretation, and communication of integrated impact and technical performance indices, resulting from analyses obtained on-farm. Field assessments were carried out on reference case studies, in cropping systems with a well-documented history of biostimulant application in different crops, distributed throughout a range of productive regions, encompassing an ample variety of soils and climatic conditions.

Materials and methods

Selection of commercial biostimulant, reference farms and experimental design

As biostimulant, we selected a well-established commercial product with usage history of over 20 years in South America. The product Microgeo® is a biostimulant applied in a wide variety of crops (Gama et al., 2014; Cardoso et al., 2017; de Almeida et al., 2018; da Silva et al., 2020; Suarez et al., 2020; Filho et al., 2021). This technology is based on a continuous liquid compost and consists of locally adapted microorganisms brewed *in situ* in a field-implemented biofactory, under the influence of the organomineral matrix Microgeo® (patent number PI 0207342-0). The product presents 10^7 to 10^9 cells ml^{-1}, diversified among fungi, yeasts, and up to 89% bacteria, the main phyla being *Actinomycetota*, *Bacteroidota*, *Cyanobacteriota*, *Bacillota*, and *Proteobacteriota*. We selected eight farms producing corn (3 farms), soybean (2), cotton (2) and sugarcane (1), located in different biomes and production contexts in Brazil and Paraguay, with history of the biostimulant use. Detailed information on location, climate and biome, size of experimental area, history of biostimulant use, planting and sampling dates are described in Table 1. In each evaluated production system, the biostimulant was tested against the control, i.e., two treatments – biostimulant *vs*

control, in neighboring commercial fields selected as to display as sole contrasting feature the application of the biostimulant. The authorization for soil sampling is registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGen) under number A11C02F.

Soil and rhizosphere microbiome assessment

Soil from crop inter-rows (bulk soil) and rhizosphere were collected from 5 to 20 cm depth. Rhizosphere samples were collected by removing the whole plant root system from soil and gently shaking to remove excess soil from the root system. Then, the root system was placed in plastic bags and vigorously shaken to obtain the soil adhered to the root system, which was used for downstream analyses. Each rhizosphere sampling replicate consisted of a single plant, and the bulk soil sample was collected in the inter-row next to the plant used for rhizosphere sampling. Therefore, two sample types (bulk soil and rhizosphere) and two treatments (biostimulant and control), considering three replicates, were collected across eight production systems (Table 1), resulting in 96 independent samples for microbiome assessment.

Soil and rhizosphere DNA isolation was performed using 0.250 g of soil, which were transferred to 2 mL cell lysis tubes containing glass microbeads (provided by the manufacturer). DNA extraction was performed using the DNeasy Powersoil Pro kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. The isolated DNA was subjected to electrophoresis on a 0.8% agarose gel for integrity analysis. Purity was evaluated on a NanoDrop1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA), using the absorbance ratios of 260/280 and 260/230.

For bacterial community analysis, the hypervariable region V4 of the 16S rRNA gene was amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2010). For fungal community analysis, the ITS1-5F (Internal Transcribed Spacer) region of the rRNA gene was amplified using the primers ITS5-1737F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2-2043R (5'-GCTGCGTTCTTCATCGATGC-3'). After purification of the PCR product with the AMPure XP Beads kit (Beckman Coulter, Life Sciences), Illumina adapters were ligated in a PCR reaction using Nextera XT Index Primer 1 (N7xx) and Nextera XT Index Primer 2 (S5xx). Subsequently, the product of this reaction was purified and quantified using a NanoDrop spectrophotometer for equimolar normalization of the

TABLE 1 Reference farms selected as case studies with a well-documented history of biostimulant adoption, with respective crops, general aspects, biostimulant usage, and sampling information.

Farm	Crop (Cultivar)	Location	Coordinates	Climate zone* and Biome	Area**	Years with Biostimulant Application	Sampling Plant Growth Stage	Planting/ Sampling Dates
Corn_MG	Corn (P3707VYH)	Pirajuba, Minas Gerais, Brazil	19°50'57.1"S 48°43'00.5"W	Cwa, Tropical savannas and shrublands	10,000 ha	3	Stage R3	Apr 21/Jul 21
Corn_GO	Corn (AG 8480)	Inhumas, Goiás, Brazil	17°19'10.4"S 50°53'06.2"W	Aw, Tropical savannas and shrublands	1,608 ha	8	Stage R2	Feb 21/Jul 21
Corn_MS	Corn (AG 8480 PRO3)	Itaporã, Mato Grosso do Sul, Brazil	22°00'30.4"S 54°46'47.3"W	Cfa, Tropical broadleaf forest	1,145 ha	15	Stage R1	Mar 21/Jul 21
Soy_SC	Soybean (NI)	Modelo, Santa Catarina, Brazil	26°45'12.4"S 53°05'51.7"W	Cfa, Tropical broadleaf forest	400 ha	7	Stage R2	Feb 21/May 21
Soy_PY	Soybean (Monsoy 6211 IPRO)	Santa Fé del Paraná, Alto Paraná, Paraguay	25°10'46.9"S 54°37'50.0"W	Cfa, Tropical broadleaf forest	450 ha	15	Stage R2	Mar 21/Apr-Jun 21
Cotton_MT	Cotton (TMG 44 B2RF)	Campo Novo do Parecis, Mato Grosso, Brazil	13°43'32.5"S 57°55'48.2"W	Aw, Tropical broadleaf forest	3,000 ha	5	Flowering	NI/Aug 21
Cotton_BA	Cotton (NI)	Luiz Eduardo Magalhães, Bahia, Brazil	11°30'19.8"S 45°44'08.2"W	Aw, Tropical dry broadleaf forest	13,500 ha	5	Reproductive	Jan 21/May 21
Cane_SP	Sugar cane (RB92 8064)	Ribeirão Preto, São Paulo, Brazil	20°49'34.3"S 47°26'55.3"W	Cwb, Tropical broadleaf forest	1,050 ha	2	Pre-maturation	Apr 18/Jul 21

* Köppen-Geiger classification.
** Total area treated with the biostimulant.
NI, not informed.

concentration. A pool was assembled and quantified by qPCR for validation and determination of the final concentration using the KAPA Library Quantification kit for Illumina (Roche). High-throughput sequencing of the amplicons was performed on the Illumina MiSeq platform (2 x 250 bp), in 2x250 bp runs.

Bioinformatics analyses and statistics

The quality of the raw sequences was checked using the program FASTQC v0.11.5 (Andrews, 2010). Sequences originating from the primers were removed using the Cutadapt v4.2 tool (Martin, 2011). Microbiome analysis was performed using the DADA2 v1.24.0 tool (Callahan et al., 2016), including: removal of low-quality reads (phread <20) and noise (denoising), joining of R1 (forward) and R2 (reverse) sequences, removal of chimeras (using the consensus method), and clustering of representative sequences based on amplicon sequence variants (ASVs). Taxonomic classification was then assigned using the SILVA ribosomal RNA gene database version 138.1 (Quast et al., 2013). Analyses were performed in the R statistical environment (v. 4.2.1) (R Development Core Team, 2014). The taxonomic table containing the count was imported along with the metadata file for analysis in the Phyloseq package (McMurdie and Holmes, 2013) of R. Principal coordinates analyses (PCoA) based on the Bray-Curtis (Bray and Curtis, 1957) distance matrices were performed to evaluate divergence between replicates and samples. Sequencing coverage was evaluated by rarefaction analysis. Alpha diversity indices based on the Chao1 richness estimator (Chao, 1984), observed species, and Shannon-Wiener H' index were calculated by the Phyloseq package of R. Microbial composition was expressed in relative abundance for all taxonomic levels.

The statistical package DESeq2 v1.36.0 (Love et al., 2014) was used to identify differentially abundant microbial groups. DESeq2 applies negative binomial distribution analyses to evaluate differences by comparing two samples in triplicate. A p-value of <0.05 was used, and heatmaps were generated for visualization of the bacterial and fungal genera that were statistically different between treated and untreated (control) samples.

Crop development, chemical, physical, and biological analyses

Sampling procedures for crop development and plant biometry were standardized according to the variables appropriate for the different cropping systems (Figure 1) and are presented here only as related to the four crop species studied. Sampling was conducted at the specific plant developmental stage as indicated in Table 1. For annuals (corn, soybean, cotton) stand quality was assessed by counting plants in five meter transects with three repetitions per treatment, 30-60 days after emergence (DAE). Perennial sugar-cane stand was assessed by counting tillers in 10 m transects with five repetitions per treatment, 120 DAE. Plant vigor indicators (1-4) were estimated by measuring/counting leaves/stems/internodes/fruits/pods in ten randomly selected plants per treatment. Rooting was checked in 10 plants per treatment for annuals, 30-60 DAE; and for sugar-cane through 50x50 cm trenches (deeper when equipment available) in three repetitions per treatment. Product quality (according to appropriate crop variables, Figure 1), production and revenue data were obtained from farm managers' administrative records. Soil samples for chemical determinations were obtained just postharvest, from five 0-20 cm depth subsamples taken from the cropping lines, combined into one

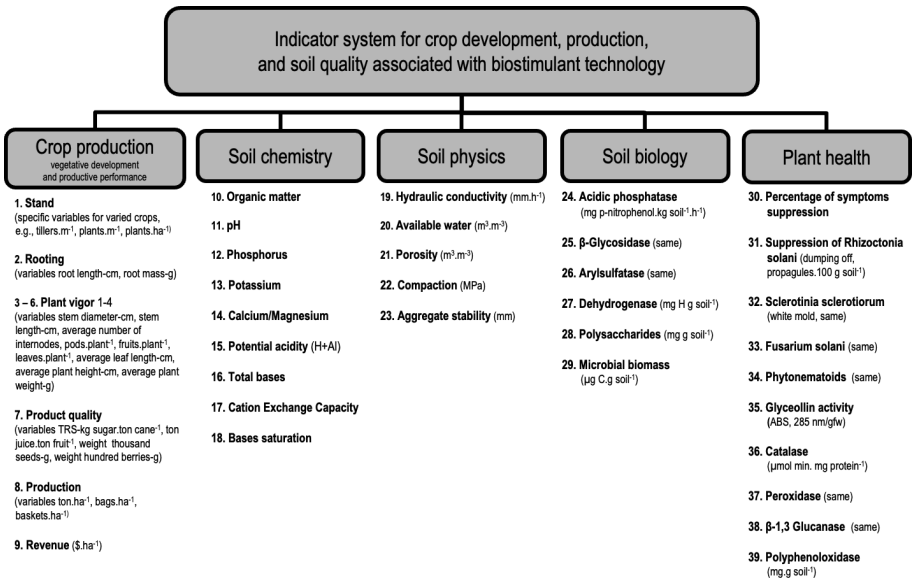

FIGURE 1
Structure of analytical themes and indicators of crop development, production, and soil quality associated with the adoption of biostimulant technology.

sample per treatment. Soil samples obtained from 0-10 cm depth were used for microbiome analysis and for enzymatic activity determinations, always observing medium soil humidity. Nutrients, organic matter, and enzymatic activity determinations were carried out in the same certified commercial laboratory (Laborsolo – Londrina, PR), conforming one single purchasing order (simultaneous). Soil compaction was determined with a penetrometer up to 40 cm depth, in five repetitions per treatment, postharvest. Bulk soil and rhizosphere samples used for microbiome analyses were taken adjacent to the sampling spots used for chemical and biological analyses.

For a better understanding of the correlations between the different indicators and how they are related to both impact values and technical performance of biostimulant technology, we conducted principal component analyses for all variables (indicators) and observations (case studies). The Kaiser criterion was used to select the principal components to retain (Kaiser, 1958).

Crop system parameters and indicator system

The system of indicators for crop development, soil physicochemical and biological quality, and plant health associated with the adoption of biostimulant technology was structured according to the multi-attribute conception of the APOIA-NovoRural method (Rodrigues and Campanhola, 2003; Rodrigues et al., 2010), according to which the analytical variables obtained in the field are expressed in a utility scale (i.e., indices 0-1, baseline modeled at 0.7). The indicators are integrated into five analytical themes, namely: i. crop production (i.e., vegetative development and productive performance, nine indicators), ii. soil chemistry (nine indicators), iii. soil physics (five indicators), iv. Soil biology (six indicators) and v. plant health (10 indicators, not assessed in the present study, Figure 1). The selection of analytical themes and associated indicators to specifically address biostimulant impacts and effects on crop performance departed from a literature review of research previously carried out on the studied biostimulant (Gama et al., 2014; Cardoso et al., 2017; de Almeida et al., 2018; da Silva et al., 2020; Suarez et al., 2020; Filho et al., 2021), and complemented by Embrapa's team institutional experience on the subject (Hungria et al., 2009; Lopes et al., 2013; Mendes et al., 2013; Mendes et al., 2015; Mendes et al., 2018; de Faria et al., 2021).

Information to resolve the indicators is obtained in field assessments, plant biometry estimations, physicochemical and biological analyses of soil samples. Analytical results are entered directly into scaling checklists designed to automatically weight the data and express the impact and technical performance indices for the indicators (Figure 2). The integrated indices are then graphically expressed for the considered analytical themes, respective to the local management conditions and productive contexts observed in the studied farms.

The scaling checklists present variable construction for each indicator, always including reference data from the control plots compared to those observed where biostimulant technology is adopted. Calculated impact values (i.e., control *vs* treatment variation) and technical performance (i.e., observed condition *vs* targeted technical standards) are associated with correspondence tables for the utility scale (0 to 1), so that different indicators have their implications properly evaluated, according to specific quantitative variables presented graphically. These matching values are then performed by best fit equations and respective coefficients, for automatic expression of impact and technical performance indices (Figure 2).

The composition of the correspondence curves between indicators and utility values is based on probability and sensitivity tests, case by case for each indicator (Girardin et al., 1999). In the probability test, the thresholds of the indicator's explanatory variable (in Figure 2, 0 to 200 mg p-nitrophenol.kg soil^{-1}.h^{-1}) and its direction (whether positive or negative) are defined in relation to its technical agronomic significance. In the sensitivity test, the value relationship between the indicator's observed amplitude and the impact/technical performance is defined, according to the

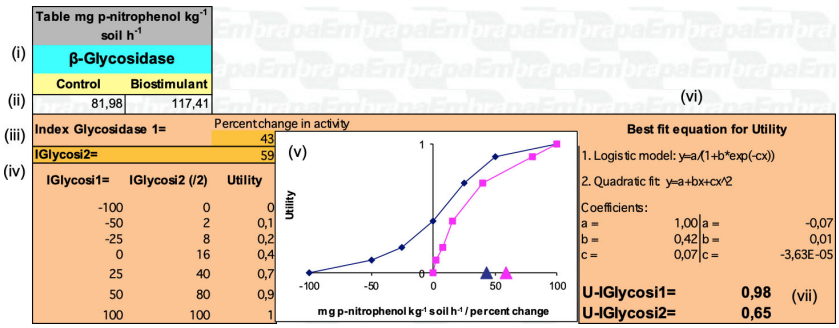

FIGURE 2
Example of a scaling checklist showing the indicator related to the β-Glycosidase enzyme, from the APOIA-Biostimulant system. The scaling checklists bring (i) the statement of the analytical variable and corresponding indicator (i.e., β-Glycosidase); (ii) cells for data entry of control and treatment samples (biostimulant); (iii) calculated values of the indicators, i.e., Glycosidase 1 index (percentage change in enzyme activity, control x biostimulant) and Glycosidase 2 index (IGlycosi2, enzymatic activity level in the treatment); (iv) correspondence table between the calculated indices (i.e., % change and enzymatic activity) and utility values (scale 0 to 1); (v) graphic expression of these correspondences, with calculated indices markers illustrated on the abscissa; (vi) best fit equations and coefficients for converting calculated indices into (vii) utility values (in this case, U-IGlycosi1 = 0.98; U-IGlycosi2 = 0.65). For additional details on the indicator system construction see Rodrigues et al., 2010.

correspondence between the occurrence and a standard of technical adequacy (baseline) established in the literature or experimentally.

The compliance value for the indicators' baseline is always modeled at 0.7, which corresponds to the situation of stability (zero change, i.e., no impact) or technical suitability for the indicator, according to agronomic standards or benchmarks of productive performance. The evaluation results obtained in the scaling checklists are aggregated by the average value of the utility indices for the set of indicators in each analytical theme and expressed in a summary chart of impact and technical performances. Figure 3 shows the baseline, the impact indices, and the technical performance indices for each component theme. Additionally, the average indices of impact and technical performance, for the whole set of indicators, are shown in the bars below. From the graph, one can verify the analytical themes that deserve attention for management improvements and those that best represent the impacts and the technical performance achieved, in the specific conditions observed in the studied farms. Specific graphs for each theme present each of the analyzed indicators, allowing the proposition of management recommendations and adoption of practices to promote soil quality and crop development.

The data set for reporting on crop development, soil physicochemical and biological quality, and plant health associated with biostimulant use, as carried out in reference farms, is presented in an Excel® file (Supplementary Material) consisting of eight worksheets: Worksheet 1, Reference: presents an explanatory summary of the methodological basis, general aspects and the main bibliographical references, with examples of the applicability of the indicator checklists, in addition to references for institutional contacts. Worksheet 2, Identification: data for the identification of the studied farm, the scale and organization of productive activities, and the space-time context defined for the field observations, selection of samples, and considerations on the objectives of the producer interested in the analyses. The following six worksheets refer to the 39 indicators' scaling checklists for the five analytical themes (Figure 1) and a results worksheet with respective graphic representations (Figure 3).

Results

Impact of biostimulant use on soil and rhizosphere microbiomes

In general, all locations and crops evaluated revealed a strong rhizosphere effect (Figures 4, 5), i.e., rhizosphere samples cluster apart from soil samples, as the plant exudate is an important driver in the microbiome assembly in the rhizosphere. The alpha diversity observed in all crop systems evaluated did not show significant variation (Supplementary Figures 1, 2 for bacterial and fungal communities, respectively). A general composition for bacterial and fungal communities associated with all treatments are shown

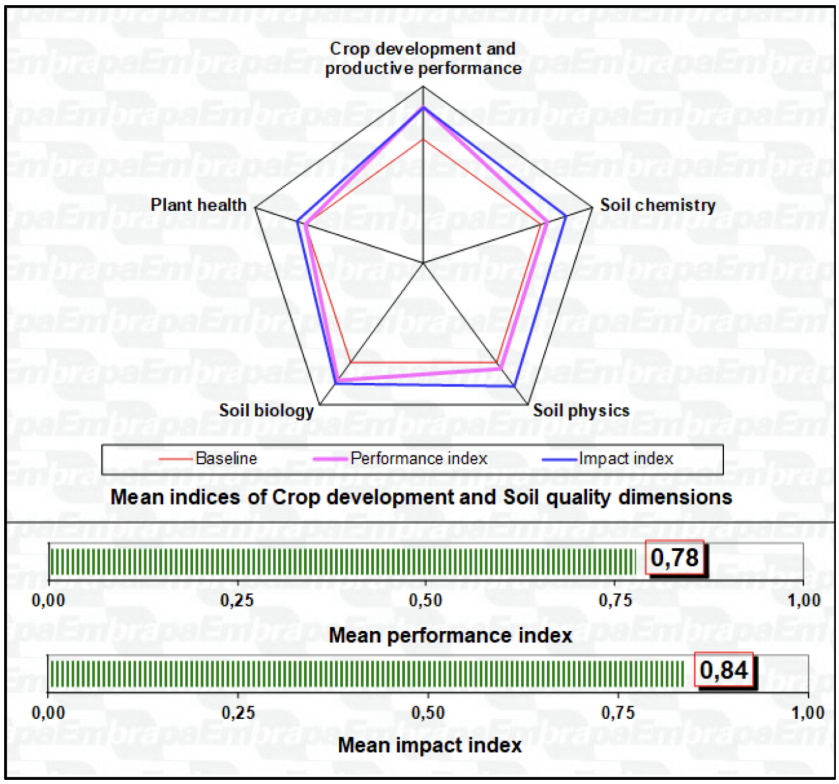

FIGURE 3
Example of expression of the APOIA-Biostimulant indicator system, showing the baseline (0.7 in red), the impact indices (in blue), and the technical performance indices (in magenta) for each component analytical theme associated with the adoption of biostimulant technology.

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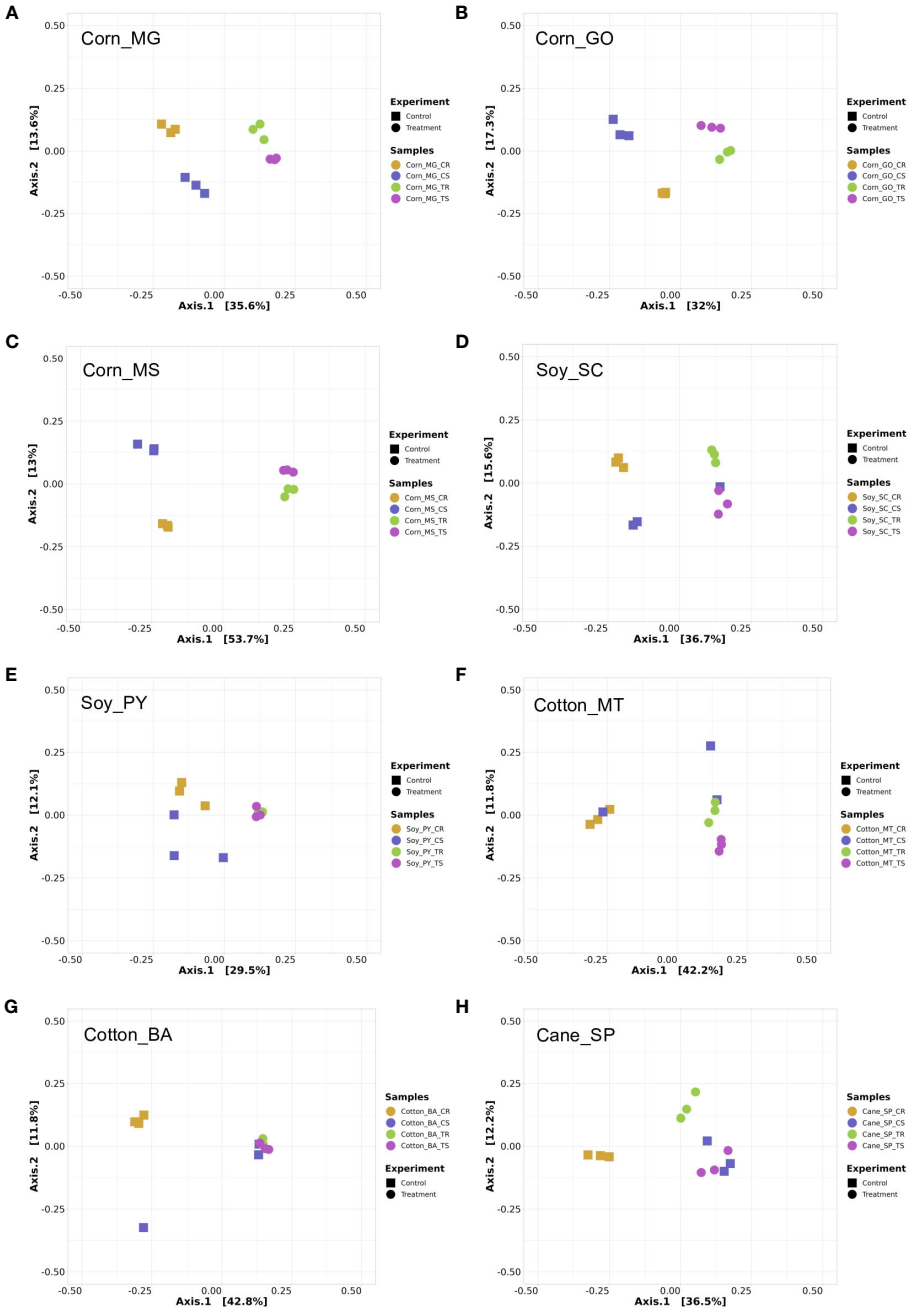

FIGURE 4
Principal Coordinates Analysis (PCoA) of amplicon 16S rRNA sequencing data based on Bray-Curtis distance matrix. Each data point represents a sample, and the sample source with different colors and shapes are indicated in the figure. Each graph shows four treatments (with 3 replicates), control rhizosphere (CR), control soil (CS), treatment rhizosphere (TR) and treatment soil (TS) for all crop systems evaluated, including corn in MG **(A)**, GO **(B)**, MS **(C)**, soy in SC **(D)** and PY **(E)**, cotton in MT **(F)** and BA **(G)**, and sugar cane in SP **(H)**. PCoA was performed using PhyloSeq package on R software.

for each crop system, i.e., corn (Supplementary Figure 3), soybean (Supplementary Figure 4), cotton (Supplementary Figure 5), and sugarcane (Supplementary Figure 6). With few exceptions, the beta diversity showed correlation between the structure of microbial communities and the use of the biostimulant, indicating that the use of the technology resulted in change of the microbiome structure in the plant rhizosphere and in the inter-row soil. Bacterial and fungal communities associated with corn showed a different clustering pattern with the biostimulant treatment in comparison with the control treatment for all three fields evaluated (Figures 4A–C, 5A–C). The same pattern was observed for bacterial and fungal communities in soybean, where the biostimulant-treated samples were grouped separated from control samples (Figures 4D, E, 5D, E), except in Soy_SC where one control sample from bacterial community grouped with samples from the biostimulants treatment (Figure 4D) and in the fungal community two samples

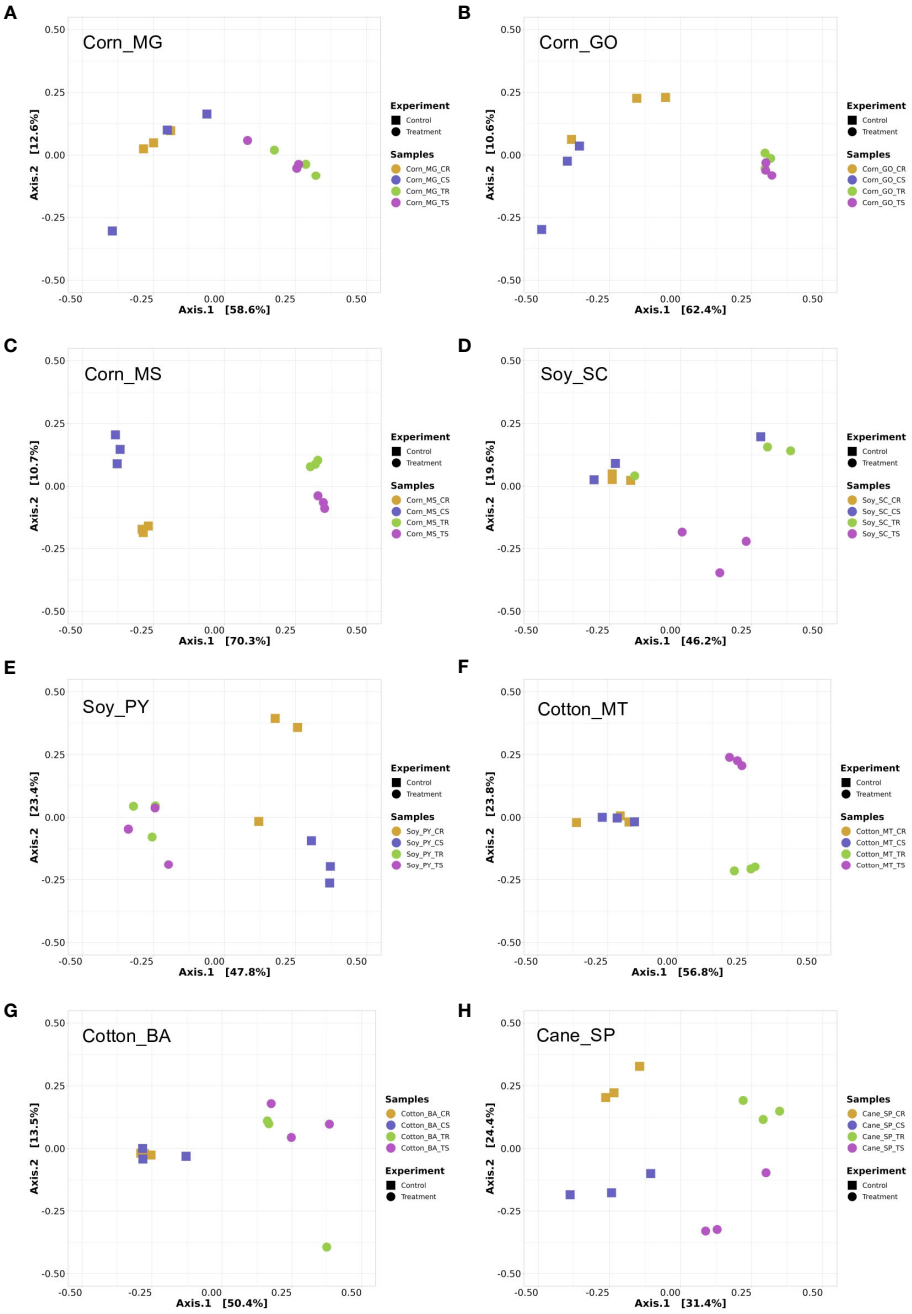

FIGURE 5
Principal Coordinates Analysis (PCoA) of amplicon ITS sequencing data based on Bray-Curtis distance matrix. Each data point represents a sample, and the sample source with different colors and shapes are indicated in the figure. Each graph shows four treatments (with 3 replicates), control rhizosphere (CR), control soil (CS), treatment rhizosphere (TR) and treatment soil (TS) for all crop systems evaluated, including corn in MG **(A)**, GO **(B)**, MS **(C)**, soy in SC **(D)** and PY **(E)**, cotton in MT **(F)** and BA **(G)**, and sugar cane in SP **(H)**. PCoA was performed using PhyloSeq package on R software.

did not cluster as expected (Figure 5D). The same general pattern discriminating biostimulant-treated samples was observed for Cotton (Figures 4F, G, 5F, G), except for bacterial communities in soil samples. For sugarcane all treatments were discriminated considering bacterial or fungal communities (Figures 4H, 5H), except bacterial communities in inter-row soil samples, that clustered biostimulant and control samples together (Figure 4G).

Further analysis was performed to identify microbial taxa significantly enriched in soil and in the rhizosphere of plants treated with the biostimulant (Supplementary Figures 7–10). Significant enrichment or depletion of bulk soil bacteria was found in all fields cultivated with corn and cotton (Supplementary Figure 7). Nine bacterial genera were enriched in corn fields where biostimulant was used, including *Longispora* and *Prauserella* (Supplementary Figure 7). *Methylovirgula* and *Novosphingobium* bacterial genera were enriched in cotton biostimulant treated fields (Supplementary Figure 7). Specific fungal genera significantly changed in abundance in all crop

systems evaluated, in total, 37 fungal genera were significantly enriched across different crop systems (Supplementary Figure 8). The most biostimulant responsive fungal genera was *Lindtneria* in corn (MS), *Leucocoprinus* in sugar cane (SP) and cotton (BA) and *Clathrus* in soybean (PY) (Supplementary Figure 8).

Considering rhizosphere samples, the microbiome analysis showed that the use of the biostimulant enriched or depleted specific bacterial genera in all crop systems, except in Corn_GO (Supplementary Figure 9). The most responsive bacterial taxon to biostimulant application was *Prevotella* in cotton (MT), *Prauserella* (MS) and *Methylovirgula* (MG) in corn, and *Methylocapsa* in sugar cane (SP) (Supplementary Figure 9). Thirty-one fungal genera were significantly enriched in the rhizosphere across all crop systems (Supplementary Figure 10), with the use of the biostimulant. *Arachnomyces* genus was the most responsive genus in soybean (PY and SC) and cotton (MT). The fungal genus *Rhizophlyctis* significantly increased in abundance in corn rhizosphere (MS) in fields treated with biostimulant.

Impact of biostimulant on crop development, production, and soil quality

All indicators were positively impacted with the biostimulant use (Supplementary Table 1). Two sets of interactions among the indicator indices were checked through Principal Component Analysis, one related to the impacts (i.e., relative change from control to biostimulant treatment) and the other relative to the performances (i.e., biostimulant index levels relative to defined technical standards), both relative to soil physicochemical and biological indicators, and to crop development and production. For the biplots of the correlation circle and the observations cloud of the Principal Component Analyses for both impact values and technical performance, refer to the Supplementary Figure 11. Among the impact indicators of soil quality, almost all associated to one PC, with significant Pearson's correlations (α=0.05) for exchangeable cations (K, Ca+Mg, CEC) and, expectedly, the associated variables Total bases and Bases saturation. The enzyme Arylsulfatase related negatively with P in a PC2 and β-Glycosidase stood in a PC3 without being strongly related to any other variable. Regarding the soil performance indices, only CEC, Total bases, and Bases saturation correlated significantly. B-Glycosidase associated positively to pH in PC1 and both correlated negatively with organic matter and Ca+Mg. Arylsulfatase associated negatively with Potential acidity (H+Al).

Interesting significant correlations were observed for the crop development and production indicators. The PC1 for the impact indices, which accounts for 40% of the variability (eigenvalue=5.26), strongly associated organic matter, soil compaction (negatively), plant vigor 2 and 3 (related to plant production and biometry), product quality and, expectedly, crop productivity and net revenue (Figure 6). Hence, organic matter showed to be relevant in preventing soil compaction and promoting crops development and production. The soil enzymes did not show significant correlation with other variables, with β-Glycosidase associated with plant vigor 4 (average plant height-cm) in a PC3 which accounts for 19.7% of the variability. Interesting negative correlations were observed between the indicator product quality (weight of 1,000 seeds for annuals; TRS for sugarcane) and soil compaction; and rooting with revenue. Regarding the crop development and production performance indices, a PC1 accounting for 38% of the variability (eigenvalue=4.96) equally associated organic matter, plant vigor 1, 2, and 3 (including pods per plant, leaves per plant, average plant height), product quality (weight of 1,000 seeds, TRS), crop productivity and net revenue. The soil enzymes did not show significant correlations, being associated with each other in a PC3, which accounted for 15% of the variability.

Discussion

Great interest has been directed toward monitoring soil-biostimulant-crop interactions, in order to improve technical and usage recommendations. Most indicator sets assembled, however, lack in scope to properly assess the impacts of the technology on the diversity of cropping systems and farming contexts, as to integrate soil physicochemical and biological properties, plant health, crop performance, and farm results. Using a multi-attribute approach, we demonstrate the positive impact of biostimulant on the microbiome associated to different crop systems and then developed integrated indicators to express these impacts of biostimulants on crop performance and soil quality.

Considering that microbial inoculants and biostimulants are screened and tested in controlled laboratory conditions, it is common to observe lack of consistency when commercial products are tested in field conditions (Kaminsky et al., 2019). After four commercially available microbial amendments failed to promote tomato growth in greenhouse experiments, Nuzzo et al. (2020) suggested that additional confounding variables can interfere in the efficacy of biostimulants evaluated under commercial fields. This fact reinforces the importance of having a reliable strategy to measure biostimulants impact in commercial validation settings, which normally consist in side-by-side comparisons instead of replicated comparisons with proper experimental design. In this sense, the innumerable local variabilities and particularities that influence crop performance, aside of biostimulant usage, are circumvented in the proposed application of the indicator system, by reducing all production environmental complexity to the immediate contrast control *vs* biostimulant treatment, in the several reference farms and crops studied.

A number of studies has demonstrated that inoculants can modify the native soil microbiome directly or indirectly through changes in plant exudates (Trabelsi and Mhamdi, 2013; Mawarda et al., 2020; Cornell et al., 2021). If these changes result in increase of microbial diversity, this could improve ecosystem functioning and consequently plant performance (Bardgett and van der Putten, 2014). In our results, no clear pattern was observed for increased alpha diversity when biostimulant was used. However, all fields treated with biostimulant showed improved plant performance. This suggests that not only increase or decrease of alpha diversity, but also microbial community structure can be correlated with changes in soil microbiome functioning, resulting in better plant

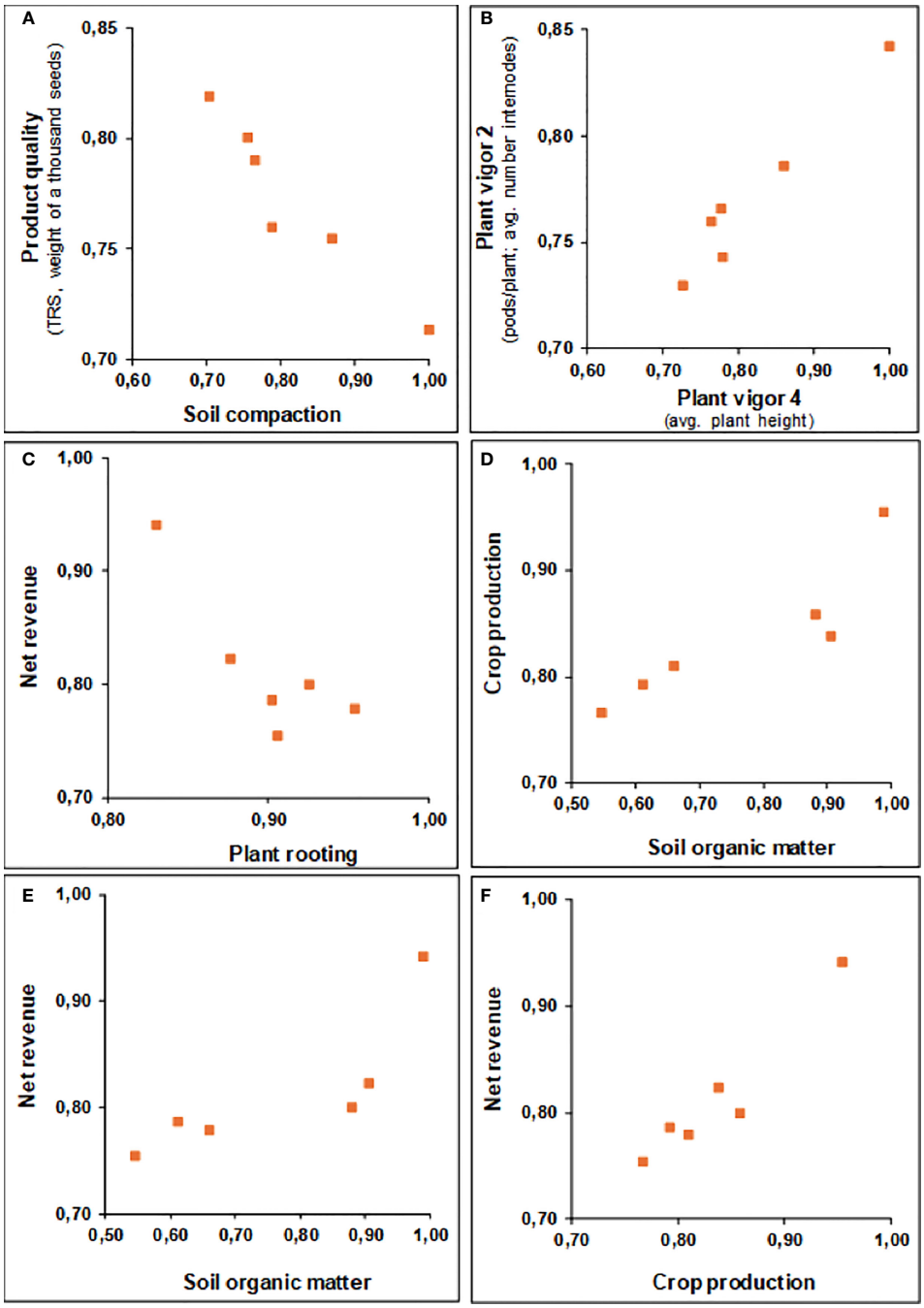

FIGURE 6
Most meaningful significant Pearson's matrix correlations (α=0.05) relating multi-attribute impact indices for crop development, production, and soil quality indicators, applied to six reference farms and four different crops (corn, soybean, cotton, sugarcane), comparing control plots against those subjected to biostimulant technology application (varied environmental and temporal contexts). **(A)** Soil compaction *vs* Product quality, **(B)** Plant vigor 4 *vs* Plant vigor 2, **(C)** Plant rooting *vs* Net revenue, **(D)** Soil organic matter *vs* Crop production, **(E)** Soil organic matter *vs* Net revenue, and **(F)** Crop production *vs* Net revenue.

development. Therefore, considering the pivotal role of the soil and rhizosphere microbiome for plant development (Mendes et al., 2013) and that the use of inoculants and biostimulants can modify native soil microbiome and consequently alter soil functioning (Mawarda et al., 2020), the assessment of the microbiome as affected by biostimulant use is an important indicator of the biostimulant effectiveness. Despite of diverse soil conditions, in farms located in different biomes and diverse crops, areas treated with biostimulants were discriminated from control areas based on the structure of the microbiome. Although a better mechanistic understanding of the mode of action of complex biostimulants is needed to finely tune their management and measure their effectiveness in field conditions, having a comprehensive set of indicators helps to tackle these challenges.

The integrated analysis of crop development, production, and soil quality associated with the biostimulant use documents the

positive impacts and the improvement in technical performance observed in the field trials. Among the main results, it was observed that the impact indices, that is the relative comparison between the controls and the areas with biostimulant, were the most expressive; mainly in the soil biology theme (index 0.84 for arylsulfatase and β-glucosidase enzymes), followed by soil physics (index 0.81 for compaction), and chemistry (index 0.75) and, in response to these positive impacts, the crop production theme (index 0.81). This preponderance of soil biology as the analytical theme of better performance confirms the important effect of those enzymes, as advocated by Mendes et al. (2015; 2018).

The improvements observed in all these analytical themes, in particular the crop production indicators, brought a series of responses of great interest to farmers, including root development (average index 0.89), plant vigor (index 0.83 in length of branches and leaves), vegetative development (index 0.82 in number of internodes, length of stems, pods or grains per plant) and product quality (index 0.77 for protein content in the grains, weight of 1,000 seeds, or TRS–kg of sugar per ton of cane). Most importantly, as an integrated result of these indicators, in areas treated with biostimulant the average productivity was greatly favored in all crops (index 0.84 for bags.ha^{-1} or ton.ha^{-1}), resulting in expressive gains in net revenue (index 0.81 for \$.ha^{-1} see Supplementary Table 1). A close correlation between the productivity and net income indicators (r^2 = 0.92), although naturally expected, attests that these gains were achieved without significant cost increases, pointing to the viability of the biostimulant program relative to the rising prices of other inputs, such as conventional chemical fertilizers.

These results of the impact indices (average of cases 0.80 on a scale between 0 and 1), which represent relative gains between treated and control areas, are of great significance, since the performance indices were more modest (cases average 0.69). As these performance indices represent the observed local condition, in relation to appropriate or desired technical benchmarks, it is indicated that there is still room for further gains, as the applications of biostimulant are repeated throughout the harvests, enhancing the expression of the observed impacts. Noteworthy is the fact that even under very contrasting situations, including four different crops in seven distinct ecoregions, soil quality and crop performances were always superior in the areas treated with the biostimulant technology. Also, significant correlations were observed between the averages of the integrated indices of soil quality and the indicators of soil biology performance (r^2 = 0.82); followed by the themes soil chemistry and crop production – the latter possibly a consequence of all others.

In conclusion, the microbiome analysis revealed that the biostimulant use consistently impacted the soil and rhizosphere microbiome assembly. The changes in community structure observed in biostimulant-treated fields correlate with better plant development and crop performance. This observation served as a proxy to assess the effectiveness of biostimulants, which was subsequently confirmed through the utilization of the integrated indicators approach. The expression of the results obtained by the use of integrated indicators suggests i) coherence between the system's analytical themes and indicators with better responses, ii) proper amplitudes of the obtained indices (expressive, but not extreme), and iii) conformity of the thresholds, weighting factors, and graphic scales. These results point to adequate calibration and sensitivity of the set of indicators, for adequately evaluating the impacts of biostimulants on crop performance.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

RM, GR, and PD'A contributed to conception and design of the study. RM and MD'A-K organized the field work, sampling strategy and data collection. RM performed microbiome analyses. GR and IB developed the integrated indicators model. GR and RM wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

PD'A and MD'A-K are employed by Microgeo®.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that this study received funding from Microgeo®. The funder had the following involvement with the study: discussed the experimental design, indicated potential farms with history of biostimulant use and gave support for soil sampling and data collection.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1214112/full#supplementary-material

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Beneficial microbial consortium improves winter rye performance by modulating bacterial communities in the rhizosphere and enhancing plant nutrient acquisition

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The beneficial effect of microbial consortium application on plants is strongly affected by soil conditions, which are influenced by farming practices. The establishment of microbial inoculants in the rhizosphere is a prerequisite for successful plant-microorganism interactions. This study investigated whether a consortium of beneficial microorganisms establishes in the rhizosphere of a winter crop during the vegetation period, including the winter growing season. In addition, we aimed for a better understanding of its effect on plant performance under different farming practices. Winter rye plants grown in a long-time field trial under conventional or organic farming practices were inoculated after plant emergence in autumn with a microbial consortium containing *Pseudomonas* sp. (RU47), *Bacillus atrophaeus* (ABi03) and *Trichoderma harzianum* (OMG16). The density of the microbial inoculants in the rhizosphere and root-associated soil was quantified in autumn and the following spring. Furthermore, the influence of the consortium on plant performance and on the rhizosphere bacterial community assembly was investigated using a multidisciplinary approach. Selective plating showed a high colonization density of individual microorganisms of the consortium in the rhizosphere and root-associated soil of winter rye throughout its early growth cycle. 16S rRNA gene amplicon sequencing showed that the farming practice affected mainly the rhizosphere bacterial communities in autumn and spring. However, the microbial consortium inoculated altered also the bacterial community composition at each sampling time point, especially at the beginning of the new growing season in spring. Inoculation of winter rye with the microbial consortium significantly improved

the plant nutrient status and performance especially under organic farming. In summary, the microbial consortium showed sufficient efficacy throughout vegetation dormancy when inoculated in autumn and contributed to better plant performance, indicating the potential of microbe-based solutions in organic farming where nutrient availability is limited.

KEYWORDS

organic farming, conventional farming, 16S rRNA gene amplicon sequencing, *Bacillus*, *Trichoderma*, *Pseudomonas*

Introduction

Soil microorganisms play a central role in almost all soil processes, as they do not only contribute to soil formation (Watteau and Villemin, 2018) but also have a significant impact on important ecosystem functions such as nutrient cycling and plant performance (Latz et al., 2016; Lori et al., 2017). Therefore, managing the soil microbial community is essential to maintain soil health and productivity. Microbial diversity has been positively associated with the resilience of microbial communities and soil fertility (van Elsas et al., 2012; Usero et al., 2021). Crops benefit from diverse soil microbial communities as they recruit and enrich beneficial microorganisms (BM) via root exudates in the rhizosphere (Berg and Smalla, 2009; Liu et al., 2021). BM can stimulate plant growth through various mechanisms, such as modulating phytohormone levels (Zubair et al., 2019) and increasing nutrient availability (Jacoby et al., 2017). Furthermore, beneficial rhizosphere microorganisms can trigger induced systemic resistance and protect the plant against below- and aboveground pathogens (Wei et al., 2020; Vlot et al., 2021). Indeed, plant pathogens can be controlled by BM either directly by producing lytic enzymes and bioactive compounds (Fira et al., 2018; Ali et al., 2020) or indirectly by competing for resources in the same ecological niche like the rhizosphere (Rai et al., 2016).

The inoculation of microorganisms with known beneficial functions for plants is a practical approach that is able to modulate the composition of indigenous microbial communities, particularly in the rhizosphere (Deng et al., 2019; Berg et al., 2021). Although many BM show promising functions on plant growth and protection under controlled conditions *in vitro* and *in vivo* (Mendes et al., 2013; Mazzola and Freilich, 2017), their efficacy in the field is often limited due to insufficient colonization of the host rhizosphere and/or unfavorable conditions (Parnell et al., 2016; Batista and Singh, 2021). To successfully colonize the rhizosphere, inoculated BM have to bypass the barrier of the indigenous soil microbial community, adapt to variable environmental conditions, and interact with the host plant using mainly root exudates as a chemoattractant and substrate (Finkel et al., 2017; Berg et al., 2021). Different plant developmental stages and changing environmental conditions shift the root exudation pattern and thereby also the composition of the microbial community in the rhizosphere (Chaparro et al., 2014; Windisch et al., 2021), which may affect the rhizosphere competence of BM. Thus, even if inoculated BM successfully establish in the rhizosphere at early plant development, persistence over an extended period is not guaranteed. Inoculation with multi-strain consortia containing two or more BM has shown increased efficacy compared to single BM species (Sun et al., 2022). Consortia of BM, comprising members with diverse functions, can occupy different ecological niches, making them more resilient to variable environments (Bradáčová et al., 2019; Tosi et al., 2020). Furthermore, the interaction among consortium members increases their ecological fitness, and complementary beneficial functions generate synergies that promote their interaction with the plant host (Moradtalab et al., 2020; Pascale et al., 2020).

Different farming practices, such as conventional and organic farming, drastically affect the structure of microbial communities (Francioli et al., 2016; Windisch et al., 2021). Chowdhury et al. (2019) showed that conventional farming using synthetic pesticides and mineral fertilizers shaped the rhizosphere microbial community composition differently from organic farming, which was associated with varying states of plant health. There is currently a limited understanding of how the farming practice impacts the efficacy of a beneficial microorganism consortium (BMc). For instance, crops can benefit more from increased nutrient bioavailability through BMc in nutrient-deficient soils (Eltlbany et al., 2019), while in soils with high nutrient level the rhizosphere colonization by the inoculated BMc might be reduced (Lopes et al., 2021).

The main goal of this study was to assess the rhizosphere competence of the inoculated BMc members in winter rye (*Secale cereale* cv. Conduct) during the growing season in dependence on different farming practices. Winter cereal crops are exposed to extreme environmental changes during the growing season, raising the question of whether the members of a BMc inoculated in autumn can establish in the rhizosphere of winter rye and maintain a high colonization density over the winter period. Furthermore, the impact of the applied BMc on plant performance may be defined by the physicochemical properties of the soil and the farming practice dependent soil microbial communities. In this context, we hypothesized that (i) early inoculation of winter rye enables sufficient colonization of each BMc member at early plant developmental stage, supporting its persistence in the rhizosphere throughout the vegetation period; (ii) the application of BMc shapes the composition of the rhizosphere bacterial community depending on the farming practice and thus differentially affects the plant performance.

To test our hypotheses, we first characterized *in vitro* the plant growth-promoting traits and the ability of each BMc member to inhibit the growth of soil-borne pathogens. Then, we used a long-time field experiment to evaluate the rhizosphere competence and efficacy of the tested BMc on plant performance under field conditions with different farming practices (organic vs. conventional farming practice). Winter rye plants were inoculated shortly after emergence and BMc colonization and plant performance were evaluated in autumn and spring. The dynamics of the bacterial community throughout vegetation dormancy were characterized by 16S rRNA amplicon sequencing.

Materials and methods

Microbial consortium used in the study

The microbial members of the consortium [*Pseudomonas* sp. (RU47; strain collection of the Julius Kühn Institute, Braunschweig, Germany), *Bacillus atrophaeus* (ABi03; strain provided by ABiTEP, Berlin, Germany), and *Trichoderma harzianum* (OMG16; strain collection of Anhalt University of Applied Sciences, Bernburg, Germany)] were selected as each strain showed beneficial effects on plant performance in previous experiments and trials (Schreiter et al., 2014; Mpanga et al., 2018; Schreiter et al., 2018; Mpanga et al., 2019; Moradtalab et al., 2020; Hafiz et al., 2022).

In vitro characterization of consortium members

To provide a more comprehensive characterization of the plant-beneficial traits associated with each consortium member (*Pseudomonas* sp. RU47, *B. atrophaeus* ABi03, and *T. harzianum* OMG16) a series of *in vitro* tests were conducted to assess their antagonistic capabilities and their capacity for plant growth promotion. The modulation of plant growth hormones by indole-3-acetic acid (IAA) production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Koo et al., 2010), as well as the increased nutrient availability by siderophore production (Schwyn and Neilands, 1987) was tested for each strain. In addition, the solubilization of potassium feldspar ($KAlSi_3O_8$) (Breitkreuz et al., 2021), calcium phosphate ($Ca_3(PO_4)_2$) (Nautiyal, 1999), zinc oxide (ZnO) (Mumtaz et al., 2017), manganese dioxide (MnO_2) (Sanket et al., 2017) and ammonia production (Cappuccino and Sherman, 2014) by the individual strains was analyzed. For nutrient solubilization assays, the medium was modified for OMG16 using Waksman-agar (Huber et al., 1987).

Cellulase (Berg et al., 2005), chitinase (Berg et al., 2001), β-1,3-glucanase (Weinert et al., 2010), protease (Berg et al., 2005), and hydrogen cyanide (HCN) (Donate-Correa et al., 2005) formation were tested as antagonistic functions. *In vitro* tests for enzyme secretion with antagonistic functions were not feasible for OMG16 due to cultivation incompatibilities. A dual culture assay modified after Lin and Ho (2021) was used to evaluate the ability of each strain to inhibit pathogen growth *in vitro*. For the two bacterial strains RU47 and ABi03, 20 µl of bacterial suspension (10^5 colony forming units (CFU) mL^{-1}) was dispensed in a centered line of a nutrient-agar I plate (Sifin diagnostics, Berlin, Germany). Freshly grown mycelia plugs (ø 6 mm) of *Fusarium culmorum* (Isolate F247), *F. graminearum* (Isolate FG66), and *Rhizoctonia solani* Isolate AG3 (Isolate Ben3) were placed at a distance of 2.5 cm on both sides of the line. For the fungal strain OMG16, precultured mycelia plugs of OMG16 and the pathogen were placed on opposite sides of a potato dextrose agar (PDA, Carl Roth, Karlsruhe, Germany) plate. Dual culture assays were cultivated in the dark at 25°C for 10 days. Antagonistic properties, i.e. formation of an inhibition zone, of the individual BMc strains were checked every second day (Supplementary Figure 1).

Field site description

The experiment was conducted from 2020-2021 in an experimental field with plots under conventional and organic farming practices as part of the long-time field trial in Thyrow (Thy_ABS; 52°15' N, 13°14' E, 44 m a.s.l.), which was established in 2005 at the Agricultural Research Institute of the Humboldt University of Berlin. Pallid brown earth (Luvisols) of sand over deep loam with low humus contents is the predominant soil type (83% sand, 14% silt, and only 3% clay) at this site. The average annual temperature (1981-2020) is 9.2°C with a mean annual precipitation of 509.8 mm.

Winter rapeseed (*Brassica napus* ssp. *napus*), winter wheat (*Triticum aestivum*), and winter rye (*Secale cereale*) were rotated in conventional farming and lucerne (*Medicago sativa*), lucerne, potato (*Solanum tuberosum*), triticale (*Triticale*), forage pea (*Pisum sativum* ssp. *arvense*), spring barley (*Hordeum vulgare*), and winter rye (*Secale cereale*) in organic farming. Mineral fertilization was used in conventional farming practice according to the agricultural standard of the region (N = 120 kg ha^{-1}, P = 21 kg ha^{-1}, K = 120 kg ha^{-1}, Mg = 12 kg ha^{-1}, S = 14 kg ha^{-1}). Nitrogen was applied as calcium ammonium nitrate (KAS) and potassium, magnesium and sulfur as equal ratios of Patentkali® (K + S Minerals and Agriculture, Kassel, Germany) and Korn-Kali® (K + S Minerals and Agriculture). Triple superphosphate (46% P_2O_5) was used for phosphate fertilization. Before growing winter rye and triticale, cattle manure was incorporated into the soil in organic farming (15,000 kg ha^{-1}), supported by the cultivation of lucerne and forage peas. Plant protection was conducted following the agricultural standards of conventional cultivation [herbicides: 2.0 L ha^{-1} Picona (BASF, Ludwigshafen, Germany), 0.25 L ha^{-1} Cadou SC (Bayer, Leverkusen, Germany); fungicides: 2.0 L ha^{-1} Ceriax (BASF)]. In organic farming, no pesticides were used, and emerging weeds were removed with a mechanical cultivator. Before sowing of winter rye (sowing density: 200 seeds per m^2 in conventional farming and 300 seeds per m^2 in organic farming), the soil was tilled, including stubble clearing with a disc harrow, plowing to a depth of about 20-25 cm and seedbed preparation with a reciprocating harrow.

Preparation of the microbial consortium and its field application

The *T. harzianum* OMG16 strain was cultivated on PDA medium (Carl Roth) supplemented with 100 mg L^{-1} penicillin (Carl Roth), 50 mg L^{-1} streptomycin sulfate (Sigma-Aldrich, St. Louis, USA), and 10 mg L^{-1} tetracycline hydrochloride (AppliChem, Darmstadt, Germany). To prepare the field inoculum, millet grains (*Panicum miliaceum*) were soaked overnight in tap water. The grains were washed intensively with cold tap water afterwards. Clean grains (500 g per bag) were transferred to bags designed for fungal culture (sun bag; Sigma-Aldrich) and autoclaved on three subsequent days. A disc with freshly grown OMG16 mycelium was added to the grains and incubated for 30 days in the dark at room temperature. After OMG16 entirely colonized the millet grains, the inoculum was freeze-dried and ground. Shortly before winter rye sowing, the powder (100 mg m^{-2}) was incorporated into the soil to a depth of 20 cm. Freeze-dried millet grain powder without OMG16 was used as a control.

The *B. atrophaeus* ABi03 and *Pseudomonas* sp. RU47 strains were used as spontaneous rifampicin-resistant mutants provided as a prepared inoculum by ABiTEP (Schreiter et al., 2018). Two weeks after winter rye emergence, each plot was drenched with 4 L of the bacterial ABi03 and RU47 suspension, mixed with tap water (each 7.5 x 10^7 CFU mL^{-1}). Control plots were drenched with tap water. Each treatment included four replicates (1.5 x 2 m) arranged in a randomized block design for both farming practices.

Sampling and verification of rhizosphere competence

Only plants from the inner plot core (0.5 x 1m) of each replicate were considered for the sampling, which was performed in autumn at seven weeks post inoculation (WPI) at EC 21, and in spring of the following year (22 WPI) at EC 25-29. A total of 20 winter rye plants were sampled per plot and treated as one sample per treatment replicate. After plant excavation, the shoots were separated from the roots. To obtain root-associated soil, the soil fraction loosely adhering to the root system was shaken off. Roots, shoots, and root-associated soil were stored immediately at 4°C. After shoot fresh mass determination, shoots were dried by lyophilization, and shoot dry mass (SDM) was measured.

Roots with the remaining tightly attached soil were washed with sterile tap water and cut into 1 cm pieces for rhizosphere sampling. After pooling the root fragments, five grams of root material were transferred into a sterile stomacher bag containing 15 mL of sterile 0.3% NaCl. The rhizosphere samples were obtained by a Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 60 s at 300 rpm. The supernatant was transferred into a falcon tube, and the Stomacher blending steps were repeated twice. The combined supernatants was used for determining bacterial BMc counts by immediately plating serial dilutions onto nutrient agar I plates (Sifin diagnostics) containing 75 µg mL^{-1} rifampicin (Serva Electrophoresis, Heidelberg, Germany) and 100 µg mL^{-1} cycloheximide (Serva Electrophoresis) and incubated in the dark for 48 h at 28°C. Given the distinct colony morphology of ABi03 and RU47, the CFU were counted and CFU per g root dry mass, used for stomacher processing, were calculated. The remaining Stomacher supernatant was further processed as described by Schreiter et al. (2014) for bacterial rhizosphere community analysis based on total community DNA extraction.

Root-associated soil was used to determine OMG16 counts. Therefore, 5 g of root-associated soil were extracted in a total volume of 50 mL NaCl (0.3%) by shaking for 30 min at room temperature on an orbital shaker (HSM-10; Hettich, Tuttlingen, Germany) at 70 rpm. Subsequently, the supernatant was immediately plated on *Trichoderma* selection medium as suggested by Williams et al. (2003) containing 250 mg L^{-1} chloramphenicol (Carl Roth), 90 mg L^{-1} streptomycin (Sigma-Aldrich), 200 mg L^{-1} quintozene (Sigma-Aldrich), 926 mg L^{-1} propamocarb (ProPlant; Arysta LifeScience, Paris, France) and 150 mg L^{-1} rose bengal (AppliChem). Following an incubation of OMG16 at 28°C in the dark for 10 days, the CFU were quantified per gram soil dry mass. Soil dry mass was obtained by drying five grams of fresh soil at 110°C until a constant weight was reached.

Winter rye ears of the whole plot were sampled at full ripeness. After counting the ears, the grain was threshed and the weight was determined. Yield per hectare was extrapolated for better comparison with literature data.

Nutrient analysis

The nutrient analysis of shoot material was conducted according to the certified protocols of the Association of German Agricultural Analytic and Research Institutes, VDLUFA, Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2011). 200-500 mg of dry plant material were solubilized in 5 mL HNO_3 (65%) and 3 mL H_2O_2 (30%) by microwave digestion at 210°C for 25 min. The extract was filtered and diluted to a final volume of 100 mL. K, P, Mg, S, Ca, Mn, Cu, and Zn concentrations were determined via inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Fisher Scientific, Dreieich, Germany); total C and N were determined via elemental analysis (Elementary Vario El cube; Langenselbold, Germany). The content of macro- and micronutrients in the root-associated soil were analyzed according to the certified protocols of VDLUFA by AGROLAB (Leinefelde-Worbis, Germany).

DNA extraction and high-throughput amplicon sequencing of the 16S rRNA gene

The total community DNA was extracted from total rhizosphere pellets using the FastPrep-24 bead-beating system and FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's recommendations. Samples were purified with the GeneClean Spin Kit (MP Biomedicals). Library construction and sequencing of the 16S rRNA gene was carried out by Novogene (Cambridge, UK) on NovaSeq PE250 using the primers Uni341F (5'-CCTAYGGGRBGCASCAG-3') and

Uni806R (5'- GGACTACNNGGGTATCTAAT-3') (Sundberg et al., 2013). Primers and adapters were removed with the software Cutadapt (Martin, 2011). Paired-end reads were processed using the DADA2 pipeline (Callahan et al., 2016). For taxonomic assignment of the obtained amplicon sequence variants (ASVs), the representative sequences were taxonomically classified down to the lowest possible taxonomic level in a Galaxy workflow (Cock et al., 2013) with an e-value cut-off of 0.001 and a percent identity cut-off of 80% against the SILVA 138.1 SSU Ref NR99 database (v.138.1, Quast et al., 2012; v138.1). Sequences identified as plastids (chloroplast or mitochondria) and sequences with less than five reads were removed. No ASV classified as archaeal taxa was present in our dataset. Rarefaction curves were generated to estimate the read coverage, and sequencing depth sufficiently covered the diversity of each sample. Amplicon sequence data has been deposited at the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA975889.

Data analysis

Statistical analysis of winter rye growth, yield, nutrient concentration, and BMc abundance was performed using R (v.4.2.2, R Core Team, 2022). The main and interaction effects between long-time farming practices and BMc use were analyzed by two-way ANOVA for SDM and shoot nutrients. Differences in the BMc abundance in the rhizosphere and root-associated soil were tested by two-way ANOVA analyzing the main and interaction effects of the sampling time and different long-time farming practices. All analyses were inspected visually for homoscedasticity and normal distribution of residuals using the Durbin-Watson test from the "car" package (v.3.1.1, Fox and Weisberg, 2019) and normal Q-Q plots. If ANOVA assumptions failed, data were transformed using the R package "rcompanion" (v.2.4.26, Mangiafico, 2023) according to Tukey's ladder of power approach. The statistical evaluation was conducted using the Sidak-test algorithm and the pairwise comparison combined with a compact letter display of the packages "emmeans" (v.1.8.3, Lenth, 2022), "multcomp" (v.1.4, Hothorn et al., 2008) and "multcompView" (v.0.1.8, Graves et al., 2019). Data were visualized using the "ggplot2" (v.3.4.1, Wickham, 2016) and "ggpattern" packages (v.1.0.1, FC and Davis, 2022). Comparisons with *p*-values below 0.05 were considered statistically significant.

All data handling and statistical analyses for ASV data were performed with the R packages "tidyverse" (v.1.3.1, Wickham et al., 2019), "ggplot2", "dplyr" (v.1.1.1, Wickham et al., 2023), "tibble" (v.3.1.8, Müller and Wickham, 2022), "reshape2" (v.1.4.4, Wickham, 2007), "vegan" (v.2.6.1, Oksanen et al., 2022) and "phyloseq" (v.3.16, McMurdie and Holmes, 2013). To account for uneven sequencing depth, we repeatedly performed 1000 rarefactions to our dataset's lowest number of sequences (49,206). Average ASV abundances were calculated based on the 1000 rarefactions. This procedure enables the representation of all observed sequences in equal proportionality and accounts for the random variation introduced by rarefaction (Cameron et al., 2021). Bray-Curtis distance was used for estimating the β-diversity by normalizing the rarefied datasets to relative abundance (%) and applying $\log_{10}$ transformation with pseudo-count addition. PERMANOVA tests were applied to evaluate how farming practice and BMc inoculation affected the bacterial community composition. A pairwise PERMANOVA test with Benjamini-Hochberg correction was performed after combining the two treatments into four groups using the "pairwiseadonis" package (v.0.4, Martinez, 2022). Significant differences in α-diversity (ASV Richness, Simpson, Shannon Index, and Evenness) were estimated with two-way ANOVA tests. If the data failed to fulfill the normality criteria based on the Shapiro test, the non-parametric aligned-rank ANOVA was performed ("ARTool" package, v.0.11.1, Kay et al., 2021).

For investigating the effect of farming practice type and BMc inoculation on the abundance of ASVs, ANCOM-BC2 (v2.1.2, Lin and Peddada, 2020) was performed as a differential abundance test with *p*-value correction via Benjamini-Hochberg method. Following the differential abundance test, we performed multiple logistic regressions with Benjamini-Hochberg correction as *post hoc* tests. Logistic regression was selected because of its similar predictive power to machine learning algorithms (e.g., random forest) and its inherent interpretability (Topçuoğlu et al., 2020). We used a logistic regression equation to predict the probability of the treatment to occur (i.e., Organic-BMc, Organic-Control, Conventional-BMc, or Conventional-Control) based on the relative abundance of the ASVs. Consequently, the logistic regression tests enabled us to evaluate whether the differentially abundant ASVs responded to a specific treatment (ASV responders). For each particular treatment, the fitted model's positive or negative model coefficient indicated higher or lower relative abundance, respectively. ASVs were assigned as positive or negative responders to each treatment based on the model coefficient.

To evaluate whether the biomass yield of rye was associated with higher proportions of the inoculated bacterial strains in comparison to the rest of the bacterial community members in the different treatments, ASV responder sequences classified as *Pseudomonas* spp. or *Bacillus* spp. were aligned to herein constructed databases. For *Pseudomonas* spp. we created a database that contained the 16S rRNA gene sequences from the six 16S rRNA gene copies of the inoculant RU47 (Kuzmanović et al., 2018). For the strain ABi03, we performed cloning and sequencing of the complete 16S rRNA region using primers U8-27 and R1494-1514 (Heuer et al., 2009), where we identified six different 16S rRNA genes. In addition, we retrieved sequences of *Pseudomonas* spp. or *Bacillus* spp. from GenBank 16S rRNA gene restricted to the type strain collection (Federhen, 2015). The V3-V4 region of the 16S rRNA gene, used for Illumina amplicon sequencing, was extracted from the database sequences using the Cutadapt software (Martin, 2011). Pairwise alignment was performed for *Bacillus* spp. and *Pseudomonas* spp., using the ASVs and the constructed databases with the package "msa" (v.1.30.1, Bodenhofer et al., 2015). A distance matrix of the alignment was calculated via the package "seqinr" (v.4.2.23, Charif and Lobry, 2007). A phylogenetic distance was calculated with the neighbor-joining algorithm (package "ape", v.5.7, Paradis and Schliep, 2019). The phylogenetic distance was visualized as principal coordinates analysis.

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Results

In vitro traits of the microbial consortium members

In vitro, the individual members of the used microbial consortium were tested for antagonistic and growth-promoting functions (Table 1). ABi03 showed cellulase, chitinase, β-1,3-glucanase, and protease activity but no HCN production. In dual culture assays, ABi03 reduced the growth of all tested pathogens by inhibiting the mycelium growth (Supplementary Figure 1). Despite the inability of RU47 to inhibit the growth of *F. culmorum* or *F. graminearum*, it is noteworthy that the RU47 colonies were not overgrown by these pathogens. Additionally, we could confirm a minor inhibition of *R. solani* growth by RU47 as well as protease and HCN production. OMG16 also reduced the growth of *F. culmorum* and *F. graminearum* by spreading faster than the pathogen on PDA. No lytic degradation of the mycelium was observed in the border region between OMG16 and the tested pathogens (Supplementary Figure 1).

RU47 dissolved K-feldspar and $Ca_3(PO_4)_2$ *in vitro* in nutrient availability assays and showed siderophore as well as IAA production (Table 1). For ABi03, ammonia and siderophores were detected, but no increase in solubility of the tested nutrients or plant hormone modulation was observed. Furthermore, IAA, low siderophore production, and ZnO solubility could be shown for OMG16.

TABLE 1 *In vitro* characterization of beneficial functions of the inoculated consortium members.

	RU47	ABi03	OMG16
Dual culture assay			
F. culmorum	–	+	+
F. graminearum	–	+	+
R. solani	+*	+	+/-
Antagonistic functions			
Cellulase	-*	+	NA
Chitinase	-*	+	NA
β-1,3-Glucanase	-*	+	NA
Protease	+*	+	–
HCN	+**	–	–
Nutrient availability			
Ammonia	–	+	NA
K-feldspar	+	–	–
$Ca_3(PO_4)_2$	+**	–	–
ZnO	–	–	+
MnO_2	–	–	NA
Siderophores	+*	+	+/-
Plant hormone modulation			
IAA	+**	–	+
ACC deaminase	–	–	–

*(Adesina et al., 2009).
**(Kuzmanović et al., 2018).
BMc members were characterized separately for beneficial functions [positive (+), negative (-), inconclusive (+/-), not applicable (N/A)]. Results marked with asterisks were taken from the indicated literature.

Density of the consortium members in the rhizosphere and soil

To evaluate the persistence of the individual BM in the rhizosphere and root-associated soil of winter rye at field scale, we estimated their density after inoculation with two subsequent samplings in autumn and spring of the following year. Both bacterial strains ABi03 and RU47 sufficiently colonized the rhizosphere of winter rye with a density of more than six Log_{10} CFU per gram root dry mass seven WPI at both farming practices (Figure 1). The fungal strain OMG16 also showed a sufficient density in the root-associated soil of more than five Log_{10} CFU per gram soil dry mass. No differences in the density of the individual BM in the respective habitat depending on farming practice were observed. At the second sampling in spring (22 WPI), no changes in the density of ABi03 in the rhizosphere of winter rye were found independent of farming practice (Figure 1, Supplementary Table 1). RU47 density decreased to approximately five Log_{10} CFU per gram of root dry mass at both farming practices compared to the first sampling in autumn. Plants grown under organic farming practice showed a significantly reduced density of OMG16 in the root-associated soil compared to the first sampling in autumn. The two different farming practices had no significant impact on the ability of the individual BM to establish in the rye rhizosphere or root-associated soil at both sampling time points.

Plant performance

The mean SDM of winter rye plants at the first sampling (EC 21) in autumn ranged from 0.07 to 0.10 g $plant^{-1}$, while the mean SDM in the second sampling (EC 25-29) in spring was between 0.26 and 0.48 g $plant^{-1}$ (Figure 2A, Supplementary Table 2A). Control plants without BMc inoculation showed no significant difference in SDM in response to farming practice (conventional vs. organic) at both sampling time points. Organically cultivated winter rye plants treated with the BMc showed a significant increase in SDM compared to those without BMc application in autumn (7 WPI, +20%) and particularly in spring (22 WPI, +45%). In contrast to organic farming, the BMc treatment did not affect the SDM of plants grown under conventional farming at both sampling time points. Two-way ANOVA further revealed a significant interaction between farming practice and BMc inoculation on SDM at both sampling time points (Supplementary Table 2B) indicating farming practice-dependent effects of BMc on SDM. At harvest, there were no significant differences in yield between organic and conventional farming, although organically grown plants had slightly lower grain

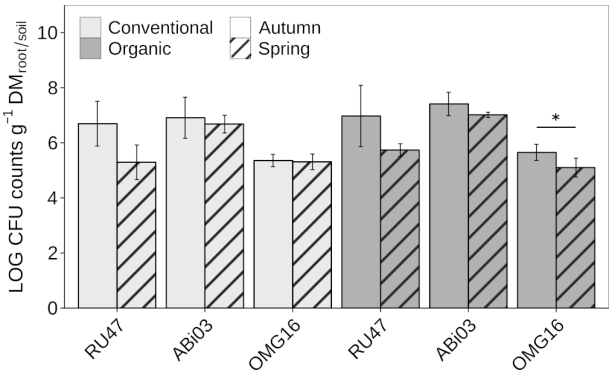

FIGURE 1
Abundance of beneficial microorganisms in winter rye (*cv.* Conduct) cultivated under different long-time farming practices (conventional vs. organic) in autumn and spring of the same growing season. Bars represent means ± standard deviation of four replicates. A two-way ANOVA was conducted to test the effect of the farming practice and the sampling time on the rhizosphere competence of each member of the consortium (Supplementary Table 1). Asterisk indicates a significant difference of the CFU between the sampling time points for each consortium member separately ($^{*}p \leq 0.05$).

weight per hectare (Figure 2B). The BMc treatment had no significant effect on the yield in either conventional or organic farming. However, a slight tendency towards higher yield per unit area was observed in organic farming after the BMc treatment.

To determine whether the nutrient status of winter rye was associated with farming practice and BMc inoculation, the concentrations of several micro- and macronutrients were measured in plants grown across all studied plots and sampling time points (Table 2). At the first sampling time in autumn, most nutrients were at the same level for both farming practices, regardless of BMc inoculation. Two-way ANOVA revealed an overall significant effect ($p \leq 0.021$) of BMc inoculation on the

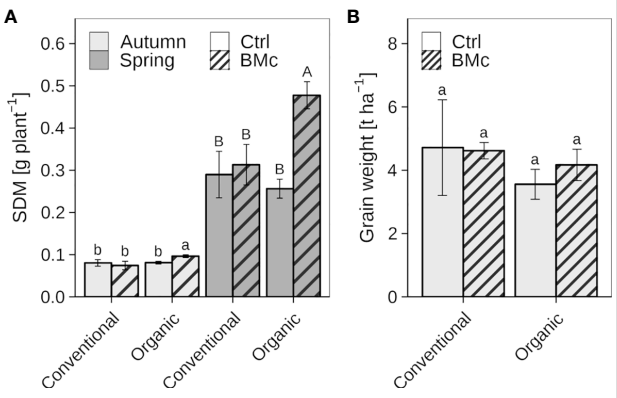

FIGURE 2
Effect of beneficial microorganisms consortium (BMc) on autumn and spring shoot dry mass (SDM) **(A)** and yield **(B)** of winter rye (*cv.* Conduct) cultivated under conventional and organic long-time farming practices. Bars represent means ± standard deviation of four replicates (Supplementary Table 2). Different letters indicate significant differences calculated by the Sidak-test ($p \leq 0.05$). The type of letter indicates a separate statistical analysis (lowercase: autumn, uppercase: spring).

nitrogen concentration (Supplementary Table 3), which was slightly increased with BMc inoculation for both farming practices. However, a pairwise comparison could not verify a significant difference between the four treatments. In contrast to the autumn sampling, the concentrations of almost all nutrients except carbon (C), copper (Cu), calcium (Ca), and manganese (Mn) were reduced in organic farming practice (Table 2) at the second sampling in spring of the following year. Based on a two-way ANOVA, the BMc treatment showed a significant effect on the majority of nutrients in both farming practices, with the exception of zinc (Zn) (Supplementary Table 3). However, a pairwise comparison across all four treatments revealed that the BMc treatment significantly increased only the phosphorus (P) concentration in organically grown plants and the sulphur (S) concentration in conventionally grown plants, in comparison to the control (Table 2). The soil properties were not affected by BMc treatment, but were mainly influenced by the farming practice (Supplementary Table 4).

Bacterial community in the rhizosphere

To elucidate whether the choice of farming practice and BMc inoculation influenced the composition of the winter rye rhizosphere bacterial communities, we performed 16S rRNA gene amplicon sequencing. The farming practice strongly influenced the composition of rhizosphere bacterial communities in the autumn (PERMANOVA, $R^2 = 0.29$, $p < 0.001$, Figure 3A) and in the spring sampling (PERMANOVA, $R^2 = 0.23$, $p < 0.001$, Figure 3B). Similarly, BMc inoculation significantly influenced the bacterial community composition of the rhizosphere in both samplings (PERMANOVA, $R^2 = 0.10$-0.13, $p < 0.001$, Figure 3). However, we did not detect any interaction effect between the choice of farming practice and the BMc inoculation (PERMANOVA, $R^2 = 0.04$-0.05). Combining the two variables and performing pairwise PERMANOVA tests showed that all treatment combinations significantly differed in the autumn sampling (Table 3). Similarly, most groups significantly differed in the spring sampling, except for the Organic-BMc and Organic-Control combination (Table 3). While both farming practices and BMc inoculation influenced bacterial community composition, they did not consistently affect α-diversity. Specifically, farming practice significantly affected ASV richness, Shannon index, and Evenness in the spring but not in the autumn sampling (Supplementary Figure 2, Supplementary Table 5). Irrespective of the sampling time, BMc inoculation did not influence bacterial α-diversity and no interaction effect between the two experimental factors was detected (Supplementary Figure 2).

We aimed to evaluate the ASVs that were affected by either the choice of the farming practice and/or the BMc inoculation. In total, 371 ASVs in the autumn and 273 ASVs in the spring sampling significantly differed between the two farming practices (Supplementary Table 6, ANCOM-BC2). Out of these differential abundant ASVs, 38 ASVs in the autumn and 16 ASVs in the spring sampling were representative of the bacterial communities investigated as they showed a relative abundance above 0.5% (Figure 4). Dominant discriminant ASVs with the highest differential abundance due to choices of farming practice

TABLE 2 Nutrient status of winter rye (*cv.* Conduct) grown under different long-time farming practices and use of beneficial microorganisms (control vs. BMc) in autumn and spring of the same growing season.

		Autumn								Spring							
		Conventional				Organic				Conventional				Organic			
	DT[#]	Ctrl		BMc		Ctrl		BMc		Ctrl		BMc		Ctrl		BMc	
Macro-nutrients [g kg^{-1} shoot DM]																	
C		411	a	407	a	400	a	390	a	331	B	367	AB	358	AB	391	A
N	25	27.0	b	30.7	ab	31.9	a	33.8	a	34.2	A	40.5	A	20.3	B	25.0	B
P	3.0	5.73	a	5.56	a	5.74	a	5.69	a	4.07	A	4.61	A	3.34	B	4.04	A
K	28	28.3	a	28.0	a	29.8	a	30.1	a	20.9	AB	23.9	A	16.4	B	20.7	AB
Mg	1.5	1.91	a	2.03	a	1.79	a	1.83	a	1.96	A	2.19	A	1.30	B	1.45	B
Ca	3.5	2.92	a	3.09	a	2.86	a	2.87	a	2.82	AB	3.30	A	2.76	B	3.00	AB
S	1.0	2.26	a	2.41	a	2.48	a	2.54	a	2.58	B	3.18	A	1.63	C	1.92	C
Micro-nutrients [mg kg^{-1} shoot DM]																	
Cu	6	6.80	a	6.84	a	6.72	a	6.88	a	5.48	A	6.04	A	5.42	A	5.97	A
Mn	25	122	ab	128	a	100	b	113	ab	209	A	180	AB	180	AB	141	B
Zn	20	29.7	a	31.6	a	26.4	a	30.4	a	34.5	A	34.2	A	20.4	B	20.4	B

[#]Deficiency threshold (DT) of macro- and micro-nutrients after Bergmann (1988).
Different letters indicate significant differences calculated by the Sidak-test ($p \leq 0.05$). The type of letter indicates a separate statistical analysis (lowercase: autumn, uppercase: spring) (BMc, Beneficial Microorganism Consortium; Ctrl, Control; DM, Dry Mass).

included ASVs taxonomically related to the *Rhizobium group* (ASV44), *Sphingobacterium* spp. (ASV418), *Sphingomonas* spp. (ASV17, ASV42, ASV53 and ASV69) and *Pedobacter* spp. (ASV14, ASV40, ASV104, ASV140 and ASV145). Two dominant ASVs belonging to *Pedobacter* spp. (ASV14 and ASV40) and one dominant ASV belonging to *Rhizobium* spp. (ASV44) differed between farming practices independent of the sampling time (Supplementary Table 6). Specifically, the relative abundance of ASV44 (*Rhizobium* spp.) increased, while the relative abundance of ASV40 (*Pedobacter* spp.) decreased due to organic farming practice (Figure 4). In contrast, the ASV14 (*Pedobacter* spp.) showed opposite trends between the two samplings under organic farming, with an increase in abundance in autumn and a decline in spring. The BMc inoculation altered the relative abundance of 21 ASVs in the autumn and 14 ASVs in the spring sampling (Supplementary Table 6), with five ASVs in the autumn and three ASVs in the spring accounting for more than 0.5% of relative abundance (Figure 4). Two ASVs classified as *Luteibacter* spp. (ASV48 and ASV61) consistently occurred in higher relative abundance in the BMc inoculated samples. Other ASVs that increased due to BMc inoculation belonged to *Mucilaginibacter* spp. (ASV37) and *Pedobacter* spp. (ASV78, ASV73). Moreover, one dominant ASVs, which belonged to *Pedobacter* spp. (ASV31) was negatively affected by BMc inoculation in the spring sampling.

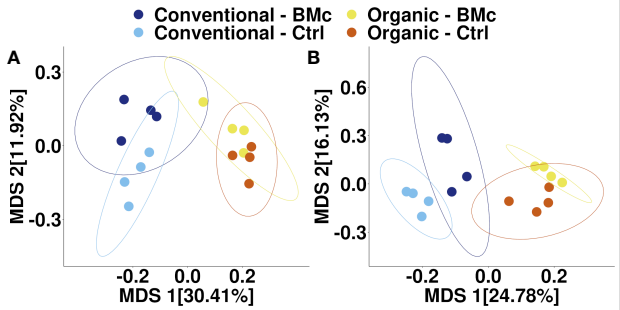

FIGURE 3
Multidimensional scaling (MDS) of bacterial community composition associated with winter rye rhizosphere under different farming practices and BMc inoculation treatment sampled in autumn **(A)** and spring **(B)**. The ellipses represent a 95% confidence interval. (BMc, Beneficial Microorganism Consortium; Ctrl, Control).

We were then interested to elucidate ASVs that significantly differed between each treatment combination. In total, 304 and 236 ASVs were found significantly different between the treatment combinations in the autumn and spring samplings, respectively (Supplementary Table 7), with 43 ASVs significantly differing independent from the sampling time (Figure 4, Supplementary Table 8). The logistic regression models revealed 277 (autumn sampling) and 210 (spring sampling) ASVs as representative predictors for each specific treatment combination (Supplementary Table 9). Of these ASVs, the relative abundance of 30 and 46 ASVs increased in the BMc-inoculated samples under organic fertilization in autumn and spring, respectively (Supplementary Table 9), where significantly higher SDM was detected (Figure 2A). In contrast, in the organic farming, BMc inoculation resulted in a decreased relative abundance of 57 ASVs at the autumn sampling and 19 ASVs at the spring sampling (Supplementary Table 9). Furthermore, three ASVs consistently responded to the BMc-inoculation in organic treatment in each sampling campaign (Supplementary Table 10).

TABLE 3 Pairwise PERMANOVA tests of the combined treatments (farming practices: conventional or organic, use of beneficial microorganisms: BMc or Control) with Benjamini-Hochberg correction (n=4).

Treatment Combination	R^2	Adjusted *p*-value
Autumn		
Conventional-Control vs. Conventional-BMc	0.23	0.029
Conventional-Control vs. Organic-Control	0.39	0.029
Conventional-Control vs. Organic-BMc	0.40	0.029
Conventional-BMc vs. Organic-Control	0.42	0.029
Conventional-BMc vs. Organic-BMc	0.36	0.029
Organic-Control vs. Organic-BMc	0.19	0.029
Spring		
Conventional-Control vs. Conventional-BMc	0.23	0.034
Conventional-Control vs. Organic-Control	0.34	0.034
Conventional-Control vs. Organic-BMc	0.44	0.034
Conventional-BMc vs. Organic-Control	0.31	0.034
Conventional-BMc vs. Organic-BMc	0.29	0.034
Organic-Control vs. Organic-BMc	0.20	0.057

Out of these differentially abundant ASV between the four treatment combinations, 28 and 12 ASVs exceeded the relative abundance of 0.5% in at least one sample in the autumn (Figure 5A) and the spring samplings, respectively (Figure 5B). From these dominant discriminant ASVs, 13 ASVs associated either positively or negatively with the BMc inoculation in the organic treatment. Negative responder ASVs were affiliated to *Chitinophaga* spp. (ASV148 and ASV88), *Duganella* spp. (ASV173 and ASV193), *Brevundimonas* spp. (ASV24), *Microbacteriaceae* spp. (ASV63), *Sphingomonas* spp. (ASV17), and *Actinoallomurus* spp. (ASV377), while positive responders were affiliated with *Sphingobium* spp. (ASV52), *Bacillus* spp. (ASV121), *Pedobacter* spp. (ASV95) and *Luteibacter* spp. (ASV61; only spring). Most notably, ASV88 (*Chitinophaga* sp.) was the single top abundant, significantly different ASV that negatively responded to BMc inoculation in the organic treatment at both sampling time points (Figure 5). Some of these ASVs were also associated with other treatments, especially in the control of conventional farming. For example, the ASV88 (*Chitinophaga* spp.) was enriched in the control samples under conventional farming and depleted with BMc inoculation in the organic treatment at both sampling time points. However, in the spring sampling, ASV61 (*Luteibacter* spp.) responded positively to BMc inoculation in the organic treatment and negatively to control in conventional farming (Figure 5).

Finally, we performed a phylogenetic clustering analysis to estimate whether ASV responders to the BMc inoculation in the organic treatment were associated phylogenetically with the inoculated BM strains (Figure 6). ASV responders classified as *Bacillus* spp. clustered more closely to other *Bacillus* spp. than the 16S rRNA genes of *B. atrophaeus* ABi03 (Figure 6A). In contrast, two out of the three ASVs classified as *Pseudomonas* spp. (ASV1123 and ASV1576), were clustered together with the six 16S rRNA gene copies of *Pseudomonas* sp. RU47 (Figure 6B). These two ASVs were positively associated with the BMc inoculation in the organic treatment in the autumn sampling but not in the spring sampling (Supplementary Figure 3).

Discussion

Successful establishment of the microbial consortium throughout winter dormancy

In this study, we hypothesized that each consortium member would colonize the rhizosphere of winter rye plants and maintain a high density over the winter dormancy independent of farming practices (organic vs. conventional). The three consortium members were selected based on their previously observed positive effects on plant performance. *Pseudomonas* sp. RU47 strain enhanced growth of tomato and maize plants in P-deficient soils (Eltlbany et al., 2019) and suppressed bottom rot (*Rhizoctonia solani*) in lettuce very efficiently under greenhouse and field conditions, although no strong antagonistic properties were found in *in vitro* assays (Adesina et al., 2009; Schreiter et al., 2018). We assumed that the disease suppression effect was mainly based on the production of HCN (Kuzmanović et al., 2018). In contrast, the *Bacillus atrophaeus* ABi03 strain showed various antagonistic functions *in vitro*. Plant growth promotion was also observed in consortia containing the *Trichoderma harzianum* OMG16 strain in tomato (Mpanga et al., 2018) and maize (Mpanga et al., 2019; Moradtalab et al., 2020). Furthermore, OMG16 reduced root infection by *Verticillium longisporum* in rapeseed as well (Hafiz et al., 2022). In this study, the strains with different modes of action were applied as a consortium in the field. Several studies have shown that using a consortium of two or more microorganisms can have a stronger and more sustainable effect on plant health and performance than a single beneficial microorganism (Sharma et al., 2020; Hafiz et al., 2022). Additive or synergistic effects can, in part, be attributed to separate habitats such as the rhizosphere, the root-associated soil, and the root cortex but also to different beneficial functions such as improved nutrient availability, phytohormone production, antagonistic functions against plant pathogens and (a)biotic stress mitigation (Jha and Saraf, 2012; De Vrieze et al., 2018; Gu et al., 2020; Santoyo et al., 2021).

A long-term coexistence between the members of a BMc in the rhizosphere or root-associated soil is crucial to ensure beneficial effects on plants in short and long periods. Seven weeks after inoculation (autumn sampling), all members of the consortium colonized the winter rye roots in a similar density compared to previous studies (Schreiter et al., 2018; Jamil et al., 2021), indicating that the combined application of the beneficial microorganisms as a consortium did not impair their rhizosphere competence. The bacterial strains ABi03 and RU47 were mainly detected in the rhizosphere and did not inhibit each other as observed in dual culture (data not shown), whereas OMG16 inhabits especially the root-associated soil but was also previously reported as an endophyte in rapeseed (Hafiz et al., 2022). Because of this spatial

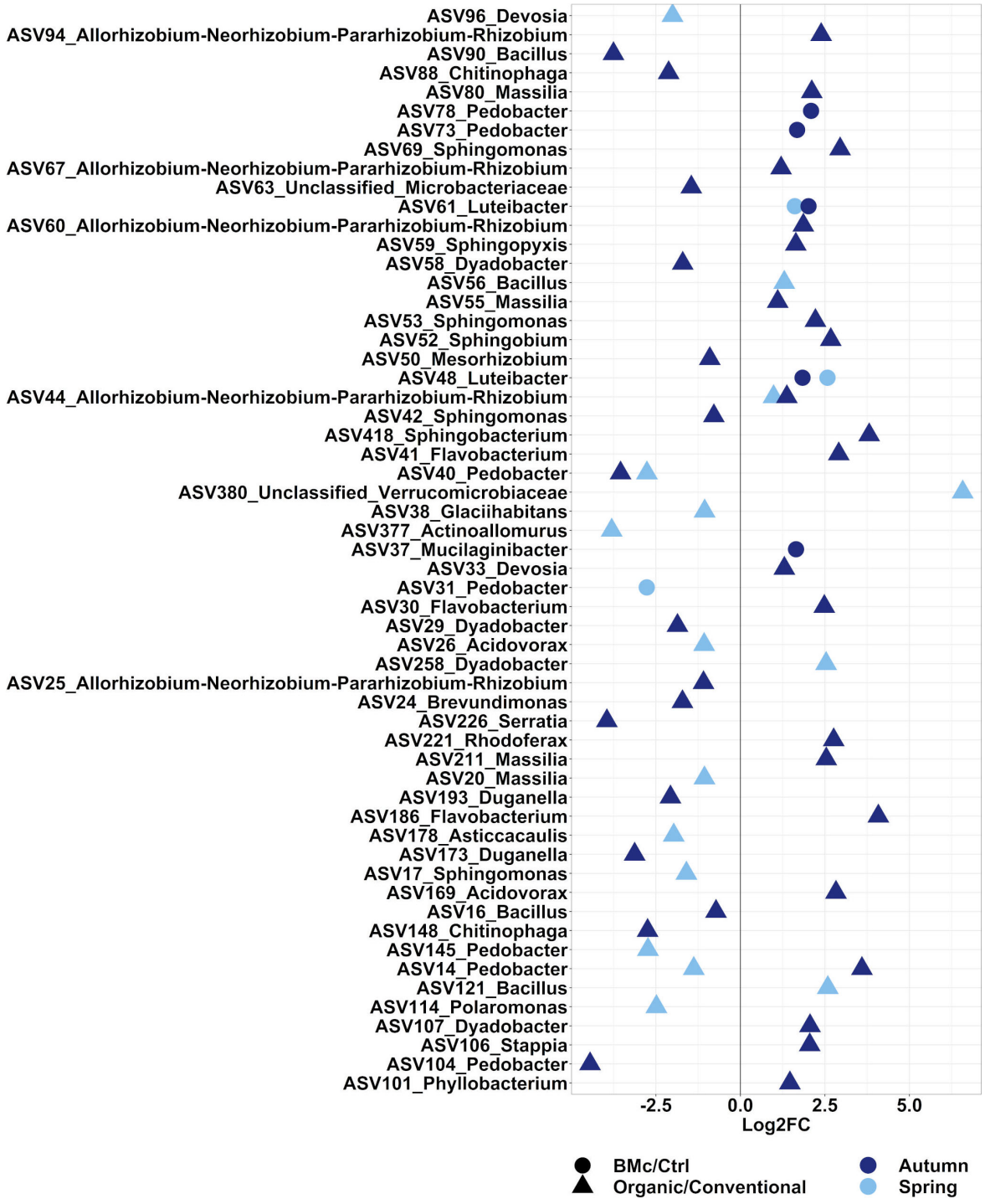

FIGURE 4
Log_2 fold-change (FC) of ASVs that significantly differed and occurred in relative abundance higher than 0.5%. The comparisons were performed between farming practices (Organic *vs.* Conventional) or BMc inoculation (BMc *vs.* Ctrl) in the autumn or the spring sampling campaign during the growth of winter rye plants. Differential abundance testing was performed on rarefied datasets via ANCOM-BC2 with Benjamini-Hochberg correction (adjusted *p*-value<0.05).

separation, potential antagonistic functions demonstrated *in vitro* for RU47 and ABi03 (Table 1) can likely not act against OMG16. Besides direct competition, the establishment of applied microorganisms is highly dependent on the composition and structure of the indigenous microbial community, as demonstrated by previous studies that linked a low rhizosphere competence of applied microorganisms to a high microbial diversity, which acts as a barrier against external invaders (Schierstaedt et al., 2020; Mawarda et al., 2022). The successful establishment of inoculated microorganisms within the rhizosphere

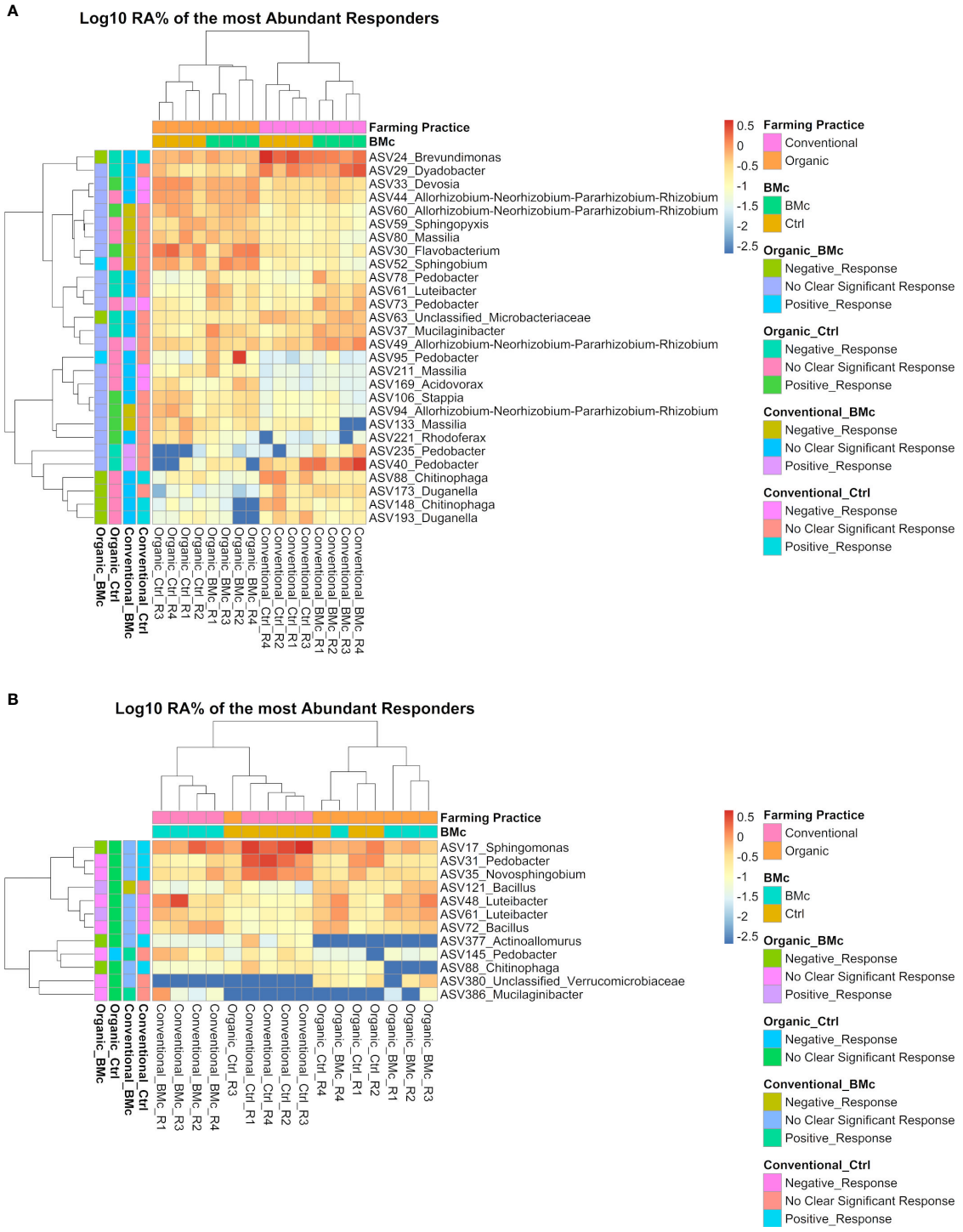

FIGURE 5
Heatmaps with the $\log_{10}$ transformed mean relative abundance of differential abundant ASVs in the autumn **(A)** and spring **(B)** sampling. The presented ASVs occurred in relative abundance higher than 0.5% in at least one sample. Significantly different ASVs were identified via ANCOM-BC2 (adjusted *p*-value via Benjamini-Hochberg <0.05, n=4). The colored legend (left side) shows whether these ASVs responded positively or negatively to a particular treatment combination (Organic-Ctrl, Organic-BMc, Conventional-Ctrl, Conventional-BMc). The responder ASVs were identified via logistic regression (adjusted *p*-value via Benjamini-Hochberg < 0.05). RA, Relative abundance.

has been linked to rapid nutrient utilization, biofilm formation, but also antagonistic capabilities (Adam et al., 2016; Kumawat et al., 2019). *In vitro* demonstrated antagonistic functions of all consortium members may facilitate their selective occupation of niches within the rhizosphere, thus promoting their integration into the microbial community (Berg et al., 2021).

