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Microbiomics in food security: Paradigm shift in omics

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Microbiomics in food security: Paradigm shift in omics

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Editorial: Microbiomics in food security: paradigm shift in omics

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Editorial on the Research Topic
Microbiomics in food security: paradigm shift in omics

Climate change is a major challenge for the agricultural sector in meeting the goals of food security and zero hunger. An increasing concern among agricultural scientists is protecting crop plants from the negative impacts of climate change activities, such as abiotic stresses and attacks by plant pathogens (Singh et al., 2023a). As a result, there is a pressing demand for eco-friendly technologies to safeguard crops against stressors and enhance field productivity, ultimately meeting the qualitative and quantitative needs of food consumers (Banerjee and van der Heijden, 2023). Relying solely on chemical pesticides poses long-term hazards for applicators, consumers, and pollinators, indirectly affecting the entire ecosystem. Excessive use of chemical pesticides can lead to the development of resistance in plant pathogens, including fungi and insects, making them even more destructive during disease outbreaks (Riedo et al., 2023; Wen et al., 2023). To address this challenging situation, a promising and environmentally friendly innovation is the use of soil microbes in the form of biofertilizers and biopesticides (Anand et al., 2022; Singh et al., 2023b). Additionally, the modulation of the rhizosphere microbial community is a well-documented approach for enhancing plant growth, yield, and immunity to combat various stress conditions (Singh and Vaishnav, 2021, 2022).

Over the past decade, microbe-based approaches for crop production have garnered significant attention in agricultural practices. Microbial products are safer options compared to agrochemicals (fertilizers and pesticides) for improving crop yield and nutritional value. Soil microbes play a crucial role in soil health, which is often adversely affected by the application of chemical pesticides in the field (Walder et al., 2022). In recent years, substantial research has been conducted by soil microbiologists to identify beneficial microbes for plants and understand their interactions with soil and host plants under adverse conditions (Edlinger et al., 2022; Jaiswal et al., 2022; Choudhary et al., 2023). The central theme of this Research Topic is to underscore the role of soil microorganisms in food security. This Research Topic offers a comprehensive overview of recent progress in plant-soil-microbe interactions and microbe-based formulations, such as mycorrhizal fungi and plant growth-promoting rhizobacteria, to establish a foundation for future research in this field. In this Research Topic collection, there are six research articles and three review articles covering the aforementioned topics.

Genome analysis of plant-associated bacteria is one of the most evaluated aspects, with three out of nine focusing on this field. This area is crucial for understanding the molecular mechanisms of bacterial interactions with host plants. One paper presents a whole genome analysis of *Fusarium udum*, a pathogen causing wilt in pigeon peas. *De novo* assembly identified a total of 16,179 protein-coding genes, with 1,060 genes (6.55%) identified as pathogenic genes involved in virulence. Moreover, different effector proteins related to cell wall degradation, pectin degradation, and host cell death were discovered. Comparative analysis revealed five common effector genes among all *Fusarium* species and one effector gene, *SIX* (Secreted in Xylem), that was validated in *F. udum* through wet lab experiments (Srivastava et al.). Whole genome analysis is also a promising approach for understanding the nature of stress-tolerant plant growth-promoting bacteria (PGPB) and their interactions with host plants to alleviate plant stress. *Pseudomonas aeruginosa* DJ06, for example, demonstrated PGP activities and successfully colonized sugarcane tissues. Complete genome sequencing of the DJ06 strain revealed a series of genes related to PGP properties and abiotic stress tolerance. In plant experiments, DJ06 strain inoculation promoted plant growth and biomass and regulated phytohormones in sugarcane (Guo et al.). Exploring the genomes of extremophiles helps understand their survival mechanisms under extreme conditions and their potential as sources of industrially important enzymes. In this context, a systematic genome analysis was conducted for *Virgibacillus halodenitrificans* ASH15, a halophilic bacterial isolate. The ASH15 strain exhibited survival and active PGP properties at salt concentrations of up to 25% (w/v) NaCl. Genome analysis revealed that a significant portion of genes was related to the synthesis of compatible solutes (glycine, betaine, ectoine, hydroxyectoine, and glutamate), which are known mechanisms of salt tolerance in bacteria. Interestingly, the ASH15 strain showed diverse genes related to antibiotics, CRISPRs, and medicinal compounds (including squalene) (Sharma et al.). Deciphering the whole genome of microorganisms is instrumental in understanding their interactions with their environment and serves as a source of novel compounds.

Plant stresses, both biotic and abiotic, pose significant challenges to achieving sustainable agricultural production. Plants host a diverse microbial community in their holobiont, aiding in their survival under stressful conditions (Vaishnav et al., 2014). Current efforts are focused on modulating this microbial diversity to enhance crop resilience. In this Research Topic, three papers are related to microbial-mediated alleviation of plant stress. Malviya et al. advocate for the use of mycorrhizal fungi (AMF) to control root-knot nematode infections in rice plants. The study describes the multifaceted effects of *Funneliformis mosseae, Rhizophagus fasciculatus*, and *Rhizophagus intraradices* in inducing plant defense responses against *Meloidogyne graminicola,* the root-knot disease-causing agent in *Oryza sativa*. Inoculating AMF strains also promotes nutrient uptake in rice plants, making AMF a biocontrol and plant growth-promoting agent for rice cultivation under normal and biotic stress conditions. On the contrary, the focus of another study is on antimicrobial compounds released by biocontrol microbes, which can be used directly in formulations for field applications where microbes cannot survive under adverse conditions. In this context, a study reported on the ability of *Bacillus subtilis* BS-58 as an antagonist for two devastating phytopathogens, *Fusarium oxysporum* and *Rhizoctonia solani*. Microscopy analysis revealed that BS-58 secretes antimicrobial metabolites, leading to perforation, cell wall lysis, and cytoplasmic disintegration in fungal hyphae. Further analysis through LC-MS and FT-IR characterized the antifungal metabolite as "macrolactin A" with a molecular weight of 402 Da. Gene analysis (*mln*) also confirmed the presence of this metabolite in the bacterial strain BS-58. In plant growth experiments, BS-58 inoculation was effective in reducing disease incidence against *F. oxysporum* and *R.* solani in *Amaranthus hypochondriacus* (Pandey et al.). Soil microbes can also be used as biofertilizers due to their nutrient solubilization activity, enhancing nutrient content in the soil for plant uptake. In this Research Topic, a study reports the biofortification of micronutrients through the application of different *Bacillus* spp. in wheat crops. A total of 42 isolates were recovered from the rhizosphere region, showing zinc solubilization activity on various zinc substrates, including zinc carbonate, zinc oxide, and zinc phosphate. 16S rRNA gene sequencing identified the bacterial strains as *Bacillus altitudinis*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, *Brevibacillus borstelensis*, and *B. xiamenensis*. Under pot and field trials, these bacterial strains were found to enhance Zn content in wheat straw and grains. Such studies underscore the importance of soil microbes in improving crop yield and nutritional value (Yadav et al.).

This Research Topic features three review papers that focus on abiotic and biotic stress alleviation in plants with the help of soil microbes. Shakeel et al. discuss "Bakanae disease" in rice plants caused by *Fusarium fujikuroi*. The *F. fujikuroi* species complex secretes toxins such as fusarins, fusaric acid, moniliformin, and beauvericin, leading to yield losses in rice and posing risks to animal and human health. Different management strategies for Bakanae disease are discussed in the article, including biocontrol microbes, resistant plants, chemical fungicides, and physical approaches. The authors emphasize biocontrol strategies for completely eradicating this disease in rice fields (Shakeel et al.). Another review article highlights the importance of endophytic fungi as sources of antimicrobial metabolites. Endophytic fungi are rich sources of phenols, polyketides, saponins, and alkaloids, contributing to plant-released metabolites. Therefore, the exploration of plant endophytes is an emerging research area for the largescale and sustainable production of bioactive metabolites (Jha et al.). Another study emphasizes abiotic stress alleviation through the phyto-microbiome. Climate change activities significantly impact abiotic components of the atmosphere, such as soil salinity, atmospheric temperature, drought, and floods. Plant-associated microbes play a vital role in maintaining plant homeostasis during abiotic stress conditions. These microbes can improve host plant tolerance through various belowground and aboveground mechanisms that ultimately modulate plant immune responses (Singh et al.). A deeper understanding through omics approaches is necessary to explore the mechanisms of plant tolerance and develop climate-resilient crops (Diwan et al., 2022).

In summary, the results of the studies and reviews mentioned above provide a substantial amount of new and relevant data on soil and plant-associated microbes and their roles in improving plant

growth, stress tolerance, and yield productivity in a sustainable manner. Despite the existing literature on this topic, the articles in this Research Topic suggest that many areas still require exploration to better harness microbial activities in sustainable agricultural practices.

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Virulence and pathogenicity determinants in whole genome sequence of *Fusarium udum* causing wilt of pigeon pea

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The present study deals with whole genome analysis of *Fusarium udum*, a wilt causing pathogen of pigeon pea. The *de novo* assembly identified a total of 16,179 protein-coding genes, of which 11,892 genes (73.50%) were annotated using BlastP and 8,928 genes (55.18%) from KOG annotation. In addition, 5,134 unique InterPro domains were detected in the annotated genes. Apart from this, we also analyzed genome sequence for key pathogenic genes involved in virulence, and identified 1,060 genes (6.55%) as virulence genes as per the PHI-BASE database. The secretome profiling of these virulence genes indicated the presence of 1,439 secretory proteins. Of those, an annotation of 506 predicted secretory proteins through CAZyme database indicated maximum abundance of Glycosyl hydrolase (GH, 45%) family proteins followed by auxiliary activity (AA) family proteins. Interestingly, the presence of effectors for cell wall degradation, pectin degradation, and host cell death was found. The genome comprised approximately 895,132bp of repetitive elements, which includes 128 long terminal repeats (LTRs), and 4,921 simple sequence repeats (SSRs) of 80,875bp length. The comparative mining of effector genes among different *Fusarium* species revealed five common and two specific effectors in *F. udum* that are related to host cell death. Furthermore, wet lab experiment validated the presence of effector genes like *SIX* (for Secreted in Xylem). We conclude that deciphering the whole genome of *F. udum* would be instrumental in understanding evolution, virulence determinants, host-pathogen interaction, possible control strategies, ecological behavior, and many other complexities of the pathogen.

KEYWORDS

***Fusarium udum*, wilt, whole genome sequence, virulence determinants, effector proteins, *SIX* genes pathogenicity determinants in Fusarium udum genome**

Introduction

The challenge of increasing food grain production is rising day by day as the global population continues to rise. It is projected to increase by another 3.5 billion people before the end of this century, reaching an estimated 11.2 billion (Roser, 2019). Pigeon pea (*Cajanus cajan* L. Millsp.) is an economically significant grain legume crop of Fabaceae family native to semi-arid tropics of the world, such as India, Bangladesh, Indonesia, Thailand, Mauritius, Kenya, Ghana, Tanzania, Uganda, Malawi, and Trinidad (Singh et al., 2016). The crop was the fifth-ranked pulse crop worldwide, accounting for 91% of global production. Pigeon pea has an average yield of 0.97 t ha^{-1} and is

cultivated globally on 7.02 million hectares of land in Asia, Africa, and Latin America (FAOSTAT, 2017). Legumes, preferably pigeon pea, is an acceptable substitute for readily available protein sources. It provides a significant amount of food protein in the Indian and African subcontinents, while requiring little in the way of cultivation care and inputs. This crop makes a significant contribution by meeting about 20% of the world population's protein needs and also serves as a substantial source of other nutrients. In India, pigeon pea stand in the second position after chickpea (Allen and Lenné, 1998). Among the various biotic stresses, vascular wilt caused by *Fusarium udum* is one of the most devastating disease in pigeon pea. It was reported to pose annual output losses of about 71 million USD in India (Reddy et al., 2012; Kashyap et al., 2015). It was later reported from other countries belonging to South Asia, Africa, and Europe (Nene and Reddy, 1976). Pigeon pea wilt can cause yield losses of up to 67% at maturity and 100% in cases of infection at the pre-pod stage (Kannaiyan and Nene, 1981). Additionally, Mahesh et al. (2010), Karimi et al. (2012), Pawar et al. (2013), Prasad et al. (2012), and Kumar and Upadhyay (2014), found comparable findings of pigeon pea wilt disease.

Fusarium udum is a soil-borne fungus with no sexual stage known. The asexual spores are of three types: microconidia, macroconidia, and chlamydospores. Micro-conidia are regularly produced under all conditions (Purohit et al., 2017). Macroconidia is a 2–6-celled sporodochia-like structure produced on the host surface. Similarly, the chlamydospores are produced in the older mycelium. Among these three asexual spores, chlamydospores survive for a considerably long time in the soil. This is an important source of inoculum for the next crop. *F. udum* causes wilting at the flowering stage and symptoms can also be seen at the seedling stage (Choudhary, 2010). Once this pathogen gets established in the vascular bundle, the mycelium, spores, and polysaccharides get filled into the xylem vessels. The water and nutrient transport in the xylem is further reduced by shortening the xylem parenchyma cells due to stimulated cell division by fungus (Agrios, 2008). Pathogen-emitted toxins are conveyed to the leaves, diminishing chlorophyll amalgamation and penetrability of the leaf cell layers and their capacity to control water misfortune through transpiration, consequently causing wilting, interveinal necrosis, yellowing, and plant death. Breeding for resistance to pigeon pea wilt mainly relies on the genetic variability present among different *F. udum* strains. Detailed information on morphological and pathogenic diversity is available (Kashyap et al., 2015), but physiological diversity at a molecular level is yet to be explored.

Whole genome sequencing (WGS) data generated from this study is currently available in the public database (Srivastava et al., 2018). *De novo* draft genome sequencing and functional annotation of *F. udum* have been done to understand the molecular function. The advancement of next-generation sequencing (NGS) technologies and the decrease in the price of sequencing for each sample have undoubtedly accelerated the process of determining emerging genes and genomic regions (Schneeberger et al., 2009). As a result, many current methods utilizing Bulked-Segregant Analysis (BSA) combined with whole-genome re-sequencing (WGRS) for rapid identification of specific genes of interest in plants called "quick forward genetics" (Mokry et al., 2011). For instance, in the model crop *Arabidopsis*, which has a genome size of about 135 Mb, was successfully used to test NGS based BSA approaches for the identification of potential genes for leaf color, next-generation mapping, and suppressor mutants (Schneeberger et al., 2009). In case of plant-pathogen interaction, genome sequencing can reveal virulence-related genes for a better understanding of host-pathogen communications. Currently, biological and chemical disease management approaches adopted for the management of wilt fungus are not successful. So far, the genes responsible for pathogenicity of *F. udum* has not yet been investigated at molecular level (Uchida et al., 2011). Therefore, through this study we performed whole genome analysis of most virulent strain of *F. udum* and identified putative virulence genes for understanding molecular basis of host-pathogen interactions in pigeon pea. The information generated from this study will be highly useful in resistance breeding against *Fusarium* wilt of pigeon pea.

Materials and methods

Pathogenicity test and selection of virulent strain

Total seven strains of *F. udum* (F-02842, F-02843, F-02844, F-02845, F-02848, F-02850 and F-02851) were collected from NAIMCC (ICAR-NBAIM) for screening and identification of the most virulent strain (Supplementary Table S1). The disease-causing ability of these strains were tested by following the protocol of Nene et al. (1981). All the strains were grown separately in potato dextrose broth and incubated at 28°C for 2–7 days. Prepared sand pigeon pea flour medium and inoculated with selected cultures of *F. udum* and incubated at room temperature for 15 days. The 200 g of fungus-infested sand pigeon pea flour medium was mixed with 2 kg autoclaved soil and the mixture was placed into the pot and kept for 2 days. After 2 days, the cell count (6×10^5 spore mL^{-1}) was optimized. In the pot experiment, four varieties of pigeon pea seeds were sown: BAHAR, BRG2, DA 11, and MAL 11. Surface sterilization of seeds was performed with 0.5% (v/v) NaOCl for 10 min, followed by washing with deionized water thrice (Sahu et al., 2022). For screening, 10 seeds of each varieties were sown separately in each pots. Plants were raised with standard agronomic practices. Wilt was observed in pigeon pea plants up to 60 days and permanent wilting of plants were recorded in each treatments.

Comparative gene analysis

The whole genome sequencing of most virulent strain *F. udum* F-02845 was performed and submitted to NCBI database (Srivastava et al., 2018). After retrieving genome sequence, a comparative analysis of orthologous gene families was carried out across *Fusarium* species. The orthologous groups among *F. udum* F-02845, *F. acutatum* (JAADJF000000000), *F. graminearum* PH 1, *F. mangiferae* (FCQH00000000), *F. oxysporum* 4,287, and *F. fujikuroi* IMI 58289 were identified using the OrthoVenn2 program (Xu et al., 2019) with a threshold E value ≤1e−5 and an inflation value 1.5. A workflow followed for the analysis followed in the present study is given in the supplementary data (Supplementary Figure S1).

Since there was one more genome of *F. udum* was reported during the analysis (*F. udum* NRL 25194), the predicted proteome of *F. udum* F-02845 was compared with the *F. udum* NRL 25194 using Orthovenn2.

Repetitive sequence analysis

RepeatMasker (v4.0.5) was used to screen the nucleotide sequences for low complexity DNA sequences and the interspersed repeat (ISR) was used to identify transposable DNA elements. Microsatellite repeats

were identified in sequence using the MISA Pearl script tool[1] (Beier et al., 2017), and results were further authenticated on the WEBSAT server[2] (Martins et al., 2009).

Gene prediction

On the basis of a database of eukaryotic genomes, GeneMark-ES and AUGUSTUS were used to predict the genes in the *F. udum* genome. With default parameters, the above programes are highly reliable for accurate gene prediction[3] (Stanke et al., 2004).

Functional annotation and pathway enrichment analysis

The BLASTx homology search tool, a component of the standalone NCBI-blast-2.3.0+, was used to perform functional annotation of the *F. udum* genes (Altschul et al., 1990). With a cut-off E value of ≤1e−06 and a similarity of 34%, BLASTx identified the homologous sequences of the genes in the NCBI non-redundant protein database. Gene ontology (GO) analysis was carried out using Blast2GO PRO 4.1.5 (Conesa and Gotz, 2008). In three different mappings, B2G performed as follows: (1) Using two NCBI-provided mapping files, blast result accessions are used to get gene names (symbols; gene info, gene 2 accessions). (2) Blast result GI identifiers were used to retrieve UniProt IDs using a mapping file from PIR (non-redundant reference protein database), which includes PSD, Swiss-Prot, UniProt, TrEMBL, GenPept, RefSeq, and PDB. The names of the identified genes were searched in the species-specific entries of the gene product table of the GO database. With the aid of the KAAS-KEGG Automatic Annotation Server, pathway analyses were carried out. This database provides functional annotation of genes using other data servers (Moriya et al., 2007). Accessions from the blast results were looked for in the DBXRef table of the GO database.

In silico mining of virulence genes

A web-based database called the Pathogen-Host Interaction Database (PHI-base; Winnenburg et al., 2006), which comprises experimentally verified pathogenicity, virulence, and effector genes from bacterial and fungal pathogens that infect hosts like plants, animals, fungi, and insects. BLASTP was employed with a cut-off E value of ≤1e−06 in the pathogen-host interaction (PHI) database to find the probable pathogenicity-related genes.

Secretome prediction and analysis of secretory effectors

In order to determine the secretory signal peptides, SignalP v4.1 (Nielsen, 2017)[4] was used to examine the 16,179 predicted proteins of *F. udum*. Further, TMHMM v2.0 was used to predict the protein sets with the existence of transmembrane domains (Krogh et al., 2001) and GPI (glycosylphosphatidyl inositol)-anchor using PredGPI (Pierleoni et al., 2008). Proteins including one transmembrane domain situated within the N-terminal signal peptide and no transmembrane domain overall were chosen. The predicted secretory proteins' cysteine content was examined. In order to functionally annotate the predicted secretome, BLAST2GO was used to assign GO keywords (Altschul et al., 1990). The dbCAN HMMs 5.0 (Yin et al., 2012) was used to find carbohydrate metabolism active enzymes (CAZymes) based on the CAZy database in the *F. udum* secretome.

PCR validation of effector genes

In order to validate the annotated genes, we designed 14 genic-SSRs targeting *SIX* family genes of different *F. udum* strains (F-02842, F-02843, F-02844, F-02845, F-02848, F-02850 and F02851). For PCR amplification of 10 µl reaction volume constituting of template DNA (50 ng µl^{-1}), Go Taq Green master mix (Promega Biotech India Pvt. Ltd) and primers (both reverse and forward) was used. Amplification was performed by following conditions of 35 cycles of 95°C for 1 min, primer annealing variable for each primer, and extension at 72°C for 2 min, followed by a hold at 72°C for 5 min. The experiment was repeated three times to confirm the results.

Results

Pathogenicity test

Out of seven strains used for pathogenicity test, strain *F. udum* F-02845 showed highest disease incidence in the BAHAR variety, suggested most virulent of all seven strains of *F. udum*. While, F-0244 had least disease incidence in pigeon pea plants (Supplementary Table S1; Supplementary Figure S2). Pigeon pea plants which were showing the highest disease incidence were taken for re-isolation of *F. udum* on PDA plates and DNA was isolated by the CTAB method (Kaul et al., 2022) and quantified by nanodrop. The most virulent strain F-02845 was chosen for whole genome sequencing.

Gene prediction and functional annotation

The detailed information about the genome assembly statistics is provided in Supplementary Table S2. Functional annotation of *F. udum* F-02845 resulted in the identification of 296 tRNAs and 53 rRNAs. A total of 14,673 and 16,179 genes were predicted by using AUGUSTUS and GeneMarkES softwares, respectively. The estimated average length of protein-coding genes was 1,365 bp. Out of 16,179 protein-coding genes, 85.54% of the genes (13,841 genes) were functionally annotated using BlastP (Supplementary Table S3). The gene annotation results indicated that, 3,436 genes belonged to biological processes (BP), with role in metabolic, cellular, localization, biological regulation, cellular component organization, response to stimulus, etc. The 4,511 genes belonged to cellular components (CC) that are part of cellular anatomical entries, protein-containing complexes and cell parts etc. The 6,260 genes belonged to molecular functions (MF) that had catalytic activity, binding, transcription regulatory activity, transporter activity, structural

1 http://pgrc.ipkgatersleben.de/misa
2 http://wsmartins.net/websat/
3 http://bioin.f.uni-greifswald.de/augustus
4 http://www.cbs.dtu.dk/services/SignaLP/

molecule activity, molecular function regulators and antioxidant activity, etc. (Figure 1).

A total of 8,928 genes were categorized into four functional groups using the KOG database. These include: KOG class A (RNA processing and modification, gene count 346), class B (chromatin structure and dynamics, gene count 136), class C (energy production and conversion, gene count 591), and class D (cell cycle control, cell division, chromosome partitioning, and gene count 257; Supplementary Figure S3; Table 1). Further, it has been predicted that the genome contained 1,439 genes encoding for secretory signal peptides, 2,858 for carbohydrate-active enzymes (CAZy), 3,682 for transporter genes, and 1,060 for putative pathogenicity or virulence genes (Table 2). The domain analysis based on InterproScan-V5 revealed the presence of 11,892 annotated genes (73.50%) and 5,134 unique Interpro domains (Supplementary Figure S4).

Comparative analysis for orthologus genes families between the predicted proteome of F-02845 and NRL 25194, identified the presence of 13,194 clusters. Out of which, 13,103 common clusters with 26,834 proteins (13,485 of F-02845 and 13,349 of NRL 25194) were shared between F-02845 and NRL 25194. Interestingly, 82 unique clusters with 188 proteins belonged to in F-02845 and 9 unique clusters with 19 proteins belonged to NRL 25194 were identified. There were 2,506 and 7,694 singletons (proteins are not in any clusters) in F-02845 and NRL 25194, respectively, were detected (Supplementary Figures S5, S6; Supplementary Table S4).

Prediction and analysis of *F. udum* secretome

Out of the 16,179 predicted protein-coding sequences, a total of 1,439 proteins represented classical secretory proteins. Out of 1,439 secretory proteins, 1,305 proteins were functionally annotated and 134 sequences had no hit in the non-redundant database (Supplementary Table S5). A total of 124 highly probable sequences containing GPI anchors were identified and 1,021 proteins had GO terms. Based on the GO, all the genes were divided into several categories that included 240 genes under biological process category, representing, cellular component organization, genesis, localization, and biological regulation. Similarly, under cellular component that included 268 genes that are part of cellular anatomical entities, cells and protein-containing complexes. In molecular function, 513 genes were identified that had catalytic activity, binding, transmembrane transporter activity, peroxidase and nutrient reservoir activity (Figure 1). Additionally, the CAZyme database was used to investigate 1,439 secretory proteins and predicted 506 secretory proteins with plant cell wall degradation functions (Figure 2A). The enzyme families related to carbohydrate metabolism and cell wall degradation were characterized such as 229 glycosyl hydrolase (GH), 44 glycosyl transferase (GT), 51 carbohydrate esterase (CE), 94 carbohydrate-binding module (CBM), 67 auxiliary activity (AA), and 21 polysaccharide lyase (PL; Figures 2B–G). In the glycosyl hydrolase class, the most common CAZymes were GH16 and GH43. Twelve of the 229 GH families demonstrated the existence of three or more genes. The GH43 family had the most genes (28 genes) followed by GH16 (21), GH18 (19), and GH10 (7; Figure 2D). The secretome of *F. udum* also contained members of 10 CE families and CE5 (11 genes) had the most genes, followed by CE6 (10), CE3 (7), and CE4 (5; Figure 2F). Additionally, 4 PL families were anticipated that included PL1 (11 genes), PL3 (5), PL4 (3), and PL9 (2; Figure 2B). The other classes like AA (10 families), CBM (16), and GT (19) that play indirect roles in the degradation of carbohydrates were also predicted (Figures 2C,E,G). The CBM-13, AA7, and GT-4 families were found highly prevalent in the analyzed *F. udum* secretome.

Mining of pathogenicity genes

A total of 16,179 predicted coding were sequences searched and validated against PHI database to identify virulence genes. As a result, 5,261 coding sequences were identified that are having active role in host-pathogen interaction. These proteins were further categorized into unaffected pathogenicity (46%), reduced virulence (34%), loss of pathogenicity (7%), mixed nature (6%), lethal (4%), increased virulence (2%), genes related to effectors (plant avirulence determinant; 1%), enhanced antagonism (~0%) and chemistry target resistance to chemicals (~0%; Figure 3A). In order to identify the putative pathogenicity-related genes, 1,439 secretory proteins were annotated against PHI database and 421 genes were identified (Supplementary Table S6). Furthermore, we confirmed that 183 genes were related to reduced virulence, 160 genes were unaffected by pathogenicity, 29 genes were of mixed nature, 13 genes related to effectors (plant avirulence determinant), 19 genes related to increased virulence, 12 genes related to loss of pathogenicity, and 5 genes were related to lethal activity (Figure 3B).

Comparative analysis for effector genes

In the secretome of F-02845, a total of 7 effector genes that are encoding Xyloglucan-specific endo-beta-1,4-glucanase 1 (*XEG1*; 2 in no.), Cellulose-growth-specific protein (*MoCDIP4*; 3), 25 kDa protein elicitor-like protein (*PaNie 3*), avirulence gene/subtelomeric avirulence effector (*AVR-Pita*; 1), *MoCDIP3* (1), *MoCDIP1* (1), and Secreted virulence factor MC69 (*CoMC69*; 1) were identified. Further, we also compared these within the secretome of four different *Fusarium* spp., i.e., *F. oxysporum* f. sp. *lycopersici* (AAXH00000000), *F. graminearum* (AACM00000000), *F. proliferatum* (*FJOF00000000*), and *F. verticilliodes* (AAIM00000000). As a result, out of 7 effectors, only 5 (*XEG1*, *MoCDIP4*, *AVR-Pita*, *CoMC69*, and *MoCDIP1*) were common in all the *Fusarium* spp. with different copy numbers. PaNie and MoCDPI3 were not common in all the studied *Fusarium* spp. Interestingly, PaNie was not observed in *F. oxysporum* f. sp. *lycopersici* and *F. proliferatum*. Similarly, MoCDIP3 is absent in *F. graminearum* and *F. proliferatum*. The highest copy numbers of PaNie and MoCDIP3 were found in *F. udum* (3) and *F. verticilliodes* (3), respectively (Table 3).

Characterization of repetitive elements

Repetitive elements in F-02845 genome were analyzed and found 895,132 bp (1.59%) of the total genome. A total of 128 transposable and 128 LTR were found in the genome. Among the LTRs, Tyl/copia (68) and gypsy/DIRS1 (60) represented more predominant in numbers (Table 4). The relative density and abundance of different SSRs were studied to get the genetic diversity of different SSRs in the F-02845 genome. A total of 4,921 SSRs representing 80,878 bp of the genome were identified. We observed relative density and abundance of 87.28 and 1434.41, respectively, for SSRs in the assembled genome. Details of

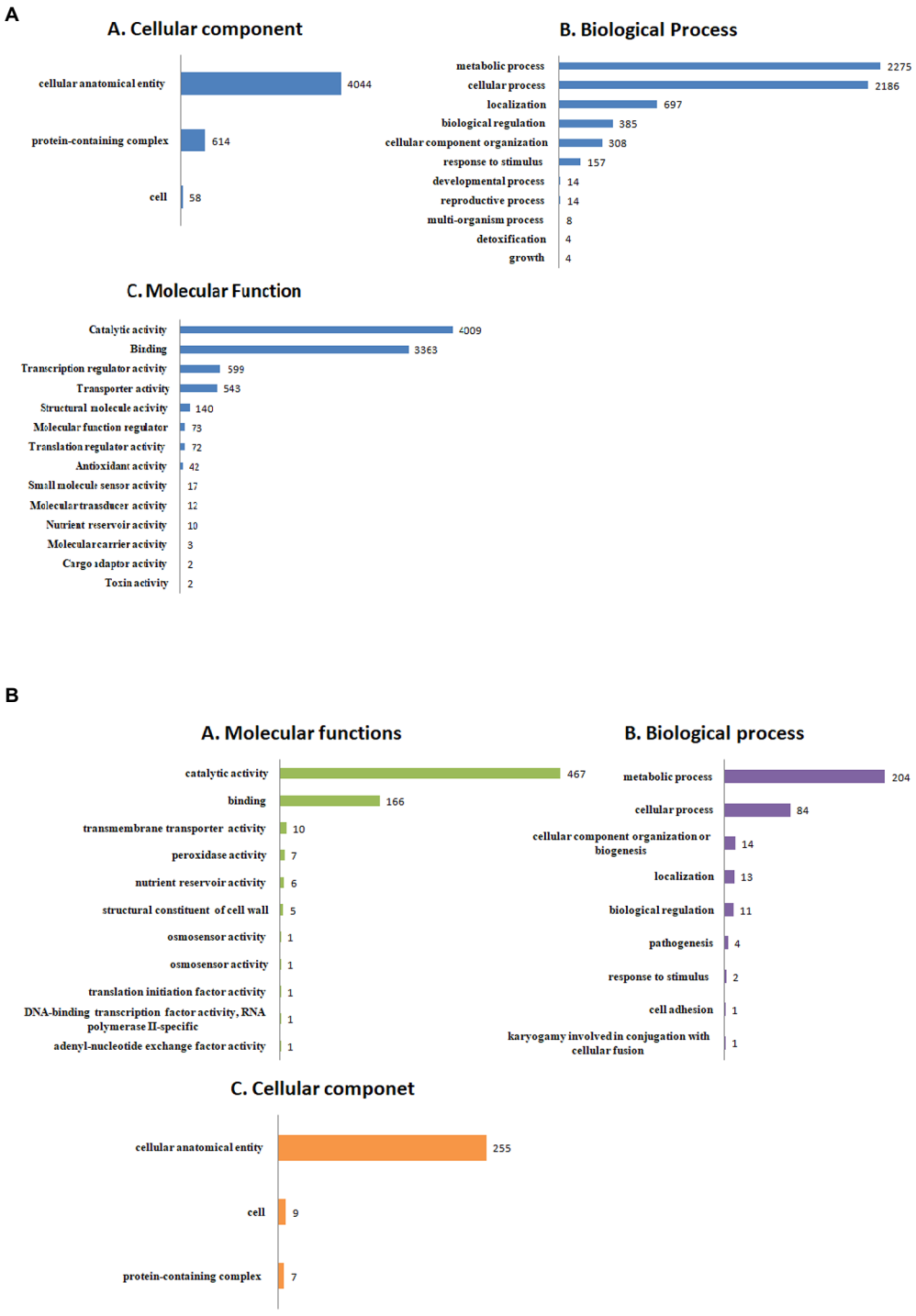

FIGURE 1
Functional annotation of the *Fusarium udum* **(A)** proteome, and **(B)** secretome showing top hits of different categories.

different types of SSRs obtained from the whole genome of F-02845 are shown in Supplementary Table S7.

Comparison of orthologous genes

Comparative genome analysis of predicted proteome of F-02845 was performed with six other species of *Fusarium* (Figure 4A). The genome statistics (size, GC%, accession number) of these genomes have been mentioned in Supplementary Table S8. Results indicated that these species formed 18,274 clusters, out of which the *F. udum F-02845* shared a total of 14,242 clusters, 9,011 common clusters, and 60 singletons. The F-02845 has shared a total of 237 common clusters with other *Fusarium* species; 161 clusters with *F. mangiferae;* 95 common clusters with *F. Fujikuroi,* 86 common clusters with *F. acutatum*; and 43 common clusters with *F. graminearum*. Further, functional annotation was done for these common clusters and a total of 202 GO terms were assigned to BP, which included biological processes (18%), metabolic processes

TABLE 1 Eukaryotic orthologous groups (KOG) classification of the predicted genes within the *Fusarium udum* F-02845 genome.

#KOG class	Count	Description
A	346	RNA processing and modification
B	136	Chromatin structure and dynamics
C	591	Energy production and conversion
D	257	Cell cycle control, cell division, chromosome partitioning
E	530	Amino acid transport and metabolism
F	125	Nucleotide transport and metabolism
G	534	Carbohydrate transport and metabolism
H	141	Coenzyme transport and metabolism
I	537	Lipid transport and metabolism
J	432	Translation, ribosomal structure and biogenesis
K	566	Transcription
L	259	Replication, recombination and repair
M	205	Cell wall/membrane/envelope biogenesis
N	5	Cell motility
O	676	Posttranslational modification, protein turnover, chaperones
P	251	Inorganic ion transport and metabolism
Q	600	Secondary metabolites biosynthesis, transport and catabolism
R	1,615	General function prediction only
S	435	Function unknown
T	789	Signal transduction mechanisms
U	602	Intracellular trafficking, secretion, and vesicular transport
V	114	Defense mechanisms
W	28	Extracellular structures
X	1	multiple functions
Y	40	Nuclear structure
Z	328	Cytoskeleton

Results are grouped into 26 functional classes according to their functions.

TABLE 2 Functional annotation of *Fusarium udum* F-02845 genome.

Sr. No.	NCBI Accession Number	NIFK00000000
1.	Genome size	56.75 Mb
2.	(G + C)%	43.44%
3.	No of gene predicted	16,179
4.	Total no. of genes annotated	13,841
5.	Total no. of repeats identified	1.59%
6.	Total no. of proteins annotated with InterPro domains	11,829
7.	Total no. of genes with gene ontology detected	8,642
8.	No of rRNA identified	53
9.	No. of tRNA identified	296
10.	Total no. of genes annotated with Cluster of Orthologous genes (KOG)	8,928
11.	Total no. of genes coding for carbohydrate active enzymes (CaZy)	2,858
12.	Total no. of pathogenic/virulence genes detected	1,060
13.	Total no. of signal peptide predicted	1,439
14.	Total no. of transmembrane helices predicted	15,649
15.	Total no. of genes coding for transporters	3,682
16.	No.of SSR	4,921

(17%), cellular metabolic processes (12%), cellular processes (10%), macromolecular metabolic processes (10%), etc. (Figure 4B). Similarly, 46 GO terms were assigned for MF, which includes oxidoreductase activity (22%), hydrolase (15%), transferase (13%), ion binding (12%), peptidase (7%), molecular function (7%) etc. (Figure 4C). Besides this, 25 GO terms were assigned for the CC, which include membrane functions (21%), cellular component (16%), cell part (16%), nucleus (10%), intracellular (10%), mitochondria (7%) etc. (Figure 4D).

Wet lab validation of few effector genes

Among 14 SSR primers designed for a few effector genes (*SIX* genes), the primer set SIX1A3 (forward primer GCCCAGGTCGT AAATAGTGAGA and reverse primer GCAGACTCAACTCCAAAT AGGC) was validated through PCR. The amplification of SIX1A3 gene with desired amplicon size (300 bp) was found in all seven strains of *F. udum* (F-02842, F-02843, F-02844, F-02845, F-02848, F-02850, and F02851).

Discussion

Pigeon pea is a herbaceous pulse crop, predominantly cultivated in tropical and subtropical climates and is vulnerable to more than hundreds of pathogens (Nene et al., 1981). Among the diseases, the major fungus affecting pigeon pea is *F. udum* is the major concern. Since, chemical and biological management of this disease has witnessed limited success against *F. udum* wilt. The whole genome sequencing and analysis of virulence-related genes could bring a better understanding of wilt disease for devising appropriate management strategies. Therefore through this study, we analyzed the first draft genome sequence of *F. udum* F-02845 for virulence related genes.

The assembled genome of F-02845 was retrieved, which constituted size of 44.62 Mb with 48.3% of G + C content and 42,598 bp N_{50} length. It is worth to mention here that, our assembled genome was near to complete and more accurate than recently reported genome of NRL 25194. In comparison to NRL 25194 strain, we found F-02845 harbors higher physiological complexity owing to its larger genome size. The larger genome size could be particularly associated with the larger biosynthetic machinery. However, this difference could not be established with respect to the virulence of the pathogen. Similarly, Dobbs et al. (2020) through whole genome sequencing of two strains of pathogen causing koa wilt (*Fusarium oxysporum* f. sp. *koae*) reported differences in genome sizes of non-pathogenic strain Fo170 (50 Mb) as compared to pathogenic strain Fo44 (48 Mb).

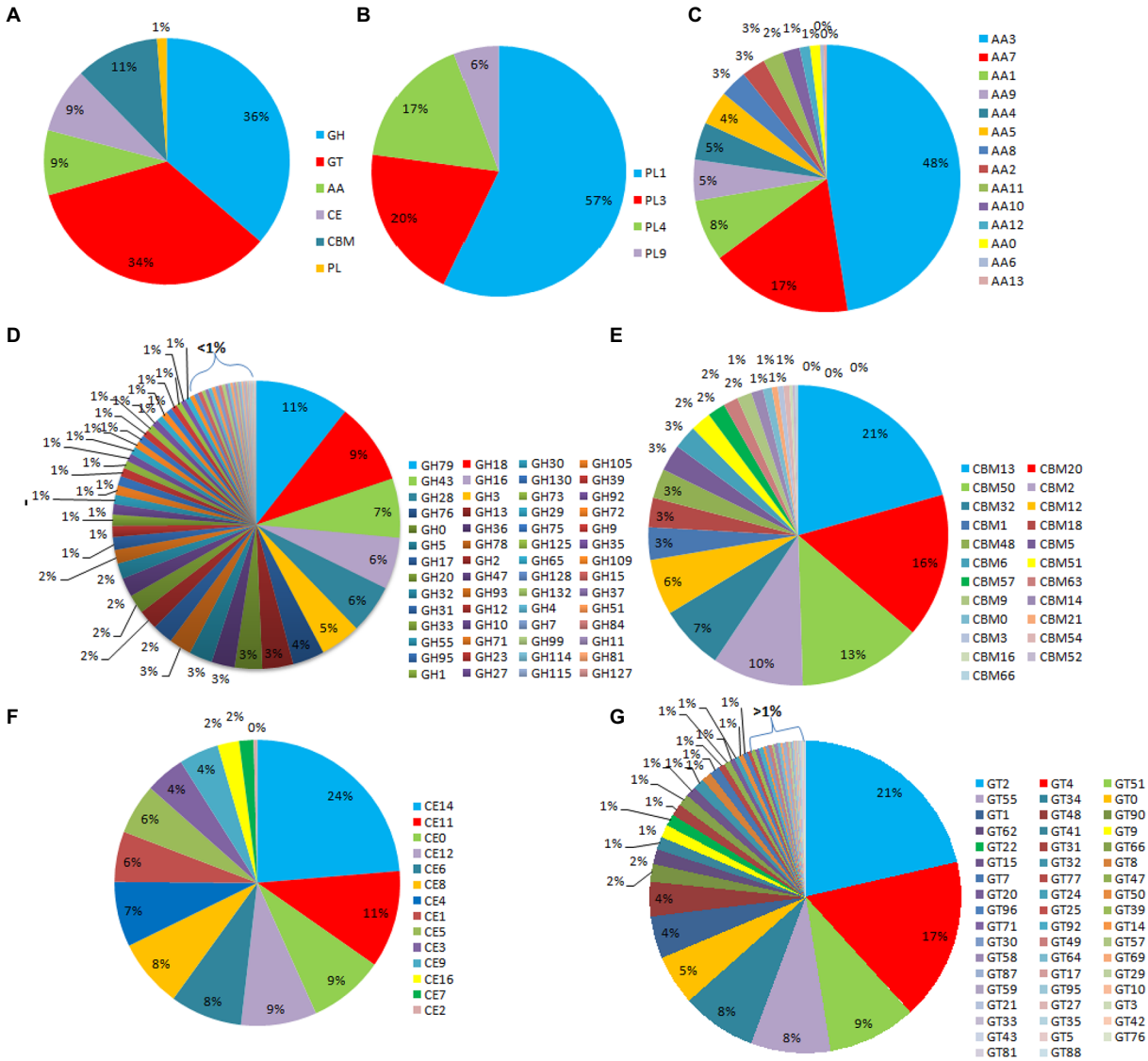

FIGURE 2
CAZymes identified in the secretome of *Fusarium udum*; **(A)** Summary of the six CAZyme categories: carbohydrate-binding modules (CBMs), carbohydrate esterases (CEs), glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), and auxiliary activities (AAs). **(B)** Distinct summaries of the CAZyme PLs, **(C)** Distinct summaries of each of the CAZyme auxiliary activities (AAs), **(D)** Distinct summaries of each of the CAZyme GHs, **(E)** Distinct summaries of each of the CAZyme carbohydrate-binding modules (CBMs), **(F)** Distinct summaries of each of the CAZyme CEs, and **(G)** Distinct summaries of each of the CAZyme GTs.

Annotation of F-02845 genome revealed the presence of genes related to cell physiology and functioning. The KOG annotation showed the presence of 1,060 putative pathogenicity genes along with other genes for cellular processes. These pathogenicity genes are of great importance when looking at the economic loss caused by the pathogen. To see early occasions in plant-pathogen (*F. udum*) interactions, it was important to investigate the pathogen secretome to recognize secreted proteins that help to organize pathogenicity. Usually, a subset of the secretome is composed of proteins whose presence is needed to initiate infection and their expulsion from the secretome would bring about pathogens with diminished or no virulence (Ranganathan and Garg, 2009). In this study, a total number of 14,673 and 16,179 protein-coding genes with an average length of 1,365 bp were predicted. These genes were further functionally annotated using BlastP and were applied to the PHI database in order to search for the virulence genes present in the fungal genome. F-02845 genome identified to contain 1,439 classical secretory proteins with successfully annotated function for 1,305 secretory proteins. It is a well-established fact that for successful invasion, pathogen secretes enzymes to destroy the plant cell walls. In addition, enzymes related to carbohydrate metabolism determine the efficacy of the pathogens to grow and their aggressiveness to cause disease. CAZymes play an important role in degrading plant biomass and have many associated families like carbohydrate esterases, glycosyl hydrolases, and polysaccharide lyases that are involved in cell wall degradation (Ospina-Giraldo et al., 2010; Zhao et al., 2013; Barrett et al., 2020). In this study, we identified 506 secretory proteins (CaZymes) with cell wall degradation functions. The presence of these secreted enzymes could contribute to active entry of pathogens into the host cells. In particular, the host-specific populations of pathogens had various enzymes which share comparative functions. For instance, the pigeon

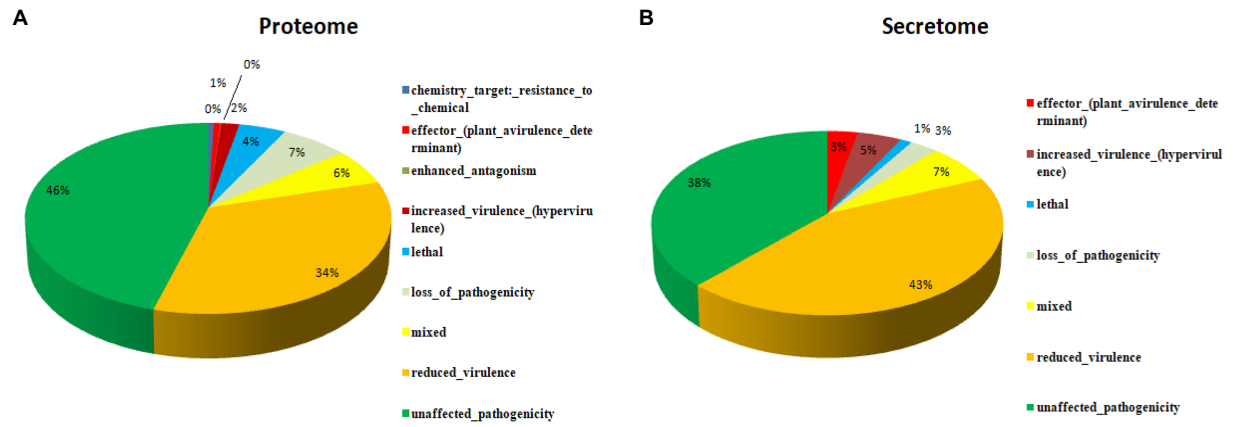

FIGURE 3
Putative pathogenicity-related genes associated with the secretome as annotated against the pathogen-host interaction database, **(A)** Proteome, and **(B)** Secretome.

TABLE 3 Effectors in secretome of different *Fusarium* species.

Sr. No.	Protein Gene id	Gene-name	*F. udum*	*F. lycopersici*	*F. graminarum*	*F. proliferatum*	*F. verticilliodes*
1.	G5A0G9	XEG1	2	2	1	2	1
2.	G4MVX4	MoCDIP4	4	2	4	6	5
3.	Q9SPD4	PaNie	3	0	1	0	1
4.	Q9C478	AVR-Pita	1	2	1	3	2
5.	H7CE70	CoMC69	1	1	1	1	2
6.	G4MX34	MoCDIP3	1	1	0	0	3
7.	G4N8Y3	MoCDIP1	1	1	1	1	1

pea wilt strains share six CAZyme copies associated with the hydrolysis of different cell wall components such as chitin, pectin, and rhamnose, along with the breakdown of glucose, xylan, and mannose (Clerivet et al., 2000). This suggests strong enzymatic capability for biomass degradation. Cell-wall breakdown discharges pectins into the xylem vessels, which could unexpectedly act as a barrier to additional microbe growth. At this moment pectin-degrading enzymes play crucial role in pathogenesis as they remove pectin barriers and help the pathogen to spread across the plant tissues and cause disease symptoms. The ability to utilize plant carbon is indicative of *F. udum*'s ability to survive inside plants before the appearance of disease symptoms (Soanes et al., 2008), which is also evident from the field infection of *F. udum*.

PHI database analysis of the secretome revealed 160 genes associated with pathogenicity loss, 29 genes of mixed nature, 19 genes associated with increased virulence, 13 genes associated with effectors (plant avirulence determinant), and 5 genes associated with lethality. Targeting these genes would open a new arena in biocontrol of this pathogen. In the F-02845 secretome, 13 genes related to effectors *viz.*, XEG1, MoCDIP4, PaNie, AVR-Pita, MoCDIP3, MoCDIP1 and CoMC69 were identified. Effectors are avirulence proteins or products involved in pathogenicity that have the ability to manipulate host cell structure and function and thereby facilitates infection. They often contribute quantitatively to pathogen aggressiveness and are dispensable for the pathogen life cycle. These effectors also play an important role in taking up of nutrients from the host tissues or pathogens self-defense (Rovenich et al., 2014; Fatima and Senthil, 2015). These could be possibly one of the key virulence determinants, which provide ecological fitness to the *F. udum*. Djamei and Kahmann (2012) analyzed the genome of biotrophic maize pathogen *Ustilago maydis* and predicted 550 secreted proteins which were upregulated during host colonization. They also reported that the *U. maydis* secretes core and organ-specific effectors. The main roles of core effectors are that they suppress plant defense during the penetration stage and organ-specific effectors infect different plant tissues (Skibbe et al., 2010; Djamei and Kahmann, 2012). The presence of core effectors could be helpful for *F. udum* to bypass the host defense system and colonize host tissues. It has been widely reported that suppressing host defense is useful in establishing disease. Effector-encoding gene clusters were found in the *U. maydis* genome. The largest effector gene cluster, 19A, contains 23 genes. They are differentially induced when different plant organs are colonized. It has been noticed that removal of the complete 19A cluster terminates tumor formation in maize plants, whereas deletion of individual genes shows a minor reduction in virulence (Kamper et al., 2006; Brefort et al., 2014). Therefore, it would be possible that controlling these *F. udum* effector encoding clusters could significantly reduce the disease symptoms.

Fusarium has a number of different *formae speciales*, and each of them is harmful to a different host plants. The diversity of well-known effector gene *SIX* is demonstrated to be strongly up-regulated during colonization of the host plant by *F. oxysporum* f. sp. *lycopersici* (Houterman et al., 2007). More recently, eight tiny secreted fungal proteins known as SIX1 to SIX8 were discovered in the xylem sap of infected plants. These effector genes are also known to play an important

TABLE 4 Identified transposable elements in *F. udum* F-02845 genome showing highest count of Ty1/Copia.

Sr. No.	Elements	Number of elements	Length occupied	Percentage of sequence
	Retroelements	128	88,783 bp	0.16%
1.	SINEs	0	0 bp	0.00%
2.	Penelope	0	0 bp	0.00%
3.	LINEs:	0	0 bp	0.00%
4.	CRE/SLACS	0	0 bp	0.00%
5.	L2/CR1/Rex	0	0 bp	0.00%
6.	R1/LOA/Jockey	0	0 bp	0.00%
7.	R2/R4/NeSL	0	0 bp	0.00%
8.	RTE/Bov-B	0	0 bp	0.00%
9.	L1/CIN4	0	0 bp	0.00%
	LTR elements	128	88,783 bp	0.16%
1.	BEL/Pao	0	0 bp	0.00%
2.	Ty1/Copia	68	29,240 bp	0.05%
3.	Gypsy/DIRS1	60	59,543 bp	0.11%
4.	Retroviral	0	0 bp	0.00%
	DNA transposons	128	61,613 bp	0.11%
1.	hobo-Activator	5	703 bp	0.00%
2.	Tc1-IS630-Pogo	22	14,498 bp	0.03%
3.	En-Spm	0	0 bp	0.00%
4.	MuDR-IS905	0	0 bp	0.00%
5.	PiggyBac	14	3,987 bp	0.01%
6.	Tourist/Harbinger	0	0 bp	0.00%
7.	Other (Mirage, P-element, Transib)	0	0 bp	0.00%
8.	Rolling-circles	0	0 bp	0.00%
9.	Unclassified:	3	423 bp	0.00%
	Total interspersed repeats		150,819 bp	0.27%
1.	Small RNA:	50	16,939 bp	0.03%
2.	Satellites:	0	0 bp	0.00%
3.	Simple repeats:	13,725	636,194	1.13%
4.	Low complexity:	1,776	91,188 bp	0.16%

role in virulence as these genes are absent in non-pathogenic *F. oxysporum* (van Dam et al., 2016). The effector SIX3 (AVR2) is established in the cell of the host and incorporated with SIX5 activates resistance and cell death in tomato plants carrying the I-2 gene (Houterman et al., 2009). SIX1 (AVR3) induced resistance in plants containing the I-3 gene from the wild tomato *Solanum pennellii* (Catanzariti et al., 2015). On parallel lines, two copies of XEG1 (Xyloglucan-specific endo-beta-1,4-glucanase 1) were also noticed in the *F. udum* genome. This observation provides an important clue regarding the probable role of this enzyme in cell wall degradation and pathogen invasion, as documented by earlier workers (Bourquin et al., 2002; Baumann et al., 2007).

In the comparison study of effectors among different *Fusarium* species, we found that out of seven secretory effectors, five were common to other *Fusarium* species and two were specific to certain species only. This could be one of the plausible reasons behind the specific pathogenicity behavior of *F. udum*. The higher copy numbers of *MoCDIP4* and *PaNie* genes are identified in the *F. udum* genome. The possible connection could be with the pathogenic establishment of *F. udum* in the host plant as MoCDIP1-MOCDIP5 secretory effectors are responsible for the host cell death (Chen et al., 2013). It may be contributing to the silencing of the host defense response. Cell death–inducing proteins of *Magnaporthe oryzae* have been reported as cell death–inducing proteins and cause necrosis in both monocots and dicots (Chen et al., 2013). They are expressed at the late infection stage of the host. Similarly, a novel protein elicitor (PaNie234) isolated from the pathogenic oomycete *Pythium aphanidermatum* activated the programmed cell death and *de novo* formation of 4-hydroxybenzoic acid in cultured cells of *Daucus carota* (Veit et al., 2001). Recently, a fungal effector protein was found to suppress the host plant's polygalacturonases-inhibiting proteins (PGIP), which inhibit fungal polygalacturonases (PG). The PG is secreted by pathogens to degrade host cell walls (Wei et al., 2022). The presence of MoCDIP4 and PaNie effectors in the *F. udum* genome indicated another mechanism of the pathogen in breaching the host's physical defense.

Apart from secretory effector proteins, comparative genome analysis suggested variations in the number of total proteins in the

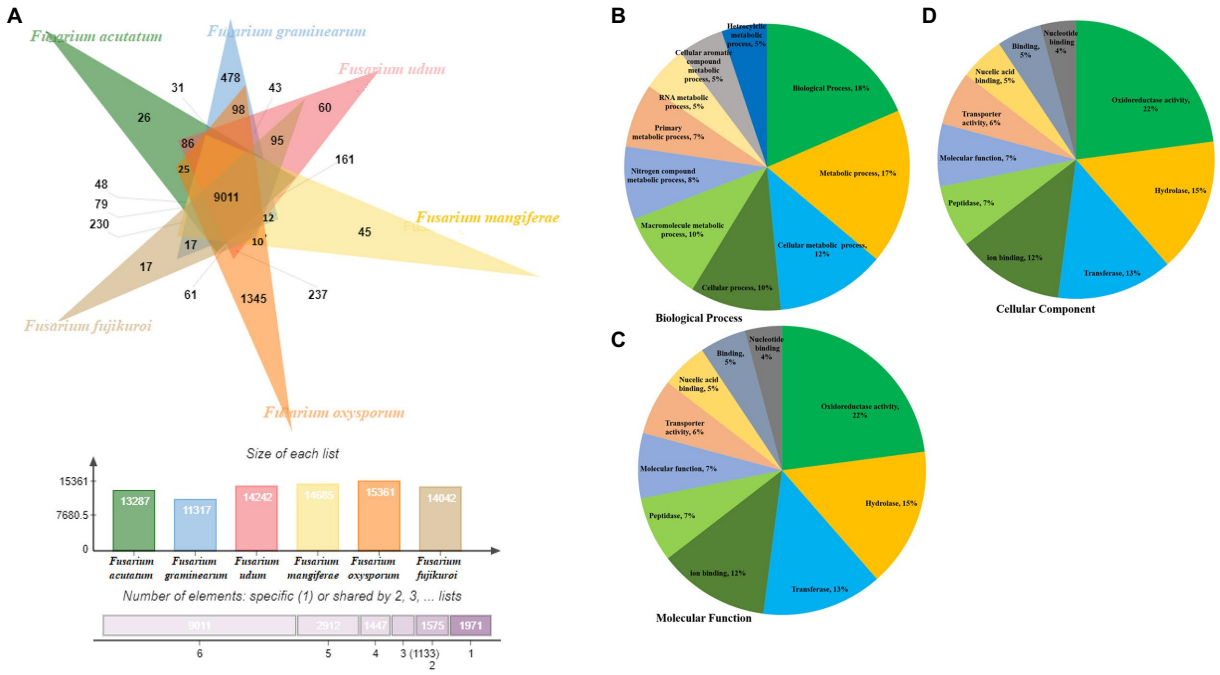

FIGURE 4
Comparison of orthologous genes between different ascomycetes fungus genome. **(A)** Functional annotation of the shared clusters showing top hits of different category, **(B)** Biological process, **(C)** Molecular function and **(D)** Cellular component.

genomes of different *Fusarium* spp. For instance, total proteins reported in *F. udum F-02845, Fusarium acutatum* (*JAADJF000000000*), *Fusarium graminearum PH 1, Fusarium mangiferae* (*FCQH00000000*), *Fusarium oxysporum 2,478, Fusarium poae FPOA1,* and *Fusarium_fujikuroi IMI 58289* genomes were 16,179, 14,081, 13,313, 15,804, 18,769, 14,740, and 15,371, respectively. These species formed 18,274 clusters, out of which F-02845 shared a total of 14,242 clusters, 9,011 common clusters, and 60 singletons. Comparative genome analysis of closely related species is the best perspective for the recognition of virulence determinants (Kamper et al., 2006). Sharing common clusters among other *Fusarium* spp. suggests that *F. udum* has common pathogenicity determinants, which could be targeted for common control strategy for all related pathogens.

Repetitive DNA plays an important role in the evolution of eukaryotic genomes. It causes genetic and beneficial changes in the evolution of pathogens (Castanera et al., 2016). In the present study, we identified 128 transposable elements. In addition to this, repetitive sequences related to epigenetic control of the expression of effector genes as part of the coordinated infection strategy (Razali et al., 2019). Simple sequence repeats (SSRs) are the repetitive DNA tracks distributed throughout the genome. These play a vital role in deciphering the genetic diversity among different fungal genera, species, and strains (Kumar et al., 2012, 2013; Singh et al., 2014; Rai et al., 2016; Kashyap et al., 2020). Here we report, identification of total 4,921 SSR with a length of 80,878 bp in the F-02845 genome, which could be instrumental in deciphering evolutionary relatedness (Rao et al., 2018; Kashyap et al., 2019).

Some of the genes and gene clusters identified in the present study could play a crucial role in pathogenesis and host-pathogen interaction for wilt disease development in pigeon pea. Such virulence genes could be used for functional characterization to recognize the infection mechanism of *F. udum* causing the wilt of pigeon pea. Establishing the molecular basis of host infection could be further utilized in marker assisted selection, CRISPR-Cas9 based genome editing, and other molecular approaches for developing disease resistance in crop plants and effective management of disease in the farmer's field.

Conclusion

Through this study, we report whole genome and secretome analysis of most virulent strain of *F. udum* F-02845, causing pigeon pea wilt. Understanding of the genes responsible for virulence and secondary metabolite production in fungus will help to explain the mechanisms for virulence functional in fungus and to develop novel strategies for disease management of *Fusarium* wilt. Various genes and their secreted proteins identified in this study, which are crucial for disease development, could be of greater significance in deciphering pathogenic determinants of *F. udum*. Comparison of orthologous genes in this study from other similar pathogen genomes resulted in identification of common set of genes, which could be used to explain the behavior of *F. udum* in respect to disease development. The information generated from this study has not only helped in deciphering virulence determinants, but also helpful in understanding the pathogen's complex ecological behavior that has yet to be discovered. This would add to the plant health promotion efforts in 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 at: NCBI GenBank – NIFK00000000; NCBI Sequence Read Archive – SRP157084.

Author contributions

AlS and PK conceived the idea and designed the experiments. RS, JY, ASi, PT, AnS, and PK performed the experiments. AlS, RS, PS, and SS analyzed the data. AlS, RS, PS, and ASi wrote and edited 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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Macrolactin A mediated biocontrol of *Fusarium oxysporum* and *Rhizoctonia solani* infestation on *Amaranthus hypochondriacus* by *Bacillus subtilis* BS-58

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Plant diseases are one of the main hurdles for successful crop production and sustainable agriculture development world-wide. Though several chemical measures are available to manage crop diseases, many of them have serious side effects on humans, animals and the environment. Therefore, the use of such chemicals must be limited by using effective and eco-friendly alternatives. In view of the same, we found a *Bacillus subtilis* BS-58 as a good antagonist towards the two most devastating phytopathogens, i.e., *Fusarium oxysporum* and *Rhizoctonia solani*. Both the pathogens attack several agricultural crops (including *amaranth*) and induce a variety of infections in them. The findings of scanning electron microscopy (SEM) in this study suggested that *B. subtilis* BS-58 could inhibit the growth of both the pathogenic fungi by various means such as perforation, cell wall lysis, and cytoplasmic disintegration in the fungal hyphae. Thin-layer chromatography, LC–MS and FT-IR data revealed the antifungal metabolite to be macrolactin A with a molecular weight of 402Da. Presence of the *mln* gene in the bacterial genome further endorsed that the antifungal metabolite produced by BS-58 was macrolactin A. Pot trial conducted in the present study showed that seed treatment by BS-58 effectively reduced seedling mortality (54.00 and 43.76%) in *amaranth*, when grown in pathogen infested soil (*F. oxysporum* and *R. solani*, respectively), when compared to their respective negative controls. Data also revealed that the disease suppression ability of BS-58 was almost equivalent to the recommended fungicide, carbendazim. SEM analysis of roots of the seedlings recovered from pathogenic attack substantiated the hyphal disintegration by BS-58 and prevention of *amaranth* crop. The findings of this study conclude that macrolactin A produced by *B. subtilis* BS-58 is responsible for the inhibition of both the phytopathogens and the suppression of the diseases caused by them. Being native and target specific, such strains under suitable conditions, may result in ample production of antibiotic and better suppression of the disease.

KEYWORDS

***Bacillus subtilis*, biocontrol, macrolactin, *mln* gene, scanning electron microscopy**

1. Introduction

Bacillus subtilis, a Gram-positive, endospore former, is able to survive under adverse conditions, and is capable of synthesizing a vast array of beneficial metabolites. The potential of *B. subtilis* strains to produce a variety of secondary metabolites is known for decades. It is also known that at least 4–5% genome of any strain of the *B. subtilis* is responsible for the production of antimicrobial compounds (Stein, 2005). This species has also been identified as a good candidate for plant growth promotion and/or biocontrol of many plant diseases by different researchers (Pandey, 2018a; Caulier et al., 2019; De la Lastra et al., 2021). These organisms enhance plant growth and suppress plant diseases by different modes of action. The most common mechanisms are phytohormone production, nutrient solubilization and suppression of phytopathogens through various means, including the production of hydrolytic enzymes, siderophores, antifungal compounds, lipopeptides, antibiotics etc. (Negi et al., 2011; Pandey et al., 2018c; Hashem et al., 2019; Ku et al., 2021). *Bacillus subtilis* is widely known for the production of antimicrobial compounds and protection of different agricultural crops by suppressing phytopathogens (Chauhan et al., 2016; Guo et al., 2019; Chakraborty et al., 2020; Mulk et al., 2022). *Bacillus subtilis* being an environmentally benign biocontrol agent, its antimicrobial metabolites and other plant growth promoting traits are adequate to increase soil fertility, plant growth and disease suppression.

Secondary metabolites produced by *B. subtilis* are classified as ribosomally synthesized peptides (bacteriocins) and non-ribosomally synthesized peptides (lipopeptide and polyketide; Moyne et al., 2001). Antibiotics such as subtilosin, subtilin, ericin A, ericin S, mersacidin, TasA, sublancin, bacilysin, surfactin, plipastatin, bacitracin, fungycin, mycosubtilin, macrolactin, corynebactin, bacillomycin, amicoumacin etc. are known to be produced by *B. subtilis* (Moyne et al., 2001; Stein, 2005). Among these, macrolactin, a polyketide responsible for antimicrobial, anticancerous and other inhibitory activities is synthesized by the action of polyketide synthase (PKS; Schneider et al., 2007).

Bacillus subtilis BS-58, a promissing plant growth promoting bacterial (PGPB) strain was isolated from the non-rhizospheric soil sample collected from Salamkhet (Tehri, Garhwal; 78°24′37″E and 30°18′13″N) during our previous study on *amaranth* (Pandey et al., 2018c). Root-rot, stem decay and damping-off are prevalent in *amaranth* in this region and adversely affect crop health and its productivity. *Fusarium oxysporum* was found to be associated with root-rot and stem decay, whereas, *Rhizoctonia solani* was found responsible for root-rot and damping-off (Post-emergence). Both the pathogens are very common and responsible for heavy crop losses (~50–60%). Since, crop losses due to the attack of different pathogens and pests result in reduced food availability, they are considered as the major threats to global food security (Savary et al., 2019). *Amaranth* is one of the nutrient rich crops and is known as a good source of proteins, essential amino acids, macro and micronutrients (Shirani et al., 2017; Pandey et al., 2018b). Therefore, effective and eco-friendly management of such diseases has to be devised. Though, fungicides have been in use for the suppression of the pathogens, but, their long term and continuous use may cause lots of side effects on humans, animals and ecosystem (Pandey et al., 2018d; Fortunati et al., 2019). Therefore, biological approaches can be an effective and eco-friendly alternative for disease management. The endospore forming ability of *B. subtilis* gives it an upper edge to be used as abiocontrol agent and plant growth promoter.

In view of the above, the present study was focused on the assessment of biocontrol ability of *B. subtilis* BS-58 towards the two important phytopathogens (*F. oxysporum* and *R. solani*) of *amaranth* and identification of the antifungal metabolite produced by BS-58.

2. Materials and methods

2.1. Bacterial and fungal cultures

Basic details of *B. subtilis* BS-58 including, its isolation, identification, and its potential to increase plant growth and yield have already been published (Pandey et al., 2018c). The important traits of this strain include, phosphate solubilization, phytase production, siderophore production, IAA production and cold tolerance up to 5.0°C (Table 1). Both the fungal pathogens (*F. oxysporum* and *R. solani*) of *amaranth* were procured from the well-characterized repository of the Microbiology laboratory of College of Forestry (VCSG Uttarakhand University of Horticulture and Forestry), Ranichauri, Tehri Garhwal (Uttarakhand), India to conduct different experiments in this study. Out of these microorganisms, *Bacillus subtilis* BS-58 was maintained on nutrient agar medium (NAM) and the fungal pathogens were maintained on potato dextrose agar (PDA) slants at 4°C.

2.2. *In vitro* antagonistic activity

The antagonistic activity of *B. subtilis* BS-58 was carried out against both the phytopathogens (*F. oxysporum* and *R. solani*) using the dual culture plate technique (Skidmore and Dickinson, 1976). Briefly, the fungal discs (6 mm dia) were excised from fully grown 5 days old cultures of both the fungi and were placed at the center of another medium plate (containing NAM + PDA in 1:1) individually. Challenge inoculation of *B. subtilis* BS-58 was done on both sides of

TABLE 1 Plant growth promotion and biocontrol potential of *Bacillus subtilis* BS-58.

Activities		Results	SEM	cd (p =0.05)
P-solubilization efficiency* (%)		165.0	0.47	1.84
Phytase production*		+		
Siderophore production efficiency* (%)		78.0	1.29	5.04
IAA production*		+		
HCN production*		−		
Cold tolerance* (up to 5°C)		+		
Antagonistic efficiency (%)	*Fusarium oxysporum*	68.25	0.42	1.65
	Rhizoctonia solani	64.50	0.84	3.29

*Data taken from Pandey (2018a).
+: Positive reaction, −: Negative reaction. cd: Critical difference. Sem: Standard error of the mean.

the fungal disc (2.0 cm apart from the disc). The plates were then kept for incubation at 27 ± 1°C for 3–5 days. Plates only with fungal growth (without challenge inoculation) were kept as control and per cent inhibition of fungal growth in dual culture plate was calculated over control plate by using the following formula:

$$\%\text{Inhibition} = \frac{C - T}{C} \text{X } 100$$

(where, C = Radius of fungal growth in control plate, T = Radius of fungal growth in dual culture plate)

2.3. Scanning electron microscopy

To understand the inhibitory action of bacterial cells on the growth of the fungal pathogens in dual culture plates SEM analysis was done by following the method of King and Brown (1983) with some modification. Briefly, small pieces of agar (~1 cm^2) from the zone of interaction were excised from each plate and transferred to the well-dried interior surface of the lid of a glass Petri dish. Fungal discs were fixed overnight at 4°C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed thrice (10 min each) in phosphate buffer. After washing, samples were serially dehydrated (thrice) in 70, 80, 90, and 100% ethanol (5 min at each step) followed by air-drying. Dried samples were mounted on stubs and coated with gold. These coated specimens were observed at 15 KV in a LEO 485 VP Scanning Electron Microscope and photographs were captured.

2.4. Inhibitory potential of cell-free supernatant

The broth medium was prepared by following the composition as described by Kumar et al. (2014) and sterilized in an autoclave. The broth was then inoculated with *B. subtilis* BS-58 inoculum and incubated at 27 ± 1°C for 72 h to reach in the exponential phase (3×10^9 cfu ml^{-1}). The cells were then harvested by centrifuging at 8000 rpm for 10 min at 4°C and the supernatant was filtered through a Millipore filter (0.22 μm) to make it completely cell free. The antagonistic activity of cell free supernatant (CFS) was assessed against *F. oxysporum* and *R. solani* by agar well method by loading 100 μl CFS in each well.

2.5. Determination of the nature of antifungal metabolite

The cell-free supernatant (CFS) was then evaluated for its stability against heat and proteinase K treatment. The heat stability of the culture supernatant was assessed at two different temperatures (70 and 100 ± 1°C) for 20 min in a water bath following the method of Deraz et al. (2005). However, proteinase K (100 μg ml^{-1}) treated sample was incubated at 37 ± 1°C in a water bath for 30 min. All the treated cell-free supernatant samples were then loaded into agar wells (100 μl in each well) made in assay plates (2.0 cm apart from the fungal disc) for the determination of antifungal activity. Development of a zone of inhibition (if any) was observed after incubation at 27 ± 1°C for 3–5 days.

2.6. Purification and identification of the antifungal metabolite

Purification of the bioactive molecule (antifungal metabolite) from CFS was done using thin layer chromatography (TLC) guided column chromatography as described below.

2.6.1. Thin layer chromatography

Thin layer chromatography was performed on silica plates using different solvent systems (Supplementary Table S1) to select the most appropriate solvent system (mobile phase) for the separation of the antifungal metabolite. The plates were then kept in an iodine chamber to develop the spots of the compound.

2.6.2. Column chromatography

Purification of the bioactive metabolite from the CFS was done by TLC guided column chromatography. For this, CFS having antifungal activity was first concentrated at 55°C using a rota-evaporator. The concentrated fraction was mixed thoroughly with silica in 1:3 and dried to prepare the loading sample. Column was prepared using silica (60–120 mesh size) and packed in the respective solvent. The sample was then placed at the top of the column and run with mobile phase (Ethyl acetate: Methanol). To elute the bioactive molecule, polarity of the solvent was increased by 5 % after each cycle. The column cycles were run with 500 ml of each solvent and elute size was kept 25 ml. Purity of the active compound in the collected elute was confirmed by obtaining a single spot on TLC plate. After identifying the elute containing pure compound, the solvent was evaporated and antifungal activity was re-assessed by agar-well method. Identity of the bioactive compound was then resolved on the basis of LC–MS and FT-IR analysis as described below.

2.6.3. Liquid chromatography-mass spectrum analysis

Liquid chromatography-mass spectrum, with an electrospray ionization (ESI) interface, was used to determine the bioactive compound(s) in *the active fraction*. LC–MS analysis of the active fraction was performed on a UPLC (Ultra performance liquid chromatography) system, attached to an ESI interface and ACCUCORE-Mass spectrometer (Bruker Daltonic, CA, United States). MS spectra were collected in the scan range 150–1,000 m/z. Analytical chromatographic separations of the *active fraction* were carried out *via* a C18 100 × 3 column (50 × 2.1 mm, 1.7 μm; Thermo Fisher Scientific). The mobile phases used in this study were (A) acetonitrile + water (5:95), (B) acetonitrile, (C) methanol, and (D) water + formic acid at a fow rate of 0.3 ml min^{-1}. Five microliters of the sample was injected, and the solvent was run by gradient elution. The positive ion mode of ESI–MS was used to acquire the mass spectra.

2.6.4. Fourier-transformed infrared spectroscopy

Fourier-transformed infrared spectra of the pure compound were recorded on 8400S, FT-IR spectrometer (Spectrum GX) equipped with a mercury-cadmium-telluride (MCT) detector and cooled with

liquid nitrogen. The extract from pure fraction was compressed into a thin pellet and analyzed at wavelengths of 400–4,000 cm^{-1}. The analysis of FT-IR spectra was carried out by using OPUS 3.1 (Bruker Optics) software (Davis and Mauer, 2010).

2.7. Detection of the *mln* gene

For this, total genomic DNA of *B. subtilis* BS-58 was isolated by the phenol-chloroform extraction method as described by Ausubel et al. (1999). The presence of the *mln* gene in *B. subtilis* BS-58 was confirmed by its specific amplification using a pair of gene specific primers (MLN-C1 ATGCTGTTGCAGGACATAGTC and MLN-C2 TAGTCAGAATGTTTCCAGGACC; Schneider et al., 2007). Reaction mixture (100 µl) for the amplification was prepared containing 25 ng DNA, 1 × PCR buffer, 400 ng of each of the primers, 2.5 mM of each of the dNTPs, 0.3 U Taq polymerase. The PCR amplification was carried out with 35 cycles of initial denaturation (at 95°C for 3 min.), denaturation (at 94°C for 1 min.), annealing (at 50°C for 1 min.), synthesis (at 72°C for 2 min.), and extension (at 72°C for 7 min.). The amplicon was eluted from the gel and sent for sequencing at Biokart India Pvt. Ltd., Bengaluru, India. The sequence homology was studied by BLASTn search program. The sequence obtained was aligned by ClustalW using the MEGA7 software and the phylogenetic tree was constructed using the neighbor-joining method. The sequence was then submitted to NCBI by using Blanklt tool.

2.8. Pot trial for disease management

A pot-trial experiment (30 days) was carried out in pots (12″ dia) to evaluate the biocontrol ability of *B. subtilis* BS-58. The pots were filled with a pre-sterilized potting mixture containing sand, soil and farmyard manure (1:1:1). Seeds were moistened with sterile distilled water and coated with talc formulation (@ 10 $g\,kg^{-1}$ seed) of *B. subtilis* BS-58. However, seeds for control set were moistened but did not receive the bacterial treatment. In case of the negative controls, soil was infested with the respective fungal pathogen (*F. oxysporum* and *R. solani*, individually) and seeds did not receive any treatment in these sets. Whereas, in positive control sets seeds were treated with carbendazim (@ 2.0 $g\,kg^{-1}$ seeds) prior to sowing in pot soil infested with the respective fungal pathogen (*F. oxysporum* and *R. solani*, individually). Ten seeds per pot were sown at a depth of 1 cm. Germination of the seeds was recorded daily until all the seeds germinated in any of the pot. The other plant growth parameters were recorded at 30 day after sowing (DAS). The percent mortality of *amaranth* seedlings was calculated by following formula:

$$\text{Seedling mortality}\,(\%) = \frac{\text{Total number of dead seedlings}}{\text{Total number of seedlings germinated}} \text{X}\,100$$

2.9. Post interaction events after pot trial

Scanning electron microscopy analysis was performed to understand the antagonistic effect of *B. subtilis* BS-58 on hyphal morphology. *Amaranth* seedlings showing characteristic symptoms (brown spots on stem and damping-off) were taken out from the respective pots. These stems and root samples were washed with sterile distilled water and dried before proceeding for SEM analysis. Dried samples were mounted on stubs and coated with gold. These coated specimens were observed at 15 KV in a LEO 485 VP Scanning Electron Microscope and photographs were captured.

2.10. Data analysis

The data recorded during the study was subjected to analysis of variance (ANOVA) using completely randomized design (CRD) to evaluate the significance by the magnitude of *F* value. Duncan Multiple Range Test (DMRT) was performed to compare the means by using SPSS Statics v26.

3. Results

3.1. *In vitro* assessment of antagonistic activity

Bacillus subtilis BS-58 displayed good inhibitory potential against *F. oxysporum* and *R. solani* with 68.25 and 64.50% inhibition, respectively (Table 1; Figures 1A,B,D,E). Scanning electron micrographs showed deformities in hyphal morphology of *R. solani* and *F. oxysporum* in post-interaction events performed with samples taken from dual culture plates. These deformities included hyphal lysis, distortion, swelling, perforation, shrinkage and mycelial shredding in both the fungal pathogens (Figures 1G,H).

3.2. Antimicrobial activity of cell free supernatant and nature of the metabolite

Cell free supernatant of *B. subtilis* BS-58 collected after 72 h of incubation showed good antifungal activity against *F. oxysporum* (65.57%) and *R. solani* (61.66%). This was also interesting to note a good antifungal activity of *B. subtilis* BS-58 even after heat treatment at both of the temperatures (70 and 100°C) against *F. oxysporum* (62.76, 58.8%) and *R. solani* (59.33, 57.6%). However, some reduction in the antifungal activity against both the fungi (*F.* oxysporum: 28.9% and *R. solani*: 22.2%) after proteinase K treatment was recorded (Table 2; Figures 1C,F).

3.3. Purification and identification of the antifungal metabolite

Results of thin layer chromatography revealed the mixture of ethyl acetate and methanol (60:40) as the most appropriate solvent system for the separation of antifungal metabolite and therefore selected as the mobile phase for column chromatography. Among all the elutes collected from column chromatography, A4, A5 (100%), F1, F5, and F7 (55:45) showed inhibitory potential against *F. oxysporum* and *R. solani* (Table 3). All the active elutes were run on TLC plates to validate the purity of the bioactive compound.

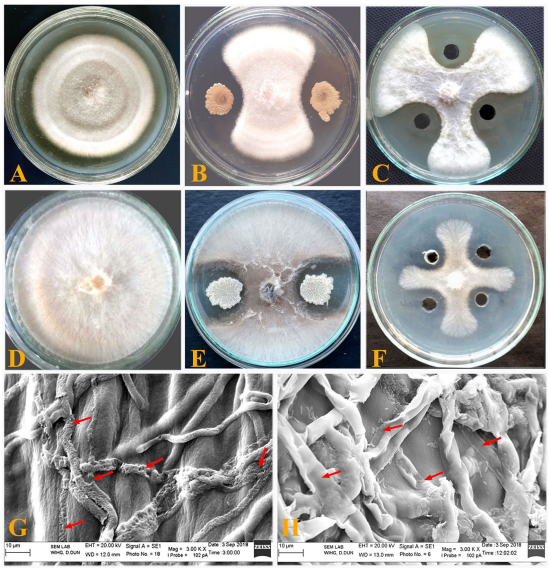

FIGURE 1
In vitro antagonistic activity of *Bacillus subtilis* BS-58 against both the fungal pathogens. **(A)**: Growth of *Fusarium oxysporum* in control plate; **(B)**: Inhibition of *F. oxysporum* by *B. subtilis*; **(C)**: Plate showing activity of elute (F7) against *F. oxysporum*; **(D)**: Growth of *Rhizoctonia solani* in control plate; **(E)**: Inhibition of *R. solani* by *B. subtilis*; **(F)**: Plate showing activity of elute (F7) against *R. solani*. **(G)**: Deformed mycelium (arrows) of *Fusarium oxysporum* upon interaction with *B. subtilis* BS-58; **(H)**: Deformed mycelium (arrows) of *Rhizoctonia solani* upon interaction with *B. subtilis* BS-58.

TABLE 2 Effect of various treatments on antifungal activity of cell-free supernatant (CFS) of *Bacillus subtilis* BS-58.

Pathogens	Treatment		Pathogen inhibition (%)	sem	cd (p =0.05)
F. oxysporum	No treatment		65.57^{a}	0.22	0.71
	Heat treatment	70^{oC}	62.76^{b}		
		100^{oC}	58.8^{c}		
	Proteinase K		28.96^{d}		
R. solani	No treatment		61.66^{a}	0.12	0.39
	Heat treatment	70^{oC}	59.33^{b}		
		100^{oC}	57.6^{c}		
	Proteinase K		22.24^{d}		

Data presented in the table is the average of three replicates. Values in the table represented with different letters are significantly different ($p < 0.05$). cd: critical difference. Sem: standard error of the mean.

3.3.1. Liquid chromatography-mass spectrum analysis

Liquid chromatography of the most active elute (F7) showed a strong retention peak at 16.57 min in its diode array chromatogram for the active metabolite along with two small peaks at 1.56 min and 2.06 min (Figure 2A). Furthermore, appearance of one major peak at 16.61 min and another on 18.31 min in the positive electrospray scan suggests the presence of cis and trans geometrical isomers of olefins in the active metabolite (Figure 2B). A positive electrospray scan determined different peaks with different m/z (mass to charge ratio)

TABLE 3 Activity of elutes and solvent system used for their separation in column chromatography.

Coloumn Elutes	Pathogen Inhibition (%)	
	F. oxysporum	*R. solani*
A4 (100% EA)	55.67^{d}	54.11^{c}
A5 (100% EA)	52.85^{e}	51.76^{d}
F1 (55% EA: 45% M)	59.17^{c}	72.94^{b}
F5 (55% EA: 45% M)	63.77^{b}	72.94^{b}
F7 (55% EA: 45% M)	71.90^{a}	75.30^{a}
sem	0.53	0.21
cd (p =0.05)	1.65	0.66

Data presented in the table is average of three replicates. EA-Ethyl acetate; M-Methanol. Values in the table represented with different letters are significantly different ($p < 0.05$). cd: Critical difference. Sem: Standard error of the mean.

value including strong peak of protonated metabolite at 403 along with its sodium adduct ion peak at 425 (Figure 2C).

3.3.2. Fourier-transformed infrared spectroscopy (FT-IR)

Fourier-transformed infrared spectroscopy spectroscopy revealed different peaks representing different functional groups including, alcohol at 3421.31, alkane at 2932.57, 2962.76, 2875.92, and 1410.34, carbonyl at 1728.01 (Figure 2D). Combining the data received from LC–MS and FT-IR analysis, identity of the molecule was found to be macrolactin A with a molecular weight of 402 Da.

3.4. Detection of the *mln* gene in *Bacillus subtilis* BS-58

The *mln* gene was isolated from *B. subtilis* BS-58 and identified by PCR amplifications using the gene specific primers. The DNA sequence retrieved from the amplicon (Supplementary Table S2) was submitted in gene bank with accession number MT726941. The sequence homology studied by BLASTn search program revealed 98.1% homology with the macrolactin genes available in the database at NCBI. Dendrogram showing the similarity of the *mln* gene isolated from *B. subtilis* BS-58 and other strains is presented in Figure 3A, and the specific amplification of the fragment is shown in Figure 3B.

3.5. Pot trial for disease management

Promising effects of seed treatment by *B. subtilis* BS-58 on seedling growth and disease suppression were observed in *amaranth* (Table 4). During this study, less seedling emergence as well as survival was observed in the pathogen infested soil (*F. oxysporum*: T-5, and *R. solani*: T-8; Figures 4A,B). Both of these treatments showed typical symptoms of infection of both the fungi including, root-rot, brown spots on stem and post emergence damping off. However, this was encouraging to note that no symptoms of infection were observed on seedlings grown out of the seeds treated with *B. subtilis* BS-58 in *F. oxysporum* infested soil (T-3). Whereas, *amaranth* seedlings grown in *R. solani* infested soil (T-6) and received seed treatment with *B. subtilis*

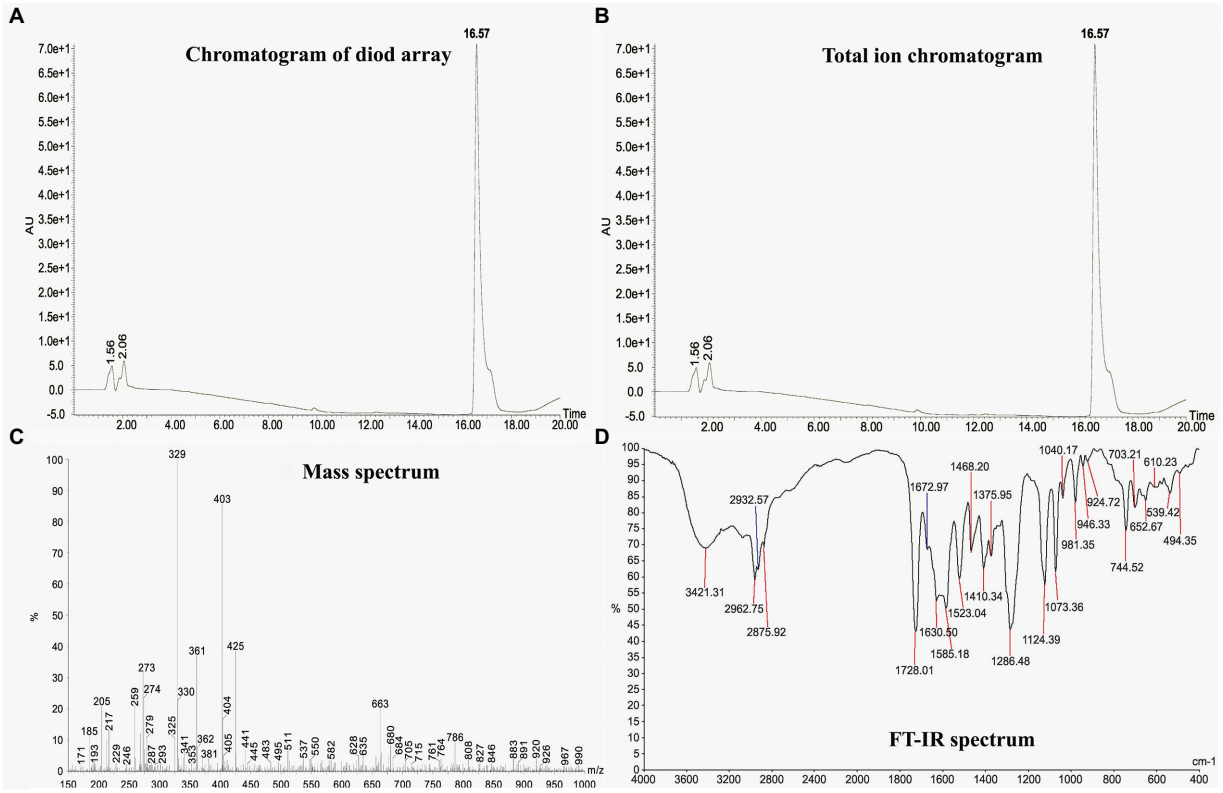

FIGURE 2
Liquid chromatography-mass Spectrum (LC–MS) and FT-IR analysis of the antifungal metabolite produced by *Bacillus subtilis* BS-58. **(A)**: Diode array chromatogram showing a major peak of the metabolite at 16.57min; **(B)**: Total ion chromatogram showing two major peaks at 16.61 and 18.31min suggesting the presence of cis and trans geometric forms of olifins in the metabolite; **(C)**: Mass spectra showing different peaks with different m/z value including strong peak of protonated metabolite (M+H) at 403 along with its sodium adduct ion (M+Na) peak at 425; **(D)**: FT-IR analysis showing different peak values for various functional groups including alcohol at 3421.31, acid at 1728.01, carbonyl at 1672.97.

BS-58 showed some early symptoms of infection (brown spots on stems). However, most of these seedlings recovered in the later stages of plant growth. Both of these treatments showed 17.85 per cent mortality. Increased shoot length, root length and other seedling growth parameters were recorded in uninfested sets (T-2 and T-4) over control (T-1). This was interesting to note that the bacterized seeds exhibited reduced percent mortality and improved growth of seedlings in pathogen infested soil. Maximum plant mortality was recorded in T-5 and T-8 (negative controls) infested with fungal pathogens (*F. oxysporum* and *R. solani*, respectively), where seeds did not receive any bacterial treatment (Table 4). However, minimum per cent mortality was recorded in T-2 (10.75%) followed by T-4 (14.81%).

3.6. Scanning electron microscopy

Effect of *B. subtilis* BS-58 treatment in the suppression of disease or restoration of health of the seedlings (after fungal infection) was also studied under the scanning electron microscope. A huge network of fungal mycelia (*R. solani*) was observed in the decayed seedlings of *amaranth* grown out of the untreated seeds, while inhibition of fungal mycelia by the swelling, fragmentation, lysis was observed in the seedlings survived from the fungal infection in BS-58 treated pots (Figures 4C,D).

4. Discussion

Extensive and sometimes inappropriate use of harmful agrochemicals adversely affects the soil ecology and disturbs the environment, as well (He et al., 2008). Therefore, the use of microbial bioagents can be a chemical-free alternative to the conventional crop protection in agriculture and dependency on fungicides (Negi et al., 2017; Singh et al., 2022). Our study revealed that *B. subtilis* BS-58 could effectively suppress the growth of two destructive fungi, *F. oxysporum* (64.7%) and *R. solani* (73.3%). Our results get support from Zhu et al. (2020), who reported 67% inhibition of *F. oxysporum* f. sp. *niveum* by *B. subtilis* IBFCBF-4. Similarly, Hussain and Khan (2020) recorded 45% inhibition of *R. solani* (causal agent of black scurf disease of potato) by *B. subtilis*. The antifungal activity of *B. subtilis* BS-58 against both the fungal pathogens in our study might be due to the production of antifungal metabolite(s) or siderophore production those have been described as effective mechanisms of pathogen suppression by several researchers (Negi et al., 2017; Ku et al., 2021; Zhu et al., 2021). *Bacillus subtilis* BS-58 has already been reported with good colonization and plant growth promoting abilities in one of our previous studies (Pandey et al., 2018c). Therefore, good antifungal activity of BS-58 may protect the host plant from diseases caused by these fungal pathogens and simultaneously can reduce the dependence on fungicides being used for disease management.

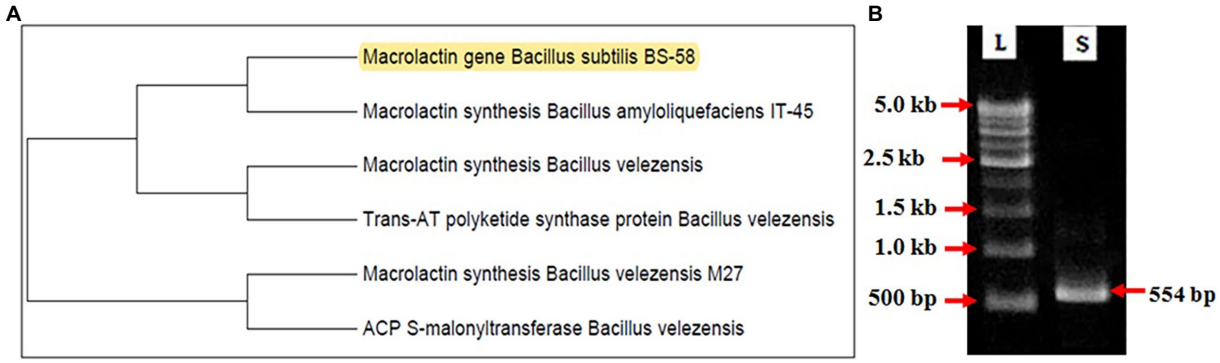

FIGURE 3
Identification of the *mln* gene in *Bacillus subtilis* BS-58. **(A)**: Figure showing relationship between the *mln* genes isolated from *B. subtilis* BS-58 and other *Bacillus* strains. **(B)**: Amplification of 554bp fragment of *mln* gene [L-DNA ladder (500–5kb); S-Amplified product of *Bacillus subtilis*].

TABLE 4 Effect of *Bacillus subtilis* on seedling growth and disease suppression.

Treatments	% Germination	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)	Mortality (%)
T1	86.67^{a}	14.88^{b}	20.34^{c}	1.50^{b}	0.52^{b}	16.67^{b}
T2	93.33^{a1}	16.44^{a}	26.00^{a}	1.70^{a}	0.58^{a}	10.73^{b}
T3	76.67^{b}	15.40^{b}	21.60^{b}	1.01^{d}	0.46^{c}	17.85^{b}
T4	90.00^{a}	15.48^{b}	18.85^{d}	1.00^{d}	0.35^{d}	14.81^{b}
T5	60.00^{d}	13.66^{cd}	16.66^{f}	0.50^{e}	0.21^{e}	38.89^{a}
T6	73.33^{bc}	15.30^{b}	20.75^{c}	1.13^{c}	0.41^{c}	17.85^{b}
T7	86.67^{a}	14.53^{bc}	19.35^{d}	1.03^{cd}	0.31^{d}	15.27^{b}
T8	66.67^{cd}	13.10^{d}	17.67^{e}	0.60^{e}	0.20^{e}	31.74^{a}
sem	2.89	0.29	0.22	0.04	0.02	3.84
cd (P = 0.05)	8.65	0.87	0.68	0.11	0.07	11.52

Data presented in the table is average of three replicates. T-1: No treatment; T-2: *B. subtilis*; T-3: *B. subtilis* + *Fusarium oxysporum*; T-4 (Positive control): *F. oxysporum* + 0.2% carbendazim; T-5 (Negative control-1): soil inoculated with *F. oxysporum*; T-6: *B. subtilis* + *Rhizoctonia solani*; T-7: (Positive control): *R. solani* + 0.2% carbendazim; T-8 (Negative control-2): soil inoculated with *R. solani*. Values in the table represented with different letters are significantly different ($p < 0.05$).
cd: critical difference. Sem: standard error of the mean.

In the present study, the loss of structural integrity of mycelia with hyphal swelling, lysis, digestion and perforation in both the fungi (*F. oxysporum* and *R. solani*) was observed in the SEM studies of the challenged fungal mycelia. Such deformities in hyphal morphology have been attributed to the production of antifungal metabolites by different biocontrol agents (Negi et al., 2011; Gomaa, 2012; Jimtha et al., 2016; Zhu et al., 2020). Similar findings were reported by Kaur et al. (2015) in post-interaction studies of *Alternaria alternata* and *B. vallismortis* R2. They observed shrunken, collapsed, empty hyphae, large depressions and loss of turgidness of *A. alternata* hyphae.

Cell free supernatant (CFS) of *B. subtilis* BS-58 was found inhibitory for *F. oxysporum* (65.57%) and *R. solani* (71.66%) in agar well diffusion assay. The results gets support from the study of Zhang et al. (2008), who found the CFS of *B. subtilis* B-FSo6 inhibitory towards *Aspergillus flavus* and suggested that the activity was due to the secretion of bacillomycin like compound by B-FSo6. Similarly, Kumar et al. (2012) showed the inhibition of *F. oxysporum* (50%), *Macrophomina phaseolina* (53.58%), *F. solani* (47.39%), *Sclarotina sclerotiorum* (47.69%) and *R. solani* (46.37%) by *Bacillus* spp. BPR7 and suggested that the antifungal activity might be due to the production of antifungal metabolites. Our results suggest that the antifungal metabolite is extracellular in nature and suppressing the fungal growth through diffusion in medium. Such metabolites when are diffused in rhizosphere may guard the crop from seed or soil borne pathogens.

Heat treatment of CFS of *B. subtilis* BS-58 at high temperatures in the present study indicated that some heat stable metabolite was present in the CFS of BS-58. Heat stability of antimicrobial protein AsR416 produced by *B. subtilis* was reported at different temperatures (30, 50, 70, and 100°C) by Kong et al. (2018). The heat stable nature of antifungal metabolite might be helpful, when processed industrially as antimicrobial formulation. High temperature will not cause any side effect in the quality of the metabolite. Considerable reductions in antifungal activity of CFS of BS-58 against both the fungal pathogens indicate towards the proteinacious nature of the active metabolite present therein. Tang et al. (2015) also found a reduced activity of AMP after protease action. They suggested that a variety of proteases could hydrolyze the carboxyl-terminal peptide bond of some proteins and destroy the spatial structure of the protein resulting in loss of antifungal activity under certain temperature conditions. However, the specific mechanisms need further verification.

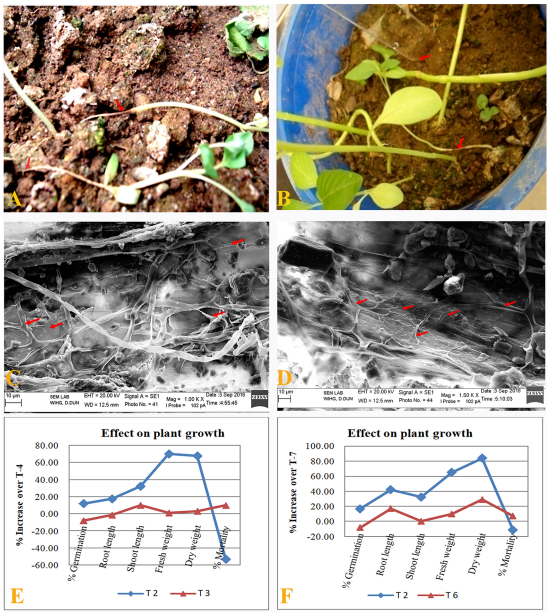

FIGURE 4
Disease suppression by *B. subtilis* BS-58 in pot assay. **(A)**: Seedlings grown in *R. solani* infested pot. **(B)**: Seedlings grown in *F. oxysporum* infested pot. **(C,D)**: SEM photomicrographs showing degradation and lysis of mycelia (note arrows) of both the pathogens (*R. solani* and *F. oxysporum*) by BS-58 under pot assay. **(E,F)**: Per cent increase in plant growth parameters calculated for different treatments over respective positive control (T-4 and T-7, respectively) [T-2: Seed treatment by BS-58; T-3: Seed treatment by BS-58+Soil infestation with *F. oxysporum*; T-4 (Positive control: Soil infestation with *F. oxysporum*+Seed treatment by 0.2% carbendazim); T-6: Seed treatment by BS-58+Soil infestation with *R. solani*; T-7: (Positive control: Soil infestation with *R. solani*+Seed treatment by carbendazim)].

Thin layer chromatography (TLC) performed with different solvent systems suggested the combination of ethyl acetate and methanol (60EA:50 M) as the best solvent system for the separation of antifungal metabolite of *B. subtilis* BS-58 in this study. The rate of migration of a particular compound depends on the absorbent and the solvent system used, therefore, the selection of the suitable solvent system is crucial (Ranjan and Jadeja, 2017). Selection of the solvent system by TLC can reduce the solvent load and thereby provide an accurate solvent system for column chromatography for a better separation and isolation of an antimicrobial metabolite.

Column chromatography is a well-established and widely used technique for the separation and purification of secondary metabolites. In this study, column chromatography revealed F7 (55EA:45 M) as the most active elute responsible for the inhibition of *F. oxysporum* and *R. solani*. Our results get support from the study of Wang et al. (2012), who isolated antifungal metabolite from *B. coagulans* by using TLC guided column chromatography and reported three fractions as active elutes for the suppression of *Phytophthora drechsleri*. Recently, Salazar et al. (2020) extracted antimicrobial metabolite (Macrolactin) from *B. amyloliquefaciens* ELI149 by silica gel column chromatography. The antifungal activity of the active elutes of *Bacillus subtilis* BS-58 in the present study might be attributed to the secretion of secondary metabolite(s).

The mass to charge ratio is measured by LC–MS through the ionization of chemical compounds to generate charged molecules or molecule fragments. In the present study, molecular weight of the antifungal metabolite was determined as 402 Da by its m/z value in LC–MS that corresponds to macrolactin A (402.5 Da). Our results are endorsed by the study of Yuan et al. (2012), who identified antifungal metabolite as macrolactin A produced by *Bacillus amyloliquifaciens* NJN-6 by LC–MS with a molecular weight of 402 Da.

The FT-IR technique is a rapid, time saving method and has been used to identify the compound present in the pure form or in the mixture of various compositions (Kowalczuk and Pitucha, 2019). In our study, different functional groups including alcohol, alkane, carbonyl were detected by the FT-IR analysis of the antifungal metabolite, which were found similar to the functional groups of macrolactin A. Likewise, Devi et al. (2010) identified an antimicrobial compound from *Bacillus licheniformis* SAB1 as 3-phenylpropionic acid by FT-IR analysis.

This study also evidenced the presence of the *mln* gene in *B. subtilis* BS-58 that is responsible for the synthesis of the polyketide of macrolactin group. Macrolactin is a polyketide that is known to inhibit bacterial as well as fungal growth (Kim et al., 2011; Chakraborty et al., 2014; Yuan et al., 2016; Salazar et al., 2020). Earlier, macrolactin type antibiotic was isolated by Yuan et al. (2012) from *B. amyloliquifaciens* NJN-6 and reported significant inhibition of *F. oxysporum* and *Ralastonia solanacearum*. The presence of the *mln* gene in *B. subtilis* BS-58 in the present study, advocates that the metabolite responsible for the effective inhibition of both the fungal pathogens belong to the macrolactin group of antibiotics.

The pot assay revealed the potential of *B. subtilis* BS-58 to suppress the diseases caused by *F. oxysporum* and *R. solani*. This was evident by decreased mortality of *amaranth* seedlings when grown in the soil infested with *F. oxysporum* and *R. solani* and treated with *B. subtilis* BS-58 in comparison to positive control. Data analysis revealed a promising performance of BS-58 for plant growth and disease suppression activities under challenged conditions (T-3), when compared with negative control (T-4; Figure 4C). Seed treatment by BS-58 could increase different growth parameters in *F. oxysporum* amended soil by 27.78 to 117.46 per cent over negative control. However, mortality in seedlings in this treatment was almost 54 per cent less than negative control-1 (T-5). This was encouraging to note that BS-58 showed a comparative performance to affect different growth parameters and disease suppression (except germination) in *F. oxysporum* amended soil (T-3), when compared with positive control (T-4). Seed germination was 23 per cent higher in positive control in comparison to T-3 (Figure 4E).

Similarly, under *R. solani* infested condition (T-6), BS-58 again presented it to be a potential candidate to enhance plant growth by 10.00 to 100 per cent and could reduce the seedling mortality by 43.76 per cent in comparison to negative control-2 (T-8). Again, BS-58 exhibited good performance to affect different growth parameters and disease suppression in *F. oxysporum* amended soil (T-3), when compared with positive control (T-7). However, seed germination was 20 per cent higher and mortality was almost 12 per cent less in positive control in comparison to T-6 (Figure 4F). This inhibition of pathogenic fungi might be due to the production of macrolactin A. Being secretary in nature, this would have spread in rhizosphere and created a non-conducive environment for these pathogenic fungi to grow. Chauhan et al. (2016) reported inhibition of *F. solani* with the reduced percentage incidence of rhizome rot in turmeric (*Curcuma longa* L.) by the treatments of *Bacillus endophyticus* TSH42 and *B. cereus* TSH77. They suggested that the antifungal activity was due to the production of certain antibiotics such as iturin, fungycin and surfactin

by this *Bacillus* sp. The above findings endorse that the strong antifungal activity of *B. subtilis* BS-58 in this study was due to the production of macrolactin A. The antifungal activity of macrolactins has previously been reported against several other plant pathogens such as *F. proliferatum*, *Moniliophthora roreri*, *Fusarium* sp., *Aspergillus niger*, *Rhizoctonia* sp. and *A. alternata* (Salazar et al., 2020). Interestingly, mycelial lysis and deformities induced by *B. subtilis* BS-58 in both the pathogens (as observed in SEM photomicrographs) further confirms the promising role of BS-58 in the suppression of fungal diseases. Thus, BS-58 promises to be a potential biocontrol agent for the management of plant diseases.

5. Conclusion

The findings of the study reveal that the antifungal activity of *B. subtilis* BS-58 against two destructive phytopathogens is due to the production of macrolactin A. Being extracellular in nature, this would have diffused in rhizosphere and effectively suppressed the fungal infection in *amaranth* seedlings grown in pathogen infested soil. Being native and target specific, such strains under suitable conditions, may result in ample production of antibiotic and greater suppression of the disease.

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

CP performed the experiments and drafted the manuscript. DP conducted *mln* gene amplification and molecular analysis. SD and MG performed the analysis of the experimental data and edited the manuscript. YN and DM conceptualized and supervised the study, and reviewed 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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An explanation of the mystifying bakanae disease narrative for tomorrow's rice

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Rice production is severely hampered by the bakanae disease (*Fusarium fujikuroi*), formerly recognized as *Fusarium moniliforme*. *F. moniliforme* was called the *F. fujikuroi* species complex (FFSC) because it was later discovered that it had some separate species. The FFSC's constituents are also well recognized for producing phytohormones, which include auxins, cytokinin, and gibberellins (GAs). The normal symptoms of bakanae disease in rice are exacerbated by GAs. The members of the FFSC are responsible for the production of fumonisin (FUM), fusarins, fusaric acid, moniliformin, and beauvericin. These are harmful to both human and animal health. This disease is common around the world and causes significant yield losses. Numerous secondary metabolites, including the plant hormone gibberellin, which causes classic bakanae symptoms, are produced by *F. fujikuroi*. The strategies for managing bakanae, including the utilization of host resistance, chemical compounds, biocontrol agents, natural goods, and physical approaches, have been reviewed in this study. Bakanae disease is still not entirely preventable, despite the adoption of many different tactics that have been used to manage it. The benefits and drawbacks of these diverse approaches are discussed by the authors. The mechanisms of action of the main fungicides as well as the strategies for resistance to them are outlined. The information compiled in this study will contribute to a better understanding of the bakanae disease and the development of a more effective management plan for it.