Although our results (Figure 3) confirm that farming practice drives the composition of microbial communities in the soil and

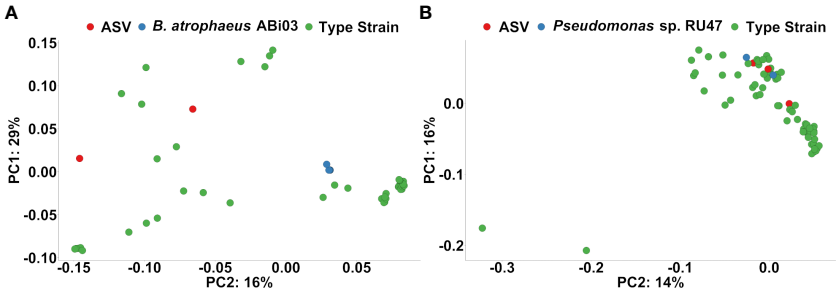

FIGURE 6
(A) Ordination plots based on phylogenetic distance following pairwise local alignment of 16S rRNA gene sequence from the ASV responders to Organic-BMc that were classified as *Bacillus* spp., 16S rRNA genes from *Bacillus atrophaeus* ABi03 and *Bacillus* spp. from the type strain collection of Genbank (Red: ASV responders, Blue: *Bacillus atrophaeus* ABi03, Green: *Bacillus* spp. type strains). The plot shows no indication that these ASV responders classified as *Bacillus* spp. could belong to *Bacillus* ABi03. **(B)** Ordination plots based on phylogenetic distance following pairwise local alignment of 16S rRNA gene sequence from the ASV responders to Organic-BMc that were classified as *Pseudomonas* spp., the strain *Pseudomonas* sp. RU47 (six 16S rRNA genes) and *Pseudomonas* spp. from the type strain collection of Genbank (Red: ASV responders, Blue: *Pseudomonas* sp. RU47, Green: *Pseudomonas* spp. type strains). The plot indicates that two ASV responders (ASV1123 and ASV1576) probably belong to the inoculum strain *Pseudomonas* sp. RU47, since they cluster with the 16S rRNA genes of the inoculum strain *Pseudomonas* sp. RU47.

rhizosphere (Schrama et al., 2018; Babin et al., 2019; Bziuk et al., 2021; Fernandez-Gnecco et al., 2022), the BMc rhizosphere competence was not affected, indicating a robust competence of the consortium members at different environmental conditions. Since only a few responder ASVs consistently differed between the farming practices at both sampling time points, we presume that the assembly of rhizosphere microbiota was a stochastic process influenced by environmental conditions and plant developmental stages. The continuous time-dependent stochastic assembly of bacterial communities in the rhizosphere could have contributed to the high rhizosphere competence of the consortium members independent of farming practices. In spring, the colonization density of RU47 in the rhizosphere decreased overall by a factor of 10, whereas the densities of ABi03 and OMG16 (root-associated soil) remained almost the same compared to the autumn sampling. The higher resilience of ABi03 and OMG16 can be attributed to their potential for sporulation, which allows higher survival under adverse winter conditions compared to RU47 (Fernández-Sandoval et al., 2012; Sella et al., 2014). Nevertheless, all three microorganisms colonized the rhizosphere of winter rye sufficiently, even 22 weeks after inoculation. This indicates a strong positive feedback between the plant and the members of the consortium since the persistence of a high colonization density depends on the one side on the root exudation profile of the plant and on the other side on the chemotaxis of each consortium member towards these metabolites and their use as a substrate (Malgioglio et al., 2022).

Consortium improved plant performance and nutrient acquisition under organic farming

Shoot growth of winter rye was promoted by BMc inoculation in autumn (+20%), but especially during regrowth after winter (+45%) exclusively under organic farming. These findings are in line with the robust rhizosphere competence shown by the investigated inoculants, which remained unaffected even during unfavorable winter conditions. Moreover, under long-time organic farming practice, BMc inoculation was associated with the enrichment of bacterial taxa with documented plant growth promoting properties. As a consequence, organic farming practice may support soil biological processes that allow more complex taxonomic and functional communities (Bender et al., 2016). Numerous studies have consistently reported positive effects on beneficial plant-microbe interactions when using manure-based fertilizers alongside inoculants such as *Bacillus*, *Pseudomonas*, and *Trichoderma* (Thonar et al., 2017; Mpanga et al., 2018; Bradáčová et al., 2019). A beneficial plant-microbe interaction under organic farming might improve the carbon supply of fast-growing copiotrophic inoculants as well as indigenous plant growth-promoting microorganisms, thereby supporting the establishment of a beneficial microbial community. This effect may be of particular importance especially on light sandy soils characterized by low organic carbon content, which were observed in this study even after long-time organic farming practice (Supplementary Table 4). Accordingly, a meta-analysis reported that responsiveness to microbial inoculants decreases with increasing soil organic carbon content (Schütz et al., 2018). Furthermore, the high availability of N and P, which is characteristic of manure-based fertilizers, can serve as a starter fertilization for the host plant, facilitating root growth and the establishment of microbial inoculants in the rhizosphere (Bittman et al., 2006; Chekanai et al., 2018).

The beneficial effects of inoculants and their impact on the rhizosphere microbiome can in part be attributed to improved plant nutrient supply. During autumn, no significant differences in shoot nutrient concentrations were initially observed among the treatments reflecting a comparable nutritional status (Table 2). However, ANOVA revealed a general impact of the BMc inoculation on the N concentration of the shoots (Supplementary Table 3). Nutrient deficiencies were only apparent for Ca (Bergmann, 1988) and may be attributed to low Ca levels of the light sandy soil at the field site (Supplementary Table 4). In contrast,

during regrowth after winter, critical nutrient concentrations were recorded, particularly under organic fertilization, including N, P, K, Mg, Ca, Cu and Zn. BMc inoculation significantly improved the P nutritional status, which is a critical nutrient in many organic farming systems (Cooper et al., 2018). However, similar trends were also recorded for all remaining nutrients except Zn and Mn (Table 2). An improved plant nutritional status due to BMc inoculation is also supported by ANOVA, which shows a general effect of BMc inoculation on the concentration of these nutrients independent of farming practice (Supplementary Table 3). As a consequence, nutrient concentrations of BMc inoculated plants reached or even exceeded the sufficiency thresholds particularly for N and P. These findings suggest a general positive effect of BMc on the nutrient acquisition, possibly mediated through the stimulation of root growth, which has been well-documented for the inoculant strains (Mpanga et al., 2018; Eltlbany et al., 2019; Moradtalab et al., 2020). However, improved plant nutrient acquisition through direct nutrient mobilizing by the BMc is a less likely scenario, although *in vitro* tests did detect solubilization of P and K. Analysis of the root-associated nutrient pools in the soil did not show any changes following the BMc inoculation, suggesting no specific effects on solubilization of available soil nutrients in spring (Supplementary Table 4). According to current literature, P-solubilizing microorganisms contribute to the host plant nutrition primarily through their long-term impact on nutrient cycling, rather than direct nutrient solubilization (Raymond et al., 2021). Interestingly, similar trends of an improved nutrient status after BMc inoculation were also shown for plants grown under conventional farming. However, compared to organic farming, conventional farming practice generally resulted in a higher nutritional status of the plants, reaching sufficiency levels for N, P, and Mg, even in non-inoculated plants. This may explain the absence of additional growth responses by BMc inoculation under conventional farming.

Although there were no significant differences among the treatments, the final yield data (Figure 2B) corresponded with the effects of the shoot biomass during spring vegetative growth (Figure 2A). The grain yield under long-time conventional farming practices was slightly higher than the 2015-2020 average rye yields (4.1 t ha^{-1}) in the district of Brandenburg (Germany). Organic farming practice tended to decrease yield by approx. 22%, similar to the reported average yield losses determined by meta-studies (de Ponti et al., 2012; Seufert et al., 2012). Although not significant, our results indicate that this yield decline by organic farming may partially be compensated by BMc application.

Inoculation-dependent modulation of rhizosphere bacterial community persists over winter dormancy

We hypothesized that the application of the BMc would affect the bacterial community composition in the rhizosphere depending on farming practice. Consequently, we anticipated that these variations would exert differential influences on the performance of winter rye. An alteration of the bacterial community composition in the rhizosphere of winter rye was found in the BMc inoculated treatments, regardless of farming practices. This effect persisted until spring, indicating a prolonged impact of the BMc (Figure 3). Similar results were observed in a study by Deng et al. (2019), where BMc inoculation affected the bacterial community composition of strawberry roots over various time points, irrespective of farming practices. This indicates that repeated application of the inoculants might not be necessary, once their effects have been established. However, further time-series experiments are needed to clarify whether the effects of BMc on the rhizosphere microbiota and plant performance persist throughout the growing season at the field scale.

In addition to BMc inoculation, we observed an effect of the farming practices on the soil microbiota, similar to our previous studies (Bziuk et al., 2021; Windisch et al., 2021; Fernandez-Gnecco et al., 2022; Sommermann et al., 2022). However, this is the first time we report that this effect persists across different plant developmental stages. While both farming practices and BMc inoculation significantly affected bacterial community composition, only a few ASVs exhibited consistent differential abundance patterns over time (Figure 4). This indicates a) that the recruitment of microorganisms in the rhizosphere differed over the developmental stages of the plant and b) that the BMc inoculation and the farming practices influenced this recruitment. A similar influence of farming practices on the recruitment of microorganisms in the rhizosphere has been demonstrated previously (Windisch et al., 2021; Sommermann et al., 2022). In summary, the BMc inoculation influence on rhizosphere microbiota persisted over the winter dormancy, indicating specific strategies such as spore formation by the consortium members to survive during winter time. In addition, changes in the root exudation during spring could have further activated dormant BMs (Windisch et al., 2017).

According to ecological theories on plant-microbe interactions, the mutualistic selection of plant beneficial microorganisms often emerges under nutrient limitations (Sánchez-Cañizares et al., 2017). Since chemical fertilizers provide a high availability of nutrients to plant and rhizosphere microbiota, they could interfere with the plant's selection processes for beneficial microorganisms (Sánchez-Cañizares et al., 2017). Interestingly, we observed increased growth performance of plants inoculated with the BMc only under organic farming, where nutrient availability was low (Supplementary Table 4). We believe that this additive effect could be associated with a higher selection pressure on both the plants and soil microorganisms due to nutrient limitations, in contrast to conventional farming with high nutrient availability. However, CFU data indicated that the farming practices negligibly altered the density of the applied BMc in the soil (Figure 1). Herein, the differential abundance testing revealed an enrichment of ASVs closely related to *Pseudomonas* spp. or *Bacillus* spp., which may correspond to our BMc inoculants. By applying phylogenetic clustering, we identified two *Pseudomonas* ASVs closely related to *Pseudomonas* sp. RU47 (Figure 6). The higher proportions (relative abundance) of *Pseudomonas* sp. RU47 under organic farming and BMc inoculation, might indicate less competition from other

bacterial taxa inhabiting the rhizosphere in organic farming. The interaction effect between farming practices and BMc inoculation on the rhizosphere bacterial community profiles was weak. Thus, the two farming practices only slightly influenced the ability of BMc to modify the rhizosphere bacterial community. Nevertheless, we attempted to identify ASVs associated with BMc inoculation under organic farming to determine which fraction of bacterial taxa are associated with the inoculated BMc. Several ASV responders were detected, but most had a relative abundance below 0.5%.

Among the dominant responders (>0.5% relative abundance), several ASVs closely classified as known potential plant-beneficial bacteria increased in relative abundance in the rhizosphere of winter rye (Figure 5). For instance, members of the genus *Luteibacter* (which belong to γ-*Proteobacteria*) are known for their plant-growth-promoting traits (Guglielmetti et al., 2013; Hoffman et al., 2013) and were enriched in the rhizosphere of the organic treatment. Interestingly, *Luteibacter* spp. can act as symbionts to fungal plant endophytes and trigger the production of IAA, which promotes plant growth. Similarly, one ASV classified as *Pedobacter* spp. (ASV95), a genus that includes several plant-beneficial species, increased in relative abundance due to BMc inoculation under organic farming (Morais et al., 2019). While the combination of BMc inoculation and organic farming might not substantially affect the overall profile of the bacterial community, it increased the relative abundance of bacterial taxa that potentially act as plant-beneficial microorganisms promoting plant growth. However, further field studies are needed to confirm whether the microbial responders due to BMc inoculation also contributed to increased plant growth.

Among the dominant responders with decreasing relative abundance due to BMc inoculation, primarily in organic farming, we identified an ASV classified as *Chitinophaga* sp. or *Duganella* sp. These ASVs have been previously associated with the onset of plant diseases (Carrión et al., 2019; Li et al., 2021). Organic farming practice and the BMc combination probably reduced bacterial taxa associated with plant diseases and stressful conditions. In contrast, some of these ASVs had higher relative abundance in conventional farming practice without BMc inoculation, indicating that the high input of nutrients through chemical fertilization might have led to the accumulation of bacterial taxa associated with plant diseases. Alternatively, plants often recruit several closely taxonomically-related bacteria in response to a pathogen (e.g., *Duganella* spp.) (Haack et al., 2016), so direct association of these taxa with plant pathogenicity is not possible, especially since no plant disease phenotype was detected.

Conclusion

This study illustrated the effects of a beneficial microorganism consortium (BMc) on the performance of a winter rye depending on different farming strategies, i.e. conventional and organic farming. The main goal of our study was to assess the ability of the consortium members to survive in a sufficient density in the rhizosphere/root-associated soil of winter rye as prerequisite for successful plant-microorganism interaction. Our findings demonstrated that the consortium members persisted in the rye rhizosphere over the vegetation period, maintaining a high density even after winter dormancy independent of the farming practice. As expected, the BMc had a positive effect on the rye performance, increasing shoot dry biomass and plant nutritional status especially under organic fertilization. These findings further confirmed the *in vitro* tested plant-promoting features that characterized the microbial members inoculated in the rhizosphere of winter rye under field conditions. Moreover, the inoculated BMc had a significant effect on the bacterial community dynamics as large shifts in rhizosphere bacterial assembly were observed after inoculation in both organic and conventional treatments, and such community shifts were also detected across different plant developmental stages. Interestingly, under organic farming we observed that in the rye rhizosphere, treated with BMc, several bacterial taxa previously associated with plant diseases were depleted, while several putative plant beneficial bacterial taxa were enriched in their relative abundance, thus further highlighting the positive effects of the BMc on the plant health by modulating the rye rhizosphere microbiome. It is noteworthy that although our field experiment indicated a positive effect of BMc on rye performance, especially under organic fertilization, the beneficial impact on rye yield was only marginal. Nevertheless, our results indicate that BMc inoculation might have the potential to compensate yield losses caused by nutrient limitation in organic farming practice. Furthermore, BMc treatment may contribute to a better yield stability by improving plant nutrient status and promoting beneficial microbiota, thus representing a sustainable approach especially in combination with organic farming. Our research emphasizes the importance of conducting additional field experiments to understand the efficacy of microbial inoculants in various cropping systems, promoting the development of efficient microbe-based solutions for sustainable agriculture.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA975889.

Author contributions

Conceptualization: JB, RG, GN, JG, KS. Experiment conduct: JB. Methodology: JB, IK, DB. Validation: JB, IK. Formal analysis: JB, IK. Data curation: JB, IK. Visualization: JB, IK. Writing—original draft preparation: JB, IK. Writing—review and editing: RG, DB, LS, DF, TK-N, SC, JG, GN, KS. Supervision: RG. Project administration: RG. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1232288/full#supplementary-material

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Limited effectiveness of selected bioeffectors combined with recycling phosphorus fertilizers for maize cultivation under Swiss farming conditions

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The use of plant biostimulants, also known as bioeffectors (BEs), has attracted increasing attention as an environmentally friendly strategy for more sustainable crop production. BEs are substances or microorganisms that are applied to plants or the surrounding soil to stimulate natural processes to enhance nutrient uptake, stress tolerance, and plant growth. Here, we tested the effectiveness of five BEs to enhance maize growth and phosphorus (P) uptake from various recycled P fertilizers in a series of pot and field experiments. First, the impact of two bacterial BEs and one soil-specific plant-based BE on crop performance was assessed in a 4-week screening experiment conducted in two arable, P-deficient soils of differing soil pH (a silty clay loam of pH 7.1 and a silty loam of pH 7.8) amended with recycled P-fertilizers (rock phosphate, biogas digestate, green waste compost, composted dairy manure, and chicken manure pellets). Then, for each soil type, the plant growth-promoting effect of the most promising BE–fertilizer combinations was re-assessed in an 8-week experiment. In addition, over a period of up to 3 years, three field experiments were conducted with maize in which up to two bacterial BEs were used either alone or in combination with a plant-based BE. Our experiments show that while BEs in combination with specific P-fertilizers can promote maize growth within the first weeks of growth under controlled conditions, the observed effects vanished in the long term, both in pots and under field conditions. In a tracing experiment, in which we tested the persistence of one bacterial BE over a period of 5 weeks, we observed a drastic decrease in colony-forming units already 2 weeks after inoculation. As previously shown in other studies, our data indicate that the plant growth-promoting effects of BEs found under controlled conditions are not directly transferable to field conditions. It is suggested that the drastic decline in inoculated bacterial strains in the tracing experiment is the reason for the decline in plant growth effect.

KEYWORDS

BIOFECTOR, bioeffectors, bacillus, field trial, humic acids, pseudomonas, seaweed

Introduction

Current agricultural practices rely on high input rates of synthetic fertilizers, pesticides, irrigation, and short-crop rotations (Tilman et al., 2002). This approach has led to a multitude of environmental problems including groundwater pollution, eutrophication of aquatic systems caused by soil erosion, nutrient leaching, and runoff (Tilman et al., 2001; Tilman et al., 2002). Synthetic fertilizer production and use also contribute significantly to greenhouse gas emissions, thus exacerbating climate change (Vermeulen et al., 2012). Additionally, soil processes can decrease the availability of some plant nutrients, such as phosphorus (P), leading to fertilizer inefficiencies and the need for surplus fertilizer application (Smil, 2000). Commonly used P fertilizers in conventional agriculture are manufactured from non-renewable resources with limited global reserves that are in addition concentrated in only a few countries. Therefore, effective recycling and judicious use of these resources are crucial for long-term sustainability (Smil, 2000; Cordell et al., 2009).

There is a growing interest in addressing the negative consequences of high-input agricultural practices, and extensive research is underway to find alternative ways to produce food in a sustainable and eco-friendly manner. Various methods have been explored to minimize fertilizer inputs in agroecosystems, such as breeding plants with superior P-uptake efficiency (Lynch and Brown, 2001), using specific fertilizer placement techniques (Dunbabin et al., 2009), and utilizing soil microorganisms and natural extracts that possess properties that enhance plant growth and nutrient acquisition (Adesemoye and Kloepper, 2009).

In the last decades, the adoption of beneficial microbes or active natural metabolites, known as bioeffectors (BEs), has gained popularity as a sustainable way to increase crop productivity and improve plant health, thereby reducing the use of agrochemicals in crop production systems (Backer et al., 2018). BEs are substances or microorganisms that, when applied to plants or the surrounding soil, stimulate natural processes to enhance nutrient uptake, stress tolerance, and plant growth. BEs are different from fertilizers in the sense that they do not directly provide nutrients to plants, but instead, they enhance the plant's ability to absorb and utilize nutrients. BEs can be derived from a variety of natural sources and can be categorized into two main types, microbial and non-microbial BEs (Du Jardin, 2015). Microbial BEs are beneficial microorganisms, such as bacteria and fungi that colonize the plants' rhizosphere or endosphere and promote plant growth and health through various mechanisms: Microbial BEs can enhance plant growth directly by producing phytohormones (Backer et al., 2018) or indirectly by producing a variety of enzymes that solubilize P and potassium (K) in the soil thereby making nutrients more available for plant growth (Wozniak et al., 2020; Sible et al., 2021). Non-microbial BEs are mainly plant extracts gained from a variety of natural sources, including humic acid or extracts from seaweed. Humic acids can increase nutrient availability by chelating micronutrients in the soil and enhance plant growth by stimulating root development and promoting plant metabolism (Jindo et al., 2020; Yang et al., 2021; Herrmann et al., 2022). Moreover, humic acids can promote microbial activity in the soil, which can enhance nutrient cycling and improve soil health (Yang et al., 2021). Seaweed extracts further contain natural growth hormones, such as auxins, cytokinins, and gibberellins that may stimulate plant growth and development (Mukherjee and Patel, 2020). They also contain trace elements, including iron, zinc, and manganese, that are essential for plant growth and development.

BEs, especially microbial BEs, have been extensively studied for their efficacy on diverse crops in various ecosystems, resulting in numerous publications summarizing their benefits. However, when applied by farmers in practice, the expected effect often fails to materialize mainly due to environmental factors, soil conditions, fertilization practice, type of BE, and crop cultivar (Schütz et al., 2018). Fertilization, i.e., the type and amount of fertilizer applied, can impact the effectiveness of BEs. Some BEs may work better in conjunction with reduced amounts of synthetic fertilizers, or in systems that incorporate organic fertilizers (Thonar et al., 2017). Similarly, soil properties, such as pH, organic matter content, and nutrient availability, were shown to have a strong impact on the effectiveness of BEs as well as the interaction between BEs and the soil microbiome (Mosimann et al., 2017). Given the complexity of these factors, it is important to carefully consider the use of BEs in a specific agricultural system and to ensure that they are applied in a way that maximizes their effectiveness. Hence, further research is required to determine the specific conditions that enable BEs to enhance plant growth more consistently and predictably. This information can be used to develop tailored BEs that could increase fertilizer efficiency and reduce agriculture's reliance on synthetic fertilizers.

To assess the effectiveness of five BEs to enhance maize growth and P-uptake from various recycled P fertilizers, we conducted a series of pot and field experiments. First, we performed a screening experiment with a combination of BEs and recycled P fertilizers in soils of differing pH. Then, for each soil type, the plant growth-promoting effect of the most promising BE–fertilizer combinations was re-assessed in a follow-up experiment. In addition, over a period of up to 3 years, three field experiments were conducted with maize in which up to two bacterial BEs were used either alone or in combination with non-microbial BEs, consisting of humic acids or algal extracts. To investigate the factors explaining the observed results, we assessed the persistence of one bacterial BE in a tracing experiment and the effects of humic acids on soil properties. This study was conducted as part of the European project BIOFECTOR (7th FP), which focused on reducing the use of mineral fertilizers in European agriculture. The project aimed to develop adapted BEs that can enhance the efficiency of alternative fertilization approaches, including organic farming, low-input farming, and the utilization of fertilizers derived from recycling waste products.

Materials and methods

Experimental design

Maize growth (variety Colisée, KWS Saat, Germany) was investigated in pots using topsoil collected from two fields of different pH and management: "Buus" soil was collected from an

organically managed arable field low in soil P content and neutral pH (pH_{H2O} = 7.1 and "Dompierre" soil from an alkaline calcareous grass clover lay (pH_{H2O} = 7.8). In addition, field experiments were conducted on two organically managed farms: the "Buus" site (47° 30′42.9″N 7°50′50.0″E, Basel-Land, Switzerland), from where also soil for experiments under controlled conditions was collected, and the "Hagenwil" site (47°31′35.4″N 9°18′28.1″E, Thurgau, Switzerland) where an on-farm experiment was conducted in collaboration with the farmer. For more details on soil properties, see Table 1. Experiments were established following a factorial design including up to three factors: P fertilization, microbial BE application (BE), and soil-specific, non-microbial BE application (from here on referred to as additive). Pot experiments were conducted with different organic recycled P fertilizers and with two mineral fertilizer controls. Table 2 gives an overview of the experiments and the tested factors applied in each experiment.

BE and additive treatments

Three microbial BE products and two soil-specific additives were tested in total. These BE treatments included the following: Proradix WP (Sourcon Padena, Germany) containing *Pseudomonas* strain DSMZ 13134 (Proradix), RhizoVital 42 fl. (Abitep, Germany) containing *Bacillus amyloliquefaciens* strain FZB42 (RhizoVital), and BEmix containing *Bacillus licheniformis*, *B. megaterium*, *B. pumilis*, *B. subtilis*, *Paenibacillus polymyxa* with $>10^9$ colony-forming units (CFU)/g product for each bacterial strain, *Trichoderma harzianum* strain OMG08 with $> 10^{10}$/g product, and 15 mg of Mn/Zn per gram product. While *Trichoderma* belongs to the fungal kingdom, all other microbial BEs used are bacteria. The initial project experiments have shown that the majority of these components are effective in enhancing crop growth. Thus, the BEmix was newly formulated by partners of BIOFECTOR to be tested within the project. The choice of Proradix and RhizoVital is based on their published ability to promote maize growth under similar soil conditions and fertilization strategies (Thonar et al., 2017). For BE application, BE suspensions were prepared under sterile conditions by diluting the products with 2.5 mM $CaSO_4$/water in pot/field experiments and inoculating at a concentration of 2×10^6 CFU per gram of substrate/soil. Additive treatments included either AgriPrime Nematec® (BioAtlantis Ltd., Ireland) containing *Laminaria digitata* (Nematec), a derived-brown alga product, applied to the microbial-rich Buus soil or humic acids extracted from artichoke residue compost (Monda et al., 2018) applied to the alkaline Dompierre soil. Nematec was selected based on the producers' experience that Nematec stimulates microbial grazers that can improve crop nutrient supply in microbe-rich soils such as the Buus soil. Humic acids were selected based on preliminary project results of improved P supply from recycled fertilizers in alkaline soils. Non-inoculated controls (noBE/A0) were included in each experiment testing BEs/additives. Further details on BE and additive application are given in Supplementary Data 1.1. All BEs and additives were provided by the EU-BIOFECTOR project partners and additional information on the BIOFECTOR project and the BEs used is available on the website: http://www.biofector.info.

Fertilization treatments

Fertilization treatments included several organic recycled P fertilizers: biogas digestate (Leureko, Rheinfelden, Switzerland) with a P content of 0.21%, sieved at 10 mm and referred to as digestate; compost from green waste (Leureko, Rheinfelden, Switzerland) with a P content of 0.281%, sieved at 10 mm and referred to as compost; composted dairy farmyard manure with a P content of 0.64% and referred to as FYM; and pelleted chicken manure (Agriges, Italy) with a P content of 1.7%, ground and sieved at 1 mm and referred to as pellets. In addition, rock phosphate (Sebald Zement GmbH, Germany) with a P content of 11.1%, ground and sieved at 1 mm and referred to as RP, and Triple Superphosphate with a P content of 20%, ground and sieved at 1 mm and referred to as TSP, were partly included as positive controls. A non-P fertilized control (noP) was included in every experiment testing different P fertilizers. Except for noP treatments, pots received P at a dose of 50 mg of P/kg of dry substrate and field plots at a dose of 50 kg of P/ha.

Experimental setup

Growth experiments under controlled conditions

The four experiments under controlled conditions followed a fully randomized design with four replicates (4-week screening

TABLE 1 Properties of soils.

Soil/ origin	Management	Soil type	Texture			Soil pH_{H2O}	Organic carbon(%)	Phosphorus (P)
			Clay	Sand	Silt			Olson[a] DL[b] (mg P/kg)
			(%)	(%)	(%)			
Buus	Organic arable field	Silty clay loam	29.9	3.90	66.2	7.1	2.64	6.5[a]
Dompierre	Conventional grass clover lay	Silty loam	14.8	43.5	41.7	7.8	1.26	10.3[b]
Hagenwil	Organic arable field	Silty loam	19.7	29.3	51.0	6.5	2.45	1.82[a]

DL, Double lactate.

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TABLE 2 Overview of the experimental design and setup of the experiments under controlled conditions (Exp. 1–4) and field conditions (Buus1,2; Hagenwil).

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Buus1	Buus2	Hagenwil
Factors tested:							
Bioeffectors							
No bioeffectors (noBE)	x	x	x		x	x	x
Proradix	x	x	x		x	x	x
Rhizovital					x		x
BEmix	x	x					
Additive							
No additive (A0)	x	x	x	x		x	
Nematec	x		x			x	
Humic acids		x		x			
Fertilizers							
No P fertilizer (noP)	x	x	x	x	x	x	
Triple superphosphate	x	x	x	x			
Rock phosphate	x	x					
Digestate	x	x		x			
Green waste compost	x	x		x	x		
Dairy farmyard manure	x	x					
Chicken manure pellets	x	x	x	x		x	
Experimental setup:							
Growing conditions	CC	CC	CC	CC	Field	Field	Field
Soil origin/location	Buus	Domp	Buus	Domp	Buus	Buus	Hagenwil
Substrate per pot (kg DW equivalent)	1	1	2.5	2.5	na	na	na
Growth period (weeks)	4	4	8	8	17, 18	17	24, 26, 27
Number of seasons	na	na	na	na	2	1	3
Number of replicates	4	4	5	5	4	4	4

CC, controlled conditions; Domp, Dompierre; na, not applicable; DW, dry weight. x indicates the selection of variants for a given experiment.

experiments: Exp. 1 and Exp. 2) and five replicates (8-week growth experiments: Exp. 3 and Exp. 4) per treatment. After sieving, the soil was mixed with quartz sand (0.6–1.2 mm) in the ratio of 2:1 [soil dry weight (DW)/sand]. Each pot contained the equivalent of 1 kg or 2.5 kg DW of the experimental substrate (Table 2). Besides P fertilizers specified in Table 2 and above, all pots received nitrogen (N) (100 mg of N/kg substrate) and potassium (K) (166 mg of K/kg substrate) in the form of calcium nitrate and potash magnesia, respectively. Where organic recycled P fertilizer containing N and K were applied (Table 2), the basal dose of mineral N and K fertilizers was reduced accordingly. The N, K, and P fertilizers were homogeneously mixed into the substrate before potting. Water addition was adjusted to reach 60–70% of the substrate's maximal water-holding capacity (WHC). Three seeds were sown per pot, and the BE suspension or water (for noBE treatments) was added to the seeding hole. After covering the seeds with the substrate, additive suspension or water (for A0 controls) was applied on the surface at a distance of 5 cm surrounding the seed. The surface of the pots was then covered by a fine layer of quartz sand to avoid the formation of surface crusts after watering and pots were covered with plastic foil until germination of maize to avoid water loss due to evaporation. The pots were randomly placed into a growth chamber with 12-h day/12-h night, 26/22°C, 30,000 lux (mercury/natrium lamps), and 60% relative humidity and watered according to the plants' needs to keep the initial water holding capacity (WHC) of 60% (increased to 70%–80% after 2 weeks). Thinning (including the root systems) was performed 1 week after sowing, leaving one plant in each pot. A second and third application of Nematec/humic acids and Nematec, respectively, were conducted (Supplementary Data 1.1). During the growth period, plant height and stem diameter were measured and the final harvest took place 4 or 8 weeks after sowing by cutting the plants shortly above the soil surface. Fresh weight was measured

before plants were dried at 60°C to determine the shoot DW and milled for elemental analyses. The shoot P-concentration was measured using the molybdate blue method (Murphy and Riley, 1962) on a Segmented Flow Analyzer (Skalar Analytical B.V., San++ Automated Wet Chemistry Analyzer, Breda, Netherlands) after incineration and acid extraction of the shoot powder. The root system was washed from the substrate, weighed, and dried to determine the total root DW.

Field experiments at the Buus site

At the Buus site, two experiments were conducted (Table 2). The selection of BE treatments of the trial "Buus 1" was based on the results presented by Thonar et al. (2017), which showed in a pot experiment that the two BE products Proradix and RhizoVital enhanced maize growth combined with organic fertilizers. To further validate this potential, we tested these two Bes combined or not with compost at the Buus site for two consecutive years (2014 and 2015). The selection of treatments for the trial "Buus 2" was based on results observed in the screening experiment using the Buus soil (experiment 1). Both field experiments were designed in randomized blocks with four replicated plots (single plot size 3 m × 8 m) per treatment. N and K were applied at a rate of 110 kg of N/ha and 220 kg of K/ha in the form of potassium sulfate (33.2% K, Landor, Schweiz) and horn meal (15.4% N, Hauert, Schweiz). Where P fertilizers containing N and K were applied, the basal dose of N and K was reduced accordingly. The N, K, and P-fertilizer composts were homogeneously spread over the plot after plowing and incorporated with a rotary harrow. Maize seeds were sown manually into seeding furrows of 10 cm depth and inoculated with BE suspension or treated with the same volume of water (noBE treatments) before closing the seeding furrow Supplementary Figure S1A). For plots receiving the additive Nematec, the diluted product was applied above the seeding furrow. To mimic under-foot fertilization, chicken manure pellets were spread manually in 15- to 20-cm-deep strips between the maize rows. A second BE application was conducted at the two-leaf stage and a second and third application of Nematec were conducted at the two- and five-leaf stage, respectively (for more details, see Supplementary Data 1.1, Supplementary Figure S1B). Total shoot biomass was harvested at the reproductive stage (R3–4) cutting the shoots 5 cm above the ground before the fresh weight was determined. Subsamples of the biomass were then dried at 60°C to calculate shoot DW and further milled for elemental analyses (as described above).

Field experiment at the Hagenwil site

The three on-farm experiments, conducted in 2014, 2015, and 2017, were arranged in a randomized strip design with four replicated strips (min. 150 m) per treatment. In these experiments, only BEs previously reported to enhance maize growth in combination with organic fertilizers (Thonar et al., 2017) were tested and compared. Thus, fertilization was the same for all strips and consisted of sheep manure (20 t/ha) and chicken or pork slurry (14 t/ha) in 2014, and sheep manure (3 t/ha) in 2015 and 2017 and spread according to farmers' practice before sowing. The BE suspensions or water (noBE treatments) were applied during sowing with a specially converted seeding machine and a second time at the three-leaf stage during mechanical weeding with a specially converted weeding machine (Supplementary Figure S1C, D; Supplementary Data 1.2). Using this innovative technique, BEs were applied directly to the maize seed in the drilling furrow. At full maturity (R6), plant density was determined from each of the two subplots and the corncobs harvested to assess the number of corncobs and their total FW. Ten representative corncobs were selected and further analyzed in the lab. The corn yield was calculated by removing the grains from the cobs of all subplots and drying both parts to assess their DW to calculate the total corncob yield.

Tracing experiment

To assess the persistence of *Pseudomonas* DSMZ 13134 contained in the product Proradix, a tracing experiment was conducted. Pots were filled with potting substrate, and maize seeds were sown and inoculated with Proradix. At five time points, each of the three pots was harvested. The persistence of the bacterial inoculum was determined by qPCR. The setting up of the experiment was in principle identical to that described for Exp. 3 in *section 2.4.1*. Fifteen 3-L pots were filled with the Buus soil and fertilized with pellets. Per pot, three maize seeds were sown and inoculated with Proradix. All pots were randomly placed on a table and randomized twice and once a week during the first and following weeks, respectively. One week after sowing, the first three pots were harvested. In the following 4 weeks, each of the three pots was harvested every week. Harvest was performed by cutting the stems directly at the soil surface and the FW was determined. After drying stems at 105°C for 12 h, the DW was assessed. The root system was carefully freed from soil and washed in a water bath. Roots were carefully dried with paper towels and the root FW was recorded. A subsample of fine roots was taken and stored at −20°C for extraction of DNA. The remaining part of the root system was dried at 105°C for 12 h before the root DW was determined. DNA extraction and qPCR analyses to quantify *Pseudomonas* DSMZ 13134 in the rhizoplane were performed as described in Mosimann et al. (2017).

Soil incubation experiment

This experiment was set up to exploit the potential of humic acids concerning its potential to promote P mobilization and the activity of microorganisms. The experiment included the same treatments as described for Exp. 4 with four replicates each. The preparation of the substrate was the same as described in *section 2.4.1*, but instead of 3-L pots, the substrate was filled in 0.5-L pots, placed into boxes, and incubated in the climate chamber for 8 weeks at 26/22°C (day/night). At the beginning and after the incubation period of 8 weeks, soil pH, resin-extractable P (resin P), and respiration of the microorganisms in the soil (soil basal respiration) were measured (for details concerning the methods, see Supplementary Data 1.3).

Statistical analyses

All data were analyzed in R version 4.2.2 (R Core Team, 2022) through Rstudio version 2023.3.0.386 (RStudio Team, 2020), and graphs were produced using *ggplot2* (Wickham, 2016). Using linear models, the effects of the factors "bioeffector," "additive," and "fertilization," as well as all of their two- and three-way interactions, were examined. Data from the field experiments (Buus 1, Buus 2, and Hagenwil) were analyzed using linear mixed-effect models through the function *nlme::lme* (Pinheiro et al, 2022 and R Core Team, 2022). We included the experimental factors as shown in Table 2 as well as all possible interactions in the model. The random factors were chosen to model the spatial and/or temporal independency of the collected data and consisted of random = ~ 1|Year/Block (Buus 1), random = ~ 1|Block (Buus 2), and random = list (Year = ~1, Block = ~1 | Year, Rep_Strip = ~1 | Block, Rep_inside.strip = ~1 | Rep_Strip) (Hagenwil). The statistical significance of the main effects in the mixed models was derived using the *anova* function. Estimated marginal means for the factors explaining a significant amount of variation in the data were, for all models, derived through the function *emmeans::emmeans* (Lenth, 2023). If the factors "bioeffectors" or "additive" (or any of the interactions involving these factors) explained a significant amount of variation in the data, we conducted the corresponding *post-hoc* tests and generate pairwise mean comparisons using the *emmeans::emmeans* (Lenth, 2023) with Bonferroni-adjusted *p*-values. All models' fit was visually verified, and if necessary, data were transformed to conform to the model residuals' variance homogeneity and normal distribution assumptions. Data analysis is available as an Rmarkdown script (https://zenodo.org/record/8169013).

Results

Plant growth experiments under controlled conditions

We conducted a total number of four plant growth experiments to evaluate the efficacy of selected BEs to enhance maize growth under controlled conditions (see Table 2 for details on the experimental setup).

In experiment 1, both BE application and fertilization explained a significant amount of variation in plant height, stem diameter, shoot DW, and root DW (Figure 1, Table 3, Supplementary Table S1). Conducting the corresponding *post-hoc* tests, we only found significant differences between levels of the factor BE application for the shoot and root DW. When compared to the control treatment without BE application, shoot and root DW increased with the application of Proradix (Table 4, Supplementary Table S2). Similarly, when compared to the control treatment noBE, root DW increased under the application of Proradix but decreased when the BEmix was applied (Table 3, Supplementary Table S2).

In experiment 2, both the application of the additive and fertilization explained a significant amount of variation in plant height, stem diameter, and root DW (Figure 2, Table 3, Supplementary Table S3) with increased values when comparing the humic acid-treated plants to the control plants A0 (Table 5). For shoot DW, there was a complex three-way interaction between BE, additive, and fertilization (Table 5, Supplementary Table S4): When humic acids were combined with compost, BE application reduced shoot DW while BE application enhanced shoot DW when humic acids were combined with FYM or pellets. Under fertilization with digestate and when no humic acids were applied, shoot DW was lower for plants inoculated

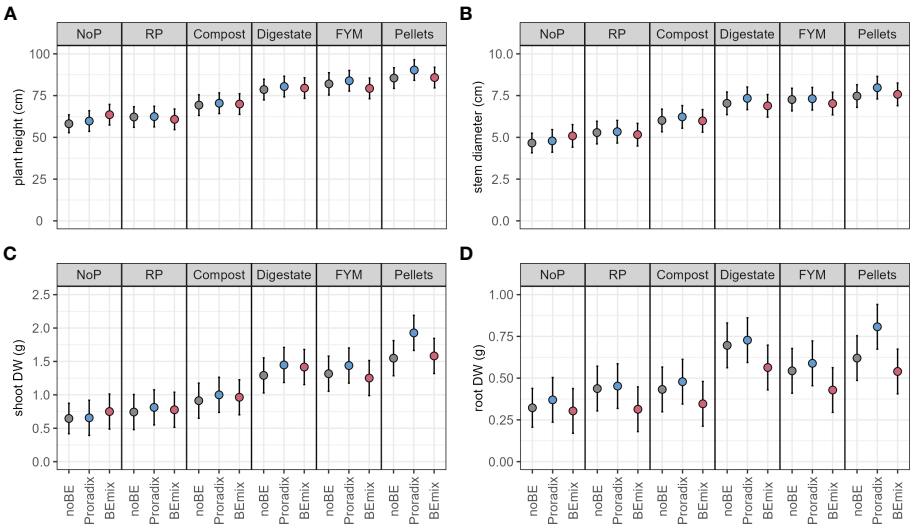

FIGURE 1
Model predictions (estimated marginal means) for experiment 1 with 95% confidence intervals of plant height **(A)**, stem diameter **(B)**, shoot **(C)**, and root **(D)** dry weight (DW) of maize inoculated with the bioeffectors (BE) Proradix or BEmix or without BE (noBE) and fertilized with rock phosphate (RP), compost, digestate, farmyard manure (FYM), pellets, or without P addition (NoP) assessed in experiment 1. Estimates are averaged over the levels of factor "Additive".

TABLE 3 Summary of significant treatment effects on plant growth parameters assessed in experiments 1–3 (Exp. 1–3) conducted under controlled conditions according to analysis of variance (ANOVA).

Experiment	Response	Source of variation	DF	Sum Sq	Mean Sq	*F* value	Pr(>*F*)
Exp. 1	Plant height (cm)	Bioeffector (BE)	2	260.137	130.069	3.979	0.021
	Stem diameter (cm)	BE	2	2.653	1.327	3.381	0.038
	Shoot dry weight (g)	BE	2	0.757	0.378	6.404	0.002
	Root dry weight (g)	BE	2	0.575	0.288	18.761	0.000
Exp. 2	Plant height (cm)	Additive	1	869.643	869.643	31.000	<0.001
	Stem diameter (cm)	Additive	1	7.809	7.809	26.278	<0.001
	Shoot dry weight (g)	Additive	1	0.352	0.352	8.947	0.003
		BE:Fertilization	10	3.920	0.392	9.960	<0.001
		Additive:Fertilization	5	1.525	0.305	7.748	<0.001
		BE:Additive:Fertilization	10	4.319	0.432	10.975	<0.001
	Root dry weight (g)	Additive	1	0.111	0.111	8.414	0.004
Exp. 3	Shoot dry weight (g)	BE:Fertilization	1	14.677	14.677	4.980	0.033

Degrees of freedom (DF), sum of squares (Sum Sq), mean squares (Mean Sq), p-value (Pr(>F)). For the complete table with all effects, see Supplementary Tables S1, S3, and S5.

with Proradix compared to plants inoculated with the BEmix, while the opposite was found under fertilization with RP. The application of Proradix promoted shoot DW under no fertilization, both in the presence and in the absence of humic acids. When combined with RP and the application of humic acids, Proradix decreased shoot DW compared to the control without BE but it increased shoot DW when no humic acids were applied.

In experiment 3, fertilization significantly affected plant height, shoot P uptake (mg P/pot), and root and shoot DW, while for the latter, there was also a significant interaction between BE application and fertilization (Table 3, Supplementary Table S5): When chicken manure pellets were used as fertilizer, there were no significant differences in shoot DW between the treatment group receiving Proradix and the control group without BE application (mean difference: 1.06, SE = 0.768, p = 0.176), while applying Proradix in the absence of P-fertilization marginally reduced plant DW when compared to the control (mean difference: −1.36, p = 0.085).

TABLE 4 Mean comparisons of plant-related data with Bonferroni-adjusted *p*-values for experiments 1 and 2.

Experiment	Contrast	Estimate	SE	DF	*t*-ratio	*p*-value	Response
Exp. 1	Proradix–no Bioeffector (noBE)	1.908	1.163	111	1.640	0.311	Plant height (cm)
	BEmix–noBE	0.523	1.163	111	0.449	1.000	
	BEmix–Proradix	−1.385	1.167	111	−1.187	0.713	
	Proradix–noBE	0.206	0.127	112	1.630	0.318	Stem diameter (cm)
	BEmix–noBE	−0.002	0.127	112	−0.016	1.000	
	BEmix–Proradix	−0.208	0.128	112	−1.629	0.318	
	Proradix–noBE	0.138	0.049	112	2.800	**0.018**	Shoot dry weight (cm)
	BEmix–noBE	0.047	0.049	112	0.959	1.000	
	BEmix–Proradix	−0.090	0.050	112	−1.822	0.213	
	Proradix–noBE	0.062	0.025	112	2.483	**0.044**	Root dry weight (cm)
	BEmix–noBE	−0.093	0.025	112	−3.707	**0.001**	
	BEmix–Proradix	−0.155	0.025	112	−6.125	**<0.001**	
Exp. 2	Humic acids (HA)–no Additive (A0)	4.358	0.877	112	4.971	**<0.001**	Plant height (cm)
	HA–A0	0.394	0.090	112	4.372	**<0.001**	Stem diameter (cm)
	HA–A0	0.046	0.019	112	2.400	**0.018**	Root dry weight (g)

Standard error (SE), degrees of freedom (DF). Bold values highlight significant contrasts.

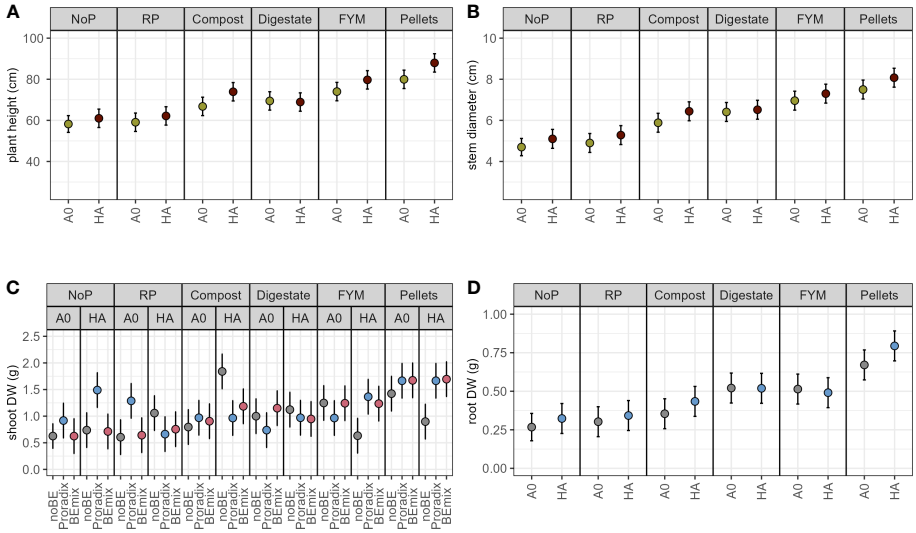

FIGURE 2
Model predictions (estimated marginal means) for experiment 2 with 95% confidence intervals of plant height **(A)**, stem diameter **(B)**, shoot **(C)**, and root **(D)** dry weight (DW) of maize inoculated with the bioeffectors (BE) Proradix or BEmix or without BE (noBE), supplemented with the additive humic acids (HA) or without additive (A0) and fertilized with rock phosphate (RP), compost, digestate, farmyard manure (FYM), pellets, or without P addition (NoP) assessed in experiment 2. Estimates in A, B, and D are averaged over the levels of factor BE.

Using a qPCR-based tracing tool for *Pseudomonas* strain DSMZ 13134, the active ingredient of Proradix, we found the bacteria to be able to colonize the rhizosphere of maize roots; however, the abundance of *Pseudomonas* strain DSMZ 13134 changed significantly over time ($F = 10.675$, $p = 0.001$). One week after inoculation, the model-based prediction was 82,385 CFU/mg root DW, yet the number of CFU/mg root DW dropped significantly within the next week to 467 CFU/mg root DW (ratio = 0.006, SE = 0.01, $p = 0.01$), corresponding to a reduction in CFU/mg root DW of around 99% (Figure 3) and leveled off within the following weeks to values close to the detection limit (Supplementary Table S6).

In experiment 4, only fertilization but not additive application explained a significant amount of variation in the response variables (Supplementary Table S7). In the accompanying incubation experiment, there were no effects of humic acids on net soil pH, while for basal respiration and net resin P measured after 8 weeks, there was significant fertilization × additive interaction (Supplementary Table S8): Applying humic acids together with

TABLE 5 Mean comparisons of maize shoot dry weight with Bonferroni-adjusted *p*-values for the screening experiment in experiment 2 conducted in the Dompierre soil under controlled conditions.

Contrast	Additive	Fertilization	Estimate	SE	DF	*t*-ratio	*p*-value
Proradix–no Bioeffector (noBE)	No additive (A0)	No phosphorus (noP)	0.291	0.121	112	2.399	0.054
Proradix–noBE	Humic acids (HA)	NoP	0.753	0.140	112	5.364	<0.001
BEmix–Proradix	HA	NoP	−0.778	0.140	112	−5.542	<0.001
Proradix–noBE	A0	Rock phosphate (RP)	0.682	0.140	112	4.865	<0.001
BEmix–Proradix	A0	RP	−0.645	0.140	112	−4.598	<0.001
Proradix–noBE	HA	RP	−0.395	0.140	112	−2.816	0.017
Proradix–noBE	HA	Compost	−0.875	0.140	112	−6.237	<0.001
BEmix–noBE	HA	Compost	−0.655	0.140	112	−4.669	<0.001
BEmix–Proradix	A0	Digestate	0.412	0.140	112	2.941	0.012
Proradix–noBE	HA	Farmyard manure (FYM)	0.732	0.140	112	5.222	<0.001
BEmix–noBE	HA	FYM	0.602	0.140	112	4.295	<0.001
Proradix–noBE	HA	Pellets	0.768	0.140	112	5.471	<0.001
BEmix–noBE	HA	Pellets	0.797	0.140	112	5.685	<0.001

Standard error (SE), degrees of freedom (DF). For the complete table with all mean comparisons see Supplementary Table S4.

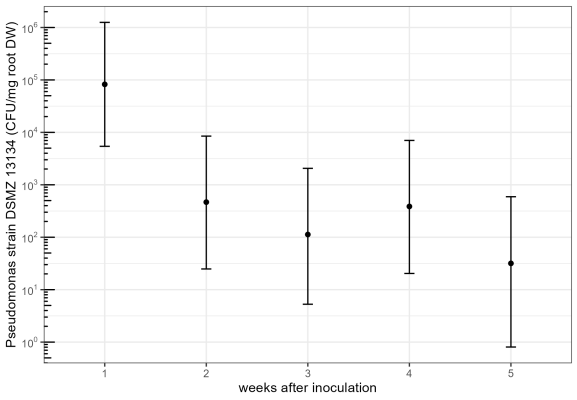

FIGURE 3
Model predictions (estimated marginal means) for the tracing experiment with a 95% confidence interval for colony-forming units (CFU) of *Pseudomonas* strain DSMZ 13134 detected per milligram of root dry weight (DW) over the first 5 weeks after inoculation. Data are log-transformed for graphical representation.

RP increased basal respiration when compared to the control without humic acids (ratio = 1.112, SE = 0.03, p = 0.001), but humic acids did not affect basal respiration when combined with any of the other fertilizers. The application of humic acids together with compost reduced the net resin p-value compared to the control (mean difference: −0.817, SE = 0.22, p = 0.001), while for the other fertilizers, it did not influence the net resin p-values whether humic acids were applied or not.

Field experiments

In experiment Buus 1, neither the factor BE nor fertilization had a significant influence on the response variables investigated (Supplementary Table S9). In experiment Buus 2, there was a significant fertilizer × BE/additive interaction on plant height (Supplementary Tables S10, S11): Under fertilization with pellets and when compared to Proradix, the application of Nematec slightly promoted plant height (mean difference: 0.193, SE = 0.069, p = 0.043) while no difference between the two BEs was detected in the absence of fertilization (mean difference: 0.110, SE = 0.069, p = 0.401). In the experiment at Hagenwil, no effects of BE application were observed (Supplementary Table S12).

Discussion

Minimal and non-reproducible plant growth-promoting effects upon BE application under controlled conditions

We observed small and soil-specific plant growth-promoting effects when maize was grown with Proradix and humic acids in the Buus and Dompierre soil, respectively. However, even these small effects vanished when repeating the experiment in larger pots and extending the growth period from 4 to 8 weeks. Our results are in contrast with other previously published studies describing improved growth of maize and other crops after the application of BE products containing *Pseudomonas* strains or microbial consortia (Schütz et al., 2018; Bradáčová et al., 2020; Li et al., 2022). In particular, microbial consortia were often shown to have larger effects on crop growth than single strains (Kumar et al., 2016; Rubin et al., 2017; Herrmann et al., 2022). A reason for this might be that diverse consortia promote the survival and function of inoculated microorganisms and consequently establish more successfully in the soil compared to single-strain BEs as the likelihood of at least one strain escaping competitive exclusion is higher (Rivett et al., 2018). Moreover, the most pronounced effects of BEs were found in the dry tropics and the Mediterranean zone, with soils low in soil organic carbon (SOC) (Schütz et al., 2018). However, in our experiments, the growth promotion of maize could not be reliably observed with none of the tested BEs.

To reveal potential factors explaining the absence of a plant growth-promoting effect, we conducted a tracing experiment in the Buus soil, characterized by a high SOC. We observed that the *Pseudomonas* strain DSMZ 13134 was initially able to colonize the roots of maize, but was no longer detectable just 2 weeks after inoculation. Potential reasons for the inefficient persistence of the *Pseudomonas* strain DSMZ 13134 after initial establishment might be competition with the resident microorganisms, e.g., because of niche overlap, priority effects, facilitation (Hawkes and Connor, 2017), resource competition (Yang et al., 2017; Mallon et al., 2018), or predation through bacteriophages and microbial predators (Otto et al., 2017; Koskella and Taylor, 2018). Processes such as competition and predation are predominantly important in SOC-rich soils since SOC can support an abundant, diverse, and active microbial community (Lori et al., 2017). Also, Schütz et al. (2018) explained the low efficacy of P solubilizing BEs by high microbial activity resulting from elevated SOC, eventually hampering the establishment of the introduced BEs. This, in turn, could potentially diminish the effectiveness of the introduced BEs, similar to what we observed in our experiments with the SOC-rich Buus soil.

Besides SOC, various other abiotic factors were shown to influence the establishment and persistence of microbial BEs and, consequently, their efficacy to enhance crop growth. These include abiotic factors such as soil pH, soil texture, moisture, and salinity (Mäder et al., 2011; Mosimann et al., 2017; Schütz et al., 2018; Herrmann et al., 2022). Also, nutrient supply via fertilization was shown to determine the efficacy of BEs in promoting crop growth (Thonar et al., 2017; Mpanga et al., 2019; Weinmann and Neumann, 2020).

To determine the factors that explain a possible mode of action of the non-microbial humic acids in the alkaline Dompierre soil, we conducted an incubation experiment and tested whether humic acid application alters P availability (measured as resin P), soil pH, or microbial activity. We only found marginal and fertilizer-specific changes in microbial respiration and P-availability; effects that did not translate into a growth promotion of maize (data not shown).

Our results are different from Yang et al. (2021) who collated the literature to explain potential modes of action by which humic acids can change various soil parameters including soil texture, cation exchange capacity, and water retention. The fact that we observed none or only minor fertilizer-specific changes upon humic acid application could point to an incompatibility between the HA and the selected organic fertilizers (Rose et al., 2014). Other possible explanations for the lack of observed effects in our plant growth and soil incubation experiments could be an inappropriate concentration of the HA solution applied or the timing of application (Rose et al., 2014). In addition, Dobbss et al. (2010) observed that differences in the sensitivity of plant species to humic acids influenced the success of humic acid application. Maize required twice the concentration of humic acids to stimulate root branching compared to the dicotyledon tomato and Arabidopsis, suggesting greater efficacy in monocotyledons.

Lack of growth response under field conditions

Besides the limited efficacy under controlled conditions, we also did not observe any growth-promoting effects of maize upon BE application under field conditions. Owen et al. (2015) also reported poor reproducibility of commercial BEs under field conditions, and this, despite decades of research on the use and application of BEs. Similarly, Richardson and Simpson (2011) observed that the agronomic potential of BEs to promote maize yield was higher in pot experiments than under field conditions. Efficient root colonization is a prerequisite for many microbial BEs (Dobbelaere et al., 2001). As seen in our screening experiment, even at optimal and controlled conditions, the *Pseudomonas* strain DSMZ 13134 only transiently colonize the maize rhizoplane. Given this, it is unlikely to expect successful colonization under field conditions where additional stress factors with potentially negative impacts on the vitality of inoculants, root growth, and activity occur (Berg et al., 2021). As mentioned above, the high SOC contents of both field soils and the associated high microbial abundance and activity (Lori et al., 2017) may also have hindered the BEs' potential to promote plant growth. We assume that this is the main reason why no stable plant growth-promoting effect was achieved in our experiments.

Perspective of BE application

According to Schütz et al. (2018), BE application tends to be more effective in dry climates due to overall lower soil fertility, including lower levels of SOC, N, and P, resulting in lower abundance and activity of native soil microbes under these conditions. Furthermore, crops in dry climates are more likely to experience stress by factors like heat, drought, and salinity. By producing various molecules such as plant hormones, enzymes, and secondary compounds, microorganisms may help alleviate stress in plants ultimately leading to stabilized yields (Ali et al., 2022; Ma et al., 2022). Also, Rubin et al. (2017) found that microbial Bes are especially effective in promoting plant growth under drought. This is supported by a recent meta-analysis of Zhao et al. (2023) who observed increased plant biomass, enhanced photosynthesis, and inhibited oxidant damage under drought. Considering that in the future global dryland areas are expected to increase, BEs might become increasingly important.

Conclusion

Although we did not observe any positive effects of BE application on soils and plants in the present study, we do not generally rule out the potential for BEs to positively affect plant growth and agricultural yields. However, our results highlight that factors including biological and chemical soil properties and climatic conditions play a fundamentally important role in determining the success of a BE application. In light of our results, we recommend against using BEs without conducting pretests. This applies particularly to arable cropping in temperate climates and fertile, SOC-rich soils often found in organically managed fields. It is crucial to carry out pretests specific to the crop, soil, and environmental conditions to identify effective products and mitigate the risk of financial losses.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

CT, DK, PM, and SS designed the experiment; PM, CL, DK, CT, and SS planned and conducted the experiment and performed the analyses; DK performed statistical analyses; DK and SS wrote the draft manuscript; and all authors revised the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1239393/full#supplementary-material

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Transcriptomic and physiological approaches to decipher cold stress mitigation exerted by brown-seaweed extract application in tomato

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Chilling temperatures represent a challenge for crop species originating from warm geographical areas. In this situation, biostimulants serve as an eco-friendly resource to mitigate cold stress in crops. Tomato (*Solanum lycopersicum* L.) is an economically important vegetable crop, but quite sensitive to cold stress, which it encounters in both open field and greenhouse settings. In this study, the biostimulant effect of a brown-seaweed extract (BSE) has been evaluated in tomato exposed to low temperature. To assess the product effects, physiological and molecular characterizations were conducted. Under cold stress conditions, stomatal conductance, net photosynthesis, and yield were significantly ($p \leq 0.05$) higher in BSE-treated plants compared to the untreated ones. A global transcriptomic survey after BSE application revealed the impact of the BSE treatment on genes leading to key responses to cold stress. This was highlighted by the significantly enriched GO categories relative to proline (GO:0006560), flavonoids (GO:0009812, GO:0009813), and chlorophyll (GO:0015994). Molecular data were integrated by biochemical analysis showing that the BSE treatment causes greater proline, polyphenols, flavonoids, tannins, and carotenoids contents.The study highlighted the role of antioxidant molecules to enhance tomato tolerance to low temperature mediated by BSE-based biostimulant.

KEYWORDS

biostimulant, brown seaweed extract, cold stress, transcriptome, plant physiology, antioxidant molecules, tomato

1 Introduction

Low-temperature stress is a common challenge faced by warm-climate plants that can dramatically impact the yield. Although temperatures are generally increasing, late and out-season frosts are also happening often, and affect the potential distribution of warm-climate-adapted plants. Warm-climate plants can be harmed by chilling temperatures (0-12°C) and critically damaged by freezing temperatures (< 0°C). When the temperature drops below 10°C, plant growth and development are inhibited, and photosynthesis is compromised (Shi et al., 2016; Ding et al., 2017), while temperatures below 0°C can cause ice formation in the intercellular spaces of plant tissues provoking cell membrane disruption (Kidokoro et al., 2022).

Plants have evolved different strategies to cope with cold stress. The first cold-induced reaction is the accumulation of reactive oxygen species (ROS) *i.e.*, OH·, O_2·, H_2O_2, which act as signal molecules in response to several stresses but also are toxic by-products that need to be worked off. To alleviate oxidative stress under cold conditions, detoxification mechanisms have been implemented such as the production of antioxidant enzymes, and a major activity of AOX (alternative oxidase) over COX (cytochrome c oxidase) (Heidarvand and Amiri, 2010; Ding et al., 2017). Low temperatures are also increasing the production of flavonoids (Jaakola and Hohtola, 2010). These pigments are divided into anthocyanins, flavones, flavonols, and isoflavonoids. They stimulate DNA repair and protect against oxidative stress (Hichri et al, 2011; He et al., 2022). Among the molecular determinants involved in cold acclimation, the *C-REPEAT BINDING FACTOR* (*CBF*) transcription factor and the *INDUCER OF CBF EXPRESSION* (*ICE*) form the *ICE-CBF* signaling pathway, which plays a pivotal role in plant acclimation to cold controlling the expression of *COLD REGULATED* (*COR*) genes (Liu et al., 2012; Shi et al., 2016; Hwarari et al., 2022; Kidokoro et al., 2022; Gusain et al., 2023).

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop in the world, next to potato. Cultivated over 5.16 10^6 ha, it produces 189 10^6 t of fresh fruit, with an annual value exceeding 90 10^9 USD (FAOSTAT, 2023). Native to Western South America (Rodríguez et al., 2011), tomato is grown all over the year, worldwide, in open field or in greenhouses. Particularly, in the Mediterranean regions tomato is generally cultivated under unheated greenhouses to obtain year-round production. Two short cycles are often completed per year (autumn and spring). In these conditions, tomato plants can frequently experience cold stress (Brazel and Graciet, 2023). Nonetheless, climate change is threatening tomato production and thus, improving tolerance towards extreme temperatures is a matter of concern.

Tomato growth is optimal with an average daily temperature ranging between 18 and 25°C, and a night temperature between 10 and 20°C. Temperatures above 32°C and below 12°C induce growth retardation and impact fruit quality (Mesa et al., 2022). Due to its geographical origin, tomato is chilling sensitive and it shows poor ability to acclimate to cold (Barrero-Gil et al., 2016).

Some of the cold response mechanisms identified in other plants have been reported in tomato as well, even though in this crop, the pathways leading to low-temperature adaptation are still mostly unknown. Liu et al. (2020) identified SlGRAS4, a transcription factor promoting cold tolerance in tomato. The Sl*GRAS4* pathway seems to work in parallel and independently from the *ICE1* one. Sl*GRAS4* has also been shown to increase the expression of antioxidant-coding genes, underlining again a very similar effect as Sl*ICE1* (Liu et al., 2020).

The potential of biostimulants in agriculture has widely been reported: these products can stimulate plant growth, sustain yield, improve crop quality, and contribute to tolerance towards environmental stressors and pathogens (Van Oosten et al., 2017; Meddich, 2022). The seaweed extracts (SWE) are forming an important class of biostimulants. Precisely, they can promote cold tolerance and prevent cold-stress damage (Van Oosten et al., 2017). Many studies claim that SWE can increase cold tolerance (Digruber et al., 2018; Ali et al., 2021; Lakshmi and Meenakshi, 2022), but very few are actually reporting a scientific study. Some examples are the use of *Ascophyllum nodosum* in barley (Burchett et al., 1998), tobacco (Zamani-Babgohari et al., 2019), and Arabidopsis (Rayirath et al., 2009; Nair et al., 2012). Thus, the mode of action of SWE in the mitigation of cold stress remains not clear, even though these products seem to enhance membrane integrity and protect chlorophyll. The micronutrients contained in SWE might also protect against oxidative stress (Van Oosten et al., 2017). Particularly, *A. nodosum* seems to help to maintain membrane integrity thus reducing electrolyte leaking, and modulating the expression of cold-responsive genes (*COR15A*, *RD29A*, and *CBF3*) (Rayirath et al., 2009; Shukla et al., 2019). A lot is at stake about biostimulants and cold-stress responses, but very little consistent information is available.

To fill this gap, the transcriptomic and physiological responses to a commercially available brown seaweed extract (BSE)-based biostimulant were investigated on tomato plants exposed to cold stress. The effect of BSE foliar application was tested on photosynthetic activity and yield components. Furthermore, molecular targets of BSE were identified by RNA-Seq analysis. Finally, the products of most representative genes were quantified. Our findings provide valuable insights for the development of sustainable and effective strategies to enhance tomato production under cold stress conditions, in open field, and in greenhouse.

2 Materials and methods

2.1 Plant material and growing conditions

Tomato (*Solanum lycopersicum* L., cv. Micro-Tom) seeds were germinated in a tray filled with a commercial substrate (TS4 Klasmann-Deilmann, Germany) consisting of 35% (w:w) white sod peat, 45% white peat, 15% perlite and 5% peat fiber. Seedlings were fertigated once a week with IDROFEED 20-20-20 NPK (Tiller, Italy) at a dose of 1 g L^{-1}. Plants with two to four leaves were transfered in pots with 1.2 L capacity and supplied with the same growth medium. The photoperiod was 15 h–19 h, with light

intensity (PFD) of 400 µmol photons m^{-2} s^{-1}, temperatures were ranging from 24°C during the day to 20°C at night and relative humidity was kept constant to 60%. Pots were supplied with 150 mL of ultra-pure water twice a week.

Plants were treated three times, at phenological stages corresponding to BBCH51 (first inflorescence visible), BBCH61 (first flower open) and BBCH65 (first flower of the fifth inflorescence open). At each of the three stages, half of the plants were treated through foliar spray with a solution containing 2.75 g L^{-1} (recommended dosage from the producer) of a brown seaweed extract (BSE) provided by Sipcam-Oxon (Pero, Italy). The remaining plants were treated with an equal volume of ultra-pure water as an untreated control. Two days after the last BSE foliar application, a sub-set of treated and untreated plants were exposed to 4°C during three successive nights in a cold chamber. During the day plants were moved back to the growth chamber. The same experiment was repeated three times for transcriptomics and biochemical analysis. The experiments were carried out during 2021.

2.2 Leaf gas exchange and yield components

Stomatal conductance and net photosynthesis were measured on the youngest fully expanded leaf of six plants per experimental condition, using an infrared gas analyzer (CIRAS 3 PP Systems, Amesbury, MA, USA) as described in Baghdadi et al. (2022). Measurements were done before the cold exposure and after 48 h, 72 h, and 96 h.

At ripening, tomato fruits were harvested. The number and diameter of fruits, as well as their fresh and dry weight were determined for each plant. The fruits horizontal diameter was manually measured with a caliper at the highest diameter along the fruit equator. Dry weights were recorded after oven-drying the samples at 105°C.

2.3 RNA extraction and library preparation

Young mature leaves of six plants per experimental condition were harvested 24 h (T1) and 48 h (T2) after cold stress exposure. 50 mg of leaf tissue were collected and immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using a Maxwell 16 LEV Plant RNA Kit (Promega Corporation, USA) from leaf tissues, ground in liquid nitrogen with a tissue homogenizer. Next, mRNA was isolated from 1 µg of total RNA, using the Dynabeads mRNA DIRECT Micro Kit (Thermo Fisher Scientific, Carlsbad, CA, United States). Transcriptome RNA libraries were prepared with the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) following the manufacturer's instructions. The yield and size distribution of cDNA barcoded libraries were checked using D1000 screen Tape (Agilent Technologies, USA) and they were normalized, pooled, and sequenced on an Ion Torrent S5 with an Ion 540 chip kit (Thermo Fisher Scientific). Single-end sequencing (200 bp) was performed to achieve an average of 8 10^6 reads per sample.

2.4 Transcriptomic data analysis

Raw single end reads were quality checked using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and mapped to the *Solanum lycopersicum* L. reference genome SL3.0 (NCBI assembly: GCA_000188115.3) using bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012). Aligned files were processed using samtools suite (Li et al., 2009) to calculate the read counts for each gene. Raw data were normalized based on library size and underwent a Variance Stabilization Transformation (VST) using DESeq2 (Love et al., 2014) R-package. The VST matrix was used to calculate the Euclidean distances between samples and to investigate the grouping of samples in reduced principal components space at each sampling time, with potential outliers identified and excluded from further analysis.

The differential expression analysis (DEA) tested each sampling time group of samples separately (Supplementary Figure 1), to avoid batch effects. It was performed to compare gene expression profiles between samples under two experimental conditions. Two T1 and T2 sampling times, were evaluated separately to determine differential expression between treated and control samples. The DESeq2 (Love et al., 2014) R-package was used to perform DEA. A Generalized Linear Model (GLM) with a Gamma-Poisson distribution was fitted to the data, and Wald's test was used to determine statistical significance. Differentially expressed genes (DEGs) were identified as those with a raw p-value ≤ 0.05 and $|\log_2(\text{fold change})| > 1$.

ShinyGO web tool v 0.75 (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020) was used to group DEGs into biological categories. Biological processes (BP) and KEGG (Kanehisa and Goto, 2000) pathway results with a significant threshold (FDR ≤ 0.05) were considered for the analysis. The resulting data were integrated with NCBI's gene description for further considerations (NCBI, 1988).

2.5 Determination of proline content

The proline content was estimated according to the method of Bates et al. (1973) (Quagliata et al., 2023). Briefly, 0.1 g fresh weight (FW) of tomato leaves were homogenized with 2 mL of 3% (w:v) 5-sulfosalicylic acid dihydrate. After a centrifugation step at 5000 rpm for 10 min, an aliquot (0.5 mL) of the supernatant was added to reaction tubes containing an equal volume of glacial acetic acid and acid-ninhydrin reagent (previously prepared by dissolving 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid). The reaction was conducted at 100°C for 1 h and stopped by cooling the samples in ice. The reaction mixture was extracted with 1.5 mL toluene and shaken vigorously for 20 sec. Subsequently, the chromophore containing toluene was separated from the aqueous phase and the absorbance read at 520 nm with an Agilent UV-Vis 8453 spectrophotometer (Santa Clara, CA, USA), using toluene as a blank. Calibration was done with 2 – 600 µL of a 1 mM L-proline (98.5 - 101.0%, pharma grade, PanReac AppliChem ITW Reagents S.R.L., Monza, Italy) stock solution, and the results were expressed as µmol g^{-1} FW. Measurements were taken from 31 different plants.

2.6 Determination of total phenolic, total flavonoid compounds, and condensed tannins content

The contents of total phenolics (TPC), flavonoids (TFC), and condensed tannins were determined in the extracts of tomato leaves, previously dried in the dark, according to Wakeel et al. (2019) with some modifications. A total of 34 plants were considered for these measurements. For the extraction, 1 g DW of leaf material was soaked in 10 mL of 80% (v:v) methanol. The samples were placed on an orbital shaker (ASAL VDRL mod. 711, Cernusco s/N, Milano, Italy) for 30 min and then incubated in the dark at 4°C. After 48 h of incubation, the samples were filtered through Whatman filter paper no. 1 and the filtrates were used for TPC, TFC, and condensed tannin assays.

The TPC was quantified using the Folin-Ciocalteu method (Al-Duais et al., 2009). Briefly, 0.125 mL of leaf extract was added to 2 mL of water, followed by the addition and mixing of 0.125 mL of the Folin-Ciocalteu's reagent. The samples were left for 3 min in the dark and then 1.250 mL of 7% (w:v) Na_2CO_3 and 1 mL of distilled H_2O were added and shaken vigorously followed by 90 min incubation in the dark. Then, the absorbance of the blue solutions was read at 760 nm with an Agilent UV-Vis 8453 spectrophotometer (Santa Clara, CA, USA). The amount of the extract was substituted by the same amount of 80% (v:v) methanol in the blank. Gallic acid (98%, Thermo Fisher Scientific Inc., Rodano, Milano, Italy) (in the 5 – 300 μg mL^{-1} concentration range) was the standard of choice and the results were expressed as gallic acid equivalent (GAE) mg g^{-1} DW of extract.

The TFC was quantified with an aluminum chloride colorimetric method (Chang et al., 2002). Briefly, 0.250 mL of leaf extract were mixed with 0.075 mL of 5% (w:v) $NaNO_2$ and 5 min later with 0.075 mL of 10% (w:v) $AlCl_3$. The samples were shaken and after 5 min of incubation in the dark were neutralized with 0.5 mL of 1 M NaOH solution. The mixtures were left in the dark for 15 min and then the readings were taken at 415 nm with an Agilent UV-Vis 8453 spectrophotometer (Santa Clara, CA, USA) against a blank of 80% (v:v) methanol. Quercetin (≥ 95%, Merck KGaA, Darmstadt, Germany) (in the 12.5 – 150 μg mL^{-1} concentration range) was the standard of choice and the results were expressed as quercetin equivalent (QE) mg g^{-1} DW of extract.

The condensed tannin content was quantified using the acidified vanillin method (Broadhurst and Jones, 1978). Briefly, 0.5 mL of leaf extract were mixed with 3 mL of 4% vanillin in methanol and 1.5 mL of concentrated HCl. The mixtures were incubated in the dark for 20 min and then read at 500 nm with an Agilent UV-Vis 8453 spectrophotometer (Santa Clara, CA, USA) against a blank of 80% (v:v) methanol. Tannic acid (ACS reagent, Merck KGaA, Darmstadt, Germany) (in the 12.5 – 900 μg mL^{-1} concentration range) was the standard of choice and the results were expressed as tannic acid equivalent (TAE) mg g^{-1} DW of extract.

2.7 Determination of leaf pigments content

The content of pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) was measured in leaves of tomato plants sampled 48 h after the chilling exposure, following the method of Prodhan et al. (2017) with slight modifications. Four mL of chilled methanol were added to 0.050 g FW of leaf material. The mixture was homogenized and incubated for 30 min in the dark at 4°C. Afterwards, the samples were centrifuged (PK110 centrifuge, Alc International S.r.l., Cologno Monzese, MI, Italy) at 3500 rpm for 20 min. The absorbance of supernantants were measured at 470, 653 and 666 nm with an Agilent UV-Vis 8453 spectrophotometer (Santa Clara, CA, USA). The specific absorption coefficient in methanol was used to calculate chlorophyll a and b and total carotenoid contents in leaves. The results were expressed as mg g^{-1} FW (Lichtenthaler and Wellburn, 1983).

2.8 Statistical analysis

A Wilcoxon rank sum test was employed to compare the physiological parameters between the experimental conditions, with a significance threshold of $p \leq 0.05$. Principal Component Analysis (PCA) was conducted on all variables related to the physiological responses. RStudio software (v. R-4.2.3) was used for statistical analysis and for plotting the results.

3 Results

3.1 The BSE treatment increases photosynthesis and fruit yield during control and cold stress conditions

Stomatal conductance and net photosynthesis were measured in tomato plants untreated or treated with BSE, in control or cold stress conditions. Measurements were taken prior cold stress application and 48 h, 72 h and 96 h after. The BSE treatment significantly ($p < 0.05$) increased both parameters (Figure 1), and this is consistent with previous report (Baghdadi et al., 2022). At the end of the treatment, the stomatal conductance increased by 69.6% and 73.8% (Figure 1A), and the net photosynthesis by 26.1% and 37.0% (Figure 1B) between untreated and BSE-treated plants in control and cold stress conditions, respectively.

Yield traits like the number, size, and weight of fruits were measured (Figure 2). The BSE treatment significantly ($p < 0.05$) increased the total number of fruits under both control (+17.4%) and cold (+25.8%) conditions (Figure 2A). In cold conditions, the BSE treatment caused a significant increment of 22.55% of the fruit diameter (Figure 2B). The BSE treatment resulted in a significant average increase in fresh weight under both control (+26.2%) and cold stress (+33.4%) conditions (Figure 2C). The average total fruit dry matter of BSE-treated plants increased under both control (+27.9%) and cold stress (+50.4%) conditions (Figure 2D). Finally, the treatment did not affect the number of cracked fruits in control conditions, since no fruits cracked, but it significantly reduced (-56.2%) the number of cracked fruits under cold conditions (Figure 2E). Other characteristics analysed were not affected by the treatment (Supplementary Table 1).

PCA was performed to obtain an overview of the global physiological change between BSE-treated plants and untreated ones, with cold stress imposition and in control conditions (Figure 3).

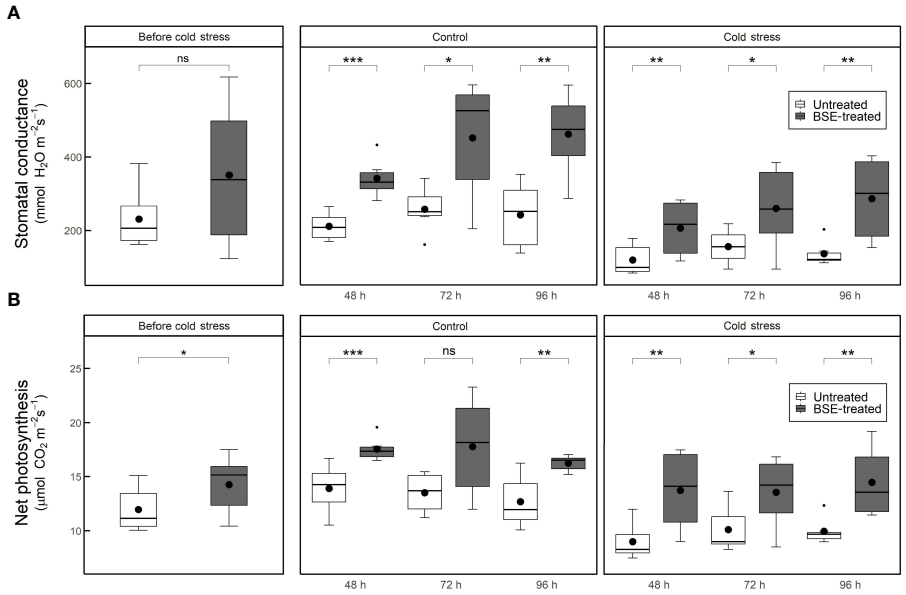

FIGURE 1
Stomatal conductance **(A)** and net photosynthesis **(B)** in cold-stress and control conditions, in treated and untreated plants. Parameters were measured at 4 different time points: before the cold exposure, and 48, 72 and 96 hours after the cold exposure. Box plots show medians, 25th and 75th percentiles, and non-outlier ranges. Small dots are considered outlier observations, big dots represent the average values. Significance is based on Wilcoxon's test: ns, not significant, *p-value < 0.1, **p-value < 0.05, ***p-value < 0.01.

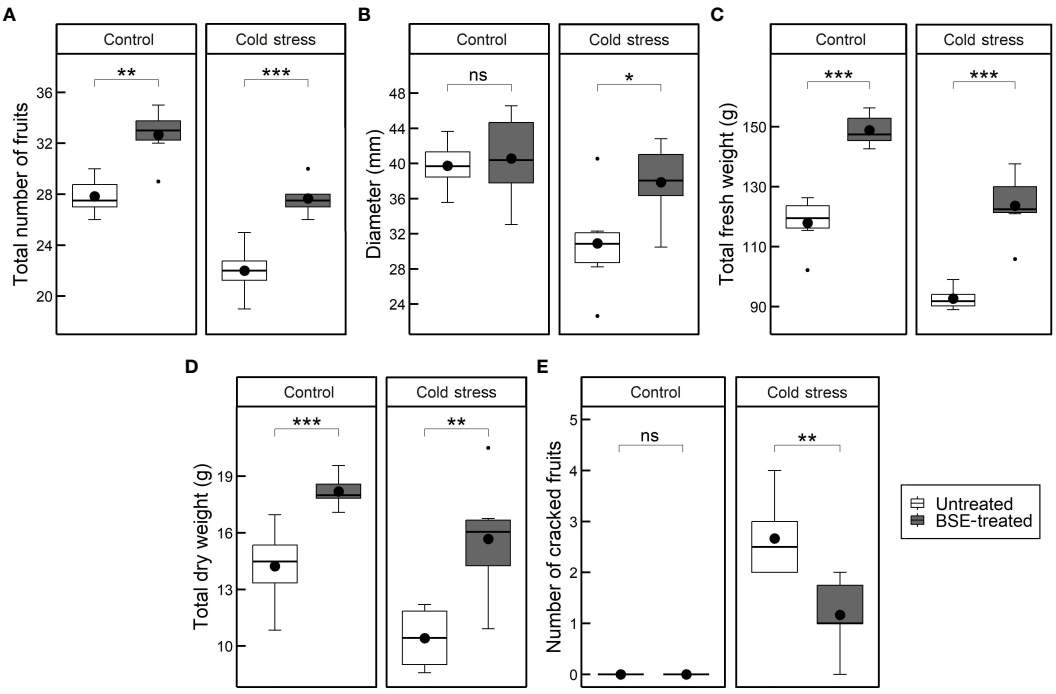

FIGURE 2
Changes in the yield. Total number of cracked fruits **(A)**, fruits diameter **(B)**, total fresh weights of fruits **(C)**, total dry matter of fruits **(D)**, and number of cracked fruits **(E)** in cold-stress and control conditions, in treated and untreated plants. Box plots show medians, 25th and 75th percentiles, and non-outlier ranges. Small dots are considered outlier observations, big dots represent the average values. Significance is based on Wilcoxon's test: ns, not significant, *p-value < 0.1, **p-value < 0.05, ***p-value < 0.01.

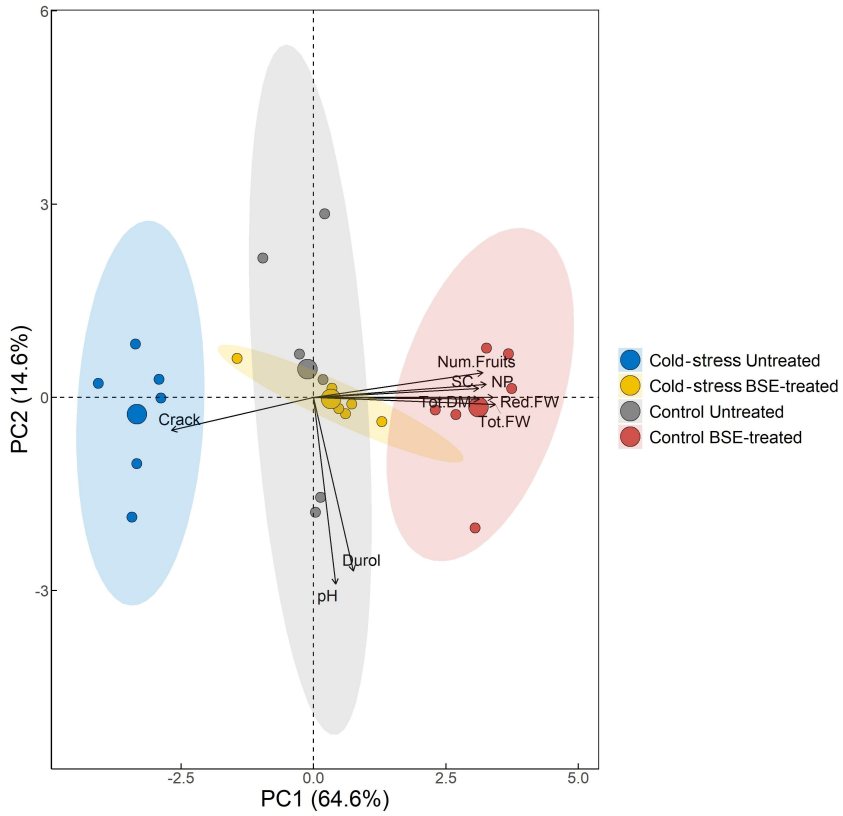

FIGURE 3
Principal Component Analysis. The variables used to compute PCs and their correlation and contribution with the firsts two PCs are shown. Points are colored by the group. The percentage in each axis shows how much variability each principal component was able to explain. The direction of the arrows and their color refers to the correlation and to the contribution of each variable have with the first two PCs respectively. In the figure: red fruits weight (Red.FW), total fruits weight (Tot.FW), frutis dry weight (Tod.DM), fruits pH (pH), fruits hardness (Durol), number of cracked fruits (Crack), stomatal conductance (SC), net photosynthesis (NP), fruits number (Num.Fruits).

PC1 explained 64.6% of the total observed variation and it is the one that describes the two variables of interest, lower values of PC1 are associated with cold-stressed samples and untreated ones, while high values refer to BSE-treated samples under control temperature conditions. It was found that bio-stimulated plants presented a distinct physiological profile with higher values of fresh weight, dry matter, SC, NP, and the number of fruits, and fewer cracked fruits. The table underlying the graph shows that the variables as fresh weight, dry matter, SC, NP, and the number of fruits are positively correlated with PC1, stating that the biostimulant application promotes higher levels of these parameters both in cold and in control temperature conditions. It is possible to follow the same logic for the Crack variable stating that it is less likely to have cracked fruits with biostimulant treatment application (Supplementary Table 1).

3.2 The BSE treatment affects proline and phenols metabolisms under cold stress

Transcriptomic analysis has been performed 24 (T1) and 48 (T2) hours after the cold stress, in BSE-treated and untreated plants. The total number of mapped reads was 96,434,292 with an average of 8,036,191 reads per sample. The average alignment rate was 62.7% (Supplementary Table 2; Supplementary Figure 2).

The BSE treatment greatly affected the transcriptome profile of cold-stressed plants. A total of 394 and 888 genes were differentially expressed after 24 h and 48 h, respectively (Figure 4). Only one gene was down-regulated and 13 genes were up-regulated, persistently at both time points. Thirty genes presented opposite expression pattern over time (Table 1).

One Gene Ontology (GO) analysis was conducted with differentially expressed genes (DEG) between BSE and control treatments under cold stress (Figure 5). Significantly enriched GO terms included proline metabolic process (GO:0006560), flavonoid metabolic (GO:0009812) and biosynthetic processes (GO:0009813), polyketide biosynthetic (GO:0030639) and metabolic (GO:0030638) processes. Four pathways related to thiamine (GO:0009228, GO:0006772, GO:0042723, GO:0042724 - thiamine biosynthetic/metabolic process, thiamine-containing compound biosynthetic/metabolic process, respectively), chlorophyll metabolic process (GO:0015994) and pigment metabolic process (GO:0042440). Genes related to these pathways are indicated in Table 2.

The KEGG results confirmed those obtained from the GO analysis, with a significant (FDR ≤ 0.05) presence of pathways

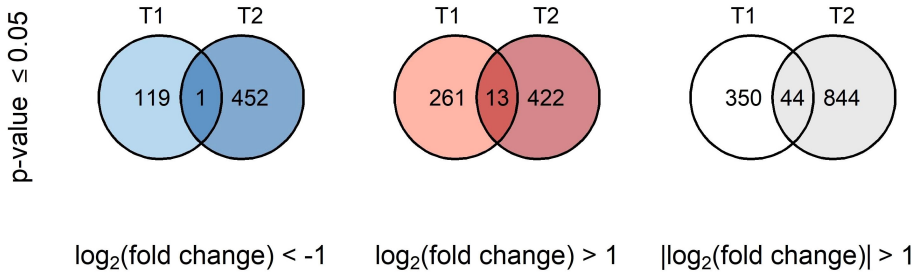

FIGURE 4
Venn's diagrams of common DEGs. Blue diagrams show downregulated genes, red ones upregulated genes while the last white diagram shows all the differentially expressed genes.

TABLE 1 Genes with inverted tendency in the expression from T1 to T2 (p-value ≤ 0.05).

Gene ID	Symbol	LFC_{T1}	LFC_{T2}	Gene annotation (Sol Genomics)
Solyc01g106620	PR1	1.614	-2.372	Pathogenesis-related protein 1a
Solyc02g085020	DFR	-1.422	1.112	Dihydroflavonol 4-reductase
Solyc02g085980	–	3.649	-2.651	Unknown Protein
Solyc02g085990	–	1.663	-2.163	Unknown Protein
Solyc02g086000	–	5.389	-2.651	Unknown Protein
Solyc02g086040	–	2.638	-2.651	Monoglyceride lipase
Solyc02g089780	SNL6	-1.267	1.383	Cinnamoyl-CoA reductase-like*
Solyc02g092550	LOB38	1.843	-1.986	Lateral organ boundaries domain protein 38
Solyc02g093070	–	1.348	-2.201	Oxoglutarate and iron-dependent oxygenase
Solyc03g006490	–	1.002	-1.053	Aluminum-induced protein-like
Solyc03g020030	–	1.177	-1.764	Proteinase inhibitor II
Solyc03g115540	bHLH024	1.035	-1.022	BHLH transcription factor
Solyc03g115930	–	1.298	-1.375	Calmodulin-like protein
Solyc03g116690	–	1.151	-1.378	Blue copper protein
Solyc04g007800	–	-1.028	1.032	C2 domain-containing protein
Solyc04g063270	PRR	1.196	-1.244	Pentatricopeptide repeat-containing protein
Solyc04g082500	–	1.084	-1.056	ATP binding/serine-threonine kinase
Solyc05g010320	CHI1	-1.083	1.510	Chalcone–flavonone isomerase 1
Solyc05g052240	CHI3	-1.368	1.729	Chalcone–flavonone isomerase 3
Solyc05g053550	CHS2	-3.332	1.368	Chalcone synthase 2
Solyc06g059710	–	1.072	-1.855	Stearoyl-acyl carrier protein desaturase
Solyc08g066050	–	1.131	-1.741	Serine/threonine-protein kinase 6
Solyc08g080590	OSM81	1.192	-1.391	Osmotin 81
Solyc08g082470	–	1.178	-3.030	Harpin-induced protein
Solyc09g006005	–	1.185	-1.384	Pathogenesis-related leaf protein 4*
Solyc09g059170	–	-1.241	1.438	Anthocyanidin 3-O-glucosyltransferase

(Continued)

TABLE 1 Continued

Gene ID	Symbol	LFC_{T1}	LFC_{T2}	Gene annotation (Sol Genomics)
Solyc09g091510	CHS1	-2.034	1.060	Chalcone synthase 1
Solyc10g083440	–	-1.632	1.166	UDP flavonoid 3-O-glucosyltransferase
Solyc11g013110	ANS	-1.610	1.418	Anthocyanidin synthase
Solyc12g011010	–	2.546	-2.564	Meiosis 5

log_2(fold change) (LFC) of each gene is shown. ITAG 3.2 IDs were obtained blasting the FASTA sequence of the mRNA to Sol Genomics website and selected the homologous with a score ≥ 200 and the highest id%.
*unavailable annotation on Sol Genomics, NCBI annotation was used.

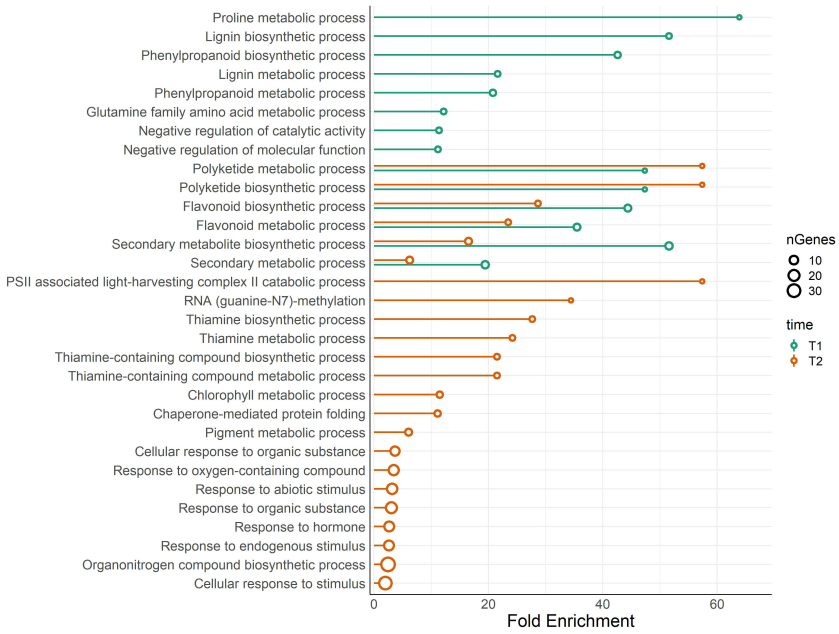

FIGURE 5
Lollipop plot of biological processes related to DEGs. The analysis compares BSE-treated plants with respect to untreated plants, in cold stress situation. Colors of the bars show the results for the DEGs at T1, and T2, while the size of the dots refers to the number of genes that belong to that ontology. X-axis shows the fold enrichment of each pathway (FDR ≤ 0.05).

related to the thiamine, proline, phenylpropanoids, and flavonoids (Supplementary Table 3). Again, flavonoids-related pathway resulted significantly enriched at both T1 and T2. Most of the genes annotated in this pathway are differentially expressed (Figure 6). Each enzyme is related to one or more genes, as well as the same gene synthesizes one or more enzymes (Supplementary Table 4).

3.3 Impact on metabolites

Because pathways reated to proline, antioxidant molecules and pigments were significantly enriched according to both GO and KEGG, such compounds were measure. All metabolites were measured at T2, 24 h after the last cold night, in the leaves.

3.3.1 Proline content is influenced by both cold stress and BSE treatment

Cold stress increased proline content significantly ($p < 0.05$) and proportionally more in untreated plants (+76.8%) than in BSE-treated ones (+28.9%) (Figure 7). The BSE treatment did not significantly alter proline content (+17.6%) in control plants, but decreased proline content (-14.2%) in cold-stressed ones. These metabolic observations are supporting transcriptomic data (Figure 7).

3.3.2 Antioxidant compounds content is influenced by both cold stress and BSE treatment

After 48 h cold treatment, the contents of antioxidant compounds significantly ($p < 0.05$) decreased both in BSE-treated plants (polyphenols: -65.6%, flavonoids: -52.3%, tannins: -61.7%, and carotenoids: -32.0%) and in untreated ones (polyphenols:

TABLE 2 DEGs with significantly enriched ontologies.

Gene ID	Symbol	LFC_{T1}	LFC_{T2}	Gene annotation (NCBI)
Genes involved in the proline metabolism				
Solyc02g089630	PDH1	-1.925	–	Proline dehydrogenase
Solyc06g019170	-	1.310	–	Gamma-glutamyl phosphate reductase
Genes involved in flavonoids metabolism				
Solyc02g083860	F3H	-1.610	–	Flavanone 3-hydroxylase
Solyc03g117600	HCT	1.062	–	Hydroxycinnamoyl-CoA shikimate
Solyc05g053550	CHS2	-3.332	1.367	Chalcone synthase 2
Solyc09g091510	CHS1	-2.034	1.060	Chalcone synthase 1
Solyc11g013110	ANS	-1.609	1.417	Anthocyanidin synthase
Genes involved in the thiamine metabolism				
Solyc06g006080	THIC	–	-1.258	Thiamine biosynthesis protein
Solyc07g064160	Thi4	–	-1.227	Thiazole biosynthetic enzyme
Genes involved in pigments metabolism				
Solyc01g086650	–	–	-1.049	Siroheme synthase
Solyc07g024000	–	–	-1.245	Dehydrogenase/reductase 3
Solyc09g065620	CLH1	–	-1.098	Chlorophyllase 1
Solyc10g006900	POR3	–	-1.450	Protochlorophyllide oxidoreductase
Solyc12g013710	AF243520S1	–	-1.289	Protochlorophyllide oxidoreductase 1

For each GO pathway enriched, the genes belonging to that pathway are shown. $\log_2$(fold change) (LFC) is reported for genes differentially expressed at T1, or T2, or both. Gene annotation is also reported.

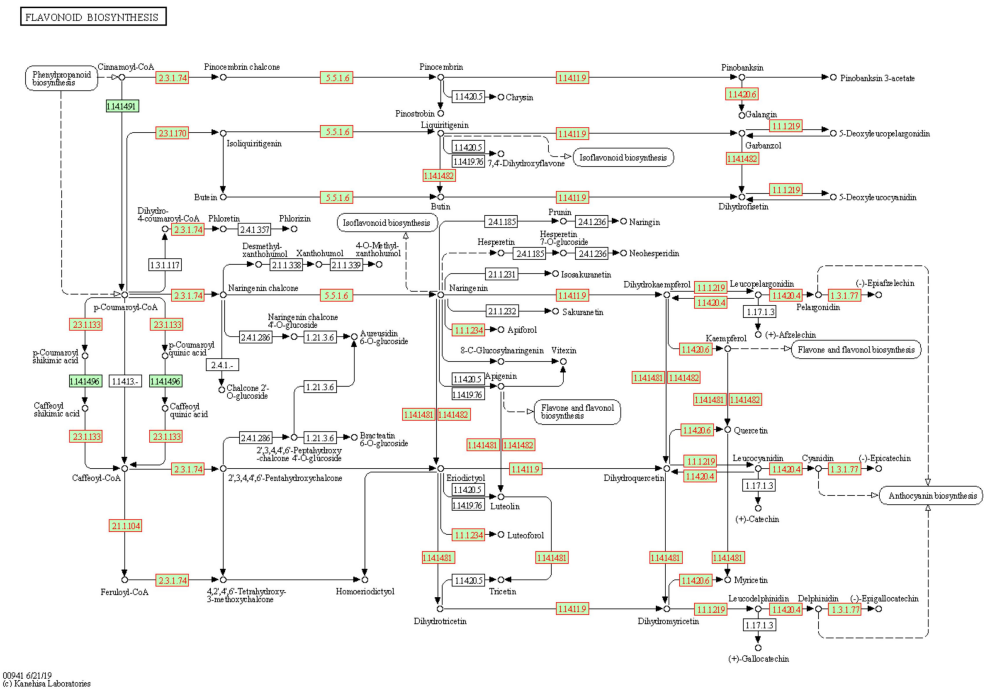

FIGURE 6
Flavonoids biosynthesis pathway in *Solanum lycopersicum*. Enzymes, chemical compounds, and molecular interactions are represented by boxes, dots, and arrows, respectively. Enzymes synthesized by DEGs are highlighted in red. Enzymes hyperlinked to GENES entries are represented from green boxes.

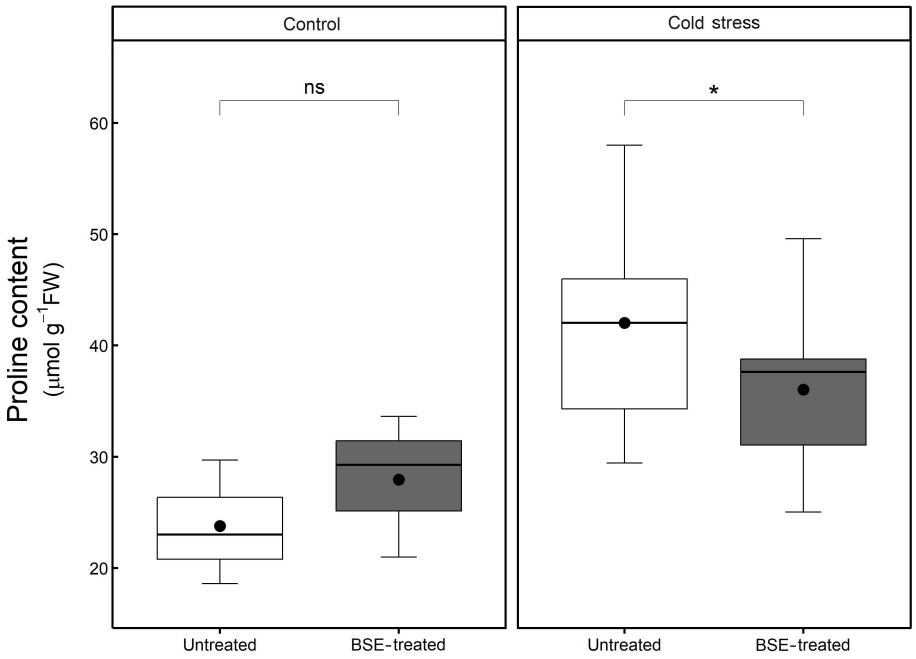

FIGURE 7
Changes in the content of proline. Proline content in the leaves of tomato plants treated and non-treated, grown in both cold stress and non-stress condition for 3 nights. Box plots show medians, 25th and 75th percentiles, and non-outlier ranges. The dots represent the average values. Significance is based on Wilcoxon's test: ns, not significant, *p-value < 0.1.

-47.8%, flavonoids: -38.0%, tannins: -23.2%, and carotenoids: -2.7%) (Figure 8).

In control conditions, the BSE treatment did not modify the contents of polyphenols (+4.7%), flavonoids (-0.5%), and carotenoids (+10.2%), but it significantly ($p < 0.05$) increased the tannins content (+32.4%). In cold stress conditions, the treatment significantly ($p < 0.05$) decreased the contents of polyphenols (-30.9%), flavonoids (-23.5%), tannins (-34.1%), and carotenoids (-23.0%) (Figure 8).

3.3.3 Chlorophyll content is influenced by cold stress but not from the BSE treatment

The chlorophyll *a* and *b* contents, significantly ($p < 0.05$) decreased during cold stress both in BSE-treated samples (chl *a*: -11.4%, chl *b*: -6.2%) and in untreated ones (chl *a*: -9.3%, chl *b*: -8.1%) (Figure 9). The BSE treatment did not significantly affect the chlorophyll content either in control (chl *a*: +0.1%, chl *b*: -2.9%) or in cold conditions (chl *a*: -2.3%, chl *b*: -0.9%) (Figure 9).

4 Discussion

Extreme temperatures may lead to important damages to plant growth and development. For instance, cellular membranes are harmed after lipid peroxidation, resulting in electrolyte and amino acid leakage from cells (Hayat et al., 2012). Such biochemical and physiological dysfunctions are mostly caused by the production of ROS. Different actions can mitigate the detrimental effects of low temperatures on crops. Biostimulant products from various origins can improve the plant capacity to tolerate chilling and freezing temperatures (Bulgari et al., 2019; Bhupenchandra et al., 2022). Specifically, algal extracts are known to enhance plant cold tolerance due to their membrane-protective and antioxidative properties (Shukla et al., 2019).

Stomatal conductance and net photosynthesis are commonly used to probe photosynthetic performance and to gauge plant health: they are crucial to determine plant growth and productivity, especially when the plant is undergoing stress conditions. These two parameters are tightly correlated since stomata pores control plant-environment gas exchanges and thus, CO_2 uptake for photosynthesis (Wong et al., 1979; Damour et al., 2010). Under control and cold conditions, the BSE treatment increased both stomatal conductance and net photosynthesis (Figures 1A, B), as previously reported (Baghdadi et al. 2022). This increase in physiological activity may explain the improved yield shown by treated tomato plants, even under cold stress. Again, the yield of cold-stressed BSE-treated plants was comparable to that of untreated plants which did not face any stress (Figure 2). At each of the three time points, physiological (stomatal conductance and net photosynthesis), and yield parameters (fruit number, diameter, fresh and dry weight) of the BSE-treated plants showed an improvement with respect to control plants.

One PCA (Figure 3) confirmed this assumption considering as a worst-case scenario untreated-cold-stressed plants (clustering at the extreme left) and as a best-case scenario BSE-treated-unstressed plants (clustering at the extreme right). The analysis exhibits a gradient that runs from the worst-case scenario to the best-case scenario: intermediate situations (BSE-treated stressed plants and untreated-unstressed plants) are overlapping.

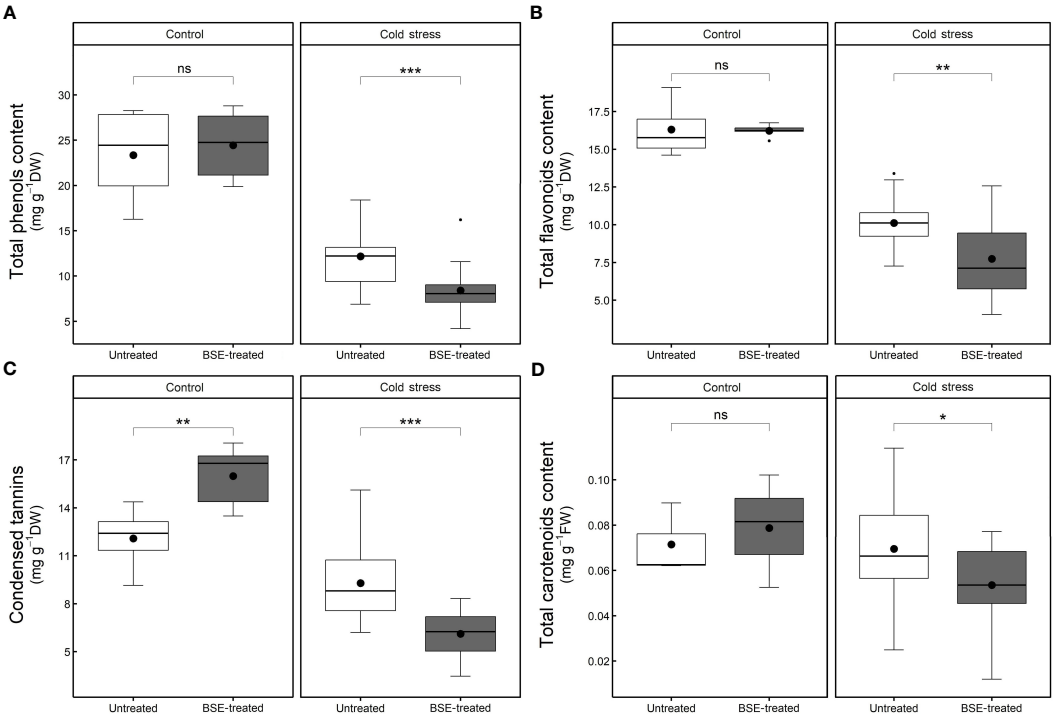

FIGURE 8
Changes in the content of non-enzymatic antioxidant compounds. Total phenols **(A)**, total flavonoids **(B)**, condensed tannins **(C)**, and total carotenoids **(D)** content in the leaves of tomato plants treated and non-treated, grown in both cold stress and non-stress condition for 3 nights. Box plots show medians, 25th and 75th percentiles, and non-outlier ranges. Small dots are considered outlier observations, big dots represent the average values. Significance is based on Wilcoxon's test: ns, not significant, *p-value < 0.1, **p-value < 0.05, ***p-value < 0.01.

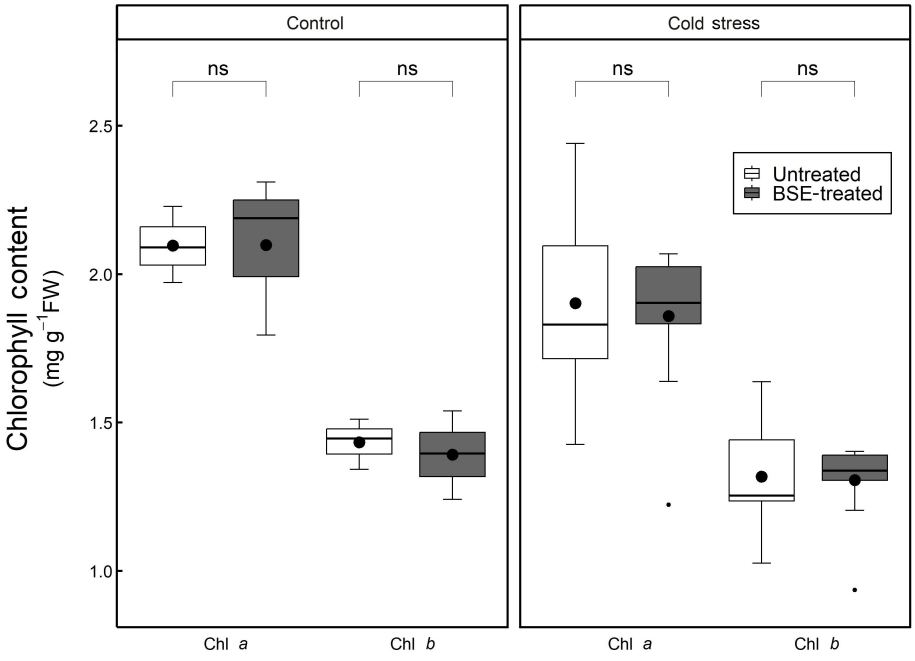

FIGURE 9
Changes in the content of chlorophyll. Chlorophyll a and b content in the leaves of tomato plants treated and non-treated, grown in both cold stress and non-stress condition for 3 nights. Box plots show medians, 25th and 75th percentiles, and non-outlier ranges. Small dots are considered outlier observations, big dots represent the average values. Significance is based on Wilcoxon's test: ns, not significant.

A global transcriptomic analysis was conducted to characterize the action modes of BSE in mitigating cold stress. A total of 1.238 DEGs were identified at two time points (T1 = 24 h and T2 = 48 h) during the stress-recovery phase. Ten genes were downregulated at T1 but upregulated at T2, while 20 genes followed the opposite pattern (Table 1). Falling into the first category, *CHALCONE SYNTHASE 1;2* (*CHS1;2*), *CHALCONE FLAVONE ISOMERASE 1;2* (*CHI1;2*), *FLAVONE 3-HYDROXYLASE* (*F3H*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) and *ANTHOCYANIDIN SYNTHASE* (*ANS*) are involved in the flavonoid biosynthetic pathway. These genes are also activating the *C-REPEAT BINDING FACTORS* (*CBFs*) pathway, leading to anthocyanin production (He et al., 2022) (Figure 10). More generally, genes in the flavonoid pathway interact with some pathogen-related (PR) genes in response to various stress conditions (Dai et al., 2022). In particular, *CHS*, *CHI*, and *F3H* are depicted as central regulators during the cold response in tomato (Han et al., 2020). The modulation of defense signaling pathways mediated by salicylic acid (SA), jasmonic acid, or ethylene is well documented after treatment with BSE in plants (Ali et al., 2021). Triggering these signaling pathways increases the expression levels of *PR* genes and genes encoding defense enzymes involved in the synthesis of polyphenolic compounds with anti-pathogenic properties (Vera et al., 2011). Nonetheless, SA plays a role in plant protection against abiotic stress, including cold (Liu et al., 2022). That hormone regulates the activity of antioxidative enzymes (Wani et al., 2017). Exogenous SA application can activate the alternative oxidase in sweet pepper exposed to cold (Fung et al., 2004), and improve chilling tolerance in cucumber through the cold-signaling pathway activation (Fu et al., 2021). Moreover, SA triggers the accumulation of soluble sugars and proline during cold or heat temperature stress, promoting tolerance through antioxidant and osmotic regulation (Soliman et al., 2018; Jahan et al., 2019). The DE genes *PR1* and *SNL6* thus, can be considered not only involved in biotic stress defence, but also in abiotic stress response (Xu et al., 2021; Akbudak et al., 2020; Yin et al., 2023) (Figure 10).

Indeed, GO analysis highlighted proline, antioxidants, and pigment pathways as highly significantly responsive to the BSE treatment.

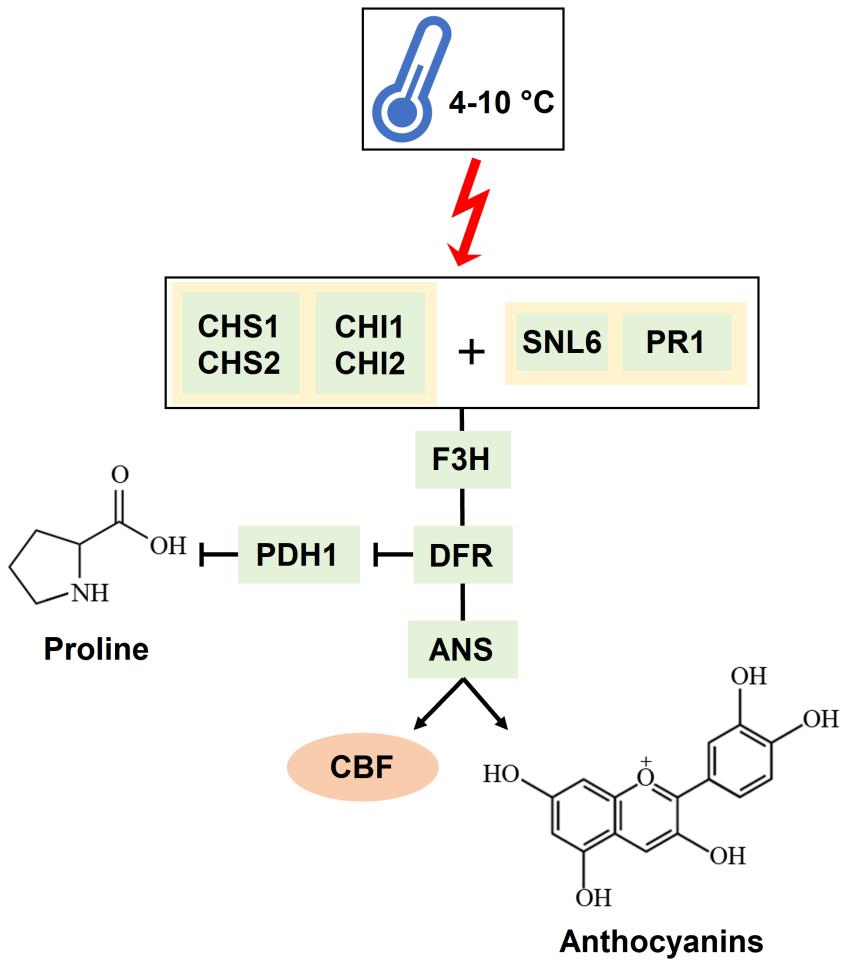

FIGURE 10
Mechanism of interaction of the genes identified as significantly involved in the cold response. A temperature ranging between 4 and 10°C triggers *CHALCONE SYNTHASES* (*CHSs*) and *CHALCONE-FLAVONONE ISOMERASES* (*CHIs*) together with *PATHOGENESIS-RELATED PROTEIN 1a* (*PR1*) and *CINNAMOYL-COA REDUCTASE-LIKE 6* (*SNL6*). These genes trigger the activation of *FLAVANONE 3-HYDROXYLASE* (*F3H*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), and *ANTHOCYANIDIN SYNTHASE* (*ANS*). *DFR* also inhibits *PROLINE DEHYDROGENASE* (*PDH1*), which degrades proline. The result of this cascade is the production of anthocyanins and the activation of the *CBF* transcription factor.

Proline is an amino acid that accumulates in plant tissue during stress (Trovato et al., 2008). Leaf proline content is following the tendency illustrated in the PCA: control plants (BSE-treated and untreated) have a lower proline content than cold-stressed BSE-treated plants, which accumulated less proline than cold-stressed untreated plants (Figure 7). We can suppose that stressed-BSE-treated plants are less affected by the low-temperature stress compared to stressed untreated plants, showing an average situation (but still statistically significant) between them and the unstressed ones. Proline plays a plethora of roles in stress response. Apart from being an antioxidant molecule, it acts as an osmolyte maintaining membranes and protein structures (Ashraf and Foolad, 2007). The present BSE-based biostimulant contains proline (Lakshmi and Meenakshi, 2022), and low exogenous proline increases plant tolerance toward various stresses (Ashraf and Foolad, 2007; Hayat et al., 2012). So the greater proline content could be a result of an exogenous application. However, in the enriched proline pathway, according to GO, a proline dehydrogenase (PDH1) is present. PDH1 has been reported to act with a mitochondrial protein, DFR1, in response to cold stress. Briefly, DFR interferes with PDH1 (and PDH2) to prevent proline degradation and increment its accumulation (Ren et al., 2018) (Figure 10). A lower amount of proline was measured in BSE-treated plants in respect to untreated ones, under cold stress. This can be due to the antioxidant power of the amino acid: in fact, during cold stress the proline content increases and then decreases (Azami et al., 2021). Or it can be caused by the modulation of the PDH1 gene. PDH1 was downregulated by the treatment under cold stress at T1, while its opposer, DFR, was downregulated at T1 and upregulated at T1 (Tables 1, 2). A quantification of the proline amount over the time of a stress would be needed to better understand the behavior of this protein in response to both the stress and the treatment.

There are no available data about the pattern of accumulation of antioxidants over time during cold exposure and recovery in tomato. At an early stage, the plant produces enzymatic and non-enzymatic antioxidants (e.g., flavonoids and polyphenols) to scavenge ROS burst (Rezaie et al., 2020). Free radicals generated during cold stress overstep the plant antioxidant capacity, and this leads to an oxidative stress (Hayat et al., 2012). The quantity of antioxidants then decreases as the stress progresses because of the reaction with the ROS: antioxidants prevent the oxidation of biomolecules by supplying the electrons needed (Azami et al., 2021). In the case of phenols, the resulting oxidized molecules, the benzoquinones, are unstable and need to be worked off (Barmaverain et al., 2022): indeed, the Folin-Ciocalteu method, quantifying the total phenols, can be considered as a measure of the antioxidant capacity of the plant (Platzer et al., 2021; Rumpf et al., 2023). Antioxidants were quantified only at T2, and they resulted significantly lower in the cold-stressed plants than in the non-stressed ones. Moreover, they resulted significantly lower in the cold-stressed BSE-treated plants, than in the cold-stressed untreated ones (Figure 8). Because the performances of cold-stressed BSE-treated plants were better than those of cold-stressed untreated ones, from the physiological point of view, this lower quantity of antioxidants in the treated plants can be the result of a higher antioxidant capacity of the BSE-treated plants. Anyway, a quantification of the antioxidant compounds over the time of a cold wave would be needed to understand tomato response to this stress. Anthocyanins are a class of flavonoids which are a class of polyphenols thus, they were included in the total phenols and flavonoids measurement. Indeed, flavonoids metabolism was found to be significantly regulated by the BSE treatment, and the genes in this GO class (CHS1, CHS2, F3H, ANS) are part of the anthocyanins biosynthetic pathway (Figure 10). Genes involved in the antioxidants biosynthetic pathways are very often inverting their expression tendency from T1 to T2, this could be due to an adjustment of antioxidant amounts after the stress. A lot of transcription factors are implied in this process (He et al., 2022) but were not highlighted in the analysis. Anyway, it was possible to identify a consistent amount of genes regulated by the BSE application.

Although the GO analysis pointed to an over-representation of the chlorophyll metabolic process, no significant difference in the chlorophyll content was measured following BSE application or cold exposure. Still, genes encoding enzymes degrading the chlorophyll were down-regulated after BSE treatment at T2 (Table 2). The free radicals generated can degrade chlorophyll (Sharma et al., 2020), but antioxidant molecules can also play an opposite role in this process, protecting the pigment from that (Leòn-Chan et al., 2017). This could be the reason for observing no alteration in chlorophyll content after cold stress. Anyway, it is hard to explain the role of downregulated chlorophyllases in our analysis. Generally, BSE-treated plants seem to have a higher content of both chlorophyll a and b, but these differences are not significant.

In conclusion, we mimicked a late cold snap, with temperature dropping at night and rising during the day. Three BSE applications until BBCH65 efficiently protected tomato plants, by increasing yield and reducing fruit cracking. The BSE treatment seems not to directly target the CBF/ICE regulatory pathway but rather the antioxidative molecule production to protect plants against cold stress. These findings on BSE treatment could have important implications for tomato cultivation, but also in a more general context, for crop productivity and protection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB62653.

Author contributions

FMan, FMag, AM, PS: conceptualization. PS, FMan, SN, AM: supervision. FMan, PS, SC, AM, AB, MCDL, MB, GB, WZ-L, CC, EP, CH, AliB: methodology. MB, MCDL and CC: writing the original draft. MB: data analysis and graphical representation. FMan, CC, SN, CH, PS, and SN: writing, reviewing, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

FMag is employed by Sipcam Italia S.p.A. belonging together with Sofbey SA to the Sipcam Oxon S.p.A. Group Pero, Italy. FMan is a former employer at Sipcam Italia S.p.A. belonging together with Sofbey SA to the Sipcam Oxon S.p.A. Group Pero, Italy. FMan was employed at Sipcam during the time of the study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1232421/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Volcano plots of the DEGs at the two time points. Volcano plot at T1 **(A)** and T2 **(B)**. Volcano plots show the DEGs in terms of LFC and p-value. Dotted lines represent the chosen thresholds: |LFC| > 1 and p-value < 0.05. Green and orange dots refer to non-significant genes. Blue and red dots refer to downregulated and upregulated genes above the p-value thresholds, while purple dots refer to DEGs which are above the LFC threshold as well.

SUPPLEMENTARY FIGURE 2
Samples overview. Euclidian distance heatmap **(A)** showing samples clustering based on their normalized expression patterns. Top colored bars show treatmen and sampling times variables. PCA at T1 **(B)** and T2 **(C)** show samples colored according with the treatment (green = untreated, orange = BSE-treated).

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Effect of microbial plant biostimulants on fruit and vegetable quality: current research lines and future perspectives

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Fruit and vegetables hold a prominent place in dietary guidance worldwide and, following the increasing awareness of the importance of their consumption for health, their demand has been on the rise. Fruit and vegetable production needs to be reconsidered so that it can be productive and, meantime, sustainable, resilient, and can deliver healthy and nutritious diets. Microbial plant biostimulants (PBs) are a possible approach to pursuing global food security and agricultural sustainability, and their application emerged as a promising alternative or substitute to the use of agrochemicals (e.g., more efficient use of mineral and organic fertilizers or less demand and more efficient use of pesticides in integrated production systems) and as a new frontier of investigation. To the best of our knowledge, no comprehensive reviews are currently available on the effects that microbial plant biostimulants' application can have specifically on each horticultural crop. This study thus aimed to provide a state-of-the-art overview of the effects that PBs can have on the morpho-anatomical, biochemical, physiological, and functional traits of the most studied crops. It emerged that most experiments occurred under greenhouse conditions; only a few field trials were carried out. Tomato, lettuce, and basil crops have been primarily treated with Arbuscular Mycorrhizal Fungi (AMF), while plant grow-promoting rhizobacteria (PGPR) metabolites were used for crops, such as strawberries and cucumbers. The literature review also pointed out that crop response to PBs is never univocal. Complex mechanisms related to the PB type, the strain, and the crop botanical family, occur.

KEYWORDS

plant biostimulants, plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi, fruit quality, vegetable quality, sustainability, bioactive compounds

1 Introduction

Fruit and vegetables hold a prominent place in dietary guidance worldwide, and a minimum consumption of 400 g (i.e., five servings) per person per day is recommended by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) (World Health Organization, 2020). Fruit and vegetables provide, in fact, the human body with an abundance of nutrients (i.e., vitamins, dietary fiber, micronutrients) and beneficial non-nutrient molecules such as bioactive compounds. Their intake has been shown to have an inverse correlation with the incidence of non-transmissible chronic diseases (NTCDs), such as cardiovascular diseases, cancer, obesity, and type 2 diabetes (Aune et al., 2017). Fruit and vegetables are the cornerstone of a healthy and varied diet (World Health Organization, 2020).

Following the increasing awareness of the important role played by fruit and vegetable consumption for the human health, their demand has increased. According to the FAOSTAT database, worldwide production of both fruit and vegetables rose by about half between 2000 and 2021: from about 572 to 909 million tons for fruits and from 687 to 1115 million tons for vegetables (FAO, 2023). Areas dedicated to fruit and vegetable cultivation have been thus extended (Hess and Sutcliffe, 2018). Nevertheless, the sustainability of their production methods must be investigated. In horticulture, the production systems are dominated by open field seasonal intensive crops with additive nutrient requirements to produce profitable marketable yields. In addition, also environments suffering from problems of water shortage are being used. Water supply, soil fertility, and pest and disease management are possible limiting factors for the sustainable production of fruit and vegetables.

Huge amounts of water are frequently required to maximize fruit and vegetable crops' growth, yield, and quality. Climate, the irregular rainfall, wind and temperature patterns owed to climate change, or the competition with other human activities (e.g., domestic or industrial water use in urbanized areas) can impact the quantity and quality of available water (Hess and Sutcliffe, 2018). Drought may lead to water stress, limiting the plant's ability to take up nutrients and resulting in a lower production of quality fruits and vegetables and, in the worst scenario, plant death (Hess and Sutcliffe, 2018). "Regulatory risks" of water scarcity also exist because of supply reallocation to sectors other than agriculture (i.e., higher priority sectors) in times of drought, as laid down by the European (EU) Water Framework Directive (European Commission, 2000). As to water quality, the sanitary status of the water used for fruit and vegetable production is of critical importance too. The use of water polluted by chemicals or heavy metals, urban waste, or sewage containing disease-causing microorganisms, as well as the salinization of freshwater resources, can have a detrimental effect (Thorslund and van Vliet, 2020).

Soil fertility and health can be further limiting factors to sustainable fruit and vegetable production. Soil fulfills, in fact, several functions. It can support plant growth by making nutrients available for root uptake; it stores and transforms compounds; it can filter, hold, and store fresh water; it prevents erosion. Soil fertility refers, therefore, to the ability of soil to maintain and sustain plant growth (Srivastava et al., 2021). Adequate salt and pH levels guarantee that the nutrients present in the soil are made available to crop plants. Evidence shows that moderately saline land is increasing yearly, limiting the possibilities for growing fruit and vegetables (Srivastava et al., 2021). A high salt concentration in the soil is detrimental for the plant as it reduces water uptake. Under salinity stress, transpiring leaves can be injured because salt enters the transpiration steam, and plant growth may be affected (Abdel-Farid et al., 2020). The decrease of water uptake and toxicity of sodium chloride may affect plant growth and thus reduce shoot length, leaf area and number (Abdel-Farid et al., 2020). Moreover, the root system is in direct contact with salt, which may harm the cell division of root tips and thus cause root length reduction.

Pest and disease management in fruit and vegetable crops is a significant issue too. Virus, bacterial, and fungi diseases and pests cause up to 40% yield losses in horticultural crops (Şener et al., 2020). For this, agricultural practices worldwide have long depended on the extensive and intensive use of chemical fertilizers and pesticides. The latter has, nevertheless, many undesirable aspects which should not be overlooked. They can remain in soil and environment for a long time and influence several biotic and abiotic factors. They adversely affect soil, microflora, other organisms, and the environment. In addition, residues remain in fruit and vegetables when, for instance, the time between the last spraying and harvest date is disregarded or when spraying over the recommended dose is carried out to achieve better results. Furthermore, pesticide residues (e.g., glyphosate, trifluralin, metazachlor, metamitron and sulcotrione, etc.) have accumulated in soils over the recent decades as "bound residues" with considerable ecotoxicological risk (Barriuso et al., 2008). This implies that their use can also be harmful to the human, animal, plant health and the environment.

Within this framework, fruit and vegetable production thus needs to be reconsidered to be productive and, at the same time, environmentally sustainable, resilient, and able to deliver healthy and nutritious diets. New technologies and approaches have been thus evaluated to achieve global food security and agriculture sustainability. The application of bio-based products, such as plant biostimulants (PBs), has thus emerged as a promising alternative to agrochemicals and a new frontier of investigation.

Plant biostimulants contribute to sustainable agriculture by exerting different beneficial effects for the plant growth and allow overcoming the detrimental effects of sub-optimal growing conditions on crops. In detail, PBs strengthen the plant root system architecture and biomass, boost nutrient absorption and utilization, increase photosynthetic activity, and improve plant tolerance to abiotic stresses, such as drought, extreme temperatures, salinity, and hypoxia (Ruzzi and Aroca, 2015; Carolina Feitosa de Vasconcelos and Helena Garófalo Chaves, 2019; Rouphael and Colla, 2020; Rakkammal et al., 2023). PBs also enhance crop quality, by fostering plant health and vigor, and increase harvestable yields. All the above thus allows for a reduction of fertilizer requirements, and this is of paramount importance in organic farming, where artificial fertilizers cannot be used.

To the best of our knowledge, no comprehensive reviews are currently available on the effects that microbial plant biostimulants' application showed to have on specific horticultural crops. This review thus aims to provide a state-of-the-art overview of plant biostimulants' application to fruit and horticultural crops, focusing on the benefits that microbial PBs can have on morpho-anatomical, biochemical, physiological, and functional traits of horticultural crops. To this aim, studies published in peer-reviewed journals and available on SCOPUS and Web of Science database were searched. Current research lines, challenges, and future perspectives of their application to horticulture are presented and discussed. Where available, the impact of biostimulants on plants via genomic, proteomic, and transcriptomic changes, is also analyzed.

2 Plant biostimulants

Plant biostimulants are defined in Regulation (EU) No. 2019/1009 as products "stimulating plant nutrition processes with the sole aim of improving: i) nutrient use efficiency; ii) tolerance to abiotic stress; iii) quality traits; and iv) availability of confined nutrients in soil or rhizosphere" (European Union, 2019). The Regulation also makes a distinction between microbial and non-microbial PBs. A microbial PB is a microorganism or a consortium of microorganisms, and this term applies only to Arbuscular Mycorrhizal Fungi (AMF) and Plant Growth-Promoting Rhizobacteria (PGPR) belonging to the *Azotobacter*, *Azospirillum*, and *Rhizobium* taxonomic groups, according to the current, but extendable, positive list in the Annex of EU Regulation No. 2019/1009 (European Union, 2019). A non-microbial plant biostimulant is a plant biostimulant other than a microbial PB. It encompasses humic substances (e.g., fulvic and humic acids), animal- or vegetal-based protein hydrolysates, seaweed extracts, and botanicals (Ruzzi and Aroca, 2015; Yakhin et al., 2017; European Union, 2019).

Among microbial PBs, AMF are soil fungi forming a mutualistic symbiosis with the roots of plants (Rouphael et al., 2015). In the presence of a host plant, AMF spores germinate, and a hyphal structure link between the plant and the fungus is formed. AMF can benefit the plant in case of abiotic stresses, such as drought, salinity, nutrient deficiency, adverse soil pH, and heavy metals. In drought, AMF creates hyphae on plant roots, implying that AMF increase the extension of the host's root system and contribute to plant growth by linking the plant and the immobile nutrients in the soil. The plant's nutrient uptake and water absorption are enhanced (Ebbisa, 2022). AMF's ability to increase plant root surface area also affects the ability of AMF to secure greater plant productivity and yield stability by enhancing nutrient uptake and promoting stress tolerance. In addition, the symbiosis between the plant and AMF has been shown to influence secondary metabolism by enhancing the synthesis of phytochemicals (Rouphael et al., 2015). This aspect is pivotal in obtaining horticultural products with improved nutraceutical value.

Plant Growth-Promoting Rhizobacteria (PGPR) have also become a significant body of research. They include strains in the genera *Agrobacterium*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pantoea*, *Pseudomonas* and *Serratia* (Verma et al., 2019; Luziatelli et al., 2020a; Luziatelli et al., 2020c; Luziatelli et al., 2021). This heterogeneous group of endophytic and epiphytic bacteria, which dwell in association with plant tissues and surfaces, can positively affect plant health and growth (Glick, 2010; de Souza et al., 2015). As any PBs, they can increase crop tolerance against abiotic stresses (Wu and Zou, 2009; Ruzzi and Aroca, 2015; Colla et al., 2017; Rouphael and Colla, 2018; Woo and Pepe, 2018) and improve nutrient use efficiency, photosynthetic activity, as well as plant health, growth, productivity and yield at different stages (Bulgari et al., 2015; Toscano et al., 2018). PGPR exert the aforesaid beneficial effects on plants through direct and indirect mechanisms. They can specifically interact with plants directly by making essential nutrients (e.g., nitrogen, phosphorus, iron) more available, by producing and regulating some compounds involved in plant growth (e.g., phytohormones), and by affecting the hormonal status stress (e.g., ethylene levels by ACC-deaminase). PGPR can also promote plant growth by inducing the expression of auxin-responsive genes in host-plant roots without producing these hormones and only by the auxin signaling pathway (Ruzzi and Aroca, 2015). The production of auxins elicits transcriptional changes in hormone and cell wall-related genes, induces longer roots, increases root biomass and decreases stomata size and density (Backer et al., 2018). In addition, PGPR can indirectly affect plant growth, which according to the current EU regulations is a trait not to be claimed for commercial plant biostimulant products, even though it is well known from the scientific literature. A legal regulation that is more according to the nature of the regulated entities therefore would be necessary to provide more adequate recommendations for their use in crop production. PGPR can protect plants against diseases, by competing with pathogens for minimal nutrients, exerting a biocontrol action of pathogens through the production of aseptic-activity compounds, synthesizing fungal cell wall lysing enzymes, and inducing systemic responses in host plants (Oleńska et al., 2020). PGPR potential to ease plant growth is therefore of paramount importance, also in case of abiotic stresses, because bacteria can enhance plant fitness, mitigate stress tolerance, or even assist in the remediation of pollutants.

3 Current research lines on the effect of microbial plant biostimulants on fruit and vegetable quality

The principal crop types, as treated by PBs in general, include row crops (cereals, oilseeds, pulses, and fiber crops), fruit and vegetables, turf ornamentals, and "others" (Critchley et al., 2021). However, the analysis performed within this study showed that the effect of microbial PBs has been so far studied mainly on horticultural crops, such as tomato (*Solanum lycopersicum* L.), cucumber (*Cucumis sativus* L.), strawberry (*Fragaria* × *ananassa* Duch.), rocket (*Eruca vesicaria* Mill.), lettuce (*Lactuca sativa* L.), and basil (*Ocimum basilicum* L.). Experimental microbial/fungal

PBs have been applied in pot, greenhouse, and field experiments through seed/root inoculation, soil treatment, foliar application, or microbial amendments.

Current research lines followed up a literature search performed in Scopus, Web of Science and PubMed databases. Studies published from 2018 to 2022 were considered for inclusion in the analysis. The following list of search terms was used: plant biostimulants, plant growth-promoting (rhizo)bacteria, Arbuscular Mycorrhizal Fungi, field experiments, greenhouse, horticultural crops, food quality, sustainability, bioactive compounds, omic sciences, transcriptomic, genomics, proteomics. This list derives from a preliminary set of scoping searches conducted to test out search terms and find possible additional terms to design the search strategy.

3.1 Tomato

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetables, with a global production of almost two hundred million tons covering a harvested area of about five million hectares in 2021. Tomato is consumed fresh and processed as pulp or sauce. Its fruits are a good source of dietary minerals and especially contain trace elements, such as iron, zinc, copper, calcium, potassium, and magnesium; they are also rich in vitamins C and E, folic acid and other organic acids (e.g., malic, ascorbic, and citric acid), and in essential amino acids (e.g., leucine, threonine, valine, histidine, lysine, and arginine) (Chaudhary et al., 2018). Tomato is also a source of bioactive compounds: carotenoids (i.e., lycopene and β-carotenoids), phytosterols, and phenolic compounds (e.g., quercetin, kaempferol, naringenin, caffeic acid) (Chaudhary et al., 2018). Bioactive compounds contribute to tomato antioxidant, anti-inflammatory, anti-mutagenic, anti-proliferative, and anti-atherogenic activity (Chaudhary et al., 2018), and make tomato an essential food of a healthy diet, such as the Mediterranean one (Naureen et al., 2022).

Tomato cropping is adapted to a wide range of growing conditions; however, worldwide productivity faces the challenge of biotic and abiotic stress. Sub-optimal temperatures after transplanting, poor soil fertility, and excessive temperature during flowering and fruiting can strongly reduce crop productivity (Cardarelli et al., 2020). Hence, an extensive use of fertilizers is required to overcome the aforesaid issues and obtain optimal plant growth and high-quality yields. Drought and salinity and various pathogens and pests are also limiting factors (Bai et al., 2018).

In identifying new approaches to implement sustainable agriculture, efforts have been made to apply PBs to tomato crops to boost yield, fruit quality, and production sustainability under environmental conditions which may limit crop growth. In addition, given this crop's commercial and nutritional value, efforts have been made to regulate the plant physiology so that the biochemical composition of the fruit, including the content of bioactive components, such as vitamin C, lycopene, among others, is also improved.

Several studies have been published over the last five years where tomato seedlings and plants were treated by seed/root inoculation, direct soil treatment, foliar application, and with PGPR strains, AMF, or a combination thereof. The effect of treatments was evaluated on different target parameters, ranging from plant morphology and crop productivity to the functional and sensory quality of the fruits (Table 1).

3.1.1 Effect of microbial PBs on tomato plant and fruit morphology and crop productivity

Tomato quality is generally defined by several physical-morphological traits, such as plant rooting system, number of flowers and fruits, fruit shape, length, diameter and weight, and total soluble solids. They are affected by many biochemical mechanisms, at the plant and fruit level, which depend on the interaction between cultural practices and genetic and environmental factors. The analysis of studies investigating the effect of microbial treatment on plant morphology and crop productivity has shown that these traits have been studied in different cultivars (e.g., *Solanum lycopersicum* L. cv. San Marzano, cv. Rio Grande, etc.) under different growing conditions.

The combined effect of mycorrhizal fungi, *Trichoderma*, and non-microbial PBs (i.e., vegetal extracts) on tomato crop productivity was investigated (Cardarelli et al., 2020). Treatments did not significantly affect the Soil Plant Analysis Development (SPAD) index, which is a non-destructive measurement of leaf chlorophyll content and an indicator of the photoinhibition of the Photosystem II (PSII); however, the general good health status of the crop and good photosynthetic activity of the tomato plants were observed after the PB treatment, compared to non-treated plants. Regarding yield, the total marketable yield was significantly higher in PB-treated plants. At the same time, no significant differences were observed for immature yield and unmarketable yield factors (e.g., blossom end rot fruits and rotten fruits; Table 1). The increase in marketable yield was related to a greater mean fruit weight than a change in the number of tomato fruits. Tomato growth was stimulated by both AMF and the ability of *Trichoderma* to release auxin-like compounds in the rhizosphere. The latter can solubilize mineral nutrients, such as phosphorus and micronutrients (e.g., Fe), and control plant pathogens through antagonistic activity and induction of systemic resistance in plants (Cardarelli et al., 2020). The effect of treatments on fruit quality was not univocal; an 18% increase was observed for fresh tomato weight, while total soluble solids and tomato juice pH were not significantly affected.

The same parameters as Cardarelli et al. (2020) were investigated by Rouphael et al. (2021) in a greenhouse experiment, where four different tomato landraces of San Marzano cultivar were treated with a plant extract (by foliar application). The application of the plant extract had a little positive effect on yield, whose increase varied among landraces. The growing conditions did not affect the shape index, calculated as a ratio of the maximum height length to maximum width relative to the longitudinal section (Table 1).

A microbial-based biostimulant containing *Rhizophagus intraradices* was applied to tomato plants cv. 'Rio Fuego' i) alone (AMF), ii) in combination with irrigation with a nutritive solution (AMF+NS), and iii) with the nutritive solution and a non-microbial-based biostimulant from *Padina gymnospora* extract

TABLE 1 Conditions and effect of microbial plant biostimulants treatment on tomato crops.

Crop	Growing conditions and location	PGPR	AMF	Treatment effect on targeted parameter(s)		Reference
Tomato (*S. lycopersicum* L., var. CXD 219 F1)	Field experiment (Italy)	*Pseudomonas* sp. 19Fv1T *Pseudomonas fluorescens* C7	AMF mix	Plant collar diameter	=	(Bona et al., 2018)
				% fruits/ flowers	=	
				Fruit length	↑	
				Fruit diameter	↑	
				Fruit weight	↑	
				Lycopene	↑	
				β-carotene	↑ (2 out of 3)	
				Lutein	↑ (2 out of 3)	
Tomato (*S. lycopersicum* L. cv. PS1313)	Open field experiment (Italy)	–	Mycorrhizal fungi and *Trichoderma*	SPAD index	=	(Cardarelli et al., 2020)
				Marketable yield	↑	
				Immature yield	=	
				Unmarketable yield	=	
				Fruit fresh weight	↑	
				TSS	=	
				Juice pH	=	
				N	↑	
				P	=	
				K	=	
				Vitamin C	↑	
				Lycopene	↑	
Cherry tomato (*S. lycopersicum* L. cv. Pomodorino del Piennolo del Vesuvio)	Field experiment (Italy)	–	*Rhizoglomus irregulare Funneliformis mosseae*	Yield	↑	(Carillo et al., 2020)
				Mineral profile	↑ (P, Ca, Mg, Na, Cu, and Zn)	
				TSS	↑	
				Fruit dry matter	↑	
				TAA	↑	
				Lycopene	↑	
				Total Ascorbic Acid	↑	
Tomato (*S. lycopersicum* L. cv. Kwangbok)	Greenhouse experiment (Korea)	*Bacillus subtilis* CBR05	–	TAA	↑	(Chandrasekaran et al., 2019)
				TPC	↑	
				TFC	↑	
				All-E-β-Carotene	=	
				All-E-Lycopene	↑	

(Continued)

TABLE 1 Continued

Crop	Growing conditions and location	PGPR	AMF	Treatment effect on targeted parameter(s)		Reference
Tomato (*S. lycopersicum* L. cv. Rio Fuego)	Greenhouse experiment (Mexico)	–	*Rhizophagus intraradices*	Aerial part	↑	(González-González et al., 2020)
				Total carbohydrate	↑	
				Total protein	↑	
				TPC	↑	
Tomato (*S. lycopersicum* L. cv. Rio Grande)	Field experiment (Greece)	*Bacillus amyloliquefaciens* B002 *Bacillus licheniformis* B017 *Bacillus mojavensis* B010 *Bacillus pumilus* W27-4 *Bacillus subtilis* Z3 *Bacillus pseudomycoides* S3 *Bacillus velezensis* B006 *Azotobacter chroococcum* A004 *Bacillus megaterium* B004	–	Plant dry weight	↓↑	(Katsenios et al., 2021)
				Photosynthetic Rate	↓↑	
				Transpiration Rate	↓↑	
				Mean fruit weight	↑ (5 out of 10)	
				Yield/plant	↑ (9 out of 10)	
				TSS	↑ (10 out of 10)	
				PME Activity	↑ (8 out of 10)	
				PG activity	↑ (5 out of 10)	
				TCC	↑ (6 out of 10)	
				TPC	=	
				Lycopene	↑ (5 out of 10)	
				TAA	↑ (4 out of 10)	
Tomato (*S.lycopersicum* L. cv. Marmande)	Pot experiment (Italy)	*Pantoea agglomerans* C1	–	Root system	↑	(Luziatelli et al., 2020b)
Tomato (*S. lycopersicum* L. cv. San Marzano)	Greenhouse experiment (Italy)	–	–	Marketable yield	↑	(Rouphael et al., 2021)
				Shape index	=	
				Juice pH	↑	
				Starch	↑ (1 out of 4)	
				Soluble sugars	↑ (1 out of 4)	
				Anthocyanins	=	
				Lycopene	↑	
				Polyphenols	↑	
				Soluble proteins	=	
				Free Amino Acids	=	
Tomato (*S. lycopersicum* L. cv. San Marzano)	Greenhouse experiment (Italy)	*Micrococcus* sp. F3 *P. agglomerans* C1 *Pseudomonas* sp. F1G	*Rhizophagus irregularis*	Root growth	↑ (PGPR; low P) ↑ (AMF; low +high P)	(Saia et al., 2020)
				Root biomass	↑ (PGPB; AMF; high P)	
				Total Root length	↑ (PGPB; AMF; high P)	

(Continued)

TABLE 1 Continued

Crop	Growing conditions and location	PGPR	AMF	Treatment effect on targeted parameter(s)		Reference
				Total Root Area	↑ (PGPB; low P) ↑ (PGPB; AMF; high P)	
				Leaf biomass	↑ (PGPB; AMF; high P)	
				No. of leaves	↑ (PGPB; AMF; high P)	
				Leaf area	↑ (PGPB; AMF; high P)	
				No. flowers	↑ (AMF; high P)	

AMF, Arbuscular Mycorrhizal Fungi; Ca, calcium; Cu, copper; Mg, magnesium; N, nitrogen; Na, sodium; P, phosphorous; PGPR, Plant Growth-Promoting Rhizobacteria; SPAD, Soil Plant Analysis Development; TAA, Total Antioxidant Activity; TCC, Total Carotenoid Content; TPC, Total Phenolic Content; TSS, Total Soluble Solids; Zn, zinc; ↑, increase; ↓, decrease; ↑↓, variable; =, no significant differences.

(AMF+NS+SE) (González-González et al., 2020). As regards root mycorrhizal colonization, an established mycorrhizal symbiosis was observed 96 days after treatment (DAT) in the AMF-treated and AMF+NS+SE plants, with a higher abundance in the second group of plants. The physiological response of tomato plants to the various applications showed that the AMF treatment favored the growth of the aerial plant. Moreover, the high polyphenol content, non-photochemical quenching, and maximum photochemical quantum yield efficiency of PSII in a dark-adapted state (F_V/F_M values) implied that AMF conferred the plant high resistance to environmental stress, as well as an increase in antioxidant and photoprotective mechanisms. The AMF biostimulant activity is related to the root biomass regulation, which may enhance nutrient uptake and translocation, increasing total carbohydrate, protein, and phenolic content (Table 1), as well as in plant-growth promotion, biomass production, stress tolerance, and disease resistance (González-González et al., 2020). The hyphal networks produced by AMF improve soil quality by increasing soil particle aggregation and reducing soil erosion. AMF also limits the amount of nutrient leaching from the soil and promotes nutrient retention (Tavarini et al., 2018). The combination of AMF, nutritive solution, and seaweed extracts allowed for an additive effect and increased foliar and root growth and protein and carbohydrate content.

A commercial microgranular inoculum containing *Rhizoglomus irregulare* and *Funneliformis mosseae* spores was placed in the planting hole of two landraces of Pomodorino del Piennolo del Vesuvio cherry tomatoes, i.e., the yellow-pigmented type "Giagiù" and red-pigmented type "Lucariello" (Carillo et al., 2020). Mycorrhization showed a beneficial effect on fruits. The number thereof per plant increased significantly, but no positive effect was observed on the mean fruit mass (Table 1). The capacity to accumulate starch was also augmented in both landraces. This is due to an increase of the photosynthetic efficiency and free amino acids. The positive effect of AMF on amino acids profiling depends on the beneficial effect of symbiosis on nutrient availability. AMF can, in fact, directly solubilize plant nutrients in the soil rhizosphere or produce siderophores; thus nutrients are directly available to the plant (Carillo et al., 2020).

Flower and fruit production was assessed in tomato (*S. lycopersicum* L., var. CXD 219 F1) plants grown in a field experiment under six conditions (Bona et al., 2018). It emerged that the treatments with the mycorrhizal inoculum (containing *Rhizophagus intraradices*, *Rhizophagus aggregatus*, *Septoglomus viscosum*, *Claroideoglomus etunicatum*, and *Claroideoglomus claroideum*), *Pseudomonas* sp. 19Fv1T, and *Pseudomonas fluorescens* C7 did not significantly affect the collar diameter and the percentage of fruits and flowers. The effect of the treatments on traits, such as the number of flowers and fruits, the total weight of mature fruits, and the percentage of non-marketable fruits, was not univocal (Table 1). Inoculation with the selected soil microorganisms significantly increased fruit size (length and diameter) and weight. This outcome is of paramount importance, since producing larger and heavier fruits is of high economic interest.

AMF has also been used with PGPR strains to investigate a possible synergistic action of the two categories of microbial PBs (Saia et al., 2020). AMF and three different strains of PGPR (i.e., *Micrococcus* sp. F3, *Pantoea* sp. C1, *Pseudomonas* sp. F1G) were applied by Saia et al. (2020) in a greenhouse experiment where tomato plants were grown under two different fertilizing conditions: low and high phosphorous. In this work, the authors evaluated the effect of the different treatments on roots biomass and traits (e.g., total length, specific length, mean diameter, total area, specific area), and leaf biomass and area, number of leaves and flowers. Whatever PGPR strains were applied, an increase in the root growth was observed under conditions of low phosphorus availability; on the other hand, AMF boosted root growth regardless of the fertilizer type. In detail, the treatment with *Pantoea* sp. C1 was the most promising, with a 121% increase in the total root length under low phosphorus conditions; *Micrococcus* sp. F3 determined an increase in the total root length as well, which was higher under low phosphorus conditions than under high phosphorus conditions;

the AMF treatment allowed for an increase of total root length. The PGPR treatment also improved leaf area, aboveground and root biomass, and nitrogen concentration but only under low-phosphorous conditions, that is, when Gafsa rock phosphate was used. This is related to PGPR's ability to produce root branching hormones, especially for the *P. agglomerans* C1 (Luziatelli et al., 2019). AMF improved the SPAD index, the total root area, and nitrogen concentration.

P. agglomerans C1 cells and metabolites were applied to tomato seedlings in pot experiments (Luziatelli et al., 2020b). The effect of the treatment on root characteristics was compared to i) sterilized distilled water (control), ii) sterilized distilled water supplemented with LB medium, iii) spent medium containing cells, iv) cell-free spent medium, and v) indole-3-butyric acid solution. When tomato shoots were treated with the spent medium containing both cells and secreted metabolites, a significant increase in the root surface area was observed, compared to the control, that is, the shoots treated with distilled water (Table 1). When the cell-free spent medium was applied, a more remarkable root growth was observed. The application of the extracellular metabolites produced by strain C1 determined an increase in the number and length of main roots of tomato cuttings; this indicates that strain C1 produces metabolites which can boost plant growth.

The effect of nine PGPR (i.e., *Bacillus amyloliquefaciens* B002, *Bacillus licheniformis* B017, *Bacillus mojavensis* B010, *Bacillus pumilus* W27-4, *Bacillus subtilis* Z3, *Bacillus pseudomycoides* S3, *Bacillus velezensis* B006, *Azotobacter chroococcum* A004, *Priestia megaterium* B004) and one mix thereof, on plant growth and physiology, yield, and quality characteristics of tomato fruits, cv. 'Rio Grande', was evaluated in a field experiment in Greece (Katsenios et al., 2021). The study outcomes showed that the soil application of the PGPR improved the industrial tomato's plant growth and physiology, yield, and quality characteristics. The effect of the treatments on dry weight, mean fruit weight and yield per plant, as well as on photosynthetic and transpiration rate was monitored at 66, 80, and 94 DAT. At the final measurement, *B. licheniformis* and *B. subtilis* treatments determined an increase in dry weight per plant of about 39.38% and 32.23%, respectively (Table 1). The application of *P. megaterium* boosted the photosynthetic rate from 5.54% at 66 DAT to 25.73% at 94 DAT; however, the highest values at the final measurement were observed in the treatment with *B. velezensis* and *A. chroococcum*. After 80 DAT, the treatment with the PGPR strains notably enhanced the transpiration rate, except for *B. amyloliquefaciens*, *A. chroococcum*, and *P. megaterium*.

3.1.2 Effect of microbial PBs on tomato functional and sensory quality

Food quality is a multi-faceted concept defined by several aspects related to commodity quality, outlined by external food attractiveness (e.g., fruit form, size, color), firmness, shelf-life, and organoleptic and functional quality. In tomato fruits, organoleptic quality is defined by physical traits, such as texture or firmness, and by biochemical traits (e.g., sugar and organic acid content and volatile compounds) determining the overall flavor; functional quality is defined by vitamins, phytonutrients (e.g., lycopene, β-carotene, polyphenols and ascorbate) and minerals (e.g., potassium, calcium, magnesium and phosphorus). As shown in Table 1, lycopene is the most studied parameter to evaluate the effect of PBs treatment on tomato functional quality.

Cardarelli et al. (2020) observed that inoculation with AMF and *Trichoderma koningii* combined with vegetal extracts determined a 14.1% increase in lycopene content and a 6.1% increase in vitamin C (Table 1). It was speculated that AMF may have promoted the activity of key enzymes involved in antioxidant homeostasis in cells. In addition, AMF can modulate host plant primary and secondary metabolism and stimulate the synthesis and accumulation of phytochemicals. Mineral concentration in tomato fruits was also investigated, and it emerged that concentration is affected positively only in the case of nitrogen; for phosphorus and potassium, no significant ($p>0.05$) differences were observed.

In the study by Rouphael et al. (2021), where four landraces of San Marzano were treated with a plant extract in a greenhouse experiment, lycopene, simple sugars, some organic acids, and macro-elements were investigated in tomato fruits. Lycopene concentration significantly increased following the PB treatment (Table 1). This result is paramount because lycopene content in mature fruits is important from a functional point of view and a critical aspect of the processing step. The increase in lycopene content determines, in fact, an increase in the fruits' red color intensity. Polyphenols, especially anthocyanins, did not vary between the control and the treated samples. As regards sugars, which are a crucial parameter as they are key contributors to tomato flavor, it was observed that the application of the plant extract positively affected glucose and fructose but not sucrose content. The biostimulation by the plant extract did not affect total protein content and free amino acids, including the essential. Citric acid increased in all landraces treated with the plant extract, but one. Either genotype or biostimulants influenced the mineral profile of tomato fruits. Interestingly, the effect of biostimulation was mineral-specific; it increased, in fact, the fruit concentration of Mg and K cations, while it decreased the concentration of the NO_3 anion. These data are important regarding the fruit's nutritional value because the abovementioned elements are essential minerals (Rouphael et al., 2021).

Carillo et al. (2020) also studied the mineral profile in the two Pomodorino del Piennolo del Vesuvio cherry tomato landraces. It was observed that AMF inoculation determined a significant increase in the content of magnesium, phosphorous, calcium, sodium, cuprum, and zinc (Table 1). The highest calcium and sodium concentrations in tomato fruits, for which a significant effect was determined by the interaction of landrace and microbial-based biostimulants, were observed in the "Giagiù" landrace. The higher calcium concentration observed in Giagiù after treating AMF is a particularly relevant trait because calcium positively affects morpho-physiological and metabolic parameters. It contributes, in fact, to increasing fruit number and yield, as well as to maintaining the firmness and turgor of fruit tissues and to extending fruit shelf-life. The increase in zinc concentration is important because it is beneficial not only for the plant and fruits but also for humans. The ion is, in fact, essential for protein and starch synthesis; it is involved in superoxide radicals scavenging and

contributes to the integrity of membranes. It regulates the expression of genes participating to cell expansion and is crucial to the correct development of tomato fruits. Zinc is finally essential for human nutrition, because it plays a key role in the growth, differentiation, and metabolism of cells. Hence, an adequate intake is crucial during human growth, e.g., prenatal and infancy stages. The effect of AMF treatment on lycopene and total ascorbic acid of the studied landraces was also investigated. The "Lucariello" landrace showed an 85% increase in lycopene compared to commercial tomatoes. Inoculation with endophytic fungi also enhanced total ascorbic acid, for which a higher content was observed (Table 1). The increase in lycopene and total ascorbic acid implies that fruit tissues are more protected from oxidative stress, and the postharvest conservation of "Lucariello" tomatoes can be extended. In addition, ascorbic acid plays a key role in maintaining fruit color and freshness, and in preventing spoilage, principally because of its antioxidant activity and low pH that limits food spoilage. AMF inoculation averaged over landrace positively affected the total soluble solids, fruit dry matter percentage, total antioxidant activity, and lycopene in fruit tissue (Table 1).

The effect of treatment with *B. subtilis* CBR05 on phytochemicals was investigated by Chandrasekaran et al. (2019). The greenhouse experiment showed that the antioxidant activity, measured by the DPPH and ABTS scavenging capacity, was positively affected by applying the PGPR strain. An increase in total phenolic content and total flavonoid content in treated plants was also observed (Table 1). A significant ($p<0.05$) higher amount of lycopene (All-E-lycopene) was also found, while no significant differences compared to the control were observed for All-E-β-Carotene (Chandrasekaran et al., 2019).

Bona et al. (2018) monitored the effect of treatment with AMF, *Pseudomonas* sp. 19Fv1T, and *P. fluorescens* C7 on glucose and fructose concentrations, and it emerged that they were highest when a combination of the three PBs was applied. A 13% and 19% increase in glucose and fructose, respectively, were found (Table 1). This finding is very important because the sweetness is a very appreciated characteristic in tomatoes for industrial use, and fructose is the most important sugar for the sweetness perception because it is rated at 1.7 times the sweetness of sucrose.

Tomatoes treated with the AMF *R. irregularis* and *F. mosseae* under optimal and low nitrogen (N) input conditions, in combination with *Trichoderma atroviride* application or alone, showed an enhanced functional quality of fruits (Ganugi et al., 2023). AMF coupling with *Trichoderma* fungal inoculations resulted in a synergistic effect on tomato fruits under sub-optimal fertility conditions. The concentration of β-carotene, Z-carotene, 13-Z-lycopene, and all-trans-lycopene increased; total phenolic content, total antioxidant activity, radical scavenging activity, reducing power and enzyme inhibitory activity were also boosted when AMF was applied with *T. atroviride* in combination with low N input and under sub-optimal fertility conditions (Table 1). These results point out that AMF inoculation helps plant growth under low nitrogen conditions by modulating the response thereof at physiological, metabolic, and molecular levels, accumulating secondary metabolites in the host plant.

3.2 Strawberry

Strawberry (*Fragaria* × *ananassa* Duch.) is the most consumed berry fruit crop worldwide, and its quality is defined by a unique flavor, its nutrients, and the antioxidant potential. Appearance, in terms of shape, color, size, and gloss; texture, in terms of firmness, crispness, and toughness; flavor, in terms of sweetness, sourness, aroma, and off-flavors; nutritional profile, in terms of vitamins and minerals; antioxidants, in terms of anthocyanins, flavonols and flavanols, phenolic acids, carotenoids, and vitamins, are quality indices important to evaluate strawberry quality (Giampieri et al., 2012; Cheng et al., 2016; Miller et al., 2019).

Strawberry (*Fragaria* × *ananassa* Duch.) cropping nevertheless requires extensive use of fertilizers at various stages of the plant life cycle. This use is detrimental in a framework of economy, environment, and human health safeguards. Recent studies have thus investigated the potential of PGPR application as an alternative to chemical fertilizers and their effect on strawberry fruit's nutritional and organoleptic quality. The application of PGPR to strawberry cropping can have valuable environmental benefits: it may help to reduce the use of chemical fertilizers, it can suppress phytopathogens, and it may help to sustain soil productivity.

3.2.1 Effect of microbial PBs on strawberry plant and fruit morphology and crop productivity

The effect of microbial PBs on strawberry plant and fruit morphological traits has been deeply investigated (Table 2). Fruit length, width, and sphericity have been widely analyzed; plant morphology was measured in a few cases.

The effect of strawberry plant treatment with PGPR strains *B. amyloliquefaciens* BChi1 and *Paraburkholderia fungorum* BRRh-411 was investigated in field conditions (Rahman et al., 2018). *B. amyloliquefaciens* is a Gram-positive spore-forming bacterium in soil, which can colonize plant rhizosphere and grow under stressed conditions. It has been studied as an eco-friendly and non-toxic agent able to stimulate plant growth without having detrimental side effects (Luo et al., 2022). *P. fungorum* is a Gram-negative environmental species commonly used as a beneficial microorganism in agriculture and as an agent for biocontrol and bioremediation. Nevertheless, its application in agriculture is controversial because there is no clear evidence about its non-harmfulness to human health. The application of the two strains in field experiments showed a beneficial effect on plant, leaf and fruit morphology and fruit antioxidant content (Table 2) (Rahman et al., 2018). Results showed that apart from plant height, for which no significant ($p > 0.05$) difference was observed between the control and the treatments (Table 2), the two PGPR determined a significant increase in all the investigated parameters: rooting apparatus, number of leaves per plant, plant length and width, and canopy diameter. Also, fruit morphology was positively affected, with an increase in the length, diameter, and weight of the fruits in treated plants.

A greenhouse experiment was carried out by Flores-Felix and colleagues, who investigated the effect of the inoculation of the type-strain of *Phyllobacterium endophyticum* (PEPV15) on strawberry

TABLE 2 Conditions and effect of microbial plant biostimulants treatment on strawberry crops.

Crop	Experiment Type and Location	PGPR	AMF	Quality parameter	Treatment Effect	Reference
Strawberry (*Fragaria* × *ananassa* Duch. cv. Honeoye)	Pot experiment (Poland)	Mixed consortia of *Peanibacillus polymyxa* sp., *B. subtilis*, *Bacillus* sp., *Streptomyces* sp., *Lysobacter* sp., and *Pseudomonas* sp.	–	Firmness	↑ (1 out of 5)	(Drobek et al., 2021)
				SSC	↑ (1 out of 5)	
				TAC	↑ (4 out of 5)	
				TPC	↑ (1 out of 5)	
Strawberry (*Fragraria* x *ananassa* Duch. var. Camarosa)	Greenhouse experiment (Spain)	*Phyllobacterium endophyticum* (type-strain)	–	Stolons number and length	↑	(Flores-Félix et al., 2015)
				Flowers/Fruit number	↑	
				Fruit weight	=	
				Vitamin C	↑	
Strawberry (*Fragaria* × *ananassa* Duch. var. Camarosa)	Greenhouse experiment (Portugal)	*Pedobacter* sp. CC1, *Bacillus safensis* B106, *B. subtilis* B167A	–	Flowering	↑	(Morais et al., 2019)
				Harvest time	↑	
				Fruiting season	↑	
				Leaves number/plant	=	
				Plant height	=	
				Fruit color	=	
				Fruit length	↑ (*B.subtilis*, *Pedobacter* sp.)	
				Fruit width	=	
				Fruit sphericity	=	
				Chlorophyll content	=	
				TCC	↓	
				TPC	↑ (all treatments)	
				TFC	↑ (*Pedobacter* sp.)	
				TAC	=	
				AA	↑ (*B.subtilis*, *Pedobacter* sp.)	
Strawberry (*Fragaria* × *ananassa* Duch. cv. Hood)	Field experiment (Oregon, USA)	*B. subtilis* *Bacillus amyloliquefaciens* *Pseudomonas monteilii*	–	TA	↓ (T1) =(T2)	(Nam et al., 2023)
				TSS	= (T1) ↑(T2)	
				Color (L)	↓ (T1, T2)	
				Color (chroma)	↓ (T1, T2)	
				VOCs	↑	
Strawberry (*Fragaria* × *ananassa* Duch. cv. Festival)	Field experiment (Bangladesh)	*B. amyloliquefaciens* BChi1 *Paraburkholderia fungorum* BRRh-411	–	Root length	↑	(Rahman et al., 2018)
				Plant height	=	
				Leaf length	↑	

(Continued)

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TABLE 2 Continued

Crop	Experiment Type and Location	PGPR	AMF	Quality parameter	Treatment Effect	Reference
				Leaf width	↑	
				Leaves number/plant	↑	
				Canopy diameter	↑	
				Fruit length	↑	
				Fruit diameter	↑	
				Fruit weight	↑	
				TAC	↑	
				TCC	↑	
				TFC	↑	
				TPC	↑	
				AA	↑	
Strawberry (*Fragaria* × *ananassa* Duch. var. Eliana F1)	Greenhouse experiment (Italy)	*Pseudomonas* sp. (19Fv1t, 5Vm1K and Pf4	*F. mosseae* *S. viscosum* *R. irregularis*	Flowers/plant	↑ (Pf4)	(Todeschini et al., 2018)
				Fruits/plant	↑ (Pf4)	
				Total fruit fresh weight/ plant	↑ (Pf4)	
				Average weight of fruit/plant	↑	
				Fruit large diameter	↑	
				Fruit small diameter	↑	
				pH	↑↓	
				Titratable acidity	↑	
				Malic acid	↑	
				Quinic, citric, fumaric and ascorbic acid	=	
				Sucrose	↑↓	
				Glucose	=	
				Fructose	↑	
				Total sugar concentration	=	
				Total anthocyanidin	↑ (*S. viscosum* +5Vm1K)	
				Pelargonidin 3-glucoside concentration	↑ (*S. viscosum* +5Vm1K)	

AA, antioxidant activity; GAC, Galacturonic Acid Content; SSC, Soluble Sugar Content; TA, total acidity; TAC, total anthocyanins content; TCC, total carotenoid content; TFC, total flavonoid content; TPC, total phenolic content; TSS, total soluble solids; VOCs, volatile compounds; ↑, increase; ↓, decrease; ↑↓, variable; =, no significant differences.

plants planted on black plastic trays with peat/vermiculite (Flores-Félix et al., 2015). The results of the experiments showed that the inoculation, six days after planting, with the strain PEPV15 determined an increase in many parameters related to plant growth. Stolons number and length were significantly ($p<0.05$) higher in the plants inoculated with the strain PEPV15; treated plants also produced a significantly ($p<0.05$) higher number of flowers and fruits. The latter's weight was nevertheless not significantly higher (Table 2).

Strawberry plants have also been treated in greenhouse experiments in Portugal with three PGPR strains, i.e., *Pedobacter* sp. CC1, *Bacillus safensis* B106 and *B. subtilis* B167A (Morais et al., 2019). Their application allowed for an earlier flowering and harvest time than the control (Table 2). A similar positive effect was observed on pear (*Pyrus communis*) seedlings by applying metabolites produced by *P. agglomerans* C1 (Valerio et al., 2023).

However, for economic reasons, it is important that the fruit's quality is increased, and a plentiful and healthy harvest is guaranteed. Strawberries are soft fruits characterized by a high and rapid loss of firm texture during ripening. The fast-softening results in a shorter shelf-life and higher susceptibility to diseases; it is thus among the main reasons for commercial loss. It is estimated that 5 to 30% of strawberry yield is lost because of over-softening and fungal decay (Posé et al., 2011). Within this framework, it is important to consider the antagonistic properties of PGPR consortia against fungal pathogens.

Regarding strawberries, one of the main postharvest diseases is grey mold, caused by *Botrytis cinerea*. Following its contamination, a grey coating appears on leaves and fruits; the plant dies off, and the fruits become dry and rot. The strawberry plant's withering is caused by *Verticillium dahliae*, which attacks the plant's vascular system, blocks the water and nutrients transport, and becomes detrimental to the plant. Drobek and colleagues hence investigated the antagonistic effect of selected bacterial consortia on four microbial pathogens (i.e., *Botrytis cinerea*, *Verticillium* sp., *Phytophthora* sp., and *Colletotrichum* sp.) causing strawberry diseases (Drobek et al., 2021). The bacterial consortia applied comprised strains belonging to *Peanibacillus polymyxa* sp., *B. subtilis*, *Bacillus* sp., *Streptomyces* sp., *Lysobacter* sp., and *Pseudomonas* sp.

The application of PGPR consortia contributed to obtaining fruits with a higher firmness when a PGPR consortium was used as an antagonist agent against a mixed pathogen group composed of *B. cinerea*, *Verticillium* sp., *Phytophthora* sp., and *Colletotrichum* sp. This outcome is of paramount importance because firmness is a property that generally allows for more extended storage periods and makes strawberry fruits more suitable for transport. The increased firmness was likely related to the production of metabolites by the PGPR strains that can limit mycelium growth by bacterial consortia. During ripening, strawberries soften, in fact, following an extensive dissolution of the middle lamella of the cortical parenchyma cells (Posé et al., 2011). The activity of fungal pathogens attacking the strawberry fruit during ripening and storage can increase the degradation. The trend of soluble solid content (SSC) after the treatment was also monitored. This parameter is connected with consumer preference for fruit. SSC measures total soluble solids, including sugars, organic acids, amino acids, and other compounds. The mean SSC in the fruits under analysis was 6%, consistent with the literature (Chen et al., 2018). The treatment of strawberries contaminated with *Phytophthora* sp. with one PGPR consortium also allowed for a higher SSC. The increase in one out of five treatments was likely because some bacteria can dissolve phosphates, which lower soil pH and increase phosphorus availability by producing organic acids. As regards phenolics and anthocyanins, which play a crucial role as bioactive compounds and are also responsible for the bright red color of strawberry fruits, it was observed that total phenolic content was higher than the control only in one treatment. At the same time, total anthocyanin content was 25–51% higher than the control in the strawberry fruits infested by *Phytophthora* sp. and treated with four out of five PGPR consortia.

3.2.2 Effect of microbial PB on strawberry functional and sensory quality

Sugars and organic acids are the main soluble components in ripe strawberry fruit, and the ratio between them affects fruit aroma and flavor (Todeschini et al., 2018). Fructose, glucose, and sucrose are responsible for fruit sweetness (Perez et al., 1997). Organic acids are important for preserving fruit's nutritional value (Mikulic-Petkovsek et al., 2012). Among them, citric acid is the most abundant and is responsible for about 92% of total acidity. Other organic acids, i.e., malic, tartaric, shikimic, quinic, and fumaric, are present at a very low concentration. Strawberry fruits are also an important source of healthy compounds such as dietary fiber, vitamin C, minerals like potassium and magnesium, and antioxidants. Among polyphenols, anthocyanins are the most important compounds in the form of pelargonidin and cyanidin derivatives. They are, in fact, responsible for the strawberry's bright red color; in addition, they play a key role in fruit tolerance to environmental stresses and the improvement of post-harvest quality and shelf life. Volatile organic compounds are also important, as they make strawberries a highly appreciated fruit and an important quality indicator for strawberries.

A consortium of three PGPR strains (i.e., *B. subtilis*, *B. amyloliquefaciens*, and *Pseudomonas monteilii*) was evaluated for its potential role as a biofertilizer by application twice a week at three different percentages (0%, 0.24% and 0.48%) to the soil in field experiments (Nam et al., 2023). The three treatments did not significantly affect total acidity (TA), which is a predictor of the impact of organic acids on food flavor and whose content declines during the fruit ripening process (Table 2). Regarding total soluble solids (TSS) determination, this parameter was higher in the strawberry sample treated with the highest percentage of biofertilizer (Table 2). The strawberry color was also affected by the treatments; it emerged that treated samples were darker than non-treated ones (Table 2). Hence, the higher values of TA and TSS and the darker color suggest that the strawberries treated with the biofertilizer were more mature. So, with equal growing periods for all three treatments, data showed that the biofertilizer had a ripening enhancer effect.

The effect of strawberry plant treatment with PGPR strains *B. amyloliquefaciens* BChi1, and *P. fungorum* BRRh-411 was also

investigated on antioxidants (Rahman et al., 2018). The application of plant probiotic bacteria significantly increased the total content of anthocyanins, carotenoids, flavonoids, and phenolics, as well as antioxidant activity compared to the non-treated control (Table 2).

Vitamin C was detected in the greenhouse experiment carried out by Flores-Felix and colleagues by inoculating the type-strain of *Phyllobacterium endophyticum* (PEPV15) (Flores-Félix et al., 2015). It emerged that its content in strawberry fruits from plants inoculated with strain PEPV15 was significantly higher (i.e., two-fold) than in fruits from non-treated plants (Table 2).

In the greenhouse experiment carried out by Todeschini and colleagues with *F. mosseae*, *S. viscosum*, *R. irregularis*, and three strains of *Pseudomonas* sp., it emerged that the treatment with AMF mainly affected the parameters associated with the vegetative portion of the plant. At the same time, the effect of PGPR was significant for fruit yield and quality. Titratable acidity, expressed as the percentage of citric acid per fresh weight unit, was significantly affected by the mycorrhizal treatment and even more by bacterial inoculation (Todeschini et al., 2018). No differences were found in organic acids for quinic, citric, fumaric, and ascorbic acid, whereas malic acid concentration was significantly affected; the treatment with *Pseudomonas* sp. Sv19Fv determined the highest concentration of malic acid. Regarding glucose and total sugars concentrations, the treatments determined non-significant differences, while the effect on sucrose and fructose was related to the combination of AMF and bacterial strains (Table 2). As regards anthocyanins, cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside, cyanidin malonyl glucoside, pelargonidin malonyl glucoside, and pelargonidin acetyl glucoside were present (Table 2). The concentration of pelargonidin 3-glucoside was the most abundant and significantly varied between the treatments (Table 2). The various treatments did not significantly affect the concentrations of the other anthocyanidins.

3.3 Leafy vegetables

Leafy vegetables are a broad group of horticultural plants cultivated for their foliar structure, constituting the plant's edible part (Alvino and Barbieri, 2015). The list of most common species of leafy vegetables includes, among others, lettuce (*Lactuca sativa* L.), rocket salad (*Eruca sativa* Miller), and common basil (*Ocimum basilicum* L.) (Alvino and Barbieri, 2015). For these crops, experimental work with PBs has been carried out. The analysis of the main outcomes of these studies is of paramount importance because consumption of salads and herbs such as basil is particularly high in the Mediterranean countries where the cultivation of the species has ancient traditions.

3.3.1 Lettuce

Lettuce (*Lactuca sativa* L.) is a leafy vegetable belonging to the *Cicoreae* tribe of the family *Compositae*. It is an excellent source of vitamin A and K, provitamin A compounds, and beta-carotene, in darker green lettuces, such as Romaine (Kim et al., 2016). It is also a good source of folate and iron and has an interesting phytochemical profile. Lettuce comes in various colors, sizes, and shapes, and because of this diversity, it can be grouped into diverse types. According to Mou (2008), six main lettuce types are identified based on leaf shape, size, texture, head formation, and stem type: i) crisphead lettuce (var. *capitata* L. *nidus jaggeri* Helm), ii) butterhead lettuce (var. *capitata* L. *nidus tenerrima* Helm), iii) romaine or cos lettuce (var. *longifolia* Lam., var. romana Hort. in Bailey), iv) leaf or cutting lettuce (var. *acephala* Alef., syn. var. *secalina* Alef., syn. var. *crispa* L.), v) stem or stalk (Asparagus) lettuce (var. *angustana* Irish ex Bremer, syn. var. *asparagina* Bailey, syn. L. *angustana* Hort. In Vilm.), and vi) Latin lettuce (Mou, 2005, Mou, 2008).

Iceberg lettuce was treated with *Trichoderma*-based biostimulants under sub-optimal, optimal, and supra-optimal nitrogen (N) fertilization levels and grown in a greenhouse (Fiorentino et al., 2018). *Trichoderma* strains *T. virens* (GV41), or *T. harzianum* (T22) were used in the inoculation. Thanks to the treatment, no visible chlorosis or necrosis symptoms were observed in treated plants. The yield was positively affected by the treatment with both strains and the supply of an optimal dose of nitrogen; moreover, no significant effect was observed between the two strains. When the soil was not fertilized with N, the inoculation of the GV41 strain gave better results than T22 for the total and marketable weight (Table 3). Ascorbic acid is an important parameter of the functional quality of horticultural crops, and the study by Fiorentino et al. (2018) showed that the *Trichoderma*-based biostimulants significantly influenced it, N availability rate and interaction thereof: the highest concentration was observed whatever strain was inoculated under no-fertilization conditions.

In the same experiment, the *Trichoderma* biostimulant was also applied to the rocket (*Eruca vesicaria* Mill.), but the inoculation had a more beneficial effect on iceberg lettuce than the rocket. Among the different reasons, including also the fact that crop response is not global but complex and generally depends on the crop botanical family, it is likely that the lettuce crop cycle was longer. This means that the higher duration allowed the root system to develop and diversify more than the rocket, following a major ability of the AMF to colonize the crop rhizosphere.

Saia and colleagues investigated the response triggered by a microbial PB containing two strains of AMF (i.e., *R. irregulare* and *F. mosseae*) and *Trichoderma koningii* on greenhouse-grown lettuce (*Lactuca sativa* L.). The experiment was performed under different water conditions: i) well-watered, ii) moderate-watered, and iii) severe deficit irrigation regimes. The study outcomes suggested that the effect of the biostimulant on lettuce's nutritional and functional quality was mainly independent of water availability. In contrast, its effect on fresh marketable and dry yields was clear under well-watered and moderate irrigation regimes through the modulation of the secondary compounds' biosynthesis (Saia et al., 2019). Under the well-watered and moderate irrigation regimes, yield, phenolic acids, and flavonoids were not affected, whereas net photosynthetic and transpiration rates were halved. In addition, the presence of AMF and Trichoderma reduced Mg and Zn concentrations in the roots, soil, and plant. After further reducing water availability, yields, ascorbic acid, total phenols, and quercetin also decreased. However, the treatment increased P, Mg, Fe, Mn, and Zn concentrations and various phenolic acids, such as chlorogenic

TABLE 3 Conditions and effect of microbial plant biostimulants treatment on leafy vegetables.

Crop	Experiment Type and Location	PGPR	AMF	Quality parameter	Treatment Effect	Reference
Lettuce (*Lactuca sativa* L. var. Iceberg cv. Silvinas)	Greenhouse experiment (Italy)	–	*Trichoderma virens* GV41 *T. harzianum* T22	Total yield	↑ (optimal N) ↑ (GV41+no N supply)	(Fiorentino et al., 2018)
				Marketable yield	↑ (optimal N) ↑ (GV41+no N supply)	
				pH	=	
				Ascorbic acid	↑ (non-fertilized conditions)	
Leaf lettuce (*Lactuca sativa* var. *crispa* L. cv. Santoro)	Field experiment (Czech Republic)	*Bacillus licheniformis* *Bacillus megatherium* *Azotobacter* sp. *Azospirillum* sp. *Herbaspirillum* sp.	–	Leaf weight	↑	(Kopta et al., 2018)
				TAA	=	
				TCC	=	
Romaine lettuce (*Lactuca sativa* L. var. *longifolia* Lam. cv. Quintus)	Field experiment (Czech Republic)	*Bacillus licheniformis* *Bacillus megatherium* *Azotobacter* sp. *Azospirillum* sp. *Herbaspirillum* sp.	–	Leaf weight	↑	(Kopta et al., 2018)
				TAA	↑	
				TCC	=	
Butterhead lettuce (*Lactuca sativa* var. *capitata* cv. Bolla)	Greenhouse experiment (Italy)	–	*R. irregulare* *F. mosseae* *T. koningii*	AMF/ *Trichoderma* presence	↓	(Saia et al., 2019)
				Plant yield	↑	
				P, Mg, Fe, Mn, Zn, Ca, Cu	↑	
				Phenolic compounds	↑	
				Luteolin glycoside	↑	
Rocket (*Eruca sativa* Mill.)	Greenhouse experiment (Italy)	–	*Trichoderma virens* (strain GV41) *T. harzianum* (strain T22)	Marketable yield	= (optimal N)	(Fiorentino et al., 2018)
				Total yield	↑ (GV41)	
				pH	=	
				Ascorbic acid	↑ (GV41)	
Basil (*Ocimum basilicum* L. Gecom)	Glasshouse experiment (Italy)	–	AMF and *T. koningii*	Leaf number	↑	(Saia et al., 2021)
				Leaf area	↑	
				Plant photosynthetic activity	↑	
				Ca, Mg, B concentration	↑	
				p-Coumaric acid	↑	
				Chicoric acid	↑	

↑: increase; ↓: decrease; ↑↓: variable; =: no significant differences.

acid, irrespective of the water availability. The increase in plant yield, calcium, copper, and isochlorogenic acid concentration was especially evident under well-watered and moderate irrigation conditions. Finally, luteolin glycoside, which is directly implied in the neighbor detection and allelopathy response of plants and is frequently associated with a plant reaction to drought stress and microbial stimulation, progressively increased in the biostimulant inoculated plant upon water availability reduction.

Leaf lettuce (*Lactuca sativa* var. *crispa* L.) cv. 'Santoro' and Romaine lettuce (*Lactuca sativa* L. var. *longifolia* Lam.) cv. 'Quintus' was treated (watering) with some PGPR strains, i.e., *Bacillus licheniformis*, *Bacillus megatherium*, *Azotobacter* sp.,

TABLE 4 Conditions and effect of microbial plant biostimulants treatment on cucumbers.

Crop	Experiment Type and Location	PGPR	AMF	Quality parameter	Treatment Effect	Reference
Cucumber (*Cucumis sativus* L.)	Pot experiments (India)	*Acinetobacter baumannii* (2 strains) *Arthrobacter* sp. *Cronobacter dublinensis* (4 strains) *Enterobacter cloacae*	–	Root length	↑ (5 out of 8, no saline stress) ↑ (2 out of 8, saline stress)	(Kartik et al., 2021)
				Root wet weight	↑ (3 out of 8, no saline stress) ↑ (4 out of 8, saline stress)	
				Root dry weight	↑ (7 out of 8, no saline stress) ↑ (2 out of 8, saline stress)	
				Shoot length	↑ (5 out of 8, no saline stress) ↑ (7 out of 8, saline stress)	
				Shoot wet weight	↑ (3 out of 8, no saline stress) ↑ (7 out of 8, saline stress)	
				Shoot dry weight	↑ (4 out of 8, no saline stress) ↑ (7 out of 8, saline stress)	
				Chlorophyll a content	↑ (4 out of 8, no saline stress) ↑ (6 out of 8, saline stress)	
				Chlorophyll b content	↑ (4 out of 8, no saline stress) ↑ (8 out of 8, saline stress)	
				Ascorbic acid	↑ (4 out of 8, no saline stress) ↑ (8 out of 8, saline stress)	
				TPC	↑ (8 out of 8, no saline stress) ↑ (8 out of 8, saline stress)	

TPC, Total Phenolic Content; ↑: increase.

Azospirillum sp., *Herbaspirillum* sp., and freshwater algae (*Chlorella vulgaris*), in a field experiment. The two lettuce varieties were cultivated at different times: spring and summer. Considering a mean trend, the weight of leaf and romaine lettuce-treated plants was significantly higher than the control (Table 3). As regards the effect of the treatment on total antioxidant capacity and total carotenoid content, no significant effect was observed between treated plants and control. However, total antioxidant capacity for the spring and summer crops was generally higher in leaf lettuce than in romaine.

3.3.2 Rocket

Rocket (*Eruca sativa* (Mill.) Thell) is a leafy green vegetable from the *Brassicaceae* family. It contains many vitamins, including vitamins A and C and folic acid (Jamal et al., 2021). It is also rich in several minerals, such as calcium, copper, iron, magnesium, phosphorus, potassium, and zinc. Rocket is well-known for its various phytochemical components, including polyphenols, flavonoids, and glucosinolates (Jamal et al., 2021).

In the same greenhouse experiment described for iceberg lettuce, Fiorentino et al. (2018) investigated the effect of inoculation with *T. virens* strain GV41 and *T. harzianum* strain T22 also on the rocket (*Eruca vesicaria* Mill.). Under sub-optimal N fertilization conditions, treatment with *T. virens* GV41 determined a 33% increase in the total yield rocket (Table 3). In rocket, ascorbic acid presented the same trend as in iceberg lettuce. Its concentration was significantly affected by the *Trichoderma*-based biostimulants, N availability rate, and interaction thereof (Fiorentino et al., 2018).

In detail, inoculation with strain GV41 under N fertilization treatments determined a significant increase in total ascorbic acid compared to T22 (Table 3).

The increase in rocket plant growth and productivity under the three fertilization conditions hints that *Trichoderma* can modify soil nutrient availability, modulate root growth, and subsequently affect the rhizosphere's various biological and chemical processes. Some strains of the *Trichoderma* species produce, in fact, secondary metabolites, which have a hormone-like behavior; hence, the exudation of molecules with auxin-similar activity has a plant growth promotion action (Fiorentino et al., 2018). *T. harzianum* strain T22 is not a remarkable producer of bioactive compounds which can stimulate plant growth. However, its application to the rhizosphere can activate plant metabolic processes involving phytohormones (auxins/cytokinins) in treated plants.

All in all, the effect of the treatment was less beneficial on rockets than on lettuce plants. As mentioned above, the rocket is a leafy green vegetable belonging to the Brassicaceae family, and Brassicaceae species are well-known for the adverse effects on many soil microbes, bacteria, and fungi following the production of inhibitory compounds such as glucosinolates, which are released in the rhizosphere (Fiorentino et al., 2018).

3.3.3 Basil

Basil is widely cultivated in pots and gardens in Europe, South-west Asia, and the USA. It is one of the most popular herbs in the cooking and food network with its wide range of applications, especially in food flavoring and preservation. The leaves are ovate and vary in size, depending on the variety; they range from the small leaves of common basil to the large leaves of lettuce leaf basil (Kokkini et al., 2003). Saia et al. (2021) demonstrated that genotype, cultivation medium, and growing conditions affect several basil properties, including i) tolerance to salinity, which is generally low; ii) parameters of economic importance, such as the leaf fraction on the total above-ground biomass; iii) the concentration, and composition of secondary compounds; the antioxidant capacity; iv) the volatile organic fractions; v) the essential oil content. The same authors evaluated, in greenhouse experiments, the effectiveness of a microbial-based biostimulant containing two strains of AMF (i.e., *R. irregulare* BEG72 and *F. mosseae* BEG234) and *T. koningii* on the growth of basil under mild salinity conditions: 25 mM (low salinity) and 50 mM (high salinity). The increase in the salinity showed detrimental effects on the plant yield, nutrient uptake and concentration, photosynthetic activity, and leaf water potential, whereas it triggered the polyphenols accumulation. The concentration of eucalyptol and β-linalool, two of its main essential oil constituents, also decreased (Saia et al., 2020). However, the inoculum showed a beneficial effect on plant growth, leaf number, and area, irrespective of the salinity stress condition; Ca, Mg, B, *p*-coumaric and chicoric acids also accumulated. The results suggested that under low-salinity conditions, the inoculum stimulated the plant's photosynthetic activity following a higher availability of iron and manganese for the plant and subsequently induced the accumulation of phenolic acids, such as caffeic and rosmarinic acids. Under high salinity conditions, the inoculum mostly sequestered Na and increased P availability for the plant; moreover, it stimulated the accumulation of some polyphenols (e.g., ferulic and chicoric acids and quercetin-rutinoside) in the shoots. The inoculum did not affect the composition of volatile organic compounds, thus suggesting the lack of interaction between its activity and essential oil biosynthesis.

3.4 Cucumbers

Cucumber (*Cucumis sativus* L.) is a member of the *Cucurbitaceae*, and among the 30 species of Cucumis, *C. sativus* has the greatest economic significance (Zieliski et al., 2016). It is very rich in water (about 96.4%) and contains other bioactive compounds, such as dietary fiber, vitamin C, phenolic compounds (e.g., flavonols and proanthocyanidins) (Zieliski et al., 2016). During cucumber fruit growth, malic acid content decreases; glucose and fructose content increases; dry matter decreases. Cucumber presents a variation in the color of ripened fruit, fruit size, and number of fruit spines (Schaffer and Paris, 2016). Fruits mainly differ in the length and not width; hence, fruit size is essentially a function of length (Schaffer and Paris, 2016).

Cucumber plants can tolerate 3% salinity stress (Ge and Zhang, 2019), and, under salinity stress conditions, photosynthetic pigments, chlorophyll synthesis, plant metabolic and physiological activities are negatively affected because primary and secondary metabolites fluxes are altered (Abdel-Farid et al., 2020). Kartik et al. thus investigated the possibility of applying microbial PBs to cucumber crops as a possible approach to tackle the abiotic stress of salinity stress (Kartik et al., 2021). Treatments were made under a saline stress condition and with no saline stress. The study showed that plant growth parameters, such as root length, root fresh and dry weight, shoot length, and shoot fresh and dry weight, generally increased compared to control plants under both growth conditions. Differences emerged depending on the applied PGPR strain (Table 4). The measured plant growth parameters, except for root length and dry weight, were significantly higher under salinity stress conditions than the control (Table 4). The enhancement of plant morphological traits by treating the salt tolerant PGPR may be related to their ability to produce phytohormones and solubilize available minerals in the soil. The production of indole-3-acetic acid and siderophores and the ability of N and P uptake by the applied PGPR may have also played a crucial role in fostering plant growth under salinity stress.

On the other hand, the beneficial effect on chlorophyll is related to induced systemic tolerance by PGPR under salinity stress. The applied PGPR isolates also showed the production of siderophores, HCN, and chitinase. Thus, the strains likely have biocontrol ability and can protect plants against pathogens.