KEYWORDS

rice, bakanae disease, mystifying, disease mechanism, management

1. Introduction

Rice is one of the world's most vital staple food crops that is cultivated in diverse environmental conditions and is therefore subjected to various biotic and abiotic stresses. Insect pests and infections due to viruses, fungi, bacteria, and nematodes are the most significant biotic stresses impacting rice production. Bakanae (also known as foot rot) has emerged as a disease of serious concern among the critically valuable diseases of contemporary significance (Bashyal et al., 2016). It has been reported that one or

more *Fusarium* species are involved in causing bakanae disease in rice. Though the bakanae pathogen is seed-borne in nature, it can cause infection at any stage of crop life from pre-emergence to maturation, leading to withering or poor germination of rice seeds (Iqbal et al., 2011). *F. fujikuroi* is one of the Asian clade members that has been studied extensively as a causal organism of the bakanae disease of rice. Generally, yield loss can be ∼10–20% due to the disease, but it can also reach more than 70% under severe infection (Fiyaz et al., 2014). Bakanae disease is widespread in Asia and episodic in other rice-producing regions. The geographic distribution of the disease can be observed in Figure 1. "Bakanae" is a Japanese term that means "bad," "kinky," or "stupid" seedling, referring to the infrequent early seedling elongation caused by the production of mycotoxin (gibberellin) during the infection cycle (Fiyaz et al., 2016; Lee et al., 2018). The pathogen can infect a broad range of plant hosts and can be found all over the world. When a plant is fully grown, it can get infected anywhere from the roots to the crown to the stems to the leaf sheaths to the panicles. After initially being identified as *Lisea fujikuroi* Sawada in 1919, the fungus was renamed *Gibberella fujikuroi* (teleomorph) in 1931 (Ito and Kimura, 1931). *Fusarium moniliforme* was identified as its anamorph stage, but it is currently known as *Fusarium fujikuroi* (Sun and Snyder, 1981). The infection in rice plants can occur even after they have been transplanted, leading to stunted growth, poor tillering, and a lack of grain fill (Fiyaz et al., 2016).

Tall, gangly, and fewer tillers with yellowish green flag leaves, sterility, and grain discoloration are the characteristic symptoms of bakanae disease. Typically, infected plants die at later stages, whereas the panicles of surviving plants become impotent to produce grains and tolerate only unfilled panicles (Lee et al., 2018, 2022), ultimately reducing the crop yield. The source of secondary infection is through infected plants or seeds that disseminate by water or wind. The low survival rate of infected plants and high sterility of spikelets can contribute to yield losses reaching up to 50% in Japan, 40% in Nepal, 28.8% in Korea, 6.7–58.0% in Pakistan, and 3.0–95% in India (Lee et al., 2022). Taking into account the economic importance of rice and the dire threat posed by bakanae to rice production and yield, this study reviewed and summarized all important information about the pathogen from disease mechanisms to various control methods.

2. Associated species

Various species complexes of Fusarium have been reported to be associated with the bakanae disease of rice (Figure 2). Among them, four species of the *F. fujikuroi* species complex (FFSC), including *F. proliferatum*, *F. fujikuroi*, *F. verticillioides*, and *F. andiyazi*, are particularly responsible for causing bakanae disease of rice. Other associated species such as *F. commune* belonging to the *F. oxysporum* species complex (FOSC); *F. asiaticum* belonging to the *F. sambucinum* species complex (FSSC); and *F. incarnatum* belonging to the *F. equiseti* species complex (FIESC) have been isolated from rice seeds in different countries (Jahan et al., 2013; Choi et al., 2018; Avila et al., 2019; Jiang et al., 2020; Lee et al., 2022). Although members of FFSC are associated with the bakanae disease, *F. fujikuroi* is considered the fundamental species responsible for the characteristic symptoms (Jiang et al., 2020). Fusarium species' propensity for isolation and variation on rice seeds is dependent on the location and variations of those seeds, highlighting their variety. However, differences in the composition of the target species in different studies may be attributable to a number of factors, such as the target organ of the plant, sample size, geographical distribution of the pathogen species, and the effect of climate on species distribution (Moreira et al., 2020). Among all *Fusarium* spp. associated with the bakanae disease of rice, *F. fujikuroi* is the most dominant one having the highest isolation frequency. According to a study conducted in China, the *Fusarium* spp. isolated from three provinces showed the highest frequency of *F. fujikuroi* (80.05%), followed by *F. proliferatum*, *F. equiseti*, *F. incarnatum*, *F. commune, F. andiyazi,* and *F. asiaticum* with a frequency 8.31%, 5.94%, 2.61%, 1.66%, 0.95%, and 0.48%, respectively (Jiang et al., 2020).

3. Morphological characterization

Fusarium isolates that produce relatively slender macroconidia lacking momentous curvature but do not produce chlamydospores are categorized as FFSC. *F. fujikuroi* produces copious ovate or club-shaped microconidia in shorter chains (false heads) from monophilalides and monophialides with a flattened base and comprises zero to one septum (Jiang et al., 2020) and medium-length macroconidia that are slightly slender and lack substantial curvature. Additionally, three to five septate, tapered apical cells and poor development of basal cells is also a characteristic feature of macroconidia produced by *F. fujikuroi*. In *F. proliferatum*, copious club-shaped microconidia in false heads and chains are produced from monophialides and polyphialides and lack septation. However, macroconidia produced by *F. proliferatum* are slightly straight and slender and comprise three to five septations, curved apical cells, and poor development of basal cells. In the case of *F. andiyazi*, clavate to ovate microconidia having a flattened base are copiously produced in long chains lacking septation from monophialides, and macroconidia produced by this species are straight to partly curved comprising three to six septations (mainly three septations) with a slightly curved apical cell and a pedicellate basal cell. The formation of pseudochlamydospores is also a characteristic feature of macroconidia produced by *F. andiyazi* (Jiang et al., 2020). In FFSC, the morphological characteristics of *F. fujikuroi* and *F. proliferatum* are very similar, and a close relationship between these two species is also evident phylogenetically. Moreover, *F. andiyazi* and *F. verticillioides* exhibit similar morphology with the only difference being in the production of pseudo-chlamydospores by the former species, evident only in FFSC. Both these species share a close relationship phylogenetically as well (Jiang et al., 2020).

Fusarium isolates producing macroconidia with a conspicuous dorsiventral curvature or crescentiform are categorized as FIESC (Avila et al., 2019; Jiang et al., 2020). *F. equiseti* lacks microconidia production on PDA, while macroconidia are produced from sporodochia with a prominent dorsiventral curvature and needle-like apical cells are formed, which are more rounded as in *F. compactum*. *F. semitectum* (formerly known as *F. incarnatum*) produces copious mesoconidia (conidia produced in the aerial mycelia with the appearance of "rabbit ears" instead

FIGURE 1
Geographical distribution of the bakanae disease.

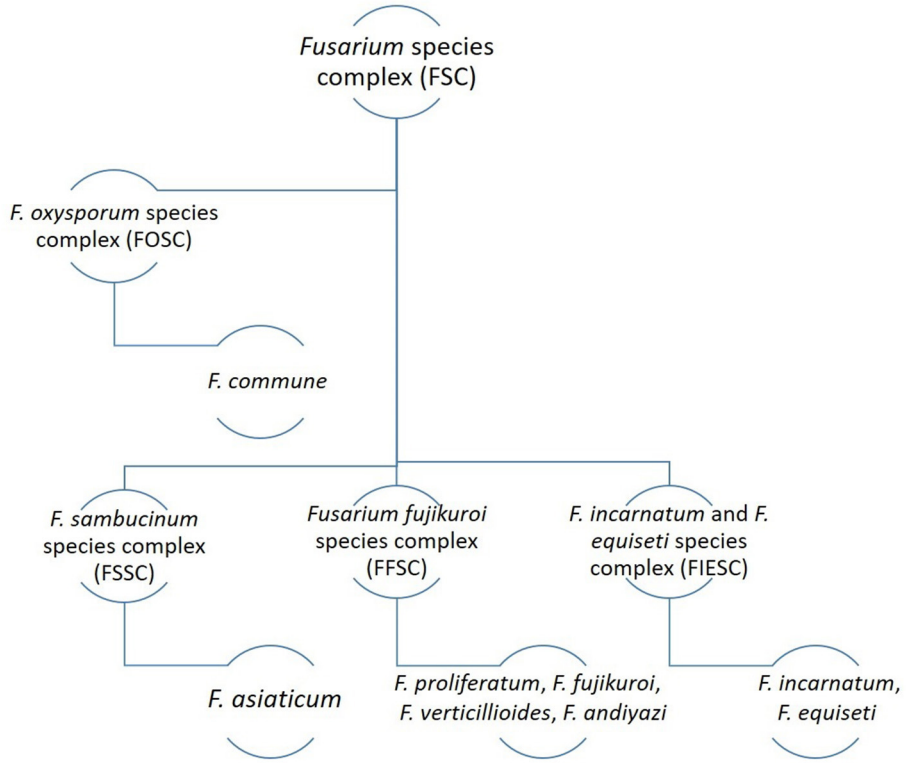

FIGURE 2
Fusarium species associated with the bakanae disease of rice.

of sporodochia) from polyphialides that are spindle-shaped and straight with three to four septations (Jiang et al., 2020). In FIESC, although the morphotype of *F. Incarnatum* and *F. equiseti* has been documented (Avila et al., 2019), it is still difficult to differentiate between some ambiguous species (Jiang et al., 2020).

Fusarium isolates that produce slightly slender, relatively straight-to-curved macroconidia comprising five septations and curved and foot-shaped cells i.e., apical and basal cells, respectively, are categorized as FSSC. In this species complex, *F. asiaticum* produces straight to slightly curved macroconidia with five septations comprising slightly curved apical cells and well-developed basal cells (Li et al., 2017). The morphology of *F. asiaticum* is very similar to *F. graminearum*, and they exhibit slightly dissimilar conidial features.

4. Molecular characterization

Advanced methods must be devised and applied for rapid plant disease diagnosis to reduce crop loss brought on by pathogen infection (Shakeel et al., 2022a). The characterization of *Fusarium* species that are associated with bakanae rice disease purely based on morphological features is a difficult task because of the high diversity in their characteristics (Choi et al., 2018). Consequently, the systemic culture media and standard methodology, in addition to multi-locus molecular records, are currently considered imperative for assertive species-level identification. Even though several studies have established that the species in FFSC associated with bakanae disease of rice, along with several other *Fusarium* species (*F. moniliforme*, *F. thapsinum*, *F. commune*, and *F. pseudonygamai*), can be differentiated using just the gene sequence of the *TEF1* gene (Wulff et al., 2010; Choi et al., 2018), but there is no evidence that the *TEF1* gene can be used to characterize all species in Fusarium species complex (FSC). Nevertheless, a combination of the *RPB1* and *RPB2* genes was employed to deduce the phylogenetic relationships of the species in FSC (O'Donnell et al., 2013), and an amalgamation of the *RPB1*, *RPB2*, and *TEF1* genes were utilized to analyze the genetic diversity of *Fusarium oxysporum* f. sp. *Cubense*; this method, therefore, has been highly endorsed (Maryani et al., 2019). Furthermore, with a lack of reference sequences available for the RPB1 gene in *F. incarnatum*, six species that were identified based on their morphological characters and by the *TEF1* gene were first categorized into the FSC through the use of just *RPB1* and *RPB2* genes because of the lack of individual reference sequences for the species. Further analysis of each FSC was further done *via* several loci sequences (Jiang et al., 2020).

5. Virulence variation

In various studies, the pathogenicity of several *Fusarium* species (*F. fujikuroi, F. proliferatum*, *F. equiseti*, *F. incarnatum*, *F. commune, F. andiyazi*, and *F. asiaticum*) was assessed to determine the virulence discrepancies among species. After 20 days of germination, rice seedlings inoculated with *F. fujikuroi* showed typical symptoms of bakanae disease (Choi et al., 2018; Jiang et al., 2020), while *F. andiyazi* and *F. proliferatum* inoculation caused the specific yellowing of leaves along with a few dying leaves to appear on normal-height rice seedlings. Comparative stunted plants with yellowish leaves were reported in response to the inoculation of *F. incarnatum* and *F. asiaticum* on seedlings. These findings from several studies showed that these six species were pathogens of rice and capable of causing the disease, but their virulence varied (Jiang et al., 2020). *F. fujikuroi* as a cause of bakanae disease of rice along with other associated species has been studied by many researchers. Numerous studies have revealed that other species of FSC (including *F. verticillioides*, *F. proliferatum*, *F. andiyazi*, *F. equiseti, F, incarnatum*, and *F. asiaticum*) were concomitant with the rice bakanae disease (Prà et al., 2010; Wulff et al., 2010; Choi et al., 2018; Jiang et al., 2020). These associated species could develop characteristic symptoms of bakanae but their symptoms varied significantly. Additionally, *F. fujikuroi* stimulated elongation in the seedlings, whereas *F. proliferatum* and *F. andiyazi* isolates were found to cause the comparative stunting of rice seedlings (Choi et al., 2018; Jiang et al., 2020). Significant stunting was observed in seedlings inoculated with *F. asiaticum*, *F. equiseti*, and *F. incarnatum*. Moreover, *F. concentricum* also can cause characteristic symptoms of bakanae disease in rice (Jeon et al., 2013), and *F. incarnatum* has also been found as a pathogen of bakanae (Song et al., 2014). *F. equiseti* is termed a saprophytic microbe (Choi et al., 2018), whereas the saprophytic or pathogenic role of *F. asiaticum* has not been recognized. The cause of virulence variation among species was determined through germination tests under *in vitro* conditions. *F. asiaticum* (73%) and *F. andiyazi* (71%) showed the maximum inhibition percentage of seed germination followed by *F. incarnatum* (54%), *F. equiseti* (44%), and *F. proliferatum* (35%), all of which also showed significant inhibition, while *F. fujikuroi* showed the least inhibition of seed germination (Zhang et al., 2015). Meanwhile, no significant difference was found between the inhibition percentages of seed germination by *F. asiaticum* and *F. andiyazi*. The cause of the inhibited seed germination mechanism was found to be associated with mycotoxins produced by the particular species (Jiang et al., 2020).

6. Mycotoxin production

A variety of mycotoxins produced by the *Fusarium* species have been associated with the bakanae disease. Within the FFSC, *F. fujikuroi* is the only species that produces Gibberellin A3, which encourages plant elongation. Among several mycotoxins [such as fumonisins (FBs), enniatins (ENN) beauvericin (BEA), and moniliformin (MON)], at least one mycotoxin has been produced by FFSC isolates (Choi et al., 2018; Saito et al., 2021). Although fusaric acid, moniliformin, and fumonisins were found to cause phytotoxicity in plants and develop characteristic symptoms (Saito et al., 2021), no information existed regarding their effect on the germination of rice seeds. Isolates of FIESC from rice seeds can produce trichothecenes, such as 4-acetylnivalenol (4-ANIV), nivalenol (NIV), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and deoxynivalenol (DON) (Avila et al., 2019). In another study, the production of T-2 toxin by *F. equiseti* was evident but not DON. Nonetheless, most of the isolates of *F. asiaticum* (member of FSSC) isolated from Jiangsu, China have been found to primarily produce 3-acetyldeoxynivalenol (3-ADON), while others produced nivalenol (NIV) (Dong et al., 2020). Various trichothecenes have been recognized as phytotoxic, while some others such as 4,15-Diacetoxyscirpenol (4,15-DAS) have been demonstrated to impede soybean seed germination (Miedaner et al., 2017; Jiang et al., 2020).

7. Disease mechanism

To prevent host plants from responding to infection, Fusarium spp. has developed sophisticated strategies. The pathogens use a wide variety of infection tactics to successfully infiltrate and colonize the host. Aside from the core genome, which is involved in fundamental metabolism, the Fusarium genome also contains virulence-related areas (adaptive genome) (Leslie and Summerell,

2013; Beccari et al., 2022). Effectors play a vital role in the interactions between the plant and the pathogenic fungus. The study of fungal plant diseases has resulted in several effectors being discovered, some of which have been described for their virulence roles (Tariqjaveed et al., 2021; Shakeel et al., 2022b). Fusarium has numerous endophytic and positive interactions with host plants, but there have been few molecular-level studies of these interactions (Pereira et al., 2019). Characterizing the secretome aids in the comprehension of pathogen virulence and host plant infection mechanisms. For instance, it has been anticipated that a subset of pathogen-secreted proteins can determine the success and progress of disease development. The size of the anticipated secretome (*F. graminearum*) is 574 proteins, or 4.2% of its total anticipated gene repertoire (Brown et al., 2012; Bashyal et al., 2017). The total anticipated secretory proteins are 1,336 in the genome of IMI58289 *F. fujikuroi* isolate (Wiemann et al., 2013; Bashyal et al., 2017). However, the investigation into the role of secretome (i.e., plant cell wall degrading enzymes) in *F. fujikuroi* has been confined to the detection of genes number. It has been indicated that, in *F. fujikuroi*, the secretome is composed of distinct proteins that interact in an organized way to inhibit various characteristics of plant immunity to effectively cause the disease. In addition, the results suggest that most of these genes are activated during the host-pathogen interaction when the genome appears to be enriched with cell envelope (CE) enzymes and glycoside hydrolases (GHs) that can penetrate the cell barrier of the host plant. The GH3 and GH5 abundance stimulate the disruption of pectin, cellulose, and hemicellulose, suggesting that these degrading enzymes play crucial roles in the genome of *F. fujikuroi* (Bashyal et al., 2017). Additionally, *F. fujikuroi* possesses one each of the GH67 and GH36 genes encoding α-galactosidases that are lacking in the majority of plant pathogens. The presence of abundant families of pectin degrading enzyme GH78 (1 gene), PL9 (2 genes), CE8 (4 genes), PL3 (5 genes), GH28 (8 genes), and PL1 (10 genes) may aid *F. fujikuroi* in root tissue colonization. The existence of CAZymes families such as GH10 (1 gene), GH36 (1 gene), GH53 (1 gene), GH62 (1 gene), GH7 (2 genes), GH12 (3 genes), PL3 (5 genes), PL1 (10 genes), and CE5 (12 genes) suggests that *F. fujikuroi* possesses an abundance of cutinases and plant biomass-degrading enzymes (Bashyal et al., 2017).

After 7–10 days of inoculation, the secretomes, PL (genes 5526, 3064), CBM (genes 13528, 12667, 11810, 5178), GH (gene 12698), CE (gene 12149), and gene 9849, associated with increased virulence were expressed maximally (Bashyal et al., 2017). This increase was stable with the richness of pectins in cell walls and could be mainly ascribed to the enhancement of pectin-degrading enzymes. This expression pattern also recommended that particular enzymes may have distinct roles at diverse infection stages. The higher stimulation of genes of secretomes in the susceptible rice genotype than the resistant genotype during the early stage of inoculation may be a result of greater colonization of the pathogen in the susceptible rice genotype and upregulation of the secretomes (Bashyal et al., 2017). Some genes, such as 12,587 (GH-72 family) and 1,530 (AA-7 family), have a greater expression in resistant genotypes than in susceptible genotypes. Maximum gene expression was observed 30 days after inoculation. It was found that the local and systemic behaviors of *F. fujikuroi* was associated with the genotype of rice during the pathogen-host interactions, which may be further involved in altering the expressed genes number and their level of expression (Matic et al., 2016a). Moreover, it was reported that the profiles of gene expression were different at different times of the infection, and it appeared that the expression of particular genes that encoded the degrading enzymes gradually deteriorated the host plant during the infection (Bashyal et al., 2017).

8. Control methods

8.1. Host resistance

The most eco-friendly and cost-efficient control method is the use of a resistant host plant. In Pakistan, *in vivo* screenings were conducted for the detection of Pakistan rice varieties having resistance against bakanae disease, which showed that DR-82, DR-83, DM-15-1-95, IR-6, IR-8, KS-133, and KS-282 were the resistant rice varieties (Iqbal et al., 2011; Lee et al., 2022). In Korea, the conidia of *F. fujikuroi* were inoculated in different rice varieties through the tissue embedding method to evaluate the resistance, and it was found that Wonseadaesoo, Erguailai, and Gwangmyeongbyeo have resistance (Hur et al., 2016). In another study, through comparative transcriptome analyses, Dorella was found susceptible while Slelnio was the resistant rice cultivar against bakanae (Matic et al., 2017). Plants activate different defense systems in response to pathogen invasion, which can lead to an increased resistance host phenotype, particularly in genotypes with resistant loci (Lee et al., 2022). In resistant varieties, the host plants resist the bakanae disease of rice by activating their defense system.

To invade the host, Fusarium species secrete cell wall degrading enzymes or secretomes including fungal enzymes (pectin degrading enzymes, cellulase, glycoside hydrolysis, biomass-degrading enzymes, cell envelop enzymes, etc.) and cutinase to disrupt the rigid plant cell wall. All these compounds are categorized as general pathogenicity factors because several Fusarium species produce such compounds to facilitate host invasion (Chang et al., 2018). After host invasion, some specific metabolites are produced by particular Fusarium species. The most important secondary metabolites produced by *F. fujikuroi* include mycotoxins, such as beauvericin, fusaric acid (Niehaus et al., 2017), and fumonisins (Suga et al., 2019), and plant hormones such as gibberellins A3 (Suga et al., 2019). Mycotoxins produced by other FFSCs include fumonisins by *Fusarium proliferatum* and *F. verticillioides* (Katoch et al., 2019). It is still unknown what function fumonisins play in the life cycle of fungi or their pathogenicity. For instance, *F. andiyazi* produces no fumonisins or gibberellins (Masratul Hawa et al., 2013). Mycotoxin production by *F. commune* is still unclear (Niehaus et al., 2017). To endure the pathogen attack, resistant cultivars exhibit two types of defense systems: First, a general host defense in which several antifungal compounds including resistance proteins, antifungal proteins, defense signaling compounds, and detoxification enzymes are produced by the host in response to pathogen attack. These compounds are not specific for a particular *Fusarium* species and are effective against various Fusarium species; second, a pathogen-specific host defense in which host plants secrete specific

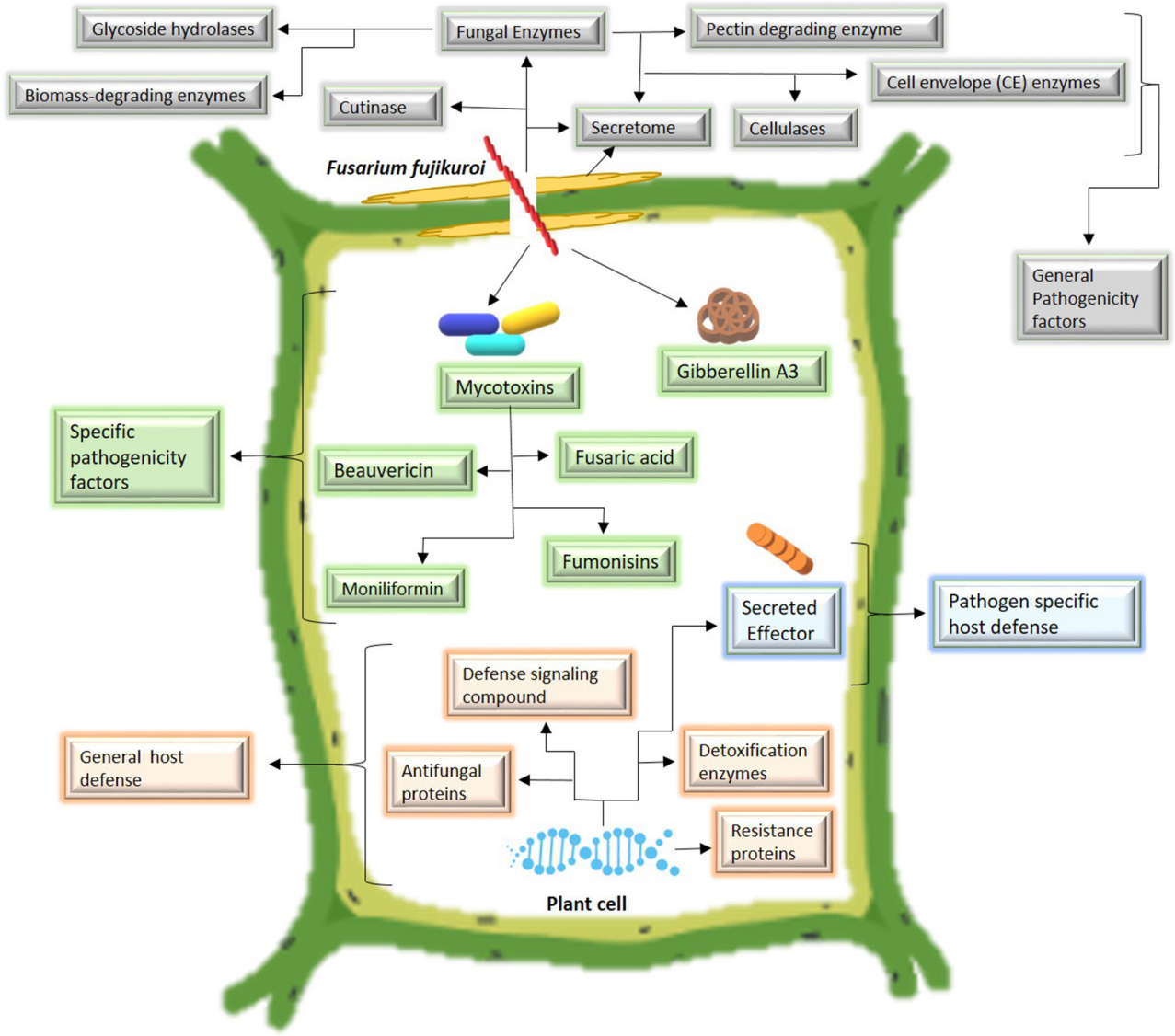

FIGURE 3
Mechanism of pathogen colonization and plant host defense.

effector proteins for a particular *Fusarium* species. Such defense mechanisms enable the host to withstand the infection. The pathogen colonization and the host-defense mechanism are shown in Figure 3.

8.2. Quantitative trait loci

Vertical resistance in rice varieties conferred by a single resistant gene (R-gene) has been reported to be gradually overwhelmed by new or resistant pathological races (Lee et al., 2018, 2019). Therefore, it is necessary to understand the resistance mechanism by identifying the resistance genes which also aids in assessing the marker efficacy used in rice breeding. Several studies have reported the resistance conferred by quantitative trait loci (QTL) against rice bakanae disease. Based on genetic mapping methods, population types, and marker systems, *qFfR1* and *qB1* (Ji et al., 2018), *qBK1* (Hur et al., 2015; Lee et al., 2019), $qBK1^{z}$ (Lee et al., 2021), $qBK1^{WD}$ (Lee et al., 2018), *qBK1_628091* (Volante et al., 2017), *qBK1.1*, *qBK1.2*, and *qBK1.3* (Fiyaz et al., 2016) on chromosome 1, *qB4* on chromosome 4 (Volante et al., 2017), *qFfR9* on chromosome 9 (Kang et al., 2019), and *qB10* on chromosome 10 (Chang-deng et al., 2006) have been identified as QTL associated with rice resistance against bakanae disease. Since *qBK1* and *qBK1.1* were found in the same genomic region, they might be the same QTL (Fiyaz et al., 2016). Recently, $qBK4^{T}$ was detected on chromosome 4 as a novel locus by genetic mapping and the Genome-Wide Association Study approach. Additionally, *qBK4.1* (Grant, 2006) and *qBK4_31750955* (Volante et al., 2017) have also been detected in chromosome 4 but their position differs from $qBK4^{T}$ (Lee et al., 2022).

8.3. Bio-control

Biocontrol is one of the most eco-friendly, convenient, and effective control methods for the bakanae disease of rice. Various bacterial and fungal biocontrol agents have been found to have higher antifungal efficacy and significantly inhibited *F. fujikuroi*. The general antifungal mechanism of biocontrol agents involved in the *F. fujikuroi* suppression can be observed in Figure 4.

8.3.1. Bacterial bio-control agents

Several bacteria have been reported to demonstrate an antagonistic effect against the bakanae disease of rice (Table 1). In a study, strains of *Pseudomonas fluorescens* and *Pseudomonas putida* were found to effectively suppress *F. fujikuroi* growth (Safari Motlagh and Dashti, 2020). It was further revealed that *Paenibacillus polymyxa*, which is an endophytic bacterium, also has the efficacy to reduce the growth of *F. moniliforme* (Zhang et al., 2010). Several Bacillus species including *B. subtilis, B. circulans* (Safari Motlagh and Dashti, 2020), *B. megaterium* (Luo et al., 2005), *B. oryzicola* (Hossain et al., 2016), and *B. cereus* (Etesami and Alikhani, 2017) were observed to successfully inhibit the *F. fujikuroi* growth (Nawaz et al., 2022). In another study, it was found that the B-44 strain of *B. subtilis* has the potential to effectively reduce the disease incidence of rice bakanae under greenhouse conditions. Moreover, the QST 713 strain of *B. subtilis* has been commercially applied as a bio-fungicide i.e., in the context of Serenade against bakanae disease of rice (Matić et al., 2014). Studies have further reported that the application of the YC7007 strain *B. oryzicola* in nursery boxes and pots to control bakanae disease decreased the disease severity from 46% to 78% (Hossain et al., 2016).

8.3.2. Fungal bio-control agents

Previously studied fungi as bio-control agents to control bakanae disease of rice are presented in Table 2. Trichoderma is a widely studied fungal bio-control agent to control several diseases. The antagonistic potential of the *Trichoderma* species against phytopathogenic fungus (*F. fujikuroi*) was evaluated by applying the SKT- 1 strain of *T. asperellum* in the form of commercialized Eco-hope, and it was found that this bio-control agent disrupted the pathogen cell wall after penetrating the pathogen hyphae (Win et al., 2021). Application of *T. virens* (Safari Motlagh and Roshani, 2020) and *T. harzianum* (Wan et al., 2015) individually suppressed the bakanae disease of rice; however, their antagonistic efficiencies were increased by applying them in combination with the chemical thiophanate-methyl 80% WP at 2 g/L. According to Ng et al. (2015), the investigation and application of all selected strains of *Trichoderma* sp. significantly suppressed the bakanae disease. Yeasts including *Pichia guilliermondii*, *Metschnikowia pulcherrima,* and *Sporidiobolus pararoseus* also show antagonistic potential against various pathogens such as *F. fujikuroi* (Zhang et al., 2011; Matić et al., 2014). Application of the R9 strain of *P. guilliermondii* and R23 strain of *M. pulcherrima* in combination with thermotherapy of rice seeds for 10 min at 60°C resulted in additionally decreasing the disease incidence of bakanae to 5% than applying these bio-control agents alone (Matić et al., 2014). *Stachybotrys atra*, *Penicillium thomii*, and *Penicillium chrysogenum* were found to have antagonistic efficacy against *F. moniliforme*. Two strains of *Chaetomium globosum,* including NR-R688 and NR-SH321, strain NR-L645 of *Fusarium* sp., and strain NR-L243 of *Penicillium* sp., decreased the incidence of rice bakanae to ∼2–6% (Ramesh et al., 2020). Furthermore, strain W5 of *Fusarium commune* was found to be non-pathogenic with the efficacy to control bakanae disease. For instance, the application of strain W5 by spraying on rice flowers and seedlings inhibited the hyphal growth of *F. fujikuroi*, and the antagonist could survive in rice seeds for ∼6 months or more (Saito et al., 2021).

8.4. Divergent invasion patterns of *F. fujikuroi* (CF283-GFP) in the early infection

Seeds of the susceptible and resistant cultivars were injected with the fungus pathogen CF283 isolate tagged with a green fluorescent protein to assess the invasion of the *F. fujikuroi* (GFP). Confocal imaging of rice seed embryo sections showed that the fungal pathogen entered both the Ilpum and Tung Ting Wan Hien1 embryos shortly after inoculation. At 3 and 7 dpis of Ilpum, the coleoptile showed robust pathogen colonization localized by the green fluorescence of the GFP signal. In contrast, under identical circumstances, the resistant Tung Ting Wan Hien1 showed no clear signs of colonization in the coleoptile. The intricate process of adhesion, penetration, and subsequent colonization inside cells and in intercellular compartments is necessary for the infection of *Fusarium* spp. (Jansen et al., 2005; Rana et al., 2017; Lee et al., 2022). According to research conducted by Lee et al. (2018), vulnerable rice cultivars have more *F. fujikuroi* in their stems than resistant ones. Similarly, the aerenchyma, pith, cortex, and vascular bundle of the rice sheath and stem were also found to be the best places for *F. fujikuroi* to thrive. In the first phase of infection, the spread of *F. fujikuroi* in seeds from susceptible and resistant cultivars was assessed. The study by Lee et al. (2022) found a comparable amount of the target fungal infection in the embryo based on the cellular localization of *F. fujikuroi* strain CF283 tagged with a GFP. Later on, it was discovered that the pathogen quickly colonized the coleoptile of Ilpum at 3 and 7 days after infection, but in the resistant cultivar Tung Ting Wan Hien1, the presence of GFP-*F. fujikuroi* in the coleoptile under the same circumstances was incredibly poor. In earlier investigations (Elshafey et al., 2018; Lee et al., 2018), *F. fujikuroi* was shown to have a similar pattern of colonization, with its presence being viewed in the vascular bundles, mesophyll, and subcutaneous tissue of infected stems in susceptible cultivars as opposed to the resistant one. This led to the hypothesis that soon after inoculating rice seeds with the virulent isolate CF283 of *F. fujikuroi* in both resistant and susceptible varieties, the pathogen colonized quickly in susceptible varieties (Ilpum), which had a defective or weak defense system, but the observed resistance of Tung Ting Wan Hien1 could be partially explained by the contribution of qBK4T.

8.5. Defense mechanism

Research on the bakanae disease highlights some of the defensive mechanisms used by the resistant cultivar C101A51 in

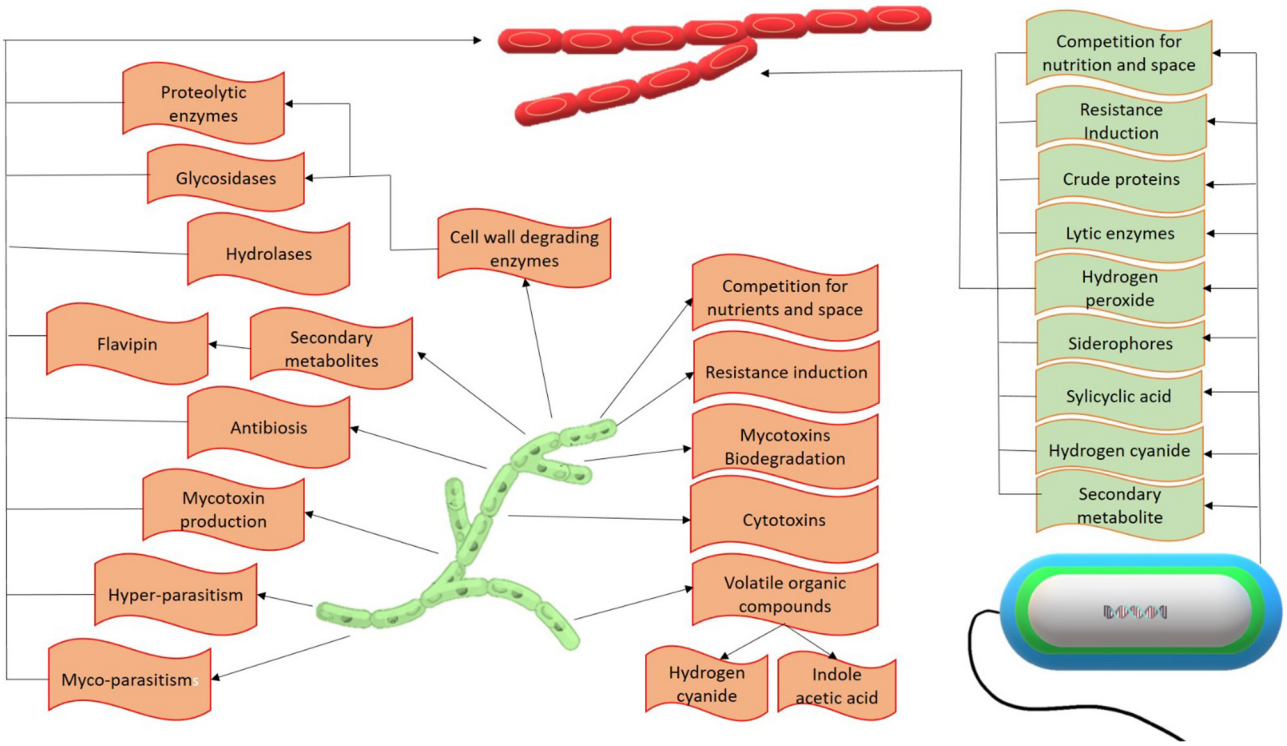

FIGURE 4
Antifungal mechanism of biocontrol agents against *F. fujikuroi*.

TABLE 1 Bacterial bio-control agents used against the bakanae disease of rice.

	Species	Conditions	Application method	Disease inhibiton	Antagonistic mechanism	References
Bacterial bio-control agents	*Bacillus circulans*	Greenhouse	Seed treatment	–	–	Safari Motlagh and Dashti, 2020
	Bacillus cereas	*In vitro*	Dual culture	66%	–	Etesami and Alikhani, 2017
	B. oryzicola	*In vivo*	Root drenching	46-78%	Hydrogen peroxide secretion, Systemic resistance induction	Hossain et al., 2016
	Bacillus megaterium	*In vitro*	–	–	–	Luo et al., 2005
	Bacillus subtilis	*In vitro*	Dual culture	76%	Stimulation peroxidase activity, production of lysis enzymes	Sarwar et al., 2018, Safari Motlagh and Dashti, 2020
	P. fluorescens	*In vitro*	Dual culture volatile antibiotics	69%	Production of volatile compounds (hydrogen cyanide), sidrophores, salicylic acid, lytic enzyme	Safari Motlagh and Dashti, 2020
			Diffusible antibiotics	77–78%		
	P. putida	*In vitro*	Dual culture	59.21%	Production of antibiotics	Safari Motlagh and Dashti, 2020
	P. polymyxa	*In vitro*	Dual culture	–	Production of crude protein	Zhang et al., 2010

response to *F. fujikuroi* infection. In comparison to the resistant genotype C101A51, a substantial number of peroxidase genes were found in the vulnerable genotype Rasi. Since peroxidases are needed to stop pathogen diffusion within cells, the limited pathogen spread in resistant genotypes may be the reason behind it. Similarly, Rasi and C101A51 both showed higher numbers of genes associated with chloroplasts. By reducing the supply of accessible carbon for pathogen development and redirecting the route toward

TABLE 2 Fungal bio-control agents used against the bakanae disease of rice.

	Species	Conditions	Application method	Disease inhibiton	Antagonistic mechanism	References
Fungal bio-control agents	*C. globosum*	*In vitro*	Dual culture	97.4%	Secretion of secondary metabolite flavipin	Ye et al., 2013; Ramesh et al., 2020
	S. pararoseus	Greenhouse	Seed dressing	40.5%	Organic volatile compounds production, competition	Huang et al., 2012; Matić et al., 2014
	P. guilliermondii	Greenhouse	Seed dressing	73%	Competition for nutrients and space, Biodegradation of mycotoxins, secretion of cell-wall degrading enzymes	Matić et al., 2014; Matic et al., 2016b
	M. pulcherrima	Greenhouse	Seed dressing	64.5%	Production of hydrolases (cell wall degrading enzymes), competition,	Matić et al., 2014; Matic et al., 2016b
	Penicillium sp. (strain NR-L243)	*In vitro*	Dual culture	92.3%	Production of mycotoxins	Ramesh et al., 2020
	P. chrysogenum	*In vitro*	Dual culture	–	Penecillin production	Mangiarolti et al., 1987
	P. thomii	*In vitro*	Dual culture	–	–	Mangiarolti et al., 1987
	Trichoderma virens	Greenhouse	Seed dressing	79%	Hyperparasitism, production of volatile compounds, IAA, hydrogen cyanide	Ng et al., 2015; Safari Motlagh and Roshani, 2020
	T. asperellum	*In vitro*	Dual culture	–	Mycoparasitism, Degradation of the cell wall by glycosidases	Yang and Xu, 2013; Win et al., 2021
		In situ				
	T. harzianum	Greenhouse	Seed dressing	83%	Hyperparasitism, antibiotic production, hydrolytic enzymes secretion	Ng et al., 2015; Wan et al., 2015; Safari Motlagh and Roshani, 2020
		In Vitro	Dual culture	>60%		
	Trichoderma viride	*In vitro*	Dual culture	–	Production of lipase, proteolytic enzymes	Rajathi et al., 2020; Safari Motlagh and Roshani, 2020
	Talaromyces sp. isolate KNB-422	*In vivo*	Seed Treatment	–	Mycoparasitism	Kato et al., 2012
	T. flavus	Glasshouse	Seed Treatment	70%	Volatile metabolite production	Naraghi et al., 2013; Rawat et al., 2022
	S. atra	*In vitro*	Dual culture	–	Production of cytotoxins e.g., trichothecenes	Mangiarolti et al., 1987; Jarvis, 1991
	Fusarium commune (strains W3, W5)	*In vivo*	Aerial Spray	–	Resistance induction	Saito et al., 2021
	Fusarium sp. (strain NR-L645)	*In vitro*	Dual culture	92.3%	–	Ramesh et al., 2020

defensive responses, it may be possible to consider a decrease in the expression of chloroplastic genes as a resistance strategy of the rice plant (Bolton, 2009; Bashyal et al., 2022). The resistant genotype showed higher levels of heat stress-related transcripts (BIP4-like protein, transcription factor A-2a-like, and BHLH148) than the susceptible genotype. Transcriptional regulators of heat stress have also been linked to resistance to fungi in many plant species. For instance, the bakanae resistant genotype Selenio has been discovered to have upregulated transcription factors associated with heat stress (Matic et al., 2016a; Bashyal et al., 2022).

Antimicrobial peptides were also expressed greater in C101A51 and were encoded by gene ID BGIOSGA033746. Plants generate antimicrobial peptides, also known as host defense peptides, as a first line of protection against possible dangerous bacteria. Several genes were upregulated in the resistant genotype C101A51, including cysteine proteinase inhibitor 10 (LOC4335551), disease resistance protein TAO1-like (LOC112939055), oleosin 16 kDa-like (LOC4336570), pathogenesis-related protein (PR1), pathogenesis-related protein (PR4), and BTB/POZ and MATH domain-containing protein 5-like (LOC112936838). One explanation for

their admission might be due to the decline in their expressiveness. CTP is a particular inhibitor of cysteine proteinases that plays a part in the control of endogenous processes as well as the defense against infections and pests. RGA3, a potential disease-resistant protein similar to TAO, was expressed more often in the resistant genotype C101A51 (Bashyal et al., 2022). In the Pseudomonas syringe-rice interaction, it was found that TAO was responsible for the expression of the pathogenesis-related protein 1 (PR1) gene. This finding is suggestive of TAO's role against *F. fujikuroi* in rice, as more expression of the oleosin-16 kDa-like protein in resistant genotypes is suggested, and more oil bodies are present in the seeds of resistant rice genotype C101A51. Compared to control plants, infected plants showed higher amylase activity. The production of storage starch granules during rice seed maturation and the motivation of the stored starch to nourish the sprouting seedlings during seed germination were both influenced by amylase isozymes, which have a direct impact on plant development and yield. Gibberellic acid content often affects amylase activity (Bashyal et al., 2022). The enzymatic activity may have been boosted by the *F. fujikuroi*-infected plant's elevated gibberellic acid concentration. Microtubule-related proteins called cytoplasmic linker associated proteins (CLASPs) are crucial for controlling the dynamics of microtubules, which are crucial for plant growth and development. Shorter internodes and semi-dwarfism in plants were linked to the inhibition of CLASP protein, according to Zhu et al. (2018). The elongated plant phenotype in rice genotype Rasi could have been caused by CLASP transcript expressions that were noticeably positive. Proteins with the BTB/POZ and MATH domains were more abundant in the resistant genotype C101A51. Broadly speaking, the BTB domain is involved in how plants react to biotic and abiotic stressors. This gene upregulation in resistant genotypes may aid in their defense against *F. fujikuroi* (Bashyal et al., 2022).

8.6. Prediction and analysis of *F. fujikuroi* secretome

SignalP version 4.1 was able to classify 1,207 proteins out of the 13,603 proteins as conventional secretory proteins, whereas TargetP version 1.1 identified 2,265 proteins as secretory proteins. We scanned 2,265 proteins using TMHMM software after the filtered sets (SignalP and TargetP) were combined and duplicate segments were eliminated. Once 574 transmembrane proteins were excluded from the protein data set, a total of 1,691 sequences were predicted to be secretory proteins. Using WoLFPSORT version 3, secretory proteins identified in the preceding phase were further screened, yielding 1,194 proteins. Approximately 985 potential secretory proteins were given GO keywords in three GO categories, including molecular function (736), biological process (670), and cellular component for the analysis of the anticipated *F. fujikuroi* secretome (280). Single-organism processes, cellular metabolic processes, cellular processes, biological regulation, regulation of biological processes, cellular component organization or biogenesis, response to stimulus, and localization were among the categories with the greatest representation under the biological process (Bashyal et al., 2017).

The most prevalent proteins in the molecular function ontology were those involved in binding, antioxidant activity, transporter activity, and structural molecule activity. Proteins for the membrane part, cell and cell part, organelle part, and macromolecular complex were significantly abundant in the cellular component category. Approximately 585 proteins out of 1,194 secretory proteins had demonstrated matches with PHI-d at a base in various categories. Of them, 38% of the proteins were associated with decreased virulence, 26% with unaltered pathogenicity, and 11% with proteins of mixed character. The presence of 5% polysaccharide lyases (PLs), 7% glycosyl transferases (GTs), 16% auxiliary activities (AAs), 11% carbohydrate-binding modules (CBMs), 20% carbohydrate esterases (CEs), and 41% glycosyl hydrolases (GHs) were predicted in the *F. fujikuroi* "F250" secretome (Figure 5) using the CAZy database and HMMER scan based on the profile compound for the six CAZy classes. Further investigations were done for the families of enzymes that break down cell walls. We found 16 of the 51 GH families to exist, which exhibited the presence of three or more genes. The GH16 family had the most genes (15), followed by GH43 (14 genes), GH5 (11 genes), and GH3 (10 genes) (9 genes) (Figure 5).

The secretome of *F. fujikuroi* contained members of 10 CE families. The most genes (i.e., 18) were found in CE10, which was followed by CE1 (16 genes), CE5 (12 genes), and CE16 (6 genes). Moreover, four PL families were predicted: PL1 (10 genes), PL3 (5 genes), PL4 (2 genes), and PL9 (2 genes). In addition, classes including AA (7 families), CBM (16 families), and GT (25 families) that play indirect roles in the degradation of carbohydrates were found (Bashyal et al., 2017). The CBM-01, AA7, and GT-34 families were the most prevalent of these. There were many oxidoreductases, transferases, hydrolases, and lyases in the secretome of F. *fujikuroi* as well. Accordingly, the research conducted by Bashyal et al. (2017) revealed that the secretome of *F. fujikuroi* contained a variety of proteins that may aid in the fungus' appropriate colonization, the breakdown of host plant materials to get nutrients, and the inactivation of the host's defenses. Following several species of *F. oxysporum*, the secretome of the *F. fujikuroi* isolate "F250" is closely connected to that of the *F. fujikuroi* isolate IMI58289.

8.7. Chemical control

8.7.1. Benzimidazoles

Currently, control of rice bakanae disease through a chemical application is the most common control method. The application of broad-spectrum fungicides (benzimidazoles) against the bakanae disease of rice has been under use for decades (Saito et al., 2021). Benzimidazoles interrupt meiosis and mitosis in pathogen cells and damage cellular processes, including cell division, formation of the cytoskeleton, and intracellular transfer. The typical benzimidazole fungicides are carbendazim, benomyl, fuberidazole, thiabendazole, and thiophanate-methyl. Under *in vitro* conditions, the application of fungicides suspension at 0.3%, such as thiophanate-methyl at 80% and carbendazim at 50%, as a seed treatment suppressed the bakanae disease (Latif et al., 2021); however, seed dipping treatment for 10 min in 10 g/L benomyl completely suppressed the

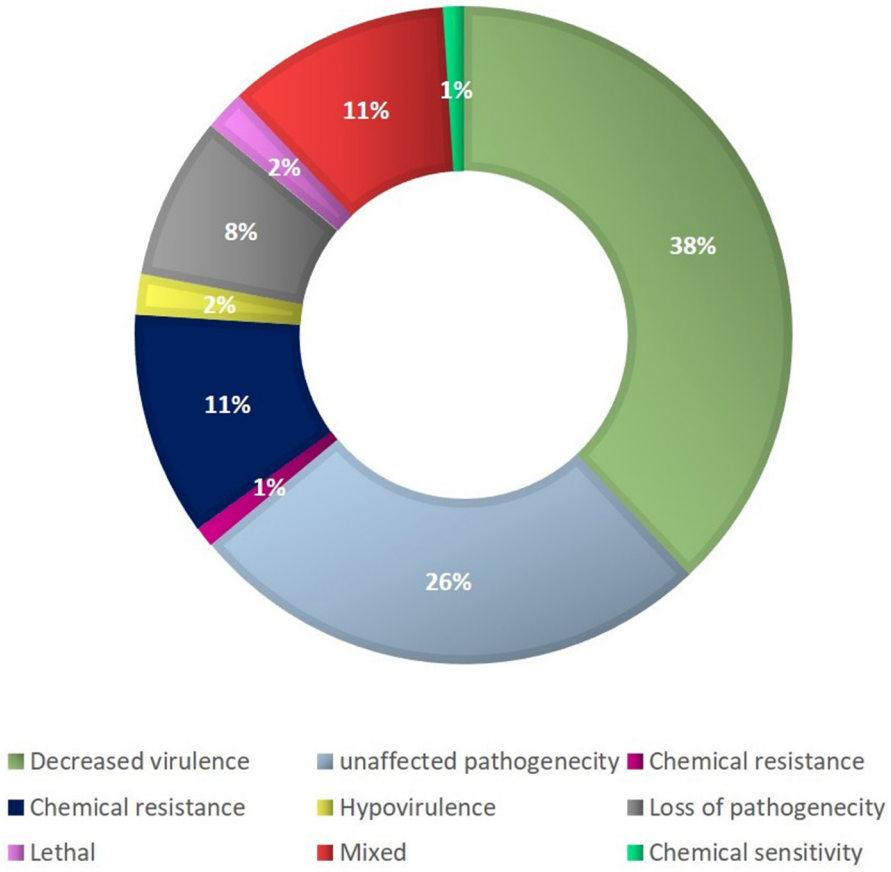

FIGURE 5
Categories of secretome genes of *F. fujikuroi* (Bashyal et al., 2017).

bakanae infection. Seed treatment with the chemical thiabendazole was also effective against the bakanae disease (Iqbal et al., 2013). However, due to excessive application of benzimidazoles fungicide, *F. fujikuroi* strains resistant to such fungicides have been reported. Potential mechanisms of resistance in phytopathogenic fungi to fungicides include measures such as (a) alteration in the structure of fungal cell wall due to which fungicide cannot enter the fungal cell wall, (b) production of excessive target molecule due to which excessive production of fungicide become unable to completely suppress the activity of target molecule, (c) modification of the target molecule due to which altered structure fungicide cannot bind itself with the target molecule, (d) secretion of fungicide whereby efflux pumps excrete the fungicide out of the fungal cell, (e) degradation of fungicide due to which fungicide loses the potential to perform its activity, and (f) production of an analogous target molecule in fungal cells due to which the fungicide cannot identify its target molecule and binds with the alternative molecule (Figure 6). In *F. moniliforme*, benomyl resistance is reported to be conferred by β-tubulin mutation D50Y (Yan and Dickman, 1996). Similarly, Chen et al. (2014) revealed that mutations F200Y and E198V in β2-tubulin produced resistance to carbendazim in Chinese *F. fujikuroi* (Table 3).