4 Evaluation of the effect of microbial PBs on horticultural crops by omic sciences

The biostimulatory effect exerted by the different microbial PBs presented above derives from the interaction between the molecular structure of plant cells and a series of external physical, chemical

and biological stimuli (González-Morales et al., 2021). Plants can, in fact, adapt to environmental conditions by exerting physiological and biochemical responses and by modifying metabolic processes. It is therefore within this framework that "omic" sciences (e.g., genomics, transcriptomics, proteomics, and metabolomics) have figured out as fundamental tools for decoding and unravelling the metabolic pathways and the key factors underlying the plant response to both endogenous and environmental stimuli.

The application of "omic" sciences to disentangle the mode(s) of action by which microbial PBs affect plant quality has turned out as informative and useful; however, a limited number of studies is still available.

Transcriptomic analysis, based on Next-Generation Sequencing, figured out as one of the most powerful tools allowing for the identification of the molecular markers that are associated with common responses of plants to the application of microbial PBs. Transcriptomic analysis has been performed to understand the mode of action of different PBs, such as fungi (Volpe et al., 2018), biostimulants based on humic acids and chitosan (Hernández-Hernández et al., 2018), biostimulants based on extracts of algae and botanicals (Goñi et al., 2018), and commercial products (Contartese et al., 2016) on horticultural crops such as tomato, lettuce and cucumbers. As regards tomato cropping, a transcriptomic approach is proposed only in a few studies on microbial PBs. The application of *Trichoderma harzianum* T22 to tomato seedlings showed that, under water stress, the strain modulates the expression of genes encoding some antioxidant enzymes, such as monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), dehydroascorbate reductase (DHAR) (Mastouri et al., 2012). The genes MDHAR1 and MDHAR2, GRc and GRp, DHARc and DHARp, and Cu/Zn-SOD*p*, Fe-SOD*p*, and APX*c* showed a higher expression in the tomato shoots, while the gene APX*c* showed a higher expression in tomato roots (Mastouri et al., 2012). These findings hint at the fact that an enhanced resistance of treated plants to water stress is due to the increased capacity to scavenge reactive oxygen species and recycle oxidized ascorbate and glutathione. The expression of genes under water deficit was also studied following the application of *Funneliformis mosseae* and *Rhizophagus intraradices* to a commercial tomato cultivar (San Marzano nano) (Volpe et al., 2018). An improvement of phosphorous uptake, transfer and delivery emerged in tomato roots, and the genes encoding for plant phosphate transporters (i.e., LePT1, LePT2, LePT3, LePT4, and LePT4) changed their expression following the treatment with AMF and under water deficit.

Genes related to plant growth promotion and biocontrol activity in tomato seedlings were found in the genome of *Pantoea agglomerans* strain C1 (Luziatelli et al., 2020b). In detail, study identified i) two genes, namely, *ipdC* and *amiE*, which encode key enzymes (i.e., indole-3-pyruvate decarboxylase and aliphatic amidase) involved in the synthesis and secretion of inole-3-acetic acid via the IPyA and IAM pathway, respectively; ii) two operons (i.e., speAB and speDE) involved in the biosynthesis of spermidine and correlated with the development of lateral roots, resistance to pathogens, as well as resistance to oxidative, osmotic, and acidic stress; and iii) several gene clusters involved in the solubilization of mineral phosphate.

As regards to the other horticultural crops analyzed in this study, it emerged that "omic" sciences have been so far applied to understand the response of plants to PBs other than microbial ones. A transcriptomic approach has been used, for instance, to understand the response of lettuce seedlings to microalgae extracts used as biostimulant agents (Santoro et al., 2023). Otherwise, the proteomic changes occurring in the antioxidant system of strawberry during ripening was investigated (Song et al., 2020). The investigation of a total of 46 proteins and isoforms by a targeted quantitative proteomic approach using multiple reaction monitoring, showed that superoxide dismutase, aldo/keto reductase, and glutathione transferase increased significantly, whereas L-ascorbate peroxidase, 1-Cys peroxiredoxin, 2-Cys peroxiredoxin, dehydroascorbate reductase, and catalase decreased significantly. These plants were not treated with biostimulants. However, a meaningful interpretation of such analyses requires reproducible effects. It might be, therefore, interesting investigating these markers in plant treated with microbial PBs.

Besides the increasing need to use "omic" sciences to understand the mechanisms that affect food quality following plant treatment with microbial biostimulants, high-throughput phenotyping technologies are necessary to identify the optimal phenological stage, the application method, time, and rate that allow improving plant performance and resilience to stress (Rouphael and Colla, 2018). They offer the advantage of an automated and non-destructive monitoring of the morpho-physiological traits of plants, as well as the possibility to carry out time-series measurements which provide information on growth progression, plant performance and stress response (Rouphael and Colla, 2018). In addition, these technologies can reduce costs, labor and analysis time (Rouphael and Colla, 2018), but are also prone to produce artefacts, when a meaningful interpretation with common human sense is missing.

5 Concluding remarks and future perspectives

This study identified the various effects that microbial PBs could have on plant and fruit morphology, crop productivity, and fruits' nutritional, functional, and organoleptic quality. Currently, most studies occurred under greenhouse conditions, where it is likely that growing conditions can be better controlled and monitored. Field trials were carried out only in a small amount. Tomato, lettuce, and basil crops have been primarily treated with AMF, while PGPR metabolites were used for other crops, such as strawberries and cucumbers.

Findings showed that crop response is never univocal and global. Complex mechanisms related to the PB type, the strain, and the crop botanical family, occur. It is necessary to continue designing other experiments where the mechanisms behind the plant growth-promoting activity are also tracked. These observations indicate the critical points to be considered in developing new PBs. First-generation PBs were produced with bioactive substances and microorganisms to stimulate plant

physiological and molecular processes and improve plant nutrient uptake and use efficiency. More recently, a second generation of PBs has been formulated thanks to the new synergistic work of chemistry, biology, and omics sciences, but the importance of agricultural and horticultural management issues is still underestimated. For the rational development of the third generation PBs, we need to better understand the molecular mechanisms allowing the modulation of plant physiology and the synergistic effect of microbial and non-microbial biostimulants. A more comprehensive knowledge of the mechanisms underlying the biostimulant activity will allow identifying the best-suited biostimulant for a specific crop and exact growing conditions. In addition, a more profound knowledge of the molecular mechanisms will be valuable for identifying the optimal dose to apply and the suitable stage of plant development and growth at which a specific biostimulant should be applied. Finally, more extensive and comprehensive knowledge, which also requires an adequate legal framework regarding efficacy testing, risk assessment and registration procedures with the respect to the actual nature of the regulated agents (Feldmann et al., 2022), might allow biostimulant manufacturers to address the product composition with a major awareness.

Author contributions

FM and VM contributed to the conceptualization and definition of the methodology and investigation of the study. MR contributed to defining the design of the study. FM and VM wrote the first draft of the manuscript. FM, VM, FL, RJ, AF, MR contributed to editing the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Inoculation with a microbial consortium increases soil microbial diversity and improves agronomic traits of tomato under water and nitrogen deficiency

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Microbial-based biostimulants, functioning as biotic and abiotic stress protectants and growth enhancers, are becoming increasingly important in agriculture also in the context of climate change. The search for new products that can help reduce chemical inputs under a variety of field conditions is the new challenge. In this study, we tested whether the combination of two microbial growth enhancers with complementary modes of action, *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22, could facilitate tomato adaptation to a 30% reduction of optimal water and nitrogen requirements. The microbial inoculum increased tomato yield (+48.5%) under optimal water and nutrient conditions. In addition, the microbial application improved leaf water potential under stress conditions (+9.5%), decreased the overall leaf temperature (-4.6%), and increased shoot fresh weight (+15%), indicating that this consortium could act as a positive regulator of plant water relations under limited water and nitrogen availability. A significant increase in microbial populations in the rhizosphere with applications of *A. chroococcum* 76A and *T. afroharzianum* T22 under stress conditions, suggested that these inoculants could enhance soil microbial abundance, including the abundance of native beneficial microorganisms. Sampling time, limited water and nitrogen regimes and microbial inoculations all affected bacterial and fungal populations in the rhizospheric soil. Overall, these results indicated that the selected microbial consortium could function as plant growth enhancer and stress protectant, possibly by triggering adaptation mechanisms via functional changes in the soil microbial diversity and relative abundance.

KEYWORDS

***Azotobacter chroococcum*, *Trichoderma afroharzianum*, nutrient stress, water stress, biostimulants, tomato rhizosphere**

1 Introduction

The global food demand is anticipated to increase from 35% to 56% in the period of 2010 to 2050, while the population at risk of hunger is projected to increase to +8% over the same time frame (van Dijk et al., 2021). To maintain a high quantity of crop production and reduce yield loss, chemical products (fertilizers, pesticides, herbicides, etc.), hormones and antibiotics are commonly used in agriculture (Savci, 2012; Pathak, 2018; Gangwar et al., 2023). Concerns over human and environmental health and negative impacts arising from chemical residues in soil, water, and food as well as exposure risks by farm workers have received considerable attention. As a consequence, in the last two decades, the scientific community is looking for innovative and eco-sustainable strategies to increase agricultural production, meet food needs, and reduce environmental impact (Comite et al., 2021; Silletti et al., 2021). The use of microbial inoculants as agricultural-probiotics, is an attractive environmental-friendly alternative strategy to agro-chemical inputs to ensure crop yield and quality (Fiorentino et al., 2018; Woo and Pepe, 2018). Probiotics are living microorganisms that offer benefits to the host plant by providing nutritional inputs, protection from pathogen-pest attack, improved fitness, enhanced growth and health also in stress conditions (Hossain et al., 2017; Van Oosten et al., 2017; Romano et al., 2020b). Biostimulant formulations containing beneficial microorganisms and/or natural substances (e.g., humic acids, seaweed and plant extracts, protein hydrolysate and silicon) can stimulate plant vigor, growth, and yield, even under sub-optimal growth conditions (Viscardi et al., 2016; Sheridan et al., 2017; Di Stasio et al., 2020; Comite et al., 2021).

Among beneficial microorganisms, plant growth promoting rhizobacteria (PGPR) emerge as key players in agricultural microbial applications, noted for their positive effects on plant growth, by favoring the absorption of nutrients, such as nitrogen and phosphate (Ventorino et al., 2007; Reddy, 2013; Ahemad and Kibret, 2014). These bacteria are commonly represented by genera such as *Bacillus*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Alcaligens*, *Arthobacter*, *Agrobacterium* and *Rhizobium*. The nitrogen-fixer *Azotobacter* is a free-living aerobic rhizobacterium, that can stimulate plant growth through nutrient supplementation or through the production of phytohormones such as auxins, gibberellins, and cytokinins (Viscardi et al., 2016; Wani et al., 2016; Van Oosten et al., 2018), as well as the production of large quantities of exopolysaccharides (Ventorino et al., 2019a; Romano et al., 2020b). Members belonging to this genus are involved in nutrient processes such as nitrogen cycling, phosphate solubilization (Wani et al., 2013), mobilization of iron (Rizvi and Khan, 2018) and the biodegradation of many commonly used chemical pesticides (Gurikar et al., 2016). *Azotobacter chroococcum* is a promising candidate for improvement of plant growth and abiotic stress tolerance (Viscardi et al., 2016; Silletti et al., 2021). Extending the biodiversity of beneficial microorganisms, it has been proven that selected fungal strains of *Trichoderma* also have PGPR-like effects, and establish positive interactions with plants including biological control, plant growth promotion, and induced plant resistance (Harman et al., 2004; Shoresh and Harman, 2008; Raaijmakers et al., 2009; Woo et al., 2023). *Trichoderma* spp. and endophytic fungi have become prominent on the agricultural scene, due to their multiple positive effects and improvement in yield properties of given crops (Harman et al., 2004; Lorito and Woo, 2015; Woo et al., 2023). *Trichoderma* spp. produce over 250 metabolic products, including secondary metabolites, peptides, proteins, and cell-wall-degrading enzymes with biostimulant or protective effects on plants (Woo and Pepe, 2018; Vinale and Sivasithamparam, 2020). Furthermore, plants inoculated with *Trichoderma* have also demonstrated effective mitigation of the negative consequences of drought stress by improving proline concentration in plant tissue and the synthesis of growth hormones (Mona et al., 2017). Considering that diverse microbial based-biostimulants are able to provide stress protection via diverse mechanisms of action (Yakhin et al., 2017), the combination of two or more selected strains can be proposed to enhance their action (Rouphael and Colla, 2018; Gemin et al., 2019).

Previous work has reported the use of microbial consortia containing both rhizobacteria and fungi as a sustainable technique for the maintenance of soil health and the increase of crop productivity (Carneiro et al., 2023). One of the main benefits of their integrated use includes the reduction in the need for water and fertilizer applications, which provides a dual benefit: i) reduced economic production costs through more efficient and resilient farming systems; ii) decreased environmental impact, due to lower contamination with biological products when compared to mineral fertilizers (Carneiro et al., 2023). Innovative microbial consortia can include *Trichoderma* strains in combinations with plant-beneficial microorganisms such as *Azotobacter* (Woo and Pepe, 2018; Woo et al., 2023). Several studies highlighted the versatile and beneficial effects of combined *Azotobacter* and *Trichoderma* inoculation on improving crop performance across diverse environmental and nutrient conditions. This synergistic interaction extends to the formation of *Trichoderma-Azotobacter* biofilm, positively impacting soil nutrient availability and overall plant growth in wheat, cotton, and chickpea (Velmourougane et al., 2017; Velmourougane et al., 2019). Despite their extensive use in agriculture, microbial-based biostimulants have mainly been tested with a focus on improving crop yield and quality aspects (du Jardin, 2015), whereas their potential role on crops exposed to biotic and/or abiotic stress needs to be further investigated. The contribution of microbial-based biostimulants as abiotic stress protectants and growth enhancers is becoming increasingly more important, also in the context of climate change, which is exacerbating the outbreak of pests and diseases (Rosenzweig et al., 2001), as well as crop exposure to extreme temperatures, drought, and soil salinization (Ahuja et al., 2010; Cirillo et al., 2018) which all have a strong negative impact on crop yield and quality.

In this work, we assessed the function of the microbial consortium containing the bacterium *Azotobacter chroococcum* 76A and the fungus *Trichoderma afroharzianum* T22 on tomato (*Solanum lycopersicum* L.) crop subjected to water and nitrogen deficiency. We hypothesized that co-inoculation of the tomato root system with these two microorganisms could facilitate plant tolerance to a combination of water and nutrient stress due to their known complementary modes of action on the host crop. Furthermore, the effects of this microbial consortium on tomato

yield and fruit qualitative, as well as the influence on the surrounding soil microbial community were also assessed. These findings could help to understand the functional link between the main components of this microbial-based biostimulant and the modulation of tomato plant response to the combined abiotic stressors.

2 Materials and methods

2.1 Experimental design and sampling

A field experiment with tomato (*Solanum lycopersicum* L.) was conducted at the experimental farm of the University of Naples Federico II, located at Bellizzi, Italy (lat. 43°31'N, long. 14°58' E; alt. 60 m above sea level) on sub-alkaline soil (pH 7.5), silty-clay-loam (Clay 334 g kg $^{-1}$, Silt 241 g kg $^{-1}$, Sand 425 g kg $^{-1}$) with low nitrogen and soil organic matter (1.2 g kg^{-1} and 18.4 g kg^{-1}, respectively). The meteorological data during the experiment are reported in Figure S1. Field plots for all treatments were moldboard plowed at 30 cm depth, followed by secondary tillage with a soil grubber and harrow for seedbed and transplanting preparation.

Tomato seeds cv. Vulcan F1 (Nunhems®—Bayer, Leverkusen, Germany) were germinated in peat planting trays and grown in the greenhouse until the 3rd–4th true leaf. Plants were transplanted with a plant density of 3.3 plants per m^2 and irrigated, starting with drip lines with emitters of 1.5 L h^{-1} flow, 0.3 m apart. The experiment was arranged in a randomized block design in plots of 50 m^2 with three replicates. Plants were treated with a microbial consortium (T) as below described, and non inoculated plants were used as controls. Nutritional input (I) included two levels of nitrogen (N) fertilization (optimal: 100%; and sub-optimal: 70% of estimated plant N requirements). Nutrients were supplied via fertigation during the whole crop cycle, providing the plant with 104 N, 124 P_2O_5 and 122 K_2O units ha^{-1} for the optimal N treatment, and 73 N, 124 P_2O_5 and 122 K_2O units ha^{-1} in the sub-optimal N plots. The fertilizers used were ammonium nitrate and potassium monophosphate. In order to impose water stress, plants were irrigated with 70% (sub-optimal, moderate stress) of the optimal water supply (100%), as estimated with the FAO-24 Pan method.

2.2 Microbial strains, inoculum preparation and tomato treatments

The microbial biostimulant treatment (A+T) used two different microorganisms: *Azotobacter chroococcum* strain 76A (Viscardi et al., 2016; belonging to the microbial collection of the Department of Agricultural Sciences, University of Naples Federico II, Portici) and *Trichoderma afroharzianum* strain T22 (ex-*Trichoderma harzianum;* Cai and Druzhinina, 2021) isolated from the commercial formulation of Trianum-P (Koppert Biological Systems Rotterdam, the Netherlands) implemented at final concentration of 10^6 spore mL^{-1}. The inoculum preparation of the bacterial strain *A. chroococcum* 76A was performed according to Van Oosten et al. (2018).

Tomato seeds were surface-sterilized and coated with a microbial cell suspension containing *A. chroococcum* 76A (1 × 10^7 CFU mL^{-1}) and *T. afroharzianum* T22 (1 × 10^6 spores mL^{-1}) to uniformly cover the seed surface. Treated seeds were air-dried and hand-seeded in styrofoam planting trays containing a peat-based substrate for germination (Tecno Grow Semina 80, TerComposti SpA, Brescia, IT). At the time of transplant, one-month old tomato seedlings were inoculated with the microbial inoculum by using a root dip method, submerging the planting trays in the microbial liquid suspension for 15 min to completely wet the roots; drained of excess liquid, then the plant-plug was removed and transplanted to pre-bored holes in the soil at the field location. Further, at 15 and 45 DAT, each plant was repeatedly inoculated at the base with 50 mL of microbial suspension containing *A. chroococcum* 76A (1 × 10^7 CFU mL^{-1}) and *T. afroharzianum* T22 (1 × 10^6 spores mL^{-1}) (A+T). Uninoculated plants were treated only with water and served as control.

2.3 Plant growth and yield measurements

Plant growth parameters were evaluated at 45 Days After Transplant (DAT), at the flowering stage. Aboveground and belowground biomass was measured in terms of shoot fresh weight and root length and width. Five plants per treatment were cut at the soil surface, and the above-ground biomass was weighted on a balance for the evaluation of shoot fresh weight (FW). Root length and width were measured as previously described in Li et al. (2020), with minor modifications. Briefly, a soil trench, 70 cm deep and 60 cm wide, was excavated beside the plots to expose the soil profile of three plants per treatment, then maximum root length and width were measured. Yield parameters were evaluated at the end of the experiment (90 DAT; harvest). Tomato fruits were harvested for the determination of the fresh biomass and the number of fruits per plant. Brix degrees were measured with a bench refractometer (ATAGO palette - ATAGO CO., LTD – Japan).

2.4 Physiological parameters

Leaf water potential was measured at 45 DAT using a dewpoint psychrometer (WP4, Decagon Devices, Pullman, WA, USA) on fully expanded leaves. At the same date, leaf temperature was measured by thermometric measurements performed with a thermal IR camera (Seek CompactPRO, Seek Thermal, Inc. 6300 Hollister Ave - Santa Barbara, CA), and soil plant analysis development system (SPAD) with a portable SPAD-502 chlorophyll meter (Konica-Minolta, Tokyo, Japan).

2.5 Enumerations of microorganisms in the tomato rhizosphere

Viable microbial counts were performed at time of flowering (45 DAT) and at harvest (90 DAT), to assess the impact of the treatment with microbial consortia on the cultivable microbial community. Soil rhizosphere samples, 9 replicates for each

treatment, were collected as previously reported (Romano et al., 2020b). Ten grams of rhizosphere composite samples (n=3) were suspended in 90 mL of quarter-strength Ringer's solution (Oxoid, Milan, Italy). After shaking for 30 minutes, a dilution series was prepared in quarter strength Ringer's solution, and aliquots were used to inoculate different solid and liquid media. Total heterotrophic aerobic bacteria were enumerated on Plate Count Agar (PCA; Oxoid, Milan, Italy) plates and incubated for 2 days at 28°C; whereas fungi were counted on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Oxoid) plates and incubated for 7 days at 28°C. To determine target microbial groups based on inoculum characteristics, free-living (N_2)-fixing aerobic bacteria were counted on the Augier medium (Romano et al., 2020a), detecting a brown patina on surface of the liquid medium of positive tube after 15 days of incubation at 28°C; selective count of *Trichoderma* was performed as described by Caruso et al. (2020).

All tests were carried out in triplicate. Microbiological data were expressed as CFU or MPN g^{-1} of soil.

2.6 Molecular analysis of tomato rhizosphere

Total DNA was extracted from composite rhizosphere samples of tomato plants using a Fast DNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions.

The primers V3f and V3r (Muyzer et al., 1993) were used to analyze prokaryotic populations. The primers NL1 (Kurtzman and Robnett, 1998) and LS2 (Cocolin, 2000) were employed for eukaryotic Denaturing Gradient Gel Electrophoresis (DGGE) analysis. A GC clamp was added to forward primers according to Muyzer et al. (1993). The PCR mixture and conditions for both amplifications were performed according to Di Mola et al. (2021). DGGE analyses were performed using a polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37:5:1)] with a denaturing gradient of 30–60% by a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories, Milan, Italy) as previously described (Ventorino et al., 2018). All tests were carried out in triplicate.

2.7 Statistical analysis

Agronomical and microbial counting data were analyzed by one-way ANOVA followed by Duncan's *post hoc* test for pairwise comparison of means (at $P < 0.05$) using SPSS 21.0 statistical software package (SPSS Inc., Cary, NC, United States).

DGGE bands were automatically detect by Phoretix 1 advanced version 3.01 software (Phoretix International Limited, Newcastle upon Tyne, England). After the matching bands confrontation, a cluster analysis was performed as previously indicated by Ventorino et al. (2013). The correlation matrix of the band patterns was performed by using the method described by Saitou and Nei (1987). Finally, the percentage of similarity (S) of the microbial community was estimated by analyzing the resulting matrix using the average linkage method in the cluster procedure of Systat 5.2.1. According to Dong and Reddy, the structural diversity of the microbial community was examined by the Shannon index of general diversity H (Shannon and Weaver, 1963).

H was calculated on the basis of peak height from the different bacterial groups (16S rDNA bands) in the densitometric curve as indicated in the equation for the Shannon index:

$$H = -\sum(n_i/N)\log(n_i/N)$$

where n_i is the height of the peak and N the sum of all peak heights of the densitometric curve. The analysis of band intensity was performed with GelAnalyzer 23.1.

3 Results

3.1 Plant growth and agronomic performance

Evaluations of the overall yield indicated that both the Input (I; water and nitrogen fertilizer) and the Treatment (T; *Azotobacter* and *Trichoderma*) factors had a significant impact, with relevant differences (Table 1; Figure 1). Under optimal water and nutritional input, the microbial inoculum of *Azotobacter* and *Trichoderma* (A+T) increased by 48.6% and 50% tomato yield and number of fruits per plants, respectively (Figure 1). In contrast, conditions in water and nutrient shortage reduced the differences for both parameters. The decrease in the water and nutrient factors reduced plant above-ground and below-ground growth by about 10% (Table 2). Root treatments with the combined inoculum of *Azotobacter* and *Trichoderma* (A+T) enhanced the shoot fresh weight by about 15%, but resulted in a 16% and 20% decrease in the root length and width, respectively (Table 2). No effects of the

TABLE 1 Productivity parameters of tomato plants grown under optimal and sub-optimal input (I) and with or without the combined inoculum of *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 (treatment A+T).

	Yield	Number of fruits
	g plant^{-1}	#
Input (I)		
Optimal	3580 a	56.0 a
Sub-optimal	1940 b	34.5 b
Treatment (T)		
Control	2410 b	40.7
A+T	3110 a	49.8
Interaction		
I	***	**
T	*	ns
IxT	*	*

Asterisks indicate significant differences according to ANOVA (ns, not significant; * = 0.05; ** = p<0.01; *** = p<0.001). Different letters after values indicate significant differences according to Duncan's post-hoc test.

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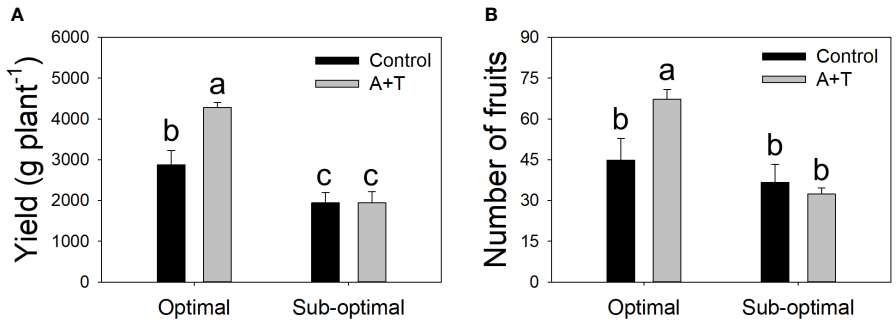

FIGURE 1
Yield **(A)** and number of fruits **(B)** of tomato plants grown under optimal and sub-optimal input (I), with or without (Control) the combined inoculum of *Azotobacter chroococcum* and *Trichoderma afroharzianum* (treatment A+T). Different letters indicate significant differences according to Duncan's *post-hoc* test.

input regime (water and nitrogen) were observed on this parameter. In contrast, a significant interaction "Input" x "Treatment" (IxT) was found in terms of aboveground plant biomass as indicated by shoot fresh weight (FW). Under optimal water/nitrogen conditions, the aboveground plant biomass of A+T treated plants was similar to untreated control plants (Figure 2). However, under sub-optimal water/nitrogen conditions, A+T plants had a 60% greater shoot biomass compared to untreated control plants (Figure 2).

In terms of fruit quality, as determined by the measurement of Brix degree of sugar content, the low input regime increased by 28% the Brix score (3.46 under optimal vs. 4.08 under sub-optimal conditions), whereas no effect of the microbial treatment variable was detected (Table S1).

TABLE 2 Growth parameters of tomato plants grown under optimal and sub-optimal input (I), with or without (Control) the combined inoculum of *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 (treatment A+T).

	Shoot FW	Root maximum length	Root maximum width
	g	cm	cm
Input (I)			
Optimal	466.5	36.65	32.00
Sub-optimal	423.2	35.25	33.15
Treatment (T)			
Control	410.5	39.90 a	37.75 a
A+T	479.2	33.55 b	30.05 b
Interaction			
I	ns	ns	ns
T	ns	***	**
IxT	*	ns	ns

Asterisks indicate significant differences according to ANOVA (ns, not significant; * = 0.05; ** = p<0.01; *** = p<0.001). Different letters after values indicate significant differences according to Duncan's post-hoc test.

3.2 Physiological measurements

The results obtained from the physiological measurements of the plant water status were consistent with the trends observed in the evaluation of the plant growth parameters, whereby, the leaf water potential was similar in A+T treated *vs* control plants under optimal input (water and nitrogen). In contrast, under reduced input (water and nitrogen deficit) the leaf water potential was slightly, however significantly, higher in A+T inoculated plants compared to untreated plants (+9.5%; Figure 3A). These results were consistent with the leaf temperature of A+T inoculated plants such that under water and nitrogen deficit there was a significantly lower value measured compared to untreated plants (-4.6%; Figure 3B), and this corresponded to the aboveground biomass production, which was 40% higher in A+T inoculated plants compared to untreated control plants (Figure 2). In respect to the SPAD values, representative of the leaf chlorophyll content, the low input treatment (I) decreased the value by 10.7% compared to optimal cultural conditions, whereas the microbial treatment increased the SPAD value by 4.9% compared to untreated control. No interaction between I and T factors was found (Table 3).

3.3 Enumerations of microorganisms in the tomato rhizosphere

Significant differences in the total heterotrophic aerobic bacteria were found between inoculated and non-inoculated plots at flowering and harvesting phase (Table 4). In optimal conditions, the microbial concentration was lower in inoculated plots compared to non-inoculated plots at time of flowering, However, in sub-optimal conditions, a significant increase (ca. 1 Log) in these populations was noted. In this case, the microbial population in the rhizosphere of inoculated plants (7.73±0.04 Log CFU g^{-1}) was greater than the control (6.70±0.22 Log CFU g^{-1}). Suggesting that the applied microbial inoculum may exert a positive effect on soil microflora especially under stress conditions. By contrast, at the end of experiment, total heterotrophic aerobic bacteria in the treated

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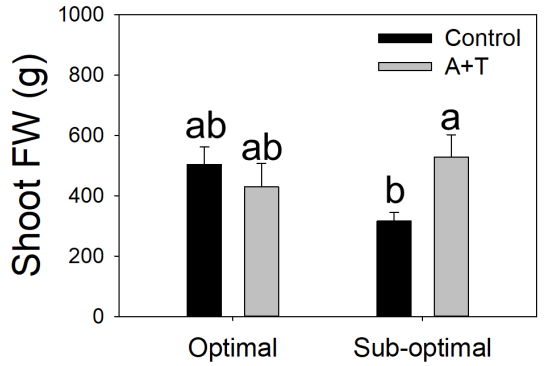

FIGURE 2
Shoot fresh weight of tomato plants grown under optimal and sub-optimal input (I), with or without (Control) the combined inoculum of *Azotobacter chroococcum* and *Trichoderma afroharzianum* (treatment A+T). Different letters indicate significant differences according to Duncan's *post-hoc* test.

plots were significantly higher than in the non-inoculated control, whereas no significant differences were found in sub-optimal plots (Table 4).

Similarly, the microbial inoculum also affected the fungal community. In fact, a significant increase in fungal counts was detected in the rhizosphere of inoculated plants (in the range of 4.58±0.08 - 6.40±0.08 Log CFU g^{-1}), in respect to the non-inoculated control (in the range of 3.74±0.04 - 4.82±0.01 Log CFU g^{-1}) in all conditions except for the condition in the sub-optimal environment at harvesting stage (Table 4).

Finally, at flowering, a significant increase, almost 1 Log CFU g^{-1}, in the free-living (N_2)-fixing bacteria was revealed in the rhizosphere of treated plants compared to non-inoculated plants subject to stress conditions (Table 4). At the end of experiment, although a drastic reduction was observed in all samples, N_2-fixers were always significantly higher in the inoculated plants cultivated under sub-optimal conditions in respect to the non-inoculated samples (Table 4). The CFU of *Trichoderma* showed a positive trend (> 1 Log CFU g^{-1}) in the rhizosphere of plants treated with microbial consortium compared to indigenous *Trichoderma* spp. of untreated plants. Moreover, at harvest a consistent decrease in indigenous *Trichoderma* spp. count was recorded from untreated plants, while a significant increase of *Trichoderma* abundance was observed in treated plants in sub-optimal conditions (Table 4).

3.4 Molecular characterization of soil microbes in tomato rhizosphere under optimal or stress conditions

PCR-DGGE was employed to obtain a qualitative fingerprint of the bacterial and fungal communities in the tomato rhizosphere receiving to the combined application effects of abiotic stress (water and nitrogen) and microbial inoculation. The main results indicated that the sampling time was the major determinant of the composition and structure of the bacteria and fungi because it, more than the cultivation conditions and inoculum, determined the clustering into groups (Figures 4, 5).

The DGGE profiles of the bacterial populations in the tomato rhizosphere were complex, producing 20-22 and 17-20 bands in inoculated samples and non-inoculated controls, respectively. Patterns indicated that microbial inoculum affected the richness of bacterial populations since the number of DGGE bands was significantly higher in the inoculated (A+T) than non-inoculated (C) plants (Table S2). Furthermore, the interaction between the sampling time and inoculum played a key role in affecting the bacterial biodiversity demonstrating that the rhizosphere of tomato plants co-inoculated with *A. chroococcum* 76A and *T. afroharzianum* T22 showed a number of bands higher than non-inoculated plants (Table S2).

Differences in the samples due to the position and intensity of the bands were evaluated by statistical analysis. It was apparent that sampling time was the main driver in determining the prokaryotic diversity. Cluster analysis (Figure 4) identified four major groups associated to the sampling time and cultivation conditions (cluster 1: samples cultivated in optimal conditions and collected at flowering; cluster 2: samples cultivated in sub-optimal conditions

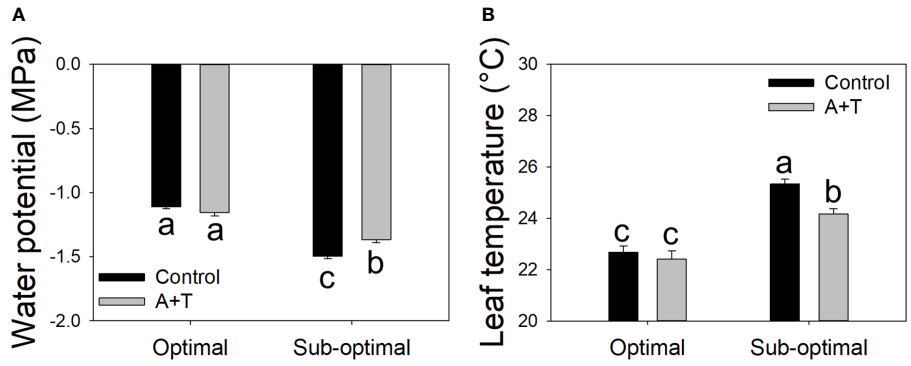

FIGURE 3
Leaf water potential **(A)** and leaf temperature **(B)** measurements of tomato plants grown under optimal and sub-optimal input (I), with or without the combined inoculum of *Azotobacter chroococcum* and *Trichoderma afroharzianum* (treatment A+T). Different letters indicate significant differences according to Duncan's *post-hoc* test.

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TABLE 3 Physiological parameters of tomato plants grown under optimal and sub-optimal input (I) and with or without the combined inoculum of *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 (treatment A+T).

	Leaf water potential	SPAD	Leaf temperature
	MPa		°C
Input (I)			
Optimal	-1.14 a	57.8 a	22.5 b
Sub-optimal	-1.47 b	51.6 b	24.7 a
Treatment (T)			
Control	-1.32	53.4 b	24.0 a
A+T	-1.29	56.0 a	23.3 b
Interaction			
I	***	***	***
T	ns	*	**
IxT	***	ns	*

Asterisks indicate significant differences according to ANOVA (ns, not significant; * = 0.05; ** = p<0.01; *** = p<0.001). Different letters after values indicate significant differences according to Duncan's post-hoc test.

and collected at flowering; cluster 3: samples cultivated in sub-optimal conditions and collected at harvesting; cluster 4: samples cultivated in optimal conditions and collected at harvesting). Clusters 2 and 3, comprising the rhizosphere samples obtained from tomato plants cultivated in stress conditions, were very similar, demonstrating a similarity of 70%; while cluster 1 had a similarity as low as 57% with the assembly of these two groups and cluster 4 was only 45% similar to these groups (Figure 4). However, within each of the major clusters, the sub-groupings of the bacterial populations were always similar and determined by the microbial inoculum applications with a high similarity level that ranged from 76% to 85% (Figure 4). The Shannon-Weaver index of bacterial populations was significantly affected by input (I), microbial treatment (T), and sampling time (ST), as well as by the interaction of the input for the phenological stage (IxST; Table 5). Specifically, this index was higher in sub-optimal conditions, in the presence of the A+T inoculum, and during the flowering stage. These findings were also observed in the interaction between input and phenological stage, with a higher value in sub-optimal conditions during the flowering stage (Table 5).

DGGE of the fungal populations showed a low complex profile producing a number of bands ranging from 11 to 15. However, fungal diversity was affected by several parameters. The number of bands of fungal populations was significantly affected by microbial

TABLE 4 Enumerations (log CFU or MPN g^{-1}) of total heterotrophic aerobic bacteria, molds, free-living (N_2)-fixing aerobic bacteria and *Trichoderma* in the rhizosphere of tomato plants grown under optimal and sub-optimal input, without microbes (Control) or with the *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 inoculum (treatment A+T) collected at phenological stages at time of flowering or harvest.

	Treatment	Sampling	Input	
			Optimal	Sub-optimal
Total heterotrophic aerobic bacteria	Control	Flowering	7.72 ± 0.02 a	6.70 ± 0.22 de
		Harvest	6.38 ± 0.04 g	6.88 ± 0.02 cd
	A+T	Flowering	7.07 ± 0.04 bc	7.73 ± 0.04 a
		Harvest	6.59 ± 0.01 e	6.87 ± 0.34 cd
Moulds	Control	Flowering	4.82 ± 0.01 c	4.79 ± 0.01c
		Harvest	3.74 ± 0.04 e	4.58 ± 0.08 d
	A+T	Flowering	4.93 ± 0.01 b	6.40 ± 0.08 a
		Harvest	4.73 ± 0.00 c	4.58 ± 0.08 d
N_2-fixers	Control	Flowering	2.65 ± 0.00 c	2.98 ± 0.00 bc
		Harvest	1.98 ± 0.00 e	1.98 ± 0.00 e
	A+T	Flowering	3.15 ± 0.12 b	3.82 ± 0.16 a
		Harvest	2.32 ± 0.09 d	2.32 ± 0.14 d
Trichoderma	Control	Flowering	2.46 ± 0.15 cd	2.59 ± 0.11 c
		Harvest	2.25 ± 0.24 de	2.20 ± 0.17 e
	A+T	Flowering	3.57 ± 0.01 b	3.74 ± 0.02 b
		Harvest	3.71 ± 0.01 b	4.01 ± 0.01 a

The measurement of the various microorganisms was determined by growth on diverse selective solid substrates indicated in the methods. Different letters after values indicate significant differences ($p < 0.05$) according to Duncan's post-hoc test.

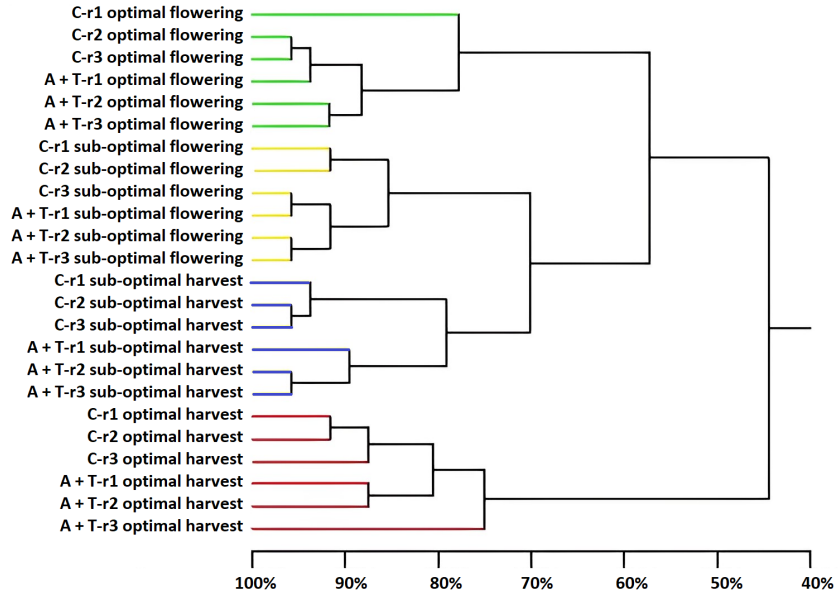

FIGURE 4
Dendrogram showing the degree of similarity (%) of the PCR-DGGE profiles of bacterial populations in the rhizosphere of tomato plants grown under optimal and sub-optimal input (I), without (C) or with the combined inoculum of *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 (treatment A+T) collected at flowering or harvest stage, from three different replicates r1, r2 and r3. Different colors indicate different major clusters.

inoculum applications since the number of DGGE bands was higher in the inoculated than non-inoculated plants (Table S2). The number of fungal bands was significantly higher under optimal than sub-optimal growth conditions. Finally, the sampling time also affected fungal biodiversity showing higher values at harvesting than flowering (Table S2).

As shown in the Figure 5, statistical analysis on the position and intensity of the bands allowed the classification of two major

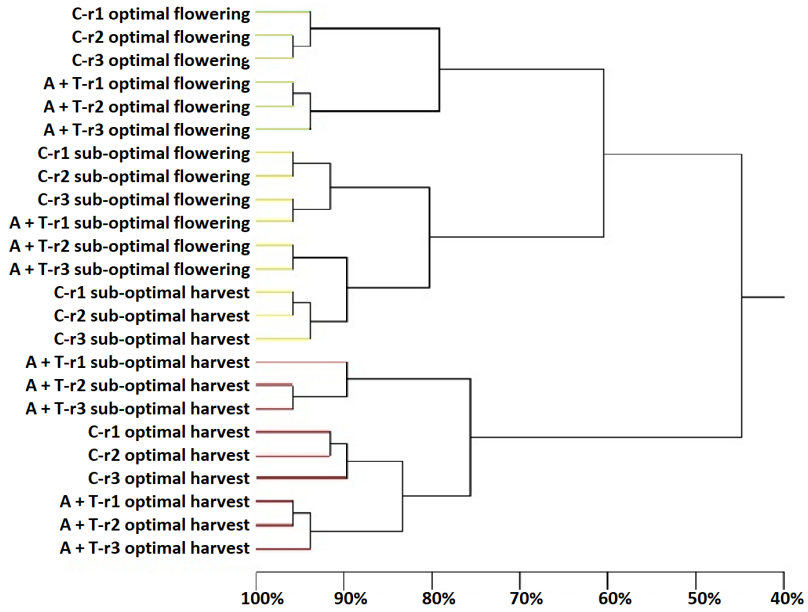

FIGURE 5
Dendrogram showing the degree of similarity (%) of the PCR-DGGE profiles of fungal populations in the tomato rhizosphere samples in the rhizosphere of tomato plants grown under optimal and sub-optimal input (I), without (C) or with the combined inoculum of *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 (treatment A+T) collected at flowering or harvest stage, from three different replicates r1, r2 and r3. Different colors indicate different major clusters.

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TABLE 5 Means and standard deviations of the Shannon diversity index (H) based on DGGE bands intensity of bacterial and fungal populations in the rhizosphere of tomato plants grown under optimal and sub-optimal inputs (I), without microbes (C) or treated with the microbial inoculum (T) of *Azotobacter chroococcum* 76A and *Trichoderma afroharzianum* T22 (A+T) collected at sampling times (ST) of flowering or at harvest.

	Source of Variance	Shannon Index Bacteria	Shannon Index Fungi
Input (I)	Optimal	0.91 ± 0.08[b]	0.78 ± 0.19
	Sub-optimal	1.05 ± 0.18[a]	0.61 ± 0.29
		***	ns
Treatment (T)	C	0.94 ± 0.17[b]	0.71 ± 0.28
	A+T	1.02 ± 0.13[a]	0.69 ± 0.24
		*	ns
Sampling Time (ST)	Flowering	1.05 ± 0.17[a]	0.73 ± 0.27
	Harvest	0.90 ± 0.09[b]	0.67 ± 0.25
		***	ns
IxC	Optimal x C	0.87 ± 0.09	0.86 ± 0.13
	Optimal x A+T	0.94 ± 0.04	0.70 ± 0.22
	Sub-optimal x C	1.00 ± 0.21	0.53 ± 0.32
	Sub-optimal x A+T	1.09 ± 0.15	0.68 ± 0.27
		ns	
TxST	C x flowering	1.03 ± 0.19	0.63 ± 0.31[ab]
	C x harvest	0.85 ± 0.09	0.81 ± 0.22[a]
	A+T x flowering	1.07 ± 0.17	0.84 ± 0.17[a]
	A+T x harvest	0.96 ± 0.03	0.55 ± 0.21[b]
		ns	*
IxST	Optimal x flowering	0.89 ± 0.08[b]	0.77 ± 0.16
	Optimal x harvest	0.92 ± 0.08[b]	0.79 ± 0.23
	Sub-optimal x flowering	1.20 ± 0.04[a]	0.69 ± 0.35
	Sub-optimal x harvest	0.89 ± 0.10[b]	0.52 ± 0.19
		***	ns
IxTxST	Optimal x C x flowering	0.87 ± 0.11	0.77 ± 0.10
	Optimal x C x harvest	0.97 ± 0.05	0.94 ± 0.12
	Optimal x A+T x flowering	0.92 ± 0.03	0.77 ± 0.24
	Optimal x A+T x harvest	0.97 ± 0.05	0.63 ± 0.23
	Sub-optimal x C x flowering	1.18 ± 0.03	0.48 ± 0.41
	Sub-optimal x C x harvest	0.82 ± 0.10	0.62 ± 0.21
	Sub-optimal x A+T x flowering	1.22 ± 0.03	0.90 ± 0.08
	Sub-optimal x A+T x harvest	0.96 ± 0.02	0.46 ± 0.18
		ns	ns

(Continued)

TABLE 5 Continued

24/11/2023 4:20:45 pmAsterisks indicate significant differences according to ANOVA (ns, not significant; * = 0.05; ** = p<0.01; *** = p<0.001). Different letters within each column indicate significant differences according to Duncan's post-hoc test.

clusters clearly associated to the two sampling times with a similarity level of 46% (cluster 1: all the samples collected at flowering stage and non-inoculated plants cultivated in sub-optimal conditions and collected at harvesting; cluster 2: samples inoculated with microbial strains and collected at harvesting and non-inoculated control samples cultivated in optimal conditions and collected at harvesting). It was interesting to note that within each of the major clusters delineated by the sampling time, the sub-groupings of the eukaryotes were similar and firstly determined by the stress conditions and then by microbial inoculum applications. In fact, three sub-clusters, with a high similarity level ranging from 76% to 81%, were delineated by cultivation conditions (optimal or suboptimal). Moreover, within these groups' other sub-groupings of the fungi were always determined by the microbial inoculum (similarity level ranging from 90% to 95%; Figure 5). Nevertheless, the Shannon-Weaver index of fungal populations was influenced by the interaction between microbial inoculum and sampling time (Table 5); indeed, Shannon index values were higher with microbial inoculum at flowering.

4 Discussion

4.1 Simultaneous application of *Trichoderma* and *Azotobacter* enhances yield in tomato and alleviates combined water-nitrogen stress

Plant biostimulants, including microorganisms such as fungi and PGPR, have been increasingly used to help crops to tolerate and/or adapt to environmental stress (Li et al., 2022; Gul et al., 2023). Microorganisms of diverse origin have been proven to protect plants from water deficit (Silletti et al., 2021), temperature extremes (Shaffique et al., 2022), salinity (Van Oosten et al., 2018), pathogens (Khan et al., 2020) and other biotic factors (Woo et al., 2023). However, most published literature refers to plant protection upon exposure to single stress, whereas how microorganisms and/or biostimulants in general could facilitate plant adaptation to multiple abiotic stresses has rarely been addressed. Coexistence of multiple stresses is a much more frequent occurrence both in nature and agricultural systems (Kissoudis et al., 2014) and it may require

the need of more complex formulations of biostimulants and beneficial microorganisms, able to simultaneously potentiate different physiological responses of the plant to specific stresses (Parađiković et al., 2019) or activate multiple resistance mechanisms (Woo et al., 2023). In our previous work (Silletti et al., 2021), it was demonstrated that although biostimulants may be capable of enhancing growth and stress tolerance, the soil nutrient availability and environmental conditions may heavily influence these responses. Furthermore, it was also shown that *T. afroharzianum* strain T22 acted mostly as a growth enhancer under optimal irrigation and moderate drought stress (50% replenishment of plant water requirements), whereas *A. chroococcum* strain 76A improved plant water relations under stronger stress conditions (25% replenishment of plant water requirements). Based on these results, it was hypothesized that together *T. afroharzianum* strain T22 and *A. chroococcum* strain 76A could reinforce the protective action not only to single abiotic factors, but also to diverse combinations of multiple stresses (water shortage and sub-optimal N availability) since these two strains were likely acting via different plant-microbe interaction mechanisms (Woo and Pepe, 2018). Such microbial consortium could offer a strategy to respond to the urgent challenges posed by sustainable agriculture and global food demand (van Dijk et al., 2021). A reduced water and nitrogen availability resulted in a 50% yield reduction in the untreated control plants, confirming that these plants were operating in sub-optimal water-nitrogen regime. The A+T treatment increased yield by 48.6% under optimal conditions, however the mixture was not able to compensate the effects of water and nitrogen shortage (Figure 1A). Treatments with *Trichoderma* spp. and *A. chroococcum* have been proven to have variable effects from other general growth enhancers (Di Mola et al., 2023), and to be more protectant to specific stress (Woo et al., 2023). This may also be a consequence of multiple, variable and complex interactions that plants establish with the surrounding environment, and not only the microbial component (Del Buono, 2021; Silletti et al., 2021). The activation of various biosynthesis functions in the plant have been attributed to *Trichoderma* interactions with the host, such as the activation of the antioxidant machinery (Mastouri et al., 2012), the regulation of phytohormones (Illescas et al., 2021), and the solubilization of phosphate and micronutrients (Li et al., 2015). Plant growth promotion effects by *Trichoderma* spp. have been noted in the increased root biomass in some crops (Macías-Rodríguez et al., 2018; Sehim et al., 2023). However, our results indicated that in combination with A+T there was a reduction in the root length and width (Table 2).

Similarly, Prajapati et al. (2008) have noted that a bacterial treatment with *A. chroococcum* in rice caused a significant decrease in root dry weight as compared to control plants. This limitation in root architecture may serve to assist the plant in tolerating the environmental stress conditions in the soil, that results in the redistribution of necessary resources to other vegetative structures. Most interestingly in our present work, the reduced root expansion due to the A+T treatment did not affect yield under sub-optimal conditions, and it was actually positively correlated to an improved yield under optimal conditions (Figure 1). Similarly, the higher SPAD values in A+T treated plants compared to control plants (Table 3) may indicate an improved nutritional status A+T plants, since SPAD values are correlated with the nitrogen status of the plant (Ghosh et al., 2023). This suggests that the microorganisms may increase nutrient availability in the rhizosphere, for example acting as siderophores or biodegraders, working in the conversion of iron, zinc or phosphorus elements into forms utilizable by the plant (Woo and Pepe, 2018; Woo et al., 2023). A+T treatments are known to improve plant tolerance to abiotic stress (Silletti et al., 2021), possibly by increasing plant root efficiency in terms of water and nitrogen uptake and/or enhancing the absorption and assimilation of water and nitrogen in the root zone. Although the physiological basis of these effects is unclear, it is at least consistent with the higher carbon allocation to root expansion in response to nutrient and water shortage (Bicharanloo et al., 2023; Wang L. et al., 2023), which is not sensed in A+T treated plants (Table 2). Moreover, an improved leaf water potential of treated plants under sub-optimal growth conditions was observed (Figure 3A) that corresponded to lower leaf temperatures (Figure 3B), and higher shoot fresh weight (Figure 2). This may indicate that the microbial consortium can act as positive regulator of plant water relations, perhaps by cooling the temperatures in the leaf reduces the physiological processes that limit transpiration and the rate of water loss by the plant particularly under limited water and nitrogen availability. Although this response was not sufficient to ameliorate plant yield under sub-optimal conditions, the positive A+T effect was clear under optimal conditions in terms of yield and fruit number (Figure 1). This was likely associated to a reallocation of plant biomass from roots to reproductive organs (Eziz et al., 2017) that may have been triggered by the A+T treatment. The higher SPAD of A+T treated plants under both input levels also confirmed an improved nutritional status of these plants. Overall, the effects of the microbial treatment appeared to have altered the physiological mechanisms that mediate tomato yield and stress adaptation in a fashion that deserves further investigation.

4.2 Rhizosphere microbial diversity is improved by *Trichoderma afroharzianum* T22 and *Azotobacter chroococcum* 76A co-inoculation in agricultural soils

Due to the close interactions with the surroundings and the high surface area to volume ratio, soil microbiota could be particularly sensitive to environmental stresses and soil perturbations compared to higher organisms (Karimi et al., 2017; Gugliucci et al., 2023). By using cultural methods, it was possible to monitor the significant impact of the inoculation with the *T. afroharzianum* T22 and *A. chroococcum* 76A consortium on the indigenous soil microbiota in the rhizosphere of tomato plants, that included heterotrophic aerobic bacteria populations, free-living (N_2)-fixing bacteria, fungi including *Trichoderma* spp. A notable increase in the microbial populations was observed in the combined stress conditions, indicating the potential of microbial inoculants to enhance the native soil microbiota abundance, possibly the beneficial microorganisms. In line with this observation, it was noted that the Shannon diversity index exhibited higher values in

the rhizosphere inoculated with the microbial consortium compared to the control, especially within bacterial populations. Whereas, for the fungal community, the effect depended on the interaction between microbial inoculum and sampling time. Similar to findings in previous research, this work has demonstrated that soil inoculation with selected microorganisms or microbial consortia can induce significant alterations in both bacterial and fungal communities (Fiorentino et al., 2018; Ventorino et al., 2018; Chouyia et al., 2020). Furthermore, the application of a *T. afroharzianum* T22 and *A. chroococcum* 76A consortium to wheat plants cultivated under stress conditions has shown a remarkable capacity to positively influence and improve the microbial community effects on the agronomic characteristics of the crop (Silletti et al., 2021). This evidence indicates a great application potential for using a microbial consortium on various crops in order to enhance microbial concentrations within the rhizosphere that also includes augmenting the presence and activities of the native beneficial microorganisms. In fact, inoculated microorganisms may synergistically collaborate within the rhizosphere, forming complex networks of interactions that affect microbial community composition and structure, resulting in beneficial outcomes for plant growth and development (Santoyo et al., 2021). Unlike single microbial inoculants, microbial consortia offer additional benefits through their wide range of functions (Ju et al., 2019) which could enhance the strength and productivity of the whole microbiota (Santoyo et al., 2021). Thus, the application of microbial consortia plays a key role in shaping and enhancing microbial communities within agricultural ecosystems, which in turn, have a significant impact on the fertility of agricultural soils and influence ecosystem function and productivity (Ventorino et al., 2019b). By harnessing the collective capabilities of multidisciplinary interacting microorganisms, these consortia promote sustainable agriculture by bolstering plant growth, reducing the dependency on agrochemicals, and preserving the health and equilibrium of the soil microbiota (Woo and Pepe, 2018; Santoyo et al., 2021; Woo et al., 2023).

Our results also highlighted the impact of sampling time as an important factor determining the composition and structure of either bacterial or fungal communities in the tomato rhizosphere. Several studies have demonstrated that the phenological stages of plant development have a great influence on microbial communities in plant-soil compartment niches (Xiong et al., 2021; Ajilogba et al., 2022). DGGE analysis revealed that both bacterial and fungal populations in the tomato plant rhizosphere exhibited differences primarily attributed to the diverse sampling times at flowering or harvest, followed by the effects of the water and nitrogen inputs, and finally the influence by the microbial inoculum application. This suggests that the impact of microbial consortium is modulated by the existing stressors in the cultivation environment, highlighting the need to understand the relationship between stress factors and microbial communities in agricultural soils. The shifts of climatic factors, such as temperature and precipitation, during seasons are often the strongest factors influencing microbial composition and dynamics (Cruz-Martínez et al., 2009; Xue et al., 2011). In open field trials, both biotic and abiotic factors, such as the presence of microbial antagonists (e.g., protists or nematodes) and the availability of a carbon source, could influence the soil microbial community composition (Fierer, 2017). On the other hand, a temporal shift in rhizosphere microbial community may be attributed to the plant interactions with specific microorganisms at a given moment, and these interactions will vary as the plant grows, be influenced by compounds released by the host such as root exudates that shape the surrounding microbiome (Santoyo et al., 2021).

However, in the tomato rhizosphere, microbial diversity was also related to nitrogen and water inputs. Nitrogen treatments have been shown to exert distinct plant-mediated effects, leading to changes in the microbial communities living in the rhizosphere (Ramirez et al., 2010; Liu et al., 2021). Different nitrogen levels have proven to have a significant impact on the distribution and composition of bacterial communities in plant monocultures such as lettuce and rocket (Li et al., 2016; Fiorentino et al., 2018). Furthermore, N fertilization may directly or indirectly alter the soil microbiome by decreasing bacterial diversity and shifting toward a more active and copiotrophic microbial community (Li et al., 2021). Wang X. et al. (2023) revealed significant alterations in the soil microbiota structure due to N fertilization likely due to the microbial adaptation to N-excess although without significant effect on microbial richness and beta-diversity. Furthermore, application of N fertilizers can stimulate the production of plant root exudates that can enhance nutrient utilization by microbiota, as previously suggested by Sørensen (1997). However, the response of agroecosystem microbiota to N fertilization can change, leading to unpredictable outcomes for nitrogen-fixing activity in the rhizosphere, as emphasized by Saraf et al. (2011).

Water input can also modify both the composition and activity of soil microbial communities, since changes in soil water content could affect the availability of soil nutrients (Li et al., 2021). Yuan et al. (2016) observed that irrigation practices had a stronger effect on the abundance, diversity, and structure of bacterial communities than fertilization, confirming the driving effect of soil moisture on shift of bacterial communities. Recently, Xu et al. (2020) observed that microbial community composition was affected by changes in water availability, showing that drought generally led to a decline in microbial biomass, while enhanced irrigation resulted in an increase, which might further translate into changes in microbial community composition (Romano et al., 2023).

5 Conclusions

Although the use of microbial-based biostimulants to aid crops in overcoming and/or adapting to single environmental stresses have been widely studied in recent years, little is known about how microbial consortia could facilitate plant tolerance to multiple stresses, a situation that is much more frequently encountered in both natural and agricultural systems. The interactions among microorganisms and between microorganisms and plants in the soil environment are complex due to various factors that determine the colonization and proliferation of these components, including overlapping needs and competition effects, the variability of field conditions, and/or other environmental stressors that may affect a

functional agroecosystem equilibrium. The development of efficient and stable multipurpose microbial consortia requires holistic investigations that address such complexity under variable field conditions, including the co-existence of multiple stresses to which crops are generally exposed. This work advances our knowledge on a new *Azotobacter* and *Trichoderma*-based inoculum, its effects on the native microbial communities and on tomato plant responses to combined water and nitrogen deficiency. The overall results demonstrate this specific consortium had significant growth and yield enhancing properties on tomato and suggest that, in low-input cropping systems, it may help to cope with environmental constraints and limited chemical fertilization.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Author contributions

VC: Formal analysis, Writing – original draft. IR: Formal analysis, Investigation, Writing – original draft. SW: Writing – review & editing, Investigation. ED: Investigation, Writing – review & editing. NL: Formal analysis, Writing – review & editing. EC: Formal analysis, Writing – review & editing. OP: Writing – review & editing, Investigation. VV: Conceptualization, Supervision, Writing – review & editing, Investigation. AM: Writing – review & editing, Investigation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Plant biostimulants as natural alternatives to synthetic auxins in strawberry production: physiological and metabolic insights

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The demand for high-quality strawberries continues to grow, emphasizing the need for innovative agricultural practices to enhance both yield and fruit quality. In this context, the utilization of natural products, such as biostimulants, has emerged as a promising avenue for improving strawberry production while aligning with sustainable and eco-friendly agricultural approaches. This study explores the influence of a bacterial filtrate (BF), a vegetal-derived protein hydrolysate (PH), and a standard synthetic auxin (SA) on strawberry, investigating their effects on yield, fruit quality, mineral composition and metabolomics of leaves and fruits. Agronomic trial revealed that SA and BF significantly enhanced early fruit yield due to their positive influence on flowering and fruit set, while PH treatment favored a gradual and prolonged fruit set, associated with an increased shoot biomass and sustained production. Fruit quality analysis showed that PH-treated fruits exhibited an increase of firmness and soluble solids content, whereas SA-treated fruits displayed lower firmness and soluble solids content. The ionomic analysis of leaves and fruits indicated that all treatments provided sufficient nutrients, with heavy metals within regulatory limits. Metabolomics indicated that PH stimulated primary metabolites, while SA and BF directly affected flavonoid and anthocyanin biosynthesis, and PH increased fruit quality through enhanced production of beneficial metabolites. This research offers valuable insights for optimizing strawberry production and fruit quality by harnessing the potential of natural biostimulants as viable alternative to synthetic compounds.

KEYWORDS

***Fragaria*, bacterial filtrate, protein hydrolysate, naphthaleneacetamide, naphthaleneacetic acid, fruit yield, fruit quality, metabolomics**

1 Introduction

One of the biggest issues facing the agriculture sector is feeding an expanding global population while minimizing its environmental impact and protecting natural resources for future generations (Devaux et al., 2021). In this context, the Food and Agriculture Organization of the United Nations (FAO) has set a vision about sustainable agriculture which is based on preservation of the natural resources and a technical transformation that is focused on ensuring the fulfilment of continual human requirements for both current and future generations (FAO, 2019). In addition, the FAO has set a strategic objective for sustainable intensification of crop production and focuses on switching to alternative intensification methods, depending on biodiversity management and natural biological processes to boost agroecosystem output while dealing with issues related to climate change and having a good influence on the environment (FAO, 2019). Synthetic hormones (e.g., napthaleneacetic acid 6-benzyladenine (NAA), gibberellins, cytokinins, abscisic acid (ABA), ethylene, brassinosteroids, and jasmonates), the so-called plant growth regulators (PGRs), are largely used in horticulture to enhance the production of vegetables and fruits (Dias, 2019). Moreover, the control mechanisms of phytohormones in fruit set have drawn a lot of attention in recent years because these technologies have been used in horticulture and agriculture to produce seedless fruits and boost crop productivity and quality. These advancements prove beneficial not only in optimal growing conditions but also in challenging and unfavorable environments (e.g., short growing seasons, non-suitable environment for fertilization, low soil fertility, and diseases) (Taglienti et al., 2011; Cho et al., 2013; Farman et al., 2019; Sharif et al., 2022). For instance, several studies elucidated the effect of synthetic auxin (NAA) and gibberellins (GAs) application on enhancing fruit set and growth (McAtee et al., 2013; Kumar et al., 2014) and on enhancing fruit size and quality in many fruits such as plum (Stern et al., 2006), apple (Devoghalaere et al., 2012), apricot (Stern et al., 2007), loquat (Amorós et al., 2004; Forlani et al., 2010), tomato (Zhang et al., 2021), strawberry (Thakur et al., 2017). On the other hand, several studies have shown that the residues of PGRs applied in agricultural crops, have been linked to genotoxicity, hepatotoxicity, and renal toxicity, all of which pose substantial risks to human health (Lin and Tan, 2011; Maslowski and Mackay, 2011; Magnone et al., 2015; Reihill et al., 2015). Being the PGRs registered as plant protection products, where rates, application methods, and safety intervals for each use between application and harvest have been defined and approved by international and national authorities to control PGR residues in crops as well as to maintain the food safety (Li et al., 2015).

Naphthaleneacetic acid is a synthetic form of auxin that improves fruit yield and quality. Studies have shown that NAA affects floral sex ratio and helps with root initiation, apical dominance, fruit setting ratio, fruit falling prevention, vascular tissue differentiation (Mehraj et al., 2015), increased fruit size (Yadav et al., 2017), induced early flowering, augmented flower production and reduced flower and fruit drop (Yamgar and Desai, 1987). In terms of fruit quality, several authors stated that by treating NAA to strawberry fruit, growers can increase anthocyanin accumulation (Yadav et al., 2017), total sugars, ascorbic acid content, and titratable acidity (Kumar and Tripathi, 2009; Bhople et al., 2020). Nonetheless, synthetic auxin can also stimulate leaf senescence and leaf and fruit abscission (Zhu et al., 2011), in addition, it can delay fruit maturation and blooming time (Yadav et al., 2017). The administration time and concentration of NAA, play a pivotal role in crop response (Suman et al., 2017).

One of the most promising new innovation in sustainable contemporary agricultural systems is the use of natural biostimulants considering their natural source and capacity to replace or reduce the use of traditional synthetic hormones. According to the Regulation (EU) 2019/1009, plant biostimulants (PBs) are microbial and non-microbial-products able to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency, (b) tolerance to abiotic stress, (c) quality traits, or (d) availability of confined nutrients in the soil or rhizosphere. PBs, whether derived from animal sources (Casadesús et al., 2019), land plant (Lucini et al., 2015; Colla et al., 2017), algae (Golubkina et al., 2022), or microbial sources (Rouphael and Colla, 2018; Cardarelli et al., 2020), are recognized for their positive impact on the hormonal balance of plants. It should not be disregarded that PBs may be a source of phytohormones, or they can modulate plant hormone homeostasis replacing the need of synthetic hormones. For instance, protein hydrolysate (PH) biostimulants are widely used in market nowadays and preferred by farmers due to their environmentally friendly application and effective role in improving crop yield (Colla et al., 2017; Xu and Geelen, 2018). Several studies demonstrated the auxin-like activity of protein hydrolysates on vegetable crops. For instance, Colla et al. (2014) reported an auxin-like activity in coleoptile elongation of corn and shoot, root dry weight, root length, and root area of tomato cuttings from the plant-derived protein hydrolysate "Trainer®". Other authors demonstrated the capacity of the tropical-plant extract biostimulant "Auxym®" in mimicking auxin-like effect on sweet cherry fruits due to the presence of peptides and free amino acids which serve as signaling molecules thus suggesting an alternative model for exogenous synthetic hormones application (Basile et al., 2021). Some authors pointed out that biostimulants derived from vegetal origin, induced a modulation of metabolites related to the regulation of the homeostasis of the auxin pool (Buffagni et al., 2021). In addition to protein hydrolysates, several studies reported that many microorganisms, such as strains belonging to the genera *Pseudomonas*, *Bacillus*, *Pedobacter*, *Pantoea*, *Luteibacter*, *Acinetobacter*, *Lysobacter*, and *Enterobacter*, have plant growth-promoting properties and capability to produce auxin compounds (Khan and Doty, 2009; Agnolucci et al., 2019; Leontidou et al., 2020). However, regulatory constraints can limit the possibility to apply auxin-producing bacterial strains as soil/crop inoculants. Moreover, inconsistent in-field success is a major problem as

microbial inoculants often fail to compete with indigenous soil microbes (Shayanthan et al., 2022). Alternatively, auxin-producing bacterial strains can be cultivated in a fermenter for producing auxin-based filtrates as plant biostimulants. Luziatelli et al. (2021) successfully developed a fermentation process with *Enterobacter* sp. strain P-36 for producing a stable auxin-based filtrate. A bacterial culture filtrate containing auxin compounds was successfully tested as foliar spray for increasing fruit weight and marketable yield of greenhouse tomato (Rouphael et al., 2021).

Omics studies, encompassing transcriptomics and metabolomics among other powerful techniques, play a pivotal role in unravelling the molecular mechanisms underlying biostimulation, providing invaluable information into the regulatory networks and metabolic pathways involved into the plant response, respectively. For example, transcriptomics has been employed to decipher global gene expression patterns, uncovering the molecular mechanisms modulated by biostimulants in enhancing plant growth, development, and/or stress tolerance (González-Morales et al., 2021; Ali et al., 2022; Baghdadi et al., 2022). Metabolomics has been employed to comprehensively analyze and identify variations relative to small metabolites, offering insights into the metabolic processes, pathways, and biochemical changes occurring in response to plant biostimulants (Lucini et al., 2015; Bonini et al., 2020a). In particular, a metabolomics investigation indicated that a foliar application of five root-promoting PHs to tomato cuttings stimulated greatly the accumulation of the IAA precursors 4-(indol-3-yl) butanoate (IBA) and tryptamine.

To be competitive, the greenhouse production of strawberries requires higher quanti-qualitative standards to satisfy the needs of the consumer and large-scale distribution and reducing production costs. This need has created a continuous search by the farmers for products that can help them in pursuing these objectives and offer the consumers healthier products respecting food safety and the environment. Starting from the above considerations, we hypothesized that biostimulants with auxin-like activity can replace the synthetic auxins improving the quali-quantitative traits of strawberry by modulating plant metabolism. Consequently, the primary objective of this study was to assess the efficacy of two bio-based products, namely an auxin-enriched bacterial filtrate and a vegetal-derived protein hydrolysate. These alternatives were investigated as substitutes for commonly used synthetic auxins (NAA and naphthaleneacetamide; NAD), with the aim of enhancing both the production and fruit quality of greenhouse-grown strawberry plants. In particular, our primary aims encompassed a comprehensive evaluation of these biostimulants' influence on the growth, development, and overall performance of the plants. Our investigation also extended into the ionomics and metabolomics responses triggered by the application of these biostimulants. To the best of our knowledge, this study represents the first exploration of biostimulants with auxin-like activity and their impact on strawberry crops, providing the foundational evidence necessary for informed decisions in the pursuit of sustainable and productive greenhouse strawberry cultivation.

2 Materials and methods

2.1 Plant material, experimental design, and growing conditions

The trial was carried out in a polyethylene greenhouse at the experimental farm 'Nello Lupori', University of Tuscia, Viterbo, Italy. The average day/night air temperatures were 24± 0.8/16± 0.9° C. Plants were grown in polyethylene bags, white on the outside and black on the inside, containing 33 litres each (22 × 100 cm). The substrate had the following characteristics: 50% perlite (granulometry 1-2 mm) and 50% coconut fiber (v:v ratio), pH of 6.5, and EC of 0.7 mS/cm. The substrate was saturated with a nutrient solution before planting. The nutrient solution contained the following nutrients: 8.5 mM N-NO_3, 2.0 mM S, 1.0 mM P, 3.2 mM K, 4.0 mM Ca, 0.9 mM Mg, 20 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo. The pH of the nutrient solution was 5.5 ± 0.2 and the EC was 1.4 ± 0.1 mS/cm. Deionized water was used for the preparation of nutrient solution.The transplant of rooted strawberry plug plants (*Fragaria* × *ananassa* Duch.– cv 'Nabila'; Salvi Vivai, Ferrara, Italy) was carried out on 02/10/2019 at a plant density of 8.3 plants/m^2 (10 plants per bag arranged as double row; bag rows were spaced 1.2 m apart). The cultivar 'Nabila' is widely used in Mediterranean countries due to the very early production and good quali-quantitative traits of fruits (high fruit size, bright red color, excellent texture, and high productivity). Nutrient solution was pumped from independent supply tanks through a drip irrigation system, with one emitter per plant of 2 L h^{-1} flow rate. The duration of each irrigation event was tuned to provide at least 35% of the nutrient solution draining from the pots.

Five foliar treatments were tested as follow: control; bacterial filtrate; synthetic auxins; and vegetal-derived protein hydrolysate. The vegetal-derived protein hydrolysate Trainer® (Hello Nature S.p.a., Rivoli Veronese, Italy) was applied at a dose of 5 ml L^{-1}. Trainer® was made by enzymatic hydrolysis of proteins from legume seeds. According to Colla et al. (2015) and Lucini et al. (2015), Trainer® contained primarily soluble peptides and free amino acids (310 g kg^{-1}). The synthetic auxin Auxyger® LG (L. Gobbi s.r.l., Campo Ligure, Italy) was applied at a dose of 0.5 ml L^{-1}; it is a liquid plant growth regulator based on pure NAD at 16.9 g L^{-1} and pure NAA at 6.7 g L^{-1}. The bacterial filtrate Capxium® (Atens, Tarragona, Spain) was a commercial product obtained by fermentation with a proprietary strain of the *Pantoea* genera in a substrate rich in tryptophan to maximize the production of indole-3-acetic acid. Capxium® was applied at a rate of 5 ml L^{-1}. Control treatment was foliarly sprayed with pure water. Foliar treatments started at flowering stage on 29/01/2020 (119 days after transplanting -DAT) and were repeated three more times: 7/02/2020 (128 DAT), 17/02/2020 (138 DAT), and 27/02/2020 (148 DAT). Tested products were uniformly sprayed with a 16-L stainless steel sprayer called Vibi Sprayer (Volpi, Piadena, Italy). Treatments were arranged in a randomized complete block design with 5 replicates. Each plot was composed by one bag with 10

plants. All early-forming runners were removed from strawberry plants in order to prolong the fruit harvest. Fungal diseases were controlled with 2 foliar sprays of a fungicide containing cyprodinil and fludioxonil (Switch®; Syngenta, Milano, Italy) at a rate of 0.8 mg L^{-1} while pests were controlled with 2 foliar sprays of an insecticide containing abamectin (Vertimec EC; Syngenta, Milano, Italy) at a rate of 0.4 ml L^{-1}.

2.2 Plant production and fruit quality analysis

Fruit harvest began on 03/03/2020 (153 DAT), followed by 06/03/2020 (156 DAT), 09/03/2020 (159 DAT), 12/03/2020 (162 DAT), 20/03/2020 (170 DAT), 26/03/2020 (176 DAT), 03/04/2020 (184 DAT), 10/04/2020 (191 DAT), 16/04/2020 (197 DAT), and 22/04/2020 (209 DAT). In each harvest, fruits were collected separately in each plot and sorted in marketable (red fruits), and unmarketable (fruits having a diameter lower than 25 mm, fruits rotten and/or deformed). Fruits of two groups were counted and weighted separately. Marketable fresh mean weight was determined dividing the marketable fruit weight by the number of marketable fruits. Early yield was calculated considering the marketable fruits collected from the first harvest (153 DAT) to the sixth harvest (176 DAT). Eight marketable fruits per experimental unit were selected on the middle of harvesting period (176 DAT) for fruit quality analyses. Fruits were oven-dried at 65 °C until constant weight for determining fruit dry matter. Fruit firmness (kg cm^{-2}) was determined using a penetrometer (Bertuzzi FT 011; Milan, Italy), fitted with a 6 mm-diameter round-head probe. Then the fresh strawberry fruits were homogenized in a blender (2 L capacity; Waring HGB140, CA, USA) for one minute at low speed. The slurry was filtered through a two-layer cheesecloth, where the total soluble solids (TSS; expressed in °Brix) content was read with an electronic Atago N1 refractometer (Atago Co. Ltd., Tokyo, Japan).

At the final harvest (209 DAT), the Soil Plant Analysis Development (SPAD) index was measured on 20 topmost fully expanded leaves per plot with the SPAD-502 instrument (Konica Minolta Europe). After SPAD readings, shoots were harvested and oven-dried at 65 °C until constant weight for determining shoot dry weight.

Fruit mineral assessment was performed on dry samples of marketable fruits harvested at 176 DAT. Dried fruits were ground separately in a Wiley mill to pass through a 20-mesh screen, then 0.5 g of the dried plant tissues were analyzed for the following mineral elements: N, P, K, Ca, Mg, Fe, Mn, Zn, Cu, B, Mo, Ni, Na, Al, As, Ba, Be, Cd, Co, Cr, Pb, Sb, Se, Sn, Ti, Tl, and V. Nitrogen concentration in the plant tissues was determined after mineralization with sulfuric acid by 'Kjeldahl method' (Bremner, 1965) while the other elements were determined by dry ashing at 400 °C for 24 h, dissolving the ash in 1:20 HNO_3, and assaying the solution obtained using an inductively coupled plasma emission spectrophotometer (ICP Iris; Thermo Optek, Milano, Italy) (Horneck and Miller, 1998).

2.3 Fruit metabolomics

On 26/03/2020 (176 DAT), 5 marketable fruits per plot were harvested and immediately frozen with liquid nitrogen and stored at -20°C. Fruits were grinded in liquid nitrogen for metabolomic analysis at oloBion Laboratory (Barcelona, Spain). Metabolites were extracted in acidified 80% methanol, as previously reported (Bonini et al., 2020a). The samples were extracted by Ultra- Turrax (Ika T-25; Staufen, Germany), centrifuged and filtered through a 0.22 µm cellulose membrane into vials for analysis. A UHPLC chromatographic system coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS) was used for the untargeted screening of metabolites (Bonini et al., 2020b). The polar metabolites were separated at 45°C on a Water Acquity UPLC BEH C18 column (100 mm length x 2.1 mm id; 1.7 µm particle size) equipped with an additional Water Acquity VanGuard BEH C18 pre-column (5 mm x 2.1 mm id; 1.7 µm particle size) using (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid as A and B mobile phases respectively, with a gradient elution starting at 0 min with 0.5% B, 0-0.1 min 0.5% B, 0.1-10 min 80% B, 10-10.1 min 99.5% B, 10.1-12 min 99.5% B, 12-12.1 min 0.5% B, 12.1-14.4 min 0.5% B, and 14.4-14.5 min 0.5% B. Mobile phase flow rate was set at 0.3 ml/min, and the injection volume was 15µl. The sample temperature was maintained at 4 °C. Following the separation, the flow was introduced by positive mode electrospray ionization (ESI) into the mass spectrometer with the following parameters: capillary voltage, ± 3 kV; gas temperature, 250°C; drying gas (nitrogen), 13 L/min, nebulizer gas (nitrogen), 50 psi; sheath gas temperature, 315°C; sheath gas flow (nitrogen), 12 L/min and acquisition rate, 1 spectra/s.

For metabolite identification, MS/MS spectra were collected at collision energies of 10, 20, and 50 eV with an acquisition rate MS^1 of 4 spectra/s (100 ms) and an acquisition rate for MS/MS of 3 spectra/s (77 ms) with 4 precursor ions per cycle.

2.4 Statistical analysis

Agronomic and mineral data were subjected to analysis of variance (ANOVA) and Duncan's test ($p = 0.05$) to determine significant differences between treatments. Before analysis of variance, homogeneity of variance was assessed using Levene's Test for equality of variances, and the percentage data of early marketable yield was subjected to arcsine transformation to make the distribution normal. All statistical analysis were performed using the SPSS software package, (SPSS 10 for Windows 2001). Metabolomic data was processed by oloMAP 2.0^2 created by oloBion Company (Bonini et al., 2020b).

3 Results

3.1 Growth, yield and fruit quality

The shoot dry biomass at the end of the cycle was the highest in plants treated with protein hydrolysate (PH), followed by the control plants, and plants treated with Auxyger® LG (SA), and

bacterial filtrate (BF) (Figure 1). No statistically significant difference was found regarding SPAD index of leaves at the end of the trial (avg. 54.6; data not shown).

Strawberry early production (first 6 harvests) was significantly influenced by treatments with the highest values recorded in plants treated with BF, and with SA, followed by the control treatment and PH (Table 1). Total marketable yield was highest in plants treated with PH followed by the untreated plants, and plants treated with BF with no significant difference among them, whereas the lowest value was obtained in plants treated with SA (Table 1). The percentage of early marketable production out of the total marketable yield was the highest with the foliar treatments of SA (52.4%), followed with plants treated with BF (47.6%), while control plants (39.9%) and especially PH treated plants (33.9%) provided the lowest values (Figure 2). Because no significant differences were recorded for unmarketable yield, total marketable yield followed the same behavior of total yield with the highest value in PH treatment (Table 1). The differences on total marketable yield were attributed to changes of fruit numbers and not to fruit mean weight (Table 1).

Fruit firmness was significantly higher in plants treated with Trainer® (PH) compared to SA treatment (Table 2); BF treatment and control exhibited intermediated values which were no significant different from the other treatments (control, SA). Fruit dry matter was not significantly affected by treatments (Table 2). The content of soluble solids in fruits treated with SA was lower compared to control and PH treatment while BF treated fruits had intermediate values (Table 2).

3.2 Mineral profiling

Treatments affected only the nitrogen (N) concentration in strawberry leaves, while no significant differences were found for the other macronutrients (Table 3). The highest N content was recorded in leaves treated with PH compared to plants treated by BF, SA, and untreated plants (Table 3). Trace elements were also affected by the different treatments (Table 3). Zinc (Zn) had a significantly higher value in plant leaves treated with BF in comparison with leaves treated by PH, with no significant differences with leaves treated by SA and control plants (Table 3). Copper (Cu) had the highest value in leaves treated with SA compared to all other treatments and untreated leaves (Table 3). Boron (B) element was significantly higher in leaves treated with SA in comparison to leaves treated by BF and PH with no significant difference compared to control leaves (Table 3). Control leaves had a significantly higher B concentration than BF and PH treated plant leaves (Table 3). Nickel (Ni) concentration was highest in leaves of the untreated plants compared to all other treatments (Table 3). Selenium (Se) concentration was significantly highest in leaves treated with PH, followed by synthetic auxins and untreated control with no significant differences with BF treated leaves (Table 3). BF treated leaves had a significantly higher (Se) concentration compared to untreated leaves (Table 3). The heavy metal cadmium (Cd) element was significantly higher in leaves of control plants and the ones treated with BF in comparison to plant leaves treated with SA, with no significant difference to leaves treated with PH (Table 3). Antimony (Sb), Thallium (TI), Vanadium (V), Cobalt (Co), Arsenic (As), Beryllium (Be) trace elements had significantly the highest values in untreated plant leaves, in comparison to all treated plant leaves (Table 3).

Macronutrients concentration (N, P, K, Ca, Mg) in strawberry fruits was not affected by treatments whereas significant differences were recorded for some trace elements (Table 4). Boron concentration was significantly highest in control treatment compared to SA and PH treatments with no significant difference with bacterial filtrate treatment. No significant difference was found

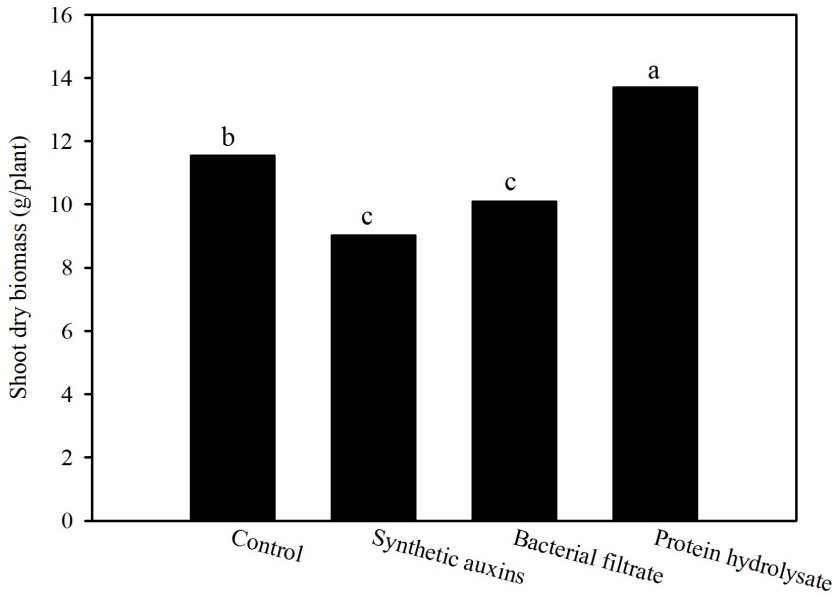

FIGURE 1
Effect of treatments on shoot dry biomass of strawberry plants at the end of the trial. Different letters correspond to statistically different values for p=0.05 (Duncan's test).

TABLE 1 Effect of treatments on early marketable (Early M), total marketable (Total M), unmarketable (U), and total yield (Total), and number and mean weight of marketable fruits in strawberry plants.

Treatment	Fruit yield (g/plant)				Marketable fruits	
	Early M	Total M	U	Total	Number (n./plant)	Mean weight (g/fruit)
Control	103.6 b	265.1 b	51.1	316.1 b	14.9 b	17.8
Synthetic auxins	119.7 a	226.6 c	44.9	271.5 c	12.7 c	17.9
Bacterial filtrate	120.1 a	255.5 b	49.2	304.7 b	14.2 b	18.0
Protein hydrolysate	102.1 b	300.3 a	48.1	348.4 a	16.6 a	18.1
Significance	**	***	ns	**	***	ns

ns, **, *** Nonsignificant or significant at $p \leq 0.01$ and 0.001, respectively. Different letters in the same column correspond to statistically different values for p=0.05 (Duncan's test).

between B concentration in fruits of plants treated with SA and PH. Aluminium concentration was highest in fruits of plants treated with BF, compared to fruits of plants treated with SA with no significant difference in comparison with fruits of plants treated with PH and control. Nickel concentration was significantly higher in fruits of control plants and SA treated plants in comparison with PH and BF treated plants with no significant differences among the two latter. Cadmium concentration was significantly higher in fruits of untreated plants in comparison to fruits treated by SA and PH while BF showed intermediate values with no significant differences from the other treatments. Lead (Pb) concentration was significantly highest in untreated plants, followed by fruits treated with BF and then by PH and SA, with no significant differences among these two latter. Titanium (Ti) concentration in fruits treated by BF and PH had a significantly higher value than fruits treated by SA with no significant difference with fruits of untreated plants. Fruits treated by SA had no significant difference with fruits of untreated plants. No significant differences were recorded for Sb, TI, V, Co, As and Be.

3.3 Metabolomic analysis

The different treatments applied to the plants clearly showed distinctive metabolomic profiles at leaf level. Fold change (FC) values were calculated comparing the treatment group against the control group. A value greater than 1 means an increase while a value below 1 means a decrease of the metabolite. Protein hydrolysate (PH) treatment induced the great increase in Phenylalanine (FC=21.77) and Tyrosine (FC=2.37), contrasting with the decrease in Chorismate (FC=0.53) and Tryptophan (FC=0.84) abundance in strawberry leaves (Figure 3; Supplementary File 1). The phenylpropanoid pathway was downregulated showing a decrease in Ferulate (FC=0.76) and

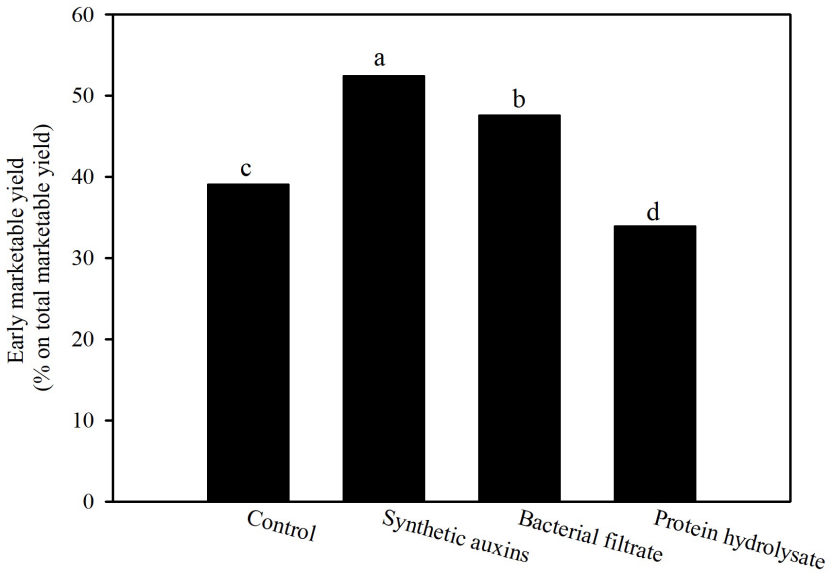

FIGURE 2
Effect of treatments on percentage of early marketable yield on total marketable yield of strawberry plants. Data are back transformed from arcosin transformation. Different letters correspond to statistically different values for p=0.05 (Duncan's test).

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TABLE 2 Effect of treatments on quality traits of strawberry fruits.

Treatment	Fruit firmness (kgf/cm^2)	Dry matter (%)	Soluble solids (°Brix)
Control	1.85 ab	6.24	5.15 a
Synthetic auxins	1.61 b	6.70	4.54 b
Bacterial filtrate	1.78 ab	6.29	5.12 ab
Protein hydrolysate	1.87 a	6.76	5.22 a
Significance	*	ns	*

ns, * Nonsignificant or significant at $p \leq 0.05$, respectively. Different letters in the same column correspond to statistically different values for p=0.05 (Duncan's test).

Syringin (FC=0.55). On the other hand, the flavonoid biosynthesis pathway showed an increase mostly due to Catechin (FC=1.37) and Kaempferol (FC=1.25), despite the decrease in Gallocatechin (FC=0.83). Purine metabolism also featured the decrease in Guanine (FC=0.56), dADP (FC=0.59), Adenosine (FC=0.53), and Deoxyadenosine (FC=0.20). The drop of Anthocyanin was also revealed by the decrease in Delphinidin 3-O-glucoside (FC=0.75) and Pelargonidin 3-O-glucoside (FC=0.07) (Supplementary File 1).

Synthetic auxins treatment showed an increase in Phenylalanine (FC=5.09), together with Phenylacetaldehyde (FC=1.32), Tyrosine (FC=2.33), Chorismate (FC=1.69), and Tryptophan (FC=3.17) in strawberry leaves (Figure 4; Supplementary File 1). The phenylpropanoid pathway featured the increase in Ferulate (FC=11.44), Scopoletin (FC=6.39), Coniferyl alcohol (FC=1.83), and Coumaroyl quinic acid (FC=1.72), and a decrease in Syringin (FC=0.69). Contrasting results were obtained for Purine metabolism with the up-regulation of Xanthosine (FC=1.63) and the downregulation of dADP (FC=0.84) as well as for Anthocyanin metabolism showing the increase in Delphinidin (FC=1.17) and a decrease in

TABLE 3 Effect of treatments on the mineral composition of strawberry leaves.

Element	Control	Synthetic auxins	Bacterial filtrate	Protein hydrolysate	Significance
	———————— g/kg d.wt.————————				
N	22.341 b	21.784 b	22.419 b	25.384 a	**
P	3.843	4.120	4.022	4.117	ns
K	24.096	25.293	25.229	23.842	ns
Ca	14.584	14.063	13.361	14.148	ns
Mg	3.175	3.285	3.228	3.121	ns
	———————— mg/kg d.wt.————————				
Fe	78.156	77.194	81.486	82.702	ns
Mn	265.653	302.317	274.683	270.110	ns
Zn	47.762 ab	51.060 ab	45.704 a	42.963 b	*
Cu	223.630 b	283.558 a	201.442 b	198.453 b	**
B	266.775 a	278.046 a	192.022 b	191.758 b	**
Mo	0.686	0.641	0.865	0.801	ns
Al	55.989	88.804	97.516	72.831	ns
Ba	63.065	65.611	70.340	59.582	ns
Ni	6.792 a	1.850 b	1.181 b	1.770 b	***
Se	0.529 c	0.773 b	0.793 ab	0.883 a	**
Cd	0.243 a	0.185 b	0.240 a	0.194 ab	*
Cr	0.250	0.098	0.567	0.141	ns
Pb	0.172	0.237	0.374	0.167	ns
Sn	0.810	0.924	0.990	0.939	ns
Ti	0.367	0.550	0.709	0.410	ns
Sb	0.016 a	0.014 b	0.013 b	0.014 b	***
TI	9.769 a	8.566 b	8.273 b	8.510 b	***

(Continued)

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TABLE 3 Continued

Element	Control	Synthetic auxins	Bacterial filtrate	Protein hydrolysate	Significance
V	10.010 a	8.777 b	8.477 b	8.720 b	***
Co	0.965 a	0.846 b	0.817 b	0.840 b	***
As	3.679 a	3.225 b	3.115 b	3.204 b	***
Be	0.181 a	0.159 b	0.153 b	0.158 b	***

ns, *, **, *** Nonsignificant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Different letters in the same row correspond to statistically different values for p=0.05 (Duncan's test).

Delphinidin 3-O-glucoside (FC=0.75) and Pelargonidin 3-O-glucoside (FC=0.38). More consistent was the enhancement of the flavonoid biosynthesis pathway with increases in Naringenin (FC=9.87), Kaempferol (FC=2.65), Gallocatechin (FC=1.68), and Delphinidin (FC=1.17) (Supplementary File 1). Bacterial filtrate treatment caused an increase in Phenylalanine (FC=4.52), as well as Phenylacetaldehyde (FC=1.83), Tyrosine (FC=2.52), Chorismate (FC=1.48), and Tryptophan (FC=2.30) in strawberry leaves

TABLE 4 Effect of treatments on the mineral composition of strawberry fruits.

Element	Control	Synthetic auxins	Bacterial filtrate	Protein hydrolysate	Significance
	———————— g/kg d.wt.————————				
N	18.316	17.193	18.524	19.582	ns
P	3.488	3.687	3.443	3.285	ns
K	19.614	18.616	20.472	19.276	ns
Ca	8.239	8.788	7.571	7.242	ns
Mg	1.769	1.676	1.667	1.554	ns
	———————— mg/kg d.wt.————————				
Fe	49.244	37.002	44.672	39.218	ns
Mn	75.329	59.431	72.974	56.067	ns
Zn	22.397	20.579	21.334	20.890	ns
Cu	9.512	6.511	8.711	8.095	ns
B	45.981 a	30.671 b	40.942 a	33.814 b	**
Mo	0.323	0.304	0.303	0.267	ns
Al	336.406 ab	285.838 b	440.211 a	375.540 ab	**
Ba	9.282	9.184	9.137	9.404	ns
Ni	5.071 a	1.761 b	3.605 a	1.514 b	***
Se	1.069	0.908	1.045	0.916	ns
Cd	0.061 a	0.043 b	0.052 ab	0.043 b	*
Cr	0.523 a	0.315 ab	0.097 bc	0.061 c	***
Pb	0.150 a	0.027 c	0.076 bc	0.034 c	**
Sn	1.113	0.959	1.012	0.949	ns
Ti	0.242 ab	0.139 b	0.278 a	0.329 a	*
Sb	0.015	0.015	0.014	0.015	ns
TI	9.242	9.491	8.833	9.346	ns
V	9.470	9.725	9.051	9.577	ns
Co	0.913	0.937	0.872	0.923	ns
As	3.480	3.574	3.326	3.519	ns
Be	0.171	0.176	0.164	0.173	ns

ns, *, **, *** Nonsignificant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Different letters in the same row correspond to statistically different values for p=0.05 (Duncan's test).

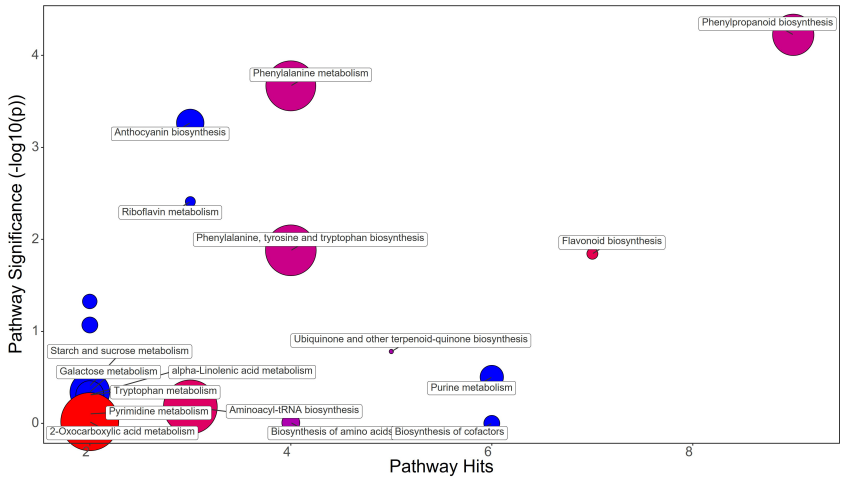

FIGURE 3
Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in protein hydrolysate treated leaves compared to untreated control of strawberry plants. Pathway significance (-log10(p)) is plotted on the y-axis, while the pathway hits are represented on the x-axis. Clusters are color-coded to indicate the proportion of compounds that have increased (in red), decreased (in blue), or exhibited mixed changes (in purple).

(Figure 5; Supplementary File 1). The phenylpropanoid pathway showed the up regulation of Ferulate (FC=11.68), Scopoletin (FC=6.98), Coniferyl alcohol (FC=1.49), and Coumaroyl quinic acid (FC=2.19), with the depletion in Syringin (FC=0.83). Regarding Purine metabolism it also featured the increase in Xanthosine (FC=2.14) and the decrease in dADP (FC=0.89). Concerning the flavonoid biosynthesis pathway compounds like Catechin (FC=11.15), Naringenin (FC=7.30), Kaempferol (FC=1.88), Gallocatechin (FC=1.46), and Delphinidin (FC=1.44) resulted in a net increase. In this case also Anthocyanin metabolism showed less consistency about the sense of change, with more abundance in Delphinidin (FC=1.44), Delphinidin 3-O-glucoside (FC=1.64), but depletion in Pelargonidin 3-O-glucoside (FC=0.07) (Supplementary File 1).

Metabolomic analysis revealed that strawberry fruits treated with BF, PH, and SA had distinct metabolic profiles (Figures 6–8). Despite this fact it has been highlighted that all treatments increased the flavonoid content in the fruits through different metabolic mechanisms.

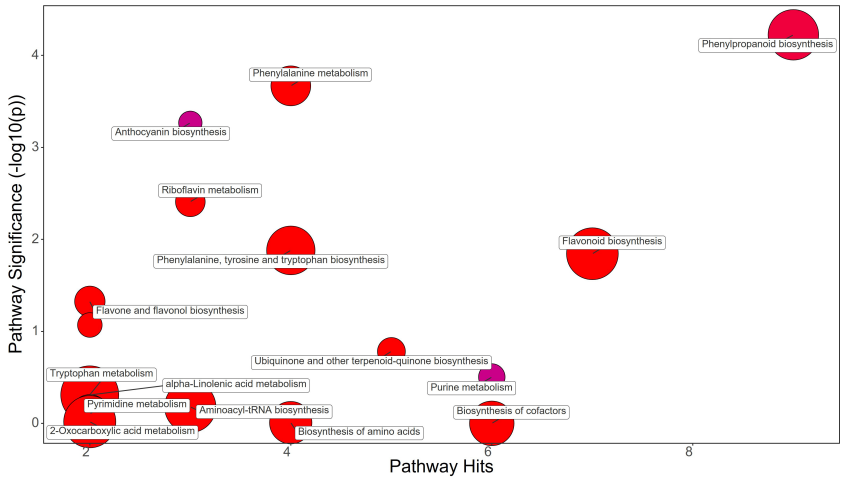

FIGURE 4
Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in synthetic auxins treated leaves compared to untreated control of strawberry plants. Pathway significance (-log10(p)) is plotted on the y-axis, while the pathway hits are represented on the x-axis. Clusters are color-coded to indicate the proportion of compounds that have increased (in red), decreased (in blue), or exhibited mixed changes (in purple).

Bacterial filtrate treatment did not alter Phenylalanine biosynthesis in fruits, but the phenylpropanoid pathway showed an increase in Cinnamaldehyde (FC=1.3) and Ferulate (FC=1.16) with a down-regulation of trans-Cinnamate (FC=0.7) (Figure 6; Supplementary File 1). Among the metabolites that also were up-regulated, Catechin (FC=1.26) and the anthocyanins Delphinidin (FC=1.21) and Delphinidin 3-O-glucoside (FC=1.12) were significantly increased by BF treatments (Supplementary File 1).

Synthetic auxins treatment did not alter Phenylalanine biosynthesis, and even the phenylpropanoid pathway showed a decrease in trans-Cinnamate (FC=0.54) in strawberry fruits (Figure 7; Supplementary File 1). However, an enhancement of Catechin (FC=1.51) and the anthocyanins Delphinidin (FC=1.46) and Pelargonidin 3-O-glucoside (FC=1.29) was recorded with the application of SA (Supplementary File 1).

Protein hydrolysate treatment significantly increased the content of Phenylalanine (fold change, FC=2.1), stimulating the phenylpropanoid pathway as evidenced by the increase in Ferulate (FC=1.4) and Coniferyl alcohol (FC=11.43) in strawberry fruits (Figure 8; Supplementary File 1). The flavonoids Kaempferol (FC=1.33) and Catechin (FC=1.33) were also increased in PH-treated fruits, as were the anthocyanins Delphinidin (FC=1.68), Delphinidin 3-O-glucoside (FC=1.21), and Pelargonidin 3-O-glucoside (FC=1.12) (Supplementary File 1).

4 Discussion

Results of the agronomic trial demonstrated that synthetic auxins (SA) and bacterial filtrate (BF) significantly enhanced early yield of strawberry fruits due to a positive effect of auxins on flowering and fruit set. Several researches reported the role of SA in inducing fruit set in many species (Srivastava, 2002; Stern et al., 2007; Sun and Hong, 2009; Yan et al., 2014) and in strawberry plants (Hunter, 1941; Thompson et al., 1969; Mudge et al., 1981) thus increasing the early fruit production. On the other hand, the protein hydrolysate (PH) favored a more gradual and prolonged fruit set over time with a greater development of the shoot biomass and total fruit number at the end of the growing cycle. This increased of shoot biomass, which is composed mainly of leaves (source tissue), could have augmented the photosynthetic capacity to sustain long-term production while the reduction in shoot biomass due to the great allocation of photosynthates on early fruit production (sink tissue) in auxin-treated plants (SA and BF) could have reduced the availability of photosynthates for new leaf development thus reducing the source activity (leaf photosynthesis) over time. Rylott and Smith (1990) also reported that application of synthetic auxin increased plant yield and made generative organs competitive over vegetative ones. It is well-known that PH 'Trainer®' bioactive compounds (peptides and amino acids) increase the plant's photosynthetic activity, which in turn increases yields (Colla et al., 2015; Rouphael et al., 2017). Particularly, the typical components of tested vegetal-PH which act as signaling molecules, may have enhance accumulation of endogenous phytohormones like auxins, cytokinin's and gibberellins (Colla et al., 2014; Ertani et al., 2017; Ceccarelli et al., 2021) to activate a signal transduction pathway, increasing crop yield (Rouphael and Colla, 2018). The prolonged total fruit production and plant biomass increase was found out similarly by some authors who observed that the use of two biostimulants derived from vegetal origin (alfalfa hydrolyzed and red grape skin), resulted in an increase in total fresh fruit weight and total fruit number along the whole greenhouse chili pepper crop cycle (Ertani et al., 2014).

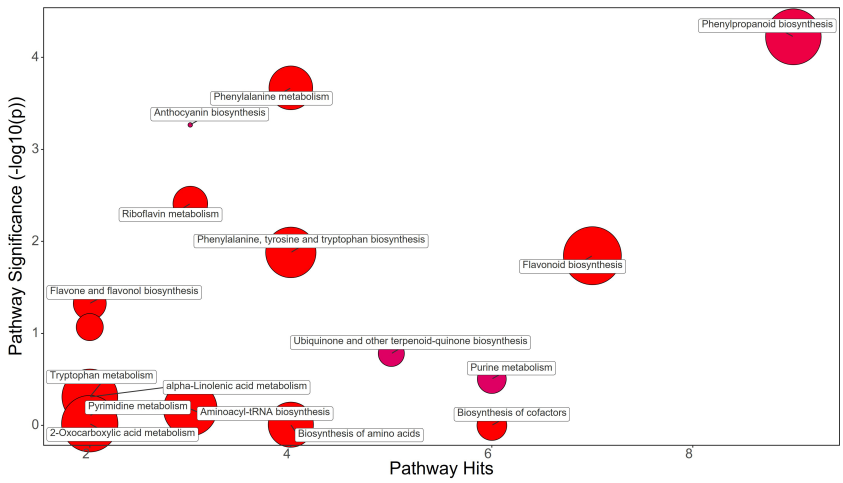

FIGURE 5
Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in bacterial filtrate treated leaves compared to untreated control of strawberry plants. Pathway significance (-log10(p)) is plotted on the y-axis, while the pathway hits are represented on the x-axis. Clusters are color-coded to indicate the proportion of compounds that have increased (in red), decreased (in blue), or exhibited mixed changes (in purple).

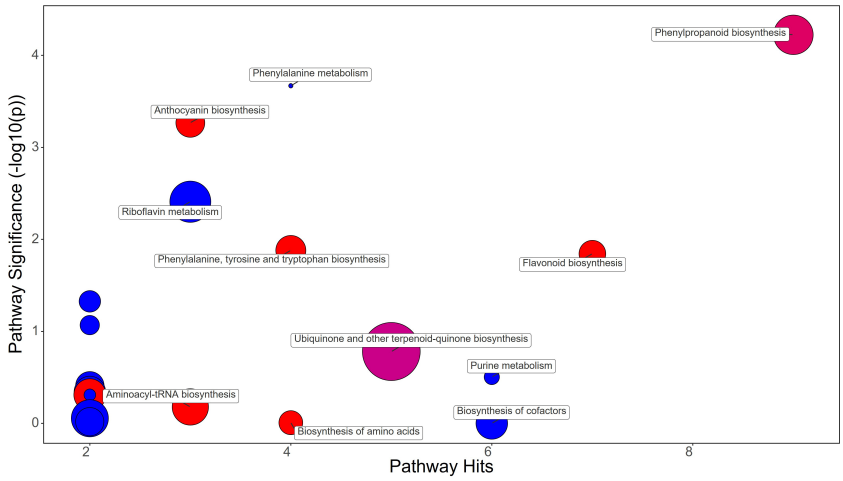

FIGURE 6
Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in bacterial filtrate treated fruits compared to untreated control of strawberry plants. Pathway significance (-log10(p)) is plotted on the y-axis, while the pathway hits are represented on the x-axis. Clusters are color-coded to indicate the proportion of compounds that have increased (in red), decreased (in blue), or exhibited mixed changes (in pink).

The increase of crop yield in PH-treated plants was associated with an enhancement of total N concentration in leaves resulting from a positive effect of PH on the absorption of mineral nitrogen (Colla and Rouphael, 2015). Moreover, Sestili et al. (2018) showed an increase in N concentration of tomato leaves after the foliar application of the PH Trainer® and referred this augmentation to the root growth stimulation and to the overexpression of genes implicated in N assimilation process. Selenium was also higher in leaves treated with PH respecting to SA treated and untreated leaves. Selenium is a crucial element for the scavenging and control of free radicals of plants and it is also an antioxidant, anti-senescent, abiotic stress modulator, and anti-senescent (Kaur et al., 2014). Therefore, the Se increase in strawberry leaves after PH foliar sprays may represent a positive attribute for maintaining an efficient photosynthetic apparatus over time. The decrease in leaf concentrations of B and Ni and trace elements such as Sb, TI, V, Co, As and Be in SA, and BF treatments can be related to a limited root activity resulting from the great allocation of photoassimilates to early fruit production whereas the decrease of leaf concentrations of B and Ni and trace elements (Sb, TI, V, Co, As and Be) in PH

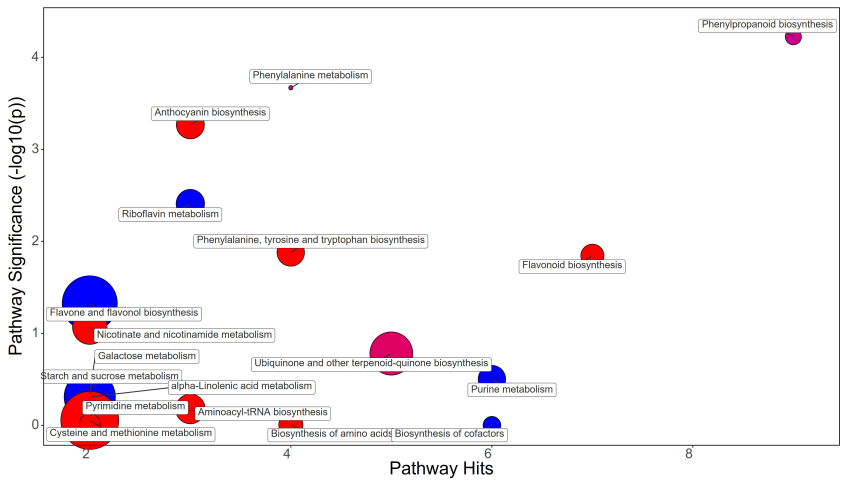

FIGURE 7
Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in synthetic auxins treated fruits compared to untreated control of strawberry plants. Pathway significance (-log10(p)) is plotted on the y-axis, while the pathway hits are represented on the x-axis. Clusters are color-coded to indicate the proportion of compounds that have increased (in red), decreased (in blue), or exhibited mixed changes (in purple).

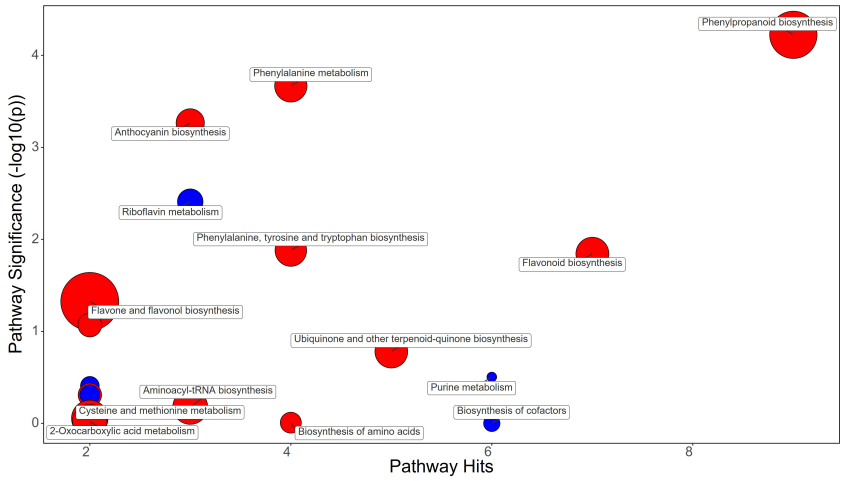

FIGURE 8
Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in protein hydrolysate treated fruits compared to untreated control of strawberry plants. Pathway significance (-log10(p)) is plotted on the y-axis, while the pathway hits are represented on the x-axis. Clusters are color-coded to indicate the proportion of compounds that have increased (in red), decreased (in blue), or exhibited mixed changes (in purple).

treatment can be attributed to the dilution effect induced by the increase of plant biomass resulting from Trainer® applications. Leaf (1973) also reported that the levels of nonlimiting growth elements in plant tissues may decrease in concentration due to an increase of plant biomass.

Moreover, the results of mineral composition indicated that macronutrients in strawberry leaves of all treatments were within the following sufficiency ranges (Mills and Jones, 1996): N (21-40 g/kg), P (2.0-4.5 g/kg), K (110-250 g/kg), Ca (6-25 g/kg), and Mg (2.5-7.0 g/kg); leaf micronutrients such as Fe (50-250 mg/kg), Mn (30-350 mg/kg) and Zn (20-50 mg/kg) were also in the sufficient ranges reported by Mills and Jones (1996), whereas Cu (6-20 mg/kg), B (25-60 mg/kg) and Mo (0.25-0.50 mg/kg) were above the sufficient ranges in all treatments. The above findings indicated that nutrients were sufficient to maximize fruit yield in all treatments.

According to Ornelas-Paz et al. (2013), fruit quality characteristics including color, firmness, and chemical composition affect customer choice. Fruit firmness is a critical quality trait that affects the postharvest shelf life and marketability of fruits (Goulao and Oliveira, 2008). El-Sharkawy et al. (2016) reported that auxin play an indirect function in stimulating fruit ripening through the up-regulation of genes encoding for several ethylene components, leading in ethylene-induced fruit ripening and softening. This was not the case in our study since fruits treated with SA showed a similar firmness of untreated control.

Total soluble solids (TSS) of strawberries represent the amount of carbohydrates, organic acids, vitamins, amino acids, and pectin in fruit pulp (Ashour et al., 2023). This fruit quality trait plays an important role in determining the fruit taste beside the total acids and their ratios (Krüger et al., 2012). The reduction of TSS content in fruits treated with SA in comparison with control and PH may be related to the sink-source imbalance causing reduction of photosynthetic capacity and total soluble carbohydrate accumulation. The lower soluble solids content observed in SA-treated fruits suggests that synthetic auxins may have a negative effect on fruit sweetness (Suman et al., 2017), probably by altering sugar metabolism or transport (Gibson, 2004). Moreover, the analysis of fruit quality traits revealed significant differences between the SA and BF treatments on mineral concentrations of B, Al and Ti. Notably, the BF, a natural auxin, tended to outperform SA in maintaining fruit firmness and soluble solids content, two key indicators of fruit quality (Døving and Måge, 2001). Fruits treated with SA and PH showed a reduction in concentrations of B and some potentially harmful elements for human health such as Ni, Cd, Pb in comparison with the untreated fruits. Foliar treatments with BF induced a reduction in Pb and Cr in comparison to the untreated fruits. Titanium concentration was decreased in fruits after the applications of SA compared to the PH and BF treatments. Considering the dry matter of the fruits (Table 3), it is possible to express the concentrations of heavy metals such as Cd and Pb in fresh weight (f.wt.) basis as follow: 0.0028, 0.0038, 0.0028 and 0.0029 mg Cd/kg f.wt. for control, SA, BF and PH, respectively; 0.0093, 0.0018, 0.0047and 0.0022 mg Pb/kg f.wt. for control, SA, BF and PH, respectively. The above Cd and Pb concentrations in all treated and untread fruits were below the maximum permissible levels (0.050 mg Cd/kg f.wt.; 0.10 mg Pb/kg f.wt) reported in Regulations (EU) 2021/1317 and 2021/1323 for strawberry. The reduction of Cd and Pb accumulation in fruits under foliar sprays with SA, and BF could be associated to a reduction of root activity in SA and BF treatments resulting from the great allocation of photosynthetates in the fruits at the expense of shoot and root growth. Moreover, the highest fruit yield in PH-treated plants could have reduced the Cd and Pb accumulation in fruits by dilution effect.

Primary and secondary metabolism have been widely categorized as two stages of plant metabolism. The first step in the process of creating primary macromolecules such proteins,

carbohydrates, nucleic acids, lipids, and hormones is the primary metabolism pathway which is responsible of supporting photosynthesis, plant growth and development (Bernards, 2010; Zhao et al., 2021). Secondary metabolites are derived from the primary metabolic pathways (Hussein and El-Anssary, 2019) and are composed of flavonoids, terpenoids, alkaloids, sterols, steroids, essential oils, lignin, carotenoids, polyphenols, anthocyanins that can act as antioxidant and defensive molecules against abiotic and biotic stress (Anjali et al., 2023). In this study, SA induced an accumulation of secondary metabolites thus reducing the carbon and sugar necessary for plant growth and production. Elmongy et al. (2020) reported that application of the synthetic auxin naphthaleneacetic acid promoted H_2O_2 production in azalea microshoots, possibly via increased peroxidase (POD) activity, because POD can participate in oxidative metabolism and in the production of H_2O_2; this may be the case in the current trial where SA promoted the accumulation of secondary metabolites such phenolic compounds for reducing the activity of reactive oxygen species like H_2O_2 in plant cells. Similar results to SA treatment on secondary metabolism were observed for BF treatments indicating that BF has the potential to replace SA for early production of strawberry fruits. On the other hand, PH promoted an accumulation of primary metabolites inducing a great support of photosynthesis and plant use of energy for plant growth over a longer period of the plant cycle resulting in a longer fruit production and higher yield. Several studies showed that the application of PH can boost crop yields and performance by activating carbon primary metabolism and N assimilation (Colla et al., 2017; Rouphael et al., 2018; Malécange et al., 2023).

A comparative metabolomic analysis was performed for discerning the metabolic pathways differentially affected by applications of PH, SA and BF. PH treatment resulted in an increase in Phenylalanine and Tyrosine, two amino acids that play crucial roles in plant growth and development. However, PH induced a decrease of Chorismate and Tryptophan, which are precursors of many plant growth regulators like auxin and salycilic acid (Erland and Saxena, 2019). This could have contributed to the delay in production observed in PH-treated plants. The decrease in Ferulate and Syringin in the phenylpropanoid pathway, along with a decrease in the anthocyanin Delphinidin 3-O-glucoside and Pelargonidin 3-O-glucoside, might have affected leaf coloration and overall plant health (Ramaroson et al., 2022). The PH-mediated effect on purine metabolism as indicated by the decreased levels of Guanine, dADP, Adenosine, and Deoxyadenosine, might have resulted from an enhancement of purine catabolism in plants which is a key process for recycling N in plant cells for remobilization to support new growth and reproduction (Zrenner et al., 2006). Moreover, several studies highlighted that purine degradation is also closely linked to plant responses and adaptation to stress. For example, several plants respond to environmental stress by inducing and activating enzymes in the purine degradation pathway (Watanabe et al., 2014). SA treatment also increased Phenylalanine, Phenylacetaldehyde, Tyrosine, Chorismate, and Tryptophan in leaf tissues. This suggests an overall stimulation of amino acid synthesis and phenylpropanoid pathway, which could have contributed to the early fruit production in SA-treated plants (Singh et al., 2010). The increase in Ferulate, Scopoletin, Coniferyl alcohol, and Coumaroyl quinic acid, along with an increase in the flavonoids Naringenin, Kaempferol, Gallocatechin, and Delphinidin, might have play a role as antioxidants in protecting plant cells from reactive oxygen species (ROS) generated by auxin application. BF treatment resulted in a similar metabolic profile to SA treatment, with an increase in Phenylalanine, Phenylacetaldehyde, Tyrosine, Chorismate, and Tryptophan. The increase in Ferulate, Scopoletin, Coniferyl alcohol, and Coumaroyl quinic acid, along with an increase in the flavonoids Catechin, Naringenin, Kaempferol, Gallocatechin, and Delphinidin, suggests that BF may also have enhanced antioxidant activity of plant cells for alleviating ROS damage.

The metabolomic analysis of strawberry fruits treated with BF, SA, and PH highlighted contrasting metabolic profiles, which were reflected in the observed differences in fruit quality. PH treatment led to an increase in Phenylalanine content of fruits, a precursor for many phenylpropanoids, and stimulated the phenylpropanoid pathway, leading to an increase in beneficial metabolites such as ferulate and coniferyl alcohol. This was accompanied by an increase in the flavonoids Kaempferol and Catechin, and the anthocyanins Delphinidin, Delphinidin 3-O-glucoside, and Pelargonidin 3-O-glucoside. These metabolites are known to contribute to fruit quality by enhancing color, flavor, and nutritional value (Parra-Palma et al., 2020). It is well known that a consumption of anthocyanins and flavonoids-rich fruits and vegetables contribute positively to human health mitigating cardiovascular disease, type 2 diabetes, non-alcoholic fatty liver disease, and neurological disorders (Kozłowska and Szostak-Węgierek, 2018; Oteizaìa et al., 2023). The increase in these metabolites suggests that PH treatment may enhance the fruit quality by stimulating the phenylpropanoid pathway and increasing the production of beneficial flavonoids and anthocyanins. SA treatment did not affect Phenylalanine biosynthesis and resulted in a decrease in trans-Cinnamate in the phenylpropanoid pathway. However, there was an increase in Catechin and the anthocyanins Delphinidin and Pelargonidin 3-O-glucoside. This suggests that SA treatment may enhance fruit quality through different metabolic mechanisms, possibly by directly stimulating the biosynthesis of beneficial flavonoids and anthocyanins. BF treatment resulted in a similar metabolic profile of SA treatment, with an increase in Cinnamaldehyde and Ferulate, and a decrease in trans-Cinnamate in the phenylpropanoid pathway. This was accompanied by an increase in Catechin and the anthocyanins Delphinidin and Delphinidin 3-O-glucoside. This suggests that BF treatment may also enhance fruit quality through similar metabolic mechanisms of SA, possibly by stimulating the biosynthesis of beneficial flavonoids and anthocyanins.

5 Conclusion

The results of the agronomic trial offer valuable insights into the effects of synthetic auxins (SA), bacterial filtrate (BF), and vegetal-derived protein hydrolysate (PH) on strawberry fruit production and quality. The foliar applications of SA and BF significantly

boosted early fruit yields, primarily attributed to their positive impact on flowering and fruit set. In contrast, PH treatment favored a gradual and prolonged fruit set, leading to increased shoot biomass and total fruit numbers over the entire growing cycle. This sustained production was likely due to the enhanced photosynthetic capacity supported by PH's bioactive compounds, such as peptides and amino acids, which are expected to activate key phytohormones. Our study also revealed differences in fruit quality among the treatments. PH-treated fruits exhibited improved firmness and soluble solids content if compared with SA treatment, but no significant differences were observed in comparison with control; fruit firmness and soluble solids contents are essential factors in determining postharvest shelf life and consumer preference. On the other hand, SA-treated fruits displayed lower firmness and soluble solids content, raising concerns about the effects of SA on fruit sweetness. Furthermore, the analysis of nutrient concentrations in leaves and fruits demonstrated that all treatments provided sufficient macronutrients for maximizing fruit yield and remained within regulatory limits for potentially harmful elements. PH treatment showed the most promise in reducing the accumulation of heavy metals in fruits, if referred to dry mass. Metabolomics indicated that the PH treatment stimulated primary metabolites, enhancing photosynthesis, and supporting long-term growth, while SA and BF treatment directly affected the biosynthesis of beneficial flavonoids and anthocyanins, contributing to enhanced fruit quality. In conclusion, this study highlights that SA may expedite early fruit production but might affect fruit firmness and sweetness, while PH treatment prolongs fruit set and supports photosynthetic capacity, leading to sustained production and improved fruit quality. BF treatment, with its natural auxin content, is a viable option to SA for enhancing fruit firmness and potentially influencing flavonoid and anthocyanin biosynthesis. Understanding the unique effects of these treatments provides valuable insights for growers and researchers seeking to optimize strawberry production and fruit quality.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

MCa: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. AE: Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. YR: Methodology, Software, Writing – review & editing. MCi: Software, Writing – review & editing. PB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. GE: Writing – review & editing. VC: Resources, Visualization, Writing – review & editing. BB: Data curation, Methodology, Software, Writing – review & editing. GCor: Methodology, Software, Writing – review & editing. SC: Methodology, Writing – review & editing. H-JK: Methodology, Writing – review & editing. GCol: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

Two authors are from Atens Company Spain who manufactured a tested product. However, they were not involved in the trial management at Tuscia University (Italy), measurements, and analysis, interpretation of the results. They role was mainly in the manuscript writing.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1337926/full#supplementary-material

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Testing plant growth promoting microorganisms in the field - a proposal for standards

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In the European Union and worldwide there are a burgeoning markets for plant growth promoting microorganisms (PGPM) and other biological agents as soil improvers, bio-fertilizers, plant bio-stimulants, and biological control agents or bio-pesticides. Microbial agents have a major share in this development. The use of such products is often advertised with the promise of contributing to sustainable agricultural practices by increasing crop growth and yield and offering an alternative or substitute to decrease the dependency of agriculture on hazardeous agrochemicals. In contrast to registered microbial plant protection products, PGPM that are marketed in the EU as soil improvers or plant biostimulants, are not strictly required to have proven minimum efficacy levels under field conditions. Manufacturers only have to ensure that these products do not pose unacceptable risks to human, animal or plant health, safety or the environment. Uniform guidelines comparable to the EPPO - standards (European and Mediterranean Plant Protection Organisation) to test the efficacy in field trials are not available. This paper attempts to fill the gap. It proposes guidelines for PGPM field trial design and implementation, as well as recommendations for the type and scope of data collection and evaluation. Selected research papers from literature were evaluated to analyze, whether and to what extent the requirements are already met. The majority of the papers had a clear experimental design followed by proper data evaluation. Frequent deficiencies were the low number of tested environments and crop species, insufficient site and agronomic management description and missing data on soil humidity and temperature. Using the suggested standards is assumed to increase the expressive power of tested microbial products.

KEYWORDS

sustainable agriculture, crop yield and quality, experimental design, biostimulants, mode of action

1 Introduction

Plant protection products (PPP) are subject to a demanding approval process including a testing of efficacy using EPPO standards (European and Mediterranean Plant Protection Organisation, Paris, France). Guidelines are available for a large range of specific indications, e.g. PP1/46(3) on efficacy evaluation of insecticides against wireworms (EPPO, 2023), or PP 1/002 (5) of fungicides against *Phytophthora infestans* on potato, foliar diseases on maize (PP 1/272 (1)), fungicides against *Gaeumannomyces graminis* causing take-all in cereal (PP 1/262 (1)), or criteria, as well as the experimental procedures, for determining the minimum effective dose of a plant protection product (PP 1/225(2)) (EPPO Bulletin, 2021b; EPPO, 2023).

Likewise, standards also exist for microbial plant protection products (PP1/276(1) published in EPPO Bullettin (2012), but without specific indications. Nowadays, various insecticides of microbial origin are well established on the market including entomopathogenic fungi such as *Beauveria bassiana* against locusts (Ranjan et al., 2021) or *Metarhizium brunneum* against wireworms (Brandl et al., 2017).

In addition to the market of strictly regulated microbial PPP, there is a burgeoning market for plant growth- promoting microorganisms (PGPM's) and other plant biostimulants based on bioactive natural compounds. They are applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits without direct effects as fertilizers (Du Jardin, 2015; Weinmann and Neumann, 2020). As summarized by O'Callaghan et al. (2022) the use of these products is often advertised with the promise of contributing to sustainable agricultural practices by increasing crop growth and yield or reducing the demand for fertilizers and agrochemicals (e.g. Lantmännen BioAgri (2021), Agriges (2023); Corteva Agriscience (2023), Syngenta Biologicals (2023).

In Europe, the Regulation (EU) 2019/1009 (EU, 2023a), laying down rules on placing fertilizing products on the EU market, defines "plant biostimulants" as product with the function to stimulate plant nutrition processes independently of its nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits, or (iv) availability of confined nutrients in the soil or rhizosphere.

The range of potential beneficial effects of living rhizosphere microorganisms, which implement their direct or indirect influence on plant performance by biological modes of actions, in particular those interfering with soil-plant-microbe interactions, is, however, scientifically well known to be much more multifaceted (Avis et al., 2008; Borriss, 2015; Weinmann, 2019).

Biostimulants have no effect against biotic stresses (e.g. pathogens and pests) and hence do not fall under the regulatory framework of pesticides. The list of biostimulants also includes PGPM such as N_2-fixing bacteria genera (e.g. *Azotobacter*, *Azospirillum*, *Rhizobium*) or mycorrhiza fungi. Any PGPM marketed for crop production purposes must be registered as either PPP, biofertilizer or biostimulant and has to fulfil the corresponding specific requirements, as compiled for different categories of EU fertilizing products including microbial and non-microbial plant biostimulants in (Table 1).

Similar to registered microbial and other PPP according to Regulation (EC) No 1107/2009 (EU, 2023b), also PGPM marketed as EU fertilizing products should be sufficiently effective and not pose a risk to human, animal or plant health, to safety or to the environment. While the obligatory and visible indicator that a EU fertilizing product including microbial plant biostimulant fulfills the safety requirements is the CE (European conformity) marking, the REGULATION (EU) 2019/1009 does not further specify the requirements for a sufficient efficacy assessment. General principles governing the CE marking and its relationship to other markings are set out in Regulation (EC) No 765/2008. Furthermore, information regarding the intended application method(s), effects claimed for each target plant, and relevant instructions related to the efficacy of the product should be given. This includes soil management practices, chemical fertilisation, incompatibility with plant protection products, recommended spraying nozzles size, sprayer pressure and other anti-drift measures, if applicable. For microbial plant biostimulant products in addition, all intentionally added micro-organisms shall be indicated (REGULATION (EU) 2019/1009).

However, elaborated guidelines for efficacy testing of PGPM used as plant biostimulants are so far not available in a comprehensive collection of standards for agronomic field experiments. Some general principles have already been suggested, but they rather focus on methods how to justify the claims of biostimulants for later submission to the admission authorities (Ricci et al., 2019). To prove such a claim the principles also allow the exclusive testing under controlled conditions. Other than the proposal here, they are not targeted on testing the practical agronomic benefit for farmers, although they include various important aspects, also considered here.

At the same time the market for PGPM is continually growing offering a wide range of products of variable performance and often unspecified composition (Figure 1).

The best-known example for the successful use of PGPM in crop production are rhizobia bacteria, which live in endophytic symbiosis with leguminous plants (Herridge et al., 2008; Lindström and Mousavi, 2019) and have been first patented as plant inoculants already in 1896 (Nobbe and Hiltner, 1896). Plant growth-promoting rhizobacteria (PGPR) may also live in less specific associations with plant roots, potentially resulting in growth-promoting effects on crops. In tropical and subtropical soils, for example, species of the genus *Azospirillum* have been shown to effectively replace N fertilizer inputs by 25-50% (Fukami et al., 2018, Santos et al., 2019). In these cases, the mode of action has been mainly linked to an improved nitrogen supply to the legume crop resulting from rhizobial atmospheric nitrogen (N_2) fixation. However, a wide range of other physiological mechanisms may affect crop growth as well. According to Hett et al. (2022), the potential functions of PGPM include (i) the facilitated acquisition of water and nutrients (primarily N, P, and Fe); (ii) the modulation of phytohormonal balances by changing the levels of auxins, cytokinins, gibberellins, abscisic, jasmonic and salicylic acids, mediating, inter alia, stimulation of root growth and

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TABLE 1 Categories of EU fertilizing products according to the REGULATION (EU) 2019/1009 and plant protection products according to the REGULATION (EC) No 1107/2009 in which plant growth promoting microorganisms (PGPM) and other biological agents for agriculture can be made available on the market.

Product Function Category (PFC)	Functional definition	Component material categories (CMCs)	Regulatory standards and product requirements
EU Fertilising Products in general	Providing plants or mushrooms with nutrient or improving their nutrition efficiency	Shall consist solely of component materials complying with the requirements for one or more of the CMCs listed in Annex II of REGULATION (EU) 2019/1009 including **CMC 7: Micro-organisms**	'EU fertilising product' means a fertilising product which is CE (European conformity) marked when made available on the market
1. Fertiliser	To provide nutrients to plants or mushrooms	**1. A: Organic Fertiliser:** shall contain organic carbon and nutrients of solely biological origin **1. B: Organo-Mineral Fertiliser:** co-formulation of 1. A and 1. C. **1. C: Inorganic Fertiliser:** shall contain macro- and/or micronutrients in inorganic form	Limits for contaminants (e.g. cadmium) and pathogens (e.g. *Salmonella*) Minimum contents for declared nutrients
2. Liming Material	To correct soil acidity	shall contain oxides, hydroxides, carbonates or silicates of the nutrients calcium (Ca) or magnesium (Mg).	Limits for contaminants (e.g. cadmium) Minimum neutralizing value, reactivity and grain size
3. Soil Improver	To maintain, improve or protect the physical or chemical properties, the structure or the biological activity of the soil	**3. A: Organic Soil Improver:** shall consist of material 95% of which is of solely biological origin (including peat, leonardite and lignite, but no other material which is fossilized or embedded in geological formations) **3. B: Inorganic Soil Improver:** other than an organic soil improver	Limits for contaminants (e.g. cadmium) and pathogens (e.g. *Salmonella*) Minimum contents for dry matter (20%) and organic carbon (7.5%) for organic soil improvers
4. Growing Medium	Products other than soil in situ, the function of which is for plants or mushrooms to grow in.	No further specification.	Limits for contaminants (e.g. cadmium) and pathogens (e.g. *Salmonella*)
5. Inhibitors	To improve the nutrient release patterns of a product providing plants with nutrients by delaying or stopping the activity of specific groups of micro-organisms or enzymes	**5. A: Nitrification Inhibitor 5. B: Denitrification Inhibitor 5. C: Urease Inhibitor**	20% reduction in the rate of ammoniacal nitrogen (NH_3-N) oxidation, release of nitrous oxide (N_2O), respectively hydrolysis of urea (CH_4N_2O), based on an analysis carried out 14 days after application at the 95% confidence level
6. Plant Biostimulant	To stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency, (b) tolerance to abiotic stress, (c) quality traits, or (d) availability of confined nutrients in the soil or rhizosphere.	**6. A: Microbial Plant Biostimulant:** shall consist of a micro-organism or a consortium of micro-organisms, including dead or empty-cell micro-organisms and non-harmful residual elements of the media on which they were produced, which have undergone no other processing than drying or freeze-drying; and are referred to in CMC 7 in Part II of Annex II (i.e.: *Azotobacter* spp., Mycorrhizal fungi *Rhizobium* spp., *Azospirillum* spp.) **6. B: Non-Microbial Plant Biostimulant:** other than a microbial plant biostimulant	Limits for contaminants (e.g. cadmium) and pathogens (e.g. Salmonella) Shall have the effects that are claimed on the label for the plants specified thereon Shall have a pH optimal for contained micro-organisms and for plants.
7. Fertilising Product Blend	Product composed of two or more EU fertilising products of PFC 1 to PFC 6	Blending shall not change the nature of each component EU fertilising product and shall not have an adverse effect on human, animal or plant health, on safety, or on the environment …	Requirements of each component EU fertilising product in the blend has been demonstrated in accordance with the conformity assessment procedure applicable to that component EU fertilising product …

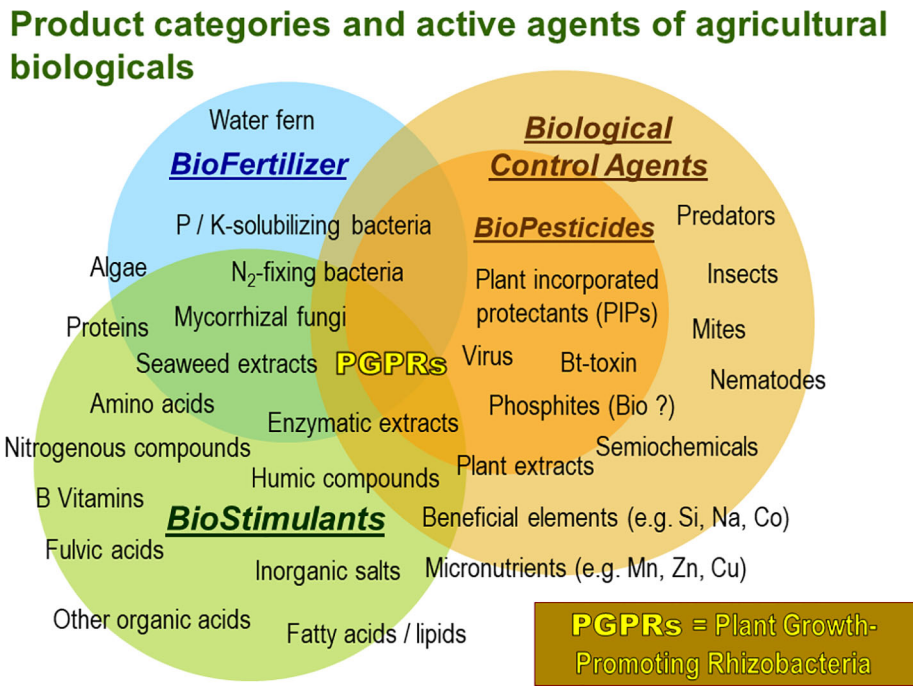

FIGURE 1
Product categories and respective types of active agents of agricultural biologicals for crop production. Especially microbial agents like plant growth promoting microorganisms (PGPMs) are characterized by multifaceted modes of actions. (Adapted from: Agricen Sciences' analysis of market analysts, survey papers on Biostimulants, www.bpia.org).

modifications of plant development; (iii) the release of volatile organic compounds and siderophores with functions in stress priming and nutrient mobilization and (iv) the reinforcement of resistance against abiotic stress factors (Vessey, 2003; Martínez-Viveros et al., 2010; Glick, 2012; Glick, 2014; Vejan et al., 2016 and Table 2).

Furthermore, in non-axenic soil systems, introduced PGPM interact directly (e.g. antagonistic or synergistic modes of action) or indirectly via their influence on plant physiology (e.g, alterations in phytohormonal balances) or morphology e.g. by more intensive fine root and root hair formation (Avis et al., 2008; Calvo et al., 2017; Weinmann and Neumann, 2020). A schematic illustration of the numerous facets of PGPR interferences with soil-plant-microbial interactions is illustrated in Figure 2.

PGPM applications may affect various soil processes or plant physiology both expected to result in improved crop growth. However, the way from proven physiological effects of PGPM applications on plants to crop yield increases in the field is far.

Doubts on the general validity of plant growth promoting effects of microbial applications under field conditions have been raised repeatedly (Mayer et al., 2010, Meyer et al., 2019, Antoszewski et al., 2022; O'Callaghan et al., 2022). Following own experiments and published data, Hett et al. (2023) stated that there is often still no unequivocal evidence for the utility of PGPM in arable farming (Gouda et al., 2018; Santos et al., 2019; dos Santos Lopes et al., 2021).

Moreover, a so-called publication bias may reflect a certain disproportion between published results with positive effects and unpublished results with no effects. In addition, most studies have been carried out under controlled conditions in pot experiments. This type of trial is characterized by constant climatic conditions (temperature and soil humidity) and a limited soil volume. These factors significantly differ from field conditions and allow a more targeted control of environmental factors relevant for the expression of PGPM effects than field experiments. Interestingly, field experiments with missing microbial effects are rarely reported in the literature (Bashan et al., 2020), although there is increasing awareness that these results are of relevance for achieving a better understanding of the factors determining the field effectiveness of PGPM applications (Hett et al., 2023). Likewise, the number of positive reports from experiments under controlled environmental conditions is likely to provide an overly optimistic impression of the intrinsic potential effectiveness of tested agents (O'Callaghan et al., 2022). At the same time the importance of product formulations, integrated applications strategies and adapted soil and crop management strategies and other external influences for the expression of beneficial traits under practice conditions remains poorly understood (Römheld and Neumann, 2006).

Other authors, in contrast, take an optimistic view claiming that PGPM will increasingly help to make crop production more sustainable and see a great future of microbial biostimulants, despite the variable efficacy under field conditions (Santos et al., 2019; Sammauria et al., 2020; Shah et al., 2022; Singh et al., 2022). This is also reflected in the results of various forecasts on market share of biostimulants with a current global market size of approx. 3.3 Bn USD and predicted annual growth rates between 11 and 12% until 2030 (Fortune Business Insights, 2023).

TABLE 2 Multifaceted effects of selected types of PGPMs as reported in the literature.

Type of PGPMs	Bio-stimulation, Bio-fertilization, Soil Improvement	Bio-Control, Plant Protection
Pseudomonas spp.	• Phytohormonal plant growth stimulation, N_2-fixation and improved nutrient acquisition (Singh et al., 2023) • Solubilization of P and other sparingly soluble nutrients (Barin et al., 2022) • Promotion of mycorrhization (Viollet et al., 2017) and legume nodulation (Soussou et al., 2017) • Soil aggregation (Sandhya and Ali 2015) and metal detoxification (Balíková et al., 2022) by release of exopolysaccharides	• Competition for space and nutrients (Miftakhov et al., 2023) • Inhibition of pathogen growth by production of iron-binding siderophores like pseudobactin and pyoverdine (Srivastava et al., 2022) • Synthesis of antibiotic and antifungal compounds, such as 2,4-diacetylphloroglucinol (2,4-DAPG), (Zhang et al., 2020) • Induced systemic resistance in plants (Reshma et al., 2018)
Bacillus spp.	. Phytormonal growth stimulation, N_2-fixation (Azeem et al., 2022) and triggering of stress responses in plants (Poveda and González-Andrés 2021) . Solubilization of P and other sparingly soluble nutrients (Saeid et al., 2018) . Promotion of mycorrhization, nutrient acquisition (Nanjundappa et al., 2019) and legume nodulation (Sibponkrung et al., 2020) . Soil aggregation (Deka et al., 2019) and heavy metal detoxification (Nazli et al., 2020) by release of exopolysaccharides	• Competition with pathogens for ecological niches and nutrients (Luo et al., 2022) • Production of secondary metabolites with antiviral, antibacterial, antifungal and nematicidal activity such as lipopeptide surfactins (Chowdhury et al., 2015; Chen et al., 2018) • Production of hydrolytic enzymes (e.g. chitinase, cellulose) with antagonistic activity against phytopathogens (Diabankana et al., 2022) • Induced systemic resistance in plants (Borriss et al., 2019; Miljaković et al., 2020)
Rhizobium spp.	• Phytormonal plant growth stimulation (Ferreira et al., 2020) • Symbiotic N2-fixation (Lindström and Mousavi, 2019) • Solubilization of sparingly soluble mineral nutrients, such as P and Zn (Verma et al., 2020) • Improved mycorrhization and increased number of newly formed mycorrhizal spores (Igiehon and Babalola 2021; Nasslahsen et al., 2022) • Production of phytohormonal compounds (indole acetic acid), exopolysaccharides	• Competition for nutrients, such for Fe as through siderophore production (Fahde et al., 2023) • Production of antibiotics, hydrocyanic acid (HCN), and hydrolytic enzymes (e.g. chitinase; Tamiru and Muleta, 2018) • Induced systemic resistance and enhance expression of plant defense-related genes (Díaz-Valle et al., 2019) • Multi-trophic plant-mediated antagonistic interactions among herbivores (aphids), pathogens (plant virus) and soil rhizobia (Basu et al., 2021)
	and siderophores (Verma et al., 2020).	
Trichoderma spp.	• enhanced nutrient efficiency (Zin and Badaluddin, 2020)	• release volatile organic compounds (Joo & Hussein, 2022) • induced systemic resistance (Tahir et al., 2017) • competition with pathogens for ecological niches (El-Maraghy et al., 2020) • release of antifungal and antibacterial compounds (Khan et al., 2020
Arbuscular mycorrhizal fungi	• increased nutrient uptake (Nadeem et al., 2017 • buffering salinity effects (Saxena et al., 2017) • increased rooth growth (Wu & Zou, 2017) • higher drought resistance (Latef et al., 2016)	• induce the synthesis of plant signal substances (Schmitz & Harrison, 2014) • promote the synthesis of plant defense hormones (Schmitz & Harrison, 2014) • slowing down the process of roots infection by pathogens by morphological changes (Basyal & Emery, 2021)

(Continued)

In any case, a proper assessment of PGPM products under field conditions according to reproducible and comparable standards is an indispensable requirement prior to any recommendation for use in commercial farming. The validity of a study mainly depends on appropriate scientific standards including a robust experimental design (O'Callaghan et al., 2022). Here we outline a set of requirements that should be considered when testing PGPM efficacy in field trials.

2 Objectives and frame setting

Based on the challenges described above, the main objective of this paper is to propose uniform criteria for agronomic field trials suitable for scientifically testing the efficacy of PGPM based biostimulants offered for use in arable crops under temperate climate conditions. These trials are an important step prior to large-scale testing on fields with farmer equipment. Lab and pot experiments will not be considered here. These methodical tools with their controllable settings may significantly contribute to uncover distinct modes of action of a PGPM or to describe and quantify physiological processes induced by PGPM. They may also allow experimental screenings for preselection of promising microbial candidates for field testing, but they are far from being a proxy for field trials. Laboratory, pot and field experiments should carefully follow the standard rules of good experimental practice (EPPO Bulletin, 2021a). Key aspects to consider include the selection of a representative dosage suitable also for later field trials and the elimination of nutritional effects resulting from PGPM application by inclusion of appropriate controls.

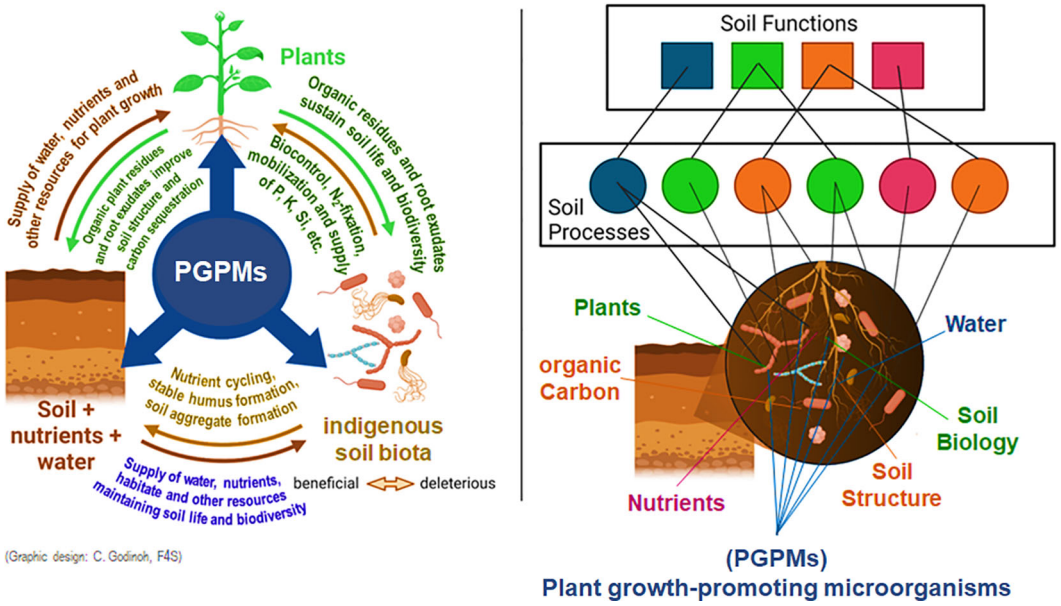

FIGURE 2
A better understanding of the synergies between soil, plants and soil life (Khati et al., 2020) and their impact on soil processes and ecosystem functions (Creamer et al., 2022) is of key importance for sustainable agriculture that is less dependent on the input of hazardeous agrochemicals by well-integrated PGPM applications (Weinmann and Neumann, 2020).

A further objective of this paper is to sharpen the view of relevant stakeholders for possible sources of error, which may result in experimental artefacts and false conclusions. In contrast to EPPO standards for PPP the proposed standards are not binding for approval, but they may support producers and users to ensure a specific product quality for the benefit of all stakeholders.

In a first step we propose standards considered suitable for testing the efficacy of PGPM applications in field trials. In the subsequent discussion, we first justify the standards by underpinning them with evidence from literature. Finally, we compare the standards to the methodology described in published papers on PGPM field trials.

3 Testing the efficacy of PGPM

3.1 Field trials on efficacy

Trials need to be implemented by a skilled person with scientific aptitude. Experimenters should strictly adhere to the specifications given in the instructions with respect to crop specific mode and frequency of application, dosage and timing. They should not know treatment allocations in the field by doing a blind study. General principles of good experimental practice as outlined in EPPO guideline PP 1/181 [5] ‚Conduct and reporting of efficacy evaluation trials including good experimental practice' (EPPO Bulletin, 2021a) should be considered. These guidelines relate to various aspects including staff qualifications, use of suitable equipment and facilities, protocols, modes of operation and recording of results. In general, the following specific requirements for PGPM testing are less demanding than EPPO guidelines for PPP, since in contrast to them, results are not used for official registration purposes.

3.2 Crop and site selection

Selection of crop species as test plants should stick to the indications listed in the instructions. To rule out genotype specific treatment effects several cultivars may be tested. In general, a standard cultivar recommended for the region and already tested in published trials with other PGPM should be used. For specific indications, e.g. strengthening of plant vigor and stress tolerance, claim related choices including cultivars with known genotypic differences in stress resilience may be considered. The seed should be certified and not chemically treated unless otherwise specified. Crops should be grown on experimental plots with fairly homogenous distribution of soil properties and a known history for crop rotation and management to be performed as far as possible under *ceteris paribus* conditions. Field history refers to at least the last two preceding crops of the crop rotation including their management such as soil tillage, fertilization and crop protection. In situations where known gradients in soil properties and topographic factors cannot be avoided, adequate experimental designs like randomized block or Latin square can be used in order to statistically compensate for such limitation in site homogeneity. Experimental designs and statistical approaches for data evaluation that are intended to compensate for restrictions in randomization by mixed models containing both fixed and random effects need to be applied with care not to cover experimental artifacts. Optimally

the persons performing the statistical data evaluations should be already involved in the planning and practical implementation of the field experiments to recognize early possible experimental biases that may lead to wrong statistical interpretations of the results.

Furthermore, a representative number of sites with well-described properties should be selected including soils with different levels of soil fertility, texture, pH, and levels of organic carbon (TOC). Selecting sites with different climatic conditions will also allow a quantitative assessment of effect stability. Annual replications may partly replace spatial variation with respect to variable weather (moisture, temperature). In case of limited test site availability, a priority might be set on testing products on less favorable soils, selected to avoid a masking of potential effects like improved root growth for better acquisition of sparingly available nutrient and water, which may not be discovered on very fertile and irrigated soils. If a specific function like improved phosphorus acquisition or enhanced tolerance to salinity stress is expected from the PGPM, site selection should consider this aspect: e.g. by conducting trials on low P or saline soils.

3.3 Treatment selection and mode of application

In the simplest case only one PGPM product is tested. In that case a minimum of two treatments would be needed (treated versus untreated). In addition to a negative control (= untreated), it is advisable to further include a positive control. Most suitable is another commercial product recommended for the same purpose. To avoid interference effects, in some cases, e.g. microbial products with additives, the inclusion of an autoclaved treatment or better the blank formulation without microbes should be taken into account. If specific effects, e.g. improved nitrogen supply or nutrient efficiency, are attributed to a PGPM - product, a second experimental factor, in that case mineral nitrogen application, should be included as a positive control, ideally in staggered treatments with lower and recommended rates. This approach allows the quantification of potential nitrogen effects resulting from PGPM application and hence the calculation of the nitrogen fertilizer equivalence (Delin et al., 2012).

Experimenters should strictly stick to the instructions on the application mode (e.g. seed coating) including dosage (in kg ha^{-1}), timing and frequency of application. In some cases, however, it may be useful to include a treatment with higher dosage than prescribed to tickle out potential effects or to identify potential phytotoxicity. A quality check of PGPM products under use should be carried out shortly before trial implementation checking the viability of the inoculum.

3.4 Experimental design and trial implementation

The design of field trials with PGPM should be as simple as possible, i.e. a fully randomized or randomized complete block design. However, lateral contamination of adjacent plots must be avoided to obtain valid results. For that purpose, an untreated buffer stripe of at least 1 m between plots is needed. The adequate distance may depend on the type of test. For example, when spore forming pathogens (e.g. powdery mildews) or PGPM (e.g. Trichoderma fungi as mycoparasite of powdery mildews) are tested, adequate distances to obtain representative results may be found in respective EPPO guidelines.

Alternatively, this can be achieved by limiting the sampling area within a plot requiring larger plot sizes. A minimum of four field replications should be implemented, if possible, even six. Important variables to be assessed are crop yield and quality. Any other effect, except crop quality, is subordinated to yield and only becomes relevant if linked to a yield increasing effect or specific application target of the tested product (e.g. crop cultivation with less or without input of agrochemicals, while maintaining plant health, crop quality, yield level). For PGPM products which are labeled as phosphorus solubilizing bacteria for instance, empirical evidence has to be provided in field trials. Likewise, higher yields may either be additive to a given amount of fertilizer input, or substitutive, by reducing the input needed for a given yield level. Otherwise, PGPM effects such as early growth promotion or increased root growth may be advantageous, e.g. for crop competition against weeds or higher water and nutrient uptake. However, it remains to be shown, whether the observed effects sustain throughout the cropping season to produce a net benefit, or will later be compensated.

Root growth promotion may be a relevant criterion, even if aboveground biomass is not affected by PGPM application. However, complete excavation of root systems in fields, is highly demanding and labor intensive and associated with high risks of losing fine root structures. For a more standardized sampling it is possible to collect a representative number of cylinder-shaped soil cores of undisturbed soil at a defined distance and depth around the plants (Helmisaari and Brunner, 2006). Soil core samples collected under field conditions are frequently used for estimating rooting densities, specific root length and biomass or fine root distribution after root washing and digital image analysis as root growth and these variables may be influenced also by PGPM inoculants.

For proper assessment of yield data, a minimum of harvested plants and hence a sufficient plot size is needed (Stockem et al., 2022). According to EPPO Standards, representative plot sizes may vary between crop species and the tested effects (e.g. testing the effectiveness in plant protection against wind carried spore forming pathogens requires larger plot sizes than testing products for improved use efficiency of phosphorus fertilizers, since phosphorus has a low mobility in soils). Specific guidelines for the conduction of efficacy trials in wheat or other cereals are given in EPPO Standard PP1/026(4) and for maize in EPPO Standard PP1/272(2). According to these, net plot sizes for wheat and other cereals should be at least 10 m^2. For maize, net plot size should be at least 2 rows x 10 m in length. The gross plot should have at least one additional treated border row on each side of the plot. With respect to accurate yield assessments it is recommended to take into account national standards for official testing of new varieties. In Germany, for instance recommended minimum plot size

(harvesting area) for cereals including maize, oil seed rape and pulses such as faba bean is 10 m². Crops with lower planting density require higher minimum plots sizes, e.g. 12.5 m² for potatoes and 12 m² for sugar beet (Bundessortenamt, 2000).Whenever possible the plot size should be 15 m².

Trial establishment, in general by sowing or planting of the test plant and application of the treatments to be assessed, is of crucial importance and a relevant source of error. Good agricultural practices such as weather dependent activities, thorough seed bed preparation and optimal sowing machine settings must be considered and accurately recorded in the experimental protocol. Cross contamination of seed lots need to be avoided in any case, for instance when PGPMs are applied by seed treatment. Therefore, negative controls should be sown first. To avoid contamination of non-target plots, the sowing device should be cleaned or even be sterilized, e.g. by ethanol before applying next treatments. Seeds treated with pesticides may interfere with PGPM application and should only be used if explicitly mentioned in the instructions. In many cases it may be useful to cover the trials with bird protection nets to ensure uniform crop emergence.

3.5 Crop management

Experimental conditions and management practices can have a strong influence on the expression of beneficial PGPM traits and henceforth on the results of any trial to test their effectiveness. They need to be as close as possible to practical farming conditions. In some special cases, however, also treatments not covered by the standard practice need to be included. This applies i.e. for evaluation of stress-protective effects. For evaluation of drought stress experiments it might be necessary to use rain shelters or compare plant performance under rainfed vs. irrigated condition, [even in cases, where irrigation is not commonly used] using a drought sensitive variety. In general, the *ceteris paribus* approach must be followed. Some management options may interfere with PGPM application. When using mechanical weed control tools, for example, carry over effects of inoculum have to be avoided. If considered relevant precautionary measures need to be taken, such as disinfection of the working tools.

Crop management may interfere with potential mechanisms of PGPM either induced directly or indirectly. Most prominent is the direct use of pesticides that may harm the applied PGPM. Unless not specified in the product instructions at least the use of fungicides should be omitted in field trials. In a two factorial design pesticide application may be included as second factor, provided that the factor level ‚no application' is included. Likewise, tillage or fertilization intensity can affect the long-term performance particularly of fungal PGPM strains applied as soil inoculants.

The experimental protocol must include all information and dates of management practices from soil tillage, sowing, fertilization, weed control, pesticide application, irrigation and harvesting.

3.6 Data collection

It is essential to generate valid and consistent data, which help to explain the overall results. Therefore, assessments and measurements need to be carried out up from the beginning preferably from the same skilled persons. Blockwise assessments may help to avoid unsystematic errors, potentially resulting from a forced interruption of measurements, e.g. by rainfall. Treatments should not be known to the data collector during assessments. Whenever possible, published and approved methods should be used. Visual ratings, e.g. on plant vigor are important, but also a possible source of bias. An internal quality check of the rating quality might be considered. An easy way to do this is a repetition of the rating in random order followed by a statistical comparison of both data sets. Name of the data collector and date of collection need to be recorded in the experimental protocol. Any management measure, e.g. irrigation, and unusual events, e.g. abiotic stress, need to be recorded as well.

3.6.1 Site (soil and climate)

Prior to the experiment soil texture should be assessed if unknown. Important soil chemical parameters include pH, soil organic matter and nutrient concentration in the topsoil (in general 0 – 30 cm). During the field trials soil temperature and moisture should be recorded in rooted soil horizon (e.g. 5 to 10 cm depth) at least during the two weeks following PGPM application. If no weather station is available on site, public data from a nearby weather station should be collected. In that case a rain gauge should additionally be installed at the trial.

3.6.2 Crop growth

The phenological stages of the crop should be recorded. Non-destructive measurements such as plant height, stem diameter and NDVI (Pettorelli, 2013) are easy to collect and may give first hints of a growth promoting effect. For destructive measurements additional area is needed. Higher plant biomass (dry matter = dm) after oven drying at 60° C and shoot nutrient accumulation in kg ha^{-1} (= above ground biomass in kg dm ha^{-1} x % NPK in shoot dm) at a given phenological stage, often the beginning of flowering, can be an indicator for the effect of a PGPM. Depending on the crop density, i.e. plants per m², representative samples sizes may vary from 0.5 to 3 m². In general, the rule should be followed that the less plants per m² are sown (e.g. maize with 10 seeds per m² vs. wheat with 350 seeds per m²) the larger the sample area should be to compensate for genotypic effects not being related to the treatments.

The occurrence of pest and diseases needs to be regularly assessed in all plots. If available, EPPO guidelines, should be used, at least for the assessment of dominant pests and diseases.

3.6.3 Crop yield and quality

Fresh and dry matter yield need to be collected from a representative (e.g. plot) area, in general a minimum of 15 m² is

compulsory for a solid assessment. It is important to analyze the yield structure as well, since it may help to better explain the obtained results. In winter wheat trials, for example, the ear density, number of grains per ear and the thousand grain weight should be reported.

Price effective quality parameters should be assessed as well. For e.g. winter wheat basic quality parameters include crude protein and gluten content of the grains. The assessment of mycotoxin contamination such as DON can be considered in justified cases. Targeted quality assessments should only be carried out, when part of the product claims.

3.6.4 Rhizosphere samples

Whenever possible rhizosphere samples should be taken in regular intervals. Metagenomic tools may help to quantify PGPM induced effects on the rhizobiome and ideally trace the fate of the inoculum after application including long-term effects on the soil microbiome and functional biodiversity. This, however, is a methodical challenge requiring considerable know-how and resources, which cannot be binding for standards.

3.7 Data evaluation and presentation of results

Prior to any statistical evaluation it is essential to check data for consistency and plausibility. The standard for field trial evaluation is Analysis of Variance (ANOVA) according to Fisher after testing the normal distribution of the residues and the homogeneity of the variances with standard tests. In general, a randomized complete block (RCB) design should be sufficient to compensate for potential field heterogeneity starting with a cross site evaluation. More sophisticated experimental designs should only be selected, if justified by an experimental factor. Testing the effect of PGPM applications on drought stress, for example, might require a split plot design with rain shelter plots. Given a sufficient number of data sets an effect size may be calculated (Hedges, 1982). Tests for mean comparisons depend on the research questions. Comparing several products among each other requires multiple comparison test such as the Tukey HSD-test. This robust *post-hoc* test can handle unequal sample sizes and variances, and controls the probability of making a type I error. If in specific cases individual treatments shall only be compared with an untreated control, the use of the Dunnett test may be considered.

Tables and figures need to be comprehensive allowing expert readers to quickly check statistical conditions. As a standard, both absolute and relative values for e.g. yield should be indicated. When showing efficacies against diseases, the absolute incidence level at least of the negative control should be indicated. Standard errors should be routinely reported as well.

4 Discussion

The guidelines for field trial testing of PGPM's presented here are targeted on gaining valid results for arable crops in temperate climate. Following the guidelines can contribute to gaining realistic assessments of the practical relevance of a given PGPM product. They try to consider the cause effect relationships from both perspectives, i.e. the manufacturer and the user. A zero efficacy of a given PGPM application can be due to a fake product or to mistakes during production, storage or application. Avoiding the former protects the user, while the latter is relevant for both stakeholders.

4.1 Important factors ensuring the validity of field trial results

To exclude the use of products with insufficient performance it is important to have a quality check prior to application in field trials. Rapid screening tests under controlled conditions working with seedlings and young plants have been described in the literature (Akter et al., 2013) for pre-evaluation of the basic effectiveness of a given product. Moreover, according to the harmonized EU legislation (EU) 2019/1009, future registration of biostimulants will comprise CE certification and a documented experimental proof of efficiency.

A second important aspect is the fate of the inoculum after application. However, inoculant tracing under field conditions is not a task which can be easily integrated into routine field testing of PGPM products. It requires strain-specific DNA primers or PGPM strains carrying resistance factors against certain antibiotics which are not widely available for many products.

With respect to the experimental design key challenges are the selection of appropriate controls and the setting of minimum plot sizes for yield determination. Using an autoclaved control, or better a blank formulation may help to avoid side effects. Mayer et al. (2010) concluded on their four years experiments using also autoclaved EM (effective microorganisms) *that the small effects observed were not caused by the EM microorganisms but rather by the nutrient inputs derived from Bokashi.* However, even the use of autoclaved PGPM controls can induce plant responses independent of nutrient effects via modifications of rhizosphere microbial communities (Nassal et al., 2018). Likewise, autoclaving does not simply affect the active microbial agent from the product, but may alter other physical and chemical product features including the release of more or degraded cell components from the microbial agents that can still have bioactive effectiveness. Marmann et al. (2014) for instance reported that the antibiotic activity of living or autoclaved bacteria on other bacteria was similar.

Therefore, appropriate controls should rather consist of blank formulations without PGPMs either provided by the manufacturers or using the filtrates of liquid or suspended product formulations after removal of microbial cells via sterile filtration.

From an agronomic point of view, it is essential to ensure an accurate quantification of the crop yield and quality effect resulting from PGPM application. Yield may be the result of PGPM effects such as e.g. improved nitrogen supply, but improved nitrogen supply does not necessarily mean higher yield. The practical use of PGPM in arable crops is only justified if a proven benefit at least compensates for the product and application costs. This can be

achieved with increases in crop yield or quality that allow for respective financial return or by improved use efficiency of fertilizers or other inputs that allow for respective cost savings. Relevance here means compensation of the product and application costs by additional revenues resulting from quality or yield increases or cost savings.

4.2 Further error sources

Soil factors can promote or restrict biological activity and effectiveness of PGPM inoculants (Figure 2). Soil pH and TOC (total soil organic carbon), but also available P and N pools have been identified as major drivers determining root traits and microbial community structures in soils (Lauber et al., 2009; Francioli et al., 2016). Accordingly, a recent meta-analysis reported that responsiveness to microbial phosphorous solubilizing PGPM and AMF inoculants decreases with increasing soil organic carbon content, whereas the response to microbial N_2-fixers shows an opposite trend (Schütz et al., 2018). An increment of soil organic carbon status is reported to increase as well autochthonous populations of agronomically beneficial microorganisms, and may suppress deleterious or pathogenic microorganisms, which may be positively correlated with a higher microbial diversity (Francioli et al., 2016). This might in turn hamper the establishment or functional relevance of additionally introduced PGPM inoculants due to increased competition from the native microbial community (Paul, 2016) or decrease the need to improve soil health with additional PGPM products, because the soil indigenous PGPM already fulfil this task. In soils with high TOC content, also increased concentrations of humic substances may induce stimulating effects, which is well documented for this class of compounds (Jindo et al., 2020).

Soil pH can exert a direct selective effect on certain microbial taxa (Rousk et al., 2010) or indirectly affect plant-PGPM interactions via effects on nutrient availability in soils (Kemmitt et al., 2006; Neumann and Ludewig, 2023). All these factors need to be considered in both, experimental design and site selection, ensuring *ceteris paribus* conditions.

Using a field with an unknown history with respect to crop rotation, fertilization and crop protection may produce inconclusive results. For example, certain agronomic practices such as applications of fungicides or glyphosate-based herbicides, intensive tillage and fertilization or crop rotations with non-mycotrophic pre-crops, can significantly inhibit the establishment and growth of mycorrhizal associations and induce harmful alterations in the soil microbiome (e.g. predomincance of phytopathogens). These processes can also interfere adversely with arbuscular mycorrhizal fungi (AMF) applied as biostimulants (Oehl et al., 2004; Oehl et al., 2005; Helander et al., 2018; Sommermann et al., 2018). However, compatibility of PGPM products with various pesticides should be usually indicated by the application instructions provided by the manufacturers.

Missing effects of PGPM application may also be due to a masking effect resulting from high nutrient availability either in pots (Hett et al., 2022) or in the field (Hett et al. 2023) and limited impact of stress factors. On the other hand, also extreme nutrient limitations or stress factors affecting root growth and activity particularly during the sensitive establishment phase of PGPM inoculants in the rhizosphere can counteract or limit the expression of beneficial effects on the host plants. Accordingly, on soils with limited nutrient availability a starter fertilization with P and N can exert a beneficial impact on the establishment of arbuscular mycorrhizal associations or the symbiosis with N_2-fixing bacteria (Bittman et al., 2006; Chekanai et al., 2018). In line with these findings also a recent meta-analysis revealed the highest efficiency of bacterial inoculants supporting plant P acquisition on soils with moderate P availability, while the benefits declined at higher and lower P levels (Schütz et al., 2018). Moreover, recent literature surveys suggest that P-solubilizing microorganisms used as inoculants contribute to the P nutrition of the host plant primarily through their long-term impact on nutrient cycling via release of sequestered P from decaying microbial biomass, rather than providing P by direct nutrient solubilization in the rhizosphere (Raymond et al., 2021).

Apart from the fertilizer dosage, also the form of fertilizer supply can affect PGPM performance. Numerous studies have reported positive PGPM effects in combination with N-rich fertilizers based on animal waste products (manures, guano, meat-, hair and feather meals) inoculants such as *Bacillus*, *Pseudomonas*, and *Trichoderma* (Thonar et al., 2017; Mpanga et al., 2018; Bradáčová et al., 2020; Behr et al., 2023). Particularly on soils with low TOC content, the use of these fertilisers might improve the carbon supply to the fast-growing copiotrophic inoculants, alongside with a starter fertilization effect promoting rhizosphere establishment. Also, the form of nitrogen supply (nitrate vs. ammonium) can affect plant-PGPM interactions. Particularly on soils with limited P-availability, ammonium-dominated fertilization promoted the acquisition of sparingly-soluble P sources and other nutrients in combination with various fungal and bacterial PGPM inoculants based on strains of *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Trichoderma* and *Penicillium* (Mpanga et al., 2018; Mpanga et al., 2019a; Mpanga et al., 2019b).

PGPM products should be screened for potential non-microbial compounds that may have plant growth promoting effects as well, e.g. micronutrients or other biostimulants such as seaweed extracts or humic acids frequently applied together with PGPM in combination products. However, even pure microbial products can contain formulation additives possessing a certain biostimulatory potential. This applies e.g. for protein-based additives, such as milk powder or soybean protein which may liberate bioactive peptides (Colla et al., 2015) during degradation in the rhizosphere.

4.3 Comparison of the proposed standards with methods in published research

In total, 18 research papers were selected and checked for their conformity to the suggested standards. Paper selection criteria

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included the testing of an arable crop in field trials preferably under temperate climatic conditions excluding experiments with Rhizobia and on salt stress. Some 22 methodical criteria were checked. The majority of the papers fulfilled important parts of the criteria including experimental design, PGPM application technique and statistical evaluation (Table 3). However, important methodological details such as information on field history and crop management, but also data on soil humidity and temperature were rarely reported. Half of the studies did not check the quality of the inoculum prior to sowing. In most cases, the number of tested environments was low (n =2) and only one crop was tested. At least some recent studies (n = 5) traced the fate of the inoculum after application (Tab 3).

Due to missing clarity of some of the criteria some arbitrary decisions may limit the specific validity of the evaluation. However, the rough evaluation suggests that a fulfilment of the listed criteria remains critical, even if the scientific quality of the selected papers tended to be high. Even though there are no prizes to be won for just publishing detailed data on crop yields and explaining management factors, they should be integrative part of future field research on PGPM.

TABLE 3 Consistency of PGPM field trial methodology with a range of criteria based on ten published research papers.

running number *	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
publication year 20–**	22	20	21	22	23	23	19	10	23	21	16	20	15	20	06	15	01	20
type of product	d	d	d	d	d	d	b	c	c	d	c	d	d	c	d	b	d	c
Number of products tested (n)	3	1	3	5	3	1	4	3	2	1	2	2	1	4	7	1	1	1
test crops (n)	1	1	1	1	1	1	1	4	1	1	1	1	1	1	1	1	1	1
number of tested environments (n)	2	2	2	1	3	2	1	1	2	1	2	2	2	2	2	92	2	2
indications on field history (**Y**es/No)	N	N	N	N	**Y**	**Y**	N	**Y**	N	N	**Y**	N	N	N	N	N	**Y**	N
negative control (yes/no)	**Y**	N	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
positive control (yes/no)	N	**Y**	N	N	**Y**	N	**Y**	N	**Y**	N	**Y**	N	**Y**	**Y**	**Y**	**Y**	**Y**	N
autoclaved or blank control (yes/no)	N	N	N	N	N	N	N	**Y**	N	**Y**	N	N	N	N	N	N	N	N
experimental design indicated (**Y**es/No)	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
number of field replications (n)	3	5	4	4	4	4	4	4	4	3	5	3	4	4	4	6	5	4
plot size (m^2)	4,8	9,6	400	15	30	3	n.i.	n.i.	24	2.5	45	13	9.6	29	13	30	15	15
clear indication of dosage (**Y**es/No)	**Y**	N	**Y**	N	**Y**	**Y**	N	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
booster dose	**Y**	N	N	N	**Y**	N	N	N	N	**Y**	**Y**	N	N	N	N	N	N	N
quality check of product (**Y**es/No)	**Y**	N	**Y**	N	**Y**	**Y**	N	**Y**	N	**Y**	**Y**	**Y**	N	N	**Y**	N	N	N
plot size for yield quantification (m^2) ***	n.i.	9,6	3	n.i.	3	0,5	n.i.	n.i.	n.i.	2.5	24	n.i.	n.i.	5.4	n.i.	30	n.i.	15
field tracing of inoculant	N	N	**Y**	N	N	**Y**	**Y**	N	**Y**	N	N	N	**Y**	N	N	N	N	N
Number of para-meters assessed (n)	>5	>5	>5	<5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	<5	>5	<5
pesticide application (**Y**es/No/not indicated)	N	n.i.	N	n.i.	N	Y&N	**Y**	N	N	n.i.	n.i.	**Y**	n.i.	**Y**	n.i.	n.i.	**Y**	**Y**
chemical soil data (**Y**es/No)	**Y**	**Y**	**Y**	**Y**	**Y**	N	**Y**	**Y**	N	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	N	**Y**	N
physical soil data (**Y**es/No)	**Y**	N	N	N	**Y**	**Y**	**Y**	**Y**	**Y**	N	N	N	N	**Y**	N	N	N	N
soil humidity and temperature data	N	N	N	N	**Y**	N	N	N	N	N	Y	N	N	N	N	N	N	N
clear indication of statistical (**Y**es/No)	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**

*: 1 = Gopalakrishnan et al., 2022; 2 = Ye et al., 2020; 3 = Nacoon et al., 2021; 4 = Frezarin et al., 2023; 5 = Hett et al., 2023; 6 = Behr et al., 2023; 7 = Bradáčová et al., 2019; 8 = Mayer et al., 2010; 9 = Symanczik et al., 2023; 10 = Mukherjee et al., 2021; 11: Nkebiwe et al., 2016; 12: Bakhshandeh et al., 2020; 13: Cai et al., 2015; 14: Gabre et al., 2020; 15: Çakmakçi et al., 2006; 16: Leggett et al., 2015; 17: Vessey and Heisinger, 2001; 18: Fröhlich et al., 2012.
: commercial (c), development (d), b = both, *: n.i. = not indicated means either plot size or smaller.
n.i. = not indicated.

Author contributions

DN: Conceptualization, Writing – original draft. GN: Methodology, Writing – review & editing. MW: Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of depth of straw returning on maize yield potential and greenhouse gas emissions

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Appropriate straw incorporation has ample agronomic and environmental benefits, but most studies are limited to straw mulching or application on the soil surface. To determine the effect of depth of straw incorporation on the crop yield, soil organic carbon (SOC), total nitrogen (TN) and greenhouse gas emission, a total of 4 treatments were set up in this study, which comprised no straw returning (CK), straw returning at 15 cm (S15), straw returning at 25 cm (S25) and straw returning at 40 cm (S40). The results showed that straw incorporation significantly increased SOC, TN and C:N ratio. Compared with CK treatments, substantial increases in the grain yield (by 4.17~5.49% for S15 and 6.64~10.06% for S25) were observed under S15 and S25 treatments. S15 and S25 could significantly improve the carbon and nitrogen status of the 0-40 cm soil layer, thereby increased maize yield. The results showed that the maize yield was closely related to the soil carbon and nitrogen index of the 0-40 cm soil layer. In order to further evaluate the environmental benefits of straw returning, this study measured the global warming potential (GWP) and greenhouse gas emission intensity (GHGI). Compared with CK treatments, the GWP of S15, S25 and S40 treatments was increased by 9.35~20.37%, 4.27~7.67% and 0.72~6.14%, respectively, among which the S15 treatment contributed the most to the GWP of farmland. GHGI is an evaluation index of low-carbon agriculture at this stage, which takes into account both crop yield and global warming potential. In this study, GHGI showed a different trend from GWP. Compared with CK treatments, the S25 treatments had no significant difference in 2020, and decreased significantly in 2021 and 2022. This is due to the combined effect of maize yield and cumulative greenhouse gas emissions, indicating that the appropriate straw returning method can not only reduce the intensity of greenhouse gas emissions but also improve soil productivity and enhance the carbon sequestration effect of farmland soil, which is an ideal soil improvement and fertilization measure.

KEYWORDS

straw returning, maize, yield potential, greenhouse gases, soil organic carbon

1 Introduction

In recent years, the impact of climate warming on natural economy and human life has become a global problem (Linquist et al., 2012). At present, it is generally believed that the increasing concentration of greenhouse gases (CO_2, CH_4, N_2O) in the atmosphere was the main cause of climate warming. Among them, 10%-20% of the total anthropogenic greenhouse gas emissions had generated by agricultural activities (Smith et al., 2007). The emission of greenhouse gases from farmland comes from the direct emission of farmland soil and the indirect emission of agricultural management measures, such as tillage, irrigation, straw returning, fertilization, etc. (Baggs et al., 2003; Toma and Hatano, 2007; Saggar, 2010; Trost et al., 2013). Therefore, agricultural production is considered to be an important source of greenhouse gas emissions.

As a carrier of material, energy and nutrients, straw is a valuable renewable natural resources (Amaya et al., 2007). China is a large agricultural country, which had produced a huge amount of crop straw every year, more than 800 million tons (Xia et al., 2014; Liu et al., 2021; Zhong et al., 2022). The content of N, P, K and other nutrient elements in straw was rich. As an organic fertilizer resource, it can be equivalent to 40% of the amount of chemical fertilizer used in China (Zhuang et al., 2020). The traditional treatments of incineration will not only cause serious environmental pollution, but also a great waste of resources. In recent years, China 's farmland farming model has changed significantly. The crop straw is no longer used as fuel, and the common agricultural practice was returned the straw to the field, which not only improves soil fertility but also reduces air pollution caused by crop straw burning (Gao et al., 2011). Straw returning can make the carbon in the straw return to the soil to participate in the carbon cycle, which can not only reduced the carbon output of the farmland ecosystem but also increased the soil organic matter content and improve the soil fertility, so as to realize the reuse of agricultural resources (Mu et al., 2016; Liang et al., 2017; Adimassu et al., 2019; Smitha et al., 2019). Some studies have shown that straw returning can stimulate the microorganisms in the soil to produce a priming effect (Kuzyakov et al., 2000), increase microbial activity, accelerate the decomposition rate of soil organic matter, and thus affect the production and emission of soil greenhouse gases. However, the current research results on the increase or decrease of greenhouse gas emissions caused by straw returning are still uncertain.

Northeast China is the main grain producing area in China. In recent years, with the increase of population growth and the improvement of living standards, higher requirements have been put forward for food production, environmental friendliness and sustainable development. People have made fruitful explorations in many fields such as high-yield cultivation, breeding and biotechnology. However, with the increase of crop yield, the biomass of straw has also increased significantly (Tian et al., 2020). In the past many years, due to the long-term shallow tillage of small agricultural machinery and the predatory production mode of large-scale application of chemical fertilizers, the comprehensive production capacity of farmland soil in Northeast China has declined sharply (Tian et al., 2019; Sui et al., 2020). Although the crop straw is the main source of organic materials for soil fertilization, straw burning is the most common straw treatment method, which was not only a waste of resources, but also caused serious environmental pollution. Therefore, aiming at the straw problem existing in the production of spring maize in Northeast China. A total of 4 treatments were set up in this study, which were no straw returning (CK), straw returning at 15 cm (S15), straw returning at 25 cm (S25) and straw returning at 40 cm (S40). By analyzing the effects of straw returning on the maize yield, physical and chemical properties of farmland soil and CO_2 and N_2O emissions, global warming potential (GWP) and greenhouse gas emission intensity (GHGI) were measured, and the regulation effect of straw returning depth on rice production potential and greenhouse gas emission reduction in paddy field was comprehensively evaluated to determine the optimal straw returning depth. It is expected that the research results will be of great significance to the scientific and rational use of straw and greenhouse gas emission reduction.

2 Materials and methods

2.1 Site description

The experiment was conducted in the Qiqihar maize experimental station of the Heilongjiang Academy of Agricultural Sciences, which is located in Qiqihar, Heilongjiang Province, China (46°52′N, 123°46′E) during the maize growing season (May to October) from 2020 to 2022. The test area belongs to the mid-temperate continental monsoon climate, which is characterized by dry and windy spring and warm and rainy summer. The annual precipitation is 477 mm, and a frost-free period of approximately 130 days. The soil type was Argosols (FAO classification) and the basic key properties are shown in Table 1.

2.2 Experimental materials and design

The experiment began in May 2020 and ended in October 2022. The test crop was maize and the variety was Nendan 29. The experiment comprised of four treatments as follows: no straw incorporation (CK), straw incorporation at 15 cm soil depth (S15), straw incorporation at 25 cm (S25) and straw incorporation at 40 cm (S40). Straw returning rate was 8000 kg hm^{-1}. The treatments were arranged into a randomized block design and replicated three times. Nitrogen rates were 180 kg ha^{-1}, and nitrogen fertilizer was applied according to the different stages, with base fertilizer and top-dressing fertilizer following a proportion of 1:2. Phosphorus (P_2O_5) rates were 90 kg ha^{-1} and potassium (K_2O) rates were 120 kg ha^{-1}, and phosphoru and potassium were applied as base fertilizer at one time. N, P, and K fertilizers was used urea, $Ca(H_2PO_4)_2$ and K_2SO_4, respectively. Other management measures were consistent with local agronomic practices including weeding and spraying insecticides throughout the experiment.

TABLE 1 The physicochemical property of composite topsoil samples (0-60 cm).

Soil layer	Organic matter	Total N content	Rapidly available N	Rapidly available P	Rapidly available K	Value of PH
	(g kg^{-1})	(g kg^{-1})	(mg $kg^{-1)}$	(mg $kg^{-1)}$	(mg $kg^{-1)}$	
0-20cm	19.12	0.75	67.52	23.21	146.8	7.23
20-40cm	18.37	0.54	64.74	22.36	140.6	7.27
40-60cm	17.37	0.53	61.74	20.36	137.6	7.25

2.3 Sampling and measurement

2.3.1 Grain yield

Yield samples of maize were collected randomly from 1 m double rows per plot at maturity. Grain yield was standardized to a moisture content of 0.14 g H_2O g^{-1}.

2.3.2 Determination of soil carbon and nitrogen content

After maize harvest, soil samples were collected in three layers (0-20 cm, 20-40 cm, and 40-60 cm) using a soil drill with a diameter of 3 cm. Five points were randomly selected from each micro-area, and the soil of the same soil layer was uniformly mixed as a sample. The soil samples were placed in a cool and ventilated place, dried and ground through a 0.15 mm sieve to determine soil organic carbon (SOC) and soil total nitrogen (TN) content. The SOC was determined by potassium dichromate external heating method (Lu, 2000), and the TN was determined by Kjeldahl apparatus (Kjeltec8400, FOSS, Denmark). The SOC and TN stock was calculated using the equal weight method (Ellert and Bettany, 1995; Xue et al., 2015), to eliminate the bias in the calculation of SOC and TN stocks caused by different plough layer thickness due to tillage. The ratio of SOC to TN was defined as soil carbon-nitrogen (C:N) ratio (Blanco-Canqui and Lal, 2008).

$$\text{Soil organic carbon stocks (SOC stocks, Mg ha}^{-1}) = \text{SOC} \times \text{BD} \times \text{soil depth} \times 100$$

$$\text{Soil total nitrogen stocks (STN stocks, Mg ha}^{-1}) = \text{TN} \times \text{BD} \times \text{soil depth} \times 100$$

2.3.3 Measurement of greenhouse gas emission fluxes

The emission fluxes of soil greenhouse gases CO_2 and N_2O were measured by static chamber method. Sampling once every 7 days during the growth period and once every 2 days after fertilization. Each treatment was placed in three static observation boxes, which were placed between two rows of corn. The sampling time was from 9:00 to 10:00 in the morning. The gas was collected every 10 min for a total of 5 times, and 30 mL of gas was collected in the trachea each time. Immediately after the sample collection was completed, the sample was taken back to the laboratory and analyzed within 24 hours using a gas chromatograph equipped with an ECD (Electron Capture Detector) and a FID (Flame Ionization Detector) detector (Agilent 7890A, Shanghai, China).The formula of CO_2 and N_2O emission flux was as follows:

$$F = \rho \times H \times \Delta C / \Delta t \times 273/(273 + T)$$

F is CO_2 emission flux or N_2O emission flux; ρ is the density of CO_2 or N_2O in the standard state; H is the height of the closed box (m); ΔC/Δt is the change rate of CO_2 or N_2O concentration in the test chamber; T is the average temperature (°C) in the chamber during the sampling process.

The formula of cumulative CO_2 or N_2O emissions during the growing season was as follows:

$$CE = \sum \left\{ \frac{F_i + F_{(i+1)}}{2} \times 10^{-3} \times d \times 24 \times 10 \right\}$$

CE is the cumulative emission of gas (CO_2 or N_2O), F_i and $F_{(i+1)}$ is the gas emission fluxes (mg m^{-2} h^{-1}) in two consecutive adjacent sampling periods, and d is the number of days between two consecutive adjacent sampling periods.

On a 100-year timescale, the warming potential of N_2O is 298. The formula of global warming potential (GWP) was as follows:

$$GWP = CE_{CO_2} + (CE_{N_2O} \times 298)$$

Greenhouse gas intensity (GHGI) is an index for comprehensive evaluation of greenhouse effect. The formula was as follows:

$$GHGI = \frac{GWP}{Grain\ \ yield}$$

2.4 Statistical analysis

Data analyzes were performed using Excel 2019 and SPSS 23.0 software. Significant differences between treatments were indicated by different letters at $p < 0.05$ level according to Fisher's LSD. Graphs were drawn with Origin 2018 software (OriginLab, Northampton, MA, USA), R software (Available online: http://www.r-project.org/) and Adobe Illustrator CS6 (Adobe Systems Inc., CA, USA).

3 Results

3.1 Grain yield

As shown in Figure 1, depth of straw returning significantly affected the maize yield. Compared with CK treatments, S15 and S25 treatments were significantly increased the maize yield, and was the highest under S25 treatments. In 2020, the maize yield increased significantly by 6.64% under S25 treatments and 5.22% under S15 treatments, respectively. In 2021, the maize yield increased significantly by 6.99% under S25 treatments and 5.49% under S15 treatments, respectively. In 2022, the maize yield increased significantly by 10.06% under S25 treatments and 4.17% under S15 treatments, respectively. While the S40 treatments had little effect on the maize yield, the maize yield increased significantly by 1.18% in 2020 and decreased by 1.51% in 2022.

3.2 Soil organic carbon and SOC stocks

The depth distribution of SOC and SOC stocks was significantly affected by depth of straw returning (Table 2). Compared with the CK treatments, at the 0-20 cm depth, the SOC was the largest under S15 treatments, which increased by 8.50~14.22%, and the SOC stock was the largest under S25 treatments, which increased by 9.71~22.34%. Compared with the CK treatments, at the 20-40 cm depth, the SOC was the largest under S25 treatments, which increased by 9.91~22.55%, and the SOC stock was the largest under S40 treatments, which increased by 4.15~16.61%. Compared with the CK treatment, at the 40-60 cm depth, the SOC was the largest under S40 treatments, which increased by 7.93~18.60%, and the SOC stock was the largest under S40 treatments, which increased by 4.88~17.30%.

3.3 Total nitrogen and STN stocks

The depth distribution of TN and STN stocks were significantly affected by depth of straw returning (Table 3). Compared with the CK treatments, at the 0-20 cm depth, the TN and STN stock was the largest under S15 treatments, which increased by 3.02~10.18% and 2.15~8.32%, respectively. Compared with the CK treatments, at the 20-40 cm depth, the TN and STN stock was the largest under S25 treatments, which increased by 7.32~12.11% and 6.77~12.65%, respectively. Compared with the CK treatments, at the 40-60 cm depth, the TN and STN stock was the largest under S40 treatments, which increased by 4.17~14.88% and 2.47~14.98%, respectively.

3.4 Soil C:N ratio

The depth distribution of C:N ratio was significantly affected by depth of straw returning. Compared with the CK treatments, at the 0-20 cm depth, the soil C:N ratio was the largest under S15 treatments, which increased by 3.66~5.32%. Compared with the CK treatments, at the 20-40 cm depth, the soil C:N ratio was the largest under S25 treatments, which increased by 2.41~9.48%. Compared with the CK treatments, at the 40-60 cm depth, the soil C:N ratio was the largest under S40 treatment which increased by 5.32% in 2020, which was the largest under S40 treatments which increased by7.24 ~8.29% in 2021 and 2022 (Figure 2).

3.5 Relationships of grain yield versus SOC, TN and C:N ratio

Correlation analysis results also showed that the grain yield was significant related to the SOC, TN and soil C:N ratio (Figure 3). The grain yield had significantly positive correlations with the SOC, TN and soil C:N ratio at the 0-20 cm and 20-40 cm depth, while was not significantly correlation with the SOC, TN and soil C:N ratio at the 40-60 cm depth.

3.6 The feature of greenhouse gases emission

The dynamic changes of the soil CO_2 flux and CO_2 emission in maize growing season under all depth of straw returning treatments

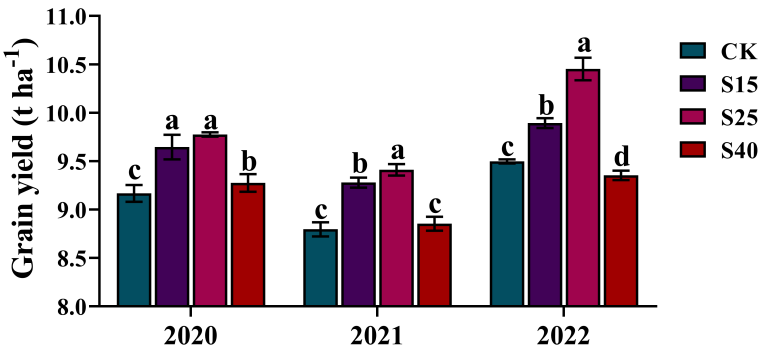

FIGURE 1
Effects of depth of straw returning on maize yield. For each year, bars followed by the different letters are significantly different at P< 0.05. CK: no straw returning; S15: straw returning at 15 cm soil depth; S25: straw returning at 25 cm; S40: straw returning at 40 cm.

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TABLE 2 Depth distribution of SOC (g kg^{-1}) and SOC stocks under different straw returning treatments.

Soil layer	Treatments	2020		2021		2022	
		SOC content	SOC stocks	SOC content	SOC stocks	SOC content	SOC stocks
0-20 cm	CK	8.12c	18.93c	8.13d	18.88d	8.09d	18.60c
	S15	8.81a	19.65b	9.02a	20.71b	9.24a	20.84b
	S25	8.63b	20.81a	8.74b	21.85a	8.96b	22.80a
	S40	8.42b	19.40b	8.43c	20.38c	8.42c	20.31b
20-40 cm	CK	6.86c	15.87b	6.79c	15.51c	6.74c	15.07c
	S15	7.12b	16.24a	7.45b	16.50b	7.55b	16.67b
	S25	7.54a	16.30a	7.86a	17.21a	8.26a	17.47a
	S40	7.03b	14.43a	7.33b	17.66a	7.36b	17.58a
40-60 cm	CK	5.55b	16.43c	5.46c	16.05c	5.27c	15.49c
	S15	5.68a	16.81b	5.81b	17.08b	5.83b	17.14b
	S25	5.70a	16.87b	6.06a	17.82a	6.11a	17.96a
	S40	5.99a	17.23a	6.22a	18.29a	6.25a	18.18a
0-40 cm	CK	7.49b	34.81c	7.46c	34.38d	7.42c	33.67c
(average)	S15	7.97a	35.90b	8.24a	37.21c	8.40a	37.51b
	S25	8.09a	37.11a	8.30a	39.06a	8.61a	40.27a
	S40	7.73b	36.53a	7.88b	38.04b	7.89b	38.19b
0-60 cm	CK	6.84b	51.23c	6.79b	50.44c	6.70b	49.17c
(average)	S15	7.20a	52.71b	7.43a	54.29b	7.54a	54.65b
	S25	7.29a	53.98a	7.55a	56.88a	7.78a	58.24a
	S40	7.15a	54.26a	7.33a	56.33a	7.34a	56.56a

Different small letters represent significant differences among treatments

TABLE 3 Depth distribution of TN (g kg^{-1}) and STN stocks under different straw returning treatments.

Soil layer	Treatments	2020		2021		2022	
		TN content	STN stocks	TN content	STN stocks	TN content	STN stocks
0-20 cm	CK	0.716c	0.711b	0.704b	1.977b	1.977c	1.944c
	S15	0.738a	0.755a	0.776a	2.020a	2.070a	2.106a
	S25	0.722b	0.741a	0.754a	1.990a	2.049a	2.070a
	S40	0.714c	0.722b	0.724b	1.965c	1.999b	1.983b
20-40 cm	CK	0.577	0.567	0.573	1.651	1.611	1.639
	S15	0.595	0.606	0.612	1.702	1.719	1.741
	S25	0.619	0.639	0.641	1.762	1.806	1.827
	S40	0.604	0.613	0.622	1.724	1.739	1.777
40-60 cm	CK	0.569	0.554	0.534	1.685	1.628	1.569
	S15	0.564	0.55	0.545	1.669	1.616	1.603
	S25	0.562	0.591	0.607	1.665	1.736	1.785
	S40	0.593	0.616	0.613	1.727	1.754	1.804

(Continued)

TABLE 3 Continued

Soil layer	Treatments	2020		2021		2022	
		TN content	STN stocks	TN content	STN stocks	TN content	STN stocks
0-40 cm	CK	0.647	0.639	0.639	3.628	3.588	3.583
(average)	S15	0.667	0.681	0.694	3.722	3.789	3.847
	S25	0.671	0.69	0.698	3.752	3.856	3.897
	S40	0.659	0.668	0.673	3.689	3.737	3.76
0-60 cm	CK	0.621	0.611	0.604	5.313	5.217	5.152
(average)	S15	0.632	0.637	0.644	5.391	5.405	5.45
	S25	0.635	0.657	0.668	5.417	5.592	5.682
	S40	0.634	0.644	0.653	5.415	5.491	5.564

were shown in Figure 4. The soil CO_2 flux of each treatments showed an obvious bimodal change trend during the whole maize growing season. In the early stage of maize growth, the soil CO_2 flux was larger and then gradually decreased, and reached the peak of emission flux in the middle stage of growth, and then the emission flux decreased. As shown in Figure 4, depth of straw returning was significantly increased the CO_2 emission, and was the highest under S15 treatments, which was increased by 7.67~19.54% compared with the CK treatments.