8.7.2. Sterol demethylase inhibitors

Site-specific fungicides such as sterol demethylase inhibitors (SDMIs) have been applied as an alternative to benzimidazole fungicides against bakanae disease (Table 3). These fungicides target the CYP51 enzyme i.e., P450-sterol 14α- demethylase. This enzyme is necessary to biosynthesize the ergosterol, which is a constituent of the cell membrane that fungi require to grow. Three genes of *CYP51* including *CYP51A*, *CYP51B*, and *CYP51C* are found in *F. fujikuroi* (Zhang et al., 2021). Demethylase inhibitors (DMIs) are chemically classified as pyrimidine, pyridine, piperazine, triazole, and imidazole. The antifungal efficacy of triazole has been assessed to control *F. moniliforme* by using 25% tebuconazole emulsifiable concentrate (EC), 25% propiconazole EC, and 2.5% difenoconazole EC (Hossain K. S. et al., 2015). Application of triazole fungicides such as propiconazole (Bagga and Sharma, 2006) and ipconazole (Tateishi and Suga, 2015; Li et al., 2018) and imidazole fungicide such as prochloraz (Kumar et al., 2020) significantly suppressed *F. fujikuroi* growth. Soaking of rice seeds for 24 h in a diluted solution of 200-folds of ipconazole wettable powder at 6% or ipconazole at 0.0472 μg/mL showed efficacy against bakanae disease. Application of 0.05% propiconazole EC on rice seedlings also effectively controlled the bakanae disease of rice; however, a side effect of its toxicity resulted

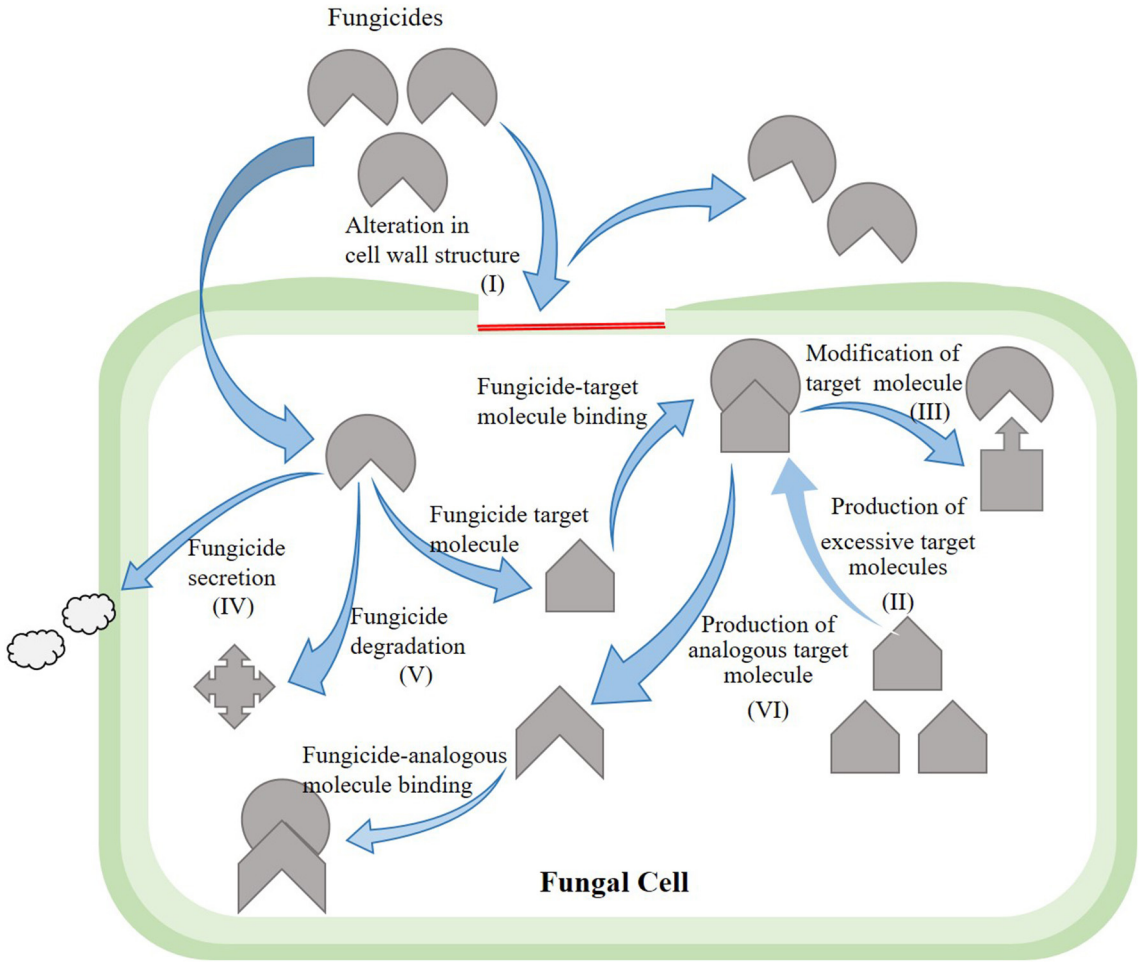

FIGURE 6
Mechanism of resistance development in fungi against fungicides.

in reduced plant height and crop yield (Bagga and Sharma, 2006). The application of prochloraz 10 μg/mL completely suppressed *F. fujikuroi* growth (Park et al., 2009). Suzuki et al. (1994) found that the application of triflumizole EC as a seed treatment was more effective against bakanae disease as compared to triflumizole WP application. The application of pefurazoate, which is an imidazole fungicide, 500 μg/mL as a seed treatment reduced 90% of disease incidence (Singh et al., 2019). Under *in vitro* tests, penconazole and difenoconazole fungicides showed high antifungal efficacy against *F. moniliforme* (Kumar et al., 2020). However, a study conducted under greenhouse conditions indicated that the application of difenoconazole fungicide as soil drenching effectively controlled the bakanae disease (Kumar et al., 2020).

The resistance mechanism of *F. fujikuroi* to SDMIs (upon their excessive use) is presented in Table 3. A Korean strain CF245 of *F. fujikuroi* showed resistance to prochloraz by degrading it (Kim et al., 2010). Consequently, an efflux transporter was responsible for resistance to prochloraz in this Korean strain (Kim et al., 2010). Furthermore, in another Korean strain of *F. fujikuroi* CF337, the alterations in fungal cell structures and changes in ATP binding cassette transporter have been revealed to play a role in prochloraz resistance (Yang et al., 2012a). In yet another study, the cause of resistance in Chinese *F. fujikuroi* against prochloraz was determined as the overexpression of *CYP51* genes and mutations S312T that occurred in CYP51B (Zhang et al., 2021). However, the link between F box or WD repeat protein and survival factor 1 with the sensitivity of *F. fujikuroi* to prochloraz fungicide was found. The resistance to prochloraz was reduced due to the disruption of genes survival factor 1 whereas, the disruption of genes in F box or WD repeat protein enhanced resistance in *F. fujikuroi* to prochloraz (Choi et al., 2017).

8.8. Other fungicides

In addition to benzimidazoles and SDMIs, other fungicides have also been applied against the bakanae disease of rice (Table 3). Phenamacril is a new cyanoacrylate chemical fungicide that restricts the class I myosin ATPase activity (Hou et al., 2018) and significantly inhibits the *F. fujikuroi* growth when applied at 0.1544 μg/mL (Li et al., 2018). However, some strains of the Chinese *F. fujikuroi* showed resistance to phenamacril fungicide (Hou et al., 2018). It has been indicated that the

TABLE 3 Fungicides used against the bakanae disease of rice.

Fungicide group	Chemical compound	Mode of action	Resistance mechanism	References
Benzimidazole	Benomyl (benlate)	Inhibit microtubule formation, apoptosis induction	D50Y mutation in β-tubulin	Yan and Dickman, 1996; Kara et al., 2020
	Thiabendazole (tresaderm, mintezol, arbotect)	Inhibit mitochondria activity, inhibit protein synthesis	–	Iqbal et al., 2013
	Carbendazim (bavistin, derosal, haydazin, knowin)	Inhibit microtubule formation	F200Y and E198V mutations in β2-tubulin	Chen et al., 2014; Latif et al., 2021
	Thiophanate-methyl (Topsin M)	Degradation of β-tubulin, interferance in Glycolysis	–	Iqbal et al., 2013; Latif et al., 2021
Others	Tebuconazole (Folicur)	Interferes in biosynthesis of ergosterol	–	Hossain K. S. et al., 2015
	Chitosan oligosaccharides	Induction of callose deposition that stimulates plant immunity	–	Luna et al., 2011; Kim et al., 2016
	Metiram (Arbatene)	–	–	Hossain M. S. et al., 2015
	Ethylenediaminetetraacetatic acid			Kim et al., 2016
	Mancozeb (Dithan)	Disturbs biochemical activities of cytoplasm and mitochondria	–	Hossain M. S. et al., 2015; Fungicide Resistance Action Committee, 2020
	Fluazinam	Restricts production of energy by deprotonating or protonating the amino acids	–	Qu et al., 2018
	Phenamacril	Interferes in activity of ATPase of fungal mayosin in domain of class I	K218T, S219L, and S219P, myosin mutations	Hou et al., 2018; Li et al., 2018; Wollenberg et al., 2019; Wu et al., 2020
	Fludioxonil (Cannonball)	Interference in fungal respiration	–	Hossain M. S. et al., 2015; US EPA, 2022
	Pydiflumetofen	Disturbs succinate dehydrogenase that inhibit energy production	–	Bai et al., 2021
	Trifloxystrobin (Flint fungicide)	Interrupt fungal respiration	–	Hossain M. S. et al., 2015
	Pyraclostrobin	Interrupt ATP production by restricting electron transportation	–	Fernández-Ortuño et al., 2012; Hossain M. S. et al., 2015
Demethylation Inhibitor	Ipconazole	Interferes in biosynthesis of ergosterol	–	Li et al., 2018
	Difenoconazole (Score)	Restricts sterol biosynthesis	–	Hossain K. S. et al., 2015
	Triflumizole (Trifmine)	Inhibits biosynthesis of ergosterol	–	Suzuki et al., 1994; Singh et al., 2019
	Penconazole (Topas)	Inhibits ergosterol synthesis	–	Kumar et al., 2020
	Pefurazoate (Healthied)	–	–	Singh et al., 2019
	Propiconazole (Protaf)	Blockage of 14-α-sterol demethylase activity	–	Gad and Pham, 2014; Hossain K. S. et al., 2015
	Prochloraz (Sportak)	–	Efflux transference, degradation of prochloraz, Alteration in cell wall structure, CYP51B and S312T mutations	Park et al., 2009; Kim et al., 2010; Yang et al., 2012a,b; Kumar et al., 2020; Zhang et al., 2021

high resistance of *F. fujikuroi* to phenamacril is associated with the mutations S219L, S219P, and K218T in myosin-5 (Hou et al., 2018; Wu et al., 2020). High antifungal activity was shown to inhibit *F. fujikuroi* by using fluazinam fungicide which belonged to the class arylaminopyridine (Qu et al., 2018), and succinate-dehydrogenase inhibitor pydiflumetofen (Bai et al., 2021). Application of pydiflumetofen at 0.1-0.2 g active ingredient/kg of this fungicide as a seed treatment showed 90%

inhibition of rice bakanae disease (Bai et al., 2021). Furthermore, high antifungal activity was found against *F. fujikuroi* in chelating agents, such as chitosan oligosaccharides and ethylene-diamine-tetra-acetic acid (Kim et al., 2016). The application of combined fungicides has also been studied and proven effective in controlling the bakanae disease. Under *in vitro* conditions, it has been found that 5% pyraclostrobin with 55% metiram, 63% mancozeb with 12% carbendazim, 2.5% celest extra 5 EC with 2.5% fludioxonil, and 100 g/L trifloxystrobin with 200 g/L tebuconazole completely suppressed the *F. moniliforme* mycelial growth (Hossain K. S. et al., 2015).

8.9. Plants and microbial extracts

The use of natural products including plants and microbes is also effective in controlling bakanae disease. Among microbial extracts, *Bacillus* sp. extract, i.e., surfactin A, has been reported to suppress *F. moniliforme* growth up to 16% upon application at 2,000 μg/mL (Sarwar et al., 2018). Extract of *P. polymyxa* in the form of crude protein restricted *F. moniliforme* activity (Khan et al., 2020). Tumescence and distortion of fungal spores were found by using crude protein extract (Zhang et al., 2010). Additionally, extracts of plants such as *Artemisia judaica*, *Eucalyptus globulus*, *Coriandrum sativum,* and *Ammi visnaga* effectively reduced *F. fujikuroi* mycelial growth (Kalboush and Hassan, 2019). Essential oils extracted from *Cinnamomum tamala* (Baria and Rakholiya, 2020), *Eucalyptus citriodora* (Gupta and Kumar, 2020), *Cymbopogon martini* (Akhila, 2009), and *Mentha piperita* (Habibi et al., 2018) were reported to have antifungal activity and effectively suppressed *F. fujikuroi* activity. Among them, the highest antagonistic potential was found in *C. martini* var. *motia* oil (Baruah et al., 1996). Though control methods for bakanae disease of rice are generally applied before the infection occurs, silica nanoparticles, formed from the husks of rice, applied as a foliar spray after the development of bakanae disease symptoms effectively decreased the bakanae incidence (Elamawi et al., 2020).

8.10. Physical control

8.10.1. Seed treatments

Cultivation of healthy (non-infected) seeds is very important for the prevention of bakanae disease because of its seed-borne nature. Immersion in hot water is useful to disinfect the seeds. The immersion of infected rice seeds in hot water for 10–20 min at 58–60°C has the potential of disinfection similar to traditional chemical applications (Kim et al., 2022). However, precise control of water temperature is a key factor in the successful disinfection of the seeds. Salt water can also be used to select non-infected rice seeds. Immersion in hot water and the selection of non-infected rice seeds using salt water significantly reduces the requirement for chemical fungicides. However, it is hard to completely eliminate the infected rice seeds by following these seed treatment methods. Irradiation with atmospheric plasma has also been reported to be efficient to disinfect the seeds; in addition, irradiation has reduced the disease severity of rice bakanae up to 18.1% more than without irradiation control (Ochi et al., 2017).

8.10.2. Agronomic practices

Conventional agronomic strategies are eco-friendly control methods. Such practices including the application of organic fertilizer, suitable planting practices, and crop rotation can be effective against rice bakanae disease. The source of infection can be minimized by burning the crop residues and infected plants (Sunder et al., 2014; Bashyal et al., 2016). Habitat manipulation is also an effective approach to controlling several soil-borne pathogens (Shakeel et al., 2022c). To prevent bakanae, a support vector machine classifier has also been developed to differentiate the infected and non-infected rice seedlings (Chung et al., 2016). Furthermore, it has been indicated that the late sowing of seedlings also reduced the incidence of bakanae. For instance, in Australia, crop rotation of rice with meadow grass and cultivation of rice seeds in late January instead of December has been highly recommended to prevent bakanae of rice in early maturing varieties (Sunder et al., 2014).

9. Conclusions and future perspectives

The emergence of the bakanae disease poses a significant threat to global rice production. The global rice industry suffers massive yield losses (up to 90%) due to the bakanae disease. The pathogen is generally seed-borne in nature and can also survive in the soil. *F. fujikuroi*, one of the several species of the genus Fusarium, reported as the causal agent of bakanae disease, has been abundantly found to be associated with the disease as the most virulent species worldwide. Trichoderma, Penicillium, Pseudomonas, and Bacillus strains are some of the natural antagonists that have all been shown to be effective to control the disease. A rapid breakout in commercial rice cultivation regions may result in considerable production losses and be very challenging to control after the bakanae disease has entered the field. Therefore, it is crucial to generate genetically-resistant kinds to ensure a high harvest. Different QTLs of bakanae disease resistance have been identified. Among them, the recently identified qBK4T on chromosome 4 is involved in the regulation of rice resistance to bakanae disease. It contains genes with novel molecular activities. Understanding the involvement of these genes in the immune response to the bakanae disease may require functional characterization. This suggests that further functional investigations may shed light on the molecular activities of qBK4T-related genes and their likely relationships with other familiar defense-related genes in the response to bakanae disease. The bakanae disease has been extensively studied from several perspectives worldwide. There is a pressing need, however, for more study of the biochemical and molecular features of pathogenesis as well as racial profiling, disease mechanism, virulence pattern, and other related topics. Disease resistance, possible antagonists, and biodegradable chemical compounds, along with a decision support system require special consideration as we worked to develop effective disease management strategies. According to projections made using climate change scenarios, the prevalence

of rice bakanae will either rise or at the very least stay the same in the future. It is crucial to first comprehend the epidemiological makeup of the target disease to reduce any future risk of bakanae sickness. In this study, thorough analyses of several components, including the pathogen, environment, and the features of the illness cycle, have been provided. The majority of bakanae management alternatives were also discussed in this study, and it encouraged the adoption of the most efficient combination of several choices, including chemical control, biological control, cultural control, resistive control, and physical control measures. Fungicides are only used with other methods in IDM when it is absolutely essential for more efficient and long-term control. Initially, during the land preparation for rice paddy fields, an ideal amount of fertilizer should be administered, with no particular element in excess or shortage. Also, the cultivation of rice types that are resistant to bakanae should be prioritized in the event of a bakanae epidemic in the field. By growing these types, bakanae production losses can be minimized with fewer fungicide treatments.

In recent years, research on non-destructive early detection of crop diseases using hyperspectral sensors has increased in response to the growing global interest in precision agriculture. A few difficulties in establishing the presence or absence of pathogens inside the thick seed coverings are anticipated given the nature of hyperspectral remote sensing, which evaluates the features of the target through spectral reflectance. It is anticipated that the illness may be identified using hyperspectral and thermal imaging, given that the field has an extended overgrowth, which is a characteristic indication of bakanae. However, the use of remote sensing imaging methods in plants to identify the bakanae symptoms is currently constrained, primarily due to its secondary symptoms, which include pigment, structure, fluorescence, and temperature. In addition, it might be challenging to distinguish whether the symptoms are brought on by bakanae alone because the physiological indications mentioned above can vary based on variables including the habitat and rice variety. Consequently, using a bakanae infection model to comprehend the epidemiological behavior of bakanae impacted by daily weather conditions across the rice growth stages would be a more practical option for the early identification of bakanae in the fields. The infection model may be used to mimic the emergence of seedling infection and the subsequent effects of common seedling symptoms including elongation, stunting, and withering. Based on empirical relationships identifying the many connections between weather, pathogen, and host plant, leading to infection and symptom development in rice seedlings, seedling infection algorithms may be created. Based on controlling meteorological conditions and pertinent infection algorithms that have been established from numerous *in vitro* and field research, floral infections that result in seed infection can also be predicted during the blooming stage. The bakanae infection model that we utilized in this study for assessing the effects of climate change can serve as a springboard for future research and be used for the early identification and efficient management of bakanae in rice fields.

Author contributions

QS, MM, and RTB: writing draft, software, and figure preparations. MAS, SA, MAA, SKU, and PKD: collection literature, formal analysis, preparation of tables, and editing. YI: validation and finalization of the review. LZ: supervision, project administration, resources, and funding acquisition. All authors have reada and agreed to the published version of the manuscript, listed have made a substantial, direct, intellectual contribution to the work, and approved it for publication.

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Conflict of interest

PKD was employed by Contec Global Agro Limited.

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

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Complete genome analysis of sugarcane root associated endophytic diazotroph *Pseudomonas aeruginosa* DJ06 revealing versatile molecular mechanism involved in sugarcane development

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Sugarcane is an important sugar and bioenergy source and a significant component of the economy in various countries in arid and semiarid. It requires more synthetic fertilizers and fungicides during growth and development. However, the excess use of synthetic fertilizers and fungicides causes environmental pollution and affects cane quality and productivity. Plant growth-promoting bacteria (PGPB) indirectly or directly promote plant growth in various ways. In this study, 22 PGPB strains were isolated from the roots of the sugarcane variety GT42. After screening of plant growth-promoting (PGP) traits, it was found that the DJ06 strain had the most potent PGP activity, which was identified as *Pseudomonas aeruginosa* by 16S rRNA gene sequencing. Scanning electron microscopy (SEM) and green fluorescent protein (GFP) labeling technology confirmed that the DJ06 strain successfully colonized sugarcane tissues. The complete genome sequencing of the DJ06 strain was performed using Nanopore and Illumina sequencing platforms. The results showed that the DJ06 strain genome size was 64,90,034 bp with a G+C content of 66.34%, including 5,912 protein-coding genes (CDSs) and 12 *rRNA* genes. A series of genes related to plant growth promotion was observed, such as nitrogen fixation, ammonia assimilation, siderophore, 1-aminocyclopropane-1-carboxylic acid (ACC), deaminase, indole-3-acetic acid (IAA) production, auxin biosynthesis, phosphate metabolism, hydrolase, biocontrol, and tolerance to abiotic stresses. In addition, the effect of the DJ06 strain was also evaluated by inoculation in two sugarcane varieties GT11 and B8. The length of the plant was increased significantly by 32.43 and 12.66% and fresh weight by 89.87 and 135.71% in sugarcane GT11 and B8 at 60 days after inoculation. The photosynthetic leaf gas exchange also increased significantly compared with the control plants. The content of indole-3-acetic acid (IAA) was enhanced and gibberellins (GA) and abscisic acid (ABA) were reduced in response to inoculation of the DJ06 strain as compared with control in two sugarcane varieties. The enzymatic activities of

oxidative, nitrogen metabolism, and hydrolases were also changed dramatically in both sugarcane varieties with inoculation of the DJ06 strain. These findings provide better insights into the interactive action mechanisms of the *P. aeruginosa* DJ06 strain and sugarcane plant development.

KEYWORDS

***Pseudomonas aeruginosa*, endophyte, complete genome, PGPB, root colonization, sugarcane**

Introduction

Sugarcane is an important C4 crop with great potential to contribute to biofuel production worldwide. It requires a large number of fertilizers, herbicides, and fungicides for plant growth and development (Li and Yang, 2015; Wayment et al., 2021). However, the application of chemical fertilizers could increase crop yields but has dramatically harmed the farmland environment and human health (Savci, 2012; Feng et al., 2020; Meftaul et al., 2020). Plant growth-promoting bacteria (PGPB) are the beneficial microorganisms that colonize plant tissues and symbiotically coexist with plants to promote plant growth through nitrogen fixation, secretion of siderophore, biocontrol, and improvement of plant resistance to stresses (Kloepper et al., 1980; Nehra and Choudhary, 2015; García et al., 2017; Singh et al., 2017; Fukami et al., 2018).

Biological nitrogen fixation (BNF) is the best way to reduce the application of chemical nitrogen fertilizer in crop production. The nitrogen contribution level in sugarcane reached 18–57.31%, verified by the ^{15}N natural abundance technique after inoculating nitrogen-fixing bacteria, including *Herbaspirillum seropedicae* IPA-CC9, *Pseudomonas* sp. IPA-CC33, and *Bacillus megaterium* IPA-CF6 (Antunes et al., 2019). The nitrogen uptake of sugarcane varieties, GT11 and B8, has significantly improved on inoculation of *Enterobacter roggenkampii* ED5 strain, and physiological enzymatic activities were also considerably changed (Guo et al., 2021). Some PGPBs were used for biocontrol due to having an effective inhibitory effect on plant pathogens. In an earlier study, Singh et al. (2021) reported that *Pseudomonas aeruginosa* B18 enhanced the growth of the sugarcane variety Yacheng 71-374 in response to the smut pathogen *Sporisorium scitamineum*. Similarly, sugarcane pathogens *Colletotrichum falcatum* and *Fusarium moniliforme* were controlled by applying *B. amyloliquefaciens* and *B. gladioli* CP2 (Bharathalakshmi and Jamuna, 2019; Pitiwittayakul et al., 2021). Improving plant resistance against abiotic stresses is one of the main growth-promoting properties of PGPB. For example, the salt tolerance of sugarcane was enhanced with *B. xiamenensis* ASN-1 inoculation (Sharma et al., 2021). The nitrogen-fixing strain *Streptomyces chartreuses* WZS021 could effectively improve the drought resistance of sugarcane (Wang et al., 2019).

In recent years, some sugarcane rhizosphere or endogenous growth-promoting strains were isolated, such as *Kosakonia radicincitans*, *Stenotrophomonas maltophilia*, *Herbaspirillum seropedicae, Enterobacter roggenkampii, P. entomophila, Klebsiella variicola*, *Paenibacillus lactis, B. xiamenensis, Klebsiella pneumonia*, *Gluconacetobacter diazotrophicus,* etc. (Mirza et al., 2001; Wei et al., 2014; Lamizadeh et al., 2016; Bhardwaj et al., 2017; Li et al., 2017; Oliveira et al., 2018; Guo et al., 2020; Singh et al., 2020; Xia et al., 2020). Whereas, *Pseudomonas* is a common PGPB with broad application prospects, and the inoculation of *P. fluorescens* promoted phosphorus uptake in sugarcane, thereby promoting its proper growth (Rosa et al., 2022).

The whole genome sequencing technology provides a suitable method for revealing the molecular mechanism of interaction between PGPB and crops. In our laboratory, the complete genome sequence of the sugarcane endophytic nitrogen-fixing bacteria *E. roggenkampii* ED5 was analyzed, and a variety of potential genes related to auxin, siderophore, nitrogen metabolism, and resistance to abiotic stresses were found in the genome of the strain, revealing the molecular mechanisms of plant growth promoting effects of the strain on sugarcane (Guo et al., 2020). The potential biocontrol mechanism of *Streptomyces griseorubiginosus* BTU6 was shown by genome sequencing (Wang et al., 2021). Although research has been conducted on *Pseudomonas* rhizosphere strains in sugarcane, most of them are from soils, but studies on the endophytic *Pseudomonas* strains are still limited (Singh et al., 2021). Meanwhile, the molecular basis of *Pseudomonas* for promoting sugarcane growth remains unclear.

In the present study, whole-genome sequencing technology was applied to analyze the molecular basis of sugarcane growth promotion by endophytic *Pseudomonas* strain isolated from sugarcane root. The purpose of this study was (i) isolation of endophytic bacterial strains from sugarcane variety GT42 roots and analyze their plant growth-promoting (PGP) properties, (ii) observation of the colonization of the *P. aeruginosa* DJ06 strain in sugarcane tissue through scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM), (iii) sequencing the complete genome of *P. aeruginosa* DJ06 and predict its potential PGP-related genes, (iv) investigation of the agronomic traits and photosynthetic responses of sugarcane varieties GT11 and B8 after inoculation of *P. aeruginosa* DJ06, and (v) the analysis of the changes in endogenous phytohormones and physiological enzymes of sugarcane responsive to *P. aeruginosa* DJ06 inoculation.

Materials and methods

Isolation of endophytic bacteria from sugarcane

Endophytic bacterial strains were isolated from the sugarcane variety GT42, which has the most extensive planting area in China

at present. The sugarcane roots were rinsed with tap water; 1 g of root sample was cut into 5 mm segments and soaked in 4% sodium hypochlorite solution for 2 min and alcohol (75%) for 2 min; and then washed with sterile water three times and used the water as the control for final washing. The cleaned root segments were put into a sterilized mortar and ground into a powder with a pestle, adding 10 ml of sterile water to dissolve it completely and placing it for 30 min. The suspension was diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}), and 100 μl of each diluted suspension was drawn and spread evenly on five different media plates, respectively (Supplementary Table 1), and incubated at 32°C for 48 h. No colonies appeared in the control plates, which means the isolation of endophytic bacteria in the sugarcane roots was successful. The single colonies were picked for purification, and the purified strains were stored at −80°C with 20% glycerol for later use.

DNA extraction from isolated strains and identification

High-quality genomic DNA of the strains was extracted with a QIAGEN QIAmp® DNA extraction kit. The 16S rRNA gene was amplified, and high-quality amplified products were obtained by 1% agarose gel detection for purification and sequencing (Li et al., 2017). The PCR primers and reaction conditions are presented in Table 1. The Trelief® DNA Gel Extraction Kit (Tsingke, China) was used for PCR product purification. The 16S rRNA gene sequences of all the endophytic strains were sequenced by Tsingke Biotechnology Co. Ltd., Beijing, China. The 16S rRNA gene was sequenced and spliced with ContigExpress software, BLAST-similar sequence alignment was performed in NCBI for each strain, and the specific sequence was submitted to the NCBI GenBank database to obtain the accession number.

Screening for PGP activities

The PGP activities including phosphate (P) solubilization, hydrogen cyanide (HCN), siderophore, DF-ACC (1-aminocyclopropane-1-carboxylic acid), and ammonia production were examined. All the inoculated strains were fresh bacteria cultured in liquid for 3 days, and all the experiments were performed in three biological replicates.

Phosphate solubilization

Pikovskaya (1948) agar plate was used to test the activity of dissolved inorganic phosphorus. A total of 5 μl of each bacterial solution was pipetted and inoculated in the center of Pikovskaya's agar culture plates, and each culture plate was sealed with parafilm and placed in an incubator at 32°C for 72 h. The appearance of a colorless transparent circle indicates that the strain can dissolve phosphorus, and the strength of the ability to dissolve phosphorus is positively correlated with the diameter of the colorless transparent circle.

Hydrogen cyanide production

The strains were inoculated into the nutrient broth (NB) tubes with 4.4 g L^{-1} of glycine, and the filter paper strip (0.5 cm × 8 cm) was soaked in 1% picric acid solution for 10 min, and then hung on the culture tubes and sealed. It was incubated at 32°C in a dark incubator for 4–7 days. If the color of the filter paper changed, it confirmed that the strain could produce HCN. The color of the filter paper indicated the strength of the produced HCN (Lorck, 1948).

DF-ACC test

A total of 3.0 mmol of 1-aminocyclopropane-1-carboxylic acid (ACC) was added to 1 L DF medium (Jacobson et al., 1994), to make culture plates for qualitative analysis. The strains were inoculated on the DF-ACC plates and cultured at 32°C for 72 h. The ability of the strain to utilize an ACC nitrogen source was positively correlated with the diameter of the bacterial circle (Li et al., 2011).

Siderophore production

The ability of siderophore production by strains was tested by the chrome azurol S (CAS) plates method (Schwyn and Neilands, 1987). A total of 5 μl sample of each bacterial solution was tested, inoculated in the center of CAS plates, sealed with parafilm, and then placed in the incubator at 32°C for 72 h for observation. The siderophore production ability of strains was positively correlated with the diameter of the measured yellow halo zone.

Ammonia production

Nessler's reagent was used to detect ammonia production by strains. A culture solution containing 10 g of peptone and 5 g of sodium chloride per liter was prepared and divided into 15 ml finger-shaped bottles, and 9.5 ml of culture solution was dispensed into each bottle and then sterilized. A total of 10 μl of each inoculated bacterial solution was tested and incubated at 32°C at 110 rpm for 48 h. Then, 0.5 ml of Nessler's reagent was added, and a change in the color of the liquid bacterial culture from yellow to reddish-brown was observed. The color change was observed, proving that the strain could produce ammonia. The darker the color, the stronger the ammonia production ability it had (Taylor et al., 1988).

Nitrogenase activity test

The nitrogenase activity of strains was determined by acetylene reduction assay (ARA), according to the protocol of Hardy et al. (1968). The ethylene with a concentration of 1,000 mg L^{-1} was used as the standard sample. The tested strain was put into a 50 ml headspace flask with 10 ml Ashby culture solution and cultivated for 48 h at 32°C at 150 rpm. A total of 5 ml of gas was taken from the culture flask using a 15 ml syringe, then supplemented with an equal volume of acetylene gas, and kept for 48 h at 32°C at 150 rpm for the detection of the content of ethylene generated by the reduction. The gas chromatograph Agilent 6890M was used to detect nitrogenase activity. The oven temperature, detector temperature, and injector temperature of gas chromatography (GC) were 80, 165, and 180°C, respectively. The ethylene and

TABLE 1 Primer sequences used for 16S rRNA gene amplification.

Gene	Primer	Sequence (5′→ 3′)	Product size (bp)	PCR conditions	Reference
16S rRNA	pA-F pH-R	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	1300 to 1600	Initial temperature (95°C for 5 min), start cycles (30), denaturation (95°C for 1 min), annealing (55°C for 1 min), elongation (72°C for 1 min), final extension (72°C for 5 min).	Edwards et al. (1989)

acetylene retention time was 0.84 min and 0.96 min, respectively. In addition, the flow rates of hydrogen (H_2) and nitrogen (N_2) were 40 and 25 ml/min, respectively. The nitrogen-fixing bacteria *Klebsiella variicola* DX120E and *E. roggenkamp*i ED5 were used as control strains, which had been confirmed to have strong nitrogen fixation effects by our previous research.

Colonization study of *Pseudomonas aeruginosa* DJ06

The strain *P. aeruginosa* DJ06 with a concentration of 1×10^6 CFU ml^{-1} was inoculated into the micropropagated plantlets of sugarcane variety GT11, provided by the Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, China. After being cultured for 3 days, sugarcane roots and stems were collected with sterile scissors, and the surface water was dried with absorbent paper. The sample was treated with 0.1 M of phosphate buffer (pH 7.4) three times for 15 min, then transferred to 1% osmic acid (O_sO_4), incubated in the dark incubator at 25°C for 1–2 h, and washed three times. The samples were soaked in alcohol solutions of 30, 50, 70, 80, 90, 95, and 100% for 15 min and then transferred to isoamyl acetate for another 15 min. All the samples were put in the sputtering ion instrument (Hitachi mc1000), sprayed gold for 30 s, and then images were observed with a scanning electron microscope (SEM, Hitachi su8100).

A single colony of strain DJ06, *E. coli* TG1/pPROBE-pTetr-TT-gfp (donor strain), and DH5a/pRK2013 (syncell) were inoculated in the C_2 medium for 12 h (Supplementary Table 2), centrifuged at 5,000 rpm (5 min), and the supernatant was discarded. Each bacterial liquid (1 ml) was centrifuged three times to remove antibiotics. Donor strains, *E. coli* TG1/pPROBE-TT-pTetr, DJ06, and DH5a/pRK2013, were mixed as 50, 100, and 50 μl (1:2:1), and a 100 μl of the mixture was taken in antibiotic-free C_2 plates and incubated at 30 °C (6 h). The bacteria were transferred into sterile water and mixed. A total of 100 μl of the mixture was added to the C_2 plate containing Sm100 and Gm15, and the growth of green fluorescent colonies was observed. The sugarcane GT11 micropropagated plantlets were used to assess the *E. coli* TG1/pprobeptetr TT GFP/DJ06 colonization. The colonization of the DJ06 strain in sugarcane roots and stems was observed by a laser scanning confocal microscope (LSCM) (Lin et al., 2012).

Genome sequencing and library construction

The genomic DNA of the DJ06 strain was extracted using Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. The purified genomic DNA was quantified by the TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA). High-quality DNA ($OD_{260/280} = 1.8 \sim 2.0$, >20 μg) was used for further research. Nanopore sequencing and Illumina sequencing platform were used for genome sequencing. The NovaSeq 6000 S4 Reagent Kit 1.5 (300 cycles) was used for Illumina sequencing. The Nanopore library preparation Kit was SQK-LSK11 and Nanopore cell R9.4.1, used to sequence with high-accuracy basecalling (HAC) as the base calling model. Illumina's data evaluation on genome heterozygosity, genome repeatability, genome size, and pollution was performed to ensure the completeness and accuracy of the assembly. The Illumina and nanopore sequencing methods were followed by Gu et al. (2020).

Genome assembly, gene prediction, and annotation

The original raw data were stored in the fastq format. For accurate assembly, the quality of the original data was checked, and the reads with low sequencing quality, the high proportion of N, and the small length after quality pruning were removed to obtain high-quality clean data. The nanopore data were assembled by Canu software (https://github.com/marbl/canu), and the reads were assembled into contigs and then manually judged into rings to obtain the genome with complete chromosomes. Finally, the assembly results were corrected by Illumina sequencing data. The coding sequence (CDS), tRNA, and rRNA were predicted by Glimmer 3.02, tRNAscan-SE 2.0, and Barrnap software, respectively. The predicted CDS protein functions were annotated from NR, Swiss prot, Pfam, Gene Ontology (GO), Clusters of Orthologous Groups (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases by using Blast2go 2.0, Diamond 0.8.35, HMMER3.1b2, and other sequence alignment tools (Guo et al., 2021).

Analysis of genome phylogeny based on average nucleotide identity

The eight similar complete genomes of *P. aeruginosa* DJ06 were selected based on five house-keeping genes (*dnaG*, *rplB*, *rpoB*, *rpsB*, *smpB*, and *tsf*) and 16S rRNA gene using the NCBI BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ANI was calculated by the OAT software program (https://www.Ezbiocloud.net/tools/orthoani), the results were exhibited in a heatmap using version 3.5.1 gplots 3.0.4 software package, and the cluster analysis was performed by vegan 2.5–6 software (Ciufo et al., 2018).

Sugarcane seedling and transplanting

To further evaluate the growth-promotion effect of strain *P. aeruginosa* DJ06 on sugarcane, two sugarcane varieties GT11 (requires much fertilizer for growth) and B8 (requires less fertilizer for growth) were tested in the greenhouse. The inoculation concentration of the DJ06 strain was 1×10^6 CFU ml^{-1}. The size of the tray for plant culturing was 40 cm in length and 30 cm in width. The red loam soil was used for the experiment. The healthy sugarcane stems were selected and cut into uniform-length segments with bud, which were soaked in hot water at 50°C for 20 min and soaked with 1% carbendazim for 30 min. The treated segments were put into the trays with organic substrates and cultivated at room temperature for approximately 15 days with routine management under greenhouse conditions. When the seedlings grew to 2–3 leaves, those with the same growth status were selected for inoculation and transplanting. The sugarcane seedlings were removed from the tray, and the root surface was washed with sterile water. The roots were soaked in the bacterial solution for approximately 1 h, and the plants were transplanted to the culture pots of a size 32 × 27 cm (H × W). The seedlings treated with sterile water were used as a control. The complete randomized block design (RBD) was applied. There were three groups of inoculation treatment and control for each variety, with 10 pots in each group and one seedling in each pot.

Evaluation of photosynthetic leaf gas exchange and plant growth parameters

The agronomic traits of sugarcane were investigated 60 days after inoculation. The photosynthesis, transpiration, stomatal conductance, intercellular CO_2, plant height, and fresh weight were observed in this study. The length from the topsoil to the ring of the top visible dewlap leaf (leaf +1) was measured as plant height. The whole sugarcane plant was removed from the pot, the soil attached to the roots was removed, the roots were rinsed with running water, and the fresh weight was observed after removing the water naturally. The photosynthetic leaf gas exchange was measured by a Li-6800 portable photosynthesis system (Li-COR Biosciences, Lincoln, NE, US) on a sunny day from 9:00 a.m. to 10:30 a.m. (Verma et al., 2020).

Determination of phytohormones

The content of phytohormones, i.e., indole acetic acid (IAA), gibberellins (GA_3), and abscisic acid (ABA), of sugarcane varieties GT11 and B8 was tested at 60 days after inoculation by high-performance liquid chromatography (HPLC). A total of 200 mg of samples were ground with liquid nitrogen, then 70–80% methanol solution was added, soaking at 4°C for 12 h, and centrifuged at 12,000 rpm for 10 min at 4°C. In total, 0.5 ml of 70–80% methanol solution was added to the residue after centrifugation and mixed up at 4°C for 2 h before leaching and centrifugation. All the supernatants were combined and evaporated to the one-third volume under reduced pressure at 4°C, and an equal volume of petroleum ether was added. After standing for stratification and repeated extraction and decolorization two to three times, triethylamine was added, and the pH was adjusted to 8.0. After adding cross-linked polyvinylpyrrolidone (PVPP), the mixture was incubated at 150 rpm for 20 min at room temperature (RT). The supernatant was collected after centrifugation, and the pH was adjusted to 3.0. After three extraction times with ethyl acetate, it was evaporated to dryness under reduced pressure at 40°C, added to the mobile phase solution, and vortexed to be fully dissolved. After filtration with a needle filter, the samples were taken and detected by HPLC (WuFeng LC-100, Shanghai, China). The chromatographic conditions were mobile phase A with 100% methanol and B with 0.1% acetic acid aqueous solution (A: B = 55:45). The chromatographic column was C18 reversed-phase chromatographic column (Supelco, United States), in which the injection volume was 20 μl, the column temperature was 30°C, and the size was 150 mm × 4.6 mm × 5 μm. IAA, GA_3, and ABA retention times were 18.36, 8.36, and 35.00 min, respectively. The ultraviolet detection wavelength was 254 nm.

Assessment of sugarcane-related enzymatic activities

The enzyme activities in the sugarcane plant were analyzed with an analytical kit 60 days after inoculation. The kits were obtained by Grice Biotechnology Co. Ltd. (Suzhou, China), and the manufacturer's instructions were followed to perform the analysis. In this study, the related oxidative enzymes, such as catalase (CAT, G0105W), peroxidase (POD, G0107W) and superoxide dismutase (SOD, G0101W), the oxidative product, i.e., malondialdehyde (MDA, G0109W), the enzymes related to nitrogen metabolism such as nitrate reductase (NR, G0402W), NADH-glutamate dehydrogenase (NADH-GDH, G0405W) and glutamine synthetase (GS, G0401W), and hydrolases enzymes (endo-1,4-β-D-glucanohydrolase-G0533W and β-1,3-glucanase-G0526W) were examined.

Data analysis

Agronomic, physiological and biochemical responses were analyzed (ANOVA) by SPSS 20.0 and Excel 2010 software. The difference significance at $p \leq 0.05$ was used to assess the comparison between the means. Bioinformatics analysis of the complete genome of the DJ06 strain was carried out utilizing the Majorbio I-Sanger Cloud Platform (www.i-sanger.com), which is a free online platform.

Results

Isolation and PGP activities of endophytic strains from sugarcane roots

A total of 53 endophytic bacterial strains were isolated using five different media from the roots of the sugarcane variety GT42. Among them, the major 18 (33.97%) strains were isolated from

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TABLE 2 PGP activities of selected endophytic strains from the sugarcane roots.

Isolations	Most similar strain	Similarity (%)	NCBI accession No.	Phosphate	Siderophore	DF-ACC	HCN	Ammonia
GA8	*Enterobacter cloacae*	99.93%	MT664177	+	+	-	+	+
GA16	*Enterobacter* sp.	99.93%	MT664178	+	+	+	-	+++
GA20	*Enterobacter* sp	99.93%	MT664179	-	-	-	+	+++
GB2	*Enterobacter* sp.	99.93%	MT664180	+	-	+	++	+
GB3	*Leclercia adecarboxylata*	99.79%	MT664194	+	-	-	-	++
GB8	*Enterobacter* sp	99.86%	MT664181	+	-	+	-	++
GB13	*Enterobacter* sp.	99.86%	MT664182	+	+++	+	-	++
GB21	*Enterobacter aerogenes*	99.79%	MT664183	+	-	+	+	++
GB22	*Enterobacter asburiae*	99.18%	MT664184	-	-	-	-	++
GB25	*Enterobacter aerogenes*	99.66%	MT664185	-	+	-	+	++
GB26	Uncultured *Enterobacter* sp.	99.93%	MT664186	-	-	-	++	+++
GB27	*Bacterium strain*	99.93%	MT664195	+	-	-	-	+
GC16	*Enterobacter cloacae*	99.93%	MT664187	+	+	-	+	++
GD3	*Enterobacter oryzae*	99.86%	MT664188	-	-	+	+	+++
GD6	Uncultured *Enterobacter* sp.	99.79%	MT664189	-	-	-	-	+++
GD7	*Enterobacter oryzae*	99.79%	MT664190	-	+++	-	+	+++
GD9	*Enterobacter* sp.	99.79%	MT664191	-	-	-	+	+
GD12	*Enterobacter* sp.	100%	MT664192	-	+	-	+	++
GD13	*Kosakonia oryzae*	99.93%	MT664196	+	+	++	+	+++
GD16	*Enterobacter* sp.	99.86%	MT664193	+	+	+	-	++
DJ06	*Pseudomonas aeruginosa*	99.45%	OP005483	++	+++	+++	+++	+++
DJ08	Uncultured bacterium	99.65%	OP005484	+	+	+	+	++

(-), No Activity; (+ + +), Strong Activity; (++), Moderate Activity; (+), Low activity.

Ashby's mannitol agar, and the most minor five (9.43%) strains from Jensen's agar, and 12 (22.64%), 10 (18.87%), and 8 (15.09%) strains from Pikovskaya's agar, Yeast mannitol agar, and Burk's medium, respectively (Supplementary Figure 1). In addition, 22 strains were found to exhibit diverse PGP activities. The 16S rRNA gene sequencing identification results of these 22 strains are shown in Table 2. The 16S rRNA sequences of the 22 strains were uploaded to the NCBI GenBank database, and accession numbers were MT664177-MT664196 and OP005483-OP005484 (Table 2). Out of them, *in vitro* tests showed that 11 (55%) strains had a positive response to dissolving inorganic phosphate on Pikovskaya's medium plates, and the DJ06 strain showed the most potent activity among them. The results of the siderophore production test found that 13 (59.09%) strains could produce an orange zone in CAS agar plates, and three strains (GB13, GD7, and DJ06) exhibited more vigorous. The growth of the strains with ACC as the sole nitrogen source test showed that 10 (45.45%) strains had positive growth in DF-ACC plates, and two of them (GD13 and DJ06) had more potent activity than other strains. In addition, all the strains had potential ammonia production capacity, and GA16, GA20, GB26, GD3, GD6, GD7, GD13, and DJ06 exhibited more robust activity. Overall, the results showed that strain *P. aeruginosa* DJ06 had more prominent PGP activities than other strains (Table 2). In addition, the nitrogen-fixing capacity of DJ06 was tested through the ARA method. The result showed that the nitrogenase activity reached 31.27 nmol C_2H_4 mg protein h^{-1}, which was higher at 3.29% and lower at 4.64% than the control strains of *Enterobacter roggenkampii* ED5 and *Klebsiella variicola* DX120E, respectively, indicating that the DJ06 strain has strong nitrogen fixation potential (Table 3).

TABLE 3 The nitrogenase activity in the DJ06 strain in comparison with the other two strains.

Strains	ARA (nmoL C_2H_4 mg protein h^{-1})
Pseudomonas aeruginosa DJ06	31.27 ± 0.23^{b}
Enterobacter roggenkampii ED5	30.24 ± 0.19^{c}
Klebsiella variicola DX120E	32.72 ± 0.73^{a}

All data points are presented as mean ± SE ($n = 3$). Different lowercase letters indicate a significant difference at $p < 0.05$ according to DMRT (Duncan's Multiple Range Test).

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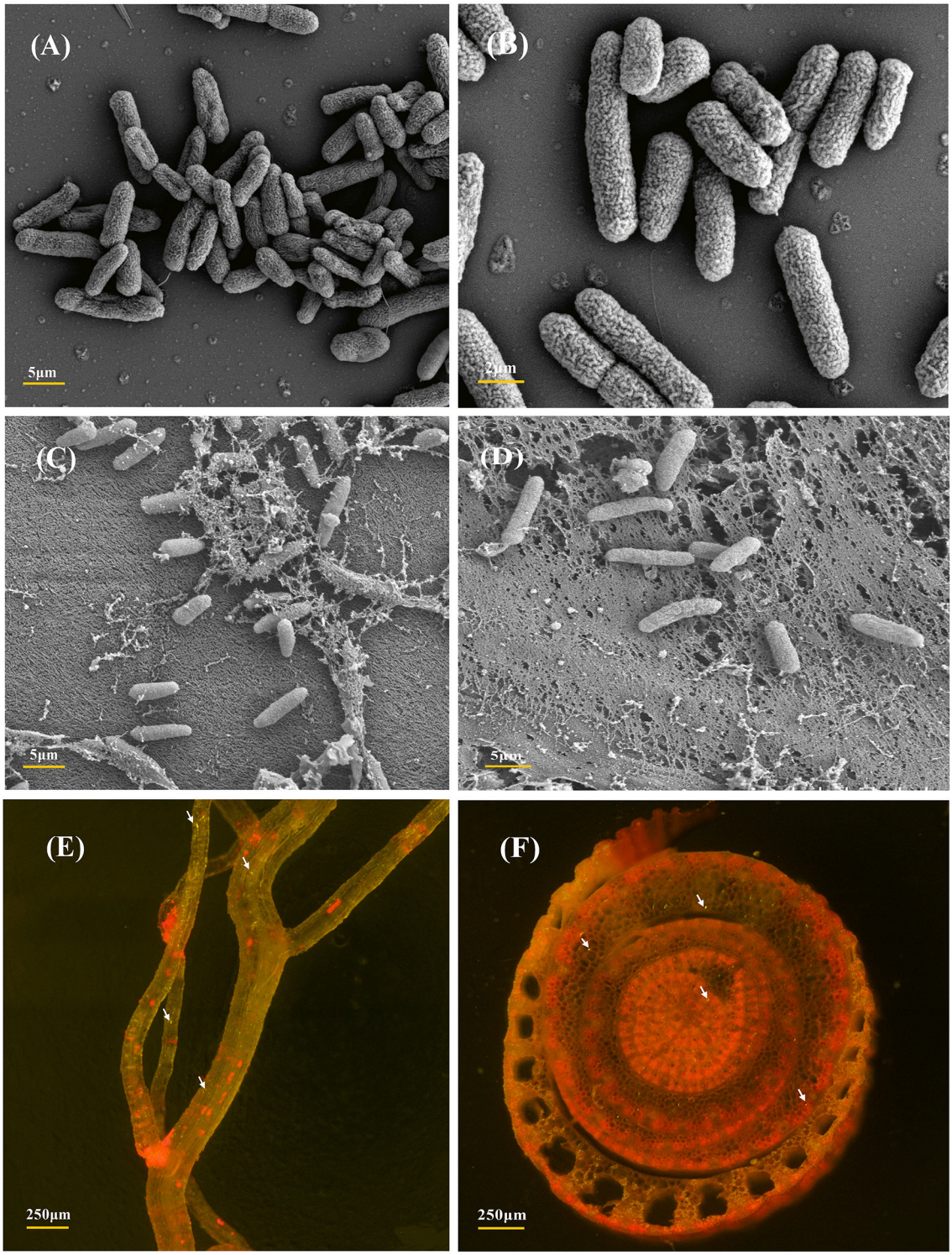

FIGURE 1
SEM and CLSM micrographs of the most efficient endophytic *Pseudomonas aeruginosa* DJ06 strain and its colonization in sugarcane plant parts at the root and stem regions. **(A, B)** Are the SEM images showing the morphology of the DJ06 strain, **(C, D)** are the colonization images obtained after the inoculation of the DJ06 strain in sugarcane root and stem, **(E, F)** show the CLSM micrographs of GFP-tagged endophytic DJ06 strain in sugarcane root and stem. Green fluorescence within the root and stem tissues indicated by white arrows indicates that the DJ06 strain has colonized in the tissue.

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TABLE 4 Genome characteristic of endophytic strain *Pseudomonas aeruginosa* DJ06.

Characteristics	Value
Genome size (bp)	6,490,034 bp
GC content (%)	66.34%
tRNA	65
rRNA (5S, 16S, 23S)	12 (4, 4, 4)
Protein-coding genes (CDS)	5,912
Genomic islands	6
CRISPR	8
Prophge	3
Genes assigned to NR	5,909
Genes assigned to Swiss-Prot	4,504
Genes assigned to COG	5,251
Genes assigned to KEGG	3,246
Genes assigned to GO	4,375
Genes assigned to Pfam	5,178

Colonization of GFP-tagged endophytic DJ06 in sugarcane tissues

The strain *P. aeruginosa* DJ06, which has various potential PGP characteristics, was used to analyze the colonization in sugarcane tissue. The sugarcane root colonization and colony morphology of the DJ06 strain were observed by SEM and CLSM (Figure 1). Colonization analysis was fundamental for further study of the interaction mechanism between the DJ06 strain and the sugarcane plant. The morphology of the DJ06 strain and colonization in plant roots and stems is presented in Figures 1A–D. It was found that the DJ06 strain could successfully colonize sugarcane tissue observed using SEM. In addition, the GFP-tagged DJ06 was observed by CLSM after inoculation in sugarcane seedlings for 3 days. The results showed that the roots and stems of the seedlings had green fluorescent bacteria, which proved that the DJ06 strain had colonized the sugarcane tissues (Figures 1E, F).

Genomic properties of the DJ06 strain

The general properties of the genome of endophytic strain *P. aeruginosa* DJ06 are shown in Table 4, with a 64,90,034 bp of circular chromosome (Figure 2). The average G+C content in the genome was 66.34% including 5,912 CDSs. In addition, the *P. aeruginosa* DJ06 genome had 12 rRNA genes with 5S, 16S, and 23S, six genomic islands, eight CRISPR, and three prophages, respectively. The CDSs were annotated in GO, COG, and KEGG databases, which were 4,375, 5,251, and 3,246, respectively, involving multiple biological processes (Supplementary Figures 2–4). The complete sequence of the strain *P. aeruginosa* DJ06 has been submitted at the NCBI/GeneBank with accession number CP080511.

Genome-based phylogeny of the DJ06 strain

The ANI results showed that the genome of DJ06 presented 99.41% ANI to *P. aeruginosa* A681 and *P. aeruginosa* AVT410 and 94.34% ANI to *P. aeruginosa* 1334/14, respectively. The ANI values of the DJ06 strain and other strains were less than 95%; the highest value was 71.95% for *P. extremaustralis* DSM17835, and the lowest was 71.43% for *P. fluorescens* SBW25. These ANI results indicated that the DJ06 strain belongs to *P. aeruginosa*, which was also confirmed by cluster analysis (Figure 3).