The dynamic changes of the soil N_2O flux and N_2O emission in maize growing season under all depth of straw returning treatments were shown in Figure 5. The soil N_2O flux of each treatment showed an obvious bimodal change trend during the whole maize growing season. In the early stage of maize growth, the soil N_2O flux was larger and then gradually decreased, and reached the peak of emission flux in the middle stage of growth, and then the emission flux decreased. It can be seen that the peak value of the soil N_2O flux is roughly the same as that of fertilization period. The first peak appears after base fertilizer, and the second peak appears after top-dressing fertilizer, indicating that fertilization is the main factor affecting the soil N_2O flux. As shown in Figure 5, depth of straw returning treatments was significantly increased the N_2O emission, and was the highest under S15 treatments, which increased by 15.41~26.56% compared with the CK treatments.

3.7 Estimation of global warming potential and greenhouse gas emission intensity

In the maize growing season, the GWP mainly comes from the CO_2 and N_2O emissions. In this study, the estimated results of the GWP and GHGI under all depth of straw returning treatments in maize growing season were shown in Figure 6. Compared with CK treatments, the GWP of S15, S25 and S40 treatments was increased by 9.35~20.37%, 4.27~7.67% and 0.72~6.14%, respectively, among which S15 treatment contributed the most to the GWP of farmland. The GHGI is an evaluation index of low-carbon agriculture at this stage, which takes into account both crop yield and global warming potential. In this study, the GHGI was shown a different trend from the GWP. Compared with CK treatments, S25 treatments was no significant difference in 2020, and was decreased significantly in 2021 and 2022. This is due to the combined effect of the maize yield and cumulative greenhouse gas emissions, indicating that the appropriate straw returning method can not only reduce the intensity of greenhouse gas emissions but also improve soil productivity and enhance the carbon sequestration effect of farmland soil, which is an ideal soil improvement and fertilization measure.

4 Discussion

4.1 Effects of different straw returning depths on soil nutrients and grain yield

Different straw returning methods will affect the distribution of straw in the tillage layer, and the position of straw will affect the spatial distribution of the SOC and TN (Puget and Lal, 2005; Du et al., 2010). In this study, the SOC and TN near the straw position were higher than those without straw. Studies have also shown that the SOC and TN in the soil profile is affected by the content of straw and soil organic matter (Turmel et al., 2015). Some studies have shown that straw returning can cause deep soil disturbance and promote the mineralization of organic matter contained in the soil itself (Devêvre and Horwáth, 2000). Therefore, the carbon and nitrogen released by straw decomposition and the mineralization of soil organic matter may be the two main reasons for the influence of the SOC and TN in the different soil layers. The soil C:N ratio directly affects the carbon-nitrogen cycle, carbon-nitrogen interaction and the stability of soil organic matter in farmland ecosystem (Russell et al., 2005; Tong et al., 2009). Similar to the SOC and TN, different tillage depths had a significant effect on the soil C:N ratio, and the soil layer near the straw had a higher C:N ratio. Consistent with previous studies (Puget and Lal, 2005), this study found that depth of straw returning treatments helped to improve the soil C:N ratio. According to the analysis, the improvement effect is mainly due to the fact that the carbon release rate of straw is higher than the nitrogen release rate. The phenomenon of carbon

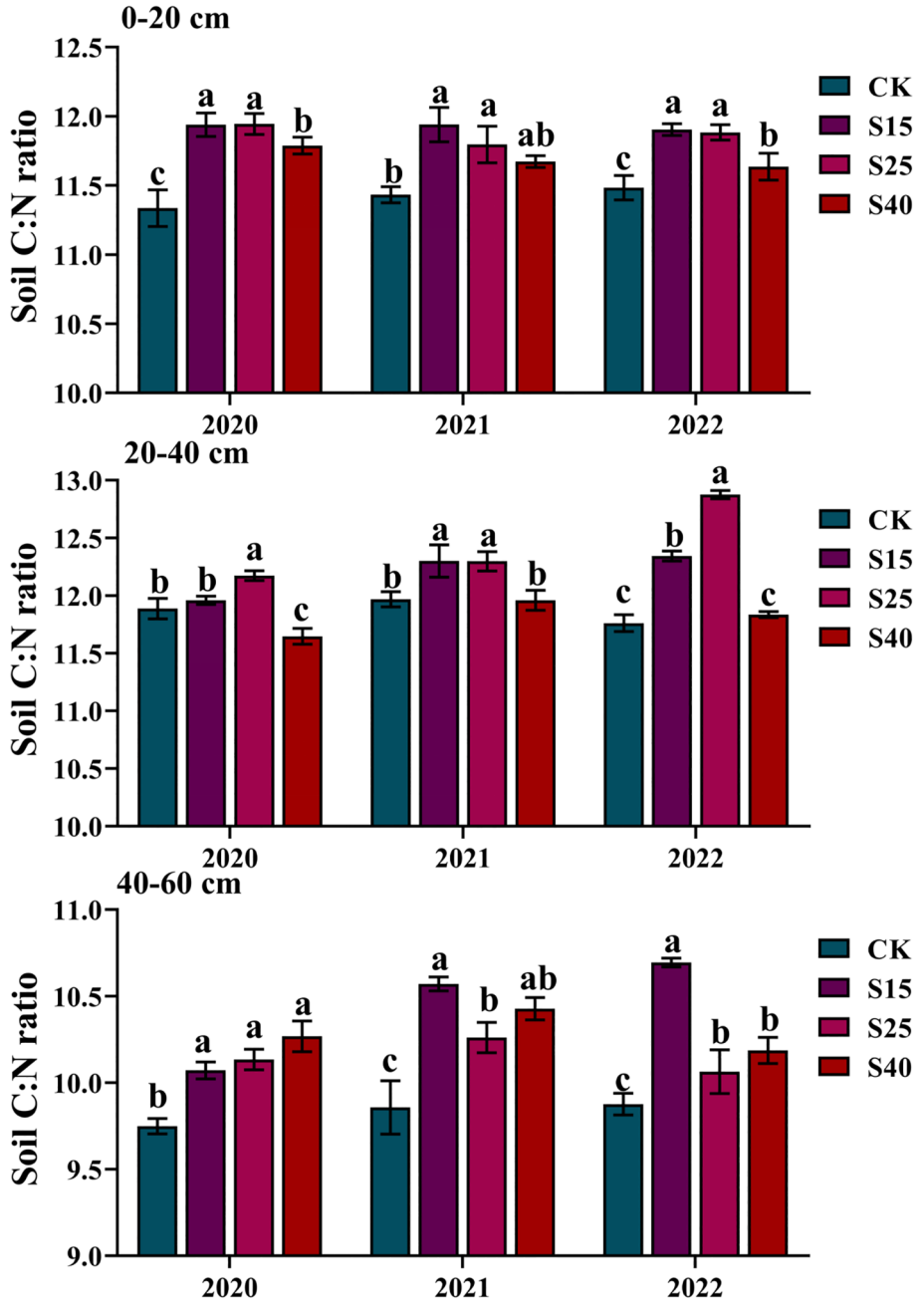

FIGURE 2
Effects of depth of straw returning on C:N ratio. For each soil layer, bars with different letters differ significantly at P< 0.05. CK, no straw returning; S15, straw returning at 15 cm soil depth; S25, straw returning at 25 cm; S40, straw returning at 40 cm.

fixation and nitrogen mineralization increase is common in depth of straw returning treatments under the environment of high carbon-nitrogen ratio, which may be mainly due to straw return treatments changed the soil carbon and nitrogen status (Kramer et al., 2013; Laird and Chang, 2013). Similar to the SOC and TN, the SOC and STN stocks were also higher in the position close to the straw returning. Compared with S40 treatments, S15 and S25 treatments significantly increased the SOC and STN stocks in the 0-40 cm layer. Previous studies have shown that this may be because under the straw returning treatments, the higher carbon and nitrogen release rate in the soil layer of straw returning promoted the significant increase of the SOC and STN content, thus increasing the upper the SOC and STN stocks.

Increasing the yield per unit area on the basis of limited cultivated land is helpful to ensure food security. Important factors affecting the crop yield include temperature, sunshine, precipitation, fertilization management and tillage pattern (Hou et al., 2012; Jat et al., 2018; Tian S. Z., et al., 2016). Improving soil nutrient status and nutrient use efficiency is of great significance to ensure high and stable yield of crops and sustainable production of farmland (Xin et al., 2019). Existing studies have shown that in most soil use types, straw returning treatments can increase the soil nitrogen content, crop

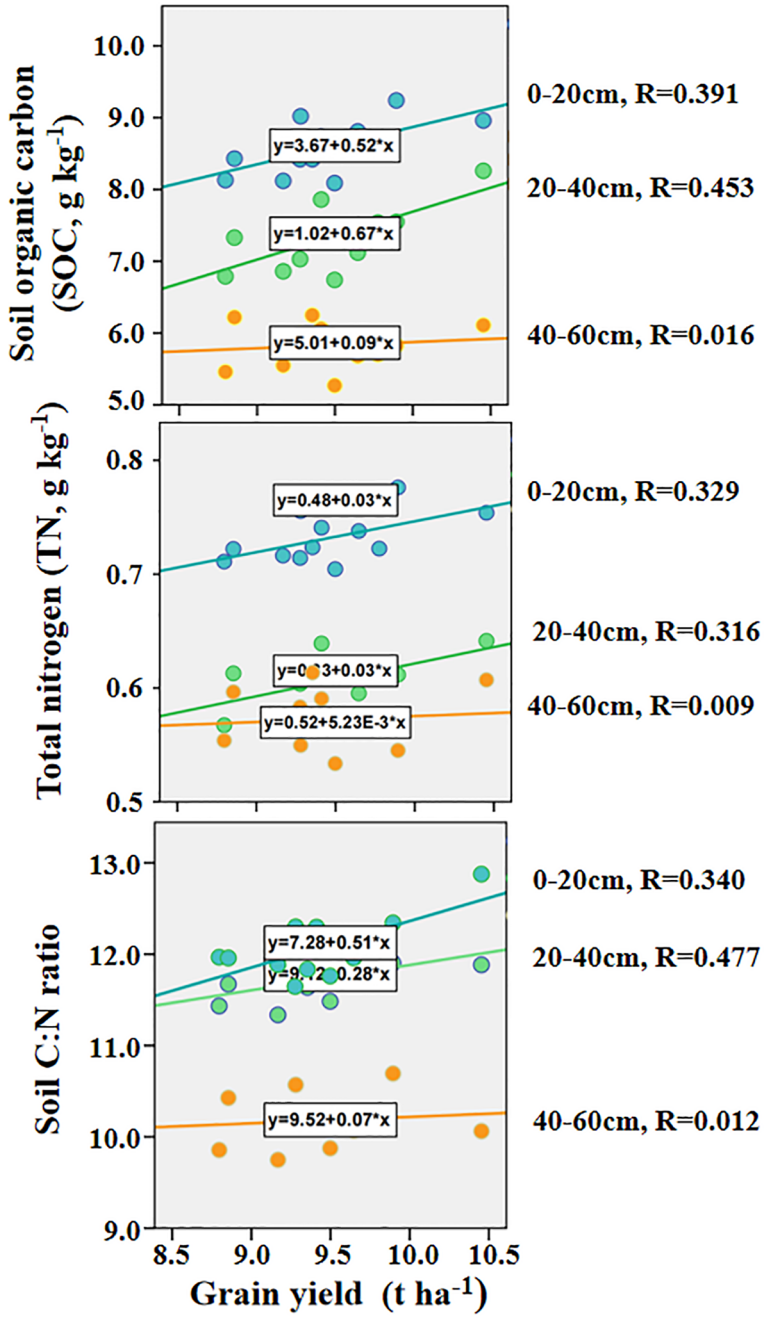

FIGURE 3
The relationship between Grain yield and SOC, TN and C:N ratio at different soil depths from 2020 to 2022. GN, grain yield, SOC, soil organic carbon, TN, total nitrogen, C:N ratio, carbon-nitrogen ratio.

nitrogen use efficiency compared with no straw returning treatments (Liang et al., 2017; Smitha et al., 2019). Under the condition of dry farmland soil environment in Northeast China, the maize yield was effectively improved under the condition of conventional shallow straw returning. In this study showed that the maize yield increased significantly with straw returning treatments, especially under S25 treatments, which is similar to some previous research results (Cai et al., 2014; Tian S. Z., et al., 2016; Zhang et al., 2017). Depth of straw returning treatments was affected soil bulk density and improved crop root architecture, thus promoted the absorption and utilization of nutrients and water to ensure the healthy growth and development of crops (Huang et al., 2013). In this study, the correlation analysis showed that the maize yield was significantly positively correlated with the STC, TN and C:N ratio in the 0-20cm and 20-40cm soil layers, and not significantly correlated with the 40-60cm soil layers. These results indicate that straw returning was beneficial to increase the fixation of the STC and TN in the plough layer, thereby increasing the maize yield. It was further explained that the SOC and TN in the 0

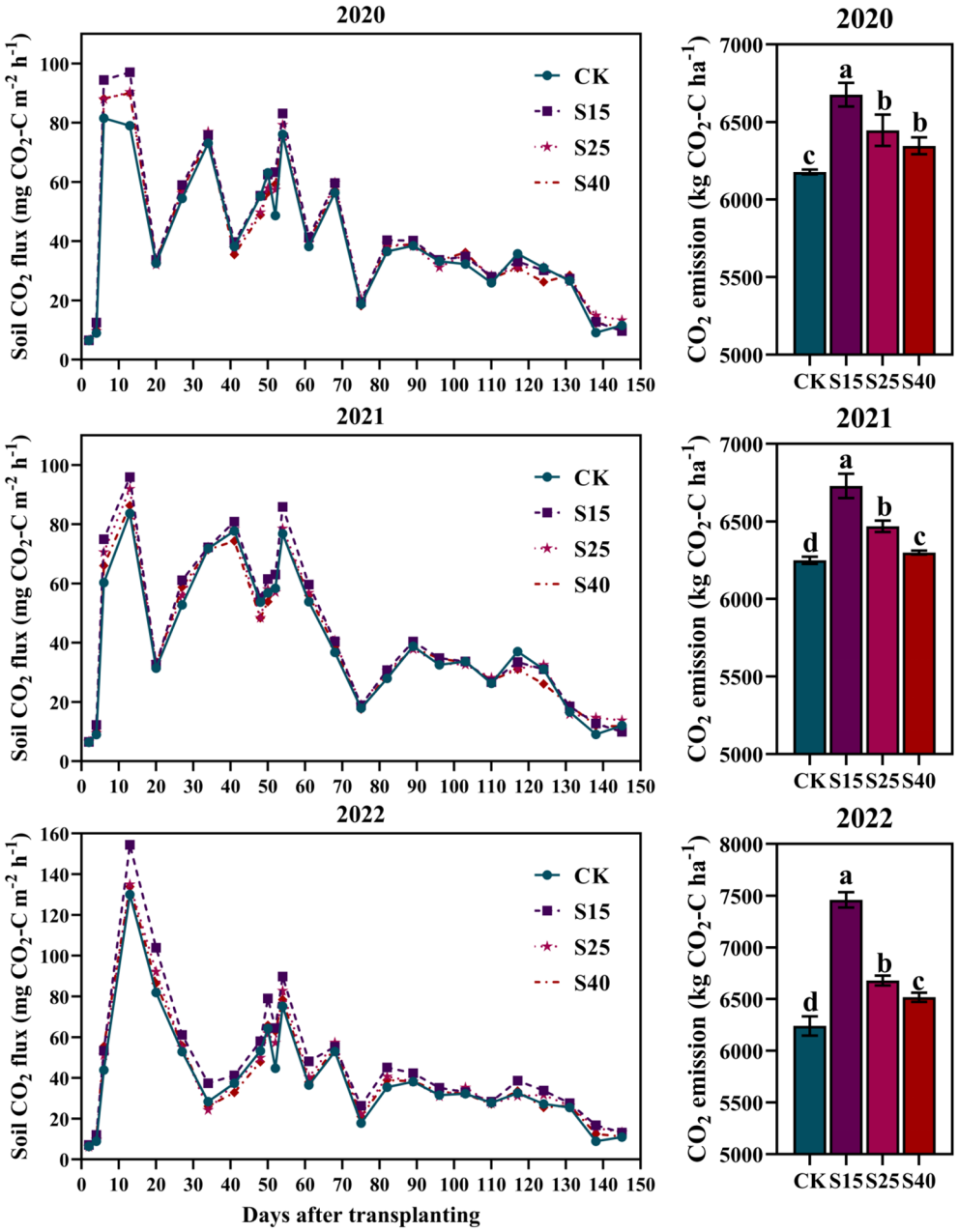

FIGURE 4
Effects of depth of straw returning on dynamics of CO_2 fluxes and cumulative CO_2 emissions. For each year, bars followed by the different letters are significantly different at $P< 0.05$. CK, no straw returning; S15, straw returning at 15 cm soil depth; S25, straw returning at 25 cm; S40, straw returning at 40 cm.

~ 40 cm soil layer could be used as key parameters for maize growth (Kautz et al., 2013; Raiesi, 2017; Zhang et al., 2018).

4.2 Effects of different straw returning depths on greenhouse gas emissions

The ultimate goal of agricultural production is to take into account both economic and environmental benefits, and to ensure the sustainable development of agriculture while increasing the economic yield of crops. In this study, the emission of soil CO_2 increased under all depth of straw returning treatments, which was the same conclusion as some study (Oorts et al., 2007; Bavin et al., 2009; Lenka and Lal, 2013). It shows that straw returning accelerates the decomposition of organic matter and the conversion rate of mineral nutrients by soil microorganisms, thus increased the emission of CO_2. Depth of straw returning treatments have different effects on the environment of different soil layers, and the effects on the CO_2 emissions were also different. In this study, the CO_2 emissions decreased significantly with the increase of straw returning depth. Compared with S15 and S25 treatments, the CO_2

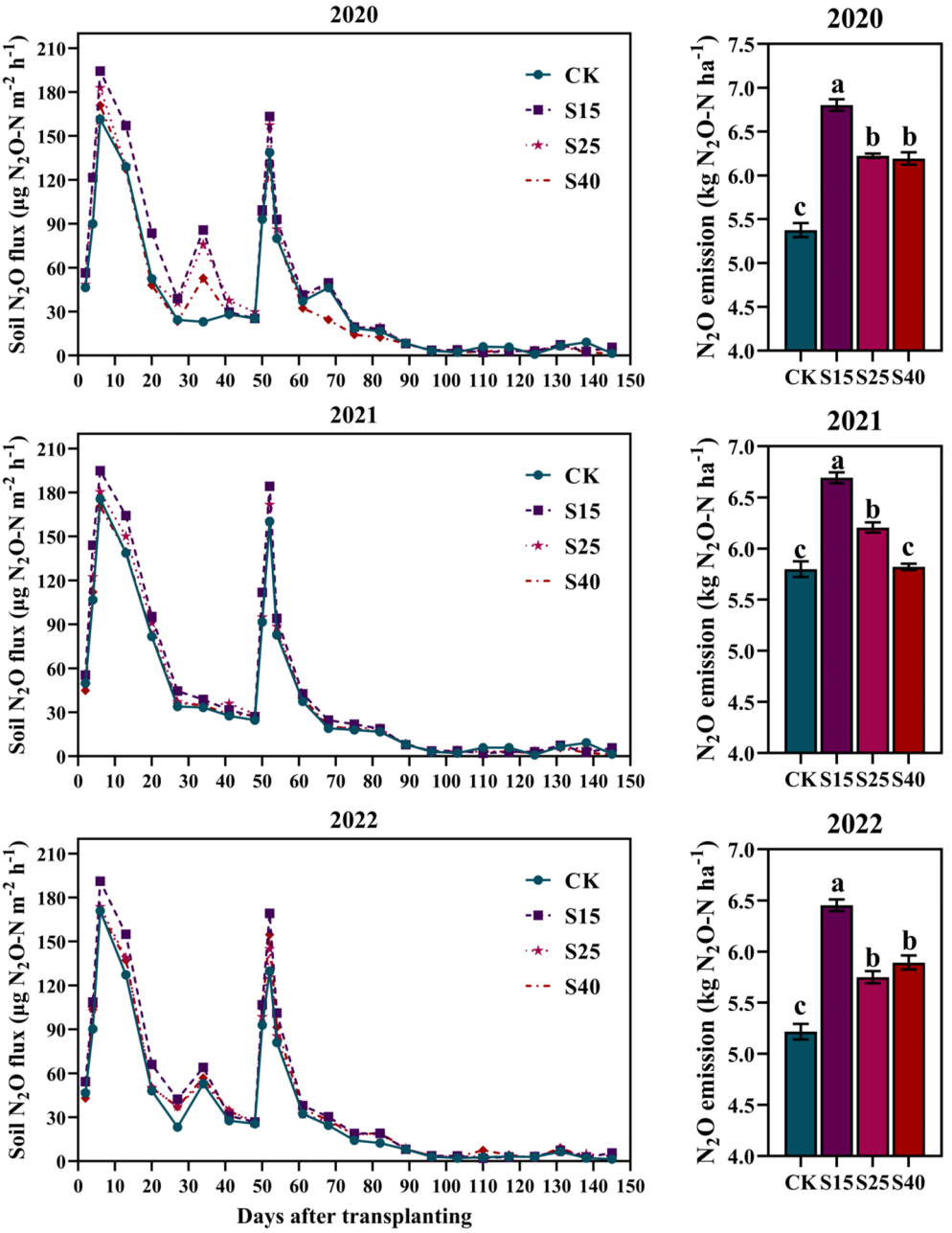

FIGURE 5
Effects of depth of straw returning on dynamics of N_2O fluxes and cumulative N_2O emissions. For each year, bars followed by the different letters are significantly different at P< 0.05. CK, no straw returning; S15, straw returning at 15 cm soil depth; S25, straw returning at 25 cm; S40, straw returning at 40 cm.

emission flux under the S40 treatment was lower under S40 treatments. This study believes that on the one hand, when the straw were returned to the 15 cm and 25 cm soil layers, the soil temperature was higher than that of the 40 cm soil layers, which promotes the CO_2 emissions. On the other hand, when the straw was returned to 40 cm, the deep water content of the soil layer greatly reduced the diffusion rate of CO_2 in the soil pores, so the diffusion of CO_2 to the ground decreased. In addition, some studies have also shown that with the increase of soil depth, soil catalase activity gradually decreased. When straw was returned to 40 cm, aerobic microorganisms increased less, respiration was relatively weak, and the CO_2 emissions were reduced.

There are different views on the impact of straw returning on the N_2O emissions. Some studies have suggested that straw returning has increased the N_2O emissions by changing soil characteristics and stimulating soil microbial activity, thereby promoting denitrification (Sey et al., 2008; Xu et al., 2017; Hu et al., 2019). This study found that depth of straw returning treatments increased the soil N_2O emissions, with significant peaks on the 6th and 52nd days depth of straw returning

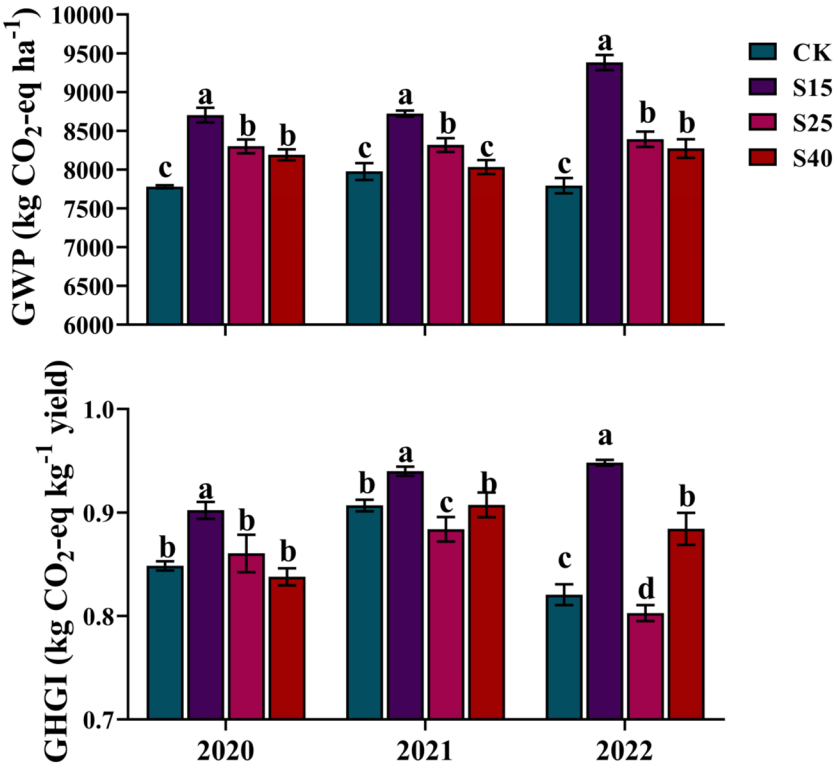

FIGURE 6
Effects of depth of straw returning on GWP and GHGI. For each year, bars followed by the different letters are significantly different at P< 0.05. CK, no straw returning; S15, straw returning at 15 cm soil depth; S25, straw returning at 25 cm; S40, straw returning at 40 cm. GWP, global warming potential, GHGI, greenhouse gas intensity.

treatments, which may be related to fertilization. Fertilization provided a large amount of available nitrogen for soil microorganisms, accelerate nitrification, denitrification and mineralization, and thus promoted the N_2O emissions (Qin et al., 2012; Hu et al., 2019). Soil NH_4^+-N and NO_3^-N are the direct substrates of nitrification and denitrification, and also directly affected the amount of the N_2O emissions (Azeem et al., 2014). Therefore, the emission of N_2O is based on the concentration of available nitrogen in the soil. Straw returning to different soil layers increased the concentration of available nitrogen and the N_2O emissions (Karen and Keith, 2003; Horváth et al., 2010; Hu et al., 2013). When straw returning to the 15 cm soil layers, the cumulative emission of N_2O was the largest, which may be due to the fact that the soil layer was close to the ground and the dry-wet alternation was frequent, and the suitable temperature was conducive to the reproduction of microorganisms, which accelerated the decomposition of straw and promoted the emission of N_2O (Jacinthe and Lal, 2003; Castro et al., 2010). When straw returning to the 15 cm soil layers, the emission of N_2O was relatively small. On the one hand, it is because deep returning reduced soil bulk density, releases nutrients to the deep layer, and increased NO_3^-N, thereby inhibited the activity of denitrifying enzymes. On the other hand, the degree of soil nutrient deficiency in the 40-60 cm soil layer was higher. After straw returning, the fixation of nitrogen by microorganisms was increased, and the concentration of available nitrogen in soil was reduced, thus inhibited the nitrification and denitrification processes and reduced the N_2O emissions (Gebauer, 2013). Some studies have shown that the emission of CH_4 in dryland soil was lower, and it is mostly absorbed (Zheng et al., 2021). This may be because the dryland soil was relatively dry, the ventilation condition was good, and oxygen was more likely to diffuse into the soil, so that the CH_4 was oxidized. It may also be due to the high decomposition rate of organic matter in dryland soil, which is not easy to accumulate organic carbon, thus affecting the production and emission of CH_4. Therefore, the CH_4 emissions were not measured in this study.

The cumulative emissions of the soil CO_2 and N_2O increased after depth of straw returning treatments, which promoted the GWP of S15, S25 and S40 treatments to be significantly higher than CK treatment. It is worth noting that the GWP was decreased with the increase of straw returning depth. The GHGI is an evaluation index of low-carbon agriculture, which takes into account both the crop yield and global warming potential. In this study, the GHGI was shown a different trend from the GWP. Compared with CK treatments, S25 treatments was no significant difference in 2020,

and decreased significantly in 2021 and 2022. This is due to the combined effect of the maize yield and cumulative greenhouse gas emissions, indicating that the appropriate straw returning method can not only be further improved crop yield without the cost of environmental benefits but also improve soil productivity and enhance the carbon sequestration effect of farmland soil, which is an ideal soil improvement and fertilization measure.

5 Conclusion

In this study, compared with CK treatments, depth of straw returning were increased the soil SOC and TN, and improved soil quality. The soil quality-related traits were highly correlated with the maize yield, among which S15 and S25 increased yield more obviously, indicating that the improvement of soil quality by depth of straw returning helped to increase maize yield. The analysis of the greenhouse gas emissions showed that the global warming potential gradually decreased with the increase of straw returning depth, and were significantly higher than that of CK treatments. In order to further evaluate the environmental benefits of straw returning, this study measured the GHGI, and the results showed that S25 treatments were decreased significantly compared with CK treatments. These results indicating that the appropriate straw returning depth of can not only be further improved crop yield without the cost of environmental benefits but also improve soil productivity and enhance the carbon sequestration effect of farmland soil, which is an ideal soil improvement and fertilization measure.

Author's note

We ensure that all maize seeds used in this study originated from Qiqihar Branch of Heilongjiang Academy of Agricultural Sciences in Heilongjiang Province, China. The legality of these seeds complies with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The maize seeds collected in the study are all cultivated maize in China rather than endangered and wild species. These varieties have passed the legal variety certification procedures in China and are licensed for production, planting, and market operations. The authors declare that the cultivation of plants and carrying out study in the Qiqihar maize experiment base of Heilongjiang academy of agricultural sciences complies with all relevant institutional, national and international guidelines and treaties.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

JW: Writing – original draft. YH: Data curation, Writing – review & editing. CZ: Formal analysis, Writing – original draft. TX: Investigation, Writing – review & editing. ZQ: Supervision, Writing – original draft. BM: Software, Writing – review & editing. MY: Methodology, Writing – review & editing. LW: Methodology, Writing – original draft. YL: Software, Writing – original draft. QL: Software, Writing – review & editing. XD: Methodology, Writing – review & editing. CQ: Software, Writing – review & editing. BXM: Software, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Synergizing biotechnology and natural farming: pioneering agricultural sustainability through innovative interventions

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The world has undergone a remarkable transformation from the era of famines to an age of global food production that caters to an exponentially growing population. This transformation has been made possible by significant agricultural revolutions, marked by the intensification of agriculture through the infusion of mechanical, industrial, and economic inputs. However, this rapid advancement in agriculture has also brought about the proliferation of agricultural inputs such as pesticides, fertilizers, and irrigation, which have given rise to long-term environmental crises. Over the past two decades, we have witnessed a concerning plateau in crop production, the loss of arable land, and dramatic shifts in climatic conditions. These challenges have underscored the urgent need to protect our global commons, particularly the environment, through a participatory approach that involves countries worldwide, regardless of their developmental status. To achieve the goal of sustainability in agriculture, it is imperative to adopt multidisciplinary approaches that integrate fields such as biology, engineering, chemistry, economics, and community development. One noteworthy initiative in this regard is Zero Budget Natural Farming, which highlights the significance of leveraging the synergistic effects of both plant and animal products to enhance crop establishment, build soil fertility, and promote the proliferation of beneficial microorganisms. The ultimate aim is to create self-sustainable agro-ecosystems. This review advocates for the incorporation of biotechnological tools in natural farming to expedite the dynamism of such systems in an eco-friendly manner. By harnessing the power of biotechnology, we can increase the productivity of agro-ecology and generate abundant supplies of food, feed, fiber, and nutraceuticals to meet the needs of our ever-expanding global population.

KEYWORDS

biotechnology, natural farming, resistance, bio-fuels, bio-fortification

1 Introduction

The term "sustainability" finds its origin from the Latin word "Sustinere", which denotes the enhancement of environmental quality and the resource base that can uphold and endure future societal development. The term "sustainable" was used for the first time at the United Nations Conference on Human Environment, Stockholm in 1972 focusing on the preservation of environment for the benefit of human beings across the globe. The major outcome of the Stockholm Conference (1972) was the establishment of the United Nations Environment Programme (UNEP), which became the leading global environmental authority for setting the global environmental agenda. Later on in 1992 in Rio de Janeiro, Brazil, the UN General Assembly called for the United Nations Conference on Environment Development (UNCED) commonly known as the Rio Summit or Earth Summit, 1992 with primary goals of socio-economic development while preventing environmental deterioration (Grubb et al., 2019). A number of multilateral environmental agreements have taken place since 1992. However, the global environment has continued to suffer in terms of loss of biodiversity, desertification, and increasing natural disasters.

Over the past two decades, there has been a growing concern about the need for sustainable agriculture to address the food and fiber requirements of society while also providing enduring solutions for both present and future generations. A fundamental prerequisite for sustainable agriculture is to guarantee social equity and economic viability for farmers and all individuals engaged in agriculture and its associated enterprises. This will encourage them to maintain a healthy environment and support the development of climate-resilient agriculture. One of the popular approaches toward sustainable agriculture is natural farming, popularly known as Zero Budget Natural Farming (ZBNF). The Indian civilization thrived on natural farming for ages and India was one of the most prosperous countries in the world. Traditionally, the entire agriculture was practiced using natural inputs where the fertilizers, pesticides, etc. were obtained from plant and animal products. This continued till the advent of colonial rule in India, which introduced plantation agriculture and turned the focus of farmers from self-sufficient crops to cash crops like indigo, jute, tea, and tobacco. Furthermore, the burgeoning population, the pressure to grow cash crops, and drastic climatic calamities led to the shift of the farming sector toward high-input agriculture.

The concept of natural farming was regained by the Japanese scientist Fukuoka in the 1970s through his book *The One Straw Revolution: An Introduction to Natural Farming*, in which he mentioned it as a do-nothing technique. The concept of natural farming revolves around the idea of self-sufficiency of the natural ecosystem without much human intervention. In India, Padma Shri recipient Mr. Subhash Palekar became the first to adopt the ZBNF system in the 1990s. His concern with the increasing indebtedness and suicide among farmers in India due to the increasing costs of fertilizers and pesticides and their long-term devastating effects on the environment compelled him to advocate the use of low-input technologies in agriculture that should be available within farmlands. He started the natural farming concept in Karnataka and subsequently converted over 50 lakh farmers into practicing ZBNF in various states of India. This method promotes soil aeration, minimal irrigation, intercropping, bunds, and topsoil mulching with crop residue and strictly prohibited intensive irrigation like flooding and deep ploughing tillage practices. However, these traditional practices will not be sufficient to provide food to the estimated 9.7 billion population in 2050. Recently, the Indian Council of Medical Research (ICMR) has set guidelines for per person per day calorie intake to achieve nutritional sufficiency (Chellamuthu et al., 2021). Incorporating modern biotechnological techniques into agriculture is the prerequisite to attaining this goal and mitigating the climate crisis (Figure 1).

However, adopting biotechnology in natural farming system is not that easy. There exists an ideological war between natural farming and biotechnology-assisted farming, leading to complete incompatibility among these two systems (Purnhagen and Wesseler, 2021).

Biotechnology in agriculture encompasses a diverse range of techniques, which may include traditional breeding methods that modify living organisms or their components to create or enhance products, improve plants or animals, or engineer microorganisms for particular agricultural applications. It is not exclusive but includes the tools of genetic engineering. It has emerged as a promising tool for crop improvement and led to significant enhancement in agricultural productivity in the 21st century through agricultural revolutions. Within the Indian biotech sector, agricultural biotechnology stands as the third largest segment (as reported by Business Standard in 2013). It is widely

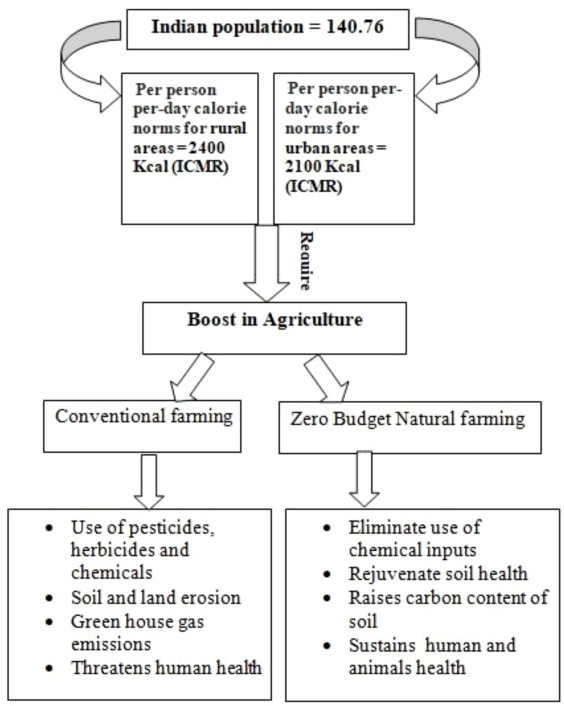

FIGURE 1
Catalyzing sustainable growth through Zero Budget Natural Farming for India's burgeoning population.

recognized as a pivotal sector that plays a significant role in driving the socio-economic development of the country (ABLE INDIA, 2013; Shukla et al., 2018; Lima, 2022). A new biotechnological revolution is estimated to revolve around deciphering the gene codes of living beings leading to "gene revolution".

Biotechnology often carries a perplexing association with industrial, commodity-based farming, monoculture practices, the extensive use of pesticides, and patented seeds. However, the most significant misinterpretation lies in conflating biotechnology—a production process—with an inherently unsafe and perilous product. This misperception forms the foundation of the stringent regulatory framework that many countries apply to biotech crops.

The current review seeks to advocate the idea that integrating biotechnology with natural farming can offer a promising solution to address key challenges in achieving sustainable agriculture. These challenges include the need to produce sufficient food within the constraints of limited arable land and finite resources, particularly in the face of stresses like drought, salinity, high temperature, and diseases. The aim is to achieve these goals while reducing reliance on synthetic fertilizers and pesticides.

2 Strategies for natural farming/ eco-agriculture

McNeely and Scherr (2001) have outlined six approaches to achieve the desired outcomes from natural farming. These are stated below:

Participation of local farmers for the creation of bio-diversity reserves. In Wayanad, Kerala, India, a "model" farm has been developed involving local farmers for the cultivation of a diversity of spices, medicinal plants, cash crops, and wild yet economically important trees (*Syzygium travancorium* and *Cinnamomum malabatrum*). The fauna in this farm consists of farm animals, honeybees, and fish. The economic sustainability of the farm is guaranteed by the consistent revenue generated from a diverse array of crops including medicinal, agricultural, and plantation crops as well as through the management of farm animals.

i. Using traditional practices of controlling pests, rain water harvesting, and soil health management using least external inputs have enabled the self-sustainability of the farm. Development of such modal farms will not only reinforce agricultural productivity but also promote the wellbeing of the ecosystem, thus helping conservation naturally.

ii. Integrating cultivated areas with natural habitats to preserve high-quality wildlife environments that are compatible with farming.

iii. Mitigating or even reversing the conversion of wild lands into agricultural use by increasing farm productivity.

iv. Minimizing agricultural pollution through the implementation of more resource-efficient methods for managing nutrients, pests, and waste.

v. Enhancing the quality of habitats in and around farms through the careful management of soil, water, and vegetation resources. Notably, the "biodiversity-rich hotspot" in Orissa, India serves as an excellent example of this approach. On the global scale, "Equator Initiative" is a worldwide movement committed to identifying and supporting innovative partnerships that alleviate poverty through the conservation and sustainable use of biodiversity.

3 Biotechnological interventions in natural farming

Biotechnology identifies and addresses multifarious aspects of agriculture, leading to a sustainable way of improving the overall productivity of agro-ecosystems. However, we can broadly classify the aspects into three major criteria: modifying plants, modifying the soil, and development of alternatives to fuel inputs for agricultural equipments (Figure 2). These aspects have been discussed in detail in the review.

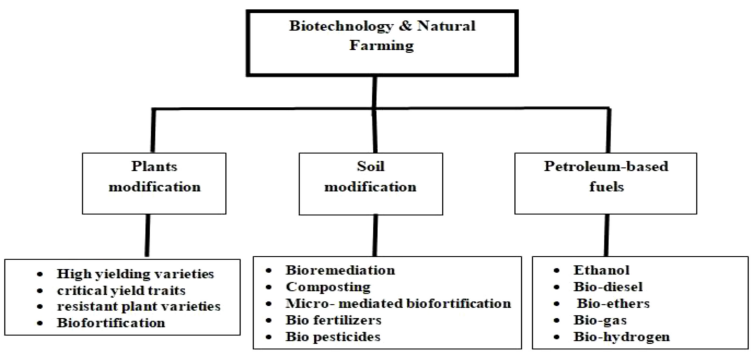

FIGURE 2
Various approaches for integrating biotechnological tools in natural farming system.

3.1 Modifying plants

Conventional plant breeding and selection techniques take much time (six to seven generations) and effort to develop plants with desirable traits. However, when supplemented with novel biotechnological tools like genetic engineering, molecular biology, and micro-propagation, such techniques may result in desirable and stable genotypes within two to four generations (Table 1).

3.1.1 High-yielding varieties

Intergeneric and interspecific hybridization followed by marker-assisted selection (MAS) enabled the development of semi dwarf high-yielding varieties, thus marking the advent of green revolution. Molecular biologists have identified the candidate genes influencing plant height, spike length, seed characteristics, and number of spikelets in wheat (Albahri et al., 2023; Jiang et al. 2023), as well as *DREB* (dehydration-responsive element binding)

TABLE 1 Some examples of successful utilization of biotechnological tools for improving plants.

S. no.	Name of plant	Trait	Candidate gene	Technique used	Reference
1.	Maize	Drought tolerance	*ARGOS8*	CRISPR/Cas9	Shi et al., 2017
		Herbicide tolerance	*IPK1*	ZFN	Shukla et al., 2009; Sedeek et al., 2019
		Northern leaf blight and southern leaf blight	*GST, Htn1, pan1, remorin*	Cloning	Ahangar et al. 2022; Wani et al., 2022; Yang et al., 2017
		Head smut	*ZmWAK*		Wani et al., 2022; Yang et al., 2017
		Maize leaf blight and ear mold	*Hm1*		Wani et al., 2022; Yang et al., 2017
		Quality protein	opaque2, *vte4*, *crtRB1*	Marker-assisted backcrossing and selection	Hossain et al., 2023
		Phytic acid content	ZmIPK	CRISPR/ Cas9, TALEN	Liang et al., 2014; Sedeek et al., 2019
		Drought	betA, TsVP, CSPs, TPP	Overexpression	Wei et al., 2011
2.	Wheat	Armyworm	*Myc transcription factor 7, Methylesterase 7, Polcalcin Phlp 7-like, Alkaline alpha galactosidase 3, Probable galactinol-sucrose*	Cloning	Hafeez et al., 2021
		Resistance to Stem rust (*Puccinia graminis* f. sp. tritici)	More than 63 genes including *Sr13, Sr21, Sr22, Sr31, Sr35, Sr45, Sr46, Sr50, Sr59, Sr60*	Wide hybridization. Backcrossing and MAS (STS, KASP)	Yazdani et al., 2023; Bouvet, 2022
		Resistance to stripe rust (*Puccinina*)	More than 80 genes including *Yr15, Yr45, Yr61, Yr81-83*	Wide hybridization. Backcrossing and MAS	Yang et al., 2023; Li J. et al., 2020
		Yield-related traits (1,000 kernel weight, spike length, spike compactness, flowering time)	*TaTAP46, TaSDIR1, QGw4B.4* QSc/Sl.cib-5A, QSc/Sl.cib-6A *FT-D1, TaCol-B5*	MAS (CASP, dCASP, STARP, KASP)	Song et al., 2023; Liu H. et al., 2022; Chen L. et al., 2022
		Grain quality (protein content, pre-harvest sprouting tolerance)	GPC, *Glu-D1*,	KASP, SSR	Jiang et al., 2021; Song et al., 2023; Rai and Han, 2023
		Heat, cold drought	*TaFER-5B, TaPYL4, ZmPEPC*	Overexpression	Ayadi et al., 2019; Zhang et al., 2019
			TaERF3, TaDREB2	CRISPR	Kim et al., 2018
3.	Rice	Rice blight	*Xa3, Xa4, Xa5, Xa7, Xa10, Xa13, Xa21, Xa23, Xa33, Xa38, Xa40*, and recessive genes	MAS (SSR)	Chukwu et al., 2020; Hsu et al., 2020; Fiyaz et al., 2022
		Grain size and weight	*GS3, Gn1a, GW2, GW5, TGW6, DEP1*	CRISPR/Cas9	Sedeek et al., 2019
		Drought tolerance	*OsPYL9, OsERA1, OsDST*	CRISPR	Ogata et al., 2020; Usman et al., 2022; Santosh Kumar et al., 2021

(Continued)

TABLE 1 Continued

S. no.	Name of plant	Trait	Candidate gene	Technique used	Reference
4.	Oilseed crops like sunflower, soybean, safflower	Oleic acid content	*FAD2*	Mutation breeding	Schuppert et al., 2006; Cao et al., 2013; Msanne et al., 2020
5.	Safflower	γ-Linolenic acid (GLA)	*Δ6DES*	Transgene expression	Nykiforuk et al., 2012
6.	Sorghum	Tiller number, grains per panicle, grain weight	*Bmr2, bmr12, SbSWEET4-3, SbVIN1, SbTST1, SbTST2*	MAS	Zhang et al., 2015; Somegowda et al., 2022
		Plant height	*Dw1, Dw2, Dw3*, and *Dw4*	MAS	Hilley et al., 2016
		Grain quality	*Sh1, SbWRKY, qGW1, KS3*	GWAS and MAS	Kimani et al., 2020
		Flowering and height	*MSD1, MSD3, y1, Wx,DGAT1, AMY3*	GWAS	Rhodes et al., 2017; Dampanaboina et al., 2019
7.	Cherry	Size	*FW2.2/CNR*, Auxin response, cell differentiation, pectin biosynthesis	Bi-parental mapping, association mapping	De Franceschi et al., 2013; Liu Z. et al., 2022
8.	Grapes	Weight	*Aux/IAA9*, DELLA protein	Bi-parental mapping, association mapping	Razi et al., 2020; Doligez et al., 2013; Ban et al., 2016
9.	Logan	Weight	*FW2.2/CNR*, *P450, EXP4*	Bi-parental mapping,	De Mori and Cipriani, 2023
10.	Walnut	Weight, size	Beta-galactosidase, *RBK1*, *BEL1*-like	Association mapping	Bernard et al., 2021

genes associated with photosynthesis, nitrogen utilization and flowering in rice (Ikeda et al., 2001; Chandler et al. 2022; Wei et al., 2022), male sterility, albino phenotype, and number and weight of kernels in maize (Chen et al., 2018; Kelliher et al., 2019). Characterization and manipulation of such genes can help transfer of these into locally adapted high-yielding cultivars by hybridization followed by MAS or by genome editing technologies.

3.1.2 Enhancing physiological efficiency of plants

Genetic manipulation offers the potential to enhance critical yield-determining traits in plants, including photosynthesis, shoot-to-root biomass ratio, inflorescence architecture, stomatal regulation, nutrient acquisition, and utilization efficiency. One effective strategy for assessing and improving photosynthetic efficiency in plants involves the examination and manipulation of key enzymes. Rubisco, a pivotal enzyme responsible for converting atmospheric CO_2 into biomass and a significant player in the global carbon cycle, has been a prime target for enhancing crop production. Methods to boost Rubisco activity encompass enhancing the enzyme's carboxylation capacity, reducing its oxygenation rates through genetic modification, and introducing the complete carbon-concentrating mechanism from cyanobacteria into crop plants via genetic engineering to enhance their photosynthetic capabilities (Hines et al., 2021; Iñiguez et al., 2021). As an example, incorporating Rubisco activase from thermophilic cyanobacteria into plants sensitive to high temperatures has shown promising results in improving crop yield by enhancing photosynthesis under elevated temperature conditions (Ogbaga et al., 2018).

Enhancing photoprotection in plants holds promise for increasing crop yield. Plants have evolved mechanisms to dissipate excess sunlight, safeguarding themselves from damage, albeit at the expense of photosynthetic efficiency (Kromdijk et al., 2016). Research into genes associated with non-photochemical quenching, such as PsbS, has revealed that modifying their expression levels can bolster photoprotection, consequently improving photosynthetic efficiency (Murchie et al., 2015). Likewise, optimizing a plant's nitrogen use efficiency (NUE) involves modulating nutrient absorption, allocation, and metabolism. Employing biotechnology to manipulate key genes governing nutrient uptake and utilization efficiency is an effective strategy for creating enhanced crop varieties. Genes such as Ammonium transport (AMT), nitrate transport (NRT), glutamine synthetase (GS), and glutamate synthase (GOGAT) play pivotal roles in nitrogen metabolism. Studies have demonstrated that transgenic crops overexpressing these genes exhibit elevated tissue nitrogen levels, increased amino acids, and enhanced biomass and greater seed production (Curatti and Rubio, 2014). For instance, the gene OsDREB1C, responsible for promoting nitrogen use efficiency and resource allocation while shortening growth, has led to a substantial increase in rice yield, ranging from 41.3% to 68.3% compared to wild types when overexpressed (Wei et al., 2022).

3.1.3 Development of resistant plant varieties

Insect resistance: The development of insect-resistant transgenic plants stands as a remarkable achievement in agricultural biotechnology, with extensive research efforts carried out by both public and private institutions. The introduction of heterologous DNA is commonly accomplished through genetic transformation methods mediated by *Agrobacterium tumefaciens*, biolistic techniques, or a combination of both (Tabashnik et al., 2013; Carrière et al., 2015). Among the most widely commercialized transgenic crops is cotton, which incorporates *cry* genes sourced

from *Bacillus thuringiensis* (Sanahuja et al., 2011). This innovation has proven highly effective in conferring insect resistance (Tabashnik et al., 2013; Carrière et al., 2015). Furthermore, various other notable examples of introducing and expressing foreign genes in crop plants include *API* (arrowhead proteinase inhibitor) in wheat, tobacco, and tomato; *OC-I* (cysteine proteinase inhibitor: *oryzacystatin*-I) in rice; *Vgb* (*Vitreoscilla hemoglobin*) in maize and tobacco; *SacB* (*levansucrase*-encoding gene) in tobacco, rye grass, and tobacco; *JERF*-36 (Jasmonic ethylene-responsive factor) in poplar trees; *BADH* (*betaine aldehyde dehydrogenase* gene) in tobacco, maize, and tomato; and *NTHK1* (*Nicotiana tabacum histidine kinase*-1) in tomato and apple (Tabashnik et al., 2008; Wang et al., 2018). Specifically, transgenic plants like cotton (*Gossypium hirsutum*), soybean (*Glycine max*), and maize (*Zea mays*) have demonstrated resistance to lepidopteran and coleopteran larvae (caterpillars and rootworms), leading to substantial reductions in pesticide usage and production costs, all while enhancing crop yields.

Disease resistance: Modifying host–pathogen interactions, signaling mechanisms, and associated proteins has led to the development of disease-resistant crop varieties. In wheat, the cloning and utilization of several adult plant resistance (APR) genes have enabled the creation of transgenic lines resistant to rust and powdery mildew pathogens at both seedling and adult stages (Krattinger et al., 2009; Risk et al., 2013; Ellis et al., 2014). The introduction of the *Lr34* allele, which codes for resistance against leaf rust, into various crops such as rice, barley, sorghum, maize, and durum wheat, as well as Lr67 into barley, has conferred resistance to a wide range of biotrophic pathogens (Risk et al., 2013; Krattinger et al., 2016; Sucher et al., 2017). Advanced techniques like Targeting Induced Local Lesions in Genomes (TILLING) and genome-editing methods such as Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs), and notably Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and Crisper-associated protein (Cas) have become powerful tools in functional genomics and crop breeding. Simultaneous modification of the three homeologs of EDR1 in wheat has resulted in powdery mildew-resistant plants (Zhang Y. et al., 2017). Moreover, rice lines with broad-spectrum resistance to *Xanthomonas* have been created by editing the promoter regions of SWEET11, SWEET13, and SWEET14 genes (Xu et al., 2019). Powdery mildew resistance has been achieved through editing MLO (Mildew Resistance Locus) in various plant species, including wheat (Wang et al., 2014; Acevedo-Garcia et al. 2017), tomato (*S. lycopersicum*) (Nekrasov et al., 2017), and grapevine (*Vitis vinifera*) (Wan et al., 2020).

Herbicide resistance: Weeds are a persistent issue in agriculture, hindering crop growth by competing for essential resources like water, nutrients, sunlight, and space. They also act as carriers for various insects and harmful microorganisms. Uncontrolled weed growth can significantly reduce crop yields, leading farmers to use methods like herbicides containing glyphosate and glufosinate, tilling, and manual weeding to manage them. Glyphosate herbicides work by inhibiting the EPSPS enzyme, vital for producing aromatic amino acids, vitamins and other plant metabolites. However, these methods can lead to problems like groundwater contamination and environmental damage, causing declines in plant and animal species (Mazur and Falco, 1989; Powles, 2018). Biotechnological advancements have given rise to herbicide-resistant crop varieties, such as those tolerant to glyphosate and glufosinate (Tan et al., 2006). These crops are engineered with genes like *CP4-EPSP synthase* and *GOX* (*glyphosate oxidoreductase*), which produce glyphosate-tolerant EPSPs and glyphosate-degrading enzymes (Shaner, 2000; Owen and Zelaya, 2005).

Abiotic stress resistance: The advancement of functional omics and computational biology software and tools has enabled the identification of candidate genes responsible for abiotic stress (AbS) from diverse gene pools. Techniques like RNA-Seq, random and targeted mutagenesis, gene shifting, complementation, and synthetic promoter trapping are valuable for analyzing AbS-responsive genes and understanding tolerance mechanisms, including post-translational modifications (PTM), protein degradation, and interactions with non-coding miRNA (Chantre Nongpiur et al., 2016). Genome-wide association studies (GWAS) have gained popularity for discovering and characterizing stress-responsive genes, which, when introduced into crop plants, enhance their tolerance to various AbS conditions (Le et al., 2021). Chan et al. (2006) reported a total of 13,022 AbS-related ESTs from *Hordeum vulgare*, 13,058 genes from *Oryza sativa*, 17,189 from *Sorghum bicolor*, 2,641 from *Secale cereale*, 20,846 from *Triticum aestivum*, and 5,695 regulators from *Z. mays* using the gene index of the TIGR database (http://www.tigr.org/tdb/tgi/) (Chan et al. 2006). Identifying these ESTs and incorporating them into widely cultivated elite cultivars through *in vitro* mutagenesis, genetic transformation, tissue culture, and MAS using omics tools have resulted in the development of several abiotic stress-tolerant plant varieties (Cassia et al., 2018). However, discovering and maintaining ESTs in a crop is very tedious and time-consuming as compared to maintaining cDNA libraries of the transcribed loci, the majority of which come from DREB/CBF, ERF, NAC, D-ZipI, and WRKY families (Noor et al., 2018; Jeyasri et al., 2021). Additionally, recent research has identified and dissected the QTLs for plant height, spike length, and seed characteristics in recombinant inbred lines by combining linkage mapping and weighted gene co-expression network analysis (WGCNA) (Villalobos-López et al., 2022; Wei et al., 2022).

3.1.4 Bio-fortification

"Bio-fortification," also known as "biological fortification," involves enhancing the nutritional value of food crops by increasing nutrient availability to the consumer population, utilizing modern biotechnology techniques, conventional plant breeding, and agronomic practices (Malik and Maqbool, 2020; Shahzad et al., 2021; Krishna et al., 2023).

Bio-fortification can be achieved by following various conventional approaches like intercropping and mixed cropping or by utilizing biotechnology in modifying rhizosphere of the crops. Intercropping or mixed cropping of cereals along with legumes employs complementation (partitioning resources or reducing competition between species) and facilitation (positive interaction between the species leading to enhanced growth, reproduction, and survival of both) as the major ecological phenomena leading to improved resource use efficiency. Complementarity of nutrient

uptake (N, P, Fe, and Zn) in cereal–legume mixed-cropping/intercropping systems provides a unique advantage for the system to be sustainable in the long run (Dissanayaka et al., 2021; Ebbisa, 2022). Furthermore, plant-growth-promoting microorganisms (PGPMs) enhance the bioavailability of nutrients like P, K, Fe, Zn, and Si to plant roots through chelation, acidification, decomposition of organic matter, and suppression of soil-borne pathogens and can replace inorganic fertilizers and pesticides (Maitra and Ray, 2019; Karnwal, 2021).

Bio-fortification is a socially, economically, and environmentally sustainable approach, especially in developing countries, as compared to alternative fortification strategies. To date, staple crops like rice, wheat, maize, sorghum, and vegetables such as common bean, potato, sweet potato, and tomato have been fortified through genetic manipulation, conventional breeding, and agronomic methods. Cassava, cauliflower, and banana have undergone bio-fortification using both transgenic and breeding techniques, while barley, soybean, lettuce, carrot, canola, and mustard have been bio-fortified through transgenic and agronomic approaches. Transgenic-based approaches offer the advantage of targeting multiple crops once a beneficial gene is identified. Notable successful examples of transgenically fortified crops include high-lysine maize, high-unsaturated-fatty-acid soybean, high-pro-vitamin A and iron-rich cassava, and pro-vitamin A-rich Golden rice. Golden rice, in particular, marked a significant breakthrough with the potential to combat vitamin A deficiency (Burkhardt et al., 1997; Ye et al., 2000; Beyer et al., 2002; Datta et al., 2003; Paine et al., 2005).

3.2 Modifying soils

3.2.1 Bioremediation

Bioremediation is a process that primarily harnesses microorganisms, plants, or microbial/plant enzymes to detoxify and degrade contaminants in various environments. In modern crop production, xenobiotics are predominantly organic compounds that do not readily break down naturally. As a result, their accumulation in the environment can lead to their entry into the food chain and water resources, posing risks to the health of animals and humans (Germaine et al., 2006; Chen et al., 2011). Plant–microbe associations, such as plant–endophytic or plant–rhizospheric partnerships, offer potential for enhancing nutrient uptake and the degradation of organic pollutants, thereby contributing to environmental restoration (Zhang et al., 2017).

Bioremediation of complex hydrocarbons can be through natural attenuation/intrinsic bioremediation (using indigenous microflora for decomposing pollutants), bioaugmentation (applying potential microbes for faster decomposition), bio-stimulation (modifying the microenvironment for facilitating microbial action), and surfactant-assisted biodegradation (Kebede et al., 2021).

Furthermore, rhizosphere microorganisms can be used to remove heavy metals from soils through biosorption (adsorption of heavy metals on the cell wall constituents, i.e., carbohydrates, proteins, and teichoic acids of microorganisms), bioaccumulation (accumulation of heavy metals inside the cytoplasm through an import-storage system mediated by metal transporter proteins), bioleaching (solubilizing metal sulfides and oxides from ore deposits and secondary wastes), biomineralization (conversion of complex metal ions into carbonates, sulfates, oxides, phosphates, etc. through metabolic pathways), and biotransformation (alteration of metal complexes into those with more polarity to make them water soluble) (Tayang and Songachan, 2021).

Examples of successful utilization of microorganisms for biosorption of complex hydrocarbons include removal of lead and cadmium by *Staphylococcus hominis* strain AMB-2 (Rahman et al., 2019); and cadmium, lead, and copper by fungi *Phanerochaeta chrysosporium* (Say et al., 2001), *Spirulina platensis, Chlorella vulgaris, Oscillatoria* sp., and *Sargassam* sp. (Leong et al., 2021). Bioaccumulation has been shown in *Pseudomonas putida* 62 BN (Rani et al., 2013), *Bacillus cereus* M116 (Naskar et al., 2020), and fungi *Monodictys pelagic* and *Aspergillus niger* (Sher and Rehman, 2019). Researchers have shown that bioleaching by microorganisms is an economic as well as eco-friendly approach toward efficient extraction of metals gold, cobalt, copper, uranium, zinc, etc. from low-grade ores (Tayang and Songachan, 2021). Even arsenic bioleaching has been possible with *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* (Zhang and Gu, 2007). Metal immobilization through biomineralization of metals from *Bacillus* sp (Zhang et al., 2019), *Acinetobacter* sp., and *Micrococcus* sp. oxidized toxic As(III) into harmless and less soluble As(III) and decreased its toxicity, as shown by Nagvenkar and Ramaiah (2010).

Rhizoremediation can bolster phytoremediation by promoting the growth of microbial communities and their associated activities, facilitated by root exudation, turnover, and the possible induction of enzymes responsible for degradation due to the secretion of secondary metabolites in plants (Didier et al., 2012). Certain common garden and ornamental plants, including *Glandularia pulchella*, *Aster amellus*, *Portulaca grandiflora*, *Petunia grandiflora*, and *Zinnia angustifolia*, have been recognized for their capacity to degrade pollutants and dyes (Khandare and Govindwar, 2015) and effectively remove polychlorinated biphenyls from the soil (Erdei, 2005; USEPA, 2005; Erakhrumen and Agbontalor, 2007; Passatore et al., 2014; Kurade et al., 2021).

Notably, *Typha domingensis*, in combination with xenobiotics effluent-degrading endophytic bacteria, achieved a substantial improvement in the removal of parameters like biochemical oxygen demand (BOD) (77%), chemical oxygen demand (COD) (79%), total suspended solids (TSS) (27%), and total dissolved solids (TDS) (59%) (Shehzadi et al., 2014). An efficient plant–bacterial synergistic system has been employed for treating substantial volumes of xenobiotic effluents in wastewater wetlands (Kabra et al., 2013) (Table 2).

3.2.2 Restructuring soil through composting

Manure fertilization is a sustainable practice by turning harmful waste into a bioavailable resource. However, improper management can also lead to serious eco-environmental concerns through release of pathogens, toxic micro-pollutants, greenhouse gases, and nuisance odors. Composting, the process of decomposition of complex waste organic matter into the simpler readily assimilable biomolecules, is a sustainable way to address the aforesaid problem but is limited by a

TABLE 2 Some examples of use of biotechnologically modified microbial formulations in agriculture.

S. no.	Trait	Microorganisms involved	Technique used	References
1.	Nutrient solubility, crop yield of soybean	*Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp., *Aspergillus* spp., and *Azotobacter* spp.	Liquid bio-inoculant based on sugar and coconut water	Neneng, 2020
2.	Seed germination in Capsicum	*Serratia liquefaciens* CPAC53, *S. plymuthica* CPPC55, *P. tolaasii* P61, and *P. yamanorum* OLsSf5	Encapsulation of biofertilizers	Quiroz-Sarmiento et al., 2019
3.	Ca alginate Diuron herbicide degradation	*Delftia acidovorans* and *Arthrobacter*	Immobilization	Bazot and Lebeau, 2009
4.	Heavy metal bioremediation	*Cronobacter muytjensii* KSCAS2	Biosorption	Saranya et al., 2018
5.	Lead and cadmium bioremediation	*Monodictys pelagic* and *Aspergillus niger*	Bioaccumulation	Sher and Rehman, 2019
6.	Arsenic bioremediation	*Acidithiobacillus ferrooxidans* and *Acidithiobacillus thio-oxidans*	Bioleaching	Zhang and Gu, 2007
7.	Bioethanol (1-butanol, isobutanol, and isopentanol as ethanol substitutes) production	Yeast (*Saccharomyces cerevisiae*), *Clostridium thermocellum*	Engineering fermentative pathways, non-fermentative keto acid pathways, and isoprenoid pathways	Lane et al., 2020
8.	Hydrogen production	*Caldicellulosiruptor*	Engineering glycolytic pathway	Cha et al., 2013

slow rate (Gautam et al., 2012; Singh et al., 2021). The microorganisms effectively contributing toward composting include fungi (*Ascomycetes*, Fungi imperfecti, Basidiomycetes, *Trichoderma*, and *Phanerochaete*), bacteria (*Bacillus* spp., *Cellulomonas, Cytophaga*, and *Sporocytophaga*), and actinomycetes (*Thermoactinomyces, Streptomyces, Micromonospora*, and *Thermomonospora*). The process of composting is mediated by extracellular production of laccase, which facilitates humification and polymerization in livestock manure. Genetically engineered microbes that produce large amounts of extracellular laccase not only enhance the fertilizer quality of end products but also manage their eco-environmental risks by inactivating pathogens, detoxifying micro-pollutants, and stabilizing organic nutrients, but the process is quite fast, thus preventing the loss of C and N into environment (Jiang et al., 2021; Niu et al., 2021).

3.2.3 Microbe-mediated bio-fortification

There are vitamins and minerals that are required in the human body in trace amounts, but their deficiency is manifested as several physiological disorders. Many of such vitamins and minerals are not even synthesized by plants. A good example is Vitamin B12, which cannot be synthesized by plants; hence, bio-fortification of this vitamin can be achieved by the help of microbes like bacteria and archea in the plant rhizosphere (Ku et al., 2019; Krishna et al. 2023). Phyto-stimulation by plant growth-promoting rhizobacteria (PGPRs) benefits the plants by increasing the nutrient availability (Kaur et al., 2020; Chouhan et al., 2021). Recent research has identified the contribution of PGPRs in the bio-fortification of iron, zinc, selenium, and other elements in several crops (Kaur et al., 2020; Singh and Prasanna, 2020; Mushtaq et al., 2021; Khanna et al. 2023).

3.2.4 Bio-fertilizers

Bio-fertilizers are formulations containing live microbes that contribute to soil fertility enhancement by nitrogen fixation from the atmosphere, phosphorus solubilization, and decomposition of organic matter. This improves nutrient bioavailability and accessibility to plants, leading to enhanced growth and productivity (Okur, 2018; Abbey et al., 2019). Utilizing bio-fertilizers offers several advantages, including cost-effectiveness, increased nutrient availability, improved soil health and fertility, protection against soil-borne pathogens, enhanced tolerance to biotic and abiotic stress, and reduced environmental pollution (Chaudhary et al., 2021; Chaudhary et al., 2022a). Researchers may follow diverse approaches like cultivation on selective media, metabolic analyses through high-performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-MS), proteomic studies using two-dimensional electrophoresis and matrix-assisted laser desorption and ionization coupled to time-of-flight mass spectrometry (MALDI-ToF/MS), and metagenomic/metatranscriptomic tools for identifying potential plant growth-promoting microbes (Pirttilä et al., 2021). Notable examples of bio-fertilizers include nitrogen-fixing microbes such as *Rhizobium*, *Azotobacter*, *Bacillus*, *Clostridium* (Sumbul et al., 2020; Gohil et al., 2022); phosphorus-solubilizing microbes like *Bacillus*, *Rhizobium*, *Aspergillus*, and *Penicillium* (Zhang et al., 2020); potassium-solubilizing microbes (*Bacillus*, *Clostridium*, and *Acidithiobacillus*) (Ali et al., 2021; Chen R. Y. et al., 2022); sulfur-solubilizing microbes (*Bacillus*, *Beggiatoa*, and *Aquifer*) (Kusale et al., 2021); zinc-solubilizing microbes (*Bacillus*, *Pseudomonas*, and *Serratia*) (Nitu et al., 2020); phytohormone-producing microbes (*B. thuringiensis*) (Batista et al., 2021); siderophore-producing microbes (*Pseudomonas* and *Bacillus*) (Sarwar et al., 2020); organic matter-decomposing microbes (*Bacillus*, *Pseudomonas*, and *Trichoderma*) (Baldi et al., 2021; Galindo et al. 2022); and PGPRs such as *Rhizobium*, *Pseudomonas*, and *Bacillus* (Khati et al., 2018; Chaudhary et al., 2022b). Bio-fertilizer

formulation includes the mixture of selected beneficial strain/s with a suitable vehicle that preserves the viability of the microorganisms in either a dormant or metabolically active state during transport, storage, and application (Schoebitz et al., 2013). A successful microbial formulation must overcome the conditions of temperature, humidity, salinity, UV radiation, and water stress present in the soil besides being effective and competitive against the native microbial populations of the soil (Glare and Moran-Diez, 2016). Classically, bio-fertilizers may be formulated and applied in the form of liquid (culture broths or formulations based mainly on water, mineral, or organic oils) or solids (mixing the microorganisms with a solid support, such as vermiculite, perlite, sepiolite, kaolin, diatomaceous earth, natural zeolite, peat, or clay). However, the failure of these to protect the microbes in drastic abiotic conditions has paved the way for introduction of bio-encapsulated microorganisms. The use of encapsulating polymers like alginate, chitosan, gellan gum, gelatine, agar, bentonite, starch, and laponite has proven to be highly effective in increasing the viability of microorganisms by protecting them against the adverse abiotic conditions (Rojas-Sánchez et al., 2022).

3.2.5 Bio-pesticides

Bio-pesticides are naturally occurring compounds or agents derived from animals, plants, and microorganisms, including bacteria, cyanobacteria, and microalgae. They are used for controlling agricultural pests and pathogens. Key advantages of bio-pesticides over chemical pesticides include their eco-friendly nature, target specificity, and non-lethality to non-target organisms. Bio-pesticides are highly effective even in small quantities and break down quickly without leaving problematic residues. They employ multiple modes of action, such as growth regulation, gut disruption, metabolic poisoning, neuromuscular toxins, and non-specific multi-site inhibition (Sparks and Nauen, 2015; Dar et al., 2021). These diverse modes of action against targeted pests reduce the likelihood of resistance development, which is common with chemical pesticides.

Additionally, when microorganisms are used as bio-pesticides in the fields, they not only combat pathogens but also contribute to plant health and soil fertility maintenance through various effects.

Major examples of bio-pesticides include microorganisms like *B. thuringiensis*, *Pseudomonas aeruginosa*, *Yersinia*, and *Chromobacterium* and fungi like *Metarhizium*, *Verticillium*, *Hirsutella*, and *Paecilomyces* (Fenibo et al., 2021). Biochemical pesticides encompass insect pheromones (Ghongade and Sangha 2021; Singh et al., 2021), plant-based extracts and essential oils (Gonzalez-Coloma et al., 2013; Ujváry, 2001), insect growth regulators (Feduchi et al., 1985; Arena et al., 1995), and genetically modified organism (GMO) products, especially RNAi-based plant-incorporated protectants (PIPs) (Parker and Sander, 2017; Wei et al., 2018; Ganapathy et al., 2021).

However, the wider adoption of biopesticides faces limitations such as high production costs, challenges in meeting global market demands, variations in standard preparation methods and guidelines, determination of active ingredient dosages, susceptibility to environmental factors, and relatively slower action.

3.3 Development of alternatives to petroleum-based fuels for agricultural equipments

Presently, a significant number of farmers rely heavily on non-renewable resources like diesel and gasoline to fuel their agricultural equipment. This dependence poses several challenges: (1) the depletion of a finite resource, (2) adverse environmental effects, and (3) vulnerability to unpredictable price fluctuations. Transitioning to biologically derived fuels, commonly known as bio-fuels, such as ethanol or biodiesel, could offer a viable solution. By utilizing crops like maize or soybean for bio-fuel production, farmers may not only insulate themselves from the uncertainties of fuel price hikes but also create an alternative revenue stream. This shift toward bio-fuels aligns with sustainable practices, fostering both economic resilience and environmental stewardship in the agriculture sector.

Bio-fuel is the fuel (solid, liquid, and gaseous) extracted from biomass (living organisms especially plants and microorganisms) (Braun et al., 2008). For the production of bio-fuels, starch-based agrowastes are prominently exploited due to their limited utility for commercial production of animal and human consumables (Nguyen et al., 2010). There are microorganisms that facilitate the production of ethanol, bio-diesel, bio-ethers, bio-gas, syngas, and bio-hydrogen from lignocelluloses degradation and subsequent glucose fermentation. These include *Kluyveromyces marxianus, Clostridium shehatae, Thermoanaerobacter* sp., *Saccharomyces cerevisae, Escherichia coli*, *Zymomonas mobilis*, *Pichia stipitis*, *Candida brassicae, Mucor indicus*, cyanobacteria (*Synechocystis* sp., *Desertifilum* sp., *Synechococcus* sp., *Phormidium corium*, *Synechocystis* sp., *Oscillatoria* sp., and *Anabaena* sp.) (Kossalbayev et al., 2020), and microalgae (*Scenedesmus obliquus*, *Chlamydomonas reinhardtii*) (Martinez-Burgos et al., 2022).

Biotechnology is revolutionizing the production of ethanol from cellulose by harnessing genetically modified yeasts and bacteria, enhancing efficiency and sustainability. However, the major constraints experienced by engineered microbial cell factories include metabolic imbalance as a result of nutrient depletion, metabolite accumulation, evolutionary pressure, genetic instability, or other stress factors. Hence, bio-prospecting (screening native strains isolated from diverse sources for novel and functional enzymes) and analyzing their genome for gene of interest and metabolome for possible alternate pathways to enhance the biofuel production can be useful (Kim et al., 2002; Adegboye et al., 2021). Successful examples include production of higher octane hydrocarbons (substitutes to ethanol such as 1-butanol, isobutanol, and isopentanol with improved fuel qualities), through engineering fermentative pathways, non-fermentative keto acid pathways, and isoprenoid pathways (Lo et al., 2017; Adegboye et al., 2021).

Furthermore, genetic engineering plays a pivotal role in developing high energy-yielding plant varieties, surpassing the output of existing strains. Additionally, biotechnological advancements open doors to the conversion of agricultural waste

into viable fuel sources, making the most of sustainable resources and minimizing environmental impact.

There are microbes like *Gluconobacter sulfurreducens, Actinobacillus succinogenes*, *Proteus* spp., *Shewanella putrefaciens*, *Rhodoferax ferrireducens*, and *D. desulfurcans*, which facilitate the production of bio-electricity (Ieropoulos et al., 2005; Capodaglio et al., 2013).

4 Conventional vs. modern natural farming

Conventional natural farming is basically a do-nothing technique that relies totally on natural inputs for the maintenance of the agro-ecosystem, thus reducing the use of artificial fertilizers and industrial pesticides. Agricultural biotechnology also exploits the natural inputs (microbes, wild relatives of cultivated plants, and agricultural wastes) but amplifies their effects with the application of technology in them. Conventional natural farming requires minimum inputs, hence called ZBNF. On the other hand, biotechnology-assisted natural farming requires financial support in research and development, but once the variety/product is ready to be used in fields, it becomes self-sustainable.

Furthermore, biotechnology is a catalyst for introducing novel concepts, methodologies, products, and procedures essential for problem-solving, particularly addressing the specific requirements of smallholder farmers in developing nations (Thompson, 2008; FAO, 2011; Yuan et al., 2011). Biotechnology-assisted breeding stands out for its unique ability to swiftly integrate advantageous traits from wild crop relatives, enhancing both yield and nutritional benefits. This approach also widens the spectrum of genes in agricultural biodiversity, enhancing crop resilience against pests, diseases, and the impacts of climate change (Asdal, 2008). The heightened efficiency in selection processes significantly accelerates breeding cycles, expediting the introduction of new plant varieties. In contrast, traditional methods often necessitate years to eliminate unfavorable traits and incorporate desired ones with elite germplasm background.

Agricultural biotechnology holds the promise of addressing critical issues in the pursuit of sustainable agriculture. These challenges include the imperative to produce an ample food supply within the constraints of diminishing arable land and finite resources, notably water, all while contending with various environmental stresses like drought, salinity, and heat.

5 Impact of biotechnology-assisted natural farming on

Environmental health: Biotechnology-derived crops have often been associated with concern regarding their potential impact on species abundance and ecosystem biodiversity. However, the utilization of bio-herbicides, as opposed to chemical herbicides, can lead to a reduction in the population and variety of targeted weeds and weed seeds within agricultural systems, all the while mitigating greenhouse gas emissions (Chamberlain et al., 2007).

Additionally, there have been worries about the loss of diversity within crop species (Gepts and Papa, 2003). Nevertheless, research focusing on cotton and soybean varieties in the USA suggests that the introduction of transgenic varieties had little to no discernible impact on genetic diversity (Bowman et al., 2003; Sneller, 2003). Furthermore, numerous public sector collections of germplasm from cultivated crops and their wild relatives exist with the purpose of preserving genetic diversity.

In comparison to conventional insecticide use, Bt crops demonstrate an ability to conserve non-target species, resulting in increased arthropod abundance and diversity (Devine, 2005; Torres and Ruberson, 2005; Cattaneo et al., 2006). They also facilitate more effective biological control of pests that are not susceptible to Bt toxins (Naranjo, 2005).

The non-restricted movements of beneficial arthropods between different cropping systems can facilitate conservation of non-target species in nearby (non-transgenic) crops (Prasifka et al., 2009). One of the major threats to sustainability is the widespread evolution of resistant pest populations. However, the limited selection pressure on insect populations by insect-resistant crops can delay the phenomenon. Furthermore, incorporating the non-biotech-derived crops known as refuges provides susceptible insects to mate with any resistant individuals emerging from Bt crops, resulting in hybrid progeny that cannot survive on insect-resistant plants (Environmental Protection Agency (EPA), 2001).

Economic status: The concept of natural farming is inherently tied to the notion of economic sustainability, emphasizing the need for agricultural practices to be financially viable and capable of generating adequate income to support the livelihoods of farmers and individuals in related sectors (Das et al., 2023). Economic incentives play a pivotal role in driving the widespread adoption of sustainable agricultural practices. Biotechnology-assisted natural farming, for instance, facilitates the efficient implementation of precision agriculture, ultimately leading to cost reduction. The diversification of crops and livestock offers a means to mitigate risks associated with weather extremes, market fluctuations, or disease/pest outbreaks.

Incorporating insect-resistant crops into cropping strategies diminishes the need for expensive chemical insecticides and pesticides. Modified soils aid in water conservation, thereby reducing erosion-induced damage within agro-ecosystems. The preservation of natural resources contributes to the reduction of irrigation costs and enhances long-term productivity.

Social system: Agriculture, as a sector deeply rooted in communities, fosters opportunities and collaborative relationships among farming families and community members. Natural farming, which relies on natural inputs and involves substantial human engagement, not only aligns with cultural traditions tied to farming but also safeguards the community's cultural identity. It acts as an avenue for job creation and wealth generation and spurs economic growth within the community.

6 Conclusion

In conclusion, biotechnology in agriculture has emerged as a multifaceted tool that encompasses a diverse range of techniques,

ranging from traditional breeding methods to advanced genetic engineering. This comprehensive approach has played a pivotal role in the 21st-century agricultural revolutions, contributing significantly to enhanced productivity and the socio-economic development of countries, with agricultural biotechnology standing as a key segment within the Indian biotech sector. The association of biotechnology with industrial farming practices has led to misconceptions and a stringent regulatory framework in many countries. It is crucial to distinguish between the biotechnological production process and the safety of the end product, addressing the misperception that underlies regulatory challenges. Biotechnology, when applied judiciously, addresses various aspects of agriculture, promoting sustainability in three major criteria: improving plants, modifying soil, and developing alternatives to fuel inputs for agricultural equipment.

The integration of functional omics, computational biology, and advanced techniques like RNA-Seq and GWAS to modify critical agro-morphological traits in plants besides altering host–pathogen interactions, signaling mechanisms, and associated proteins holds promise for disease-resistant high-yielding varieties. These advancements are crucial for addressing contemporary challenges, including climate change and resource constraints, in the pursuit of sustainable agriculture.

As we anticipate a new biotechnological revolution focused on deciphering gene codes and the "gene revolution," it is imperative to foster a balanced understanding of biotechnology's potential in synergy with natural farming practices. This synergy holds the key to pioneering agricultural sustainability through innovative interventions, encompassing microbe-mediated bio-fortification, bioremediation, restructuring soil through composting, and developing alternatives to petroleum-based fuels for agricultural equipment. By embracing these innovative approaches, we can pave the way for a sustainable future in agriculture that maximizes productivity while minimizing environmental impact and ensuring food security for generations to come.

In terms of environmental sustainability, genetically engineered crops have proven to be advantageous over conventional insecticides, conserving non-target species, enhancing arthropod abundance and diversity, and promoting more effective biological control of pests. The incorporation of insect-resistant crops not only reduces the need for expensive chemical inputs but also contributes to soil modification for water conservation, decreasing erosion-induced damage and lowering irrigation costs. The implementation of refuges alongside insect-resistant crops serves as a strategic measure to delay the evolution of resistant pest populations, emphasizing the importance of maintaining a balanced ecosystem.

The economic sustainability of natural farming is underscored by its inherent link to financial viability and income generation for farmers. Biotechnology-assisted natural farming facilitates precision agriculture, reducing costs and offering a diversified approach to mitigate risks associated with weather, market fluctuations, and disease/pest outbreaks. On a societal level, the social system surrounding agriculture is positively influenced by the adoption of natural farming practices. The alignment of natural farming with cultural traditions fosters a sense of identity and community resilience. It serves as a source of job creation, wealth generation, and economic growth within the community, reinforcing the interdependence of agriculture with social wellbeing.

In conclusion, the impact of biotechnology-assisted natural farming on environmental health, economic status, and social systems demonstrates the potential for a harmonious integration of technological advancements with sustainable agricultural practices.

Author contributions

AB: Conceptualization, Investigation, Writing – original draft. RM: Conceptualization, Writing – review & editing. RR: Funding acquisition, Resources, Visualization, Writing – review & editing. RS: Investigation, Writing – original draft. AW: Conceptualization, Writing – review & editing. TR: Writing – review & editing, Methodology. SM: Writing – review & editing, Data curation. DKJ: Resources, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effectiveness of bio-effectors on maize, wheat and tomato performance and phosphorus acquisition from greenhouse to field scales in Europe and Israel: a meta-analysis

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Biostimulants (Bio-effectors, BEs) comprise plant growth-promoting microorganisms and active natural substances that promote plant nutrient-acquisition, stress resilience, growth, crop quality and yield. Unfortunately, the effectiveness of BEs, particularly under field conditions, appears highly variable

and poorly quantified. Using random model meta-analyses tools, we summarize the effects of 107 BE treatments on the performance of major crops, mainly conducted within the EU-funded project BIOFECTOR with a focus on phosphorus (P) nutrition, over five years. Our analyses comprised 94 controlled pot and 47 field experiments under different geoclimatic conditions, with variable stress levels across European countries and Israel. The results show an average growth/yield increase by 9.3% (n=945), with substantial differences between crops (tomato > maize > wheat) and growth conditions (controlled nursery + field (Seed germination and nursery under controlled conditions and young plants transplanted to the field) > controlled > field). Average crop growth responses were independent of BE type, P fertilizer type, soil pH and plant-available soil P (water-P, Olsen-P or Calcium acetate lactate-P). BE effectiveness profited from manure and other organic fertilizers, increasing soil pH and presence of abiotic stresses (cold, drought/heat or salinity). Systematic meta-studies based on published literature commonly face the inherent problem of publication bias where the most suspected form is the selective publication of statistically significant results. In this meta-analysis, however, the results obtained from all experiments within the project are included. Therefore, it is free of publication bias. In contrast to reviews of published literature, our unique study design is based on a common standardized protocol which applies to all experiments conducted within the project to reduce sources of variability. Based on data of crop growth, yield and P acquisition, we conclude that application of BEs can save fertilizer resources in the future, but the efficiency of BE application depends on cropping systems and environments.

KEYWORDS

meta-analysis, PGPMs, biostimulants, biofertilizers, phosphorus, maize, wheat, tomato

1 Introduction

Over the past century, improvements in agricultural productivity have mainly been driven by the introduction of high-yielding crop varieties combined with the intensive use of agrochemicals (Tilman et al., 2002). However, excessive use of nitrogen (N), phosphate (P) fertilizers, and pesticides has created an array of environmental problems such as groundwater pollution, eutrophication of surface waters, and increased emissions of ammonia (NH_3) and nitrous oxide (N_2O) (Tilman et al., 2002). For P fertilizers, the large mining efforts for rock phosphate precursors and the high rates of P fertilization carried out during the last century led to a "high risk" perturbation of the P cycle (Steffen et al., 2015). At the same time, rock phosphate is a finite resource and high-quality reserves with low co-contamination by toxic heavy metals, are concentrated in a few places around the world (Cordell et al., 2009). Due to historical surpluses in P inputs, large quantities of P have accumulated in most agricultural soils in Europe (Withers et al., 2015). However, 99% of the total P in soil is present in P fractions with strongly limited availability for root uptake, which requires the presence of phosphate anions in the soil solution (Zou et al., 1992; Richardson, 2001). Major soil P fractions comprise inorganic P (P_i) and organic P (P_o) sequestered in soil organic matter (SOM). P_i may be adsorbed to mineral surfaces with Fe/Al oxides and hydroxides, precipitated with calcium (Ca), aluminum (Al), and iron (Fe) or adsorbed to SOM (Hinsinger, 2001; Richardson and Simpson, 2011). Furthermore, a large proportion of soluble P added with fertilizers rapidly becomes unavailable via fixation and will no longer be directly available for plant uptake. Nutrient acquisition can be further impaired by stress factors affecting root development, with increasing impact related to climate change.

Strategies for decreasing the input of N and P fertilizers in agroecosystems and enhancing nutrient use efficiencies include the use of fertilizers based on products of waste recycling (Möller et al., 2018), appropriate timing and placement of fertilizers (Dunbabin et al., 2009; Nkebiwe et al., 2016a), crop genetic potential (van de Wiel et al., 2016) and bio-effectors (BEs) with plant growth-promoting properties (Herrmann et al., 2022). BEs lack significant amounts of nutrients and include a diverse group of living microorganisms and active natural compounds (Weinmann, 2017). To evaluate the potential of BE-assisted production

strategies, the integrated project BIOFECTOR (www.biofector.info; located within the EU 7th framework program) was initiated in 2012 with the aim to investigate perspectives for reducing the input of mineral fertilizers (especially P) and to improve stress resilience in European crop production. The BEs tested included viable plant growth-promoting microorganisms (PGPMs), natural active substances based on extracts from seaweed, plants or compost preparations, humic acids, as well as amino acids, protein- or chitin-hydrolysates (Backer et al., 2018; Halpern et al., 2015).

The term "bio-effector" was coined to cover the whole range of plant growth-promoting properties by microorganisms (PGPMs) and natural active substances (non-microbial biostimulants). The separation of plant growth-promoting properties into categories of bio-control agents acting against pests and pathogens and bio-stimulants with other beneficial functions was intentionally avoided. A whole suite of different mechanisms may be responsible for the plant growth-promoting effect of BEs (de et al., 2015), acting directly or via interactions with native soil organisms. Common modes of action of both microbial and non-microbial BEs, are the induction of plant defense mechanisms against abiotic and biotic stress factors via elicitor-based signaling events (Backer et al., 2018; Thoms et al., 2021) and the stimulation of root growth via direct or indirect interactions with plant hormonal balances (Richardson, 2001; Mäder et al., 2011; Richardson et al., 2011; Bradáčová et al., 2019b; Mpanga et al., 2019b; Moradtalab et al., 2020). Adaptive changes in root morphology are particularly important for the absorption of nutrients with low solubility and mobility in soils such as P (Vacheron et al., 2013). Shifts in the plant hormonal balance can alter root branching, fine root production and root hair development and thus improve plant nutrient acquisition not only due to an increased root surface (Richardson, 2001; Mäder et al., 2011), but also through increased root exudation (Richardson et al., 2011).

Plant-available soil nutrients are an important determinant of the function of BEs (Leggett et al., 2015; Egamberdiyeva, 2007) and the combined application of fertilizers (mineral or organic) with BEs may increase nutrient availability (Gómez-Muñoz et al., 2017; Nkebiwe et al., 2017). Soil pH (Sánchez-Esteva et al., 2016), SOM (Schütz et al., 2018) and the size, composition and activity of the native soil microbial community (Mäder et al., 2011) are important. Wide differences of the effects of BEs on the performance of different plant species and cultivars (Marasco et al., 2013; Timmusk et al., 2014), BE source and application rate (Rose et al., 2014) and across geoclimatic regions (Rose et al., 2014) are observed. Combinations of different strains of PGPMs or non-microbial BEs with complementary and synergistic properties may lead to a larger effect than application of single BEs (Omar, 1997; Han and Lee, 2006; Borriss, 2015; Barea et al., 2005; Bona et al., 2017). BEs may improve plant tolerance to abiotic and biotic stresses (van Oosten et al., 2017).

A steadily increasing number of reviews and meta-analyses on different types of BEs suggests effectiveness of *Azospirillum* spp (Veresoglou and Menexes, 2010)., plant growth-promoting rhizobacteria (PGPR) (Rubin et al., 2017) as well as other microbial and non-microbials BEs such as humic substances (Herrmann et al., 2022; Rose et al., 2014). But there is a large variation in the effects observed after BE application (Schütz et al., 2018; Rose et al., 2014). This may be because systematic meta-studies based on published literature commonly face the inherent problem of *publication bias* where the most suspected form is the selective publication of statistically significant results (Rosenberg, 2005). Furthermore, it has often been reported that effects observed in pot experiments under controlled conditions could not be translated to the field (Richardson and Simpson, 2011). Part of the challenge lies in the fact that the mechanisms behind the observed positive effects are often not known (Yakhin et al., 2017). Therefore, in our contribution to close these knowledge gaps, we have conducted a meta-analysis in which the results obtained from all experiments within the BIOFECTOR project are included. Therefore, it is free of *publication bias*. Moreover. In contrast to reviews of published literature, our unique study design is based on a common standardized protocol which applies to all experiments conducted within the project to reduce sources of variability. The overall hypothesis of the study was that environmental conditions can be identified that favor BE effectiveness. Special emphasis was placed on P as a critical macronutrient for the following reasons: a) It has limited plant availability; b) BEs can induce physical, chemical and biological modifications in plant roots and rhizosphere to favor adaptation to P limitation, which may be beneficial for the acquisition of other nutrients (e.g. stimulation of root growth, rhizosphere acidification, promotion of mycorrhizal associations). Using a common experimental protocol, experiments were primarily conducted with three important crop species representing the European crop production systems: maize (*Zea mays L.*); wheat (*Triticum aestivum L.*) and tomato (*Solanum lycopersicum L.).* Furthermore, a wide range of BE treatments, soils and fertilizers were used in different locations and climatic conditions across Europe and Israel. Data were produced during the years 2013 – 2017 and mean effects of BE application were quantified. Using moderator analysis, we identified experimental conditions under which positive BE effects are most likely to be observed.

2 Materials and methods

2.1 Data source

Experiments were conducted during the years 2013-2017 by 16 BioFector partner institutions (Supplementary Table S1, Supplementary materials). Experimental data were collected directly from the doctoral students and staff responsible for conducting the experiments. This setup enabled us to obtain a considerable amount of background information on the experiments and to cross-check data inputs. Data were entered in a database made in Microsoft Excel. A description of the structure of the database is included in the supplementary information (Supplementary Figure S1). A total of 141 experiments (94 pot and 47 field) were performed. For field trials, an experiment was defined as a one-year growing season. So, if the same experiment was carried out during more than one year, the results from the different growing seasons were regarded as separate experiments.

The eligibility criteria for inclusion in the meta-analysis were: (i) experiments had to include both treatments with addition of BEs and a corresponding control where all conditions were identical except that no BE was added (negative control for BE addition), (ii) data on at least one of the following yield variables must have been reported: shoot dry matter (DM), fruit DM, fruit fresh matter (FM) or grain DM, (iii) one of the three model crops (maize, tomato or wheat) were included. This led to the exclusion of observations from experiments that for instance only reported data on plant height and not DM (Figure 1). From 141 experiments, 136 experiments (89 pot and 47 field) met the eligibility criteria. These 136 experiments yielded 945 observations. An observation is defined as a unique control (untreated)-to-BE (treated) data pair. For each of these observations the number of replicates of the control and BE treatment were recorded and for any response variable (shoot biomass, grain yield etc.), the mean and standard deviation of the control and BE treatment were also recorded.

2.2 BE treatments

A large variety of different BE treatments were applied in the BioFector project (Supplementary Table S2, Supplementary materials). Here BE treatments included both experimental strains/formulations and already marketed products and combinations of single strains and/or extracts. The treatments were grouped according to four overall BE categories (Table 1): Single strain of bacteria; Single strain of fungus; Mixture; and Non-microbial. Investigations on arbuscular mycorrhizal Fungi (AMF) was not within the scope of the BIOFECTOR project. For this reason, there were no experiments conducted explicitly to investigate for AMF effects on crop performance, although AMF effects on root colonization by native AMF fungi were considered in some studies.

2.3 Crops

Three crops with importance for European agriculture were selected in the project representing C3 (wheat, *Triticum aestivum L.*) and C4 (maize, *Zea mays L.*) grain crops as well as fruiting crops (tomato, *Solanum lycopersicum L.)*. Maize was specifically selected due to its early sensitivity to P limitation (Colomb et al., 2000). A full list of cultivars used in the experiments is included as supplementary material (Supplementary Table S3, Supplementary materials). A comprehensive list of fertilizers applied is also given on Supplementary Table S4 under supplementary materials.

2.4 Soil data

Soil characteristics (Supplementary Table S5) were generally obtained on air-dried soils analyzed at the *Landesanstalt für Landwirtschaftliche Chemie* (now renamed Core Facility) at the University of Hohenheim, Stuttgart, Germany. The standard

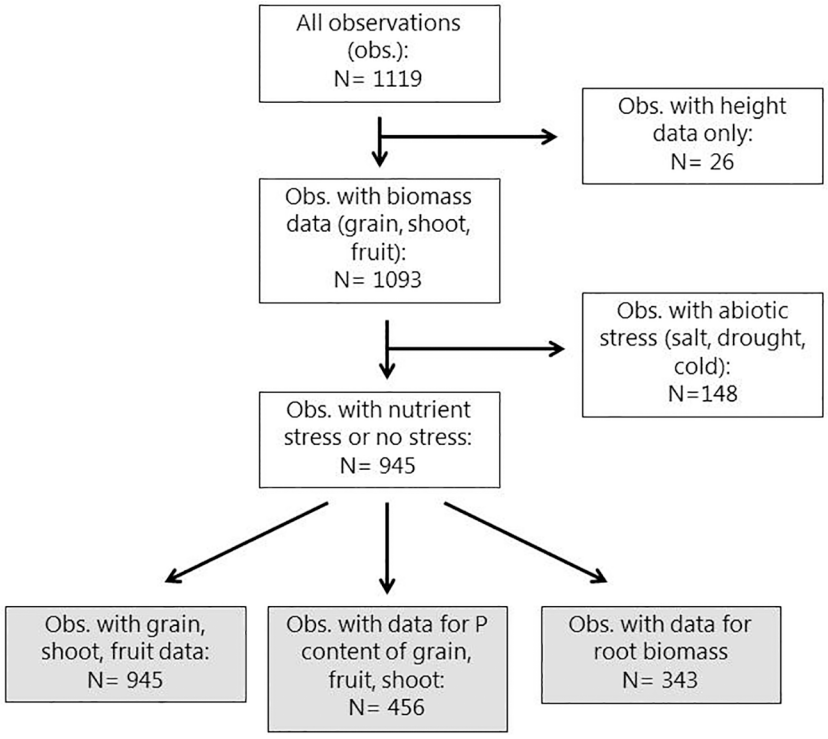

FIGURE 1
Diagram showing the flow of observations in initial handling of data for the meta-analyses. The grey boxes represent subsets of observations used for meta-analyses in the present paper. After excluding the 26 datasets containing observations of plant height only, the remaining 1093 datasets were produced from 136 experiments (47 field and 89 pot) by 16 project partners across the European Union and Israel from 2013 – 2017.