Potential PGP-related genes in endophytic strain DJ06 genome

Some essential PGP-related genes were predicted in the genome of the DJ06 strain, such as nitrogen fixation (*iscU*), ammonia assimilation (*gltB*), nitrosative stress (*fpr*, *hmp*, *gbcB*, and *vanB*), siderophore (*tonB*, *fabY*, *fepA*, and *fhuE*), IAA production (*trpCEGS*), phosphate metabolism (*pit*, *pstABCS*, and *PhoABDUR*), hydrolase (*bglXB*, *folE*, *folE2, ribA, and ribBA*), HCN (*hcnABC*), phenazine (*phzA_B*, *phzF*), chemotaxis (*cheABDVRWAZ*, *pilIJK*, *aer*, and *mcp*), sulfur metabolism (*cysABCDEG, HI, JK, SP, UWYZ*, and *cysNC*), and biofilm formation (*flgABCDEFGHIJKLMN*, *motAB*) (Table 5).

In addition, some key genes in the DJ06 strain genome involved in plant resistance to abiotic stresses, for example, those coding for a cold-shock protein (*cspA*), heat shock proteins (*htpRX*, *hslJR*, and *ibpA*), magnesium transport (*corAC*), copper homeostasis (*copABZ*, *cusRS*), zinc homeostasis (*znuABC*), chromium homeostasis (*czcACD*), and drought resistance (*nhaB*, *kdpABCDE*, *proABCSVWX*, *betABT*, and *trkAH*), were categorized (Table 6). By using the software of antiSMSAH 4.0.2, the genome was predicted to contain various secondary metabolites such as phenazine, bacteriocin, and bifunctional 3,4-dihydroxy-2-butanone-4-phosphate synthase/GTP cyclohydrolase II (Figure 4).

Plant growth parameters

In the present study, the DJ06 strain had a growth-promoting effect on sugarcane varieties GT11 and B8 at 60 days after inoculation compared with the control (Supplementary Figure 5). The photosynthetic rate in the two varieties was significantly increased by 64.35 and 59.18%, respectively. The transpiration rate in variety GT11 was significantly decreased, whereas that in B8 was enhanced nearly 2-fold. The stomatal conductance in GT11 and B8 was reduced by 24.27 and 68.33%, respectively. However, the intercellular CO_2 in GT11 and B8 was enhanced by 41.69 and 116.22%, respectively. In addition, the plant height in GT11 and B8 increased significantly by 32.43 and 12.66 %, and the fresh weight was improved by 89.87 and 135.71% as compared with the control (Figure 5).

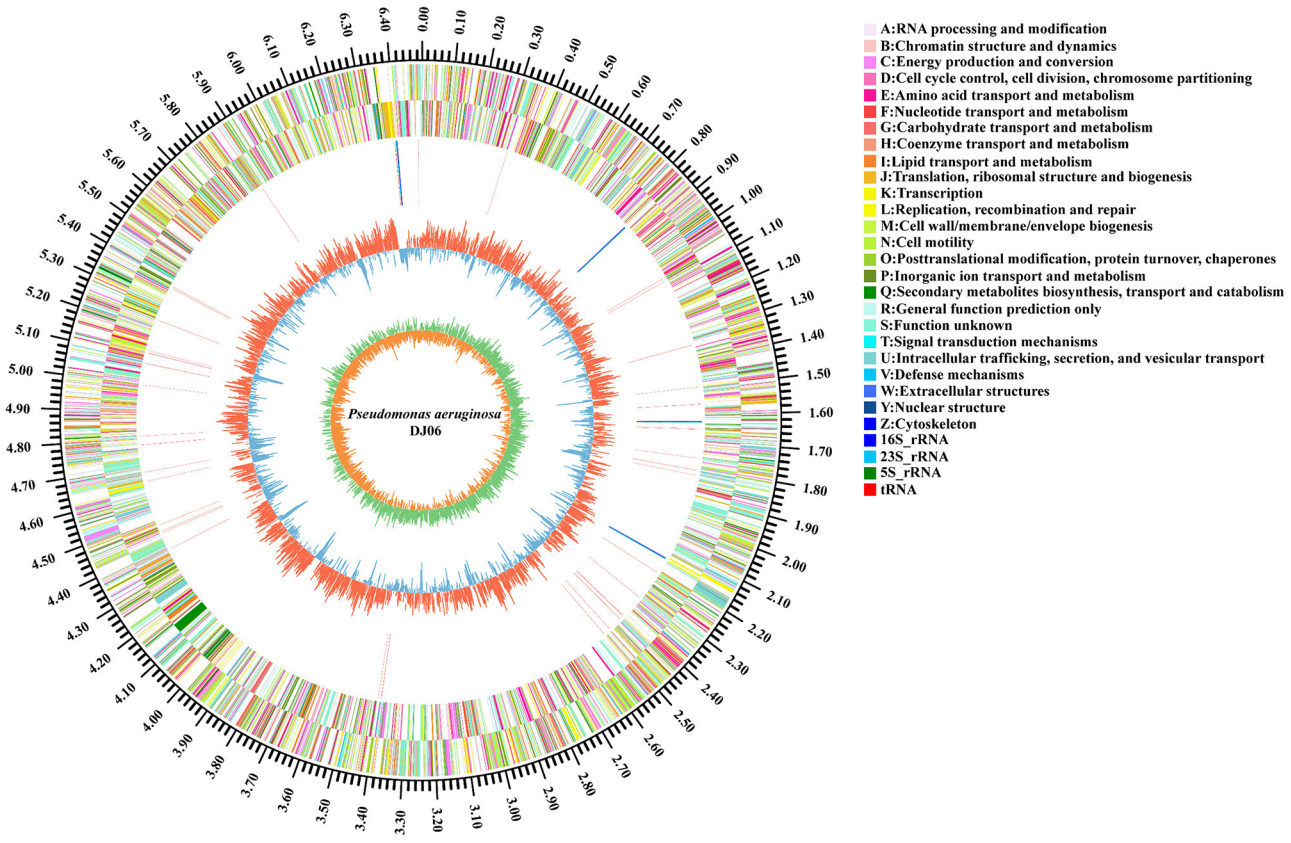

FIGURE 2
Circular representation of chromosome of endophytic bacteria *Pseudomonas aeruginosa* DJ06 strain isolated from sugarcane root. The inner and innermost rings display the GC content and skew. Circles 1–2 show the functional classification of the CDS genes in the chromosome with the colors of the COG database, and in circles 3, different colors indicate different RNA types.

Plant endogenous hormones

A total of three endogenous plant hormones, namely IAA, GA_3, and ABA, were examined. The IAA content in sugarcane varieties GT11 and B8 was increased by 37.38 and 8.01%, whereas the contents of GA_3 and ABA in GT11 and B8 significantly decreased at 60 days after inoculation with the DJ06 strain as compared with the controls (Figure 6).

Plant physiology relevant enzyme activities

The activities of four oxidative enzymes were tested, and the results showed that the SOD activity in sugarcane variety GT11 rose by 11.35% whereas it decreased by 40.79% in B8 as compared to the control. However, the CAT activity in two sugarcane varieties exhibited the same change trend and was enhanced by 102.77 and 179.08% in GT11 and B8, respectively. The POD activity in the two sugarcane varieties was inconsistent, specifically that in B8 was higher but that in GT11 showed no change when compared to the control. The change of the MDA content in B8 was similar to the POD activity, which was enhanced by 24.43% at 60 days after inoculation with the DJ06 strain. However, that in GT11 was lowered by 14.48% (Figures 7A–D).

Nitrogen metabolism-related enzyme activities were also assessed in the present study. The DADH-GDH activity in sugarcane variety GT11 decreased significantly by 7.54% at 60 days after DJ06 inoculation when compared to the control, and there was no significant difference in B8. However, the GS and NR activities significantly increased at 60 days in two sugarcane varieties, that is, GS activity was enhanced by 39.77 and 37.33%, and the NR activity was increased by 18.61 and 119.08%, respectively (Figures 7E–G). A total of two hydrolases, endo-1,4-β-D-glucanohydrolase and β-1,3-glucanase, expressed similar results. The activities of two hydrolases in GT11 significantly increased by 31.20 and 104.11% at 60 days, while those in B8 were not significantly different in the inoculation treatment as compared to the control (Figures 7H, I).

Discussion

Sugarcane is a sugar and energy crop, and farmers apply a large amount of chemical fertilizers to increase sugarcane yields. However, the excessive application of chemical fertilizers not only enhances the cost of sugarcane production but also causes severe pollution to the soil environment and groundwater (Bokhtiar and Sakurai, 2005; Jiang et al., 2012; Li et al., 2016, 2020). Nitrogen is an essential element for plant growth, whereas the source of nitrogen for crops mainly depends on synthetic fertilizers.

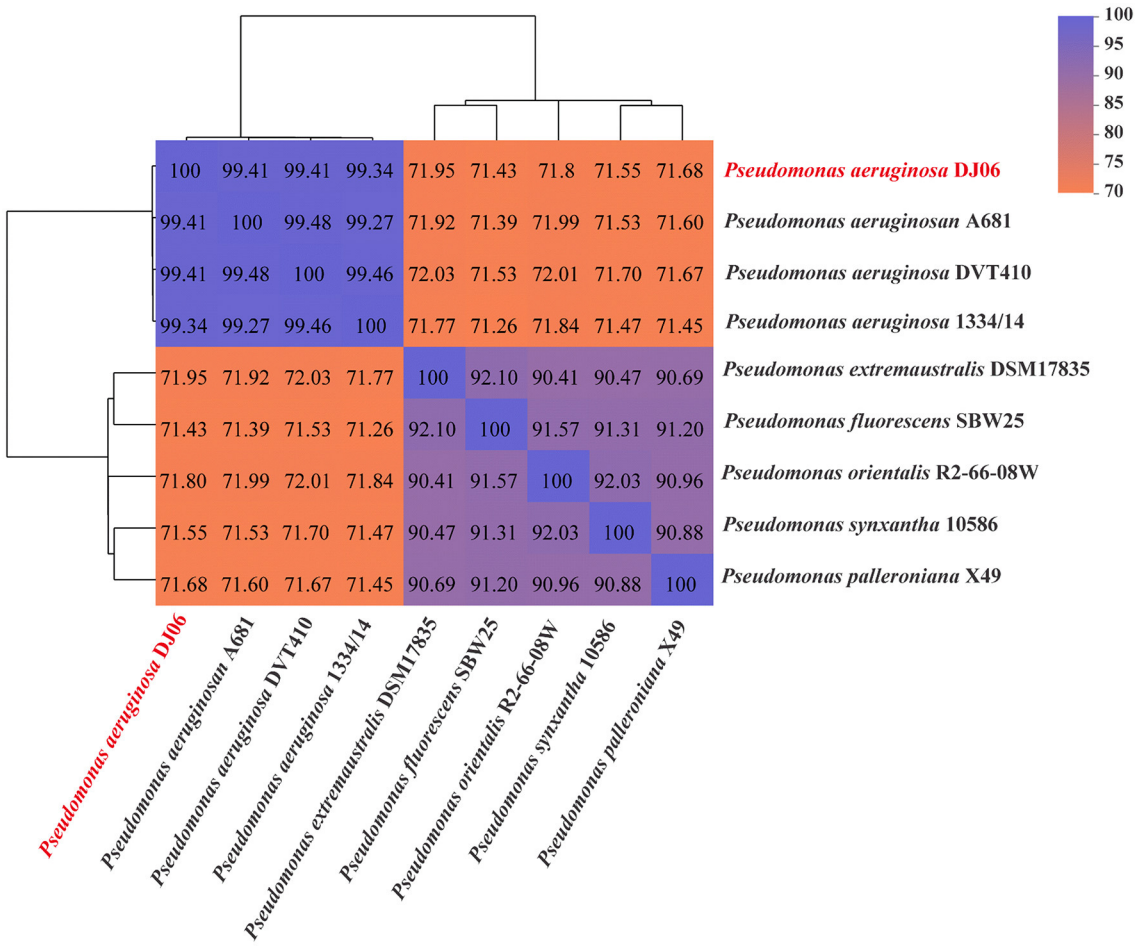

FIGURE 3
Phylogenetic analysis of *Pseudomonas aeruginosa* DJ06 genome based on ANI.

Biological nitrogen fixation (BNF) is an ecological approach to improve crop production and reduce the application of chemical nitrogen fertilizers (Rosenblueth et al., 2018). In this study, the *P. aeruginosa* strain DJ06 has vigorous nitrogenase activity. A series of nitrogen metabolism-related genes were found in the genome of *P. aeruginosa* DJ06, which were mainly involved in nitrogen fixation, ammonia assimilation, and nitrosative stress. Nitrogen fixation mainly involves four genes, i.e., *iscU* and three unknown genes. iscU protein is necessary for biological nitrogen fixation and plays a significant role in Fe-S cluster aggregation because the NifU nitrogen fixation protein is encoded by the *iscU* gene (Smith et al., 2005; Crooks et al., 2012). According to Andrés-Barrao et al. (2017), nitrogenase-encoding gene *nifHDK* was also found in strain *Enterobactor* sp. SA187 genome. The genomes of *Klebsiella variicola* GN02, *K. variicola* DX120E, *Enterobacter roggenkampii* ED5, and *streptomyces chartreuses* WZS021 also contain several nitrogen fixation-related genes, such as *nif* gene clusters, *nifHDK*, *iscU,* and *nifLA*; nitrogen metabolism regulator genes, *ntrBC* and *glnD*; and ammonia assimilation cycle gene, *amtB* (Lin et al., 2015, 2019; Wang et al., 2019; Guo et al., 2020). In this study, DNDH-GDH, GS, and NR activities were examined with the inoculated strain DJ06, and the results showed that sugarcane nitrogen metabolism enzyme activities were changed after inoculation of the DJ06 strain. The nitrate reductase activity was affected by nitrogen-fixing bacteria *Herbaspirillum seropedicae* when inoculated at two nitrogen application levels (3.0 and 0.3 mM) (da Fonseca Breda et al., 2019). Similar to this study, the inoculation of *Bacillus tequilensis* SX31 in cucumber for 3 weeks increased the activities of nitrogen metabolism-related enzymes (nitrate reductase, glutamine synthase, glutamine-2-oxoglutarate acid aminotransferase, and glutamate dehydrogenase) in cucumber (Wang et al., 2022).

Like nitrogen, phosphorus is another major element for plant growth. Phosphorous usually exists in the soil as insoluble and cannot be directly absorbed by plants. Some PGPB strains can dissolve phosphate in the soil as orthophosphate (PO_4^{3-}) to provide growth and development for plants (Etesami, 2020). In this study, 11 endophytic strains exhibited the phosphate solubilization trait, and the DJ06 strain showed the most potent ability. Similar to our research, other *Pseudomonas* stains, such as *P. sativum* L (Oteino et al., 2015), *P. aeruginosa* KUPSB12 (Paul and Sinha, 2017), and *P. plecoglossicida* (Astriani et al., 2020), were also reported as phosphate solubilizers. The genome of the DJ06 strain contains 11 genes related to phosphorus metabolisms, such as *phoADBRU*, *pit*,

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TABLE 5 Genes associated with PGP traits in *Pseudomonas aeruginosa* DJ06 genome.

PGP activities description	Gene name	Gene annotation	E.C. number	Chromosome location
Nitrogen fixation	-	Nitrogen fixation protein FixS OS	-	3119172–3118963, -
	-	Nitrogen fixation protein FixS OS	-	3123549–3122134, -
	iscU	nitrogen fixation protein NifU and related proteins	-	5861277–5860891, -
	-	Nitrogen fixation protein FixG OS	-	6223989–6225419, +
Ammonia assimilation	*gltB*	Glutamate biosynthetic process; ammonia assimilation cycle	1.4.1.13	1212098–1216543, +
	-	Glutamate biosynthetic process;;ammonia assimilation cycle	-	5621876–5623486, +
Nitrosative stress	*fpr*	Is involved in NO detoxification in an aerobic process	1.18.1.2 1.19.1.1	1720673–1721449, +
	-	Response to nitrosative stress	-	4540178–4539921, -
	hmp	Response to nitrosative stress	1.14.12.17	4541414–4540233, -
	fpr	Is involved in NO detoxification in an aerobic process	1.18.1.2 1.19.1.1	5402930–5402154, -
	gbcB	Is involved in NO detoxification in an aerobic process	-	795365–794265, -
	vanB	Is involved in NO detoxification in an aerobic process		1381843–1380890, -
	-	Ferredoxin reductase	-	1399767–1400867, +
ACC deaminase	-	1-aminocyclopropane-1-carboxylate deaminase	3.5.99.7	5071988–5071089, -
Siderophore	-	TonB-dependent siderophore receptor	-	88310–90703, +
	-	tonB-dependent siderophore receptor	-	134496–132304, -
	tonB	Siderophore transmembrane transporter activity	-	389670–388858, -
	-	Siderophore uptake transmembrane transporter activity	-	446014–443627, -
	tonB	Siderophore transmembrane transporter activity	-	656442–657473, -
	fabY	Siderophore biosynthetic process; cytosol	2.3.1.180	1058808–1056904, -
	-	Siderophore uptake transmembrane transporter activity	-	1456126–1458252, +
	fur	Ferric iron uptake transcriptional regulator	-	1542620–1543024, +
	tbpA	Siderophore transmembrane transport	-	1605480–1603186, -
	-	TonB-dependent receptor	-	1651522–1649294, -
	fiu	TonB-dependent siderophore receptor	-	1858562–1860823, +
	-	siderophore transmembrane transport	-	2267255–2265192, -
	fepA	TonB-dependent siderophore receptor	-	2442960–2445188, +
	fiu	TonB-dependent siderophore receptor	-	2862829–2865027, +
	-	TonB-dependent receptor	-	2906036–2908477, +
	-	TonB-dependent siderophore receptor	-	3525154–3522740, -
	fecI	Siderophore transport	-	3526815–3526309, -
	-	Hypothetical protein	-	3661863–3662819, +
	fhuE	TonB-dependent siderophore receptor	-	4124341–4121894, -
	mbtH	Chain X, Hypothetical Protein Pa2412	-	4160575–4160357, -
	-	Outer membrane receptor protein, mostly Fe transport	-	4258973–4256511, -
	fepA	Siderophore enterobactin receptor PfeA	-	4567882–4570122, +
	pfeE	Ferric enterobactin esterase PfeE	3.1.1.108	4570142–4571056, +
	-	TonB-dependent receptor	-	4868884–4871040, +
	fecA	TonB-dependent receptor family protein	-	5251782–5249617, -
	-	TonB-dependent copper receptor	-	5840255–5838096, -
	fecA	TonB-dependent receptor family protein	-	5953771–5956125, +

(Continued)

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TABLE 5 (Continued)

PGP activities description	Gene name	Gene annotation	E.C. number	Chromosome location
	-	TonB-dependent receptor	-	6256838–6254754, -
	fhuE	TonB-dependent siderophore receptor	-	6273393–6275801, +
	-	RhtX/FptX family siderophore transporter	-	6332484–6331240, -
	fhuE	TonB-dependent siderophore receptor	-	6336427–6334265, -
IAA production	*trpC*	Indole-3-glycerol phosphate synthase TrpC	4.1.1.48	6420339–6419503, -
	trpS	Tryptophanyl-trna synthetase	6.1.1.2	1970955–1972301, +
	trpE	Anthranilate synthase component I	4.1.3.27	2499727–2501298, +
	trpG	Anthranilate synthase component II	4.1.3.27	2501276–2501878, +
	trpG	Aminodeoxychorismate/anthranilate synthase component II	4.1.3.27	6421992–6421387, -
	trpE	Anthranilate synthase component I	4.1.3.27	6440605–6439127, -
Auxin biosynthesis	-	Auxin Efflux Carrier	-	727207–726263, -
	-	Auxin Efflux Carrier	-	3320366–3321253, +
	-	Auxin Efflux Carrier	-	402421–401540, -
Phosphate metabolism	*pit*	Inorganic phosphate transporter	-	2129593–2128124, -
	pstS	Phosphate-binding protein PstS OS	-	4352689–4354074, +
	pstS	Phosphate ABC transporter substrate-binding protein	-	843290–844261, +
	pstC	Phosphate ABC transporter permease	-	844432–846717, +
	pstA	Phosphate ABC transporter permease PstA	-	846737–848413, +
	pstB	Phosphate ABC transporter ATP-binding protein	7.3.2.1	848429–849262, +
	phoU	Chain A, Phosphate-specific Transport System Accessory Protein Phou Homolog	-	849358–850086, +
	phoR	Phosphate regulon sensor histidine kinase PhoR	-	854931–853600, -
	phoB	Phosphate regulon transcriptional regulator PhoB	-	855693–855004, -
	phoA	MULTISPECIES: alkaline phosphatase	3.1.3.1	5283151–5284581, +
	phoD	Alkaline phosphatase	3.1.3.1	5966444–5964882, -
Hydrolase	-	Chitinase	-	3999213–3997762, -
	-	Chitinase	-	4393909–4393280, -
	-	Chitinase	-	4699259–4698630, -
	-	Glycoside hydrolase family 19 protein	-	6423582–6422953, -
	-	Cellulase activity	-	3829462–3830538, +
	pslG	Cellulase family glycosylhydrolase	-	3928966–3930294, +
	bglB	Family 1 glycosylhydrolase	3.2.1.21	1352806–1351265, -
	bglX	Beta-glucosidase BglX	3.2.1.21	3311378–3309084, -
	folE2	GTP cyclohydrolase IB	3.5.4.16	650698–649802, -
	folE	GTP cyclohydrolase 1 2	3.5.4.16	3268888–3268343, -
	folE	Dihydromonapterin reductase	3.5.4.16	5443637–5444197, +
	ribA	MULTISPECIES: GTP cyclohydrolase II	3.5.4.25	6135458–6134841, -
	ribBA	GTP cyclohydrolase II	4.1.99.12 3.5.4.25	6140653–6139556, -
HCN	*hcnA*	Cyanide-forming glycine dehydrogenase subunit HcnA	1.4.99.5	3843114–3843428, +
	hcnB	Cyanide-forming glycine dehydrogenase subunit HcnB	1.4.99.5	3843425–3844819, +
	hcnC	Cyanide-forming glycine dehydrogenase subunit HcnC	1.4.99.5	3844822–3846075, +

(Continued)

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TABLE 5 (Continued)

PGP activities description	Gene name	Gene annotation	E.C. number	Chromosome location
Phenazine	*phzA_B*	Phenazine biosynthesis protein PhzA 2	-	3511140–3511628, +
	phzA_B	MULTISPECIES: phenazine biosynthesis protein phzB 2	-	3511664–3512152, +
	phzE	Phenazine-specific anthranilate synthase component I	2.6.1.86	3514010–3515893, +
	phzF	PhzF family phenazine biosynthesis protein	5.3.3.17	3515907–3516743, +
	phzA_B	Phenazine biosynthesis protein	-	6323470–6323958, +
	phzA_B	Phenazine biosynthesis protein	-	6323988–6324476, +
	phzF	PhzF family phenazine biosynthesis protein	5.3.3.17	6328241–6329077, +
Chemotaxis	*cheY*	Chemotaxis protein CheY	-	3019761–3020135, +
	cheZ	Chemotaxis protein CheZ	-	3020155–3020943, +
	cheA	Chemotaxis protein CheA	-	3021144–3023405, +
	cheB	CheB methylesterase	-	3023459–3024565, +
	cheW	Purine-binding chemotaxis protein CheW	-	3027272–3028162, +
	cheW	Chemotaxis protein CheW	-	3028208–3028687, +
	cheR	Chemotaxis protein methyltransferase 1 OS	2.1.1.80	5354447–5353623, -
	cheV	Chemotaxis protein CheV1 OS	-	5355456–5354524, -
	cheY	Chemotaxis protein CheY OS	-	412592–412957, -
	cheA	Chemotaxis protein CheA	-	412985–414913, +
	cheW	Chemotaxis protein CheW OS	-	414900–415385, +
	cheR	Chemotaxis protein methyltransferase 2	2.1.1.80	417530–418372, +
	cheD	Chemoreceptor glutamine deamidase CheD	3.5.1.44	418378–418980, +
	cheB	Chemotaxis response regulator protein	3.5.1.44	419000–420049, +
	aer	Aerotaxis receptor Aer	-	3134157–3132592, -
	mcp	Methyl-accepting chemotaxis protein	-	411222–412394, +
	pilK	Chemotaxis protein methyltransferase OS	-	166216–165341, +
	pilJ	Methyl-accepting chemotaxis protein (MCP)	-	168325–166277, -
	pilI	Chemotaxis protein CheW	-	168946–168410, -
Sulfur metabolism	*cysW*	Sulfate ABC transporter permease subunit CysW	-	304421–305290, +
	cysA	Sulfate ABC transporter ATP-binding protein	7.3.2.3	305294–306283, +
	cysU	Sulfate ABC transporter permease subunit CysT	-	303592–304410, +
	cysJ	PepSY domain-containing protein	1.8.1.2	1861039–1863591, +
	cysD	Sulfate adenylyltransferase subunit CysD	2.7.7.4	1966763–1967680, +
	cysNC	Sulfate adenylyltransferase subunit CysN	2.7.7.4 2.7.1.25	1967692–1969593, +
	cysZ	Sulfate transporter CysZ OS	-	2349639–2348899, -
	cysK	PLP-dependent cysteine synthase family protein	2.5.1.47	2564594–2565691, +
	cysC	Adenylyl-sulfate kinase	2.7.1.25	2950058–2949468, -
	cysP	Sulfate ABC transporter substrate-binding protein	-	3055525–3054527, -
	cysB	HTH-type transcriptional regulator CysB OS	-	3337225–3338199, +
	cysH	Phosphoadenylyl-sulfate reductase	1.8.4.8 1.8.4.10	3339402–3338668, -
	cysS	Ysteine–tRNA ligase	6.1.1.16	3387721–3389103, +
	cysI	Nitrite/sulfite reductase	1.8.1.2	3439778–3438120, -
	cysK	PLP-dependent cysteine	2.5.1.47	3751289–3752206, +
	cysG	Uroporphyrinogen-III C-methyltransferase	2.1.1.107	4479697–4478300, -

(Continued)

TABLE 5 (Continued)

PGP activities description	Gene name	Gene annotation	E.C. number	Chromosome location
	cysE	Serine O-acetyltransferase	2.3.1.30	5863969–5863193, -
	cysI	Nitrite/sulfite reductase	1.8.1.2	6223592–6221919, -
Biofilm formation	*flgB*	flagellar basal body rod protein FlgB	-	2580028–2580435, +
	flgC	flagellar basal body rod protein FlgC	-	2580441–2580881, +
	flgD	flagellar hook assembly protein FlgD	-	2580894–2581607, +
	flgE	Flagellar hook protein FlgE	-	2581635–2583023, +
	flgF	Flagellar basal-body rod protein FlgF	-	2583241–2583990, +
	flgG	Flagellar basal-body rod protein FlgG	-	2584037–2584822, +
	flgH	Flagellar L-ring protein precursor FlgH	-	2584868–2585563, +
	flgI	Flagellar basal body P-ring protein FlgI	-	2585575–2586684, +
	flgJ	Flagellar assembly peptidoglycan hydrolase FlgJ	-	2586695–2587897, +
	flgK	Flagellar hook-associated protein FlgK	-	2587916–2589964, +
	flgL	Flagellar hook-associated protein FlgL	-	2590003–2591304, +
	flgA	Flagellar biosynthesis protein FlgA	-	5355549–5356286, +
	flgM	Flagellar biosynthesis anti-sigma factor FlgM	-	5356431–5356754, +
	flgN	Flagellar protein FlgN	-	5356809–5357279, +
	motA	Flagellar motor protein MotA	-	1324368–1325219, +
	motB	Flagellar motor protein MotB	-	1325239–1326282, +
	motA	flagellar motor protein	-	3024654–3025394, +
	motB	flagellar motor protein MotD	-	3025407–3026297, +

pstABCS, and one unnamed gene. The *Pit* system is constitutive (Jansson, 1988). However, the *Pst* transporter is phosphate-inhibited and induced by phosphate-limiting conditions. Kwak et al. (2016) documented that *P. lutea* OK2T has potential PGP characteristics, including the ability to dissolve phosphate, and a large-scale value. The *PhoB* transcriptional regulator, part of the *Phog-PhoR* two-component signaling system, senses inorganic phosphate restriction, thereby turning on the expression of the *vreA*, *vreI*, and *vreR* genes that make up the operon (Faure et al., 2013).

The secretion of IAA is another direct plant-promoting characteristic of PGPBs such as *P. fluorescens* (Gravel et al., 2007), *P. aeruginosa* TQ3 (Khare and Arora, 2010), and *P. putida*1290 (Leveau and Lindow, 2005). In this study, the genome sequencing revealed that the genome of the DJ06 strain included the gene *trpCGES* encoding the enzyme related to the IAA synthesis pathway and three unknown genes for auxin efflux carrier. In addition, similar to our study, the presence of tryptophan-related genes is associated with IAA production in previously published bacterial genomes (Naveed et al., 2015; Liu et al., 2019). It was reported that *P. aeruginosa* 6A (BC4) (Marathe et al., 2017) and *P. putida* UB1 (Bharucha et al., 2013) enhance plant IAA levels and stimulate plant development. Previous research demonstrated that the tryptophan biosynthesis gene *trpABD* was involved in IAA production in the genome of *Sphingomonas* sp. LK11, and the siderophores secreted by PGPR strains are essential for plant growth under plant iron nutrient limitations (Asaf et al., 2018). In addition, the indole-3-pyruvate decarboxylase gene *ipd* was found in the genome of the strain *Acinetobacter calcoaceticus* SAVSo04, which produced high IAA *in vitro* (Leontidou et al., 2020). In the present study, the endogenous hormone IAA in the plant was significantly enhanced in two sugarcane varieties, GT11 and B8, as compared to the control at 60 days after inoculation.

Kurepin et al. (2015) reported that the root growth of potatoes correlated with the levels of phytohormones, IAA and GA_1, after inoculation with *Burkholderia phytofirmans* PsJN. Under cadmium (Cd) stress, *Brassica nigra* L. was inoculated with high IAA-producing strains *Lysinibacillus varians* and *P. putida*, and the results showed that the plant germination rate, root and stem length, chlorophyll content, and other growth parameters were all improved (Pal et al., 2019). The contents of GA_3 and ABA in the plant of sugarcane varieties, GT11 and B8, were decreased with the inoculation of the DJ06 strain in the present study. However, during salinity stress with inoculation of *Bacillus subtilis*, the contents of IAA, ABA, N, P, K+, Ca^{2+}, and Mg^{2+} enhanced significantly in radish (*Raphanus sativus*) in relation to control plants. In contrast, the contents of ABA, Na^+, and Cl^- significantly decreased, indicating that the inoculation with PGPB significantly changed the endogenous plant hormones (Mohamed and Gomaa, 2012).

The ability of the DJ06 strain to siderophore secretion was confirmed in the PGP test. Its encoding genes are mainly involved in siderophore transmembrane transporter activity, siderophore biosynthetic process, and TonB-dependent receptor, including

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TABLE 6 Genes involved in different abiotic stresses in *Pseudomonas aeruginosa* DJ06 genome.

Activities description	Gene name	Gene annotation	E.C. number	Chromosome location
Cold-shock protein	*cspA*	Cold-shock protein	-	2472057–2471443, -
	cspA	Cold-shock protein	-	5247298–5247507, +
	cspA	Cold-shock protein	-	106158–105949, -
	cspA	Cold-shock protein	-	2675545–2675754, +
	cspA	Cold shock domain protein CspD	-	4491483–4491755, +
Heat shock proteins	*htpX*	Heat shock protein HtpX	3.4.24.-	4785931–4786890, +
	hslR	Heat-shock protein	-	1034089–1034490, +
	ibpA	16 kDa heat shock protein B OS	-	5112206–5111757, -
	hslJ	Heat shock protein HslJ	-	2436184–2435777, -
Magnesium transport	*corA*	Magnesium transport protein CorA	-	947776–948807, +
	corC	HlyC/CorC family transporter	-	6048076–6048915, +
Copper homeostasis	*copB*	P-type Cu2+ transporter	7.2.2.9	3121586–3119151,-
	copZ	Heavy-metal-associated domain-containing protein		5518289–5518095, -
	copA	Copper-translocating P-type ATPase	7.2.2.8	5974427–5976805, +
	cusS	Heavy metal sensor histidine kinase CusS	2.7.13.3	1404894–1403503, -
	cusR	Copper resistance phosphate regulon response regulator CusR	-	1405670–1404873, -
	cusR	Heavy metal response regulator transcription factor	-	3001305–3001994, -
	cusS	Heavy metal sensor histidine kinase CusS	2.7.13.3	3002002–3003417, +
	cusR	Heavy metal response regulator transcription factor	2.7.13.3	4317562–4318236, +
	cusR	Heavy metal sensor histidine kinase CusS	-	4760863–4761543, +
	cusS	Heavy metal response regulator transcription factor	2.7.13.3	4761540–4762871, +
Zinc homeostasis	*znuB*	High-affinity zinc uptake system membrane protein znuB	-	688886–688098, -
	znuC	Zinc ABC transporter ATP-binding protein ZnuC	7.2.2.-	689688–688879, -
	znuA	Zinc ABC transporter substrate-binding protein	-	690261–691184, +
	znuA	Zinc ABC transporter substrate-binding protein	-	4155971–4156924, +
	znuC	Zinc ABC transporter substrate-binding protein	7.2.2.-	4156921–4157676, +
	znuB	Zinc transport system permease protein	-	4157673–4158578,+
Chromium homeostasis	*copB*	Multispecies: cadmium-translocating P-type ATPase	7.2.2.9	3121586–3119151, -
	-	Cadmium-translocating P-type ATPase	-	4198337–4196352, -
	czcA	Cobalt-zinc-cadmium resistance protein CzcA OS	-	4314233–4311078, -
	czcC	Cobalt-zinc-cadmium resistance protein CzcC OS	-	4317049–4315763, -
	czcD	Cobalt-zinc-cadmium efflux system protein	-	179617–180516, +
Drought resistance	*nhaB*	Na(+)/H(+) antiporter NhaB OS	-	3422913–3421411, -
	kdpA	Potassium-transporting ATPase subunit KdpA	-	3210235–3211929, +
	kdpB	Potassium-transporting ATPase subunit KdpB	7.2.2.6	3211941–3214013, +
	kdpC	Potassium-transporting ATPase subunit KdpC	-	3214067–3214618, +
	kdpD	Two-component system, OmpR family, sensor histidine kinase KdpD	2.7.13.3	3214726–3217383, +
	-	Citrate-Mg2+:H+ or citrate-Ca2+:H+ symporter, CitMHS family	-	725624–724320, -
	GDT1	Ca2+/H+ antiporter, TMEM165/GDT1 family	-	1697520–1696942, -

(Continued)

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TABLE 6 (Continued)

Activities description	Gene name	Gene annotation	E.C. number	Chromosome location
	kdpE	Response regulator	-	3217484–3218176, +
	proC	Pyrroline-5-carboxylate reductase	1.5.1.2	183803–184624, +
	proX	Glycine betaine/proline transport system substrate-binding protein	-	584983–585906, +
	proX	Glycine betaine/proline transport system substrate-binding protein	-	815673–816611, +
	proX	Glycine betaine/proline transport system substrate-binding protein	-	826054–826992, +
	proW	Glycine betaine/proline transport system substrate-binding protein		827034–827873, +
	proV	Glycine betaine/proline transport system ATP-binding protein	7.6.2.9	827877–829055, +
	proB	Glutamate 5-kinase	2.7.2.11	1779627–1780745, +
	proS	Chain A, Proline–tRNA ligase	6.1.1.15	2468134–2466419, -
	proA	Glutamate-5-semialdehyde dehydrogenase	1.2.1.41	6071938–6070673, -
	betA	Choline dehydrogenase	1.1.99.1	3771488–3773125, +
	betT	Choline/glycine/proline betaine transport protein	-	5994121–5996082, +
	betB	Betaine-aldehyde dehydrogenase	1.2.1.8	831810–833282, +
	trkA	Trk system potassium transporter TrkA	-	599227–600600, +
	trkH	Potassium uptake protein TrkH	-	5192548–5194002, +

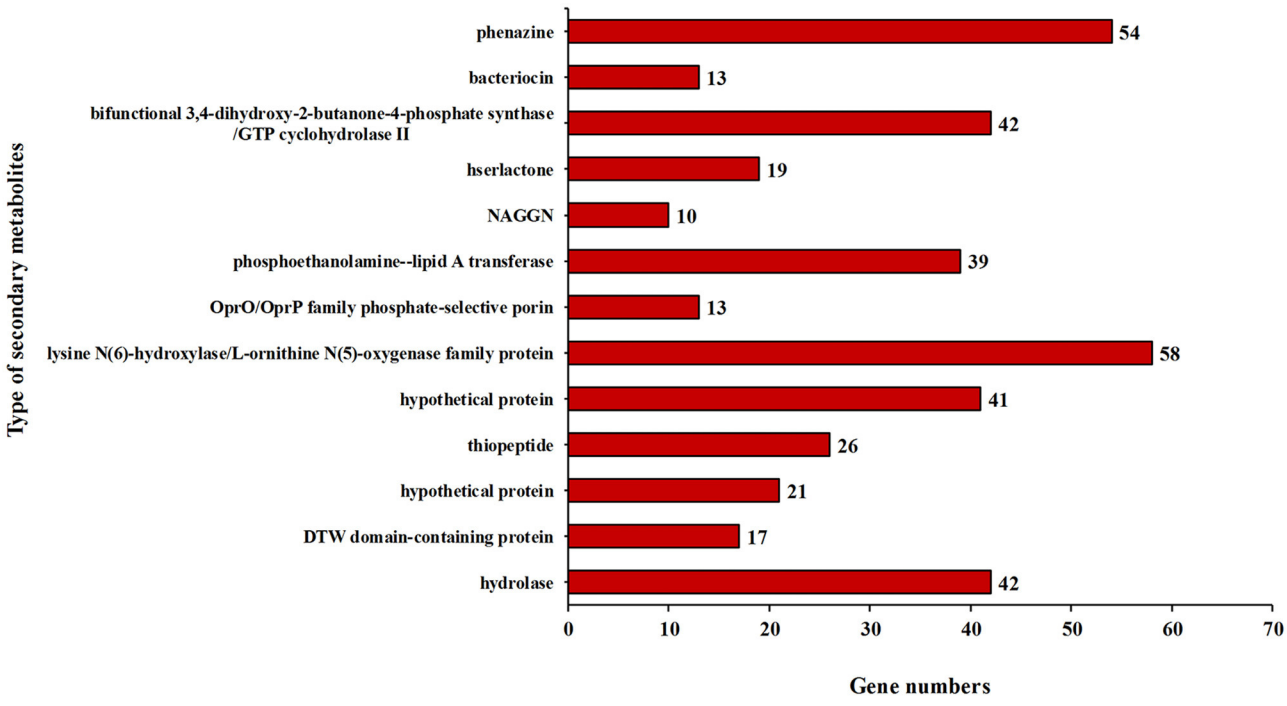

FIGURE 4
Secondary metabolic gene clusters in the genome of strain *Pseudomonas aeruginosa* DJ06.

various genes such as *tonB*, *fabY*, *fur*, *fepA*, *fecAEI*, and *fhuE*. Similar to the present study, the siderophore synthesis pathway-related gene *fepEGDC* also exists in the genome of strain *B. subtilis* EA-CB0575 (Franco-Sierra et al., 2020).

In the present study, we predicted the HCN and phenazine synthesis-related genes in the DJ06 strain genome. HCN is a volatile secondary metabolite that is naturally produced by bacteria and has an antagonistic effect against phytopathogenic fungi. It

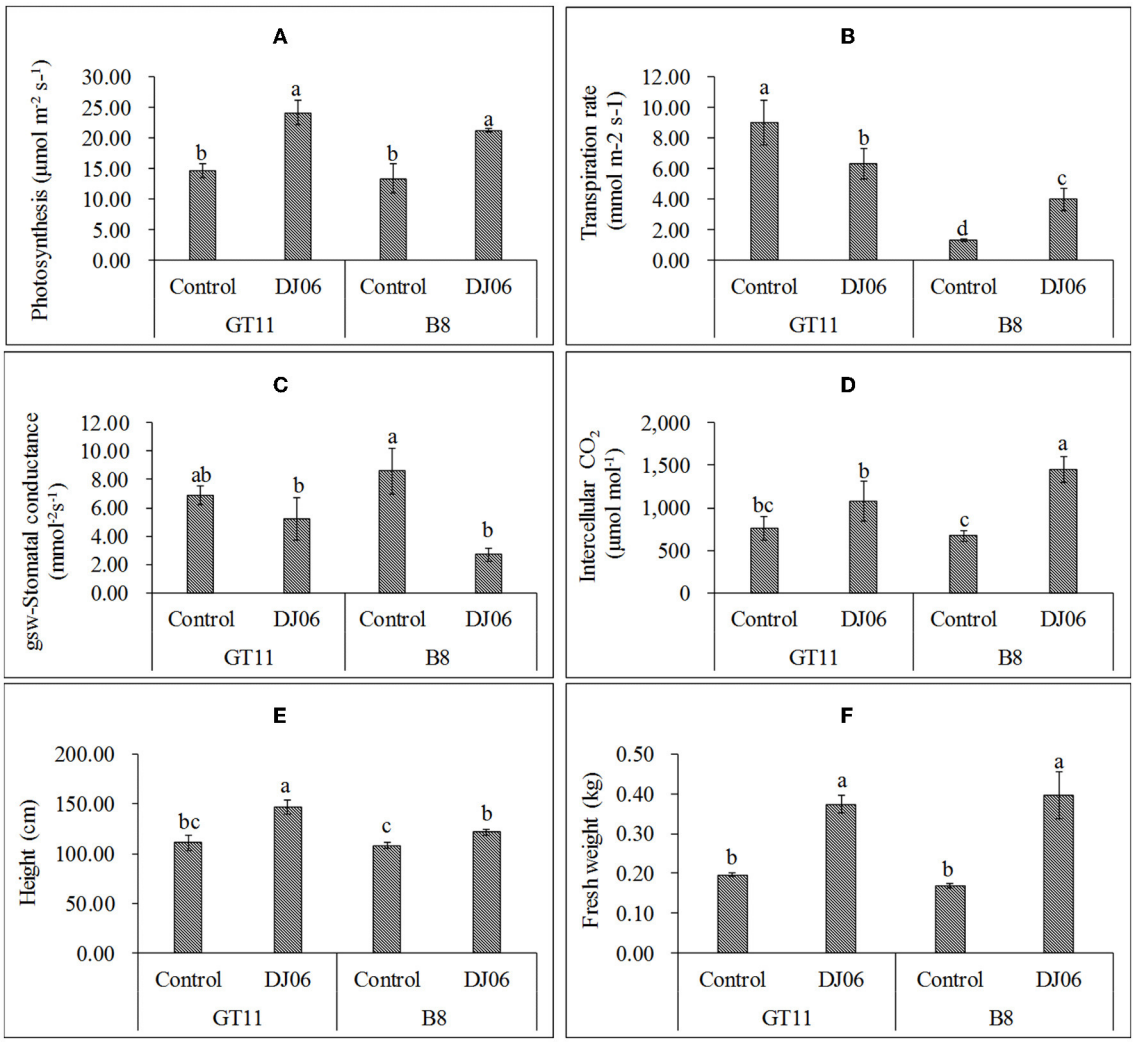

FIGURE 5
The agronomic growth parameters in sugarcane varieties GT11 and B8 60 days after inoculation with *Pseudomonas aeruginosa* DJ06 compared with the control. **(A)** Photosynthesis; **(B)** transpiration rate; **(C)** stomatal conductance; **(D)** intercellular CO_2; **(E)** height; and **(F)** fresh weight. The same letter indicated that no significant difference was detected at Duncan's multiple range test, $P \leq 0.05$ ($n = 3$).

has also been suggested that HCN has a fixative effect on iron, thereby directly increasing the availability of phosphate to promote plant growth (Sagar et al., 2018). Currently, the molecular data of HCN synthase are mainly related to *Pseudomonas* strains from the GenBank database (Rijavec and Lapanje, 2017). This study found cyanide-forming glycine dehydrogenase subunit-coding gene *hcnABC* in the DJ06 strain genome. Real-time reverse transcription PCR (qRT-PCR) analysis indicated that the expression of the *hcnC* gene in *Pseudomonas sp*. LBUM300 inoculated to strawberries was significantly stimulated by the infection of *Verticillium dahlia,* and the number of *Pseudomonas* strains was increased (DeCoste et al., 2010). Phenazine is another class of nitrogen-containing pigment secondary metabolite secreted by *Pseudomonas* with a significant ability to inhibit phytopathogens. Approximately 100 different phenazine derivatives have been identified recently, and 6,000 related compounds have been synthesized (Bilal et al., 2017). A total of two redundant operons, *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*), in *P. aeruginosa* have been reported to encode nearly identical proteins, which are precursors to several phenazine derivatives (Recinos et al., 2012). In this study, the phenazine synthesis-coding genes, *phzA_B* and *phzF,* were found in the genome of the DJ06 strain. Similarly, these genes were also predicted in *P. chlororaphis* GP72 (Shen et al., 2013), *Pantoea agglomerans* C1 (Luziatelli et al., 2020), and *P. aeruginosa* B18 (Singh et al., 2021). In addition, we observed several gene clusters related to hydrolase synthesis in the DJ06 strain genome, including *pslG*, *bglBX*, *folE2*, *folE*, *ribA*, *ribBA,* and some unknown genes. These genes encode the proteins of chitinase, cellulase, β-glucosidase, etc., which effectively destroy the cell wall of phytopathogenic fungi to realize biocontrol. Similar genes have also been predicted in other strain genomes (Shariati et al., 2017; Luo et al., 2018).

Abiotic stress can cause severe damage to plant productivity and sometimes total mortality (Mittler, 2006). Currently, a variety of PGPB strains have been successfully applied to alleviate crop abiotic stresses. In this study, we predicted a large number of drought resistance and heavy metal toxicity-related genes in the

genome of the DJ06 strain related to magnesium (Mg) transport (*corAC*), copper (Cu) homeostasis (*copABZ*, *cusSR*), zinc (Zn) homeostasis (*znuABC*), chromium homeostasis (*copB*, *czcACD*), etc. These genes encode the proteins associated with the tolerance to divalent cations of Mg, Cu, Zn, and chromium due to their ability to automatically efflux metal ions (Mima et al., 2009). Cu is an essential component of biological evolution and a metal cofactor for several enzymes such as monooxygenase, dioxygenase, and SOD (Giner-Lamia et al., 2012). Excess Cu harms plants by inhibiting shoot and root growth, reducing respiration and photosynthesis, and altering enzymatic activities (Rajput et al., 2018). Zn is transferred in the form of Zn^{2+} and absorbed by plants for maintaining biological structural and functional integrity, promoting protein synthesis, gene expression, enzyme structure, energy production, and positively impacting crop plants, especially in plant productivity (Mousavi et al., 2013). However, excess Zn may inhibit the activity of plant photosystem II (PS II), decrease RUBP carboxylase, and induce Zn toxicity when the concentration exceeds 300 ppm (Prasad et al., 1999). The ability to resist Zn is different in diverse plants (Vitosh et al., 1994). Therefore, various metal ion metabolism-related genes in the DJ06 genome may play an essential role in mitigating heavy metal toxicity. In addition, the transpiration rates in the two sugarcane varieties, GT11 and B8, showed differences after inoculation with the DJ06 strain. We speculate that it may be due to the genetic differences between sugarcane varieties.

Some PGPB strains could alleviate drought stress in the plant growth process. It was reported that PGPB strains secreted 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4), which catalyzes ACC to α-ketobutyric acid when plants respond to biotic/abiotic stresses and ammonia to regulate ethylene stress to protect plants from damage (Danish et al., 2020; Ullah et al., 2021). The ACC deaminase-encoding gene was also found in the DJ06 genome, and similar genes also appeared in the genomes of strains ED5 and B18 (Guo et al., 2020; Singh et al., 2021). Currently, the PGPB strains, which produce ACC deaminase, are widely used in the study of drought resistance of crops, such as *B. amyloliquefaciens* (Danish and Zafar-Ul-Hye, 2019), *Agrobacterium fabrum* (Munir and Zafarul-Hye, 2019), and *Pseudomonas* spp. (Shaharoona and Mahmood, 2008). In addition, drought stress-related genes, such as *nhaB*, *kdpABCDE*, *GDT1*, and *proCX*, were also predicted in the DJ06 genome. At the same time, it was found that the activities of oxidative stress enzymes (SOD, POD, and CAT) and the content of oxidative product MDA in sugarcane varieties, GT11 and B8, inoculated with the DJ06 strain was significantly changed as compared with the controls in this study, indicating that the DJ06 strain stimulated the occurrence of physiological responses of sugarcane, and then enhance the ability to adapt to the external environment. The variations in SOD, POD, and MDA activities after inoculation of the DJ06 strain may differ from the genetic variability in the two sugarcane varieties (Guo et al., 2021). In addition, this study found that there were mobile genetic elements (MGEs) in the genome of the DJ06 strain, such as the CRISPR system and prophage, which play an important role in PGPB strains for adaptation to the environment, and similar results were also reported in *Enterobacter roggenkampii* ED5 (Guo et al., 2020).

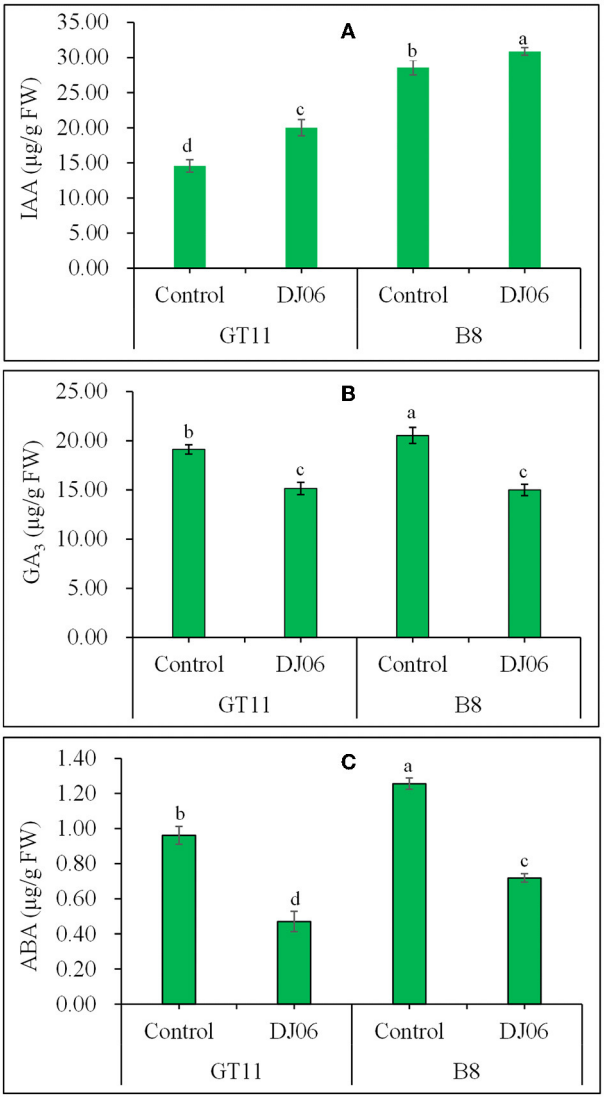

FIGURE 6
The endogenous hormones in sugarcane varieties GT11 **(A)** and B8 **(B)** 60 days after inoculation with *Pseudomonas aeruginosa* DJ06 compared with the control. **(A)** IAA; **(B)** GA_3; **(C)** ABA. The same letter indicated that no significant difference was detected at Duncan's multiple range test, $P \leq 0.05$ ($n = 3$).