TABLE 1 Overview of BE categories used in this study.

Name of BE* category	Number of datasets	Examples of contents	Type BEs selected in BIOFECTOR	
			Examples Organism/origin	Product names
Single strains of bacteria	471	Isolates of soil bacteria (PGPR)	*Pseudomonas, Bacillus, Paenibacillus, Azotobacter*, etc.	Proradix, Rhizovital,
				Rhizovital42, ABiTEP
Single strain of fungi	163	Isolates of soil fungi	*Trichoderma*, Penecillium,	Trianum-P, Koppert
Mixture	183	> 1 strain PGPM + non-microbial´ BMs + Si, Zn, Mn	*T. harzianum* + *Bacillus* strains + Mn/Zn	Combifector A, AUAS
Non-microbial	128	Humic acids artichoke	N/A	N/A
		Extracts of seaweeds of the genera *Ascophyllum, Laminaria*	*Ascophyllum nodosum*	SuperFifty, BioAtlantis
		Extract of Sorghum roots, killed bacteria	N/A	N/A
	Total 945**			

*There were 139 different BEs of which 106 were tested in experiments included in the meta-analysis database.
**These 945 datasets exclude 148 datasets from experiments conducted under abiotic stress conditions (salinity, drought and cold). Put together, 1093 datasets were obtained from 136 experiments (47 field and 89 pot) by 16 project partners across the European Union and Israel from 2013 - 2017.

methods of the *Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten* (VDLUFA) were used to analyze soil texture, organic carbon content and pH. Soil texture was analyzed according to the VDLUFA standard method C 2.2.1 (Vdlufa-Methodenbuch, 1991); soil organic carbon (SOC) content according to the VDLUFA standard method A 4.1.3.1 (Vdlufa-Methodenbuch, 1991); and soil pH in 0.01 M $CaCl_2$ according to the VDLUFA standard method A 5.1.1 (Vdlufa-Methodenbuch, 1991). Finally, the plant-available soil P was measured using the calcium-acetate lactate-extractable P (P_{CAL}) according to the VDLUFA standard method A 6.2.1.1 (Vdlufa-Methodenbuch, 1991), the Olsen-P (P_{Olsen}) method (Olsen, 1954) or by water extraction method (P_{water}) (Sissingh, 1971). Unlike residual soil P (P_{resid}), which can only be extracted only by strong acids (e.g. HCl and H_2SO_4), P_{CAL}, P_{Olsen} and P_{water}, represent soil P fractions that are apparently available to plant roots and taken up. When data were available for more than one method, P_{CAL} was chosen if the pH was below 7.5 and P_{Olsen} was chosen if the pH was 7.5 or above. P_{water} was only chosen if data was not recorded using any of the two other methods. In pot experiments sand was added in most of the experiments (Supplementary Table S1) to ensure good substrate drainage in the pots. Therefore, the level of available P in the pot experiments was corrected for the addition of sand by assuming a simple dilution effect according to Equation 1:

$$P_{growth\ \ medium} = P_{soil} \times \left(\frac{100\,\% - \%\ \ sand\ \ added}{100\,\%}\right) \quad (1)$$

The same calculation was performed for SOC content.

2.5 Response variables

Meta-analyses were conducted on the following response variables: (i) mass of grain, fruit or shoot, (ii) total P content of grain, fruit or shoot; (iii) root mass. In some experiments more than one yield parameter was measured (for instance straw biomass and grain yield). In these cases, one of the yield types was chosen for each experiment using the following precedence: grain>fruit>shoot biomass.

2.6 Meta-analyses

The response ratio was used as the effect size (Hedges et al., 1999). For each observation, the response ratio (RR) was calculated for the response variable in question according to Equation 2:

$$RR = \frac{\bar{X}_{BE}}{\bar{X}_{control}} \quad (2)$$

where and $\bar{X}_{control}$ are the means of the BE treatment and the corresponding control treatment, respectively. This number is log-transformed according to Equation 3 to maintain symmetry in the analysis (Olkin et al., 2009):

$$\ln(RR) = \ln\left(\frac{\bar{X}_{BE}}{\bar{X}_{control}}\right) = \ln(\bar{X}_{BE}) - ln(\bar{X}_{control}) \quad (3)$$

Calculations of effect size and variance of the individual observations were carried out with the escalc() function of the metafor package for R (Viechtbauer, 2010). Observation, cluster and experiment were included as random factors in multi-level model meta-analysis using the rma.mv() function of the metafor package. Either random (for main effects) or mixed-effects (for moderator analyses) meta-analyses were carried out using the restricted maximum likelihood (REML) estimator. A basic assumption when conducting meta-analysis is independence of data (Borenstein et al., 2011). However, in multiple treatment studies that all refer to one common control the effect sizes will

be correlated (Olkin et al., 2009). Since all the experiments included in our study contributed with more than one observation in the analysis, these observations will therefore not all be independent, thus violating the assumption of independence. This may be handled by aggregating data within experiments (Gattinger et al., 2012), which is often advised (Del Re, 2015) but then information is lost in the analysis. An alternative would be to ignore the dependence of observations in the analyses, which has also been practiced (Schütz et al., 2018; Gattinger et al., 2012; Lori et al., 2017; Skinner et al., 2014). However, we took the dependence of effect size estimates with shared controls into account by not only including the variance of the individual effect size estimates but also the covariances of the dependent effect size estimates (belonging to the same control group). The covariance for each cluster of observations was calculated using data from the shared control according to Lajeunesse et al (Lajeunesse, 2011):

$$covariance = \frac{(sd_{control})^2}{N_{control} \cdot \bar{X}^2_{control}} \quad (4)$$

A variance-covariance matrix was then constructed with the variance estimates from escalc() and the covariances calculated using Equation 4. The resulting variance-covariance matrix was then used as argument in the rma.mv() function.

After analyzing overall BE effects, moderator (subgroup) analyses were carried out using the following moderators: crop type (maize, tomato, wheat); growing conditions (A) (for all crops: controlled, controlled nursery + field (Seed germination and nursery under controlled conditions and young plants transplanted to the field) and field); and growing conditions (B) (for maize only: controlled, field); BE type (four levels: single bacterium, single fungus, mixtures, microbial and non-microbial); fertilizer type based on different P forms and fertilizers based on products of waste recycling: (Control-no P fertilizer, ashes, biochar, compost, digestates, animal waste products, Rock P, sewage sludge, soluble P); soil pH (four levels:<5.5, 5.5-6.5, 6.5-7.5, 7.5-8.5; substrate plant-available P (three levels: low, moderate, optimal, high) and substrate concentration (% OC, five levels: 0-0.5, 0.5-1.0, 1.0-1.5, 1.5-2.0, 2.0-3.0); type of N fertilizer (three levels: organic N, other mineral N, stabilized ammonium); N or P fertilizer application method (four levels: No fertilizer, Fertigation, Placement, Broadcast). For plant-available soil P, we only looked at observations that originated from plots/pots that were not amended with a P fertilizer because the addition of P fertilizers is expected to influence the level of plant-available P in the soil. The models in most cases generated residuals, which were non-normally distributed. Although this is a violation of the assumptions behind the models, but the works of Kontopantelis & Reeves (Kontopantelis and Reeves, 2012; Kontopantelis and Reeves, 2010) indicates that this does not have the potential to fundamentally alter the conclusions. To avoid any selection biases that may occur in our case by rejecting datapoints considered as influential outliers (Habeck and Schultz, 2015), all datapoints were included in the meta-analysis as long as the criteria for experiments in that particular analysis were met.

3 Results

3.1 Geographic distribution of trials

The location of BioFector project partner institutions across Europe and Israel is shown in Figure 2 together with the number of experiments conducted and the resulting number of datasets or observations (BE versus control comparisons) provided by each partner. A total of 141 experiments were conducted from 2013 – 2017 (94 pot and 47 field) leading to 1119 observations (Figure 1). Excluding experiments where only plant height was recorded led to 1093 observations, originating from 136 experiments (89 pot and 47 field) (Figures 1, 2). Out of these, 148 observations with abiotic stresses (cold, drought or salt) other than nutrient (P) limitation were pooled aside for separate analysis.

3.2 BE effects in the context of nutrient acquisition

The RR for yield (e.g. shoot biomass, grain, fruit) from 945 observations of 73 pot and 41 field experiments that were ordered in 290 clusters was 1.093 (P< 0.0001; 95% C.I.: 1.053-1.135) (Figure 3). Observations within the same cluster were not independent (see details in Methods), as a cluster was defined as a group that shares a common control (Olkin et al., 2009). The RR of P content in grain, fruit or shoot from 456 observations belonging to 168 clusters and 53 experiments was 1.083 (P<0.001, 95% C.I.: 1.037-1.131). Furthermore, root biomass (343 observations belonging to 118 clusters and 48 experiments) tended to be positively affected by BE addition (RR= 1.11, P=0.079, 95% C.I.: 0.99-1.24).

Subgroup or moderator analyses identified that crop type (maize, tomato or wheat) significantly affected BE effects (F=8.63; P<0.001). The effect of BE addition on yield was largest in tomato (RR = 1.27, P<0.001, 95% C.I.: 1.17-1.37), smaller in maize (RR=1.06, P<0.01, 95% C.I.: 1.02-1.11) and insignificant in wheat (RR=1.02, P=0.70, 95% C.I.: 0.93-1.11) (Figure 3). The same overall trend (tomato > maize > wheat) was observed in separately analyzed pot experiments, although the effect was less pronounced and not significant (P=0.067).

There was a significant effect of the growing condition on yield (F=3.13, P<0.05). The largest effect on yield (although highly variable) was observed in the controlled nursery (under greenhouse conditions) + field combination (RR=1.35, P<0.05, 95% C.I.: 1.00-1.81), it was smaller under controlled conditions (RR=1.12, P<0.001, 95% C.I.: 1.07-1.17) and only insignificant for field experiments (RR=1.03, P=0.48, 95% C.I.: 0.96-1.10) (Figure 4A). The controlled nursery + field combination was restricted to experiments with tomato. To further separate the effects on yield under controlled conditions versus field conditions, we performed a separate analysis using only results for the BE Proradix (*Pseudomonas* sp. DSMZ13134) tested in maize (Figure 4B), which was the crop/BE combination with the highest number of observations (n=158). A significant effect of the type of

Project partners: Academic institutions and Small and Medium Enterprizes (SMEs)	Experi-ments	Data-sets
UHO a, University of Hohenheim, Group a	37	178
UHO b, University of Hohenheim, Group b	14	83
UHO c, University of Hohenheim, Group c	3	8
UHO d, University of Hohenheim, Group d		
JKI, Julius-Kühn-Institut	5	18
CZU, Czech University of Life Sciences	16	188
BUAS, Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara	12	64
CUB, Corvinus University of Budapest	5	21
UNAP a, University of Naples, Group a	6	78
UNAP b, University of Naples, Group b	6	58
UCPH, University of Copenhagen	5	72
AUAS, Anhalt University of Life Sciences	3	53
FiBL, Research Institute of Organic Agriculture	11	120
AFBI, Agri-Food and Biosciences Institute	4	54
ABI, ABiTEP GmbH	2	15
HKKalke, Arbeitsgemeinschaft Hüttenkalk e.V.	3	54
ARO, Agricultural Research Organization (Volcani)	4	29
DLO, Stichting Dienst Landbouwkundig Onderzoek, research institute		
BIOAT, BioAtlantis Ltd		
Madora, DR.RAUPP E.K. & madora gmbh		
GABO:mi, Gesellschaft für Ablauforganisation: milliarium mbH & Co. KG		
PROPH, Prophyta Biologischer Pflanzenschutz GmbH (Bayer CropScience Biologics GmbH)		
SP, Sourcon Padena GmbH		
FiBL-P, FiBL Projekte GmbH		
AGRIGES, AGRIGES s.r.l.		
KomTek, KomTek Miljø		
Total	**136***	**1093**

FIGURE 2
Geographical distribution of 16 project partners who provided 1093 datasets from 136 experiments across the European Union and Israel during the period 2013 – 2017.*136 experiments after excluding 5 experiments containing only plant height data from 141 experiments in total.

the growing condition (F=4.1, P<0.05) with a positive and significant effect of BE addition was seen under controlled conditions (RR=1.07, P<0.001, 95% C.I.: 1.03-1.10), but not under field conditions (RR=1.00, P=0.87, 95% C.I.: 0.96-1.05) (Figure 4B).

Remarkably, all BE types (single bacteria, single fungi, non-microbials, mixtures) promoted very similar yield improvements without induced abiotic stress (P=0.947; Figure 5A), but with induced abiotic stress (salinity, drought and cold), which also increased experimental variability (P=0.65; Figure 5B).

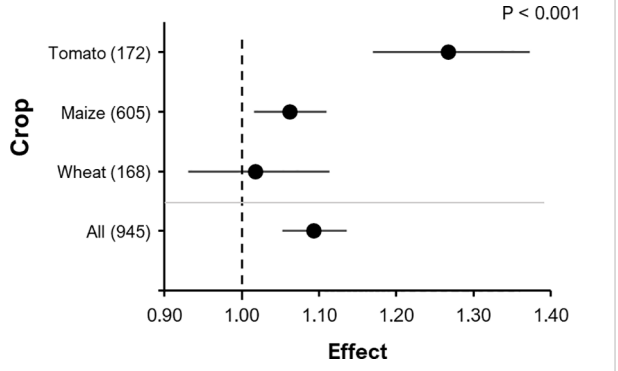

FIGURE 3
The effect of BE addition on either: grain DM, fruit DM, fruit FM or shoot DM according to different crops within all experiments. A total of 945 observations from 290 clusters were included in the analysis. For each crop, the number inside the brackets represent the number of observations included, the point indicates the mean effect while the horizontal line represents the 95% C.I. The p-value indicates whether there was a significant effect of crop type.

Unlike the positive effect of BE addition on yield, the effect on root biomass was not significant but showed only a positive trend (Figure 6A, RR= 1.11, P= 0.079; 95% C.I.: 0.99-1.24). Here, the effect on BE addition of root biomass tended to increase in the following order of BE type: Mixture< Single fungus< Single bacterium< Non-microbial. There was a significant positive effect of BE addition on P content in above-ground biomass (Figure 6B, RR= 1.083, P< 0.001; 95% C.I.: 1.037-1.13). The effect of BE type on above-ground biomass P content was also significant (F=3.5, P = 0.016). Comparably to the effect of BE type on root biomass, the effect of BE type on P content in above-ground biomass increased in the following order of BE type: Single fungus< Mixture< Single bacterium< Non-microbial.

Although the yield RR was not significantly affected by the type of fertilizer applied (manure, ashes, soluble P, control (no P fertilizer), municipal waste composts, rock P, sewage sludge, digestates, biochar) (P=0.155; Figure 7), animal waste products tended to have the strongest increase in the RR of BE addition, whereas Biochar even showed a negative trend. Comparing more specifically, different types of mineral and organic N-fertilization had no significant BE effect on the RR for yield, but a similar trend for highest performance of organic N fertilizers (Supplementary Figure S2). The application method for N-fertilizers (P = 0.96, Supplementary Figure S3A) or P-fertilizers (P= 0.39 Supplementary Figure S3B), including broadcast and localized placement techniques, did not have a significant effect on the RR of yield.

The effectiveness of BE addition on yield was related to substrate properties: pH, % organic carbon (%OC) and plant-available P (Figure 8). There was a tendency towards an increase

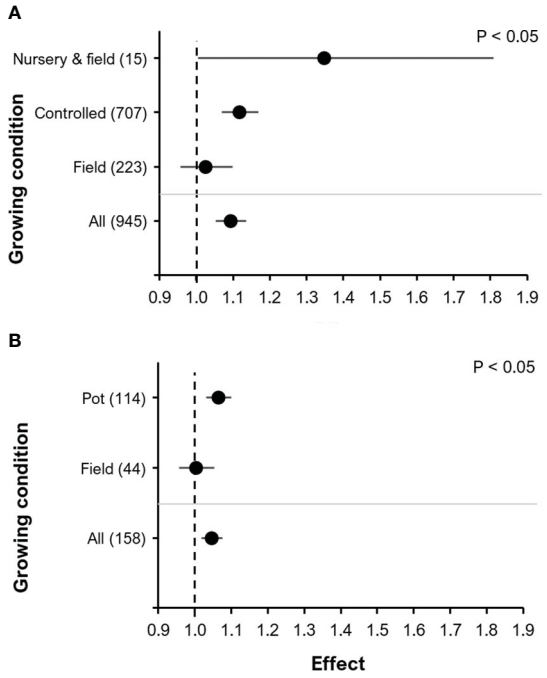

FIGURE 4
The effect of BE addition on yield according to the crop growing condition: Analysis including all observations **(A)**; Analysis only of maize in combination with the BE Proradix **(B)**. In **(A)** the combination "Nursery & field" is included which was only included in experiments with tomato (*Solanum lycopersicum L)*. For each part, the overall mean effect for the given subset of data is included at the bottom (All). For each growing condition, the number inside the brackets represent the number of observations included, the point indicates the mean effect while the horizontal error line represents the 95% C.I. The p-value indicates whether there was a significant effect of crop growing condition.

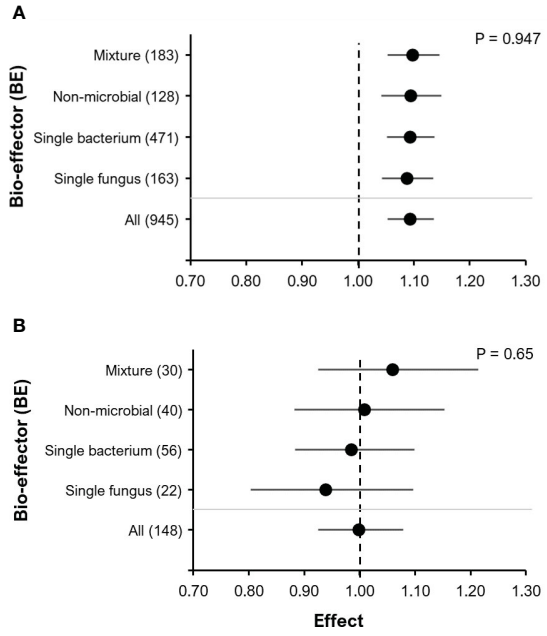

FIGURE 5
The effect of BE addition on yield according to BE type: Experiments without induced abiotic stress **(A)**; Experiments with induced abiotic stress (cold, drought/heat or salt stress) **(B)**. For each part, the overall mean effect for the given subset of data is included at the bottom (All). For each BE type, the number inside the brackets represent the number of observations included, the point indicates the mean effect while the horizontal error line represents the 95% C.I. The p-value indicates whether there was a significant effect of BE type.

in the effect of BE addition on yield with an increase in soil pH (F=1.44, P=0.23) i.e. BE addition tended to have the strongest effect in soils or substrates with an alkaline pH range of 7.5 – 8.5 (Figure 8A). We found a significant effect of %OC on the RR of BE addition to yield with an increase in BE effect on yield with decreasing substrate %OC (F= 3.74, P< 0.01; Figure 8B). Finally, there was also a trend of increasing RR of BE addition on yield with decreasing plant-available P in soils or substrates (Figure 8C) (F=0.33, P= 0.718).

4 Discussion

4.1 Main observations

In the experiments on improved nutrient acquisition, there was a positive relative effect of BE application on crop yield (9.3%) in comparison to the no BE control (Figure 3). The overall mean effect was not as strong as those of other meta-analyses (Herrmann et al., 2022; Schütz et al., 2018; Rubin et al., 2017), most likely because we considered all the results in our project, including also those lacking a positive growth response of BEs. This suggests the possibility of a considerable *publication bias* in more conventional meta-studies on the use of BEs. Rubin et al. (2017) reported a mean effect size of PGPRs of 28% on crop shoot biomass with the highest responsiveness under drought conditions. Similarly, Schütz et al. (2018) observed a mean effect size of approximately 16% on yield response of microbial inoculants applied in dry, tropical or continental climate zones, of which the smallest effect of 8.5% was recorded for trials in temperate continental climate zones. The overall effect size of 9.3% from this meta-analysis may be comparable to the latter (8.5%) possibly because the majority of the field observations in our study were also from temperate continental regions (Figure 2). This also points to a significant impact of the geo-climatic conditions, determining the efficiency of BE-assisted production strategies. In a global network meta-analysis, Herrmann et al. (2022) reported a 25% and 30% BE-induced increase in plant growth and yield, respectively. In comparison, only trials that were conducted within the EU and Israel were included in our current meta-analysis, whereas the study of Herrmann et al. was composed largely of trials conducted in lower- and upper middle-income countries such as India and China.

4.2 Pot versus field effects

Frequently, it is observed that effects of BEs are more reliably obtained under controlled laboratory conditions, where plants are

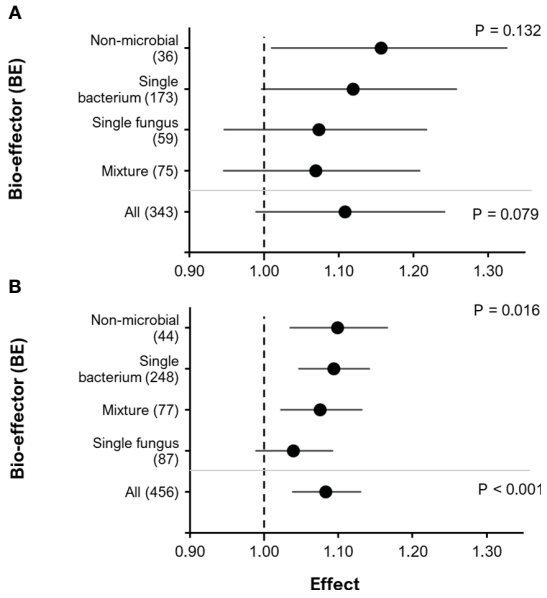

FIGURE 6
The effect of BE addition on root biomass **(A)** and P content in above-ground biomass **(B)** as a function of BE type. For each BE type, the number inside the brackets represent the number of observations included, the point indicates the mean effect while the horizontal error line represents the 95% C.I. The upper p-values indicate whether or not there was a significant effect of BE type on root biomass or P content in above-ground biomass. The lower p-values indicate whether or not there was an overall significant effect of BE addition for all BE types combined.

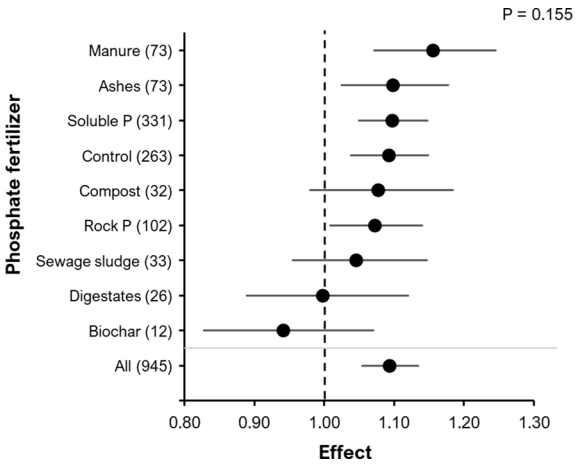

FIGURE 7
The effect of BE addition on yield as a function of the type of fertilizer added in the experiment Manure, guano hair-, feather-, meat and bone meal fertilizers were summarized in the category animal waste products (73); P = phosphate; Control = no P fertilizer applied). The analyzed data are on either: grain DM, fruit DM, fruit FM or shoot DM. A total of 945 observations from 290 clusters and 114 experiments were included in the analysis. For each phosphate fertilizer type, the number inside the brackets represent the number of observations included, the point indicates the mean effect while the horizontal line represents the 95% C.I. The p-value indicates whether there was a significant effect of phosphate fertilizer type.

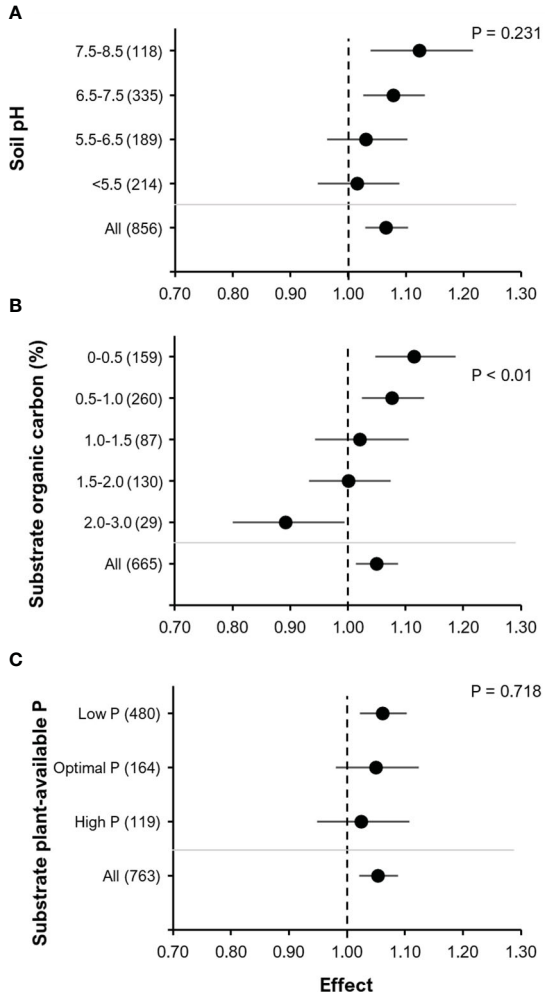

FIGURE 8
The effect of BE addition on yield as a function of the chemical properties of the soil or substrate used: pH **(A)**; % organic carbon concentration (% OC) * **(B)**; plant-available P** **(C)**. For each part, the overall mean effect for the given subset of data is included at the bottom (All). For each level of pH, % OC or available-P, the number inside the brackets represent the number of observations included, the point indicates the mean effect while the horizontal line represents the 95% C.I. The p-values indicate whether there was a significant effect of the chemical property. *The category OC 3.0 – 4.0% is has been excluded because it contains only three datasets (Effect= 1.079, 95% C.I. = 0.8062 – 1.445). ** Substrate plant-available P (mg P (kg dry soil)$^{-1}$): Low P = P_{CAL}< 45 or P_{Olsen}< 20 or P_{H20}< 10; Optimal P = P_{CAL} 45 - 90or P_{Olsen} 20 - 40 or P_{H20} 10< 20; High P = P_{CAL} ≥ 91 or P_{Olsen} ≥ 40 or P_{H20} ≥ 20.

grown in pots, compared to field experiments, where effects are more variable and often insignificant (Richardson and Simpson, 2011). The agronomic potential of biofertilizers for maize yield in pot experiments was higher than in field conditions (Schmidt and Gaudin, 2018). Similarly, we found that the mean effect size was higher in experiments conducted under controlled conditions compared to those conducted under field conditions (Figure 4A). The exception was the specific set of growing conditions starting with a controlled nursery and subsequent transplantation to the field, used for field-grown tomato, which had the largest mean BE effect on yield, but also the largest variability, potentially related to

the lower number of observations. This large effect can most likely not be ascribed solely to the growing conditions. It might at least partially also be influenced by a crop-type induced effect of tomato, as described earlier. Nevertheless, a clear differentiation is not possible based on the available datasets. To further isolate the effect of pot experiments versus field experiments, we investigated the observations originating from the same crop and the same BE treatment. The BE/crop combination with the largest number of observations was the BE Proradix (*Pseudomonas* sp. DSMZ13134) applied in maize (Figure 4B). Similarly, to the complete dataset, we saw a significant effect in pot but not in field experiments. This is in accordance with the stronger yield increase observed for maize inoculated with *Pseudomonas* sp. under pot (24.9%) compared to field (13.8%) conditions (Schmidt and Gaudin, 2018). Particularly for rhizosphere-microbial BEs investigated in our study, efficient root colonization is a prerequisite for the expression of beneficial BE effects (Dobbelaere et al., 2001; Berg et al., 2021). This is achieved more easily under controlled conditions, excluding external stress factors with negative impact on vitality of inoculants, root growth and activity, which did not apply for field conditions. Moreover, pot experiments allowed repeated inoculations of small, densely rooted-soil volumes during the culture period, known to promote root colonization (Nkebiwe et al., 2017). This is not the case for most field experiments, where seed treatments or seeding row inoculations at the begin of the culture period are frequently the only technically and economically feasible options. However, the potential benefits on seedling establishment and early growth do not necessarily translate into comparable yield effects under field conditions (Mpanga et al., 2019b; Dobbelaere et al., 2001). In contrast to our finding that the effect of BEs on crop yield under field conditions was not significant, in another meta-analysis, Li et al. (2022) showed an overall yield increase of 17.9% attributed to biostimulants applied to open field crops. This result is very promising and the difference to the results of this paper can be explained by the type of biostimulants applied. Whereas only 128 of the 945 datasets used in our study is from the application on non-microbial biostimulants (13.5%), the meta-analysis by Li et al. (2022) was focused solely on non-microbial biostimulants (100%). This again highlights efficient root colonization as a prerequisite for the expression of beneficial BE effects for microbial biostimulants under field conditions (Dobbelaere et al., 2001; Berg et al., 2021).

4.3 Crop-specific effects

We observed a larger mean BE effect in tomato compared to the two monocot crops (Figure 3). Rubin et al. (2017) also reported a difference in the effects of PGPR in different crops. They found strong effects (~40% increase) in forbs, legumes and C4 grasses on shoot biomass and insignificant effects in C3 grasses. This is to some extent supported by our data, as we did not observe an overall positive effect of BE addition on the yield of wheat (a C3 grass), whereas we found a significant positive effect in the yield of maize (a C4 grass). The lack of effect in wheat in the present analysis is in accordance with a series of field trials reported by Karamanos et al. (2010), in which inoculation with *Penicilium bilaii* resulted in an increase in wheat P uptake in only a few cases (4 out of 33 experiments). As in our analysis, Schütz et al. (2018) observed stronger effects of BE addition in vegetables as compared to cereals. Similarly, Rho et al. (2018) observed C4 plants to be more responsive to endophyte inoculations than C3 plants when subjected to drought stress conditions. This has also been reported for diazotrophic bacteria used as inoculants (Dobbelaere et al., 2001). This may be attributed to the higher efficiency of C4 photosynthesis under tropical and subtropical conditions, mediating a more efficient carbon supply to microbial inoculants (Bennett et al., 2020). In an unweighted meta-analysis of published studies, also Megali et al. (2015) found large plant-specific differences in the effect of Effective Microorganisms®. Only considering humic substances as BEs, Rose et al. (2014) observed a higher responsiveness of monocots compared to dicots in terms of shoot dry weight increase, while the opposite was true for root dry weight. Focusing on the effects of AMF, also strong growth promotion effects in wheat can be observed (Omar, 1997; Kucey, 1987). Apart from differences between species, there may also be important differences between the effect in different cultivars as shown by Harman et al (Harman, 2006). for *Trichoderma* in maize or by Valente et al. (2020) for *Pseudomonas* in wheat.

4.4 BE-specific effects

Interestingly, there were no significant differences between BE categories, all with a very similar effect size (9-10%) (Figure 5). The meta-analysis by Herrmann et al. (2022) also found no significant differences between BE categories. Overlapping beneficial effects reported for many microbial and non-microbial BEs, based on root growth promotion, scavenging of reactive oxygen species (ROS) or effects on hormonal balances (van Oosten et al., 2017; Sani and Yong, 2022), might partially explain the observed similarities. Furthermore, testing only the most promising BE-crop combinations in the field, based on the results from greenhouse experiments, represents a possible experimental bias, which could explain why in our case the RR differed not as strong as it might have been expected when compared to other studies (Schmidt and Gaudin, 2018; Ansari et al., 2015; Stamford et al., 2007).

In addition to the yield benefits, the application of BE led to a non-significant trend for increased root biomass production (Figure 6A), which was associated to improved BE-induced nutrient acquisition was reflected by increased P accumulation in the shoot tissues (Figure 6B), This may reflect a contribution of BE-mediated root growth promotion to P acquisition as demonstrated in numerous studies on BE functions, conducted within the project (Bradáčová et al., 2019b; Mpanga et al., 2019b; Mpanga et al., 2018; Mpanga et al., 2019a; Weber et al., 2018; Eltlbany et al., 2019), although root length rather than root biomass would be a more reliable indicator in this context. By contrast, mobilization of sparingly soluble P sources by microbial inoculants could not be identified as an important mechanism contributing to P acquisition in most experiments addressed in this meta-analysis (Bradáčová et al., 2019b; Mpanga et al., 2019b; Mpanga et al., 2019a; Lekfeldt et al., 2016; Thonar et al., 2017). This was confirmed also on a more

general basis in a recent review covering the scientific literature on P solubilizing microorganisms as plant inoculants since 1948, coming to a final conclusion that despite significant long-term contributions of native Phosphate Solubilizing Microorganism (PSM) populations in soil to P cycling in ecosystems, PSM inoculants do not mobilize sufficient P to change the crops' nutritional environment under field conditions (Raymond et al., 2021).

When comparing BE responses with other meta-studies, we found that single bacterial strains (Figure 5A) showed a lower mean effect size (9.3%) in our study as compared to that of Rubin et al. (2017), who reported an effect size of 32%. However, the single bacteria effect size of 9.3% is comparable to the results obtained by Veresoglou & Menexes (Veresoglou and Menexes, 2010), who observed increases in wheat grain yield of 8.9% after inoculation with *Azospirillum* sp. The effect size for shoot yield with the same inoculum was higher 17.8%. In comparison to our observations for single fungal strains (8.8%), Leggett et al. (2015) found a more moderate effect of up to 3.7% for the inoculation with *Penicilium bilaii* on the yield in maize (Figure 5A).

Some authors have observed a larger effect when more than one microbial isolate or combinations of microbial and non-microbial BEs were applied (BE consortia) (Bradáčová et al., 2019b; Kumar et al., 2016; Singh et al., 2014). Rubin et al. (2017) observed a superior performance of microbial consortia in enhancing shoot dry weights across several crop species. Also, the meta-study of Schütz et al. (2018) found that a PGPM consortia composed of N-fixers and P-solubilizers were more effective than single inoculations with P-solubilizers.

In contrast, we did not observe a larger effect of using BE combinations as opposed to single BE products in the experiments on improved nutrient acquisition (Figure 5A). However, under conditions with induced abiotic stress (cold, drought/heat or salt stress), a trend of increasing RR according to the BE type was recorded in the order single fungus< single bacterium< non-microbial< mixture (Figure 5B). The low and partially even negative mean effect size of single strain microbial inoculants in this case may reflect the well-documented sensitivity of many beneficial plant-microbial interactions to stress conditions acting during the establishment phase (Backer et al., 2018); which may be compensated by BE combinations with complementary or synergistic stress-protective functions (Bradáčová et al., 2019b; Moradtalab et al., 2020).

4.5 Effect of fertilizers

Special emphasis was put on P acquisition and fertilizer-based products of organic and inorganic waste recycling. Although there was no significant effect of P (Figure 7) or N fertilizers (Supplementary Figure S1) on the BE effect size on yield, we observed trends among different fertilizer types. Largest BEs effects were obtained in combination with fertilizers derived from N and P rich animal waste products, such as manure-based fertilizers, hair-, feather-, meat- and bone-meals. This was confirmed particularly for microbial BEs in numerous studies conducted within the project (Mpanga et al., 2018; Thonar et al., 2017; Li et al., 2018; Vinci et al., 2018b; Vinci et al., 2018a; Bradáčová et al., 2019a; Cozzolino et al., 2021). The supply of organic C from fertilizers might have promoted this effect since many PGPMs are characterized as fast-growing copiotrophic microorganisms with a high demand for easily available carbon sources. Accordingly, Windisch et al. (2021) demonstrated that low rhizosphere abundance of PGPMs in lettuce was associated with limited availability of low molecular weight sugars in the rhizosphere soil solution. Moreover, due to high N and P availability, the respective organic fertilizers could provide a starter fertilization effect, which is a well-documented measure to promote the establishment of symbiotic plant-microbial interactions (Bittman et al., 2006; Chekanai et al., 2018), and likely applies similarly to other PGPMs. Root growth promotion, interactions with the plant hormonal status and mineralization of nutrients in the organic fertilizers induced by the microbial inoculants and/or related soil microbiome shifts are potential modes of action in this context (Richardson, 2001; Eltlbany et al., 2019; Cozzolino et al., 2021). However, we did not find evidence to suggest a larger RR with the application of organic fertilizers in general, which again indicates that the interaction of many factors influences the effectiveness of the BEs.

N-fertilizer form has been shown to influence the effects of BEs on crop yield with stabilized ammonium, leading to the highest increases in yield related with improved P acquisition (Bradáčová et al., 2019b; Mpanga et al., 2019b; Nkebiwe et al., 2017; Mpanga et al., 2018; Mpanga et al., 2019a; Nkebiwe et al., 2016b; Mpanga et al., 2020). The effect of ammonium on BE-induced yield increase could not be captured adequately by this meta-analysis (Supplementary Figure S1) probably because few observations and large variability was associated with the category stabilized ammonium fertilizer) in comparison to other mineral N-fertilizer forms (n = 800). Moreover, the ammonium effect was limited to soils with low P availability and moderate pH buffering capacities, which would not counteract ammonium-induced rhizosphere acidification by plant roots (Bradáčová et al., 2019b; Mpanga et al., 2020).

Although there is some evidence that localized placement of root growth-stimulating stabilized ammonium fertilizers in soil may enhance root colonization of microbial BEs and improve yield (Nkebiwe et al., 2017; Bradáčová et al., 2019a), N-fertilizer (Supplementary Figure S2A) or the P fertilizer (Supplementary Figure S2B) application method did not influence the effect of BE addition. There was large variability in the effect sizes of the different fertilizer application methods. Regarding alternative P fertilizer sources, there is some evidence that the combination of a sparingly soluble P fertilizer like rock phosphate and compost increases P bio-availability (Redel et al., 2019), which may be further improved with BE addition. Additionally, direct use of sewage sludge showed a low RR on yield after BE addition (Figure 7). Alternatively, pyrolyzed sewage sludge (ash) may be used to partially replace rock phosphate in in the production of P fertilizers to improve its plant availability (You et al., 2021). This would also contribute to closing the P cycle and alleviating environmental problems associated with high P losses through unrecycled waste materials.

4.6 Effect of soil properties

A trend towards an increased yield response to BE application with increasing soil pH suggest that P availability, as influenced by soil pH, may play an important role in the mode of action (Egamberdiyeva, 2007). The majority of observations in our study comprised soils with neutral to slightly alkaline pH, limiting P solubility by precipitation of Ca-phosphates and this applied also for many of the tested P fertilizers, such as superphosphate, rock-phosphates, ashes and slags. This may represent a major nutrient limitation mitigated by BE applications (Figure 8A). In addition, Rousk et al. (2009) observed an inhibiting effect of decreasing soil pH on bacterial activity. This may explain why we observed an increase in crop yield with effect of BE addition only at elevated soil pH (6.5 – 8.5) but not at low pH (<6.5) conditions. Also, Schütz et al. (2018) found a positive relationship between soil pH and yield response in their meta-analysis for P solubilizers in combination with N fixers, while for N fixers alone and P solubilizers alone, no and only a weak trend was found respectively. For AMF, there was a tendency towards a bell-shaped curve and related this to an increased availability of macronutrients at an intermediate pH (~7.5). Investigating the effect of the fungus *P. bilaii* in maize, Leggett et al. (2015) did not find a significant correlation between yield response and soil pH. In contrast, Sánchez-Esteva et al. (2016) reported that the effect of *P. bilaii* on wheat plant was affected by soil pH. So, it seems that soil pH might affect the size of BE effects but that the impact of pH seems to depend on other factors such as crop type and their inherent nutrient acquisition strategies.

In accordance with the results obtained in the meta-analysis of Schütz et al. (2018), we observed a decrease in the RR for yield with an increase in the soil/substrate organic carbon content (%OC, Figure 8B). This might be related to a general increase in microbial abundance, diversity and activity with increasing soil organic carbon/matter status as revealed by the meta-analysis of Lori et al. (2017). An increase in soil organic carbon status is reported to increase populations of plant beneficial microorganisms (Francioli et al., 2016). This might in turn hamper the establishment of introduced microorganisms due to increased competition from the native microbial community (Paul, 2016). Moreover, the expected benefits of BE applications may be triggered already by the higher abundance of indigenous beneficial microbes in soils with high organic carbon content. The stimulatory effects of organic carbon supply on microbial BEs might at least partially explain also the observed benefits of combined application with selected organic fertilizers based on manures and animal waste products (Figure 7).

In contrast to Schütz et al. (2018), who observed significant effects of the level of plant-available soil P on the BE RR of yield, we could not find a significant effect of this soil property. Schütz et al. (2018) found that plant-available soil P status (extraction and analytical methods: Olsen, Bray, Mehlich, and AB DTPA-ammonium bicarbonate-diethylenetriaminepentaacetic acid) triggering best performance differ depending on the type of biofertilizers applied. N fixers preferred higher soil P concentrations than P solubilizers. In all cases, BE responses declined at the lowest P availability, but also at higher P levels. Since we evaluated the effect of plant available soil P across different types of BEs on non-legumes, we might have masked or missed BE type specific effects for legumes as reported by Schütz et al. (2018). Nonetheless, similar to Schütz et al. (2018), we still observed a trend of decreasing RR with increasing soil plant-available P status (Figure 8C). This is well explained if under elevated plant available P levels plants can independently acquire sufficient amounts of P for optimal growth and are then less dependent on the support by BEs for nutrient acquisition. However, our data on BE relationships with native soil available P are not directly comparable, since Schütz et al. (2018) considered both, native soil available P and fertilizer P. The trend we observed of an increasing effect of BE addition on yield with decreasing plant-available soil P may be linked to BE-assisted mobilization of naturally inherent soil P or legacy soil P, which may constitute a substantial amount of P after a history of P-fertilizer application in agricultural soils (Yu et al., 2021).

Given the elaborate and transdisciplinary nature of the project (Figure 2), a comprehensive list of 107 biostimulants (microbial and nonmicrobial, Supplementary Table S2) were evaluated on 24 crop*cultivar combinations (Supplementary Table S3), in 94 soils (Table S5) and fertilized with 145 fertilizers (Supplementary Table S4) in 136 different experiments (Supplementary Table S1), explicitly excluding a publication bias. This comprehensive design was well-suited to generate overall summary effects as a first overview to determine the global effectiveness of biostimulants under different conditions. However, a potential limitation of this approach was the high degree of heterogeneity brought in, which made it sometimes challenging to uncover statistically significant differences between levels of different moderators/groupings. To reduce the variability, it may be recommended for future studies to focus now on fewer, more defined classes of biostimulants evaluated with more repetitions and a more specific focus on selected physico-chemical soil or geoclimatic conditions. This may also be well suited for more targeted mode of action studies in addition to agronomic evaluation with the final goal to define conditions and indicators for successful application of biostimulants in agricultural practice.

5 Conclusion and outlook

Bio-effector-based production strategies can offer perspectives to improve plant productivity without acting as direct nutrient sources (Halpern et al., 2015; Du Jardin, 2015). Nevertheless, our study identified limitations for their successful agronomic use. The global biofertilizers market size steadily increased in recent years with several new commercial products emerging every year. In 2019, it was valued at USD 1.0 billion and is anticipated to witness a compound annual growth rate of 12.8% from 2020 to 2027 (Grant review research, 2020). Our results suggest that all BEs stimulate plant growth to a similar extent under conditions representative for European agriculture. Horticultural crops, such as tomato, grown under greenhouse conditions at least during a nursery phase used

for BE inoculation are most promising. To a limited extent, similar benefits were recorded for maize as a field crop, especially when the soil is characterized by a low organic carbon content and a neutral to alkaline pH value. BEs appeared to exert strongest effects when combined with manures and organic N fertilizers and their efficiency declined with increasing soil nutrient status. The similar overall performance of microbial and non-microbial BEs (i.e. seaweed/plant extracts and humic acids) offers flexibility for application strategies and points to improved root growth as a common stimulation mechanism for crop growth. Rhizosphere microbial BEs seem most promising as starter applications promoting seedling establishment and early growth, while non-microbial BEs can be applied more flexible by soil drenching and also as foliar sprays in later stages of the culture period. As all BEs had a similar growth effect, this potentially indicates that common physiological plant growth stimulation mechanisms were involved. Combinations of different BEs with complementary properties may provide an additional option for improved performance under conditions of mild cold stress, drought or salinity, but stronger stress appears to impair beneficial effect.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors through the permanent repository PANGAEA (https://www.pangaea.de/) without undue reservation.

Author contributions

PN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. JL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SS: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. CT: Investigation, Supervision, Writing – review & editing. PM: Supervision, Writing – review & editing. AB-T: Supervision, Writing – review & editing. MH: Investigation, Writing – review & editing. BB: Supervision, Writing – review & editing. KB: Investigation, Writing – review & editing. PC: Investigation, Writing – review & editing. KC: Investigation, Writing – review & editing. VC: Supervision, Writing – review & editing. ED: Investigation, Writing – review & editing. SD: Investigation, Writing – review & editing. JG: Supervision, Writing – review & editing. AL: Investigation, Writing – review & editing. BG-M: Investigation, Writing – review & editing. EK: Supervision, Writing – review & editing. FK: Investigation, Writing – review & editing. ZK: Investigation, Writing – review & editing. MK: Supervision, Writing – review & editing. FM: Investigation, Writing – review & editing. GT: Investigation, Writing – review & editing. NM: Investigation, Writing – review & editing. AP: Supervision, Writing – review & editing. AM: Supervision, Writing – review & editing. DN: Investigation, Writing – review & editing. MS: Supervision, Writing – review & editing. KJ: Investigation, Writing – review & editing. CF: Supervision, Writing – review & editing. AF: Investigation, Writing – review & editing. GP: Supervision, Writing – review & editing. KL: Supervision, Writing – review & editing. BT: Investigation, Writing – review & editing. PT: Supervision, Writing – review & editing. IM: Investigation, Writing – review & editing. NW: Investigation, Writing – review & editing. MW: Funding acquisition, Investigation, Supervision, Writing – review & editing. UY: Supervision, Writing – review & editing. JM: Supervision, Writing – review & editing. TM: Funding acquisition, Supervision, Writing – review & editing. GN: Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. UL: Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. AdN: Conceptualization, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

PN is employed since 01.10.2022 at Yara GmbH Co & KG., Germany. NM is employed since 01.06.2021 at Yara GmbH Co & KG., Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1333249/full#supplementary-material

SUPPLEMENTARY TABLE 1
Overview table with experiments and clusters (control groups) within experiments

SUPPLEMENTARY TABLE 2
List of BE treatments applied. Both single treatments and combinations. Includes information on contents and producers.

SUPPLEMENTARY TABLE 3
List of crops included + cultivars + information on suppliers

SUPPLEMENTARY TABLE 4
List of P and N fertilizers applied

SUPPLEMENTARY TABLE 5
List of soils used with basic info on pH, P content, texture, %C.

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Research on methods for estimating reference crop evapotranspiration under incomplete meteorological indicators

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Background: Accurate estimation of reference crop evapotranspiration (ET_0) is crucial for farmland hydrology, crop water requirements, and precision irrigation decisions. The Penman-Monteith (PM) model has high accuracy in estimating ET_0, but it requires many uncommon meteorological data inputs. Therefore, an ideal method is needed that minimizes the number of input data variables without compromising estimation accuracy. This study aims to analyze the performance of various methods for estimating ET_0 in the absence of some meteorological indicators. The Penman-Monteith (PM) model, known for its high accuracy in ET_0 estimation, served as the standard value under conditions of adequate meteorological indicators. Comparative analyses were conducted for the Priestley-Taylor (PT), Hargreaves (H-A), McCloud (M-C), and FAO-24 Radiation (F-R) models. The Bayesian estimation method was used to improve the ET estimation model.

Results: Results indicate that, compared to the PM model, the F-R model performed best with inadequate meteorological indicators. It demonstrates higher average correlation coefficients (R^2) at daily, monthly, and 10-day scales: 0.841, 0.937, and 0.914, respectively. The corresponding root mean square errors (RMSE) are 1.745, 1.329, and 1.423, and mean absolute errors (MAE) are 1.340, 1.159, and 1.196, with Willmott's Index (WI) values of 0.843, 0.862, and 0.859. Following Bayesian correction, R^2 values remained unchanged, but significant reductions in RMSE were observed, with average reductions of 15.81%, 29.51%, and 24.66% at daily, monthly, and 10-day scales, respectively. Likewise, MAE decreased significantly, with average reductions of 19.04%, 34.47%, and 28.52%, respectively, and WI showed improvement, with average increases of 5.49%, 8.48%, and 10.78%, respectively.

Conclusion: Therefore, the F-R model, enhanced by the Bayesian estimation method, significantly enhances the estimation accuracy of ET_0 in the absence of some meteorological indicators.

KEYWORDS

reference crop evapotranspiration, Penman-Monteith, FAO-24 radiation, meteorological indicators, Bayesian estimation

1 Introduction

Agriculture stands as the largest consumer of freshwater (Food, and Nations, A. O. o. t. U, 2017; Boretti and Rosa, 2019). Efficient freshwater resource utilization in agricultural product production is a pivotal concern for sustainable development (Tunalı et al., 2023). Particularly in arid or semiarid climates, irrigation plays a critical role in food production systems and economies. However, limited available water may not meet the demands of food production, necessitating effective scheduling methods to optimize crop yields with constrained water resources (King et al., 2020; Gu et al., 2021; Zhang et al., 2021). There is a growing emphasis on enhancing water productivity by improving evapotranspiration (ET) efficiency in food production (Xu et al., 2018; Qiu et al., 2021). This shift toward sustainable and efficient water use in agricultural systems underscores the need for precise estimations of crop transpiration and soil ET (Yong et al., 2023a).

Accurate estimation of crop ET is instrumental in on-farm irrigation management, facilitating improvements in irrigation practices and systems (Nyolei et al., 2021). This enhances water productivity, enabling more farmers to derive benefits from limited water resources and achieve increased food production (Perry et al., 2009; Akumaga and Alderman, 2019). Crop water requirement holds a pivotal role in the farm water cycling system. Modern water-saving irrigation theory advocates for deficit-regulated irrigation based on crop water requirement. This approach maximizes yields while maintaining optimal water levels in the root zone and minimizing nutrient losses, disease susceptibility, and operating costs (Tunalı et al., 2023). Reference crop evapotranspiration (ET_0) forms the basis for calculating crop water requirements. Over nearly a century, the estimation methods for ET_0 have been extensively studied globally. Although lysimeters are one of the most accurate tools for direct calculation of ET_0, they are not suitable for this purpose due to their relatively higher cost, the time required for the complex measurements, and their limited accessibility at most sites (Chia et al., 2020). Another common strategy is to calculate ET_0 indirectly using experimental formulae and meteorological factors (Salam and Islam, 2020). The Penman-Monteith model, widely utilized, comprehensively describes ET processes, incorporating meteorological and vegetation physiological characteristics (Monteith, 1965). This model estimates ET as water vapor diffusing from the canopy surface through aerodynamic and gradient methods (Monteith and Unsworth, 2013). Although the ET_0 obtained by the PM model is reliable, it faces limitations due to the stringent requirements for climate data at specific locations (Alam et al., 2024).The Priestley-Taylor (PT) model, a radiation-based approach, calculates actual evapotranspiration using an empirically derived potential ET coefficient α (Kohler et al., 1955). This model minimizes differences in land cover and soil moisture (Priestley and Taylor, 1972). Hargreaves and Samani (1985) introduced the Hargreaves (H-A) model, utilizing maximum and minimum temperatures and extraterrestrial radiation to estimate ET_0. Recognized for its simplicity and accuracy, the H-A model is considered one of the most reliable methods for ET_0 estimation (Jensen et al., 1997). The Mc-Cloud method, relying on average daily air temperature, treats potential ET as an exponential function of temperature. This method is particularly suitable for regions with large temperature variations (Valipour, 2015). The FAO-24 Radiation method, derived from the Makkink formula, exhibits variable accuracy based on altitude (Hauser et al., 1999). Each of these methods contributes to the rich landscape of ET_0 estimation, offering diverse options for addressing the complexities of agricultural water management.

The Penman-Monteith (PM) model has demonstrated applicability to various surfaces across diverse spatial and temporal scales (Allen et al., 2006; Matejka et al., 2009). In order to exclude the impact of climate change on reference evapotranspiration (ET_0), it is necessary to fully consider the impact of different annual rainfall on the evapotranspiration model. Therefore, it is necessary to select a representative hydrological year to verify the model to reflect the universality of the model (Yong et al., 2023b; Latrech et al., 2024). It is recommended as the standard method for estimating ET_0 and serves as a benchmark for validating other evapotranspiration models (Allen, 1998). The PM method exhibits versatility across environments and climates, eliminating the need for local calibration. Extensive validation in various climates, including the use of lysimeter facility, supports its reliability (Landeras et al., 2008; Shiri et al., 2012). Reference evapotranspiration relies on meteorological factors such as radiation, air temperature, humidity, and wind speed, with temperature being the most influential. The PM model, chosen as the standard method for ET_0 estimation, requires daily maximum and minimum temperatures, relative humidity, solar radiation, and wind speeds (Luo et al., 2014). However, a notable limitation of PM models is their demand for an extensive array of

uncommon meteorological data, including relative humidity, solar radiation, and wind speed (Droogers and Allen, 2002; Almorox et al., 2015). In the absence of comprehensive meteorological information, accurately calculating ET_0 using PM models becomes challenging (Feng et al., 2017). Public weather forecasts typically include only weather conditions, maximum and minimum temperatures, wind levels, and wind directions. To address this, four widely used ET_0 estimation models with lower meteorological data requirements have gained prominence. The PT model omits the need for wind speed and humidity data, the H-A model calculates ET_0 based on temperature and solar radiation, the M-C model simplifies ET_0 calculation based on temperature, and the F-R model primarily uses sunshine duration data. An ideal ET_0 estimation method should minimize the number of required meteorological variables without compromising accuracy (Shih, 1984; Traore et al., 2010). Recent studies (Choi et al., 2018; Gao et al., 2021; Yamaç, 2021; Dimitriadou and Nikolakopoulos, 2022; Elbeltagi et al., 2022) have achieved superior ET_0 estimation results compared with traditional methods with limited climate data. As a result, there is a pressing need to comprehend the temporal distribution of crop ET and anticipate its future changes using constrained meteorological information.

In the current study, the calculation of ET_0 is based on the PM model with more meteorological data, or the model with less meteorological data to blur the calculation, but the accuracy is not high. Therefore, in order to accurately calculate ET_0 to successfully monitor crop water requirements and prevent excessive or insufficient irrigation. The primary aim of this study is to conduct a comparative analysis of different ET_0 estimation models under conditions of incomplete meteorological indicators. Additionally, the study seeks to enhance the optimal estimation model to better suit the requirements for ET_0 estimation in the presence of insufficient meteorological data. The most important studies are listed below:

1) Conduct a comparative performance analysis of the PM model and four alternative ET_0 calculation models (H-A, PT, F-R, and M-C), which require fewer meteorological data inputs. Evaluate their effectiveness in estimating ET across various hydrologic years.
2) Investigate and identify a simplified method for calculating ET_0 distinct from the PM model. Explore alternative models or approaches that offer simplicity while maintaining accuracy in ET_0 estimation.
3) Employ Bayesian estimation to rectify the empirical parameters of the optimal ET estimation model.

2 Materials and methods

2.1 Overview of the study area

The Haihe Plain (34°48′–41°3′N, 112°33′–119°50′E), situated in the northern part of the North China Plain, encompasses the plain areas of Beijing, Tianjin, and Hebei, as well as the northern regions of Henan and Shandong Provinces (Figure 1). Renowned as a primary grain-producing region, our study specifically focuses on the large and medium-sized cities of Baoding, Xinji, and Handan within the plain part of Hebei Province. The climate of the Haihe Plain is characterized by a temperate semi-humid and semiarid continental monsoon climate. This climate exhibits four distinct seasons, featuring a dry and windy spring, a hot and rainy summer, a mild and cool autumn with slightly more cloudiness and rain in early autumn, and a cold winter with minimal rain and snow. These pronounced seasonal variations contribute to noticeable changes in ET_0 within the study area.

2.2 Data preparation

The study is conducted in Baoding, Xinji, and Handan cities in Hebei Province, China. Meteorological data were sourced from the Meteorological Information Center of the National Meteorological Administration (http://www.nmic.cn/). The time span covered by the meteorological data is 1991–2019 for Baoding, 2000–2021 for Xinji, and 1991–2019 for Handan. The comprehensive meteorological datasets encompass information such as station name, elevation of the meteorological station, observation time, mean barometric pressure, mean water vapor pressure, mean air temperature, daily maximum temperature, daily minimum temperature, mean relative humidity, 8–8-h rainfall (24-h cumulative rainfall from 8 a.m. to 8 a.m. the next day), mean wind speed, and sunshine hours.

2.3 Selection of typical hydrological years

To mitigate the impact of annual rainfall variations on ET model estimation, a specific hydrological year was carefully chosen for the test area. Separate validations were conducted for each identified typical hydrological year to uphold model accuracy. The selection of typical hydrological years followed a process whereby cumulative annual rainfall data for Baoding City (1991–2019), Xinji City (2000–2021), and Handan City (1991–2019) underwent frequency exclusion. The annual rainfall was then ranked in descending order, and cumulative frequencies were computed for accurate year selection.

$$p = m/(N + 1) \quad (1)$$

where p represents the cumulative frequency, m is the ordinal number of years for rainfall after the treatment of rainfall frequency ranking, and N is the total number of years of rainfall. Utilizing the Pearson Type III curve for fitting, the rainfall values corresponding to p = 25%, 50%, 75%, and 90% are typically considered as the design values for high flow, median water, low flow, and special dry years.

2.4 ET_0 calculation method

The Haihe Plain region experiences four distinct seasons, marked by significant climatic variations. To assess the calculation accuracy of different ET_0 models during each fertility

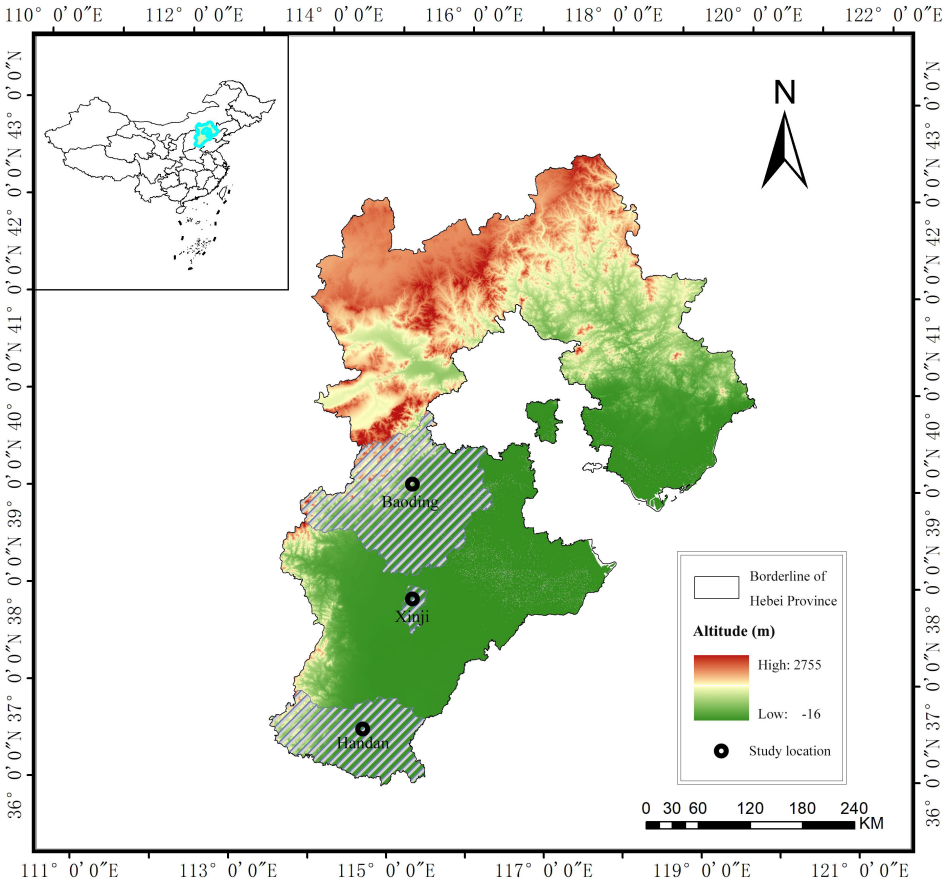

FIGURE 1
Study area.

period of crops, daily ET_0 values for each identified typical hydrological year were computed using five ET_0 models, calculating daily ET_0 values for each typical hydrological year using five ET_0 models, and further obtaining monthly and 10-day ET_0 values.

(1) The Penman-Monteith model

The meteorological data utilized in the model encompass insolation, radiation, temperature, humidity, and wind speed. The Penman-Monteith equations are formulated to accurately predict ET_0 across diverse locations and climatic conditions, although they exhibit high demands for meteorological data. Previous studies applied the Penman-Monteith model in controlled environments (Doorenbos, 1977; Smith et al., 1991), emphasizing the importance of determining evaporative losses in the presence of various natural and anthropogenic land cover interventions. This approach aids in identifying the contributors to evaporative losses. The FAO Penman-Monteith model employed in this study is derived from the original Penman-Monteith equation, aerodynamic drag equation, and surface drag equation, as follows:

$$ET_0 = \frac{0.408\Delta(R_n - G) + \gamma \frac{900}{T+273} u_2(e_s - e_a)}{\Delta + \gamma(1 + 0.34u_2)} \quad (2)$$

where R_n is the net radiation at the crop surface (MJ m^{-2} day^{-1}), G is the soil heat flux (MJ m^{-2} day^{-1}), T is the air temperature at a height of 2 m (°C), u_2 is the wind speed at a height of 2 m (ms^{-1}), e_s is the saturated water vapor pressure (kPa), e_a is the actual water vapor pressure (kPa), $e_s - e_a$ is the difference in saturated water vapor pressure (kPa), Δ is the slope vapor pressure curve (kPa $°C^{-1}$), and γ is the psychrometric constant (kPa $°C^{-1}$).

(2) The Priestly-Taylor model

The meteorological data utilized in the model consist of insolation, radiation, and temperature (Priestley and Taylor, 1972). The PT model establishes a relationship between heat flux and evaporation. Notably simpler than the PM model, it eliminates the need for wind speed and humidity data, rendering it more convenient for application over large areas. However, subsequent studies have indicated that the PT model is better suited for humid areas (Priestley and Taylor, 1972; Pereira et al., 2007) and may not perform as well in arid regions. The formula is as follows:

$$ET_0 = \alpha \frac{\Delta}{\Delta + \gamma}(R_n - G) \quad (3)$$

where α coefficient is mainly considered the influence of aerodynamic factors, in general, taking 1.26; Δ is the slope vapor pressure curve (kPa $°C^{-1}$), γ is the psychrometric constant (kPa $°C^{-1}$), R_n is the net radiation on the surface of the crop (MJ m^{-2} day^{-1}), and G is the heat flux of the soil (MJ m^{-2} day^{-1}).

(3) The Hargreaves model

The Hargreaves model, introduced by Hargreaves and Samani (Hargreaves and Samani, 1985), simplifies the estimation of ET_0. This model necessitates only the average daily maximum and minimum temperatures along with solar zenith radiation (Hargreaves and Allen, 2003), thereby reducing the need for extensive raw data. This characteristic makes it feasible to utilize observations for estimating ET_0 in regions where meteorological data are limited. The formula is as follows:

$$ET_0 = C_0 R_a (T_{mean} + 17.8)\sqrt{T_{max} - T_{min}} \quad (4)$$

where T_{mean}, T_{max}, and T_{min} represent the daily mean, daily maximum, and daily minimum temperatures, respectively; R_a is the atmospheric upper boundary solar radiation; and C_0 is the conversion factor, taken as 0.0023.

(4) The Mc-Cloud model

The Mc-Cloud model, introduced by McCloud in 1955, offers a simplified equation for estimating ET_0 based solely on temperature (McCloud, 1955). The formula is as follows:

$$ET_0 = KW^{1.8T_{mean}} \quad (5)$$

where K and W are constant terms, 0.254 and 1.07, respectively, and T_{mean} is the average temperature, °C.

(5) The FAO-24 Radiation model

The FAO-24 Radiation model, derived from the Makkink formula (Hauser et al., 1999), calculates ET_0 exclusively from solar radiation data. The formula is as follows:

$$ET_0 = a + b\left(\frac{\Delta}{\Delta + \Upsilon} R_s\right) \quad (6)$$

where a and b are empirical coefficients with values of 0.18 and 0.50, respectively; Δ is the slope vapor pressure curve (kPa $°C^{-1}$); γ is the psychrometric constant (kPa $°C^{-1}$), and R_s is the incoming short wave solar radiation, ($MJ{\cdot}m^{-2}{\cdot}day^{-1}$).

2.5 Modifying evapotranspiration models using Bayesian estimation

The ET_0 values for each typical hydrological year were computed using the aforementioned five ET_0 models (Equations 2–6). Simulated values from the PM model served as the standard for analyzing the performance of the H-A, PT, F-R, and M-C models. The objective is to identify the most suitable and recommended model for simplified ET_0 estimation in the Haihe Plain region. Employing Bayesian theory, which involves both prior and posterior distributions, possible outcomes were obtained by reestimating the probability of an event occurring based on estimates of existing data. Bayesian estimation was iteratively applied to infer the model parameters, correcting the empirical parameters of the ET model. This iterative process enhances the model's adaptability and accuracy in the study area.

2.6 Model performance statistics

Utilizing the original eight meteorological data inputs (daily minimum temperature, daily maximum temperature, daily average temperature, geographic latitude and longitude, altitude, average relative humidity, actual sunshine duration, and wind speed), the ET_0 inputs of the PM model were selected as the model's calibration values. Statistical measures, including the R^2, RMSE, MAE, and WI (Equations 7–10) were employed as key factors for evaluating the model. These evaluation metrics are calculated as follows:

$$R^2 = \left[\frac{\sum_{i=1}^{N}(P_i - \bar{P})(Q_i - \bar{Q})}{\sqrt{\sum_{i=1}^{N}(P_i - \bar{P})^2} - \sqrt{\sum_{i=1}^{N}(Q_i - \bar{Q})^2}}\right]^2 \quad (7)$$

$$RMSE = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(P_i - Q_i)^2} \quad (8)$$

$$MAE = \frac{1}{N}\sum_{i=1}^{N}|P_i - Q_i| \quad (9)$$

$$WI = 1 - \frac{\sum_{i=1}^{N}(Q_i - P_i)^2}{\sum_{i=1}^{N}(|Q_i - \bar{P}| + |P_i - \bar{P}|)^2} \quad (10)$$

where N is the number of data series; P_i and Q_i (mm/d) are the simulated and PM model ET_0 values, respectively; and $\bar{P}$ and $\bar{Q}$ (mm/day) are the average of the simulated and PM model ET_0 values, respectively.

3 Results and analysis

3.1 Selection of hydrological year

Based on the rainfall data from Baoding (1991–2019), Xinji (2000–2021), and Handan (1991–2019), the selection of typical hydrological years was carried out sequentially using Equation 1. The identified years for Baoding are 2008, 2009, 1992, and 1997, representing the high flow year (p = 25%), median water year (p = 50%), low flow year (p = 75%), and special dry year (p = 90%), respectively. Similarly, for Xinji, the years are 2004, 2010, 2007, and 2006, and for Handan, the years are 1993, 2014, 2006, and 2017, corresponding to the same hydrological conditions (Figure 2 and Table 1).

3.2 Comparative analysis of daily ET_0 values for different typical hydrologic years

In Figure 3, the day-by-day ET_0 trends of the five models across the three regions under various typical hydrological years exhibit patterns approximating monotonically increasing and decreasing parabolas. The upward segment spans from January to July, followed by a downward segment from July to December, with peak values occurring in the months of June and July for all five models. Comparatively, the H-A model consistently produces higher ET_0 results than the PM model throughout the year. In contrast, the PT and F-R models consistently yield lower ET_0 results than the PM model throughout the year. The M-C model produces higher ET_0 results than the PM model in the months of June–September but lower values in the remaining months.

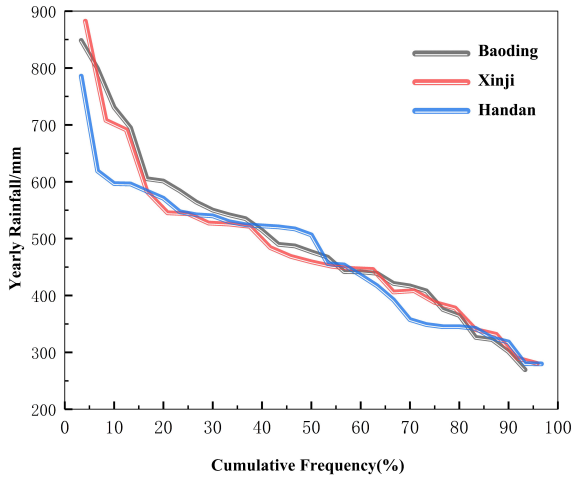

FIGURE 2
Typical hydrological year selection.

Using the PM model-calculated ET_0 values as the standard, a comparative analysis of daily ET_0 values for the remaining four ET models is conducted under different typical hydrological years. At the daily scale, the H-A model yielded slightly larger results than the PM model while the F-R and PT models produced slightly lower values. The remaining three models showed relatively close results to the PM model, except for the H-A model. The PM model and the other four models were used to calculate the RMSE, MAE, and R^2 for each typical hydrological year. The results are presented in Table 2. At a significance level of 0.01, the PT, H-A, and F-R models exhibited good correlation with the PM model's standard values. The average R^2 for PT, H-A, and F-R models in typical hydrological years were 0.710, 0.703, and 0.748 in Baoding; 0.707, 0.718, and 0.746 in Xinji; and 0.644, 0.664, and 0.644 in Handan, respectively. All three models had R^2>0.6, demonstrating their predictive effectiveness at the daily scale. However, the M-C model showed an average R^2 of 0.500, 0.480, and 0.471 in Baoding, Xinji, and Handan, respectively, with R^2<0.6, indicating a lower prediction effectiveness. Moreover, in each typical hydrological year, the F-R model consistently exhibits smaller RMSE and MAE compared with the PT and H-A models. Moreover, the WI is higher for the F-R model, indicating its superior predictive performance for daily ET_0 values under varying hydrological conditions.

3.3 Comparative analysis of monthly ET_0 values for different typical hydrologic years

In Figure 4, the monthly ET_0 trends of the five models across the three regions under various typical hydrological years exhibit patterns resembling monotonically increasing and decreasing parabolas. The upward segment spans from January to July, followed by a downward segment from July to December, with peak values occurring in the months of June and July for all five models. Similar to the daily trends, the H-A model consistently produces higher ET_0 results than the PM model throughout the year. In contrast, the PT and F-R models consistently yield lower ET_0 results than the PM model throughout the year. The M-C model produces higher ET_0 results than the PM model in the months of June–September but lower values in the remaining months.

Using the PM model-calculated ET_0 values as the standard, a comparative analysis of monthly ET_0 values for the remaining four ET_0 models is conducted under different typical hydrological years. At the monthly scale, the H-A model yielded slightly larger results than the PM model while the F-R and PT models produced slightly lower values. The remaining three models showed relatively close results to the PM model, except for the H-A model. The PM model and the other four models were used to calculate the RMSE, MAE, and R^2 for each typical hydrological year. The results are presented in Table 3. At a significance level of 0.01, the PT, H-A, and F-R models exhibited good correlation with the PM model's standard values. The average R^2 for PT, H-A, and F-R models in typical hydrological years were 0.852, 0.900, and 0.879 for Baoding, Xinji,

TABLE 1 Selection of typical hydrological years in some areas of the Haihe Plain region.

Study area	Year	Annual precipitation	Cumulative frequency	Hydrological year type
Baoding	2008	564.3	25%	High flow year
	2009	476.9	50%	Median water year
	1992	375.4	75%	Low flow year
	1997	301.3	90%	Special dry year
Xinji	2004	543.9	25%	High flow year
	2010	459.3	50%	Median water year
	2007	387.8	75%	Low flow year
	2006	290.2	90%	Special dry year
Handan	1993	541.9	25%	High flow year
	2014	506.6	50%	Median water year
	2006	345.2	75%	Low flow year
	2017	318.2	90%	Special dry year

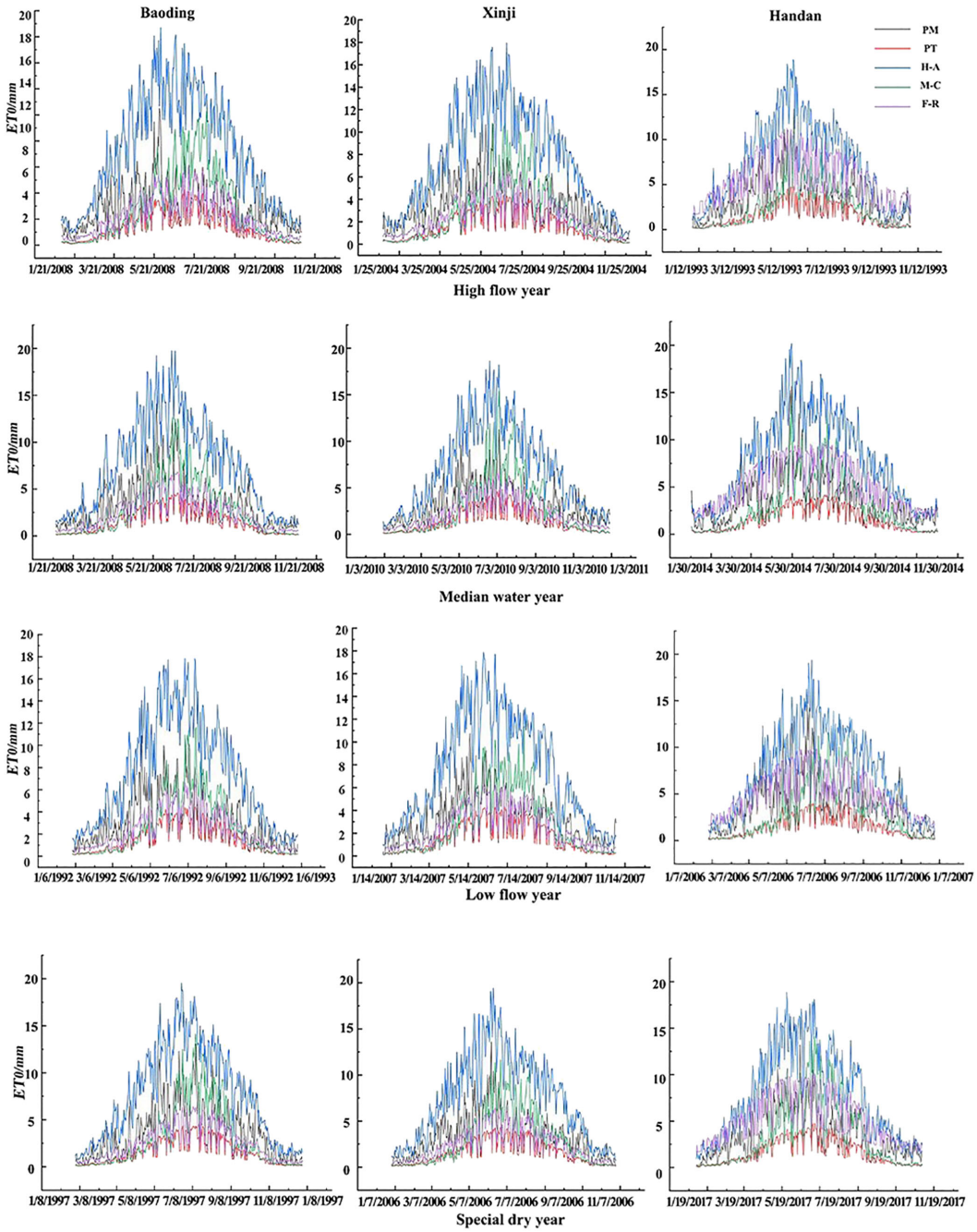

FIGURE 3
ET_0 values of different models under typical hydrological year at daily scale.

and Handan, respectively. All three models had $R^2 > 0.8$, indicating better prediction effects at the monthly scale. However, the M-C model showed an average R^2 of 0.622, 0.607, and 0.554 in Baoding, Xinji, and Handan, respectively, with R^2 around 0.6, signifying poorer prediction compared with other models. Simultaneously, in each typical hydrological year, the F-R model consistently exhibits smaller RMSE and MAE compared with the PT and H-A models. Additionally, the WI is higher for the F-R model, indicating its superior predictive performance for monthly ET_0 values under varying hydrological years.

3.4 Comparative analysis of 10-day ET_0 values for different typical hydrologic years

In Figure 5, the trends of 10-day ET_0 values from the five models across the three regions under various typical hydrological years exhibit patterns resembling monotonically increasing and decreasing parabolas. The overall trend indicates an increase from January to around early July and a subsequent decrease from around early July to the end of December, with peak values occurring around early June to early July. Similar to the daily and

TABLE 2 Comparison the performances of different models under typical hydrological year at daily scale.

Hydrological year type	Evaporation model	Baoding				Xinji				Handan			
		R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI
High flow year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.637	1.886	1.554	0.670	0.659	2.082	1.659	0.698	0.626	2.385	1.784	0.672
	H-A	0.600	5.222	4.226	0.485	0.660	5.178	4.225	0.551	0.649	5.045	4.068	0.607
	M-C	0.359	2.115	1.714	0.731	0.415	2.073	1.668	0.774	0.455	2.107	1.666	0.803
	F-R	0.685	1.227	0.891	0.853	0.707	1.312	0.958	0.874	0.606	2.856	2.515	0.723
Median water year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.755	2.483	1.839	0.680	0.722	2.099	1.705	0.691	0.612	2.611	1.957	0.632
	H-A	0.747	4.965	4.050	0.661	0.711	4.929	3.945	0.587	0.663	5.211	4.235	0.611
	M-C	0.566	2.056	1.649	0.856	0.500	2.262	1.792	0.795	0.553	2.028	1.633	0.848
	F-R	0.799	1.626	1.103	0.867	0.753	1.356	0.975	0.868	0.585	2.373	1.990	0.781
Low flow year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.695	2.161	1.606	0.698	0.727	1.899	1.424	0.738	0.597	2.922	2.186	0.601
	H-A	0.715	5.249	4.293	0.598	0.749	5.334	4.454	0.573	0.623	4.739	3.803	0.651
	M-C	0.486	2.105	1.577	0.819	0.496	1.979	1.530	0.819	0.329	2.624	2.032	0.741
	F-R	0.724	1.441	0.990	0.867	0.768	1.166	0.787	0.908	0.632	2.151	1.820	0.821
Special dry year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.754	2.721	2.052	0.669	0.719	2.174	1.665	0.700	0.742	2.473	1.931	0.684
	H-A	0.751	4.978	4.036	0.675	0.751	5.093	4.208	0.605	0.719	5.181	4.223	0.631
	M-C	0.591	2.231	1.736	0.859	0.511	2.052	1.600	0.827	0.547	2.259	1.746	0.836
	F-R	0.784	1.876	1.277	0.837	0.757	1.384	0.935	0.880	0.751	2.172	1.840	0.834

monthly trends, the H-A model consistently produces higher ET_0 results than the PM model throughout the year. In contrast, the PT and F-R models consistently yield lower ET_0 results than the PM model throughout the year. The M-C model produces higher ET_0 results than the PM model from mid-late June to early September, and the remaining 10-day ET_0 values are lower than those of the PM model.

Using the PM model-calculated ET_0 values as the standard, a comparative analysis of 10-day ET_0 values for the remaining four ET_0 models is conducted under different typical hydrological years. At the 10-day scale, the results of the H-A model are slightly larger than the standard value of the PM model, the results of the F-R model and the PT model are slightly lower than the standard value of the PM model, and the results of the other three models are relatively close to those of the PM model except for the H-A model. The PM model and the other four models were used to calculate the RMSE, MAE, and R^2 for each typical hydrological year, and their corresponding results were analyzed. The results are shown in Table 4. At significance of 0.01, the analytical results of the three models, PT, H-A, and F-R, have good correlation with the standard values of the PM model, among which the average coefficients of determination (R^2) of the three models, PT, H-A, and F-R, in typical hydrological years are 0.806, 0.835, and 0.840 in Baoding, Xinji, and Handan cities, respectively; 0.818, 0.885, and 0.851, respectively; and 0.743, 0.799, and 0.815 respectively. The R^2 of the three models is >0.8, which proves that the three models have better prediction effects at the decadal scale. In contrast, the average coefficients of determination (R^2) of the M-C model were 0.593, 0.560, and 0.521 in Baoding, Xinji, and Handan, respectively, with R^2< 0.6, and the model predicted poorly. Meanwhile, during each typical hydrological year, the F-R model consistently demonstrates smaller RMSE and MAE in comparison with the PT and H-A models. Furthermore, the F-R model exhibits a higher WI, implying superior predictive accuracy for 10-day ET_0 values across varying hydrological years.

In conclusion, among the three models analyzed (PT, H-A, and F-R), all show predictive ability under different typical hydrological years, excluding the M-C model. The PT model demonstrates good correlation at daily, monthly, and 10-day scales across different regions. Specifically, the daily scale R^2 in Baoding, Xinji, and Handan are 0.710, 0.707, and 0.644, respectively; the monthly scale R^2 are 0.852, 0.852, and 0.789, respectively; and the 10-day scale R^2 are 0.806, 0.818, and 0.743, respectively. The H-A model exhibits better correlation at different scales with daily scale R^2 values in Baoding, Xinji, and Handan of 0.703, 0.718, and 0.664, respectively. The monthly scale R^2 are 0.900, 0.924, and 0.857, while the 10-day scale R^2 are 0.835, 0.885, and 0.799. However, the H-A model has larger RMSE and MAE values compared with the PT and F-R models, indicating higher prediction errors.

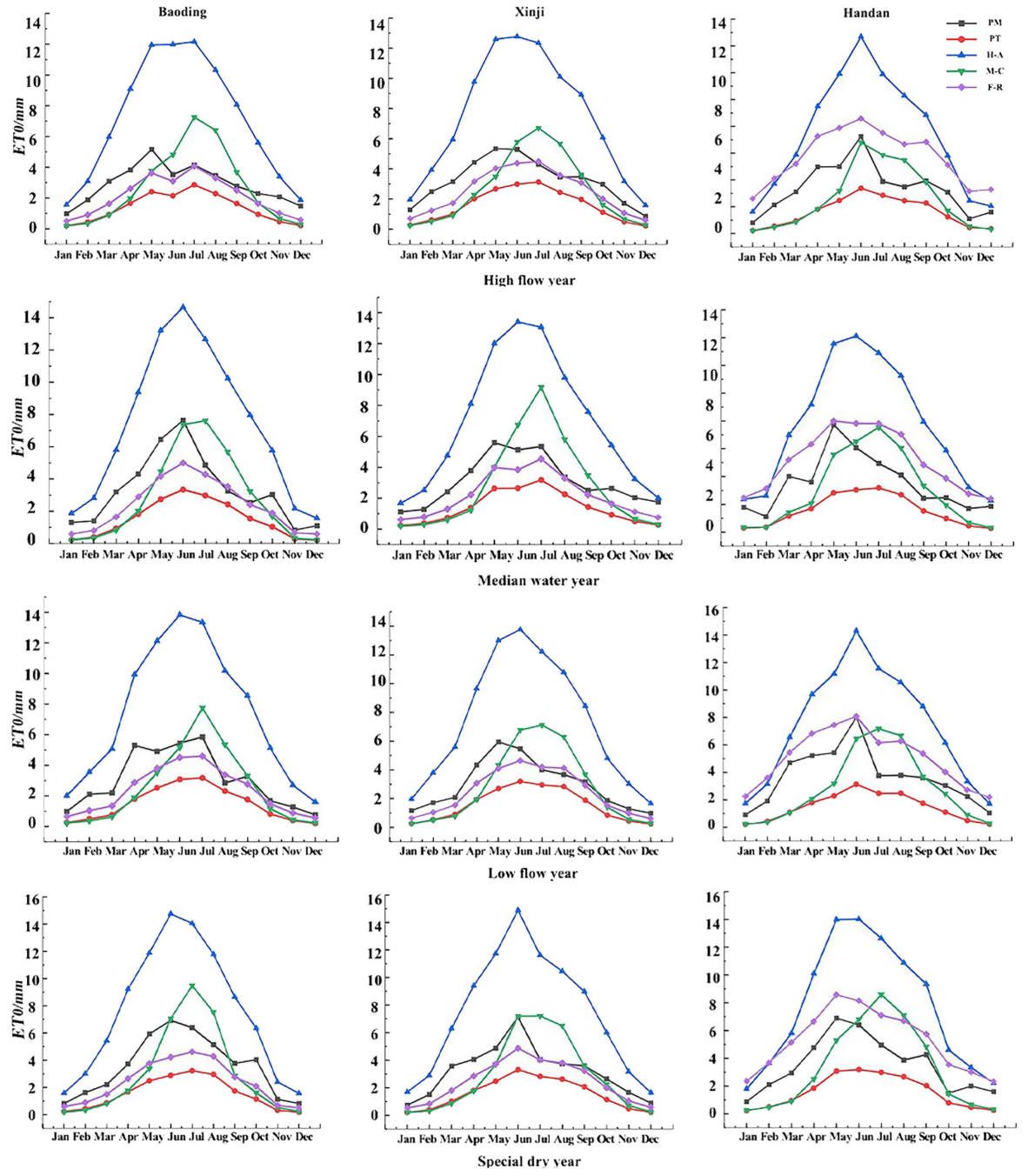

FIGURE 4
ET_0 values of different models under typical hydrological year at monthly scale.

The F-R model shows good correlation at different scales with daily scale R^2 values in Baoding, Xinji, and Handan of 0.748, 0.746, and 0.644, respectively. The monthly scale R^2 are 0.879, 0.877, and 0.822, while the 10-day scale R^2 are 0.840, 0.851, and 0.815. Compared with other models, the F-R model demonstrates higher R^2 values, along with lower RMSE and MAE. Additionally, its WI is consistently higher across various time scales. Consequently, the F-R model shows superior applicability in the Haihe Plain region, particularly after correction, making it more suitable for predicting ET_0 in this area.

3.5 FAO-24 Radiation improvement

The Bayesian estimation method is utilized to iteratively infer the empirical parameters a and b in the F-R model, leveraging meteorological data from Baoding City (1991–2014), Xinji City (2000–2016), and Handan City (1991–2014). The process entails computing posterior distributions of coefficient b using prior data, followed by iteratively calculating coefficient a by incorporating adjusted b values into the prior data. This iterative procedure refines

TABLE 3 Comparison the performances of different models under typical hydrological year at monthly scale.

Hydrological year type	Evaporation Model	Baoding				Xinji				Handan			
		R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI
High flow year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.804	1.642	1.579	0.646	0.836	1.773	1.754	0.676	0.791	2.029	1.835	0.667
	H-A	0.855	5.065	4.198	0.406	0.923	4.982	4.196	0.471	0.870	4.820	4.026	0.550
	M-C	0.493	1.785	1.611	0.720	0.552	1.648	1.467	0.783	0.581	1.671	1.439	0.826
	F-R	0.840	0.905	0.768	0.864	0.865	0.893	0.773	0.901	0.892	2.399	2.311	0.705
Median water year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.842	2.162	1.920	0.674	0.888	1.825	1.726	0.673	0.764	2.240	1.985	0.652
	H-A	0.886	4.816	4.014	0.614	0.919	4.757	3.886	0.530	0.831	5.002	4.207	0.566
	M-C	0.641	1.685	1.476	0.866	0.650	1.910	1.724	0.799	0.645	1.671	1.450	0.862
	F-R	0.874	1.271	0.994	0.881	0.909	0.998	0.883	0.887	0.822	1.883	1.660	0.811
Low flow year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.839	1.842	1.692	0.711	0.851	1.612	1.485	0.743	0.729	2.513	2.314	0.611
	H-A	0.893	5.058	4.275	0.551	0.933	5.183	4.419	0.517	0.796	4.056	3.759	0.586
	M-C	0.608	1.608	1.294	0.848	0.621	1.645	1.417	0.836	0.369	2.164	1.823	0.746
	F-R	0.860	1.015	0.822	0.904	0.875	0.784	0.631	0.937	0.870	1.564	1.391	0.854
Special dry year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.925	2.325	2.172	0.687	0.835	1.897	1.759	0.697	0.873	2.153	1.990	0.689
	H-A	0.964	4.772	4.006	0.635	0.922	4.932	4.192	0.546	0.932	4.955	4.188	0.580
	M-C	0.745	1.768	1.510	0.883	0.603	1.712	1.334	0.837	0.619	1.878	1.552	0.836
	F-R	0.942	1.398	1.173	0.867	0.861	1.029	0.780	0.902	0.943	1.807	1.727	0.832

model parameters, enhancing the accuracy of ET_0 estimation. The specific procedure is as follows:

In accordance with the original F-R model, the two parameters can be expressed as:

$$b = \frac{ET_0 - a}{\frac{\Delta}{\Delta+\gamma}R_s} \tag{11}$$

$$a = ET_0 - b\frac{\Delta}{\Delta+\gamma}R_s \tag{12}$$

(2) The distribution of b and a values follows a normal distribution. The coefficient b was calibrated using Equations 11-13 using day-by-day meteorological data for a typical hydrological year.

$$E = \frac{\alpha_o\widehat{\delta^2} + \widehat{\Theta}0.81^2}{\widehat{\delta^2} + 0.81^2} \tag{13}$$

where E is the mathematical expectation, α_o is the corresponding initial value, and $\hat{\Theta}$ is the estimated mean as well as the variance $\hat{\delta}^2$. Following the same procedure, the mathematical expectation of a is calculated by Equation 12 and Equation 13. The obtained expectations of parameters b and a are substituted into the F-R model in order to obtain the Calibrated F-R model as shown in Table 5.

3.6 Validation of improved F-R model

Following Shiri et al.'s recommendation (Shiri et al., 2015), validation with a distinct dataset was employed to ensure unbiased results. The original and calibrated models were evaluated using meteorological data from Baoding and Handan (2015–2019) and Xinji (2017–2021). ET_0 values were computed for both monthly and 10-day periods derived from the daily values.

After comparing the error analysis results in Table 6 and Table 7, it is evident that under a significance level of $P < 0.01$, R^2 remained unchanged. However, Figure 6 shows significant decreases in RMSE and MAE across daily, monthly, and 10-day scales, accompanied by further improvements in WI. In Baoding City, Xinji City, and Handan City, the average coefficients of determination (R^2) at the daily scale are 0.632, 0.746, and 0.693, respectively. At the monthly scale, the average R^2 values are 0.769, 0.871, and 0.905, respectively, and at the 10-day scale, the average R^2 values are 0.790, 0.838, and 0.852, respectively. There is good correlation at all three scales. Comparing the ET_0 values before and after modification, the modified F-R model reduced RMSE by 15.81%, 29.51%, and 24.66% at the daily, monthly, and 10-day scales, respectively. MAE decreased by 19.04%, 34.47%, and 28.52% at the daily, monthly, and 10-day scales, respectively, while WI increased by 5.49%, 8.48%, and 10.78% at the daily, monthly,

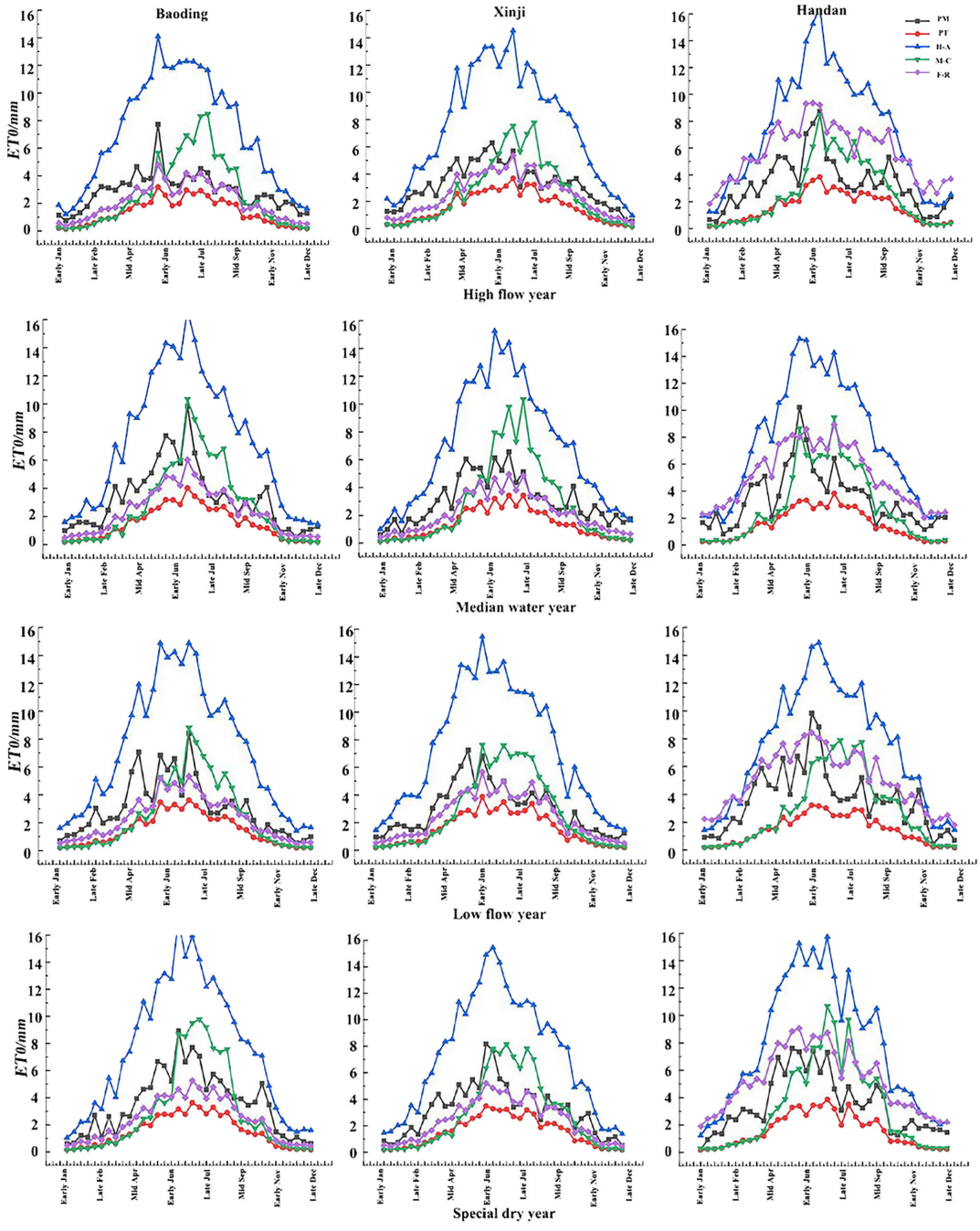

FIGURE 5
ET_0 values of different models under typical hydrological year at the ten-day scale.

and 10-day scales, respectively. Therefore, the modified model can be effectively used for calculating reference crop evapotranspiration in the Haihe Plain region.

4 Discussion

This study compared and evaluated the applicability of four evapotranspiration models—Priestley-Taylor (PT), Hargreaves (H-A), Mc-Cloud (M-C), and FAO-24 Radiation (F-R)—that use incomplete meteorological data. The Penman-Monteith (PM) model's ET_0 values were used as a benchmark for comparison. Overall, the simulation results highlight the superior performance of the F-R model among the four models.