Sulfur is a vital nutrient element for plant growth and development, which is related to plant stress resistance (Gill and Tuteja, 2011), and sulfur deficiency in crops will lead to severe yield losses. Some scholars have found the function of the *cysP* gene in *Bacillus subtilis* by transforming *Escherichia coli* with the plasmid of the *cysP* gene through the sulfate transport mutant; that is, the *cysP* gene operon in *B. subtilis* is responsible for sulfur metabolism (Aguilar-Barajas et al., 2011). Some transporter-related genes, such as *cysABCDEGKHIUWSPZ*, were found in the DJ06 genome. It has been reported that these genes may be involved in the transport of thiosulfate or inorganic sulfate into cells (Duan et al., 2013) and possibly the oxidation of sulfur and sulfur-conjugated secondary metabolites (Kwak et al., 2014). In addition, sulfur oxidation affects soil pH and gradually increases the solubility of nutrients, such as

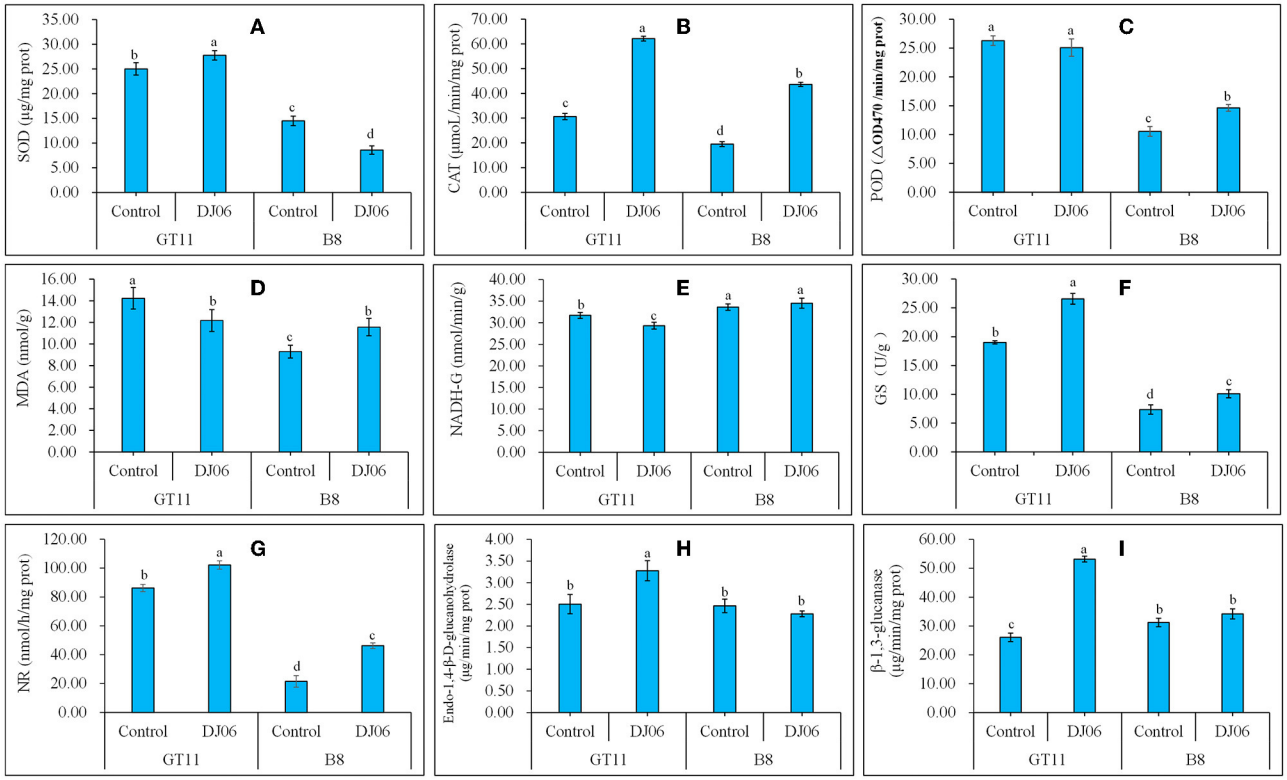

FIGURE 7
The activities of various physiological enzymes and the content of MDA in sugarcane varieties GT11 and B8 60 days after inoculation with *Pseudomonas aeruginosa* DJ06 compared with the control. **(A)** SOD; **(B)** CAT; **(C)** POD; **(D)** MDA; **(E)** NADH-GDH; **(F)** GS; **(G)** NR; **(H)** Endo-1,4-β-D-glucanohydrolase; **(I)**, β-1,3-glucanase. The same letter indicated that no significant difference was detected at Duncan's multiple range test, $P \leq 0.05$ ($n = 3$).

N, P, K, Mg, and Zn, which can enhance plant uptake of mineral nutrients (Wainwright, 1984).

Plant growth-promoting bacteria (PGPB) usually colonize inside different parts of plants, such as roots, stems, and leaves, and perform beneficial functions (Liu et al., 2011). The colonization of PGPB in plants is a prerequisite for mutualistic symbiosis. In this study, the SEM and GFP labeling techniques were used to confirm that the DJ06 strain colonized in sugarcane tissues successfully. The analysis of *Pseudomonas aeruginosa* DJ06 revealed that many colonization-related and signal transduction chemotaxis genes, such as *cheABDRVWYZ*, *pilIJK*, *aer,* and *mcp*, play essential roles in microbe–plant interactions (Drr et al., 2010). Similar results were also found in the genome of the strain *P. fluorescens* PCL1751 (Cao et al., 2015).

Biofilms are constrained by polymers, such as autogenic exopolysaccharides, extracellular DNA, and biological surface-associated proteins (Bogino et al., 2013; Teschler et al., 2015). PGPBs in plants' interior or rhizosphere region form biofilms to coexist with plants. PGPBs mutually beneficially coexist with plants by forming biofilms inside plant roots or in the rhizosphere region. Previous reports showed the antagonistic activities of *Paeni bacillus*, *B. cereus,* and *P. stutzeri* against phytopathogens by initiating biofilms (Xu et al., 2014; Salme et al., 2015; Wang et al., 2017). In biofilms, cell-to-cell communication enhances gene upregulation and downregulation, thereby improving the fitness of microorganisms in both biotic and abiotic environments. In the genome of the DJ06 strain, several flagellar motility protein-encoding genes related to biogenesis were also found, such as *flgABCDEFGHIJKLMN* and *motAB*, which indicated that the DJ06 strain has potential plant growth-promoting properties.

Conclusion

This study showed the plant growth-promoting effects of endophytic PGP strain *P. aeruginosa* DJ06 isolated from sugarcane root. It was confirmed that the DJ06 strain could successfully colonize sugarcane tissue. The complete genome study of the DJ06 strain exhibited the presence of various PGP genes in its genome. The inoculation of the DJ06 strain inoculation also significantly increased some agronomic parameters, i.e., plant height, fresh weight, and photosynthesis in sugarcane varieties GT11 and B8 as compared with the control under greenhouse conditions. In addition, the endogenous plant hormones and physiological enzyme activities in the two sugarcane varieties also changed significantly after the inoculation. Our findings provide a reference for the molecular interaction mechanism between the DJ06 strain and sugarcane.

Data availability statement

The datasets presented in this study can be found in the NCBI repository, accession number CP080511: https://www.ncbi.nlm.nih.gov/nuccore/CP080511.

Author contributions

D-JG, D-PL, RS, and Y-RL: planned the proposal and experiments. D-JG and D-PL: accomplished the experiments. KV, QK, YQ, T-SC, B-QZ, and X-PS: data examination. Y-RL: study and resources. D-JG and PS: writing the original manuscript. BY and Y-RL: 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

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1096754/full#supplementary-material

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Arbuscular mycorrhizal fungi-mediated activation of plant defense responses in direct seeded rice (*Oryza sativa* L.) against root-knot nematode *Meloidogyne graminicola*

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Rhizosphere is the battlefield of beneficial and harmful (so called phytopathogens) microorganisms. Moreover, these microbial communities are struggling for their existence in the soil and playing key roles in plant growth, mineralization, nutrient cycling and ecosystem functioning. In the last few decades, some consistent pattern have been detected so far that link soil community composition and functions with plant growth and development; however, it has not been studied in detail. AM fungi are model organisms, besides potential role in nutrient cycling; they modulate biochemical pathways directly or indirectly which lead to better plant growth under biotic and abiotic stress conditions. In the present investigations, we have elucidated the AM fungi-mediated activation of plant defense responses against *Meloidogyne graminicola* causing root-knot disease in direct seeded rice (*Oryza sativa* L.). The study describes the multifarious effects of *Funneliformis mosseae, Rhizophagus fasciculatus,* and *Rhizophagus intraradices* inoculated individually or in combination under glasshouse conditions in rice plants. It was found that *F. mosseae, R. fasciculatus* and *R. intraradices* when applied individually or in combination modulated the biochemical and molecular mechanisms in the susceptible and resistant inbred lines of rice. AM inoculation significantly increased various plant growth attributes in plants with simultaneous decrease in the root-knot intensity. Among these, the combined application of *F. mosseae, R. fasciculatus,* and *R. intraradices* was found to enhance the accumulation and activities of biomolecules and enzymes related to defense priming as well as antioxidation in the susceptible and resistant inbred lines of rice

pre-challenged with *M. graminicola*. The application of *F. mosseae, R. fasciculatus* and *R. intraradices*, induced the key genes involved in plant defense and signaling and it has been demonstrated for the first time. Results of the present investigation advocated that the application of *F. mosseae, R. fasciculatus* and *R. intraradices*, particularly a combination of all three, not only helped in the control of root-knot nematodes but also increased plant growth as well as enhances the gene expression in rice. Thus, it proved to be an excellent biocontrol as well as plant growth-promoting agent in rice even when the crop is under biotic stress of the root-knot nematode, *M. graminicola*.

KEYWORDS

AM fungi, root-knot nematode, rice (*Oryza sativa* L.), *Meloidogyne graminicola*, *Funneliformis mosseae*, *Rhizophagus fasciculatus*, *Rhizophagus intraradices*, plant defense

Introduction

Rice (*Oryza sativa* L.) is one of the most important and staple food crops in South East Asia including India plays a vital role in the food security, livelihood wellness and country's economy (Mahajan et al., 2017). Rice is grown on an area of 43.78 mha in India with an annual production of 127.9 million tonnes approximately (Anoymous, 2022). Direct-seeded rice covers 23% of the area worldwide (Rao et al., 2007; Marasini et al., 2016; Devaraja et al., 2017). Aside from the benefits of direct-seeded rice, the productivity of direct-seeded rice is hindered by various biotic and abiotic stresses (Singh et al., 2012a,b,c, 2016, 2021). Nematode infestation is one of the major biotic factors that can cause yield losses of up to 72% in rice (Khan and Ahamad, 2020). *Ditylenchus angustus*, *Meloidogyne graminicola*, *Aphelenchoides besseyi*, *Hirschmanniella* spp. and *Heterodera oryzicola* are the prevalent nematode species which invade rice (Walia and Bajaj, 2003; Wesemael et al., 2011; Singh et al., 2012c). Among them, *M. graminicola,* an endo-parasitic sedentary nematode causing root-knot disease is the most notorious pathogen of rice. It has been reported from every rice-producing regions of the world and causes a 10.54 per cent production loss in direct-seeded rice in India, costing about 779.30 million rupees every year (Singh et al., 2007; Kumari et al., 2016; Zhou et al., 2020). When environmental conditions are favorable, the infectious juvenile stage (J_2) emerges from the egg, locates the root, and moves into the meristematic zone and feeds continuously to stimulate the production of huge galls which leads to impairment in the nutrient and water uptake and translocation (Devaraja et al., 2017; Sacchi et al., 2021). *M. graminicola* is an obligate biotroph causing stunting, wilting and decreased tillering; juvenile plants show chlorosis, and mature spikelets with empty florets, resulting in a substantial loss in growth and yield in rice leading to huge losses of foreign exchange (Singh et al., 2007; Jain et al., 2012; Khan and Ahamad, 2020). The direct impairment caused by root-knot nematodes can be intensified by secondary infections of the wounded plant tissues by other pathogens (Walia and Bajaj, 2003; De Waele and Elsen, 2007; Upadhyay and Dwivedi, 2008).

Conventionally root-knot nematode (RKN) is controlled by integrating chemical nematicides with cultural methods and resistant cultivars (Walia and Bajaj, 2003). Chemical nematicides are very expensive and have a negative impact on the useful microorganisms and fauna found in agricultural soil as well as could lead to the development of resistant pathogenic strains (Walia and Bajaj, 2003; Singh et al., 2007, 2012a; Upadhyay and Dwivedi, 2008). In recent decades, a number of compounds, including methyl bromide and aldicarb, have been taken off the market because of risks to the environment, human health, and non-target organisms (Kim et al., 2018; Xiang et al., 2018). Resistance development in the pathogens, residual toxicity of chemical nematicides on the environment and animal health, with the possible withdrawal of pesticides specially nematicides and soil fumigants from the schedule of pesticides and the demand for residue-free produce have obligated researchers and rice growers to explore suitable alternatives strategies for the management of RKN in agriculturally important crops including rice (Singh et al., 2007, 2012a,b,c, 2013a,b, 2017). Among the possible alternatives, the development of resistant cultivars with a high degree of resistance to RKN is of great importance (Walia and Bajaj, 2003; Kumari et al., 2016; Zhou et al., 2020). Moreover, availability of resistance genes in the suitable donor parents is not an easy task. As the resistance is polygenic which further increases the difficulties associated with resistance breeding programme. Further, the pyramiding/transfer of desired gene(s)/quantitative trait loci (QTLs) into commercial cultivars using a resistance breeding programme is a great challenge to rice breeders (Singh et al., 2013a; Forghani and Hajihassani, 2020; Dash et al., 2021; Sun et al., 2021). Under these circumstances, the use of microbe-based strategies is a more environment-friendly, residue-free, safer, and emerging approach to combat RKN effectively (Walia and Bajaj, 2003; Singh et al., 2012a,b,c, 2013a,b, 2017). In recent past, several workers evaluated and used biological control agents of microbial origin to control RKN in many crops including rice. Among them, fungal bioagents (*Arthrobotrys oligospora, Monacrosporium eudermatum, Drechslerella dactyloides, Dactylaria brochopaga, Trichoderma harzianum, T. asperellum, T. virens*), and bacterial (*Bacillus subtilis, Paecilomyces lilacinus, B. licheniformis, B. cereus, Pseudomonas fluorescens,* and *Streptomyces cacaoi*) are noteworthy (Singh et al., 2012a,b,c, 2013a,b, 2017, 2020; Abd-Elgawad and Askary, 2018; Haque et al., 2018; Zhao et al., 2018;

DiLegge et al., 2019; Molinari and Leonetti, 2019; Seo et al., 2019; Singh U. B. et al., 2019; Forghani and Hajihassani, 2020; Sun et al., 2021). Similarly, few studied were made on the application of AM fungi for controlling nematodes in agriculturally important crops (van der Heijden et al., 2015; Bagyaraj et al., 2022).

Recently, several studies reported that arbuscular mycorrhizal fungi (AMF) may offer a safer alternative to pesticide use as the demand for environmental and agricultural safety grows (Harrier and Watson, 2004; Dong et al., 2006; Yang et al., 2014; Bagyaraj et al., 2022). AMF, which are found naturally in soil and behave as bio-stimulators and bio-protectors, may be extremely advantageous to sustainable agriculture by preserving plant productivity and alleviating soil-borne plant pathogens while causing no harm to the environment (Newsham et al., 1995; Smith and Smith, 2011; Koffi et al., 2013; da Silva Campos, 2020). Though there are different kinds of mycorrhiza, the most common mycorrhizal association occurring in crops important in agriculture is the arbuscular type (Babikova et al., 2013; Ceballos et al., 2013; van der Heijden et al., 2015; Bagyaraj et al., 2022). AMF are obligate root symbionts, appraised to colonize more than 80% of all land plant species and they are beneficial for the growth of host plants (van der Heijden et al., 2015). In exchange for photosynthetic carbon from their host, they boost plant growth and development by enhancing nutrient uptake (Bonfante and Genre, 2010; Smith et al., 2010; Bender et al., 2015; Bagyaraj et al., 2022) and also help plants to cope with various stresses imposed by abiotic and biotic elements, including parasitic nematodes on plants (Singh et al., 2012c; Schouteden et al., 2015; van der Heijden et al., 2015). Typically found in the rhizosphere, phytopathogenic nematodes including RKN colonize the roots of their host plants and have opposing effects on the health of those plants (Kumari et al., 2016; Zhou et al., 2020). However, few reports indicated that AMF inoculation reduces the infestation of plant roots by phytopathogenic nematodes (Agrios, 2005; Elsen et al., 2008). Growing plants with AMF inoculation in the nursery can increase their growth and safeguard them from infection caused by soil-borne phytopathogens including phytopathogenic nematodes (Elsen et al., 2008). The AMF can function as biocontrol agents through direct or indirect processes, increasing nutrient uptake and mitigating the harm caused by nematodes (Bagyaraj and Gautam Chawla, 2012). Direct impacts of AMF on the pathogen, such as competition for space or nutrients, or indirect, plant-mediated effects, can be implicated in AMF-mediated biocontrol. The latter can be further sub-divided into AMF's influence on plant tolerance, plant defense induction, and altered plant exudation, all of which result in modifications in rhizosphere interactions (Kuila and Ghosh, 2022). The various mechanisms cannot be considered totally independent of one another, and biocontrol is most likely the outcome of a mix of mechanisms (Bell et al., 2016). Usually, AMF exhibit an antagonistic effect on plant-parasitic nematodes, and several studies have shown a significant reduction in nematodes when healthy mycorrhizal diversity present in the rhizosphere (Heinen et al., 2018; Gough et al., 2020; Poveda et al., 2020). In recent years, many investigations have been reported where AMF showed protective effects against plant parasitic nematodes (PPN) in various crop plants (Bagyaraj and Gautam Chawla, 2012; Vos et al., 2012, 2013; Alban et al., 2013; Bagyaraj, 2016; Sharma et al., 2021). However, only a few studies have been conducted so far to investigate the applications of AMF for the biocontrol of PPN in India (Bagyaraj and Gautam Chawla, 2012; Bagyaraj, 2018), and it is a need of the hour to explore these fungal symbionts for control of PPNs, especially RKN. Further there is no information available on AMF inducing key genes involved in plant defense and signaling for the biocontrol of RKN. With this in mind, the current study was conducted to investigate the role of selected AMF in the activation of plant defense responses against root-knot nematode *Meloidogyne graminicola* in rice (*Oryza sativa* L.) under direct seeded conditions.

Materials and methods

Source of media and reagents

Corn meal agar, potato dextrose agar and other media reagents were procured from HiMedia Private Limited (Mumbai, India). Other chemical reagents including analytical grade solvents and chemicals were purchased from E. Merck, Mumbai, India. Oligo nucleotide primers used in the present investigation were synthesized from Eurofins Genomics Services, Hyderabad, India. However, molecular grade chemicals were procured from BioRAD, India, Thermo Scientific, USA, and Bangalore GeNi, India.

Source of AM fungi and inoculation techniques

The AMF fungal (*Funneliformis mosseae, R. fasciculatus,* and *R. intraradices*) inocula obtained from Centre For Natural Biological Resources and Community Development, Bangalore, India were maintained in pots with sterilized coco peat mixture (Ajay Kumar & Company, New Delhi, India) as the substrate and maize (*Zea mays* L., *cv*. Sujata) as the host according to Barea and Azcon-Aguilar (1983) with slight modifications. The AMF inoculum needed for the pot experiments, coco peat mixture was sterilized by autoclaving (at 121 °C for 20 min) twice at 24 h intervals. Thereafter, the coco peat mixture was kept as it is for the next 3 days to maintain the equilibrium of the cations and anions. The sterile coco peat mixture (1 kg) was filled in plastic pots (10 × 12 cm). To each pot, 10 g of AMF inoculum consisting of chopped AM-colonized root pieces along with the substrate containing spores and hyphal bits was added and mixed with the sterile coco peat. Ten healthy seeds of maize (*cv*. Sujata) were sown in each pot and watered as and when required. A total of 50 mL of Hoagland's solution (without KH_2PO_4) (Hoagland and Arnon, 1950) was added to each pot at 15-days interval. After 75 days of sowing, the above-ground parts of the plants were cut and the roots separated from the substrate, cut into approximately 1 cm pieces, mixed with the substrate containing spores and hyphae, air-dried and used as the inoculum. The percent of AM root colonization and AM spore counts was calculated using the method of Gerdemann and Nicolson (1963) and Phillips and Hayman (1970) at 90 days of inoculation.

Preparation of pathogen inoculum

Meloidogyne graminicola infested rice plants were collected from Research farm, Botanical Research Unit, Dhangain,

Bikramganj, Rohtas; Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and farmers' field, Rohtas districts of Southern Bihar, India and brought to the laboratory. Infested roots and galls were separated from plants using sharp scalpel and forceps. Infected roots and knots/galls were washed gently under running tap water. The 2nd stage juveniles (J_2) were extracted following the protocols described by Singh et al. (2013a) with slight modifications. In brief, separated and cleaned root knots/galls were cut into small pieces with a sharp scalpel and placed on moist qualitative filter paper (Grade 4) mounted on cavity blocks containing sterilized distilled water with streptocycline (75 mg l^{-1}) and cycloheximide (25 mg l^{-1}). These cavity blocks were incubated at 27 ± 2°C for a week. A hemocytometer and nematode counting disc were used to count the number of second-stage juveniles (J2) (Singh, 2007) which were further used as nematode inoculum (2,500 J_2 pot^{-1}) (Singh et al., 2012c).

Soil collection, preparation, and analysis

The experimental soil used in pot experiments was collected from the Research farm, Botanical Research Unit, Dhangain, Bikramganj, Bihar, India (coordinates: 25°21′2752″N 84°25′1975″E, Elevation 77 m) and brought to the laboratory. To eliminate the extra moisture, the soil was sieved (2 mm pore size) and air-dried. Vermiculture (obtained from ICAR-Indian Institute of Seed Sciences, Kushmaur, India) and river sand were blended into the soil in a 2:1:1 ratio (w/w). Required quantities of fertilizers for 3 kg soil were calculated and applied in liquid form using 0.314 g, 0.195 g, and 0.150 g of urea, diammonium phosphate and muriate of potash per pot, respectively. This represented the recommended dose of 120 kg ha^{-1} N, 60 kg ha^{-1} P_2O_5, and 60 kg ha^{-1} K_2O for direct seeded rice. Thereafter, experimental soil was sterilized by steam-sterilization (autoclaving) at 121°C for 30 min twice at 24 h intervals. After the second sterilization, the soil was kept as it is for the next 4–5 days to maintain the ionic balance/equilibrium in the cations and anions. Analysis of the physico-chemical characteristics of experimental soil was done using standard protocols described by Sparks et al. (2020).

Planting materials and growth conditions

Susceptible and resistant breeding lines of rice (Pusa Basmati-1 and Jasmine 85, respectively) were obtained from the Department of Plant Breeding and Genetics, Veer Kunwar Singh College of Agriculture (Bihar Agricultural University-BAU, Sabour), Dumraon, Buxar, Bihar, India. Seeds were planted in pots (20 cm × 25 cm) under glass house conditions and each pot contained 3 kg of experimental soil with and without inoculation of AMF. The trials were conducted from July to September with a 13/−11 h light/dark photoperiod and relative humidity between 85 and 95 per cent. The annual average temperature in the region ranges from 27 to 32°C and the annual rainfall from 750 to 825 mm.

Experimental set-up

Biocontrol efficacy of the AMF *F. mosseae, R. fasciculatus* and *R. intraradices* against root-knot diseases of rice were evaluated under glasshouse conditions at Veer Kunwar Singh College of Agriculture (Bihar Agricultural University-BAU, Sabour), Dumraon, Buxar, Bihar, India and ICAR-National Bureau of Agriculturally Important Microorganisms, Kushmaur, Maunath Bhanjan, India. The treatments were: *M. graminicola* (alone) (T_1), *F. mosseae* + *M. graminicola* (T_2), *R. fasciculatus* + *M. graminicola* (T_3), *R. intraradices* + *M. graminicola* (T_4), *F. mosseae* + *R. fasciculatus* + *R. intraradices* + *M. graminicola* (T_5), and untreated Control (T_6). In glasshouse trials, there were ten replications of each treatment using a completely randomized block design (CRBD).

To each pot, 10 g of AMF inoculum consisting of chopped AMF-colonized root pieces of maize, along with substrate containing about 75–100 AM spores g^{-1}, was added. Rice seeds (inbred lines, PB-1 and Jasmine 85) were soaked in a brine solution (5% NaCl) for 10 min to remove the undersize and unhealthy seeds which floated on the surface. The healthy seeds, thus obtained, were surface-sterilized with sodium hypochlorite solution (NaOCl, 1% v/v) according to Singh et al. (2016). Ten seeds were sown in each pot with different treatments, later thinned to five when the third leaf appeared. After 30 days of sowing, second-stage juveniles (2,500 kg^{-1} of soil) were introduced near the root system. Nematode-free soil without AMF inoculum served as a control. The pots were randomly arranged according to CRBD in the glasshouse. Further, the positions of pots was re-randomized at weekly interval. To maintain the soil moisture, pots were watered on alternate days. The moisture was maintained at 60–70% of water holding capacity. The leachate came out from the hole was collected in saucer and reintroduced to the same pot. During the experimentation, temperature ranged from 25 to 32°C with 13/11 h photoperiod.

Sampling and analysis

AM root colonization

After 30 and 45 days of sowing, the plants were up-rooted randomly from each treatment, washed in running tap water and brought to the laboratory. Five replicates per treatment were used. The mycorrhizal parameter, per cent root colonization was estimated following standard procedures (Phillips and Hayman, 1970; Giovannetti and Mosse, 1980).

Effect of AM inoculation on physio-biochemical parameters and antioxidant enzymes

The quantitative estimation of total chlorophyll content in the plant leaves was done according to Ferjani et al. (2003). The changes in the accumulation of total soluble sugar (TSS), total protein (TP), and total phenolic compounds (TPC) in the leaves and roots of plant primed with AMF fungal inocula and pre-challenged with the *M. graminicola* was estimated as per methods described by Sadasivam and Manickam (1996). Further, the activities and accumulation of phenylalanine ammonia-lyase (PAL), peroxidase (POx), ascorbate peroxidase (APx), polyphenol oxidase (PPO),

superoxide dismutase (SOD), and catalase (CAT) were measured spectrophotometrically in the leaves and roots of AMF inoculated plants as per the protocols described by Thimmaiah (2012) at 30 days after pathogen inoculation (DAPI).

Further, AMF inoculation is known to affect the lignin content in plants challenged with biotic stresses. Hence, the rice root samples were collected 45 DAPI, washed thoroughly in running tap water and air-dried. The lignin content in the root samples was estimated spectrophotometrically as per the protocol defined by Yang et al. (2018).

Effect of AM inoculation on activation of defense-related pathways/cascades in roots

To see the effect of AM inoculation on the activation of defense-related pathways/cascades in the resistant and susceptible cultivar/inbred lines of rice pre-challenged with *M. graminicola,* quantitative real-time PCR analysis was carried out. In the present investigation, the expression of key genes involved in the induced systemic resistance, signaling processes, jasmonate biosynthesis, ethylene biosynthesis, BR biosynthesis, PR proteins, and lignin biosynthesis was studied in the rice plant under different treatments using qPCR. Sequences of 9 key genes regulating the phenylpropanoid cascade in rice were retrieved from National Centre for Biotechnology Information (NCBI) database. Gene-specific primers were designed for qPCR analyses and *in silico* validation of these primers was done. The 9 key genes analyzed were: Phenylalanine ammonia-lyase (*OsPAL*), Phenylalanine/tyrosine ammonia-lyase (*OsPAL/TAL*), 4-Coumarate-CoA ligase (*Os4-CL*), Cinnamoyl-CoA reductase (*OsCCR*), Cinnamyl-alcohol dehydrogenase (*OsCAD*), Peroxiredoxin 6 (*OsPOx*), Ferulate-5-hydroxylase (*OsF5H*), Caffeoyl-CoA O-methyltransferase (*OsCCoAOMT*), and Coniferyl-aldehyde dehydrogenase (*OsCALDH*). After 30 DAPI, plants from each treatment were harvested and brought to the laboratory in cool packs. The root samples were properly rinsed under running water to totally eliminate any traces of dirt. The clean root samples were quick-frozen in liquid nitrogen and ground and total RNA was extracted using a Total RNA isolation kit (Agilent, India) following the steps defined in the manufacturer's protocols. There were three biological replicates. The cDNA was synthesized using a cDNA Synthesis Kit (BioRAD, India) following the manufacturer's protocols. The quality and quantitative estimation of cDNA was determined using Nanodrop 2000c (Thermo Scientific, United States). The expression of these key genes was analyzed using gene-specific primers (**Supplementary Table 1**). The housekeeping gene *Actin* and *Glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) was used as an endogenous standard to normalize the quantitative expression data. The qRT-PCR was performed according to Malviya et al. (2022) using BioRAD Real Time PCR System (MJ MiniOpticon, BioRAD). The relative transcript levels (fold change) were enumerated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) over the housekeeping gene *Actin* and GAPDH.

Effect of AMF inoculation on plant growth attributes and disease dynamics

To see how AMF inoculation affects plant growth characteristics in the rice plants pre-challenged with *M. graminicola,* plants were harvested from each treatment at 30 and 45 days after pathogen inoculation and taken to the workroom in cool packs. In order to completely remove the adhering dirt particles, the roots were extensively rinsed under running water. Root and shoot length and their fresh weight were determined.

Further, five plants from each treatment were sampled randomly to count the average records of root galls per root system, egg mass per root system, eggs per egg mass and J_2 per root system in terms of numbers at 30 and 45 days after pathogen inoculation. The average number of J_2 present in the root system was enumerated as per the method described by Bridge et al. (1981) with slight modifications. In brief, for enumeration of J_2 population in the roots, root samples were stained for 30 min in a solution of boiling acid fuchsin (0.1 per cent w/v) in lactic acid, glycerol, and distilled water (1:1:1), macerated in distilled water and the number of J_2 was counted under a light stereomicroscope.

Statistical analysis

The laboratory experiments were laid out in a completely randomized design. The glasshouse experiments were laid out in a completely randomized block design (CRBD). Experiments were repeated twice. Data were subjected to analysis of variance (ANOVA) and compared with Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ using statistical package for Social Sciences Version 16.0 (SPSS Inc, 2007) programme. The root colonization data were transformed using an arcsine transformation and statistically analyzed using a CRBD using the SPSS version 16.0. Graphs were prepared using statistical software Origin (Version 9) and Microsoft Office Excel (2010).

Results

AM root colonization

To assess the effectiveness and potentiality of AMF inoculants to control the RKN infection and disease development, root colonization ability is an important attribute for any of the bioinoculants and it provides a clue for a commensal association between the two partners mediated through root exudates. Root colonization results indicated that rice roots were found to be colonized by all three strains tested at 30 and 45 DAPI. The per cent mycorrhizal root colonization was different for the three test species, *F. mosseae, R. fasciculatus* and *R. intraradices* in both the cultivars grown under glasshouse conditions and pre-challenged with *M. graminicola*. Among the three species studied, significantly higher root colonization was observed in the plants inoculated with *R. intraradices* at 30 and 45 DAPI (**Table 1**). However, per cent root colonization significantly increased when plants were inoculated with all the three AMF species together across the rice cultivars/inbred lines. Interestingly, Pusa Basmati-1 was found to be the best host as compared to Jasmine 85 (**Table 1**).

TABLE 1 Percent root colonization of *F. mosseae, R. fasciculatus,* and *R. intraradices* in rice cultivars at 30 and 45 days of sowing under glasshouse conditions.

Treatments	Pusa Basmati-1		Jasmine 85	
	30 DAPI	45 DAPI	30 DAPI	45 DAPI
T_1- *M. graminicola*	00.00 (0.00)^e	00.00 (0.00)^d	00.00 (0.00)^d	00.00 (0.00)^d
T_2- *M. graminicola* + *F. mosseae*	60.25 (50.91)^c	78.10 (62.10)^b	59.47 (50.46)^c	68.60 (55.92)^c
T_3- *M. graminicola* + *R. fasciculatus*	55.74 (48.30)^d	74.20 (59.47)^c	62.40 (52.18)^c	72.50 (58.37)^b
T_4- *M. graminicola* + *R. intraradices*	66.05 (54.36)^b	80.26 (63.62)^b	66.10 (54.39)^b	74.10 (59.41)^b
T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*	75.47 (60.31)^a	89.25 (70.86)^a	70.50 (57.10)^a	86.29 (68.27)^a
T_6- control (untreated)	00.00 (0.00)^e	00.00 (0.00)^d	00.00 (0.00)^d	00.00 (0.00)^d

Where, DAPI represents days after pathogen inoculation, Data are mean ($n = 5$). Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test. Figures in parenthesis indicate arcsine transformed averages.

Effect of AM inoculation on physio-biochemical parameters and antioxidant enzymes

To assess the mechanisms of tolerance in the susceptible and resistant inbred lines/cultivars of rice (Indica type) to RKN infection, accumulation and activities of defense-related biochemical and antioxidant enzymes, respectively, were measured spectrophotometrically at 30 DAPI. With respect to chlorophyll content, significantly higher content was reported in the absolute control (neither AMF inoculation nor pathogen challenge) at 30 DAPI (Figure 1A). Among the different treatments, plants inoculated with *F. mosseae, R. fasciculatus,* and *R. intraradices* in combination showed maximum chlorophyll content in both the inbred lines which were more close to the values reported in absolute control. However, *R. intraradices* inoculated plants performed better and compared to either *F. mosseae* or *R. fasciculatus* inoculated inbred lines pre-challenged with *M. graminicola*. The least amount of total chlorophyll was recorded in *M. graminicola* alone inoculated plants. In general, slightly higher chlorophyll content was reported in the resistant inbred line, Jasmine 85 as compared to PB-1 across the treatments (Figure 1A). In the content of TSS and TP, more or less a similar trend was observed in both susceptible and resistant inbred lines as recorded for total chlorophyll content in the absolute control and AM inoculated plants pre-challenged with *M. graminicola* in the glasshouse experiments (Figures 1B, C, respectively). In contrast, significantly higher accumulation of TPC was reported in the plants inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* in combination and pre-challenged by *M. graminicola* as compared to individually inoculated and pathogen alone inoculated plants (Figure 1D). However, the least TPC was reported in the absolute control. As reported for chlorophyll content, slightly higher TSS, TP, and TPC were recorded in the resistant inbred line, Jasmine 85 as compared to PB-1 across the treatments (Figures 1A–D). The TSS, TP, and TPC content was slightly lower in the roots of susceptible inbred line, PB-1 as compared to shoots across the treatments. However, the pattern was similar to shoot (Figures 2A–C). Further, the TSS and TP content in the roots of resistant line, Jasmine 85 was also lower as compared to shoot, while TPC was significantly higher in the roots as compared to shoot across the treatments (Figures 2A–C).

In order to gain an insight into the differential response of AM inoculated susceptible and resistant inbred lines upon *M. graminicola* infestation, the activity of antioxidant enzymes involved in the ROS-scavenging activities and plant defense was measured in the infested roots at 30 DAPI using UV vis spectrophotometer. Results revealed that significantly higher activity of PAL (26.25), POx (10.47), APx (16.76), PPO (18.50), SOD (15.26), and CAT (28.10) was recorded in the resistant inbred line, Jasmine 85 inoculated with consortia of *F. mosseae, R. fasciculatus* and *R. intraradices* and pre-challenged by *M. graminicola* as compared to individually inoculated and pathogen alone inoculated plants. The least activity of these antioxidant enzymes was observed in the absolute control (Figures 3A–F, respectively) as reported in case TPC. Interestingly, significantly less activity of these antioxidant enzymes was reported in the susceptible inbred line, PB-1 as compared to the resistant inbred line, Jasmine 85 across the treatments (Figures 3A–F). In contrast, the activity of PAL, POx, APx, PPO, SOD, and CAT was significantly higher in the roots of susceptible inbred line, PB-1 and resistant line, Jasmine 85 as compared to shoots across the treatments. However, the pattern was similar to shoot (Figures 4A–F).

Effect of AM inoculation on activation of defense-related pathways/cascades

Expression of key genes involved in the MAPK pathway

In order to gain an insight into the differential response of AM inoculation in susceptible and resistant rice inbred lines/cultivars upon RKN infestation, the expression of key genes involved in the signaling process in root tissues was investigated in infested root using qRT-PCR at 30 DAPI. Mitogen-activated protein kinases (MAPK) involved as phosphorylation cascades in both pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) and play a key role in signaling process during the early response to pathogens. Results indicated that RKN infestation drastically arrests the signaling process and in general, down-regulated the key genes involved in the signaling process in root tissues of susceptible inbred line, PB-1. Surprisingly, in the resistant inbred line, Jasmine 85, *OsCERK1*, *OSCEBiP,* and *OsWRKY70* genes were down-regulated

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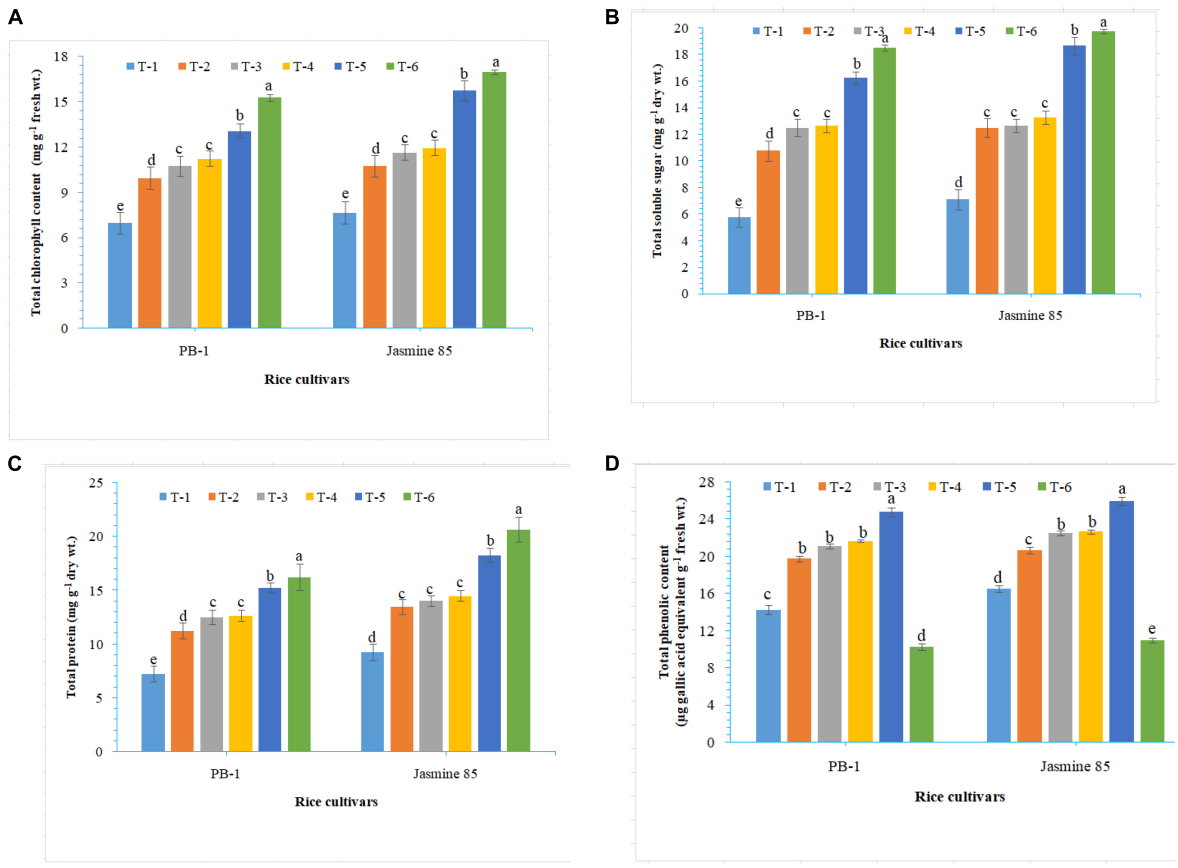

FIGURE 1
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on **(A)** total chlorophyll, **(B)** total soluble sugar, **(C)** total protein, and **(D)** total phenolic content in the shoot of susceptible inbred line, PB-1 and resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola*, T_2- *M. graminicola* + *F. mosseae*, T_3- *M. graminicola* + *R. fasciculatus*, T_4- *M. graminicola* + *R. intraradices*, T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*, and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

slightly, while other genes tested returned to basal or even more expression levels in the infested roots of Jasmine 85 in comparison to the susceptible inbred line, PB-1 and untreated control plants (Figure 5). When we compared closely all the key genes taken into consideration up-regulated manifold upon inoculation of AMF fungi (2.50 to 11.74-fold). When consortia of *F. mosseae*, *R. fasciculatus*, and *R. intraradices* was inoculated to the roots of PB-1 pre-challenged by *M. graminicola* the mRNA levels of these genes were markedly increased. as compared to individually AMF inoculated, and pathogen alone inoculated plants (Figure 5).

On the other hand, transcript accumulation of *OsCERK1*, *OSCEBiP*, *OsWRKY25*, *OsWRKY29*, *OsWRKY70*, *OsMYB15*, *OsMAPKKK1*, *OsMAPKKK5*, *OsMAPK5*, *OsMAPK6*, *OsMAPK15*, and *OsMAPK17*, in general was significantly higher in resistant inbred line, Jasmine 85 as compared to susceptible line PB-1. The mRNA levels of these genes were considerably increased in the roots of Jasmine 85 upon inoculation of AMF consortia and pre-challenged with *M. graminicola* (3.5 to 16.10-fold) as compared to AMF and *M. graminicola* individually inoculated and untreated control plants (Figure 5). Conversely, a non-significant expression of *OsWRKYs*, *OsMYB*, *OsMAPKKKs*, and *OsMAPKs* was recorded in the root of untreated control plants (PB-1 and Jasmine 85 both) suggesting the key role of *MAPKs* and other genes in induced systemic resistance mechanisms of rice against *M. graminicola* infection (Figure 5).

Expression of key calcineurin B-like protein-interacting protein kinases genes

The protein kinase, 'calcineurin B-like protein-interacting protein kinase' genes play important role in the signaling process upon pathogen-challenged and activated downstream biochemical pathways lead to plant defense. Differential expression was recorded in susceptible and resistant inbred lines upon RKN infection. The elevated transcriptional expression of the *CIPK* genes was verified by qRT-PCR analyses (Figure 6). The qRT-PCR results clearly indicated that nematode infestation suppresses the calcineurin B-like protein-interacting protein kinases-mediated signaling in the rice which was clearly evidenced from qRT-PCR analyses. Results clearly indicated that AM inoculation up-regulated the expression of *OsCIPK* genes, *OsCIPK5*, *OsCIPK8*, *OsCIPK9*, *OsCIPK11*, *OsCIPK14*, *OsCIPK23*, *OsCIPK24*, and *OsCIPK33* in both susceptible and resistant inbred lines across the treatments. Interestingly, like *OsMAPKKK*, *OsMAPKK*, *OsMAPKs*, *OsWRKYs*, and *OsMYB*, the higher expression was reported in

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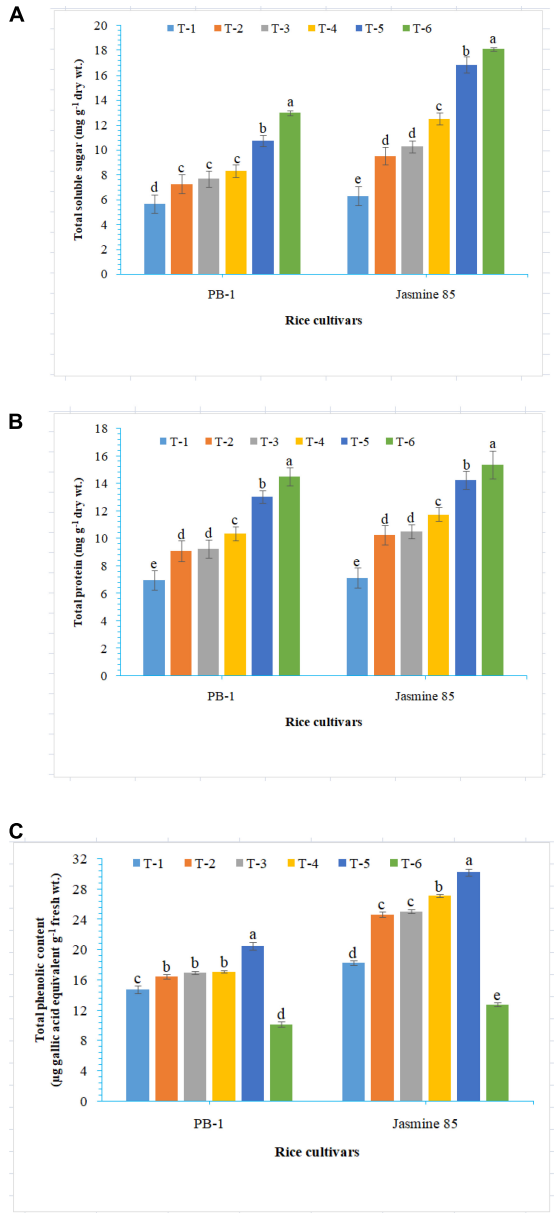

FIGURE 2
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on **(A)** total soluble sugar, **(B)** total protein, and **(C)** total phenolic content in the roots of susceptible inbred line, PB-1 and resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola*, T_2- *M. graminicola* + *F. mosseae*, T_3- *M. graminicola* + *R. fasciculatus*, T_4- *M. graminicola* + *R. intraradices*, T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*, and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

the resistant line, Jasmine 85 inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* in combination and pre-challenged by *M. graminicola* as compared to individually inoculated and pathogen alone inoculated plants (Figure 6). A more or less similar trend was reported in the susceptible line, PB-1. However, the expression level (fold change) in PB-1 was significantly less as compared to Jasmine 85 across the treatments (Figure 6). In individual inoculation, the differences in the number of transcripts accumulated were not significant ($p > 0.05$) except for *OsCIPK8* in PB-1. The least expression was recorded in untreated controls where only basal level expression was recorded in both susceptible and resistant inbred lines (Figure 6).

Expression of key genes involved in the BR signaling and regulation

Despite CIPK and MAPK genes, genes involved in the BR signaling and regulation were induced by AMF inoculation in rice under pathogenic challenge. To examine the expression level of BR signaling and biosynthesis gene in response to *M. graminicola* infection, the key biosynthesis genes, *OsBRI1* (LOC_Os01g52050), *OsBAK1* (LOC_Os08g07760), *OsD2* (LOC_Os01g10040) and *OsD11* (LOC_Os04g39430) were down-regulated manifold in the susceptible line, PB-1 (−1.25 to −3.66 fold), whereas this value was slightly less in the resistant line, Jasmine 85 (−1.10 to −1.76 fold). However, AM inoculation up-regulated the *OsBRI1, OsBAK1, OsD2,* and *OsD11* genes in the root of rice plants pre-challenged with *M. graminicola*. Results revealed that significantly higher expression of *OsBRI1* (4.19-fold), *OsBAK1* (6.96-fold), *OsD2* (3.90-fold), and *OsD11* (2.66-fold) genes was recorded in the rice cultivar, PB-1 inoculated with consortia of *F. mosseae, R. fasciculatus* and *R. intraradices* and pre-challenged by *M. graminicola* as compared to individually inoculated and pathogen alone inoculated plants (Supplementary Figure 1). A more or less similar trend was recorded in the resistant cultivar/inbred line, Jasmine 85. Interestingly, in the resistant line, the effect of RKN on the expression of BR biosynthesis genes was significantly less (Supplementary Figure 1). It was also observed that basal level expression of BR biosynthesis genes always recorded in the susceptible and resistant plants which clearly indicated that these genes were not only involved in the plant defense but also played another role in plant development and reproduction (Supplementary Figure 1).

Expression of key genes involved in the jasmonate biosynthesis

During nematodes and vertebrates' invasion, jasmonate-dependent pathways played important role in the plant defense and coping with the negative impact of these invaders. Among different key genes of jasmonate biosynthesis and signaling, *OsAOS2* (a key enzyme in JA biosynthesis), *OsJMT1* (converts JA to volatile MeJA), and *OsJAMYB* (JA-inducible MYB transcription factor) were used as the key marker genes to investigate the JA-dependent responses in plants including rice. Results of the present investigation clearly indicated that RKN infestation down-regulated the expression of *OsAOS2* (−1.66 fold) and *OsJMT1* (−1.96 fold) in the susceptible line, PB-1. While AM inoculation alone or in combination significantly up-regulated the genes involved in the JA biosynthesis and downstream signaling in both susceptible and resistant lines under the pathogenic stress of *M. graminicola* (Supplementary Figure 2). It was clearly observed that significantly higher expression of *OsAOS2, OsJMT1,* and *OsJAMYB* genes was recorded in the rice cultivars, PB-1 and Jasmine 85 inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* in combination and pre-challenged

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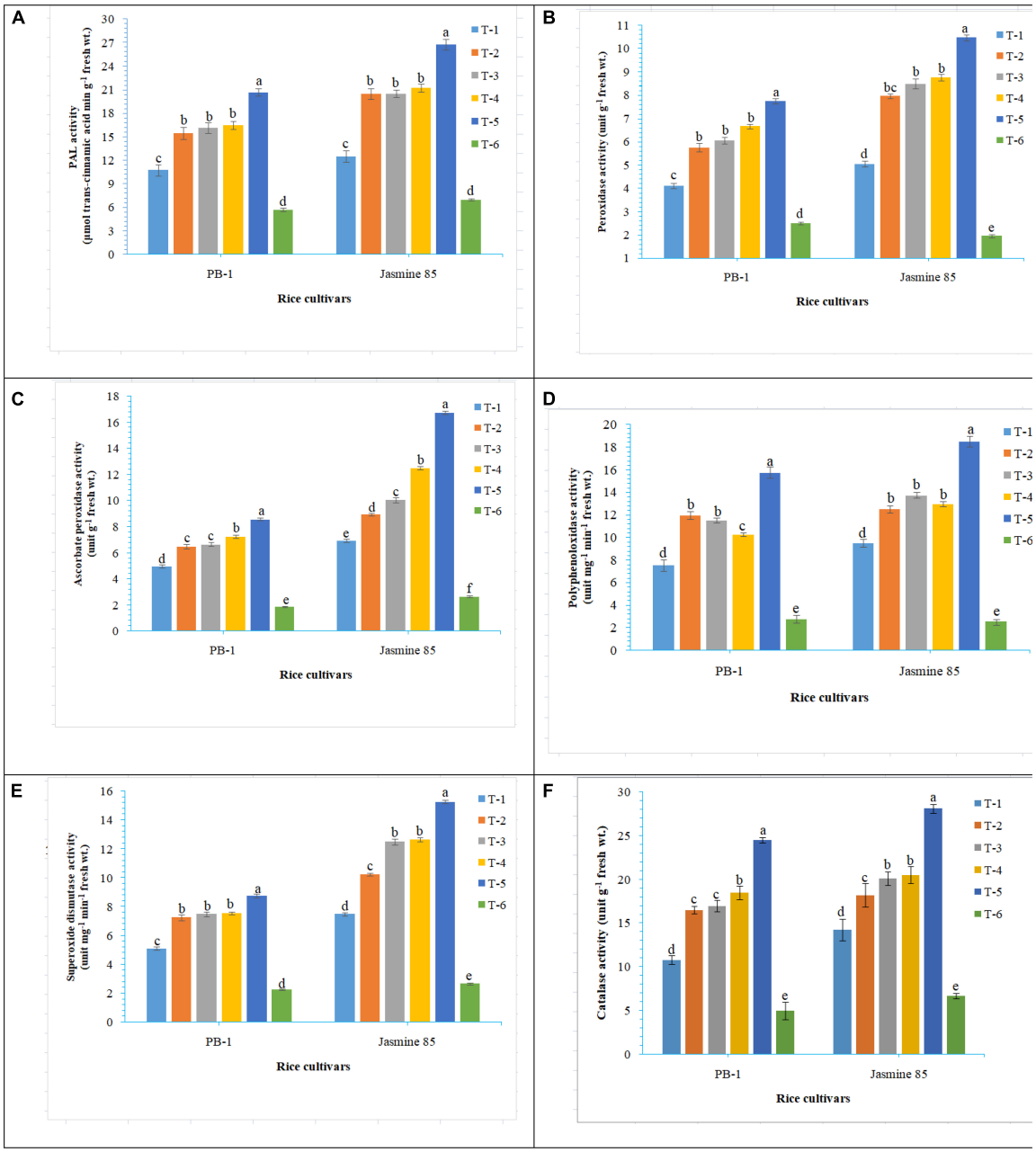

FIGURE 3
Effect of *F. mosseae*, *R. fasciculatus*, and *R. intraradices* inoculation on **(A)** PAL, **(B)** peroxidase, **(C)** ascorbate peroxidase, **(D)** polyphenol oxidase, **(E)** superoxide dismutase, and **(F)** catalase activity in the shoot of susceptible inbred line, PB-1 and resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola*, T_2- *M. graminicola* + *F. mosseae*, T_3- *M. graminicola* + *R. fasciculatus*, T_4- *M. graminicola* + *R. intraradices*, T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*, and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

by *M. graminicola* as compared to individually inoculated and pathogen alone inoculated plants (Supplementary Figure 2). The least expression was reported in untreated control plants.

Expression of key genes involved in the ethylene biosynthesis

Ethylene is the key phytohormone playing an important role in signaling, plant defense and plant growth as a whole. Ethylene, at lower concentrations induced IAA-mediated root development and downstream regulation of plant defense under pathogenic stresses. In general, AM-mediated induction of ethylene biosynthesis and downstream signaling in rice has not been reported so far. ET biosynthesis and signaling genes were either down-regulated or up-regulated differentially in the roots of the susceptible and resistant plant at 30 DAPI. However, in the present investigation, AM inoculation up-regulated key genes involved in ethylene biosynthesis and signaling. Among them, *OsACS1*, *OsACO7* (two major catalytic enzymes involved

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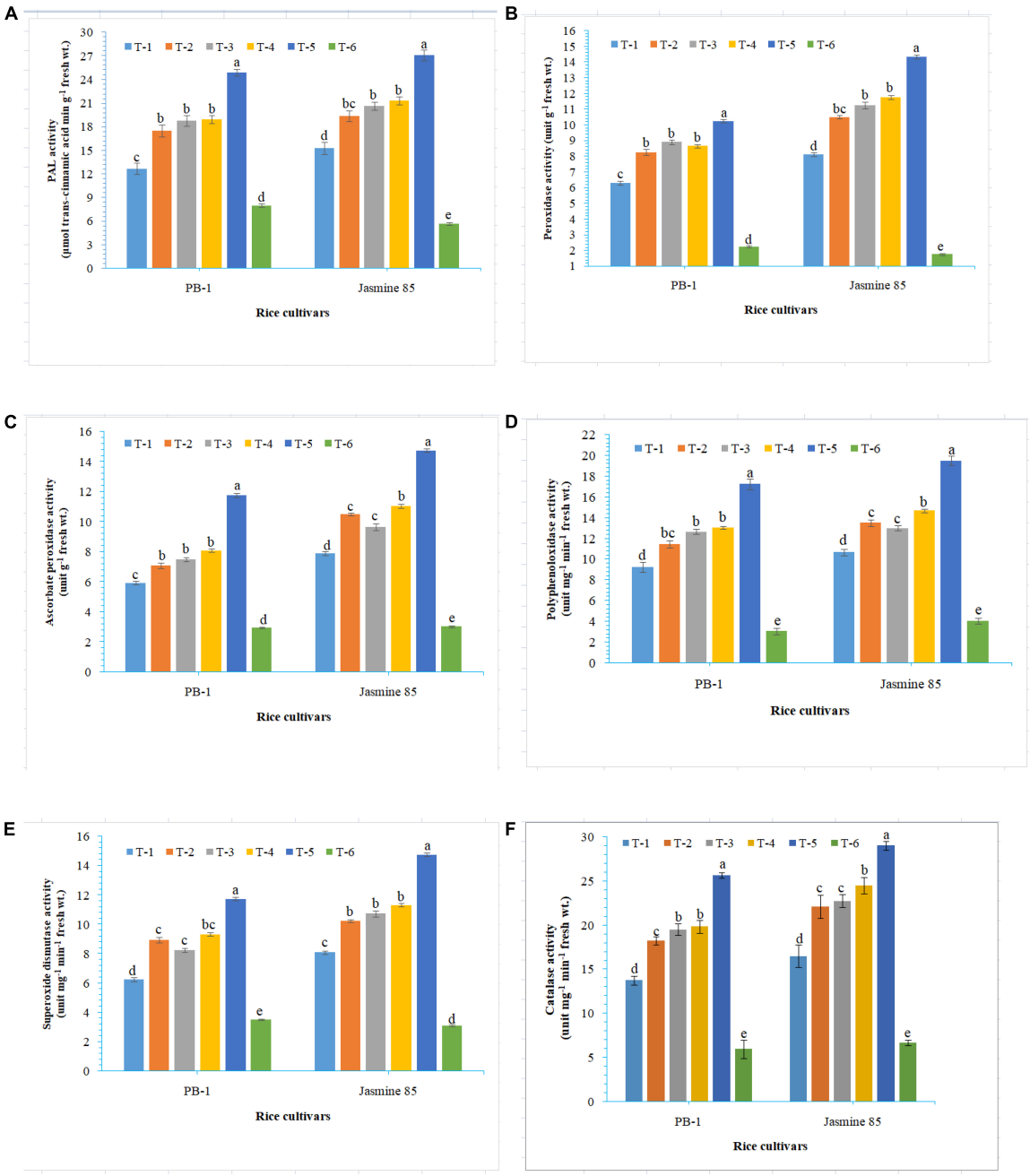

FIGURE 4
Effect of *F. mosseae*, *R. fasciculatus*, and *R. intraradices* inoculation on **(A)** PAL, **(B)** peroxidase, **(C)** ascorbate peroxidase, **(D)** polyphenol oxidase, **(E)** superoxide dismutase, and **(F)** catalase activity in the root of susceptible inbred line, PB-1 and resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola*, T_2- *M. graminicola* + *F. mosseae*, T_3- *M. graminicola* + *R. fasciculatus*, T_4- *M. graminicola* + *R. intraradices*, T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*, and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

in the biosynthesis of ET from methionine), *OsEIN2* (central signal transducer in ET signaling pathway), and *OsERF1* (ET-inducible gene) are the most important and were used as the marker genes to demonstrate the ET-related responses. Results of the present investigation clearly indicated that *M. graminicola* attenuated the expression of these genes in the infected root of the susceptible line, PB-1 (−1.25 to −4.67 fold) as compared to the resistant inbred line, Jasmine 85 (−1.25 to −1.66 fold). Inoculation of AM fungi, *F. mosseae*, *R. fasciculatus*, and *R. intraradices* individually or in combination up-regulated and mRNA levels of *OsACS1* and *OsACO7* were increased manifold (~2-fold) as compared to *OsEIN2*, and *OsERF1* in the infected root of PB-1 at 30 DAPI. A near baseline expression of *OsACS1*, *OsACO7*, *OsEIN2*, and *OsERF1* was documented in the root of uninoculated control line PB-1 at 30 DAPI (**Supplementary Figure 3**). On the contrary, a strong induction

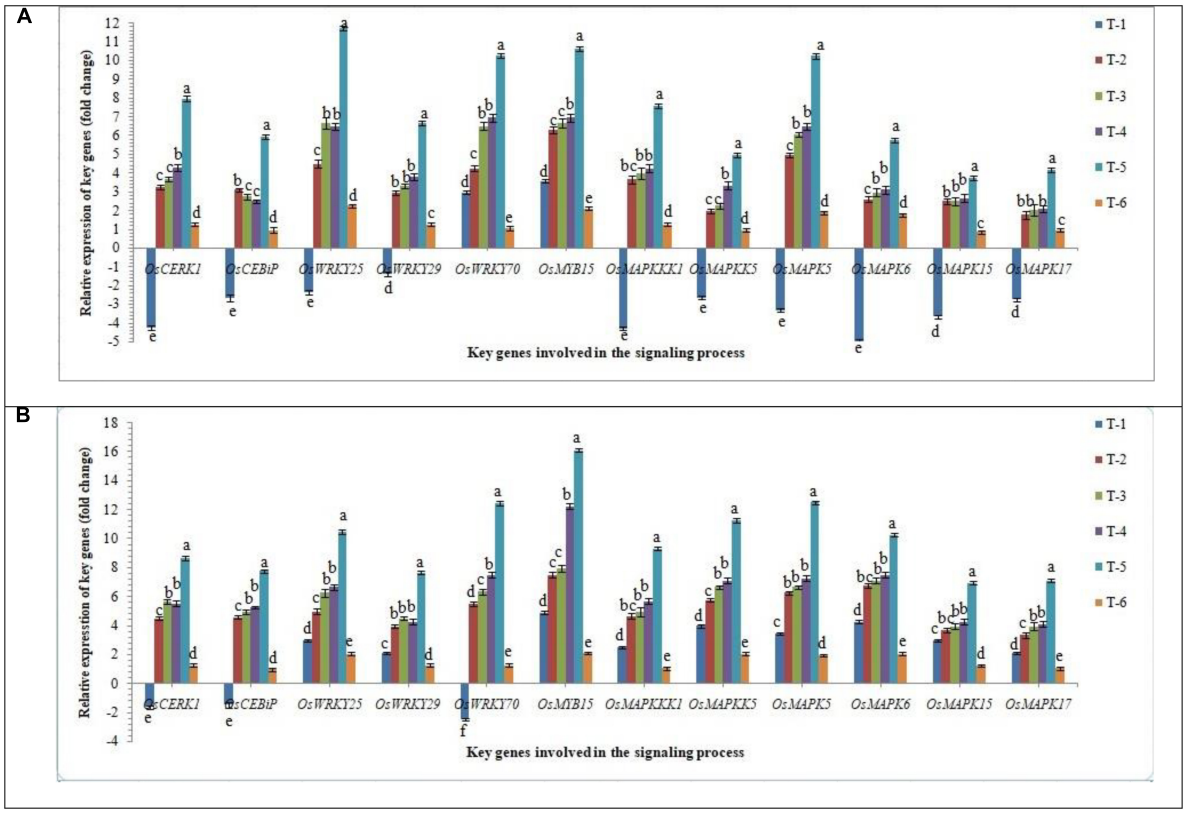

FIGURE 5
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on expression profile of key genes of involved in the signaling process in the **(A)** susceptible inbred line, PB-1 and **(B)** resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola,* T_2- *M. graminicola* + *F. mosseae,* T_3- *M. graminicola* + *R. fasciculatus,* T_4- *M. graminicola* + *R. intraradices,* T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices,* and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

of *OsACO7* was observed in the roots of Jasmine 85 inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination at 30 DAPI. However, the transcripts of *OsACS1, OsEIN2,* and *OsERF1* were significantly induced in the roots of Jasmine 85 inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination 5 at 30 DAPI (**Supplementary Figure 3**). Collectively, a consistent overexpression of ethylene-responsive genes throughout the course of nematode infection in the resistant and susceptible plants suggests a positive correlation between ET-inducible gene expression in rice co-inoculated with AM fungi and overall defense to *M. graminicola*.

Expression of genes encoding pathogenesis-related proteins

General defense responses in rice inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination upon *M. graminicola* infection were investigated. To elucidate the molecular mechanisms sustaining the general defense response of rice triggered upon AMF inoculation and nematode infection, the differential expression of PR genes, *OsPR1, OsPR2, OsPR5,* and *OsPR10* was evaluated. Despite differential expression at 30 DAPI mRNA levels of *OsPR1, OsPR2, OsPR5,* and *OsPR10* were attenuated in the infected root of PB-1 inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination. An increased and approximate consistent transcript abundance of *OsPR1, OsPR2, OsPR5,* and *OsPR10* was recorded in the roots of susceptible inbred line, PB-1 at 30 DAPI (**Figure 7**). However, the least expression was reported in untreated control plants. The strong and differential up-regulation of *OsPR1, OsPR2, OsPR5,* and *OsPR10* genes found in the roots of resistant line, Jasmine 85 inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination at 30 DAPI confirms the defense-inducing capabilities of PR genes in rice roots in response to RKN attack. However, the strongest up-regulation of *OsPR1* was recorded in Jasmine 85 at 30 DAPI. Conversely, expression of *OsPR1, OsPR2, OsPR5,* and *OsPR10* was markedly down-regulated in the infected root of Jasmine 85 as compared to AM-inoculated plants (**Figure 7**).

Expression of key genes involved in the phenylpropanoid pathway

Phenylpropanoid is the key pathway induced in plants under biotic and abiotic stresses and modulates the downstream defense/tolerance mechanisms in a different manner. Similar to other signaling mechanisms discussed above, differential expression of crucial phenylpropanoid pathway-related genes was reported in the roots of susceptible and resistant inbred lines

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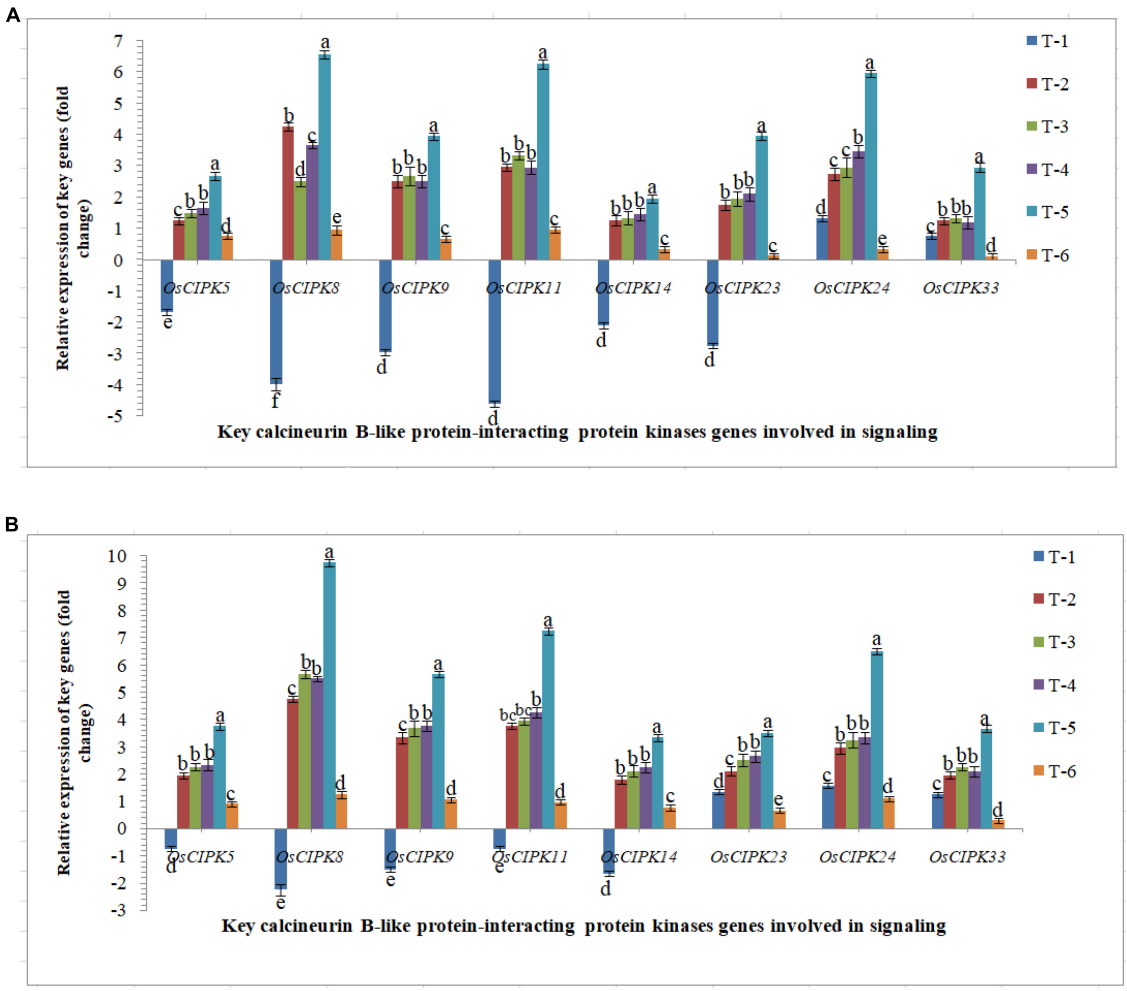

FIGURE 6
Effect of *F. mosseae*, *R. fasciculatus*, and *R. intraradices* inoculation on expression profile of key calcineurin B-like protein-interacting protein kinases genes involved in signaling in the **(A)** susceptible inbred line, PB-1 and **(B)** resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola*, T_2- *M. graminicola* + *F. mosseae*, T_3- *M. graminicola* + *R. fasciculatus*, T_4- *M. graminicola* + *R. intraradices*, T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*, and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

inoculated with AM fungi upon RKN infection. Nine marker genes, *OsPAL*, *OsPAL/TAL*, *Os4-CL*, *OsCCR*, *OsCAD*, *OsPOx*, *OsF5H*, *OsCCoAOMT*, and *OsCALDH* were used as the marker genes to demonstrate the phenylpropanoid-related responses. While in roots of PB-1, transcripts of *OsPAL*, *OsPAL/TAL*, *Os4-CL*, *OsCCR*, *OsCAD*, *OsPOx*, *OsF5H*, *OsCCoAOMT*, and *OsCALDH* were either unaltered or attenuated under RKN infection at 30 DAPI (Figure 8). The transcripts of *OsPAL*, *OsPAL/TAL*, *Os4-CL*, *OsCCR*, *OsCAD*, *OsPOx*, *OsF5H*, *OsCCoAOMT*, and *OsCALDH* were strongly up-regulated in Jasmine 85 in contrast to significantly less expression in PB-1 inoculated with *F. mosseae*, *R. fasciculatus* and *R. intraradices* individually or in combination at 30 DAPI (Figure 8). Conversely, a non-significant expression of these genes was documented in the RKN-infected root of PB-1 and Jasmine 85 inoculated with *F. mosseae*, *R. fasciculatus*, and *R. intraradices* individually at 30 DAPI, suggesting the equivocal role of these genes in induced systemic defense of rice against RKN (Figure 8). In concordance with these findings, it appeared that key genes involved in the phenylpropanoid pathway had a major positive effect in activating the systemic defense of susceptible and resistant inbred lines of rice in response to RKN attack.

Expression of key genes involved in the lignin and callose biosynthesis

Lignin and callose deposition in plant roots play a crucial role in plant defense against biotic stresses. Significantly higher accumulation reinforces the plant tissues by conferring mechanical strength to cell walls against invading pathogens including RKN. In the present investigation, the expression of key genes involved in the lignin and callose biosynthesis was studied in the roots of susceptible and resistant inbred lines, PB-1 and Jasmine 85, respectively, inoculated with AMF. As revealed in Figure 9, the expression of *OsC4H*, *OsCAD6* (two lignin biosynthesis genes), *OsGSL1* (callose synthase genes) and *OsGNS5* (callose hydrolyzing gene) was down-regulated significantly in the roots of PB-1 challenged with *M. graminicola*. However, inoculation

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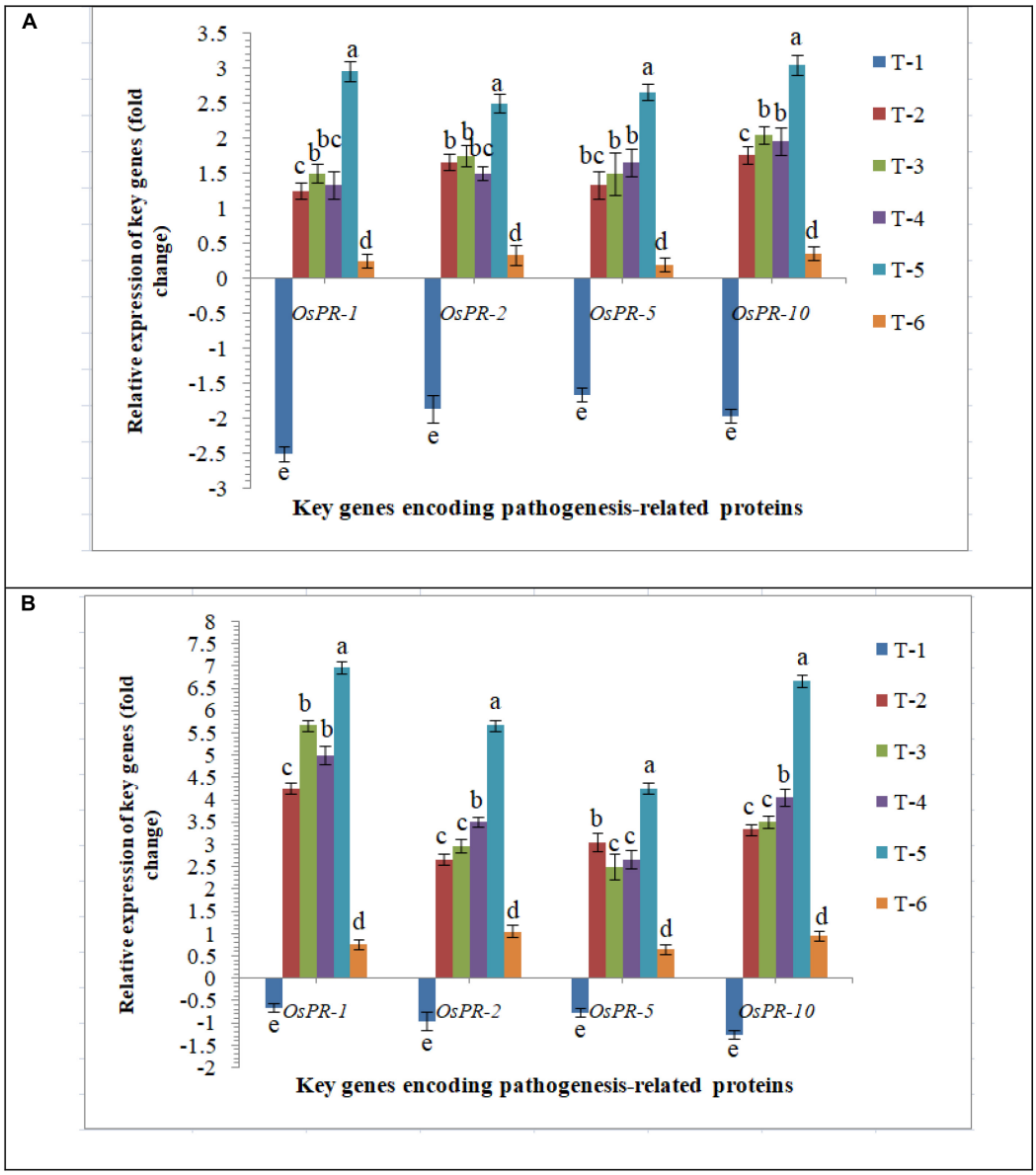

FIGURE 7
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on expression profile of genes encoding pathogenesis-related proteins in the **(A)** susceptible inbred line, PB-1 and **(B)** resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola,* T_2- *M. graminicola* + *F. mosseae,* T_3- *M. graminicola* + *R. fasciculatus,* T_4- *M. graminicola* + *R. intraradices,* T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices,* and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

of *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination strongly up-regulated the expression of *OsC4H, OsCAD6, OsGSL1, OsGSL3, OsGSL5* and *OsGNS5* in the roots of susceptible and resistant inbred lines (Figure 9). In agreement with these results, significantly higher expression of these genes was observed in the RKN-infected root of resistant plants compared to susceptible ones at 30 DAPI (Figure 9). These findings supported the notion that AM fungi modulated the expression of key genes involved in lignin and callose deposition and might play the pivotal role in inhibiting nematode penetration and consequently, the delayed development and reproduction of RKN occured in the resistant inbred line, Jasmine 85. This finding is attributable to the possible role of lignin and callose-related genes in rice basal defense against nematodes (Figure 9).

Effect of AM inoculation on lignin content

Quantitative analysis revealed that inoculation of *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination enriched lignin synthesis and accumulation in susceptible and resistant inbred lines, PB-1 and Jasmine 85. Results of the present investigation clearly indicated that RKN infection hampered

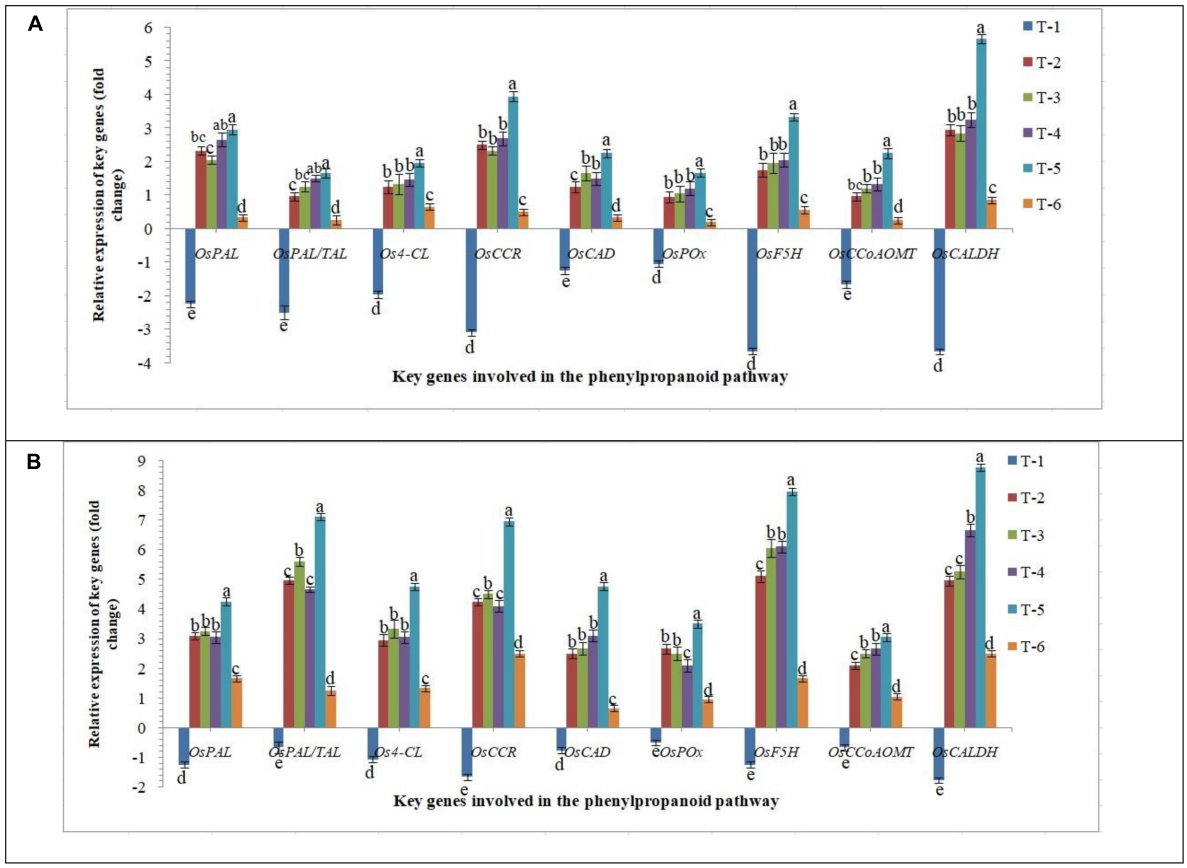

FIGURE 8
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on expression profile of key genes involved in the phenylpropanoid pathway in the **(A)** susceptible inbred line, PB-1 and **(B)** resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola,* T_2- *M. graminicola* + *F. mosseae,* T_3- *M. graminicola* + *R. fasciculatus,* T_4- *M. graminicola* + *R. intraradices,* T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices,* and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

the lignin synthesis and accumulation in a significant way (Figure 10). As compared to other treatments, significantly higher accumulation of lignin was reported in the plants inoculated with consortia of the three AMF. However, the least lignin was recorded in the roots of PB-1 followed by Jasmine 85 pre-challenged with *M. graminicola* (Figure 10).

Effect of AMF inoculation on disease dynamics

The effect of AMF inoculation on the development of root gall, production of egg mass and enumeration of J_2s in the root system was investigated in the susceptible and resistant inbred lines of rice pre-challenged with *M. graminicola.* Significantly higher number of galls was recorded in the inbred line PB-1 inoculated with *M. graminicola* alone (16.25 galls/root system) as compared to the *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculated plants (10.47, 11.25, and 10.66 galls/root system, respectively). However, the least number of galls was reported in the roots of PB-1 inoculated with consortia of the three AMF (7.25 galls/root system) at 30 DAPI (Figure 11A). Further results revealed that the total number of egg mass per root system was higher in the roots of PB-1 infected with *M. graminicola* (14.25 egg mass/root system) alone than in plants inoculated with *F. mosseae, R. fasciculatus* and *R. intraradices* individually (9.27, 9.05, and 8.10 egg mass/root system, respectively) or in combination (6.50 egg mass/root system) under pathogenic stress of *M. graminicola* (Figure 11B). Results revealed that the number of eggs per egg mass varied in different treatments. The maximum of eggs per egg mass was recorded in the roots of PB-1 pre-challenged with *M. graminicola* (36.50) at 30 DAPI (Figure 11C). However, the sum of eggs per egg mass decreased significantly in the root of PB-1 inoculated with *F. mosseae, R. fasciculatus,* and *R. intraradices* individually (25.75, 26.10, and 24.47 eggs/egg mass, respectively) or in combination (18.25 eggs/egg mass). At 30 DAPI, J_2 populations were significantly higher in the roots of PB-1 infected with *M. graminicola* alone (1076.25 J_2/root system) as compared to *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation individually (759.25, 702.10, and 715.21 J_2/root system, respectively) or in combination (501.25 J_2/root system) inoculated plants under pathogenic stress of *M. graminicola* (Figure 11D). After 45 DAPI, 2nd observation on the average number of galls per plant, egg mass per root system, eggs per egg mass and J_2/root

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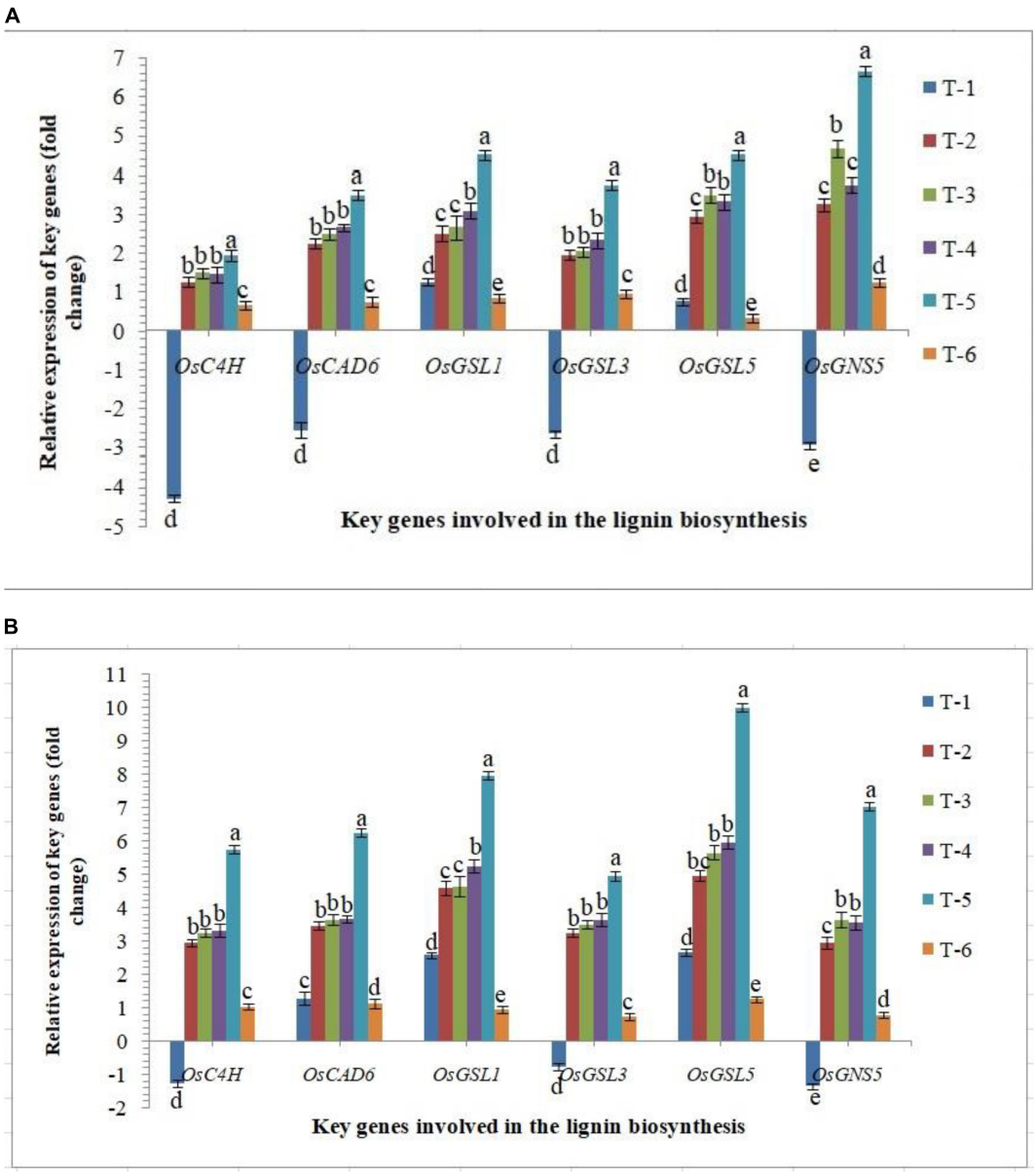

FIGURE 9
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on expression profile of key genes involved in the lignin and callose biosynthesis in the **(A)** susceptible inbred line, PB-1 and **(B)** resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 30 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola,* T_2- *M. graminicola* + *F. mosseae,* T_3- *M. graminicola* + *R. fasciculatus,* T_4- *M. graminicola* + *R. intraradices,* T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices,* and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

system was taken. Results revealed that the average number of galls per plant increased by 1.50 to 2.00 times, egg mass per root system by 2.00 to 7.75-fold, and J_2/root system by 1.25 to 1.50-fold in the roots of PB-1 under different treatments. However, eggs per egg mass did not change significantly at 30 and 45 DAPI (Figures 11A–D). Further analysis showed that the average number of galls per plant, egg mass per root system, eggs per egg mass and J_2/root system were significantly lower in the roots of the resistant inbred line, Jasmine 85 as compared to the susceptible line, PB-1 at 30 and 45 DAPI (Figures 11E–H).

Effect of AM inoculation on plant growth attributes

To assess how AM inoculation affects plant growth characteristics, glasshouse experiments were conducted in susceptible and resistant inbred lines of rice. The plant growth attributes, i.e., shoot and root length and fresh weight of shoot and root increased significantly in plants inoculated with the three AMF individually or in combination in both the susceptible and resistant inbred lines as compared to pathogen alone treated plants. In general, plant growth attributes increased 1.50 to 2.00 times in

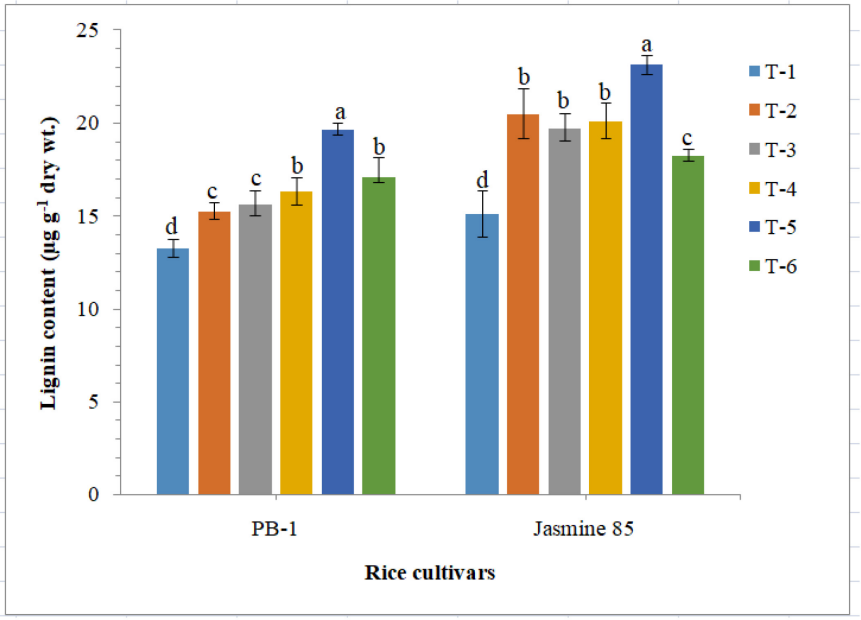

FIGURE 10
Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on lignin content in the susceptible inbred line, PB-1 and resistant inbred line, Jasmine 85 of rice pre-challenged with *M. graminicola* at 45 days of sowing under greenhouse conditions. Treatments were: T_1- *M. graminicola,* T_2- *M. graminicola* + *F. mosseae,* T_3- *M. graminicola* + *R. fasciculatus,* T_4- *M. graminicola* + *R. intraradices,* T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices,* and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

the plants inoculated with the consortia of three AMF as compared to pathogen alone treated plants at 30 and 45 DAPI (**Table 2**).

Discussion

The current day emphasis is on sustainable agriculture, which uses less of chemical inputs having adverse effect on soil health, and environment. The arbuscular mycorrhizal fungi (AMF) and other microbial inoculants play an important role in sustainable agriculture (Smith and Read, 2008; Akhmetzhanova et al., 2012; Babikova et al., 2013; Kammadavil Sahodaran et al., 2019; Lakshmipathy et al., 2019). There are several reports on AMF-soil-borne plant pathogen interactions (Bagyaraj, 2016; Singh A. et al., 2019; Singh U. B. et al., 2019). Most of the studies on AMF-root pathogens suggest that AMF decreased or mitigated the disease severity. Consistent reduction of disease symptoms has been described for fungal, bacterial and nematode pathogens. Studies conducted so far suggest that the mechanisms of suppression may be due to morphological, physiological and biological alterations in the host (Ijdo et al., 2011; Nahar et al., 2011; Khan et al., 2012; Frac et al., 2018). It includes thickening of the cell walls through lignification preventing penetration of pathogens and activation of specific defense mechanisms such as production of phytoalexins, chitinases, pathogenesis related proteins and enhanced antagonists in the mycorrhizosphere (Nahar et al., 2012; Bagyaraj, 2018). In the present investigation, inoculation of AM fungi, *F. mosseae, R. fasciculatus,* and *R. intraradices* individually and in combination under RKN challenge modulated physio-biochemical cascades in rice led to downstream signaling. Application of AM fungi not only modulates physio-biochemical pathways but also reduced the infestation, colonization, and invasion of RKN, *M. graminicola*. Results indicated that *F. mosseae, R. fasciculatus,* and *R. intraradices* when inoculated individually or in combination, colonized the rice roots to a greater extent (55.74 to 89.25%) thereby occupy maximum area of the root along with increased hyphal network in soil and thereby helping in enhanced plant nutrition (Brundrett, 2002, 2009; Johansson et al., 2004). Enhanced AMF colonization increased lateral root formation (secondary and tertiary rooting) and modulates the root morphology of rice which is beneficial for plant growth and development (Kyndt et al., 2012a; van der Heijden et al., 2015). During the course of screening of rice germplasm, PB-1 was found to be the most susceptible cultivar and Jasmine 85 the most resistant cultivar to *M. graminicola* infection at 30 DAPI. Hence, the rice-*M. graminicola* pathosystem with the susceptible and resistant inbred line was taken as a model system to elucidate the interactions between RKN and the host (Kyndt et al., 2012a,b; Nguyễn et al., 2014). In plants, immune system responses are in general regulated by effector-triggered immunity (ETI) and pattern-triggered immunity (PTI). PTI is generally activated when plant perceive microbial structures, in general, referred to as pathogen-associated molecular patterns (PAMPs), *via* the transmembrane pattern recognition receptors (PRRs) (Lee et al., 2004; Gheysen and Jones, 2006; Hewezi et al., 2010). However, ETI is activated when plants recognize specific effector molecules produced by pathogens *via* intracellular nucleotide-binding leucine-rich repeat (NLR) receptors, called resistance (R) proteins (Sanz-Alferez et al., 2008; Hamamouch et al., 2011; Molinari et al., 2014) and thereby effectively up-regulate the defense mechanisms inside plant cells in response to pathogen infection

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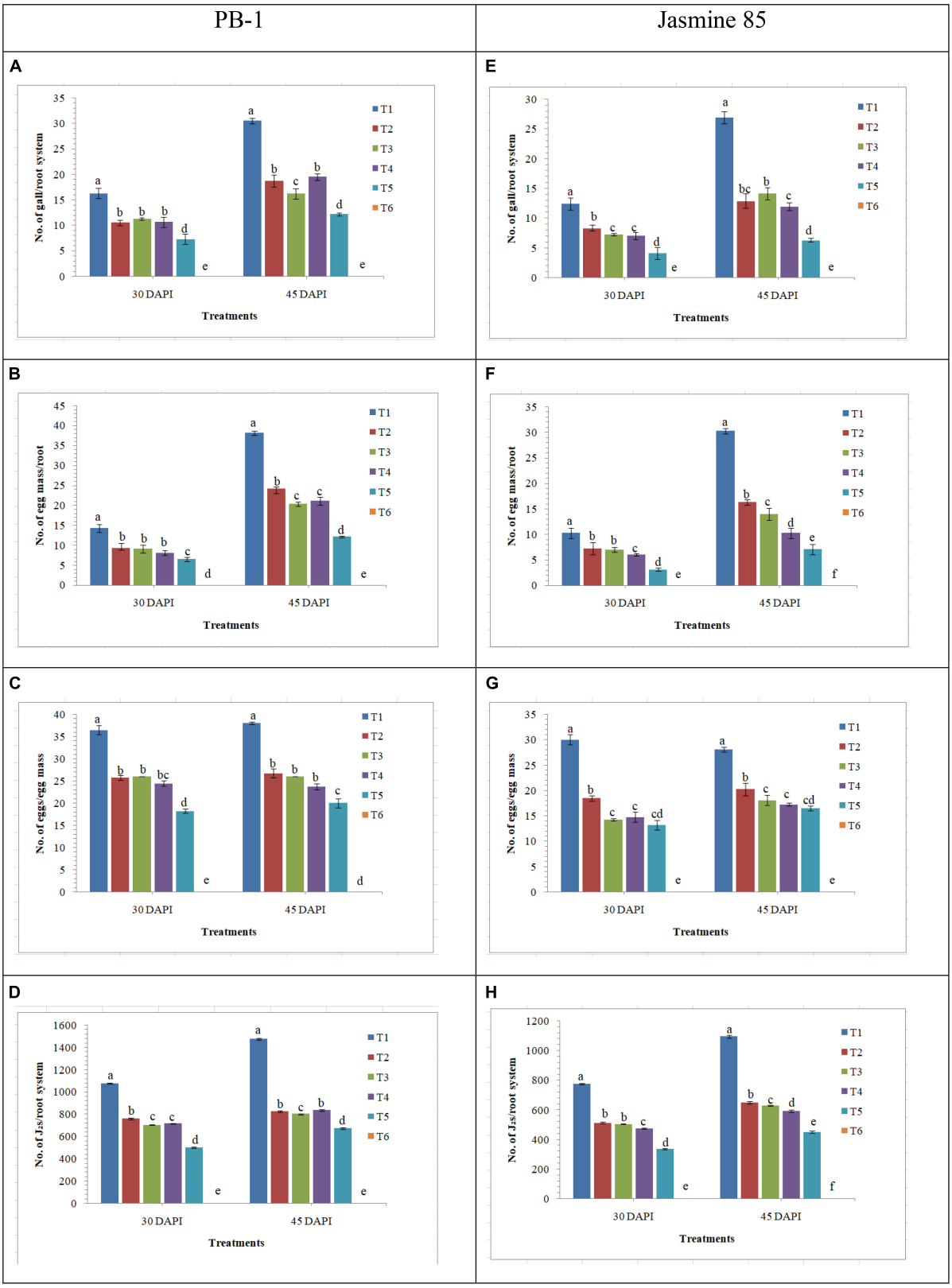

FIGURE 11

Effect of *F. mosseae*, *R. fasciculatus*, and *R. intraradices* inoculation on root gall development under biotic stress of *M. graminicola* in the susceptible (PB-1) **(A–D)** and resistant (Jasmine-85) **(E–H)** inbred lines of rice at 30 and 45 days of pathogen inoculation under net house conditions. Treatments were: T_1- *M. graminicola*, T_2- *M. graminicola* + *F. mosseae*, T_3- *M. graminicola* + *R. fasciculatus*, T_4- *M. graminicola* + *R. intraradices*, T_5- *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*, and T_6- Control (untreated). Column data are mean ($n = 5$) and vertical bar represents standard deviation. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

TABLE 2 Effect of *F. mosseae, R. fasciculatus,* and *R. intraradices* inoculation on plant growth attributes under biotic stress of *M. graminicola* in rice at 30 and 45 days of pathogen inoculation under net house conditions.

Treatments	Shoot length (cm)		Root length (cm)		Shoot fresh wt. (mg)		Root fresh wt. (mg)	
	30 DAPI	45 DAPI	30 DAPI	45 DAPI	30 DAPI	45 DAPI	30 DAPI	45 DAPI
Rice cultivar: Pusa Basmati-1								
T_1 - *M. graminicola*	28.25^{d}	36.20^{d}	16.50^{e}	20.89^{e}	130.33^{d}	145.20^{d}	21.50^{c}	29.47^{d}
T_2 - *M. graminicola* + *F. mosseae*	43.46^{c}	55.25^{c}	22.47^{d}	30.25^{d}	166.25^{c}	190.50^{c}	24.46^{b}	30.75^{d}
T_3 - *M. graminicola* + *R. fasciculatus*	40.75^{b}	59.21^{b}	25.50^{c}	34.45^{c}	176.50^{b}	199.25^{b}	24.05^{b}	32.10^{c}
T_4 - *M. graminicola* + *R. intraradices*	39.50^{b}	60.47^{b}	27.10^{b}	38.50^{b}	175.10^{b}	202.15^{b}	23.50^{b}	32.50^{c}
T_5 - *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*	46.91^{a}	66.25^{a}	31.50^{a}	41.50^{a}	189.50^{a}	226.75^{a}	26.25^{a}	34.15^{b}
T_6 - control (untreated)	47.50^{a}	60.50^{b}	30.45^{a}	42.75^{a}	190.25^{a}	225.20^{a}	25.10^{ab}	36.27^{a}
Rice cultivar: Jasmine 85								
T_1 - *M. graminicola*	26.46^{e}	32.10^{e}	18.50^{e}	25.47 ^{d}	135.20^{e}	150.25^{e}	22.45^{e}	31.75^{b}
T_2 - *M. graminicola* + *F. mosseae*	34.25^{cd}	46.25^{d}	25.25^{d}	34.29^{c}	175.50^{d}	210.20^{d}	26.29^{d}	29.10^{c}
T_3 - *M. graminicola* + *R. fasciculatus*	35.75^{c}	49.11^{c}	28.75^{c}	38.50^{b}	181.25^{c}	215.33^{c}	26.75^{d}	28.75^{c}
T_4 - *M. graminicola* + *R. intraradices*	36.05^{c}	50.25^{c}	28.07^{c}	40.10^{b}	180.75^{c}	216.50^{c}	24.10^{c}	29.35^{c}
T_5 - *M. graminicola* + *F. mosseae* + *R. fasciculatus* + *R. intraradices*	38.10^{b}	55.33^{b}	34.20^{a}	45.50^{a}	190.10^{b}	225.15^{b}	30.50^{b}	32.12^{b}
T_6 - control (untreated)	40.50^{a}	56.05^{a}	32.40^{b}	46.05^{a}	195.50^{a}	230.33^{a}	31.05^{a}	33.90^{a}

Where, DAPI represents days after pathogen inoculation, Data are mean ($n = 10$). Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

and invasion (Harman et al., 2004; Singh U. B. et al., 2019; Malviya et al., 2022).

Further, mitogen-activated protein (MAP) kinase (MAPK) signaling pathways play a crucial role in plant defense, hypersensitive response (HR) reaction, immune responses, and oxidative burst to pathogen attack (Zhang and Klessig, 2001; Bari and Jones, 2009; Pieterse et al., 2009). Moreover, the microbe-mediated activation of MAPKs and downstream signaling networks in plants has not been completely defined. In general, 74 MAPKKK, 8 MAPKK, and 17 MAPK genes have been reported so far in the rice genome (Hamel et al., 2006; Reyna and Yang, 2006; Rao et al., 2010; Yang et al., 2015). They are playing a different role in signaling cascades underlying the physiological and cellular responses in rice (Tamogami et al., 1997; Schweizer et al., 1998; van Loon et al., 2006; Singh et al., 2012a). In the present investigation, RKN infection down-regulated the expression of key genes involved in the MAPK-mediated signaling pathways in the susceptible cultivar, PB-1 (−1.39 to −4.29 fold) as compared to the resistant inbred line, Jasmine 85. Further inoculation of AM fungi, *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination up-regulated the expression of these genes in both susceptible and resistant inbred lines, PB-1, and Jasmine 85, respectively, pre-challenged with *M. graminicola*. This advocates a key role for MAPK signaling in rice response to RKN infection (Kumari et al., 2016; Zhou et al., 2020). To the best of our knowledge and the literatures available, this is the first report on the role of *F. mosseae, R. fasciculatus,* and *R. intraradices* in the activation of MAPK signaling in rice under pathogenic challenge of RKN *M. graminicola* in rice, which is further confirmed by the enhanced systemic resistance in susceptible and resistant inbred lines of rice (Pozo and Azcon-Aguilar, 2007). According to Rodriguez et al. (2009), "Plant MAPK cascades proceed through three central kinases: MAPK kinase kinase (MAPKKK); MAPK kinase (MAPKK), also known as MAPK and ERK (extracellular signal regulated kinase) kinase (MEK); and MAP kinase (MAPK or MPK)." In the present investigation, *OsCERK1*, *OSCEBiP, OsWRKY25, OsWRKY29, OsWRKY70, OsMYB15, OsMAPKKK1*, *OsMAPKKK5*, *OsMAPK5*, *OsMAPK6*, *OsMAPK15,* and *OsMAPK17* were highly expressed in the rice plant primed with the selected strains of AM fungi, *F. mosseae, R. fasciculatus,* and *R. intraradices* individually or in combination. The MAPKs further activated *OsWRKYs,* and *OsMYB* transcription factors cause HR reaction and programmed cell death by modulating the generation of reactive oxygen species (ROS) at the infection site and thereby restricting the penetration, invasion and further colonization of RKN (Nahar et al., 2011; Kyndt et al., 2012a,b). Similar results were reported on the MAPK-mediated defense-priming in soybean (*Glycine max*) in response to *Heterodera glycines* infection (Bekal et al., 2003; Zhang et al., 2017; McNeece et al., 2019) and in rice against *M. graminicola* (Kumari et al., 2016; Zhou et al., 2020). Asai et al. (2002) clearly demonstrated the role of MAPKs signaling cascades (*MEKK1, MKK4/MKK5*, and *MPK3/MPK6*) and WRKY transcription factors (*WRKY22/WRKY29*) in the activation of innate immune responses in *Arabidopsis thaliana*. It was further reported that *AtMPK3* and *AtMPK6* are involved in ETI in *Arabidopsis thaliana* (Tsuda

et al., 2009; Meng and Zhang, 2013; Choi et al., 2017; Su et al., 2018; Zhang et al., 2018) which was further endorsed by several other researchers (Pitzschke et al., 2009; Rasmussen et al., 2012; Meng and Zhang, 2013). Similarly, qRT-PCR analysis clearly indicated that RKN infection suppresses the expression of CIPKs genes (−0.75 to 4.60 fold). However, inoculation of AM fungi up-regulated the CIPKs genes in roots of susceptible and resistant inbred lines (1.20 to 9.67 fold). CIPK9 repression augmented the total RKN population in roots of susceptible inbred lines. Previous reports clearly indicated that calcium sensor (Cbl10) together with its interacting partner *CIPK6* regulates plant immunity in tomato plants (De la Torre et al., 2013; Zhu et al., 2019; Huang et al., 2020). Similarly, *CIPK9* regulate the downstream signaling in rice to RKN invasion (Zhou et al., 2020). Further, RKN infection significantly altered the expression level of key genes involved in the BR biosynthesis and downstream signaling in rice root. Manifolds suppression was reported in the expression of the gene involved in the BR signaling in susceptible inbred line PB-1 as compared to the resistant line, Jasmine 85. However, AM inoculation up-regulated the BR biosynthesis genes in both susceptible and resistant inbred lines at 30 DAPI signifying the presence of diverse BR functions responding to RKN infection and disease development (Song et al., 2018). Our findings clearly signify a negative correlation between the activation of BR biosynthesis genes and RKN population on rice roots. These findings are also in agreement with the observation of earlier researchers (Kumari et al., 2016; Song et al., 2018; Zhou et al., 2020). In the present investigation, it was observed that plants inoculated with AM fungi showed significantly higher accumulation of antioxidants and defense-related mediator molecules/enzymes in them leading to increased cell wall lignification and reduction in the RKN invasion. qRT-PCR results also showed that RKN invasion negatively regulates the jasmonates biosynthesis genes, *OsAOS2, OsJMT1*, and *OsJAMYB*. However, these genes were highly up-regulated in the AM-inoculated plant which clearly indicated that AM inoculation has a positive impact on JA-mediated defense priming in rice (Seo et al., 2001; Kyndt et al., 2012a,b; Liu et al., 2013; Li et al., 2015; Zhou et al., 2020). Similar to BR biosynthesis and signaling, jasmonate biosynthesis and accumulation of jasmonic acids significantly affect the phenolics content in many crops (Wang and Zheng, 2005; Heredia and Cisneros-Zevallos, 2009). In the present study, the key genes involved in the ET biosynthesis (*OsACS1, OsACO7, OsEIN2,* and *OsERF1*) were suppressed upon RKN invasion in rice, while AM inoculation up-regulated these genes in resistant and susceptible inbred lines. ET biosynthesis and ET-mediated downstream signaling positively regulate the defense cascades in crop plants under biotic and abiotic stresses (Leon-Reyes et al., 2009; Molinari et al., 2014; Yuan et al., 2018; Zhou et al., 2020). Along with defense cascades, ET biosynthesis play a crucial role in IAA-mediated lateral root development, tissue differentiation, photosynthesis and overall plant growth and development. Earlier reports also emphasized that ET signaling is known to regulate plant response to RKN invasion (Yuan et al., 2018; Zhou et al., 2020).