The F-R model calculates ET_0 mainly based on solar radiation data (Hauser, Gimon, Horin, & TX, 1999), which mainly uses the actual sunshine duration to obtain the solar magnetic declination, the atmospheric upper boundary solar radiation, and thus further the actual solar radiation. By analyzing the results of this study, under the condition of incomplete meteorological data, the

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TABLE 4 Comparison the performances of different models under typical hydrological year at 10-day scale.

Hydrological year type	Evaporation Model	Baoding				Xinji				Handan			
		R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI
High flow year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.744	1.700	1.549	0.936	0.897	1.806	1.661	0.934	0.752	2.120	1.781	0.919
	H-A	0.739	5.103	4.205	0.422	0.944	5.014	4.199	0.490	0.809	4.852	4.025	0.575
	M-C	0.466	1.875	1.617	0.729	0.718	1.748	1.559	0.777	0.522	1.823	1.584	0.814
	F-R	0.787	0.980	0.780	0.862	0.916	0.940	0.803	0.902	0.831	2.463	2.313	0.723
Median water year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.822	2.239	1.836	0.920	0.840	1.887	1.708	0.930	0.711	2.342	1.946	0.910
	H-A	0.854	4.830	4.017	0.629	0.881	4.779	3.891	0.549	0.766	5.059	4.214	0.582
	M-C	0.634	1.770	1.520	0.868	0.597	2.022	1.751	0.794	0.625	1.735	1.521	0.863
	F-R	0.861	1.352	1.001	0.879	0.871	1.074	0.891	0.882	0.734	2.024	1.792	0.801
Low flow year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.777	1.980	1.601	0.935	0.816	1.680	1.426	0.952	0.671	2.647	2.192	0.869
	H-A	0.814	5.095	4.273	0.571	0.885	5.215	4.417	0.533	0.739	4.548	3.759	0.614
	M-C	0.566	1.733	1.404	0.842	0.567	1.741	1.446	0.826	0.341	2.319	1.929	0.738
	F-R	0.809	1.190	0.869	0.886	0.848	0.872	0.634	0.931	0.789	1.715	1.557	0.846
Special dry year	PM	—	—	—		—	—	—		—	—	—	
	PT	0.882	2.416	2.048	0.912	0.809	0.352	1.671	0.932	0.835	2.238	1.937	0.921
	H-A	0.935	4.800	4.007	0.652	0.881	1.123	4.190	0.566	0.884	4.995	4.194	0.608
	M-C	0.705	1.877	1.591	0.877	0.562	0.021	1.497	0.830	0.596	2.016	1.634	0.835
	F-R	0.903	1.509	1.175	0.858	0.844	0.166	0.813	0.898	0.907	1.862	1.730	0.844

simulated values of the F-R model at three scales of daily, monthly, and decadal under different typical hydrological years in three areas of the Haihe Plain before the modification have good correlation with the standard values of the PM model, and the results of the error analyses are also satisfactory. By further correcting the F-R model calculations, as shown in Table 6 and Table 7, the R^2 of the corrected F-R model did not change at a significance level of $P < 0.01$, whereas the RMSE and the MAE in the study area decreased substantially. Therefore, the predictions of the modified F-R model were more satisfactory and can be used for the calculation of ET of local reference crops.

TABLE 5 F-R correction model.

Study area	a	b	Calibrated F-R model
Baoding	0.15	0.74	$ET_0 = 0.15 + 0.74\left(\frac{\Delta}{\Delta + \Upsilon} R_s\right)$
Xinji	0.13	0.69	$ET_0 = 0.13 + 0.69\left(\frac{\Delta}{\Delta + \Upsilon} R_s\right)$
Handan	0.21	0.33	$ET_0 = 0.21 + 0.33\left(\frac{\Delta}{\Delta + \Upsilon} R_s\right)$

The use of historical data for model calibration may lead to instability over time due to changing climate conditions. To address this, a suitable calibration method is essential. In this study, the simulated values of the Penman-Monteith (PM) model were employed as standards for the comparative analysis of four models: Priestly-Taylor (PT), Hargreaves (H-A), Mc-Cloud (M-C), and FAO-24 Radiation (F-R). The F-R model was identified as the most suitable for the Haihe Plain region with incomplete meteorological data. Considering geographical differences in the original F-R model's applicability, a modification was performed using the Bayesian principle. This method utilizes known data as the prior distribution and recalculates data as the new posterior distribution, improving the reliability of the calculation by overcoming empirical data uncertainty and considering spatial-temporal variability. The Bayesian approach ensures a systematic and adaptive calibration method, enhancing stability and reliability in different scenarios. Beck et al. introduced Bayesian theory into model correction for the first time and clarified the basic idea of the correction (Beck and Katafygiotis, 1998), and also put forward a kind of adaptive MH algorithm-based Markov chain Monte Carlo method based on the MH algorithm (Beck and Au, 2002). Cheung et al. introduced and improved the hybrid Monte Carlo (HMCMC) method to solve the problem of Bayesian model

TABLE 6 Error analysis of the original F-R model.

Study area	Year	Daily scale				Monthly scale				Ten-day scale			
		R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI
Baoding	2015	0.652	1.625	1.103	0.801	0.782	1.219	0.973	0.825	0.877	1.321	1.006	0.862
	2016	0.609	1.580	1.097	0.781	0.725	1.321	0.956	0.788	0.849	1.363	0.991	0.823
	2017	0.670	1.774	1.191	0.778	0.859	1.374	1.065	0.809	0.812	1.465	1.085	0.837
	2018	0.630	1.575	1.060	0.824	0.778	1.097	0.908	0.856	0.727	1.211	0.965	0.888
	2019	0.600	1.600	1.200	0.822	0.701	1.247	0.972	0.859	0.673	1.337	1.065	0.887
Xinji	2017	0.805	1.250	0.906	0.906	0.926	0.856	0.706	0.934	0.894	0.963	0.789	0.945
	2018	0.743	1.267	0.910	0.894	0.880	0.798	0.670	0.934	0.849	0.894	0.685	0.945
	2019	0.771	1.323	0.881	0.886	0.876	0.979	0.675	0.912	0.852	1.055	0.717	0.927
	2020	0.754	1.179	1.145	0.910	0.903	1.023	0.658	0.960	0.856	1.050	0.700	0.957
	2021	0.656	1.260	1.120	0.861	0.768	0.896	0.773	0.898	0.741	0.925	0.760	0.917
Handan	2015	0.675	2.043	1.699	0.842	0.909	1.465	1.345	0.874	0.810	1.677	1.490	0.778
	2016	0.693	2.327	1.989	0.782	0.885	1.999	1.884	0.765	0.848	2.062	1.895	0.548
	2017	0.751	2.172	1.845	0.834	0.943	1.807	1.727	0.832	0.907	1.869	1.741	0.715
	2018	0.664	2.252	1.923	0.804	0.890	1.814	1.685	0.800	0.838	1.886	1.688	0.637
	2019	0.681	1.985	1.660	0.853	0.896	1.408	1.290	0.897	0.856	1.492	1.345	0.837

correction for high-dimensional uncertainty parameters (Cheung and Beck, 2009). Currently, there is a recommended application of the modified Hargreaves model using Bayesian estimation method to calculate the ET of de-measured reference crops in the Sichuan Basin area (Feng et al., 2017). The modification of the F-R model using the Bayesian principle in the experimental area ensures the model's applicability, providing a more accurate ET_0 calculation. This enhanced model can serve as a scientific foundation for future farmland moisture management in the Haihe Plain area.

TABLE 7 Error analysis of the calibrated F-R model.

Study area	Year	Daily scale				Monthly scale				Ten-day scale			
		R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI	R^2	RMSE	MAE	WI
Baoding	2015	0.652	1.334	0.968	0.894	0.782	0.806	0.598	0.939	0.877	0.904	0.717	0.936
	2016	0.609	1.305	0.939	0.877	0.725	0.898	0.748	0.918	0.849	0.939	0.759	0.916
	2017	0.670	1.361	0.990	0.894	0.859	0.733	0.662	0.955	0.812	0.887	0.700	0.940
	2018	0.630	1.451	1.060	0.887	0.778	0.888	0.670	0.929	0.727	1.017	0.799	0.921
	2019	0.600	1.600	1.200	0.872	0.701	1.197	0.901	0.905	0.673	1.282	0.924	0.896
Xinji	2017	0.805	1.106	0.816	0.944	0.926	0.632	0.512	0.973	0.894	0.746	0.570	0.967
	2018	0.743	1.251	0.900	0.923	0.880	0.762	0.499	0.954	0.849	0.856	0.637	0.950
	2019	0.771	1.121	0.833	0.936	0.876	0.686	0.506	0.966	0.852	0.783	0.600	0.960
	2020	0.754	1.117	0.934	0.918	0.903	0.934	0.674	0.945	0.856	1.042	0.736	0.939
	2021	0.656	1.260	0.965	0.882	0.768	0.871	0.687	0.928	0.741	0.939	0.707	0.915
Handan	2015	0.675	1.728	1.218	0.822	0.909	0.939	0.656	0.914	0.810	1.324	0.904	0.862
	2016	0.693	1.288	1.036	0.876	0.885	0.691	0.629	0.943	0.848	0.810	0.689	0.930
	2017	0.751	1.365	1.014	0.886	0.943	0.667	0.547	0.958	0.907	0.854	0.668	0.941
	2018	0.664	1.462	1.019	0.857	0.890	0.680	0.415	0.947	0.838	0.856	0.605	0.925
	2019	0.681	1.884	1.244	0.801	0.896	1.296	0.811	0.866	0.856	1.416	0.967	0.853

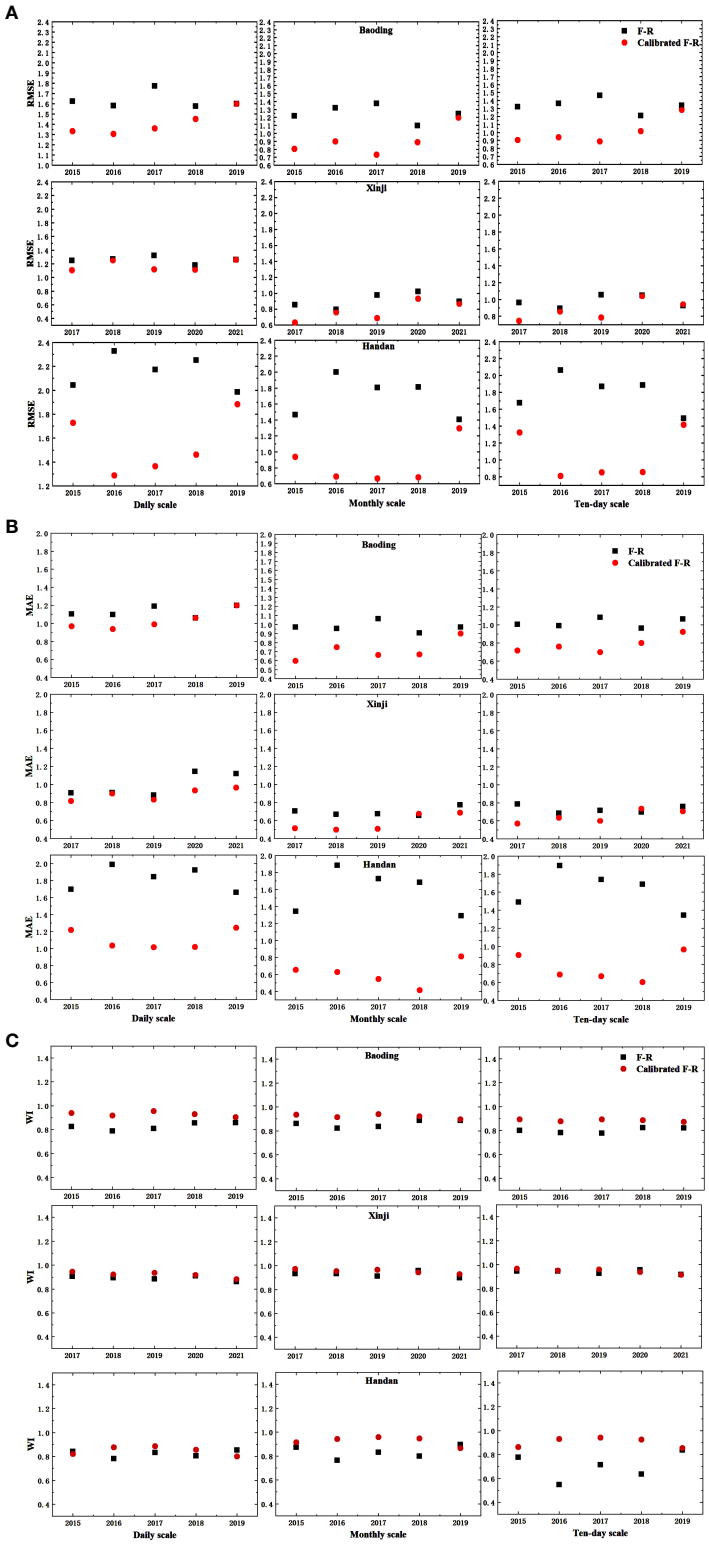

FIGURE 6
The comparisons of RMSE, MAE, and WI before and after the F-R model calibrated; **(A)** RMSE; **(B)** MAE; **(C)** WI.

Different types of models have different sensitivities to meteorological data and are adapted to different regions. The PT model does not require wind speed and humidity data (Priestley and Taylor, 1972), and by comparing with the standard values of the PM model, the overall PT model simulation values are lower than those of the PM model, and the three scales of daily, monthly, and decadal are all well correlated under different typical hydrological years in the three regions of the Haihe Plain, and the error analysis. The results

are relatively satisfactory. It can be used to calculate the ET_0 in the Haihe Plain if the error is within the allowable range. In contrast to the PM model method for calculating reference ET, the PT model ignores the effect of water vapor deficit on reference ET, thus generating the assumption that ET_0 depends only on solar radiation and temperature (Wu et al., 2021). This allows for PT modeling where PM modeling is not possible due to lack of data (Utset et al., 2004). It has been demonstrated that the simple and less data-demanding PT model is a good choice in many climatic regions (Jamieson, 1982; Pereira and Nova, 1992; Sau et al., 2004).

The M-C model is a simplified calculation method of ET_0 based on temperature (McCloud, 1955), which just uses the daily mean temperature as meteorological data, and by comparing with the standard value of PM model, the correlation of this model is low, $R^2 < 0.6$, indicating that this model is not good at predicting in the sea–river plain area. However, the M-C model is based on the daily mean air temperature, which is easy to calculate and especially suitable for areas with large differences in temperature variations (Valipour, 2015). The H-A model is suitable for the lack of radiative data and just uses the daily mean air temperature, daily maximum and daily minimum air temperature, and the atmospheric upper boundary solar radiation calculated through the daily ordinate. The simulated values of this model are compared with the PM model. The model simulated values are compared with the standard values of the PM model, and although there is a high correlation, the results of the error analysis are less satisfactory, with larger values of RMSE and MAE, and the model is not effective in predicting in the test area. However, many studies have confirmed that the H-A model is also a good predictor in some regions, and model optimization is continuously performed to better adapt to climate change (Gavilán et al., 2006; Tabari and Talaee, 2011; Berti et al., 2014; Cobaner et al., 2017). These calibrations are site-specific and cannot be extrapolated to some sites with completely different meteorological conditions.

This study warrants further validation, especially considering the absence of measured ET_0 data. While the PM model served as the standard for calibrating the F-R model based on Bayesian theory, it is essential to verify the conclusions with measured data. Relying solely on model calculations, as highlighted by Martí et al (Martí et al., 2015), may yield unreasonable or incorrect conclusions. Therefore, incorporating measured ET_0 data from lysimeters for calibration and evaluation is crucial. Moreover, while Bayesian theory allows for updating model parameters based on new sample data, it is important to note that this method is purely mathematical and overlooks the physical basis of the evapotranspiration process. Consequently, future research should emphasize calibrating the model using measured solar radiation data to enhance its accuracy.

5 Conclusions

In this study, we conducted a comparative analysis of four evapotranspiration models using incomplete meteorological data across various hydrological conditions to enhance ET_0 estimation accuracy. The results revealed consistent spatial distribution trends among the models, with the F-R model demonstrating superior accuracy and predictive performance, particularly in terms of R^2 and WI. Furthermore, the calibrated F-R model, refined through Bayesian theory, achieved higher accuracy, with R^2 reaching 0.85 and WI reaching 0.9. The calibrated FAO-24 Radiation model offers valuable insights for precise ET_0 estimation and irrigation decision-making in the Haihe Plain region, suggesting avenues for further accuracy improvements in future research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

XS: Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation. BZ: Writing – original draft, Methodology, Data curation. MD: Visualization, Writing – original draft. RG: Writing – original draft, Visualization. CJ: Writing – original draft, Formal analysis. KM: Writing – original draft, Visualization. SG: Writing – original draft, Methodology. LG: Writing – review & editing, Supervision, Funding acquisition. WZ: Writing – review & editing, Supervision, Funding acquisition. XG: Methodology, Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Protective potential of selected microbial and non-microbial biostimulants against *Zymoseptoria tritici* leaf blotch in winter wheat as affected by the form of N supply

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Introduction: The production of high-quality food for the growing world population on the one hand and the reduction of chemical-synthetic pesticides on the other hand represents a major challenge for agriculture worldwide. The effectiveness of a combination of microbial and non-microbial biostimulants (BSs) with various nitrogen (N) forms in pathogen defense is discussed as a promising, but still poorly understood bio-based alternative for crop protection.

Methods: For this reason, nitrate and stabilized ammonium fertilizer both combined with a consortium of *Pseudomonas brassicacearum*, *Bacillus amyloliquefaciens*, and *Trichoderma harzianum* as soil treatment or with a mixture of seaweed extract (*Ascophyllum nodosum*) together with chitosan-amended micronutrient fertilizer as foliar spray application were compared under controlled greenhouse conditions. Furthermore, a combination of microbial and different non-microbial BSs (seaweed extracts + chitosan) and micronutrients with nitrate or with stabilized ammonium fertilizer was tested under field conditions to improve nutrient availability, promote plant growth, and suppress *Zymoseptoria tritici* (*Zt*) in winter wheat.

Results and discussion: While plant-protective effects against *Zt* by the microbial consortium application could be observed particularly under ammonium fertilization, the application of seaweed extract–chitosan mixture expressed plant defense against *Zt* more strongly under nitrate fertilization. In the field trial, the combination of microbial consortium with the seaweed extract–chitosan mixture together with micronutrients zinc (Zn) and manganese (Mn) showed positive effects against *Zt* under ammonium fertilization, associated with increased levels of defense metabolites. Furthermore, the additional input of Zn

and copper (Cu) from the chitosan application improved the micronutrient status by minimizing the risk of Zn and Cu deficiency under controlled and field conditions. The use of BSs and the inoculation of *Zt* did not show any effects on plant growth and yield neither under controlled greenhouse conditions nor in the field. Summarized, microbial and non-microbial BSs separately applied or even combined together as one treatment did not influence plant growth or yield but made a positive contribution to an N form-dependent promotion of pathogen defense.

KEYWORDS

biotic stress, *Zymoseptoria tritici*, winter wheat, seaweed extracts, microbial consortium, agriculture, nitrate, ammonium

1 Introduction

One of the most critical fungal pathogens in wheat is *Zymoseptoria tritici* (*Zt*) (Quaedvlieg et al., 2011), which leads to yield losses of 5%–10% and reduction of food quality worldwide (Fones and Gurr, 2015). Intensive farming with the application of pesticides against pathogens such as *Zt* and chemical fertilizers is often regarded as necessary to feed the world's growing population and to guarantee food security, but at the same time, this industrialized style of agriculture has a negative impact on the environment. Many of those pesticides are assumed to be readily degraded and removed from the soil. It was later shown that they can form bound residues, formerly undetected, and are even found as pesticide residues in food (Pogacean and Gavrilescu, 2009; Raliya et al., 2018). This agricultural challenge points out the relevance of an improved and sustainable cropping system avoiding excessive use of chemical-synthetic pesticides for environmental protection and high-quality food supply (Zimmermann et al., 2021). Targeted mineral fertilization with the focus on nitrogen (N) is most common for high plant growth and crop yield, even though its pathogen-suppressing effect is not less relevant (Huber and Haneklaus, 2007) but has been neglected in the past. However, it has been confirmed that adequate mineral N fertilization can improve plant tolerance to biotic and abiotic stresses (Fernandes and Rossiello, 1995; Walters and Bingham, 2007; Geisseler and Scow, 2014; Ding et al., 2021). Other studies proved the dependence of the effect of N fertilization on plant tolerance to the disease susceptibility of the crop varieties (Huber and Watson, 1974) or the importance of the source of N and the rate of application (Baker and Martinson, 1970; Huber and Watson, 1974). On the one hand, the impact of nitrate fertilization on plant tolerance correlates with the applied amount. The higher the N application is, the higher the susceptibility to certain diseases. Nevertheless, it has to be mentioned that the effect of nitrogen depends on the biology of the pathogen and the response of the crop plant. For example, a dense canopy formation can create a microclimate favorable for fungal infections. Furthermore, the nitrogen effect depends on the difference between biotrophic or saprophytic fungi and the influence of the nitrogen form (Weinmann et al., 2023). On the other hand, Sun et al. (2020) mentioned in their research that both ammonium and nitrate fertilization can promote or suppress plant diseases equally. This may depend on the type of pathogen and whether the ammonium fertilizer was stabilized or not, because of fast nitrification in aerated soils ammonium fertilizer show almost the same effect as nitrate. To combine the different N forms with microbial biostimulants (BSs), a consortium of plant-growth-promoting microorganisms (PGPMs) is supposed to be more effective than single PGPMs to strengthen plants against biotic and abiotic stresses (Mamun et al., 2024). Furthermore, a microbial consortium offers a broader range of usage than single strains due to the versatile compounds which are present in the consortium (Sarma et al., 2015; Bradáčová et al., 2019). Bradáčová et al. (2020) and Mamun et al. (2024) additionally identified that the form of N supply can be decisive for the efficiency of microbial BSs. Stabilized ammonium nutrition in combination with microbial consortium of *Pseudomonas* spp., *Bacillus* spp., and *Trichoderma* spp. improved the uptake of ammonium-N and led to increased phosphorus (P) concentrations in maize shoot tissues compared to nitrate supply. Increased root length was also observed with stabilized ammonium supply compared to nitrate supply. In addition, Halpern et al. (2015) recorded many positive abilities of a diversity of microorganisms, such as increased root growth due to the production of phytohormones, higher phosphate availability through mineralization of organic P, solubilization of iron (Fe) through chelating siderophores produced by the bacteria, and enhanced accumulation of Zn and potassium (K) in the plant tissue by excreting organic acids resulting in higher plant uptake and yield. Non-microbial BSs such as chitin, chitosan, or seaweed extracts also perform more efficiently as combined treatments to improve disease tolerance according to several studies (Nanda et al., 2021). Apart from stress priming effects, seaweed extracts in higher concentrations can contribute to enhanced solubility of micronutrients chelating them by large organic molecules and affect the nutrient uptake (Halpern et al., 2015). Furthermore, it is reported that chitosan can reduce the water content in cells due to its hydrophilic nature to mitigate stress and

increases root length by acting as an additional source of carbon, resulting in improved nutrient uptake (Shahrajabian et al., 2021). Regarding the application of the micronutrients Zn and Mn, a Mn-dependent superoxide dismutase (MnSOD) is produced as a defense reaction by plants due to biotic and abiotic stresses (Kumar et al., 2020). Furthermore, Mn is involved in the synthesis of toxic phenols and lignification by forming a physical barrier against fungal pathogens. Similar to Mn, Zn acts as a cofactor for enzymes such as superoxide dismutase, and can increase the production of total phenolics or total antioxidants resulting in protective plant responses (Silva et al., 2022). In this multicomponent approach, the effect of microbial (*Pseudomonas brassicacearum*, *Bacillus amyloliquefaciens*, and *Trichoderma harzianum*) and non-microbial (seaweed extracts + chitosan) BSs and micronutrients (Zn and Mn) on *Zt* affected winter wheat with different N supplies was investigated. To examine both, the plant growth promoting potential and the plant protecting potential of biostimulants (BSs) combined with ammonium or nitrate fertilization, nutrient analyses, and metabolic assessments were performed. As complex treatments were tested in this study, it is hypothesized that beneficial effects on plant performance, nutrient acquisition, and tolerance of wheat plants result from a complexity of interactions of these treatments with the plant and its environment. However, the experimental setups do not allow for an isolation of effects that could be clearly attributed to certain components of the complex treatments.

We hypothesized that:

1. the application of different N forms together with a microbial consortium or seaweed extract in combination with chitosan improves plant growth and development by enhanced root growth and nutrient uptake under biotic stress conditions in the greenhouse.
2. the combination of stabilized ammonium fertilizers with microbial BSs, non-microbial BSs, and micronutrients leads to increased plant growth and enhanced disease suppression of *Zt*-inoculated wheat plants in the field.
3. different N forms combined with various BSs alleviate the effects of *Zt* infestation by inducing increased production of defense metabolites under greenhouse and field conditions.

The overall objective of this study was to examine the effect of different N forms in combination with different BSs on the suppression of *Zt*.

1.1 Pot experiment

1.1.1 Experimental setup

Winter wheat *Triticum aestivum* L. Asory (SECOBRA Saatzucht GmbH, Unterschleißheim, Germany), a medium-late ripening variety and showing a moderately tolerance to *Zt*, was cultivated from 05/05/2022 to 29/06/2022 (55 days) in a greenhouse at a mean temperature of 28.7°C and a mean relative humidity of 45.7%. Plastic cylinders with a diameter of 95 mm (pot surface 71 cm^2), and a height of 210 mm were used as pots. Each pot contained 1,173 g soil (Filderlehm 2015) from the experimental station Heidfeldhof of the University of Hohenheim) and 587 g quartz sand mixture (0.6 – 1.2 mm) (66.5:33.5 w:w). An overview on physical and chemical soil properties is shown in Supplementary Table 1. Per pot, 12 wheat seeds were sown. A 100-g layer of washed quartz sand (0.6 – 1.2 mm) was added on top of the soil surface to reduce evaporation and pest pressure and avoid siltation by irrigation. Each pot was fertilized with 120 mg P kg^{-1} DW in the form of $Ca(H_2PO_4)_2$, 150 mg K kg^{-1} DW in the form of K_2SO_4, and 50 mg Mg kg^{-1} DW in the form of $MgSO_4$ and sprayed on the soil–sand mixture while the substrate was mixed by hand before filling into the pots. The two different nitrogen treatments, calcium nitrate (YaraTera® CALCINIT®, YARA GmbH & Co. KG, Dülmen, Germany) and ammonium sulfate (NovaTec® Solub 21, COMPO EXPERT GmbH, Münster, Germany) with a nitrification inhibitor [3,4-dimethyl-1H-pyrazole phosphate (DMPP)], were fertilized each with 100 mg kg^{-1} DW as milled powder mixed with the soil–sand mixture before filling into the pots. The pots were regularly watered by weight up to 70% of the water holding capacity (WHC) when the water content in the soil/sand substrate fell below 50% of the WHC. The pots were arranged according to a split-plot design with two real repetitions and two main plots per repetition (Supplementary Figure 1). In total, 50 pots were prepared, 10 different variants with five repetitions per treatment (Supplementary Table 2). Within the main plots, treatments were arranged on two tables (table one with two replicates and table two with three replicates) as randomized complete block design (RCBD). The model for this design was

$$y_{ijkl} = \mu + t_i + r_{ij} + \tau_k + gt_{li} + e_{ijkl}$$

with μ as the overall effect, t_i as the fixed/random effect of the ith table, r_{ij} as the fixed effect of the jth block on the ith table, τ_k as the effect of the kth treatment, gt_{li} as the random effect of the tth main plot in the jth block on the ith table, and e_{ijkl} as the error of y_{ijkl}.

1.1.2 Application of biostimulants

As biostimulant products, a microbial consortium composed of different microorganisms and a mixture of seaweed extract with chitosan was tested. The consortium (*Pseudomonas brassicacearum*: 2×10^{10} cfu g^{-1}, *Bacillus amyloliquefaciens*: 2×10^{10} cfu g^{-1}, and *Trichoderma harzianum*: 1×10^{8} cfu g^{-1}, SP Sourcon Padena GmbH, Tübingen, Germany) formulated with milk powder that served as a carrier and nutrient medium was applied as a soil application on the sowing day shortly before sowing. The 10-g package of consortium powder (for the 10^7 cm^2 soil surface) was dissolved into 500 ml of distilled water. From the stock solution (SL), 35.5 µl (0.05 µl cm^{-2}) were pipetted and mixed in a beaker with 100 ml of distilled water using a magnetic stirrer. For each pot, 10 ml SL (0.14 ml cm^{-2}) was dropped onto the top layer of the soil and then carefully mixed into the soil by hand. The seaweed extract (*Ascophyllum nodosum* extract, BioAtlantis Ltd., Tralee, Ireland) together with chitosan as a micronutrient formulation with surfactants containing the mixture of micronutrients together with copper (Cu), manganese (Mn), molybdenum (Mo), and zinc (Zn); 7.1 N (0.55 NH_4-N+6.55 NH_2N) + 11.8 K + 3.4 S + <0.1 Cl + 1.42 Cu + 2.84 Mn + 2.23 Zn + 0.028 Mo w/v [g 100 ml^{-1} chitosan

formulation] (Wuxal® Micromix plus Chitosan; A7116, AGLUKON Spezialdünger GmbH & Co. KG, Düsseldorf, Germany), were applied as foliar spraying. The total amounts of mineral nutrients applied to the plants via chitosan are shown in Supplementary Table 3. In accordance with the BioAtlantis' application recommendation, the seaweed + chitosan mixture was applied 2 days prior to a biotic stress event and every 5 days thereafter if the disease intensity was >25% of infested leaf area or every 7 days thereafter if the disease spread was <25% of infested leaf area with 2 l seaweed extract ha^{-1} and 2 l chitosan ha^{-1} (0.02 µl cm^{-2}) in 250 l water ha^{-1} (2.5 µl water cm^{-2}). In total, five applications have been performed during the pot experiment. An SL was prepared as follows: 15.62 µl of the seaweed extract was pipetted in one 2 ml Eppendorf tube and the same amount of chitosan in another 2 ml Eppendorf tube, both filled up with distilled water to 1 ml. Both tubes were properly mixed by use of an orbital shaker, then 968 µl of distilled water was added in each and stirred again. Finally, the contents of both Eppendorf tubes were combined with 200 ml of distilled water in a glass beaker and mixed with a magnetic stirrer. Of this application mixture, 18.2 ml was sprayed onto the leaves of the plants of each pot.

1.1.3 *Zymoseptoria tritici* cultivation, inoculation, and disease assessment

YMDA–agar YMB–liquid culture medium as the most efficient culture media for inoculum production according to Saidi et al. (2012) was selected for the *Zt* cultivation. 4 g Bacto™ Yeast Extract Technical (A288620; Becton, Dickinson and Company, Franklin Lakes, United States), 4 g Bacto™ Malt Extract (A218630; Becton, Dickinson and Company, Franklin Lakes, United States), 10 g (D (+)-glucose 1-hydrate), (A143140.1211; AppliChem GmbH, Darmstadt, Germany), and 15 g Bacter Agar (A0949, 1000; AppliChem GmbH, Darmstadt, Germany) were dissolved in 1 l of distilled water. The solution was mixed properly with a magnetic stirrer and autoclaved (121°C, 20 min). Afterwards, the solution was poured into sterile Petri dishes and solidified during cooling. With a flame-sterilized spatula, a piece of *Zt* fungal mycelium was gouged out and placed *vice versa* on the agar-medium, and the Petri dishes were locked with parafilm and stored in a light cabinet for 1–3 weeks at room temperature (18°C–20°C), with the culture medium facing the above. The YMB–liquid culture medium was prepared, consisting of 4 g YE (Bacto™ Yeast Extract Technical, A288620; Becton, Dickinson and Company, Franklin Lakes, United States), 4 g ME (Bacto™ Malt Extract, A218630; Becton, Dickinson and Company, Franklin Lakes, United States), and 10 g glucose (D (+)-glucose 1-hydrate) (A143140.1211; AppliChem GmbH, Darmstadt, Germany) filled up to 1 l with distilled water. The solution was mixed with a magnetic stirrer and autoclaved (121°C, 20 min). In a 1 l Erlenmeyer flask, two complete *Zt* plates were mixed with 500 ml of liquid YMB medium. In a 0.5 l Erlenmeyer flask, one complete *Zt* plate was mixed with 333 ml liquid YMB medium. The *Zt* fungi on the plates were gouged out with a flame-sterilized spatula and cut into pieces before adding them to the liquid medium. Everything together was mixed in a sterile cabinet and put on a shaker with a frequency of 100 - 125 rounds per minute (rpm) for 2 weeks at 18°C–20°C. Thereafter, the YMB medium was poured through an autoclaved, two-layered gauze bandages into an autoclaved vessel. The residue represented the mycelium, and the filtrate contained the *Zt* spores. The filtrate was centrifuged at 20°C for 10 min and 6,000 min^{-1} (17,307×*g*) (Sorvall RC 6 Plus centrifuge, Thermo Electron LED GmbH, Osterode, Germany), and the supernatant was discarded. The remaining *Zt* spores were then diluted with autoclaved water, filled into tubes, and mixed with an orbital shaker. The spore suspension was filled from the tubes into 0.5 l PET bottles which were stored at −40°C until they were used. The *Zt* spores were counted with a "Fuchs-Rosenthal" cell chamber. Three 2 ml Eppendorf tubes with a 1:1,000 concentration of *Zt* spores were used, and 3.2 µl was pipetted in the counting chamber. There were 16 group squares existing in the chamber; one group square included 16 small squares. For every sample, five group squares were counted and the mean value was determined. Lastly, the mean value from all three counts was 374.8×10^7 spores ml^{-1}. *Zt* was applied with a small sprayer and a concentration of 1×10^7 spores ml^{-1} *Zt* suspension 15 days after sowing (DAS). 355 µl *Zt* suspension/pot (5 µl cm^{-2} soil surface) was applied. The pots were covered for 3 days with four different foil tunnels with wet towels inside to keep a high relative humidity. For keeping the humidity even higher, a transparent plastic foil was additionally placed over the tunnels. Visual assessment of *Zt* infestation was done 29, 34, 43, 49, and 55 DAS. The percentage of leaf area covered with *Zt* for six youngest, fully developed leaves from six different plants per pot were evaluated based on the method of James W.C. described by Eyal et al. (1987).

1.1.4 Plant analyses

The dry weight of the aboveground plant tissue and root samples per pot was determined after harvest at 55 DAS. Aliquots of washed root samples were stored in 70% (v/v) ethanol and analyzed for root length and morphological structure (i.e., root diameter classes) with an Epson Expression 10000XL scanner (Seiko Epson K.K., Japan) using WinRHIZO root analysis software package (Regent Instruments Inc., Quebec, Canada) (Moradtalab et al., 2020). For estimating the shoot and root dry weights, the plant tissue and root samples packed in paper bags were oven-dried at 60°C for 4 days and then weighed.

1.1.5 Determination of stress metabolites

Fresh leaf samples from 27 DAS and 55 DAS were used for the determination of selected physiological stress indicators such as hydrogen peroxide (H_2O_2), ascorbate peroxidase (APX; EC 1.11.1.11) activity, and guaiacol peroxidase (GPX; EC 1.11.1.7) activity after homogenization of 0.1 g of plant tissues shock-frozen in liquid nitrogen in 1.5 ml of 50 mM potassium phosphate extraction buffer, followed by centrifugation for 10 min at 14,000 min^{-1} (20,160×*g*) (Hettich centrifuge MIKRO 24-48 R, Tuttlingen, Germany). APX activity was recorded spectrophotometrically at 290 nm according to the method of Boominathan and Doran (2002). H_2O_2 levels were determined spectrophotometrically at 390 nm according to the method described by Moradtalab et al. (2018). GPX activity was performed spectrophotometrically at 470 nm using the tetra-guaiacol assay described by Moradtalab et al. (2020). For the determination of total antioxidants, leaf samples from 27 DAS were

shock-frozen and homogenized in liquid nitrogen to use 0.1 g of fresh matter for methanolic extraction (80% v/v methanol) followed by centrifugation for 10 min at 14,000 min^{-1} (20,160×*g*) (Hettich MIKRO 24-48 R centrifuge, Tuttlingen, Germany). The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH)-modified method was used to evaluate the free radical scavenging activity of antioxidants in the plant tissue (Moradtalab et al., 2020). A U-3300 spectrophotometer (Hitachi, Tokyo, Japan) was used for all spectrophotometric measurements.

1.1.6 Analysis of mineral nutrients

The concentrations of the essential nutrients N, P, K, Ca, Mg, S, Zn, Mn, Fe, and Cu were determined in the oven-dried and milled plant tissues of final harvest (55 DAS). The shoots were ground in a disc-oscillating agate stone mill (SIEBTECHNIK GmbH, Mülheim-Ruhr, Germany) for 3 min – 4 min to a fine powder. 0.2 g of powdered plant tissue per sample was weighed into a quartz glass beaker, and 1 ml ultrapure water and 2.5 ml nitric acid (ROTIPURAN® ≥65%, p.a., ISO; Carl Roth GmbH & Co. KG) were added. After microwave ("ultraCLAVE III"; Fa. MLS Leutkirch) digestion was accomplished, elemental concentrations were measured by inductive coupled plasma optical emission spectrometry (ICP-OES; "Agilent 5110", Santa Clara, United States). Total nitrogen, carbon, and sulfur were determined with the Vario MAX CNS elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) (VDLUFA, 2012).

1.1.7 Statistical evaluation

Statistical analyses were performed using SAS/STAT software package of SAS® 9.4 (2016) (SAS Institute Inc., Cary, USA). A one-way ANOVA followed by a Tukey test ($p < 0.05$ significance level) was used to compare means for statistically significant differences. Data are presented as mean values. Normal distributions and variance homogeneities of the residuals were checked by the Shapiro–Wilk test and by Levene's test, respectively, and graphically against the predicted values by QQ plots, histograms, and graphs of the residuals according to Kozak and Piepho (2017).

1.2 Field experiment

1.2.1 Experimental setup

Similar to the pot experiment, winter wheat of the variety Asory (SECOBRA Saatzucht GmbH, Unterschleißheim, Germany) was the test plant in the field experiment. This variety is described as moderately tolerant to *Zt* with medium plant length and standability, and medium-late ripening quality. It was cultivated from 20/10/2021 to 27/07/2022 (280 days) with a sowing density of 330 grains m^{-2} at the experimental station Heidfeldhof of the University of Hohenheim, Filderhauptstraße 201, 70599 Stuttgart, Germany (48°42′56.21 N, 9°11′15.64 E, 402 m above sea level, mean temperature of 8.8°C, mean relative humidity of 76.6%). A 3.28 ha field was used for the experiment with a plot size of 6 m × 8 m (48 m^2). The sowing was performed with Amazone AD 303 (Amazonen-Werke H. Dreyer SE & Co. KG, Hasbergen, Germany) with a 3 m working width and front cultivator, a 3.5 cm sowing depth, and a 12.5 cm row spacing. The soil texture in the field was loamy clay, and the soil type was Cambisol. The field was tilled with a rotary harrow twice before sowing. Five days after sowing and during field emergence (BBCH 23), weeding/hoeing was undertaken to control the weeds, except for the negative control variant 27. Soil sampling for N_{min} measurements was performed during the vegetation period for the whole field for fertilizer requirement calculation on 03/03/2022 (N_{min} = 38.46 kg N ha^{-1}). Furthermore, after harvesting, plot-specific sampling for N_{min} analyses was performed on 11/08/2022. One sampling was performed to collect soil samples from 0–30-cm, 30–60-cm, and 60–90-cm depths according to VDLUFA (2023). The results of the soil analyses are shown in Supplementary Table 4. The fertilizer "P, K, Mg plus S – fertilizer" containig 4.4 P +12.5 K + 3 Mg + 12 S (w/w) [kg 100 kg^{-1}] (Beiselen GmbH, Ulm, Germany) was applied, broadcast twice: once 380 kg fertilizer ha^{-1} before tillage in September 2021 and 324 kg fertilizer ha^{-1} at the beginning of the vegetation (BBCH 22) on 03/03/2022 with Rauch Aero 1110 pneumatic spreader (Rauch Landmaschinenfabrik GmbH, Rheinmünster, Germany). In total, 31 kg P ha^{-1}, 88 kg K ha^{-1}, 21 kg Mg ha^{-1}, and 84 kg S ha^{-1} were fertilized. The two different nitrogen treatments, calcium nitrate (YaraTera® CALCINIT®, YARA GmbH & Co. KG, Dülmen, Germany) and ammonium sulfate (NovaTec® Solub 21, COMPO EXPERT GmbH, Münster, Germany) with nitrification inhibitor [3,4-dimethyl-1H- pyrazole phosphate (DMPP)], were fertilized each with application rates of 240 kg N ha^{-1} also with the Rauch Aero 1110 pneumatic spreader. The application of calcium nitrate was divided into three applications according to farming practice. The first one with 40% of the total amount of calcium nitrate (80.6 kg N ha^{-1}) at BBCH 23 on 10/03/2022, the second one with 43% (86.7 kg N ha^{-1}) at BBCH 30 on 22/04/2022, and the third one with 17% (34.3 kg N ha^{-1}) at BBCH 33–37 on 20/05/2022.

The application of ammonium sulfate was divided into two applications: the first one with 60% of the total amount of ammonium sulfate (120.9 kg N ha^{-1}) at BBCH 23 on 10/03/2022, and the second one with 40% (80.6 kg N ha^{-1}) at BBCH 33–37 on 20/05/2022. The herbicide Artus® (Cheminova Deutschland GmbH & Co. KG, Stade, Germany) was sprayed with 50 g ha^{-1} at BBCH 23; the herbicides Biathlon® 4D + Dash® E.C. (70 g ha^{-1} + 1 l ha^{-1}; BASF SE, Limburgerhof, Germany) were sprayed together at BBCH 30. The herbicides were mixed with 300 l of water using an Amazone UF 901 (Amazonen-Werke H. Dreyer SE & Co. KG, Hasbergen, Germany) field sprayer (nozzle type IDKN 120-03, 3–4 bar, application volume 300 l ha^{-1}, driving speed 5 km/h; Lechler GmbH, Metzingen, Germany). Variants treated with herbicides were also treated with the calcium nitrate fertilizer and therefore performed as a positive control treatment. The experimental design was a row-column design latinized in columns and blocks (Richter et al., 2009) (Supplementary Figure 2). There were 28 different variants with four repetitions for each sharing 112 plots in total (Supplementary Table 5). Ten variants were investigated in this study.

1.2.2 Application of biostimulants

As BS products, a combined treatment of seaweed extract + chitosan mixture, micronutrients Zn and Mn, and a milk powder as a carrier medium for the microbial consortium of different microorganisms was used. The consortium (*Pseudomonas brassicacearum*: 2 × 10^{10} cfu g^{-1}, *Bacillus amyloliquefaciens*: 2 ×

10^{10} cfu g^{-1}, and *Trichoderma harzianum*: 1×10^8 cfu g^{-1}, SP Sourcon Padena GmbH, Tübingen, Germany) formulated with milk was applied as a soil application on the sowing day shortly before sowing. A 5 g package of pure milk powder (for 500 m^2) as a blank control and a 14 g package of milk powder mixed with the consortium (for 1,400 m^2) were each dissolved into 500 ml of distilled water and filled up to 20 l of water to prepare the SL. The quantities applied were 1,920 ml SL milk powder $plot^{-1}$ and 685.7 ml SL milk powder + consortium $plot^{-1}$ each with three watering cans/plot. Each watering can was filled with 640 ml SL milk powder or 228.6 ml SL milk powder + consortium both along with 10 l of water applied simultaneously per plot in the longitudinal direction at first, and then perpendicular to the first application in the transverse direction of the plot, to ensure an even distribution of the products. The seaweed extract (*Ascophyllum nodosum* extract, BioAtlantis Ltd., Tralee, Ireland) together with WUXAL® MICROMIX plus Chitosan (AGLUKON Spezialdünger GmbH & Co. KG, Düsseldorf, Germany) were applied as foliar application with an Amazone UF 901 (Amazonen-Werke H. Dreyer SE & Co. KG, Hasbergen, Germany) field sprayer (nozzle type IDKN 120-03, 3–4 bar, application volume 300 l ha^{-1}, driving speed 5 km/h; Lechler GmbH, Metzingen, Germany). The total amounts of mineral nutrients applied to the plants via chitosan are shown in Supplementary Table 6. In accordance with AGLUKON's application recommendation, the seaweed + chitosan mixture to be applied at shooting (BBCH 30) and at ear pushing (BBCH 51) was 2 l seaweed extract ha^{-1} and 2 l chitosan ha^{-1} (0.2 ml m^{-2}) in 250 l water ha^{-1} (25 ml water m^{-2}). An SL for 49 half plots was prepared as follows: 235.2 ml of seaweed extract and 235.2 ml of chitosan were filled up with distilled water to 1 l. The seaweed + chitosan solution got mixed with a magnetic stirrer and was filled in the tank of an Amazone UF 901 field sprayer with 29.4 l of water and 0.6 l seaweed + chitosan solution (25 ml m^{-2}). Then, the SL was sprayed on the leaves of the plants per plot. Micronutrients Zn and Mn (Lebosol®-Zink^{700}SC; 40% total Zn as zinc oxide 700 g Zn l^{-1}; Lebosol®-Mangan^{500}SC; 27.9% total Mn as manganese carbonate 500 g Mn l^{-1}; Lebosol® Dünger GmbH, Elmstein, Germany) were used for foliar application with an Amazone UF 901 field sprayer (nozzle type IDKN 120-03, 3–4 bar, application volume 300 l ha^{-1}, driving speed 5 km/h; Lechler GmbH, Metzingen, Germany). In accordance with the manufacturer's application recommendation, the Zn and Mn to be applied about 10 days after the start of vegetation, at BBCH 14 (3–4 days before *Zt* inoculation) were each 0.3 l Zn ha^{-1} and 0.5 l Mn ha^{-1} in 200 l of water (1.4 ml Zn $plot^{-1}$ and 2.4 ml Mn $plot^{-1}$ in 1 l of water), at BBCH 16 and at shooting/extension (BBCH 31) each 0.5 l Zn ha^{-1} and 0.75 l Mn ha^{-1} in 200 l of water (2.4 ml Zn $plot^{-1}$ and 3.6 ml Mn $plot^{-1}$ in 1 l of water).

1.2.3 *Zymoseptoria tritici* cultivation, inoculation, and disease assessment

The process of *Zt* cultivation was performed as described in the pot experiment (see 1.1.3) based on Saidi et al. (2012). *Zt* was applied with a Hege 76 field sprayer (device carrier from Wintersteiger, Ried im Innkreis, Austria; field sprayer from Kubota, Osaka, Japan) at BBCH 23-24 (160 DAS) and a concentration of 1×10^7 spores ml^{-1} *Zt* suspension. 100 ml *Zt* suspension m^{-2} (4.8 l $plot^{-1}$) was applied. The *Zt* disease incidence was determined visually by evaluating the percentage of infested plants inside a 60 × 40 cm rectangle formed with a meter stick at BBCH 30-31 (190 DAS), BBCH 31-33 (204 DAS), and BBCH 75 (259 DAS) modified according to Moll et al. (2000).

1.2.4 Plant analyses

The dry weight of five fully unfolded leaves from five different plants per plot at BBCH 33-37 (209 DAS) and three root samples per plot at BBCH 99 (278 DAS) were determined. To ensure the extraction of representative root samples, the shovel was placed carefully leaving sufficient space along the side and the puncture was created deep to the full height of the shovel blade (30 cm) to dig fresh roots out. The determination of the leaf and root dry weight as well as of the root length and morphological structure took place as in the pot experiment (see 1.1.4). Furthermore, for estimating grain nutritional analyses, grain samples (400 - 500 g per plot) from harvest at BBCH 99 (280 DAS) were oven-dried at 40°C for 4 days and then weighed.

1.2.5 Determination of stress metabolites

Five fully unfolded leaves without visible disease symptoms from five different plants per plot from BBCH 75 (245 DAS) were used for the determination of selected physiological stress indicators with the methods as described in the pot experiment (see 1.1.5).

1.2.6 Analysis of mineral nutrients

The concentrations of the essential nutrients N, P, K, Ca, Mg, S, Zn, Mn, Fe, and Cu were determined in oven-dried and milled leaf samples from BBCH 33-37 (209 DAS) and in 400 g–500 g oven-dried and milled grain samples per plot from harvest at BBCH 99 (280 DAS). Grinding of the leaf samples was the same process as for the pot experiment (see 1.1.6). 0.2 g of powdered leaf material and powdered grain material was weighed into a quartz glass beaker followed by the process of element analysis, which took place as described in the pot experiment (see 1.1.6).

1.2.7 Statistical evaluation

Statistical analyses were performed using SAS/STAT software package of SAS® 9.4 (2016) (SAS Institute Inc., Cary, USA). A one-way ANOVA followed by a Tukey test ($p < 0.05$ significance level) was used to compare means for statistically significant differences. Data are presented as mean values. Normal distributions and variance homogeneities of the residuals were checked by the Shapiro–Wilk test and by Levene's test, as well as graphically against the predicted values by QQ plots, histograms, and graphs of the residuals according to Kozak and Piepho (2017).

2 Results

2.1 Pot experiment

2.1.1 Disease severity affected by different N supplies and microbial and non-microbial biostimulants

The *Zt* disease severity (DS) increased over time following a biphasic pattern with a slower relative increase of 25%–27%

between 14 and 28 days after inoculation (DAI) and a steeper increase by 48%–53% over the next 12 days, finally reaching comparatively low absolute DS levels of 15% at the end of the culture period without N-form-dependent differences (Figure 1).

Protective effects of microbial consortium (MC) were particularly expressed under the ammonium nutrition [average DS suppression rate 40% (NH_4^+) vs. 22% (NO_3^-)] over the culture period. Furthermore, a clear trend although not significant at all time-points was detected (Figures 2A, B).

Protective effects of seaweed extract + chitosan (SC) appeared particularly under nitrate nutrition [average DS suppression rate 47% (NO_3^-) vs 37% (NH_4^+)]. Similar to MC, differences declined by the end of the culture period (Figures 2C, D).

2.1.2 Defense metabolites

MC: At final harvest (55 DAS, 40 DAI), a trend of increased leaf H_2O_2 concentrations by 47% (Figure 3A) was associated with a similar but significant increase in APX activity (47%, Figure 3B) mediating H_2O_2 detoxification in MC-inoculated plants with NH_4^+ fertilization. No stimulatory effects of MC inoculation on H_2O_2 accumulation and APX activity were recorded under NO_3^- supply (Figures 3A, B), but APX activity was generally significantly increased under NO_3^- supply compared with plants supplied with NH_4^+ fertilization (Figure 3B). No comparable effects were detected at earlier developmental stages (27 DAS, 12 DAI), for GPX activity or for total antioxidants mediating non-enzymatic detoxification of reactive oxygen species (ROS) (Supplementary Figure 3).

SC: In contrast to MC-inoculated plants, earlier changes in defense metabolites were detectable already at 27 DAS (12 DAI). In plants with NO_3^- fertilization, H_2O_2 accumulation in the leaf tissue tended to increase by 30% (Figure 3C). This was associated with an increase (27%) in the activity of GPX (Figure 3D). No comparable effects were detectable under NH_4^+ fertilization (Figures 3C, D), at later developmental stages (55 DAS, 40 DAI) or for APX activity. Also, no significant differences were detectable for total antioxidants (Supplementary Figure 4).

2.1.3 Mineral nutritional status

For all treatments, the nutritional status of N, P, S, Mg, Fe, and Mn at final harvest was sufficient for adequate growth of wheat plants. Critical nutrient concentrations close or even below the reported deficiency threshold values (Bergmann, 1992) were recorded for K, Ca, Zn, and Cu. N form effects were detected for P, S, and Zn with increased leaf concentrations under NH_4^+ fertilization and for Ca, Mg, K, and Mn concentrations promoted under NO_3^- supply (Supplementary Table 7).

No nutritional benefits were recorded in response to inoculation with MC. By contrast, SC applications increased the concentrations of Zn and Cu (Figures 4A, C) above the deficiency thresholds (Bergmann, 1992). The plants with nitrate fertilization exhibited particularly high Mn shoot concentrations, which were further increased by SC application (Figure 4B).

2.1.4 Plant growth

Neither the pathogen inoculation nor the application of biostimulants affected shoot and root biomass production, total root length, or root diameter (Figure 5). A significant decline in shoot biomass (14%) was recorded in pathogen-infected plants with NO_3^- supply as compared with NH_4^+ fertilization (Figure 5A).

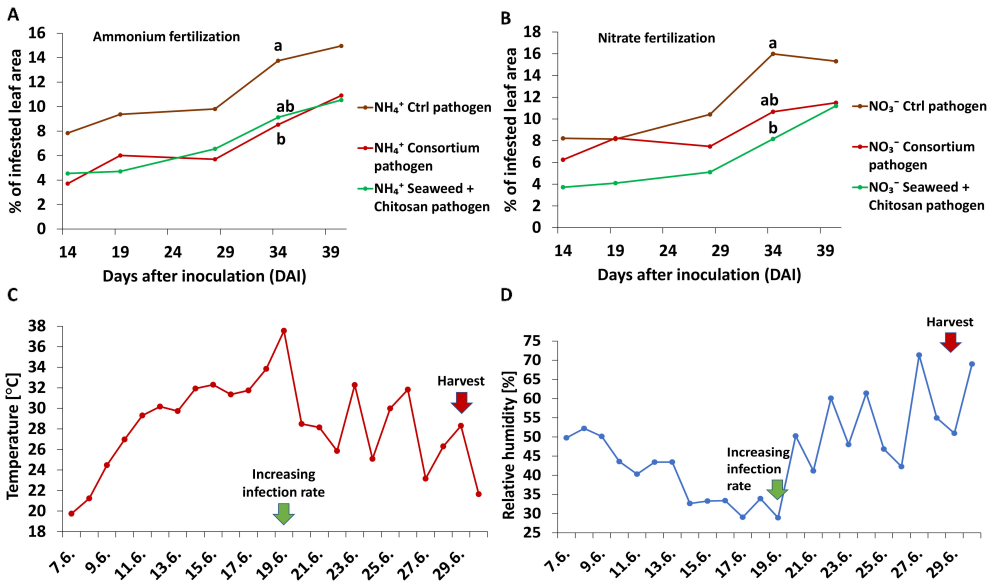

FIGURE 1
(A, B) Time course of disease spread of *Zymoseptoria tritici (Zt)* [% of infested leaf area] on winter wheat plants in the greenhouse treated with ammonium sulfate or calcium nitrate under control condition (brown lines) or with different biostimulants (microbial consortium, red lines; seaweed extract + chitosan, green lines) both inoculated with *Zt*. **(C)** Temperature course [°C] and **(D)** relative humidity course [%] over the plant growth period. Increasing infection rate from 19.06.2022 on. **(A, B)** represent mean values of five replicates per treatment. Mean values with at least one same or without lowercase letters within graph **(A, B)** are not significantly different according to Tukey test (α=0.05).

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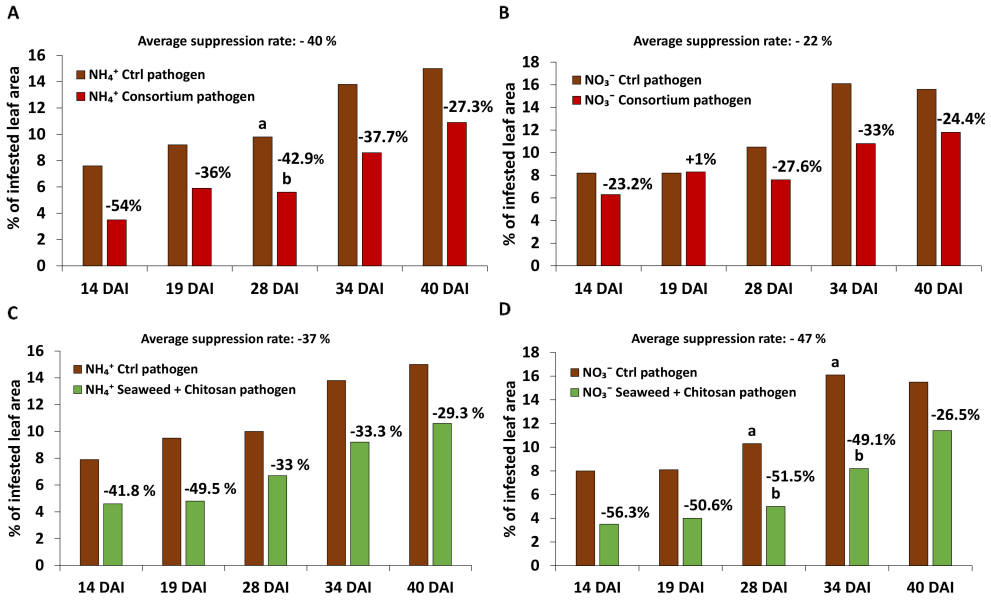

FIGURE 2
Disease severity [% of infested leaf area] of *Zymoseptoria tritici (Zt)* on winter wheat plants in the greenhouse 14, 19, 28, 34 and 40 days after inoculation (DAI) treated with **(A, C)** ammonium sulfate and **(B, D)** calcium nitrate, both under control condition (brown bars) and with different biostimulants (microbial consortium, red bars; seaweed extract + chitosan, green bars) inoculated with *Zt*. **(A–D)** represent mean values of five replicates per treatment. Mean values with the same or without lowercase letters within each graph are not significantly different according to Tukey test (α=0.05).

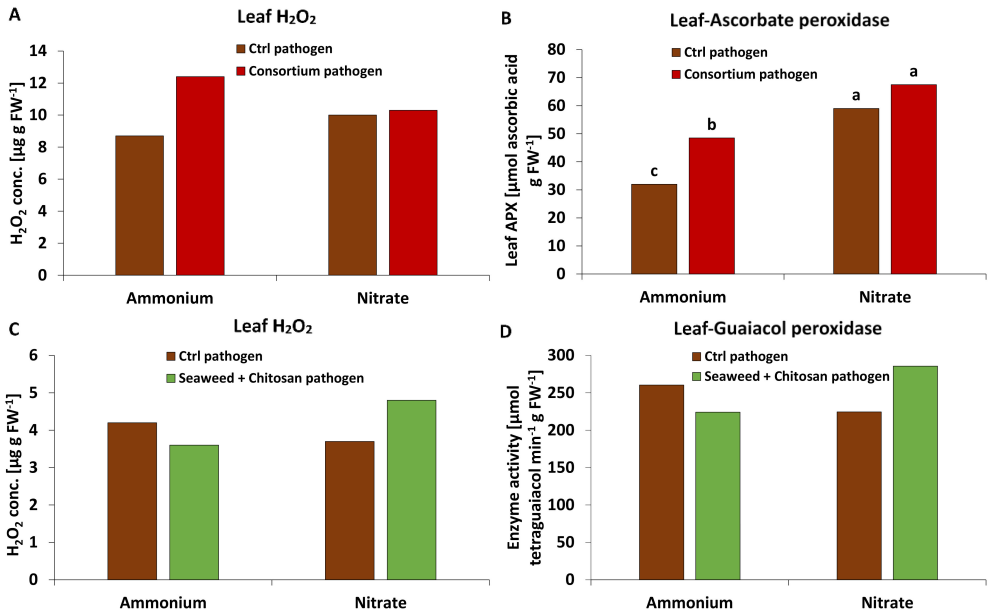

FIGURE 3
(A, C) Hydrogen peroxide (H_2O_2) concentration [ug g FW^{-1}], **(B)** ascorbate peroxidase (APX) activity [µmol ascorbic acid g FW^{-1}] and **(D)** guaiacol peroxidase (GPX) activity [µmol tetraguaiacol min^{-1} g FW^{-1}] in the leaf tissue of winter wheat plants in the greenhouse treated with ammonium sulfate or calcium nitrate under control condition (brown bars) or with different biostimulants (microbial consortium, red bars (55 DAS); seaweed extract + chitosan, green bars (27 DAS)) both inoculated with *Zymoseptoria tritici (Zt)*. A represents mean values of three replicates in the ammonium control and ammonium- consortium treatments, five replicates in the nitrate control and nitrate-consortium treatments. **(B)** represents mean values of three replicates in the ammonium control and ammonium-consortium treatment, five replicates in the nitrate control and four replicates in the nitrate-consortium treatment. **(C, D)** represent mean values of five replicates per treatment. Mean values with the same or without lowercase letters within each graph are not significantly different according to Tukey test (α=0.05).

2.2 Field experiment

2.2.1 Disease incidence affected by combination of biostimulants

Due to the fact that no significant differences in *Zt* disease incidence (DI) between the *Zt*- and non-infected plants could be determined, all results with applied *Zt inoculum* and natural infestation were combined to a pool of *Zt*-inoculated and naturally infested variants in the following graphs. Independent of *Zt* inoculation, at 190 DAS (April, BBCH 30-31), first leaf blotch symptoms with a disease incidence of 5%–6% became detectable in all treatments with the exception of the control without N fertilization where DI reached already 13% (Figure 6A). A massive increase of DI was recorded within the next 10 weeks until 259 DAS (July, BBCH 75). Maximum DI values of approximately 65% were recorded in plants without N fertilization but similarly also under nitrate fertilization with or without application of biostimulants. Ammonium fertilization significantly reduced DI by 10% and the lowest DI values below 50% were recorded for BS-treated plants with NH_4^+ supply (Figure 6B).

2.2.2 Defense-related metabolites

The leaf H_2O_2 accumulation significantly increased by approximately 50% in the BS-treated plants as compared with the remaining treatments associated also with the highest APX activity (Figures 7A, B). Compared with plants supplied with nitrate fertilization, ammonium fertilization tended to increase also GPX activity and accumulation of total antioxidants (Figures 7C, D).

2.2.3 Mineral nutritional status

With the exception of Zn, the mineral nutritional status was sufficient for all investigated nutrients (N, P, K, S, Ca, Mg, Fe, Mn, Cu) in all plants which received N fertilization. However, negative controls without N supply showed multiple nutrient deficiencies with respect to N, K, Ca, Mg, and Zn but also decreased leaf concentrations of the remaining nutrients except Cu. Compared with NO_3^- fertilization, NH_4^+ fertilization decreased the leaf concentrations of N, K, Mg, and Ca (summarized in Supplementary Figure 5). The only recorded BS effect was reflected by a significantly increased Zn concentration in grains in the ammonium-treated variant. Additionally, NH_4^+ fertilization significantly increased grain concentrations of Mn (Figure 8).

2.2.4 Grain yield, grain protein, and root growth characteristics

A high grain yield with an average of 9.2 t ha^{-1} was achieved in all treatments except the negative control without N supply, which showed a 40% reduction (Figure 9A). In all treatments receiving N fertilization, approximately 12% grain protein content was recorded with the lowest values in BS-treated plants under NH_4^+ fertilization. In the negative control without N fertilization, the grain protein content reached only 8% (Figure 9B).

For the characterization of root morphological characteristics, root length in different root diameter classes was investigated from excavated root systems. For all treatments, the proportion of fine roots (0 - 0.2 mm diameter) was very similar and comprised between 30% and 40% of the total root length (Supplementary Figure 6).

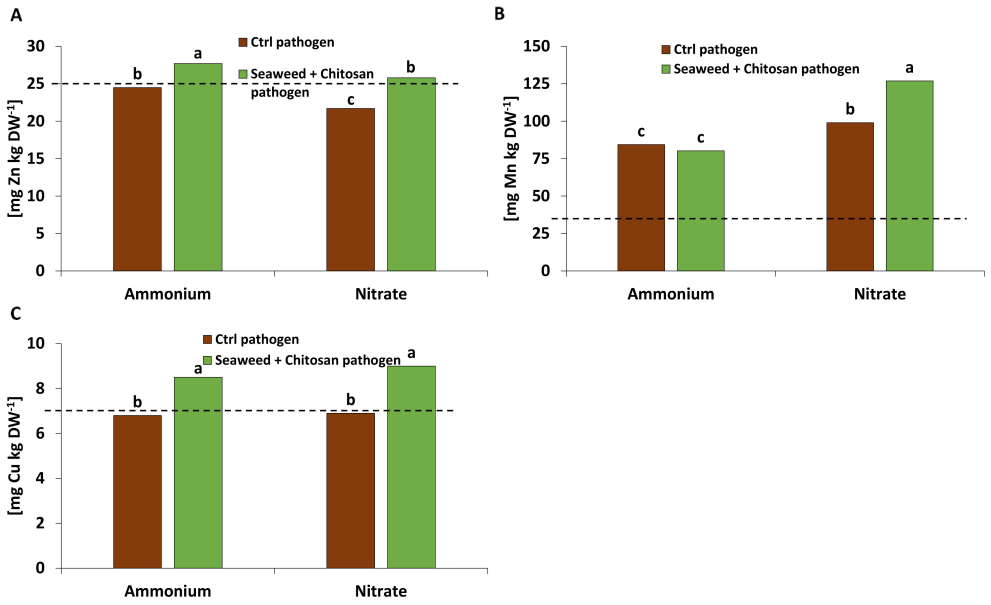

FIGURE 4
(A) Zinc (Zn), **(B)** manganese (Mn), **(C)** copper (Cu) concentration [mg kg DW^{-1}] in the shoot tissue of winter wheat plants in the greenhouse 55 days after sowing (DAS) treated with ammonium sulfate or calcium nitrate under control condition (brown bars) or with seaweed extract + chitosan (green bars) both inoculated with *Zymoseptoria tritici (Zt)*. The dashed lines show the nutrient deficiency limits according to Bergmann (1992). **(A–C)** represent mean values of five replicates per treatment. Mean values with the same lowercase letters within each graph are not significantly different according to Tukey test (α=0.05).

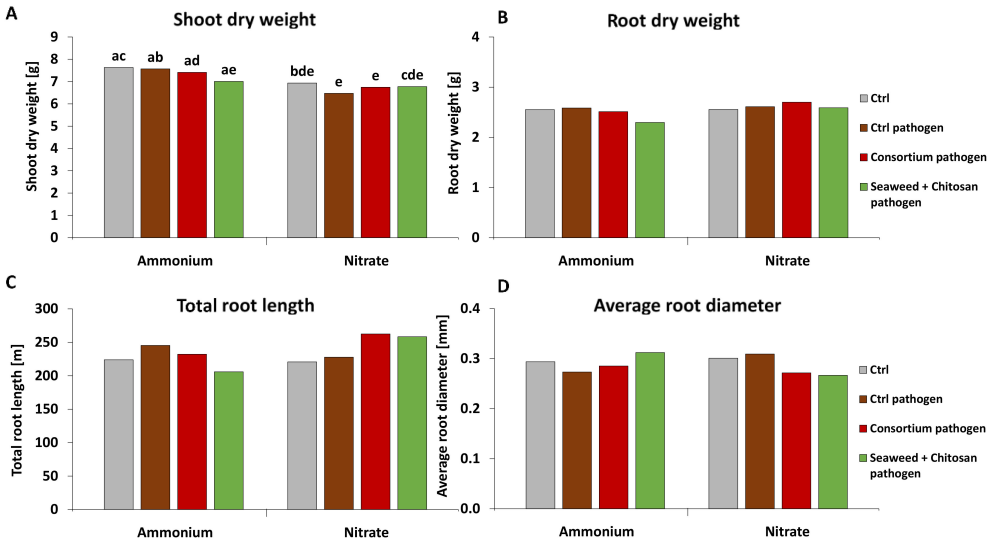

FIGURE 5
(A) Shoot and **(B)** root dry weight, **(C)** total root length and **(D)** average root diameter of winter wheat plants in the greenhouse 55 days after sowing (DAS) treated with ammonium sulfate or calcium nitrate under control condition with or without *Zymoseptoria tritici (Zt)* inoculum or with different biostimulants (microbial consortium, red bars; seaweed extract + chitosan, green bars) both inoculated with *Zt*. **(A–D)** represent mean values of five replicates per treatment. Mean values with at least one same or without lowercase letters within each graph are not significantly different according to Tukey test (α=0.05).

3 Discussion

3.1 Disease spread affected by form and amount of N supply

In the greenhouse experiment, disease spreading showed a biphasic pattern, starting with a slow increase of approximately 25% during 14 DAI–28 DAI followed by a stronger spreading of approximately 50% during the next 12 days (Figures 1A, B). This may be attributed to unfavorable conditions with respect to temperature (25°C–36°C) and relative humidity (<50%) during the initial phase of pathogen infection (Eyal et al., 1987; Shaw, 1991; Fones et al., 2017). Accordingly, disease spread subsequently increased with declining temperature (<25°C) and increasing relative humidity (>50%) (Figures 1C, D), finally reaching a moderate disease severity (DS) of 15% independent of the N form supply (NO_3^- vs NH_4^+ during the first 8 weeks of plant development).

Similarly also under field conditions, a low disease incidence (DI) of 5%–6%, which was independent of the applied N form (Figure 6A), was observed during early growth of winter wheat in spring until BBCH 30-31, associated with generally suboptimal temperatures of <15°C for *Zt* infection during this time period (Eyal et al., 1987). During the next 10 weeks, a steep DI increase (Figure 6B) coincided with conductive conditions, characterized by increasing temperatures, relative humidity values of 60%–90%, and precipitation above the long-term average (164%–168%) in April and June (Supplementary Figure 7). However, in later stages

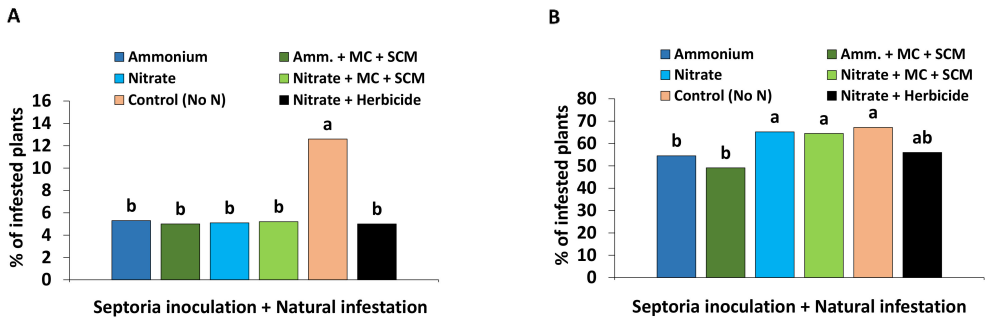

FIGURE 6
Disease incidence **(A)** 190 days after sowing (DAS) and **(B)** 259 days after sowing (DAS) [% of infested plants] of winter wheat plants in the field treated with either ammonium sulfate (Amm.) or calcium nitrate (Nitrate). In addition, both fertilizers were combined with a microbial consortium (MC) and seaweed extract + chitosan + micronutrients zinc + manganese (SCM). **(A, B)** depict a pool of *Zymoseptoria tritici (Zt)* inoculated and natural infested treatments. A negative control without nitrogen (N) (orange bars) and a positive control (black bars) both with natural infestation are included. **(A, B)** represent seaweed extract + chitosan not yet applied at the time of bonituring. **(A, B)** represent mean values of eight replicates per treatment. Mean values with at least one same lowercase letter within each graph are not significantly different according to Tukey test (α=0.05).

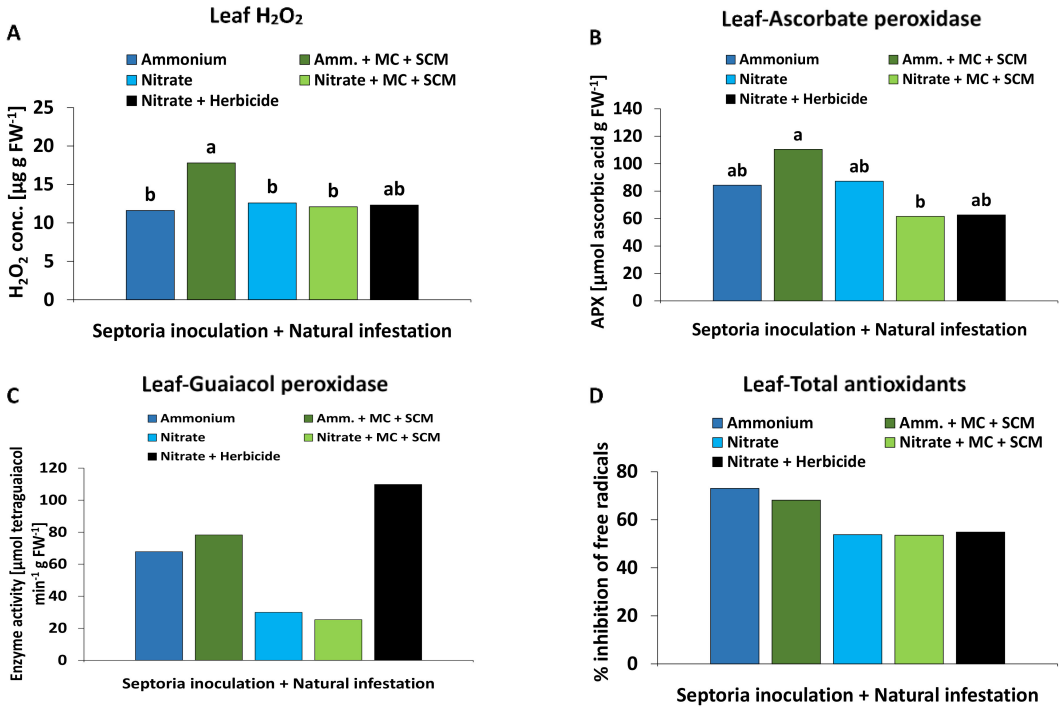

FIGURE 7
(A) Hydrogen peroxide (H2O2) concentration [µg g FW^{-1}], **(B)** ascorbate peroxidase (APX) activity [µmol ascorbic acid g FW^{-1}], **(C)** guaiacol peroxidase (GPX) activity [µmol tetraguaiacol min^{-1} g FW^{-1}] and **(D)** total antioxidant potential [% inhibition of free radicals] in the leaf tissue of winter wheat plants in the field 245 days after sowing (DAS) treated with either ammonium sulfate (Amm.) or calcium nitrate (Nitrate). In addition, both fertilizers were combined with a microbial consortium (MC) and seaweed extract + chitosan + micronutrients zinc + manganese (SCM). **(A–D)** depict a pool of *Zymoseptoria tritici (Zt)* inoculated and natural infested treatments. A positive control (black bars) with natural infestation is included. **(A–D)** represent mean values of eight replicates per treatment. Mean values with at least one same or without lowercase letters within each graph are not significantly different according to Tukey test (α=0.05).

of plant development (BBCH 75), a clear N form effect was detectable with a maximum DI of 65% and the highest leaf N concentrations in plants with NO_3^- fertilization. By contrast, a significantly lower DI (55%) and a lower leaf N status were recorded in plants with NH_4^+ supply (Figure 6B; Supplementary Figure 5A). High levels of N fertilization can improve the N-nutritional status and promote plant growth, but at the expense of reduced formation of lignin and waxy cuticles acting as physical barriers for pathogen penetration (Sun et al., 2020). Accordingly, also Simón et al. (2003) and Harrat and Bouznad (2014) reported increased disease spread for *Zt* in wheat, associated with increased N supply. In our experiment, the lower N status of plants supplied with stabilized NH_4^+ fertilization may be related to reduced N availability due to stronger adsorption and lower mobility of NH_4^+ in soils compared to NO_3^- fertilizers (Marschner and Rengel, 2023). Accordingly, a stimulation of oxidative stress defense, lignification, and accumulation of epicuticular waxes under NH_4^+ fertilization was reported by Wang et al. (2010) and Blanke et al. (1996).

Increased DI values were similarly recorded also in wheat plants without N fertilization both during early growth (BBCH 30-31, Figure 6A) and in later stages of plant development (BBCH 75, Figure 6B). A massive decline in grain yield (Figure 9A) associated with leaf concentrations of N, K, Mg, Ca, and Zn below the reported deficiency thresholds (Bergmann, 1992) suggests that the respective plants were obviously affected by multiple nutrient deficiencies, weakening the expression of defense responses against pathogens.

3.2 Disease spread affected by microbial and non-microbial biostimulants

A microbial PGPM consortium (MC) derived from a combination of bacterial and fungal PGPM strains (*Pseudomonas*, *Bacillus*, *Trichoderma*) and a non-microbial BS combination product (SC) based on *Ascophyllum nodosum* seaweed extract, chitosan, and stress-protective micronutrients (Zn, Mn, Cu) were used for the experiments. The selection was based on literature reports suggesting synergistic or complementary benefits of BS combinations to protect plants against various biotic and abiotic stress factors (Zaim et al., 2018; Bradáčová et al., 2019; Gunupuru et al., 2019; Karuppiah et al., 2019; Moradtalab et al., 2020) to cover a wider range of environmental conditions.

In the greenhouse experiment, protective effects against *Zt* leaf blotch were recorded for both MC and SC treatments (Figure 2). In contrast to the untreated controls (see. 4.1), disease severity was differentially affected by the form of N supply in MC- and SC-treated plants. While the MC formulation was more effective in plants with NH_4^+ supply, the non-microbial SC combination

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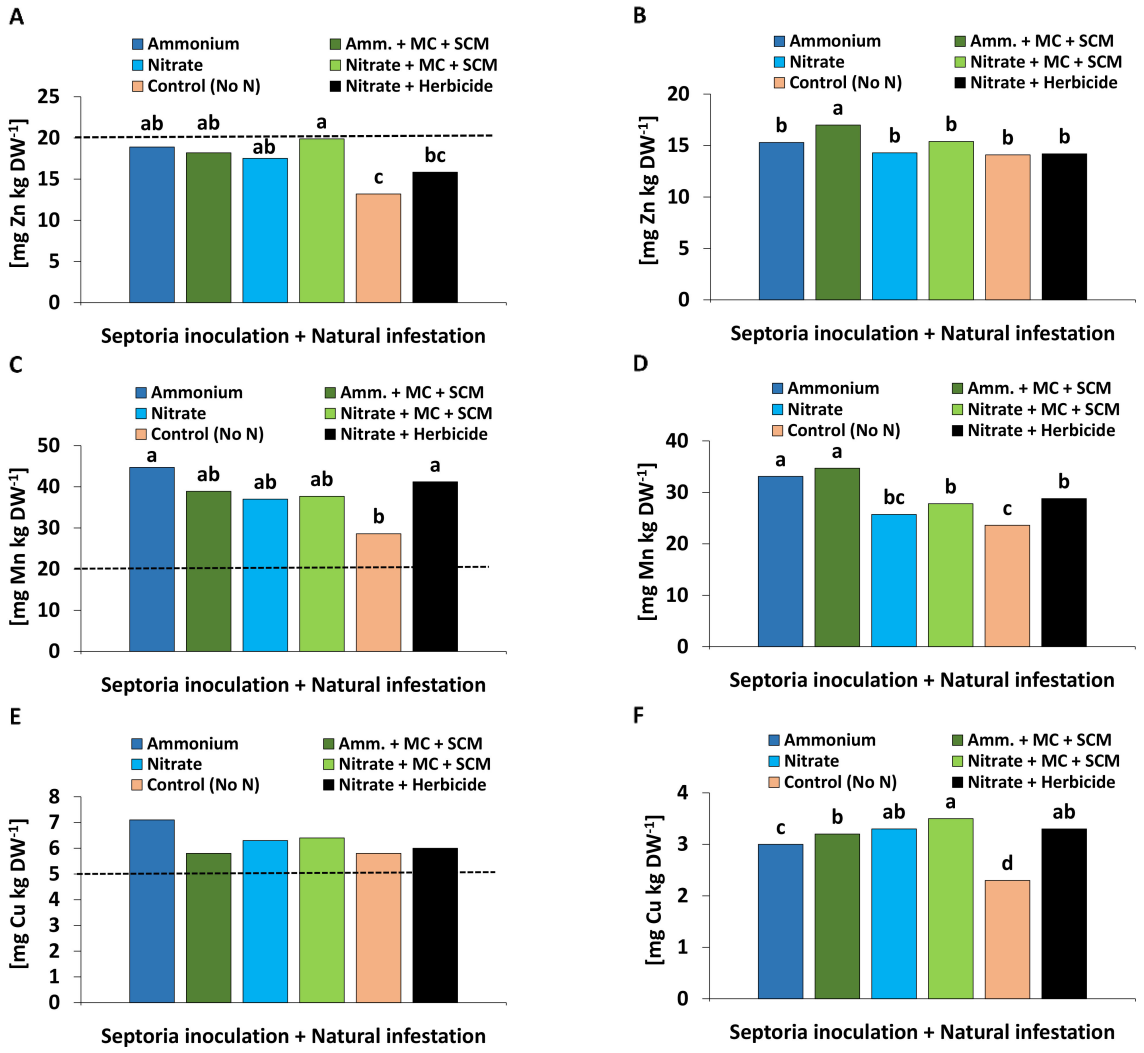

FIGURE 8
(A) Zinc (Zn), **(C)** manganese (Mn), **(E)** copper (Cu) concentration [mg kg DW^{-1}] in the leaf tissue 209 days after sowing (DAS) and **(B)** Zn, **(D)** Mn, **(F)** Cu concentration [mg kg DW^{-1}] in the grain 280 days after sowing (DAS) of winter wheat plants in the field treated with either ammonium sulfate (Amm.) or calcium nitrate (Nitrate). In addition, both fertilizers were combined with a microbial consortium (MC) and seaweed extract + chitosan + micronutrients zinc + manganese (SCM). A-F depict a pool of *Zymoseptoria tritici (Zt)* inoculated and natural infested treatments. A negative control without nitrogen (N) (orange bars) and a positive control (black bars) both with natural infestation are included. **(A, C, E)** represent seaweed extract + chitosan not yet applied at the time of bonituring. The dashed lines show the nutrient deficiency limits according to Bergmann and Neubert (1976). **(A–F)** represent mean values of eight replicates per treatment. Mean values with at least one same or without lowercase letters within each graph are not significantly different according to Tukey test (α=0.05).

responded faster compared with MC and showed stronger suppressive effects under NO_3^- fertilization (Figure 2).

3.2.1 Oxidative burst

Similar to DS, also physiological stress defense responses to BS applications were differentially influenced depending on the form of N supply. Ammonium-fertilized plants specifically responded to MC application with a selective increase in leaf H_2O_2 accumulation by almost 50%. A similar trend was recorded after SC application in plants with NO_3^- supply (Figure 3). This may reflect the locally increased ROS (H_2O_2) production of pathogen-infected tissues (oxidative burst), frequently recorded as a first defense line against invading pathogens (Choudhary et al., 2017). Accordingly, the capacity for H_2O_2 production during the oxidative burst seems to be one factor determining *Zt* resistance in wheat genotypes (Shetty et al., 2003). Interestingly, after application of PGPMs or non-microbial biostimulants, the opposite response, characterized by improved ROS detoxification and declining tissue concentrations of H_2O_2, is frequently reported in plants affected by abiotic stress (Orzali et al., 2017; EL Mehdi EL Boukhari et al., 2020). However, in the presence of pathogens, also defense priming via increased PGPM-induced H_2O_2 production (mediated, e.g., by bacterial surfactants), has been described (Zhu et al., 2022). This scenario applies similarly also for the SC components chitosan (Vasil'ev et al., 2009) and *Ascophyllum nodosum* seaweed extract (Cook et al., 2018), suggesting selective effects of the investigated microbial and non-microbial BSs, depending on biotic vs abiotic stress factors.

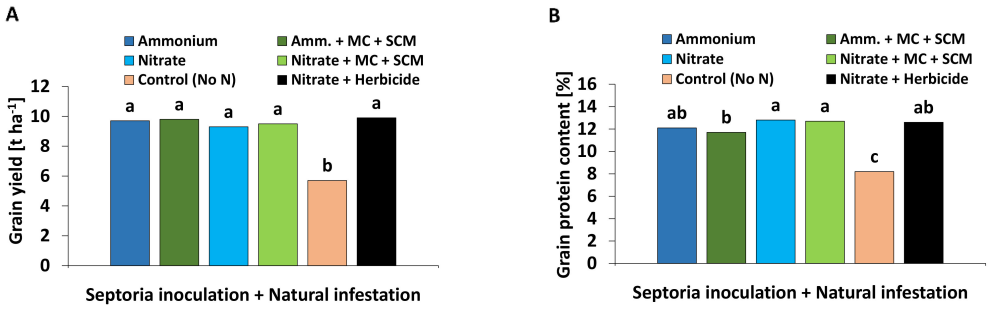

FIGURE 9
(A) Total grain yield [t ha^{-1}] and **(B)** grain protein content [%] 280 days after sowing (DAS) of winter wheat plants in the field treated with either ammonium sulfate (Amm.) or calcium nitrate (Nitrate). In addition, both fertilizers were combined with a microbial consortium (MC) and seaweed extract + chitosan + micronutrients zinc + manganese (SCM). **(A, B)** depict a pool of *Zymoseptoria tritici (Zt)* inoculated and natural infested treatments. A negative control without nitrogen (N) (orange bars) and a positive control (black bars) both with natural infestation are included. **(A, B)** represent mean values of eight replicates per treatment. Mean values with at least one same lowercase letter within each graph are not significantly different according to Tukey test (α=0.05).

3.2.2 Detoxification of reactive oxygen species

In parallel with the stimulation of H_2O_2 accumulation, MC inoculation also increased the activity of APX mediating the enzymatic degradation of H_2O_2 (Caverzan et al., 2012) preferentially in wheat plants with NH_4^+ fertilization (Figure 3B). This effect may protect non-infected tissues located close to the infection sites from oxidative stress associated with the oxidative burst. Systemic induction of APX and other enzymes involved in ROS detoxification is a well-known response not only to inoculation with PGPM strains of *Pseudomonas*, *Bacillus*, or *Trichoderma* (reviewed by Kumudini and Patil, 2021) but also to treatments with non-microbial BSs such as seaweed extracts (EL Mehdi EL Boukhari et al., 2020) or chitosan (Orzali et al., 2017). In contrast to MC-treated plants, no comparable increase in APX activity was recorded after SC application. However, under NO_3^- fertilization, the activity of GPX, known to play a central role in pathogen defense (Prakasha and Umesha, 2016), was increased in SC-treated plants. Similar to APX, also GPX activity might be expected to reduce the level of ROS by metabolizing H_2O_2. However, GPX is also capable of catalyzing various oxidase reactions leading to H_2O_2 generation and is involved in lignification, biosynthesis of ethylene, wound healing, and polysaccharide cross-linking (Sharma et al., 2012).

3.2.3 Micronutrient status

SC application increased the plant micronutrient status, thereby reducing Zn and Cu deficiencies (Bergmann, 1992) of the investigated plants (Figure 4). However, this effect was not detected in response to MC inoculation (Supplementary Figure 8). Deficiency of Zn and Cu was likely related to the neutral soil pH promoting the fixation of these micronutrients (Marschner, 1995). Supplementation by foliar micronutrient supply via the SC mixture/treatment obviously provided these micronutrients as important cofactors for enzymatic ROS detoxification and the GPX-mediated reactions described above (Datnoff et al., 2007).

An extraordinarily high plant-Mn status, far above the reported deficiency threshold (Bergmann, 1992), was recorded in the pot experiment, which was even further increased by SC application under NO_3^- fertilization (Figure 4B). In plants with NH_4^+ fertilization, this increase was likely counteracted by cation competition between uptake of NH_4^+ and Mn^{2+} (Marschner, 1995). The high Mn status may reflect an exceptionally high Mn availability in the respective soil, which was probably caused by long-time exposure to high greenhouse temperatures of 25°C–35°C (Figure 1C) reported to increase soil Mn availability (Reid and Racz, 1985). This may be mediated by stimulation of reductive processes such as increased respiratory oxygen consumption and modifications of microbial communities involved in Mn mobilization at higher soil temperatures especially when there is too much water and/or soil substrate compaction in the pot (Sparrow and Uren, 1987). A protective effect of Mn nutrition on controlling root or foliar diseases of plants (e.g., powdery mildew, downy mildew, take-al) is well documented (reviewed by Datnoff et al., 2007). Accordingly, also Eskandari et al. (2018) and Eskandari et al. (2020) reported improved resistance of cucumber to powdery mildew and anthracnose after foliar application of Mn, reaching similar Mn leaf concentrations >100 mg kg^{-1} DM as recorded in SC-treated plants in our study (Figure 4). The protective effects were attributed to improved lignification associated with increased activities of guaiacol peroxidase and phenol oxidase and increased callose production as mechanical barriers against fungal infection (Eskandari et al., 2018, 2020).

Taken together, the results suggest complementary protective effects of the MC and SC formulations against *Zt* leaf blotch, influenced by different forms of N fertilization. The defense responses, systemically induced by the MC formulation under NH_4^+ supply may be related to beneficial effects of NH_4^+ fertilizers on the establishment of plant microbial interactions. Therefore, PGPM inoculants, based on strains of *Pseudomonas*, *Bacillus*, and *Trichoderma* may promote pathogen suppression, e.g., via improved root colonization, increased auxin production or proliferation of root hairs as potential infection sites (Bradáčová et al., 2019; Mpanga et al., 2019; Moradtalab et al., 2020). The SC formulation additionally provides a source of micronutrients (Zn, Mn, Cu) with essential functions as cofactors for various

physiological defense responses to pathogen infection. This is particularly important under conditions of limited micronutrient solubility at neutral to alkaline soil pH, further promoted by root-induced rhizosphere alkalization induced by NO_3^- fertilization (Marschner, 1995).

3.2.4 Field experiment

Under field conditions, a combined application of MC and SC with the addition of micronutrients zinc + manganese (MC-SCM application) was performed to exploit the potential benefits arising from complementary effects detected in the greenhouse experiment. At 259 DAS (BBCH 75), the highest DI of 65% was recorded under NO_3^- fertilization without any benefits by MC-SCM application (Figure 6). By contrast, MC-SCM-treated plants supplied with NH_4^+ fertilization showed the lowest DI (<50%) associated with increased H_2O_2 accumulation and APX activity in the leaf tissue (Figure 7), similar to the MC responses in the greenhouse experiment. However, compared with NO_3^- fertilization, the DI significantly declined to 55% even in untreated controls with NH_4^+ supply, demonstrating only a small additional non-significant effect of MC-SCM (Figure 6), at least during the investigated later stages of plant development. This applied similarly also for an improved micronutrient status (Zn, Mn, Cu) detectable in the leaf tissue and finally also in the grains (Figure 8).

Similarly, also in the greenhouse experiment, the protective effects of the MC/SC inoculants declined with increasing age of the plants and increasing DS (Figure 2). This may indicate that the applied BS products mainly promoted the early stages of pathogen defense. It may also be speculated that this reflects a downregulation of plant defense reactions induced by fungal effector proteins, characteristic for many biotrophic and hemibiotrophic pathogens including *Zt* (Yang et al., 2013; Brennan et al., 2019).

3.3 Plant growth and grain yield

Despite mitigation effects on DI and DS recorded in the experiments, plant growth indicators such as shoot and root biomass, root length, root diameter (Figure 5; Supplementary Figure 6), and also final grain yield (Figure 9) remained largely unaffected, both by pathogen inoculation or application of the microbial and non-microbial BSs. High grain yields of 9 t ha^{-1}–10 t ha^{-1} reaching baking quality with grain protein contents around 12% in all treatments supplied with N fertilizers even in the presence of high DI values (50%–65%) may reflect a certain inherent disease tolerance of the investigated wheat cultivar (Asory). Asory is claimed to be a variety of medium-high resistance against *Zt* with a rating of 7.5 (Danko Saatzucht Deutschland GmbH, 2024). The high grain yields, far above the 2022 average of 7.5 t ha^{-1} in Baden-Württemberg (Statistisches Landesamt Baden-Württemberg, 2023), may indicate that there was no relevant impact of stress factors other than the *Zt*-related pathogen pressure. This could also be a reason for the lack of expression of relevant effects on plant growth in response to application of the investigated microbial and non-microbial BS products, of which benefits have been frequently proven under abiotic stress conditions (Bradáčová et al., 2016; Mpanga et al., 2019; Moradtalab et al., 2020; Rasul et al., 2021).

3.4 Concluding remarks

The BS-assisted fertilization strategies investigated in this study could not fully prevent but clearly slowed down *Zt*-induced disease spread, depending on the stages of plant development and the form of N fertilization. The applied BS products promoted early defense responses to pathogen attack with preferences for the microbial MC formulation if combined with NH_4^+ fertilization and the non-microbial SC formulation with NO_3^- supply. Benefits of NH_4^+-dominated N fertilization correlated with an improved micronutrient status but were detectable also in later stages of plant development under field conditions. Thus, the combined application of MC and SC with the addition of micronutrients zinc + manganese (MC-SCM application) reduced the pathogen pressure with NH_4^+ fertilization in the field experiment by inducing increased H_2O_2 and APX activities. Furthermore, plant growth remained largely unaffected, under both greenhouse and field conditions. It remains to be investigated to which extent these effects can be used to replace fungicide applications during the respective time periods as part of a strategy for integrated pest management. Additional benefits may arise from protective effects reported for the applied BS products against abiotic stress factors.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MG: Writing – original draft, Writing – review & editing. SD: Writing – review & editing. LS: Writing – review & editing. MW: Writing – review & editing. AM: Writing – review & editing. AA: Writing – review & editing. NS: Writing – review & editing. KM: Writing – review & editing. GN: Writing – review & editing. TM: Writing – review & editing. KB: Writing – review & editing.

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Conflict of interest

Author NS was employed by BioAtlantis Ltd. Author KM was employed by Sourcon Padena GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1407585/full#supplementary-material

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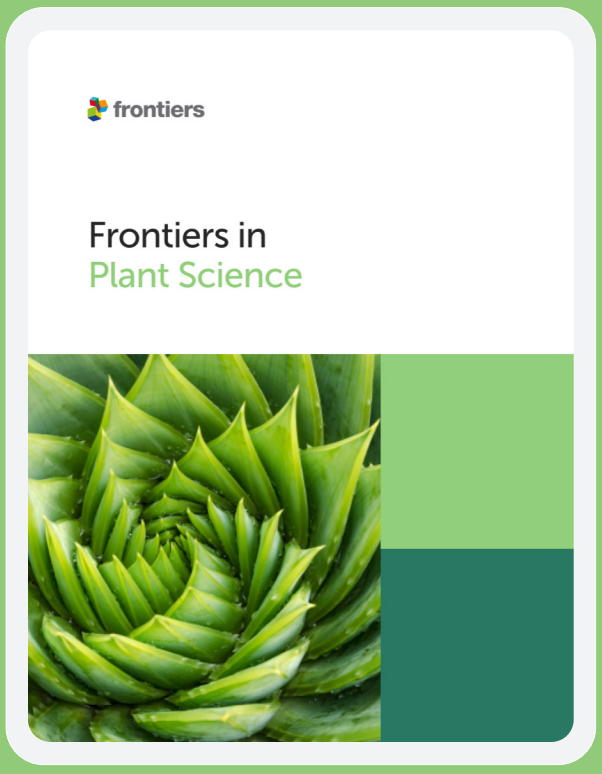

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