Despite being stress-inducible, systemic mRNA levels of pathogenesis-related proteins, *OsPR-1, OsPR-2, OsPR-5,* and *OsPR-10* did not significantly change in the roots inoculated with AM fungi individually and pre-challenged with *M. graminicola* (Xiong and Yang, 2003; Leon-Reyes et al., 2009; Goto et al., 2013; Goverse and Smant, 2014). In contrast, significantly higher up-regulation was observed in the roots inoculated with consortia of AMF. The systemic mRNA levels of *OsPR-1, OsPR-2, OsPR-5,* and *OsPR-10* were consistently suppressed in the roots of PB-1 under RKN invasion at 30 DAPI (**Figure 7**). Among all defense mechanisms, PR proteins are the indispensable component of innate immune responses in plants under biotic or abiotic stress conditions (Sarma et al., 2015; Lv et al., 2016; Yuan et al., 2018; Zhou et al., 2020). The biosynthesis and accumulation of PR proteins protect the plants from further infection by not only accumulating locally in the infected and surrounding tissues but also in remote uninfected tissues. PR proteins are also involved in the synthesis of phytoalexins, regulation of oxidative burst, HR response or SA-mediated systemic acquired resistance against pathogenic infection including phytopathogenic nematodes (Harman et al., 2004; Sanz-Alferez et al., 2008; Pieterse et al., 2009; Vlot et al., 2009; Singh et al., 2020). Further structure, biochemistry, source, regulation of gene expression and nature of stress, define their role in defense mechanism of various PR proteins (Sanz-Alferez et al., 2008; Bari and Jones, 2009; Hamamouch et al., 2011; Kumari et al., 2016). Phenylpropanoid pathway is the first line of plant defense imparting host resistance by reprogramming the downstream signaling involving activation and accumulation of antioxidant enzymes (PAL, TAL, Pox, APx, etc.) and various defense-related biomolecules such as phytoalexins, callose, pectin, lignin derivatives and other metabolites toxic to the pathogens (Wuyts et al., 2006, 2007; Singh U. B. et al., 2019; Singh et al., 2020). Cell wall lignifications and deposition of pectin substances between the cells give mechanical strength to the plant tissues (Singh et al., 2020). In the present study, results clearly showed that RKN invasion repressed the expression of key genes of phenylpropanoid cascades and took benefit of that during the penetration and invasion of the root tissues. At the same time, plants bioprimed with AM fungi alone or in combination upregulated the expression profile of key genes of phenylpropanoid pathways and increased the activities of PAL, TAL, Pox, APx, etc. While in the resistant inbred line, the fold expression was significantly higher as compared to the susceptible cultivar. Similar effects were also observed in terms of the expression profile of key genes involved in lignin and callose biosynthesis (Wuyts et al., 2006, 2007; Hao et al., 2008; Portillo et al., 2013). Increased lignifications and callose deposition in response to RKN invasion provide additional adoptive mechanisms and strength to resistant inbred lines. Due to the antimicrobial and non-degradable nature of lignin, pectin and callose restrict the entry of pathogens including phytopathogenic nematodes and protect plants even under conducive conditions (Dixon and Paiva, 1995; Wuyts et al., 2006, 2007; Hao et al., 2008; Portillo et al., 2013; Singh U. B. et al., 2019; Singh et al., 2020).

Apart from this, AMF have increased the accumulation of total chlorophyll, total soluble sugar and total protein in rice plants even under pathogenic stress. It is a well-established fact that AMF have the ability to mobilize diffusion-limited nutrients and water from distant places and supple to plants those helping plant growth and productivity (Opik et al., 2006; Parniske, 2008; Bagyaraj et al., 2022). Increased uptake and translocation of mineral nutrients contribute to improved total chlorophyll, total soluble sugar and total protein content in the plants which lead to better plant growth and vigor (Plett et al., 2014). It has also been reported that RKN invasion led to the overproduction of reactive oxygen species (ROS) at

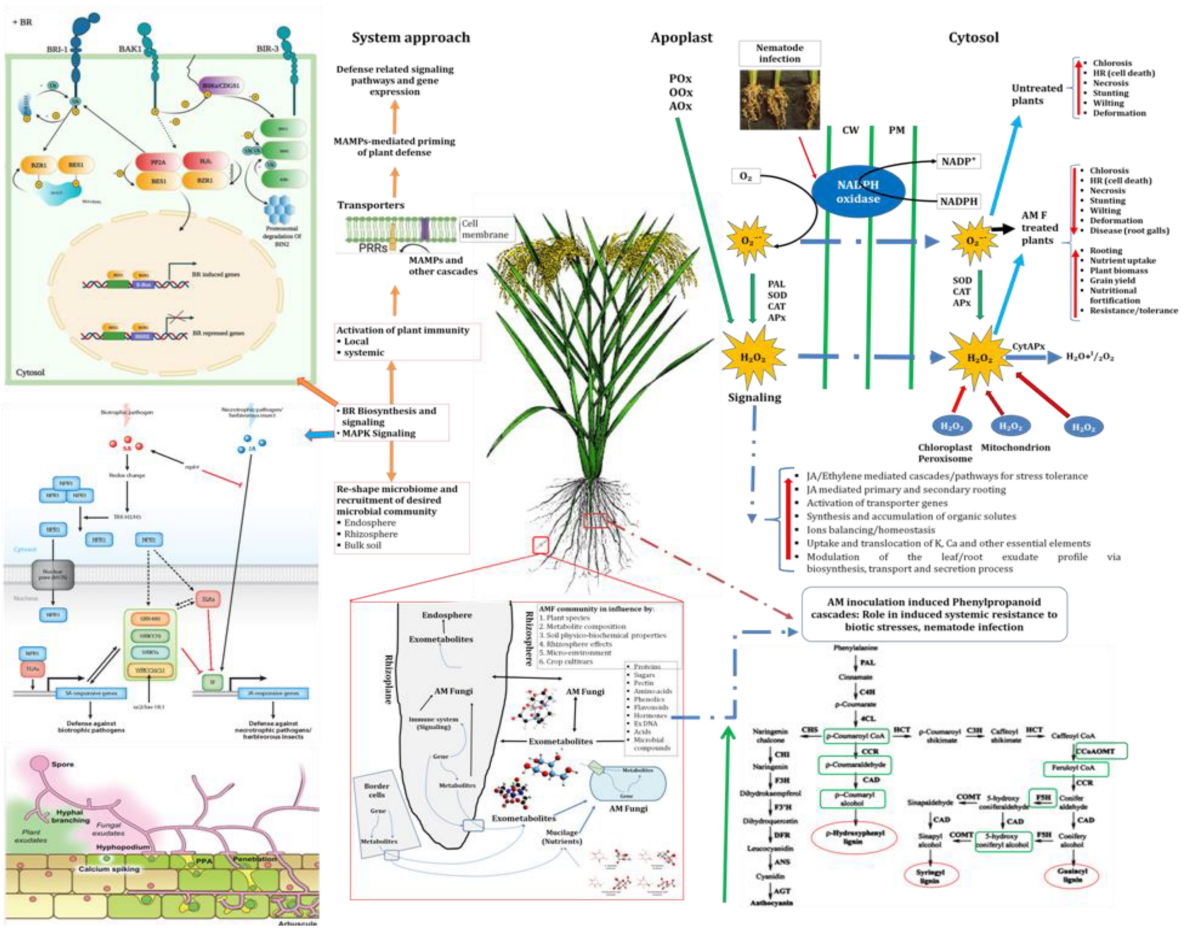

FIGURE 12
A schematic overview of AMF-mediated induction of defense cascades in rice under pathogenic stress of *M. graminicola*.

the cellular level (Singh U. B. et al., 2019; Singh et al., 2020). In the present investigation, manifold increases in the activities of antioxidant and defense-related enzymes were recorded in plants primed with consortia of three AMF *F. mosseae, R. fasciculatus* and *R. intraradices* compared to plants primed individually with each of them and conferring resistance to RKN invasion. Among them, ascorbate peroxidase, peroxidase, catalase, and superoxide dismutase are the most important. These antioxidant enzymes are known to reduce ROS more efficiently. These results are in agreement with the findings of other earlier researchers (Lorito et al., 2010; Singh et al., 2011). Unlike AM fungi, roots primed with plant growth-promoting microorganisms modulated different cascades related to defense related metabolome in plant systems over a time period and activated plant growth and defense biome directly and/or indirectly (Ahn et al., 2007; Bakker et al., 2013; Facelli et al., 2014; Alori et al., 2017; Singh A. et al., 2019; Singh U. B. et al., 2019). As revealed by infection bioassay in the present study (**Table 1**), a significantly higher number of galls/root system, egg mass/root system and J_2s/root system was reported in the roots of susceptible inbred line, PB-1 compared to the resistant inbred line, Jasmine 85 during early stage of infection (30 DAPI). A similar trend was also recorded at 45 DAPI. It is well established that the resistant inbred line, Jasmine 85 contains several resistant genes/QTLs as compared to susceptible ones which protect plants from severe invasion and development of root gall (Ryan and Graham, 2002; Selosse and Rousset, 2011). Further, inoculation of AM fungi individually or in combination primed the plant and activated several genes involved in the plant defense which gives addition support to the plant defense even under RKN invasion. Increased lignifications and callose deposition restrict the entry of J_2s and the invasion caused by them. AM inoculation not only reduced the RKN invasion but also increased plant growth attributes under the pathogenic stress of *M. graminicola*. In short, the present study describes well-coordinated mode of action of AM fungi during RKN invasion, expression of defense-related genes regulating several pathways, accumulation and activities of mediator molecules/enzymes, callose deposition and cell wall lignification ultimately leading to the reduction in RKN invasion, disease development and improved plant growth under biotic stress condition (**Figure 12**).

Conclusion

Root priming with AMF, *F. mosseae, R. fasciculatus* and *R. intraradices* individually or in combination significantly increased the transcript accumulation of key genes involved in

the signaling and plant defense in the susceptible and resistant inbred lines, PB-1 and Jasmine 85 pre-challenged with RKN, *M. graminicola*. The present study, has summarized the detailed investigation of AM fungi-induced activation and accumulation of defense-related biomolecules in rice plants even under pathogenic stress conditions. These AMF could be very promising microbial inoculants for the activation of defense pathways in a cooperative manner under pathogenic stress. Inoculation of AMF also increased lignin content in roots which further reduces the pathogen infection, colonization and invasion. Results clearly indicated that inoculation of AM fungi alone or in combination significantly reduced the number of gall/root system, egg mass/root system and J_2s/root system at 30 and 45 DAPI. Inoculation of AM fungi to rice led to modifications in the root architecture that supports efficient uptake of nutrients and water and promotes plant growth even under pathogenic stressed conditions. With the help of these findings, we concluded that AMF inoculation could be a potential alternative to toxic chemical nematicides and could be applied at a larger scale to manage root-knot disease in rice at experimental plots and commercial production after extensive field evaluation at farmers' fields.

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 authors.

Author contributions

DM, PS, US, JR, and HS conceived and designed the experiments. DB supplied the AM fungi. PS, RV, and AK given the resistant and susceptible inbred lines/cultivars of rice. DM, PS, and US performed the experiments. PS, PKB, US, and RAF analyzed the data. DM, SP, PK, PS, and US wrote the manuscript. AK, DB, JR, MA, and SD edited and given final touch to the manuscript. All authors reviewed the manuscript and given approval to the final 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/fmicb.2023.1104490/full#supplementary-material

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Zinc-solubilizing *Bacillus* spp. in conjunction with chemical fertilizers enhance growth, yield, nutrient content, and zinc biofortification in wheat crop

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Micronutrient deficiency is a serious health issue in resource-poor human populations worldwide, which is responsible for the death of millions of women and underage children in most developing countries. Zinc (Zn) malnutrition in middle- and lower-class families is rampant when daily calorie intake of staple cereals contains extremely low concentrations of micronutrients, especially Zn and Fe. Looking at the importance of the problem, the present investigation aimed to enhance the growth, yield, nutrient status, and biofortification of wheat crop by inoculation of native zinc-solubilizing *Bacillus* spp. in conjunction with soil-applied fertilizers (NPK) and zinc phosphate in saline soil. In this study, 175 bacterial isolates were recovered from the rhizosphere of wheat grown in the eastern parts of the Indo-Gangetic Plain of India. These isolates were further screened for Zn solubilization potential using sparingly insoluble zinc carbonate ($ZnCO_3$), zinc oxide (ZnO), and zinc phosphate $\{Zn_3(PO_4)_2\}$ as a source of Zn under *in vitro* conditions. Of 175 bacterial isolates, 42 were found to solubilize either one or two or all the three insoluble Zn compounds, and subsequently, these isolates were identified based on 16S rRNA gene sequences. Based on zone halo diameter, solubilization efficiency, and amount of solubilized zinc, six potential bacterial strains, i.e., *Bacillus altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-22, *B. licheniformis* MJW-38, *Brevibacillus borstelensis* CHW-2, and *B. xiamenensis* BLW-7, were further shortlisted for pot- and field-level evaluation in wheat crop. The results of the present investigation clearly indicated that these inoculants not only increase plant growth but also enhance the yield and yield attributes. Furthermore, bacterial inoculation also enhanced available nutrients and microbial activity in the wheat rhizosphere under pot experiments. It was observed that the application of *B. megaterium* CHW-22 significantly increased the Zn content in wheat straw and grains along with other nutrients (N, P, K, Fe, Cu, and Mn) followed by *B. licheniformis* MJW-38 as compared to other inoculants. By and large, similar observations were recorded under field conditions. Interestingly, when comparing the nutrient use efficiency (NUE) of wheat, bacterial inoculants showed their potential in enhancing the NUE in a greater way, which was further confirmed

by correlation and principal component analyses. This study apparently provides evidence of Zn biofortification in wheat upon bacterial inoculation in conjunction with chemical fertilizers and zinc phosphate in degraded soil under both nethouse and field conditions.

KEYWORDS

microbial inoculants, nutrient use efficiency, wheat (*Triticum aestivum*), Zn biofortification, PGPR, rhizosphere, biological yield

Introduction

Globally, wheat (*Triticum aestivum* L.) is the most important staple crop, and it is considered a main source of food and income for millions of smallholder farmers (Shewry and Hey, 2015). It is grown in nearly every part of the world. Wheat-based foods are therefore critical for food and nutritional security worldwide (FAOStat, http://faostat.fao.org/site/291/default.aspx). In addition, wheat is considered an important staple food as well as a major source of food, feed, fiber, fuel, starch, and energy (Paloma et al., 2016; Acevedo et al., 2018; Singh et al., 2019; Igrejas and Branlard, 2020). It also provides substantial amounts of other nutritional components, which are essential or beneficial for health. Among them, vitamins (notably the B group of vitamins), dietary protein, fiber, minerals, and phytochemicals are noteworthy (Igrejas and Branlard, 2020; Erenstein et al., 2022). There is a well-established relationship between consumption of cereal dietary fiber and reduced risk of cardiovascular disease, type 2 diabetes, and forms of cancer (notably colorectal cancer) (Pipero et al., 2015). Besides these, zinc (Zn) deficiency in wheat is rampant and causes Zn malnutrition in resource-poor human populations especially in women and underage children in most developing countries (Mottaleb et al., 2022). It has been reported that one-third (3 billion) of the global population is at high risk of Zn deficiency (FAO, 2014). Such an inadequate dietary status of Zn induces diseases such as immune deficiency, cancer, memory disorder, pneumonia, cardiovascular disorder, respiratory issues, and diarrhea in humans (Yadav et al., 2022). Therefore, mitigating Zn malnutrition poses a big challenge for researchers, policymakers, and other stakeholders. Zinc is a vital micronutrient for plants regulating the production of phytohormones, synthesis of starch, chlorophyll, protein, maintenance of membrane integrity, carbohydrate metabolism, auxin metabolism, cell growth, and multiplication (Alloway, 2008). Furthermore, it is also a cofactor of all six classes of enzymes, namely hydrolases, isomerases, ligases, lyases, oxidoreductases, and transferases (Rehman et al., 2019; Ullah et al., 2020). Normal plant growth and development require around 15–55 $\mu g\ g^{-1}$ of zinc in the tissues (Al Jabri, 2022). Zn deficiency in plants induces chlorosis, retards growth, affects root development, water uptake and transport, and grain yield, and affects the immune responses against biotic and abiotic responses in plants (Alloway, 2004). The application of an optimal dose of Zn improves the quality of cereal grains by improving proteins, carbohydrates, nucleic acid, and lipid synthesis (Sharma et al., 2013).

There are several factors, such as agronomic, edaphic, environmental, and anthropogenic, responsible for zinc deficiency in 50% of the world's soils (Alloway, 2009; Ramesh et al., 2014). Similarly, there are soil factors responsible for the low availability of Zn to plants, such as low organic matter contents, high calcium carbonate content, extremely high and low pH, significantly higher concentrations of cations, and bicarbonate and phosphate concentration being the noteworthy ones (Alloway, 2009; Rehman et al., 2018a,b; Singh et al., 2021b). Increasing the uptake of Zn from soil and accumulation in the grain can reduce the problem of Zn deficiency. Grain enrichment with Zn can be enhanced through agricultural approaches such as agronomic, plant breeding, and transgenic approaches (Prasad et al., 2010). Of these, continuous application of Zn fertilizers in the soil leads to its fixation into unavailable form in soil subject to the soil types and chemical reactions (Cakmak, 2008). Such insoluble/unavailable Zn compounds in the soil can be transformed back to soluble/available form by the processes governed by zinc-solubilizing bacteria (ZSB) (Saravanan et al., 2007; Aloo et al., 2022; Yadav et al., 2022). The use of ZSB is an environmentally friendly, cost-effective, and sustainable approach for Zn biofortification in cereal grain crops. Several ZSB strains have been reported to solubilize unavailable forms of Zn for improving plant growth, yield, and grain quality (Ramesh et al., 2014; Yadav R. et al., 2020). Among these, *Bacillus* spp., ubiquitous in nature, are widely studied that possess a multitude of PGP traits, including zinc solubilization. *Bacillus* spp. solubilize insoluble forms of Zn compounds by the secretion of organic acids in general, proton extrusion, and production of chelating ligands (Zhao et al., 2011; Rashid et al., 2012; Ramesh et al., 2014; Yadav et al., 2022). Production of 2-ketogluconic acid and gluconic acid by ZSB is of particular mention as these are the main acids responsible for Zn solubilization (Zhao et al., 2011; Desai et al., 2012; Rashid et al., 2012; Kumari et al., 2016; Yadav et al., 2022). According to earlier studies, inoculation of wheat with *Bacillus aryabhattai* MDSR14 and *B. thuringiensis* FA-4 enriched grain's Zn up to 38 and 46%, respectively (Ramesh et al., 2014; Abaid-Ullah et al., 2015).

Biofortification by inoculation of ZSB is a widely accepted approach for enriching nutrient concentration in the edible portions of the crops to improve human and animal health (Aloo et al., 2022). In view of the abovementioned facts, it is imperative to evaluate the effectiveness and efficacy of ZSB in a sustainable way to increase the bioavailability of Zn in soil, which ultimately helps in maintaining plant and human health (Igrejas et al., 2020). The information on the effects of rhizobacteria especially bacilli in conjunction with the recommended dose of fertilizer (RDF) and zinc phosphate on Zn biofortification in wheat is lacking, and this study was undertaken in order to develop an agronomic practice for wheat cultivation in degraded soil of Eastern Uttar Pradesh and also

other parts of the country. Hence, the objectives of the study were to identify and characterize bacteria isolated from wheat rhizosphere with zinc-solubilizing ability and to further evaluate the influence of single inoculation of potential zinc-solubilizing rhizobacterial in conjunction with soil-applied RDF and Zn phosphate in degraded soil on growth, yield, and Zn biofortification of wheat crop under both pot and field conditions.

Materials and methods

Bacterial strains

During the course of the investigation, 32 soil samples from the rhizosphere of wheat were collected from different parts of Eastern Uttar Pradesh, India into cool packs and brought to the laboratory. The moist soil samples were stored in a refrigerator until further analysis. For the isolation of bacterial isolates, serially diluted soil samples were plated on nutrient agar, Bacillus agar, and Pseudomonas agar (HiMedia Pvt. Ltd., Mumbai, India) with incubation at 28°C for 2-3 days. One hundred seventy-five isolates with diverse morphotypes were recovered from 32 soil samples. The pure bacterial cultures were maintained on a nutrient agar medium at 28°C till further use. Later on, another duplicate set of cultures was preserved in 20% glycerol stock at−20°C in a deep freezer (Blue Star, India).

In vitro screening of zinc-solubilizing ability of rhizobacteria

These rhizobacterial isolates were screened for their zinc-solubilizing ability on tris-minimal agar medium with D-glucose (10 gl^{-1}) and separately supplemented with zinc oxide (1.244 g l^{-1} = 15.23 mM), zinc phosphate (1.9882 g l^{-1} = 5.0 mM), and zinc carbonate (1.728 g l^{-1} = 5.2 mM) to prepare three separate media (Khande et al., 2017) with slight modifications (Yadav et al., 2022). Briefly, freshly grown bacteria were spot-inoculated with doubled sterile toothpicks on Petri plates containing tris-minimal agar medium separately amended with zinc oxide, zinc phosphate, and zinc carbonate. The Petri plates were incubated in the dark at 28°C for 7 days to observe the formation of a clear halo zone around colonies. Subsequently, the colony diameter and the diameter of the halo zone (mm) formed around the colony were measured after 7 days of inoculation.

Identification of zinc-solubilizing rhizobacteria

Based on the zinc solubilization potential of the isolates, 42 isolates were shortlisted and subjected to identification on the basis of 16S rRNA gene sequence homology. Bacterial DNA was isolated using Nucleo-pore® gDNA Fungal/Bacterial Mini Kit (Cat.# NP-7006D, Genetix Biotech Asia Pvt. Ltd., New Delhi, India). According to Singh et al. (2021a), the amplification of 16S rRNA gene was performed using Peqlab peqSTAR, Thermal Cyclers (VWR Lab Products Pvt. Ltd., Bangalore, India). The molecular grade chemicals used were procured from Merck Specialities Private Limited, Mumbai, India. The sequences were aligned using the EzTaxon server for the identification of the bacterial isolates. The phylogenetic analysis of sequences was performed using Molecular Evolutionary Genetics Analysis (MEGA- 10), and 16S rRNA gene sequences were submitted to the NCBI GenBank.

Selection of potential Zn-solubilizing rhizobacteria

The isolates which solubilized all three zinc compounds were considered potential zinc solubilizers (Yadav et al., 2022). Based on the zone diameter formed on all three solid media, six potential zinc solubilizers were further evaluated for their ability to release soluble zinc in a liquid medium as well as a reduction in medium pH in Erlenmeyer flasks (Ramesh et al., 2014; Khande et al., 2017). Briefly, 1.0 ml culture (10^8 CFU ml^{-1}) of each bacterium was inoculated in tris-minimal broth containing 0.1% Zn as zinc oxide, zinc phosphate, and zinc carbonate, separately. The tris-minimal broth supplemented with inorganic zinc but without bacterial inoculation served as an uninoculated control. After 10 days of incubation at 28°C in a shaker at 120 rpm, the samples were withdrawn and centrifuged at 10,000 rpm for 10–12 min to obtain clear supernatant, which was directly fed to an atomic absorption spectrophotometer for estimation concentration of soluble zinc (μg Zn ml^{-1}). The fall in pH of the broth medium of all the treatments including uninoculated was also measured. Entire experiments were performed in triplicates. Furthermore, Zn solubilization efficacy was calculated according to Vazquez et al. (2000) with slight modification (Ramesh et al., 2014). The gluconic acid was quantified using high-performance liquid chromatography (Shimadzu, Separon SGX C18 column) equipped with a quaternary pump, auto-sampler, DAD detector, and degasser following the procedure described by Larcher et al. (2009) with slight modifications (Sunithakumari et al., 2016).

These strains were further evaluated for their plant growth-promoting attributes, such as solubilization of potassium and phosphate; and production of indole-3-acetic acid (IAA), siderophore, ammonia, and HCN of potential zinc solubilizers were assayed using standard methods. The potassium solubilization was estimated by the method of Rajawat et al. (2016), whereas phosphate solubilization was performed on Pikovskaya medium containing 0.1% tri-calcium phosphate according to Olsen and Sommers (as cited in Penrose and Glick, 2003). The IAA production by zinc solubilizers was estimated as per the method of Ahmad et al. (2020). The siderophore production on chrome azurol S (CAS) agar by zinc solubilizers was estimated as per the method described by Schwyn and Neilands (1987). HCN production was estimated as per the method of Kremer and Souissi (2001). Ammonia production of zinc solubilizers was estimated by adding 1 ml Nessler's reagent to 72-h-old cultures grown in peptone water broth. Production of the brown color from yellow was treated as a positive test. ACC deaminase activity of zinc solubilizers was determined as per the method of Bal et al. (2013).

The catalase, oxidase, amylase, protease, and lipase enzyme potential of zinc solubilizers was tested using standard methods (Cappuccino and Welsh, 2017). Cellulase production was tested by growing bacterial cultures on LB agar supplemented with

1% carboxymethyl cellulose (CMC) using the method described by Lin et al. (2012), whereas lipase was detected on LB agar supplemented with Tween-20 (Sierra, 1957). Skimmed milk agar was used for the production of proteases (Kumar et al., 2005).

Evaluation of potential zinc solubilizers under pot and field conditions

Planting materials and growth conditions

Wheat seeds (cv. HD-2967) were procured from ICAR-Indian Institute of Seed Sciences, Kushmaur, Mau, India. Experiments were conducted during the winter season (2020–2021 and 2021–2022) to investigate the impact of zinc-solubilizing bacteria on wheat growth and development and Zn biofortification. The weather conditions during the growing period were as follows: mean temperature of 22-25°C and relative humidity of 70–75% with 11-/13-h photoperiod.

Preparation of inoculants of zinc solubilizers

Six selected zinc-solubilizing bacterial strains, *viz*., *B. altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-22, *B. licheniformis* MJW-38, *Brevibacillus borstelensis* CHW-2, and *B. xiamenensis* BLW-7, were used for their evaluation on plant growth, yield, biofortification of nutrient content, and rhizosphere properties of wheat crop. The bacterial inoculums were prepared using 0.85% sterile saline solution as per the method described by Ramesh et al. (2014) with slight modifications (Singh et al., 2016). The population count (CFU) of inoculums was adjusted to 2×10^8 cfu ml^{-1} before application to the seeds.

Experimental setup

The six selected zinc-solubilizing bacterial strains were evaluated in the presence of RDF and with or without zinc phosphate under the nethouse at ICAR-NBAIM, Mau (25°53"56.99//N 83°29"18.29//E; elevation 74 m) as well as under farmers' field conditions near District Jail, Mau (25°55.828 N 83°28.390 E; elevation: 52m), Uttar Pradesh, India, to assess their impact on plant growth, yield, and biofortification of nutrient content especially Zn in wheat grains. The eight treatments were: T_1–Absolute control, T_2–RDF, T_3–RDF + *B. altitudinis* AJW-3, T_4–RDF + *B. subtilis* ABW-30, T_5–RDF + *B. megaterium* CHW-22, and T_6–RDF + *B. licheniformis* MJW-38, T_7–RDF + *Brevibacillus borstelensis* CHW-2, and T_8–RDF + *B. xiamenensis* BLW-7. The experiment was performed in a completely randomized block design (CRBD)/randomized block design (RBD) containing eight different treatments with five replicates each in the nethouse and three replicates each in the field experiment. Recommended doses of fertilizers for N: P: K (120:60:60) were applied in all treatments. The experiment was divided into two parts, the first part with the above eight treatments where the wheat (variety HD-2967) seeds were treated with zinc-solubilizing bacterial strains in soil without zinc phosphate [Zn_3 $(PO_4)_2$]. Another part of the experiment included similar treatments but the soil was applied with 5 kg/ha zinc phosphate to study the effect of combined inoculation of soil-applied zinc and inoculation of zinc-solubilizing bacteria on plant growth yield and nutrient content in soil and plant. Moisture content in the pots was maintained at field capacity by adding sterile distilled water as and when needed.

Evaluation of zinc-solubilizing *Bacillus* spp. in pots under nethouse conditions

The experiment was laid out with eight treatments and five replications each in a complete randomized design block (CRBD) with or without zinc phosphate application. The treatments are the same as mentioned in the previous section, "Experimental setup." Soil for the pot experiments was collected from farmers' fields near the District Jail, Mau (25°55.828 N 83°28.390 E, Elevation: 52m), Uttar Pradesh, India, and each pot was filled up with 3 kg soil. The characteristics of the experimental soil were as follows: pH 8.2 ± 0.2, EC 4.2 ± 0.2 dS m^{-1}, OC 0.37 %, available-N 142.50 kg ha^{-1}, available-P 13.96 kg ha^{-1}, available-K 205.56 kg ha^{-1}, DTPA-Zn 0.52 μg g^{-1}. Recommended doses of fertilizers for N: P: K (120:60:60) kg/ha were applied as basal doses. The soil was further mixed with 6.6 mg Zn_3 $(PO_4)_2$ (at the rate of 2.2 mg Kg^{-1}) as a Zn source in each pot containing 3 kg soil in one set of experiments. Furthermore, seeds were surface-sterilized with 1% NaOCl for 60 s and later washed with sterile distilled water 3-4 times to make seeds free of NaOCl. A population of 10^8 CFU ml^{-1} bacterial suspension was prepared using 0.85% sterile saline solution as mentioned earlier.

Seeds were bioprimed with 20 ml of bacterial suspension/kg^{-1} seeds for overnight (12 h) at ambient room temperature followed by the sowing of four seeds per pot. After germination, only two seeds were maintained during the entire experimentation following all standard agronomic practices. All the pots were irrigated as per the requirement with sterile distilled water to maintain moisture at field capacity.

Evaluation of zinc solubilizers in the field

A field experiment with wheat was conducted in the plot (dimension 3×2 m^2) with plot-to-plot spacing of 1 m during the winter season (2020–2021 and 2021–2022). The randomization of plots with three replications was maintained. Each plot was basely dressed with N: P: K (120:60:60 kg ha^{-1}), and zinc phosphate was added as described in the previous section. The field soil was deep and well-drained silty clay loam with pH 8.2 ± 0.2, EC 4.2 ± 0.2 dS m^{-1}, OC 0.37 %, available-N 142.50 kg ha^{-1}, available-P 13.96 kg ha^{-1}, available-K 205.56 kg ha^{-1}, and DTPA-Zn 0.52 μg g^{-1}. The same wheat variety HD-2967 that was grown in the pot experiment was used in the field study. Seeds were sown at a rate of 43 seeds m^{-2} and a depth of 4–5 cm. The treatments were the same as mentioned in the section on "Experimental setup". Bacterially treated seed @ 120 kg ha^{-1} was sown to each plot in line. All the standard agronomic practices prescribed for wheat crop were followed. Four irrigations were given at critical stages, i.e., crown root initiation stage (21 DAS), tillering stage (45 DAS), flowering

stage (70 DAS), and grain filling stage (95 DAS) to fulfill the water requirement of the crop under field conditions.

Evaluation and analysis

Effects of seed biopriming on plant growth and yield parameters

The wheat plants were uprooted from both pot and field carefully, and data were recorded on the agronomic parameters such as plant height (cm) and dry matter accumulation (g pot^{-1} and g m^{-2} under field conditions) at 30DAS, 60DAS, and 90DAS (days of sowing) as the indicative of plant growth according to Kumar et al. (2014), and yield attributes such as number of effective tillers, spike lengths (cm), spikelet $spike^{-1}$, number of grain $spike^{-1}$, test weight, and grain yield of wheat were measured after harvesting as per the methods described by Rana et al. (2014). The grains were dried in sunlight for 4–5 days, carefully weighed, and the yield was calculated in terms of tones $hectare^{-1}$. The grain yield was noted at 10% moisture content. The straw yield and biological yield were measured in tons per hectare, and the Harvesting Index (HI) was calculated in percentage as per the method given by Kumar et al. (2014).

Effects of seed biopriming on chemical and biological properties of soil

Soil samples were collected after harvesting wheat plants under both pot and field conditions and subjected to analysis of soil chemical and biological properties. Before analysis, all the soil samples were air-dried and passed through a 1-mm sieve. The soil's chemical properties such as soil pH (1:2.5 soil: water) were measured using a glass electrode pH meter (Richards, 1954), and the EC (dS m^{-1}) of soil was measured using a conductivity bridge (Piper, 1950). The SOC was determined as per the method described by Walkley and Black (1934). Soil-available macronutrients N, P, and K were determined by the standard method described by Subbiah and Asija (1956), Olsen (1954), and Jackson (1973), respectively. The micronutrients, *viz*., Zn, Fe, Mn, and Cu concentrations in soil were determined by the DTPA extraction method using an atomic absorption spectrophotometer (Lindsay and Norvell, 1978). The soil biological properties such as soil microbial biomass (SMBC mg $kg–^{1}$ soil), soil dehydrogenase activity (DHA, μg TPF g^{-1} soil $day–^{1}$), soil alkaline phosphatase activity (APA, μg *p*-nitrophenol g^{-1} soil h^{-1}), and fluorescein diacetate (FDA, μg fluorescein released g^{-1} soil h^{-1}) were measured following the standard method described by Casida et al. (1964), Tabatabai and Bremner (1969), Nunan et al. (1998), and Green et al. (2006), respectively.

Effects of seed biopriming on macro- and micronutrients in plant samples

At maturity, the wheat plants under pot and field experiments were sampled for the estimation of macro- (N, P, and K) and micronutrient (Zn, Fe, Cu, and Mn) contents. The plant samples (grains and straw) were dried, ground to fine powder, and digested in a di-acid mixture containing nitric acid and perchloric acid (5:4 v/v) at 320°C for 1 h, and Zn, Fe, Cu, and Mn content (μg Zn g^{-1} plant material) was measured using an atomic absorption spectrophotometer at the most sensitive wavelengths for Zn (213.7 nm), Fe (248.7 nm), Cu (324.6 nm), and Mn (279.5 nm). Potassium concentration was analyzed using a flame photometer and compared with standards ranging from 0 to 100 ppm of KCl. Phosphorous concentration was measured using the method of Jackson (1967). The total N of plant samples was estimated using the Kjeldahl method (Jackson, 1967).

Effects of seed biopriming on nutrient use efficiency of wheat plant

The nutrient use efficiency such as partial factor productivity, agronomic efficiency, apart nutrient recovery, and physiological efficiency of wheat plants was calculated as per the method described by Piper (1966).

Statistical analysis

The nethouse and field experiments were repeated twice in two consecutive years (2020–2021 and 2021–2022), and pooled analysis was performed. The data on various parameters were analyzed in triplicates for the plant study and subjected to an analysis of variance (ANOVA) in accordance with the experimental design (completely randomized block design) using Statistical Package for Social Science (SPSS) version 11.5 to quantify and evaluate the source of variation. The treatment means were compared at a significance level of 0.05. Furthermore, all the data obtained from this study were statistically analyzed using the F-test as per the procedure described by Gomez and Gomez (1984). Least significance difference (LSD) values at p=0.05 were used to determine the significance of differences between means. Correlation analysis was performed using SPSS 11.5. Heatmaps were constructed using Package 'Heatplus' version 3.7.0 (Ploner, 2020), 'RColorBrewer' (Kanno et al., 2011), and 'g plots' (Warnes et al., 2016) packages in R (version 3.6.1) using Ward's hierarchical clustering (Strauss and von Maltitz, 2017). The PCA was conducted using the biplot method in Matlab R2019b version 9.7 (Math Works Inc., USA) to detect the effect of different treatments on the Zn and other nutrient concentrations (N, P, K, Fe, Cu, and Mn) in grains and straw of wheat crops. The PCA was carried out according to Mishra et al. (2017). PCs with high eigenvalues best signify variation in the systems, so only the PCs with eigenvalues ≥ 1 were retained (Kaiser, 1960). The extracted outcomes of a PCA are presented in terms of component scores, also known as factor scores and loadings (Wold et al., 1987).

Results

Isolation and identification of zinc-solubilizing rhizobacteria

During the course of isolation, 175 distinct bacterial morphotypes were isolated from different parts of Eastern Uttar Pradesh. These isolates were screened for Zn solubilization

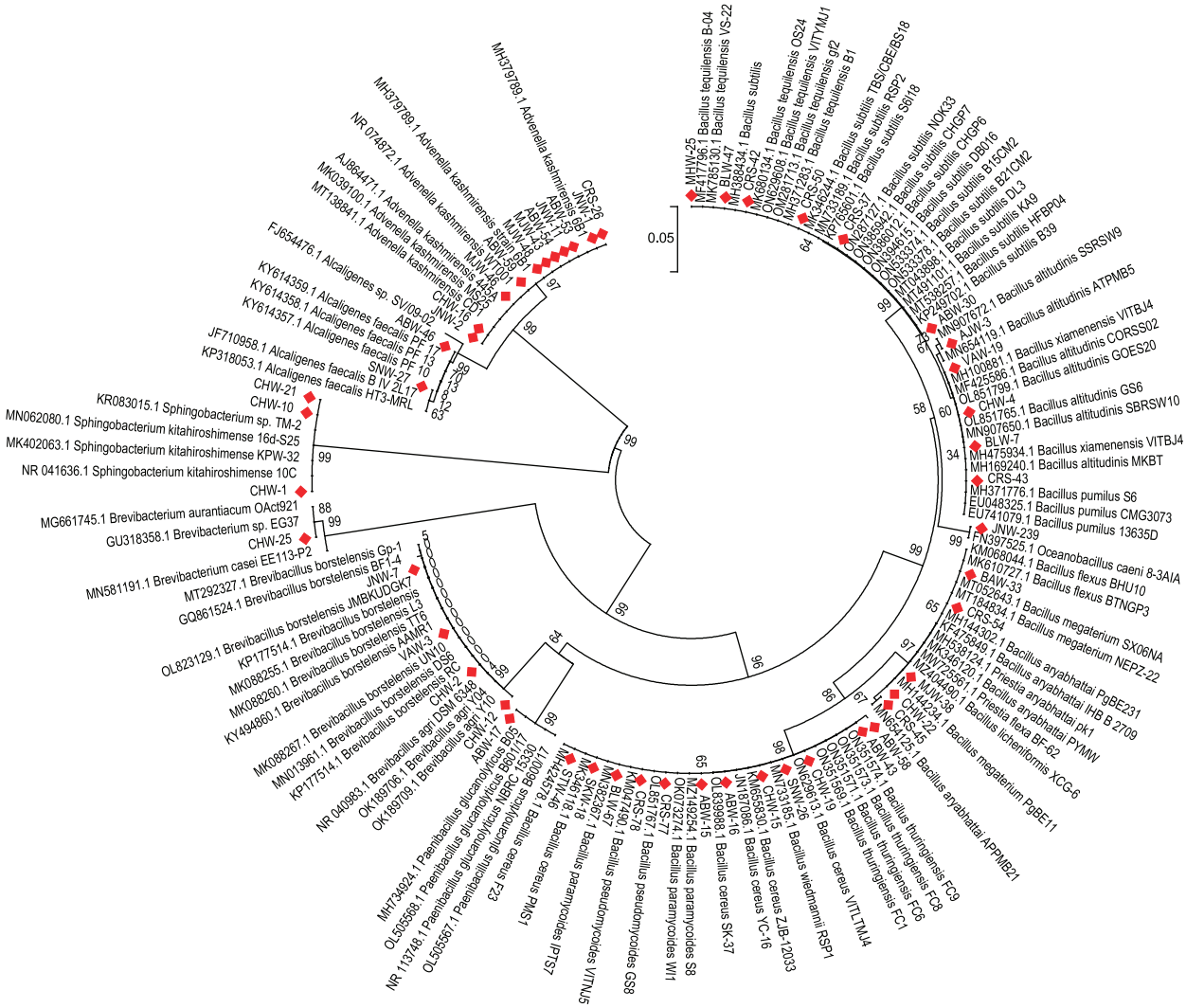

FIGURE 1
Neighbor joining tree derived using CLUSTAL W and MEGA X using analysis of 16S rRNA gene sequences. The numbers at nodes indicate bootstrap support values, as calculated using MEGA 5.2.

on tris-minimal agar medium supplemented with three different Zn sources, i.e., zinc oxide, zinc carbonate, and zinc phosphate. Of the 175 morphotypes, 42 rhizobacteria solubilized either of the zinc compounds on plate assay and were shortlisted for identification (Supplementary Table 1). The isolates were designated as CHW-12, ABW-30, CHW-21, JNW-2, VAW-19, ABW-43, ABW-46, CHW-4, JNW-23, BLW-7, ABW-17, SNW-27, CHW-16, CHW-2, MJW-46, ABW-59, CHW-19, MJW-48, MJW-43, ABW-54, CHW-25, SNW-26, ABW-58, CHW-15, CHW-10, CHW-22, JNW-11, ABW-16, ABW-53, ABW-15, JNW-1, VAW-3, MJW-38, JNW-7, AJW-3, CHW-1, BLW-47, BAW-33, BLW-67, SKW-18, MHW-25, and STW-46 (Supplementary Table 2). These 42 isolates were identified based on 16S rRNA gene sequence homology and percentage similarity during BLAST analysis using EzBioCloud, a public database of type strains (Supplementary Table 2). The 16S rRNA gene sequences were submitted to NCBI GenBank, and accession numbers were obtained. The results of BLAST homology analyses yielded 19 different bacterial species, including *Brevibacillus agri* (1), *Bacillus subtilis* (1), *Sphingobacterium kitahiroshimense* (3), *Advenella kashmirensis* (10), *Bacillus xiamenensis* (2), *Bacillus thuringiensis* (1), *Alcaligenes faecalis* (2), *Bacillus altitudinis* (2), *Oceanobacillus caeni* (1), *Paenibacillus glucanolyticus* (1), *Brevibacillus borstelensis* (3), *Bacillus cereus* (6), *Brevibacterium aurantiacum* (1), *Bacillus wiedmannii* (1), *Bacillus megaterium* or *Priestia megaterium* (1), *Bacillus paramycoides* (2), *Bacillus licheniformis* (1), *Bacillus tequilensis* (2) and *Bacillus flexus* or *Priestia flexa* (1). It was further observed that *Advenella kashmirensis* followed by *Bacillus cereus, Sphingobacterium kitahiroshimense* and *Brevibacillus borstelensis,* were the most dominant species (Figure 1, Supplementary Table 2).

Characterization of zinc-solubilizing *Bacillus* spp.

It was observed that some of the strains solubilize all three zinc compounds tested. Based on the size of the halo zone formed around the bacterial colony and isolates solubilized by all three zinc compounds, six potential strains, *B. altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-22, *B. licheniformis* MJW-38, *Brevibacillus borstelensis* CHW-2, and *B. xiamenensis* BLW-7, were selected for further in-depth investigation (Supplementary Table 3).

The zinc solubilization efficacy was calculated on the basis of the diameter of the halo zone formed on a growth medium supplemented with three different zinc compounds, *viz*., zinc oxide, zinc phosphate, and zinc carbonate. Among the six strains selected, *B. licheniformis* MJW-38 showed maximum zinc phosphate solubilization efficiency (411.76%) followed by *B. megaterium* CHW-22 (375.00%) and *B. altitudinis* AJW-3 (325.00%). However, the least zinc phosphate solubilization efficiency was reported in *Brevibacillus borstelensis* CHW-2 (273.68). Similarly, *B. megaterium* CHW-22 (375.00%) showed maximum zinc oxide followed by *B. subtilis* ABW-30 (244.44%) and *B. xiamenensis* BLW-7 (225.00%). However, in the case of zinc carbonate, maximum solubilization efficacy was recorded in *B. megaterium* CHW-22 (244.95%) followed by *Brevibacillus borstelensis* CHW-2 (240.00%), *B. subtilis* ABW-30 (216.22%), and *B. xiamenensis* BLW-7 (214.29%) (Table 1).

In general, the maximum reduction in the media pH was reported in the case of *B. megaterium* CHW-22 across the zinc compounds used. The quantitative assays indicated that all six isolates are efficient zinc solubilizers. The maximum amount of solubilized zinc was recorded in a liquid medium inoculated with *B. licheniformis* MJW-38 (40.00 μg Zn ml^{-1}) and supplemented with zinc phosphate at the lowest mean pH value of 4.2 (Table 1). In the case of zinc oxide, the maximum amount of solubilized zinc was recorded in a liquid medium inoculated with *B. megaterium* CHW-22 (29.00 μg Zn ml^{-1}) and *B. subtilis* ABW-30 (28.00 μg Zn ml^{-1}) and at the lowest mean pH value of 4.4 and 4.2, respectively. A more or less similar observation was recorded in the case of zinc carbonate where maximum zinc solubilization was reported in the liquid medium inoculated with *B. megaterium* CHW-22 (24.00 μg Zn ml^{-1}) as compared to other strains tested (Table 1).

The production of gluconic acid in the presence of zinc phosphate, zinc oxide, and zinc carbonate by the rhizobacterial strains used in the study was determined using HPLC. All the strains were found to produce gluconic acid in the medium supplemented with zinc phosphate, zinc oxide, and zinc carbonate. The HPLC results clearly revealed that a significantly higher amount of gluconic acid was produced by *B. borstelensis* CHW-2 (171.57 μg ml^{-1}) followed by *B. megaterium* CHW-22 (150.92 μg ml^{-1}) and *B. xiamenensis* BLW-7 (149.89 μg ml^{-1}) in the presence of zinc phosphate. A similar trend was recorded in the case of zinc oxide and zinc carbonate (Table 1).

Selected strains were further characterized for PGP traits, viz. P solubilization, K solubilization, IAA production, ACC deaminase activity, siderophore, HCN, and ammonia production. Similarly, these strains were also tested for the production of enzymes such as catalase, oxidase, amylase, protease, lipase, and cellulose (Supplementary Table 4). All six strains solubilized P and K and exhibited strong PGP traits. These strains were found to produce varying levels of IAA in culture filtrate with the maximum being by *B. megaterium* CHW-22 (18.61 μg ml^{-1}) followed by *B. xiamenensis* BLW-7 (12.70 μg ml^{-1}) and *B. licheniformis* MJW-38 (10.16 μg ml^{-1}). Except for *B. altitudinis* AJW-3, *B. subtilis* ABW-30, and *B. megaterium* CHW-22, the other three strains did not produce ACC deaminase in the medium. Differential activities were recorded for siderophore, HCN, and ammonia production. A more or less similar trend was recorded for enzyme assay except for oxidase. None of the bacterial strains was found positive for the oxidase test (Supplementary Table 4).

Evaluation of microbial inoculants under nethouse conditions

Plant growth and yield attributes

The effect of rhizobacterial inoculation on plant height and dry matter accumulation was recorded at 30, 60, and 90 DAS in the wheat plants grown under nethouse conditions supplemented with and without zinc phosphate. In general, all the strains considerably increased the plant height and dry matter accumulation as compared to absolute control plants and RDF-treated plants. When compared among the microbial inoculants, significantly higher plant height and dry matter accumulation were recorded in the treatments inoculated with *B. megaterium* CHW-22 closely followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other inoculants at 30, 60 and 90 DAS. A more or less similar trend was recorded in the plants grown with and without zinc phosphate. However, these parameters were slightly higher in the plants amended with zinc phosphate (Table 2).

Similarly, a significantly higher number of effective tiller $plant^{-1}$ (3.84), spike length (9.32c m), spikelet $spike^{-1}$ (19.87), number of grain $spike^{-1}$ (32.55), and test weight (38.48 g) were recorded in the plants inoculated with *B. megaterium* CHW-22 without zinc phosphate followed by *B. licheniformis* MJW-38 (number of effective tiller $plant^{-1}$—3.82, spike length-—9.22 cm, spikelet $spike^{-1}$—19.43, number of grain $spike^{-1}$—32.43, and test weight-—38.41 g) and *B. altitudinis* AJW-3 (number of effective tiller $plant^{-1}$—3.76, spike length-—9.15 cm, spikelet $spike^{-1}$—19.08, number of grain $spike^{-1}$—32.32, and test weight-—38.37 g) as compared to other inoculants, RDF alone, and untreated absolute control. A similar pattern was reported in the plants amended with zinc phosphate and inoculated with microbial inoculants. However, these values are slightly higher as compared to treatments without zinc phosphate (Table 2).

Furthermore, grain yield (g pot^{-1}), straw yield (g pot^{-1}), biological yield (g pot^{-1}), and harvest index (%) were also recorded in the plants inoculated with selected microbial inoculants supplemented with and without zinc phosphate under nethouse conditions. The results indicated that maximum grain yield (4.32 and 4.28 g pot^{-1}), straw yield (6.49 and 6.49 g pot^{-1}), biological yield (10.81 and 10.77 g pot^{-1}), and harvest index (39.95 and 39.73%) were recorded in the plants inoculated with *B. megaterium* CHW-22 and *B. licheniformis* MJW-38, respectively, without zinc

TABLE 1 Zinc solubilization halo zone on agar medium, solubilization efficiency, zinc solubilization, pH, and gluconic acid production in liquid cultures containing zinc as inorganic insoluble form as influenced by inoculation of zinc-solubilizing *Bacillus* species.

Isolates	Dia. of zinc solubilization halo zone (mm)	Solubilization efficiency (%)	Amount of solubilized zinc (μg Zn ml^{-1})	pH of culture medium	Gluconic acid (μg mL^{-1})
Zinc phosphate					
Uninoculated control	NDc	ND	ND	6.8 ± 0.17^{a}	ND
Bacillus altitudinis AJW-3	26.00 ± 1.72^{c}	325.00 ± 13.8^{c}	30 ± 0.90^{c}	4.9 ± 0.13^{c}	110.64 ± 2.35^{e}
Bacillus subtilis ABW-30	24.00 ± 1.39^{d}	282.00 ± 14.5^{d}	28 ± 0.73^{c}	5.2 ± 0.27^{b}	120.87 ± 4.42^{d}
Bacillus megaterium CHW-2	30.00 ± 2.72^{a}	375.00 ± 9.82^{ab}	38 ± 1.23^{a}	4.3 ± 0.08^{d}	150.92 ± 1.52^{bc}
Bacillus licheniformis MJW-22	28.00 ± 1.65^{ab}	411.76 ± 23.3^{a}	40 ± 1.17^{a}	4.2 ± 0.11^{d}	129.93 ± 3.81^{c}
Brevibacillus borstelensis CHW-38	26.00 ± 1.10^{c}	273.68 ± 12.5^{d}	25 ± 1.12^{d}	5.4 ± 0.16^{b}	171.57 ± 7.45^{a}
Bacillus xiamenensis BLW-7	24.00 ± 0.98^{d}	282.35 ± 8.35^{d}	36 ± 1.43^{b}	4.7 ± 0.22^{c}	149.89 ± 2.38^{bc}
Zinc oxide					
Uninoculated control	ND	ND	ND	6.9 ± 0.37^{a}	ND
Bacillus altitudinis AJW-3	15.00 ± 0.45^{d}	166.67 ± 6.42^{d}	18 ± 0.73^{c}	5.3 ± 0.39^{bc}	141.61 ± 2.53^{c}
Bacillus subtilis ABW-30	22.00 ± 0.92^{a}	244.44 ± 9.35^{a}	28 ± 0.65^{a}	4.2 ± 0.15^{ef}	119.95 ± 4.22^{d}
Bacillus megaterium CHW-2	20.00 ± 1.42^{ab}	250.00 ± 11.3^{a}	29 ± 0.98^{a}	4.4 ± 0.13^{e}	164.75 ± 3.85^{a}
Bacillus licheniformis MJW-22	14.00 ± 0.39^{d}	215.38 ± 14.2^{c}	24 ± 0.47^{ab}	4.9 ± 0.08^{d}	161.02 ± 3.25^{ab}
Brevibacillus borstelensis CHW-38	16.00 ± 0.67^{cd}	160.00 ± 7.22^{d}	15 ± 0.52^{d}	5.6 ± 0.25^{b}	119.50 ± 5.42^{d}
Bacillus xiamenensis BLW-7	18.00 ± 0.98^{c}	225.00 ± 13.5^{ab}	25 ± 1.33^{ab}	4.8 ± 0.11^{d}	161.47 ± 7.13^{ab}
Zinc carbonate					
Uninoculated control	ND	ND	ND	6.8 ± 0.29^{a}	ND
Bacillus altitudinis AJW-3	18.00 ± 0.53^{c}	180.00 ± 5.42^{c}	15 ± 0.62^{d}	5.6 ± 0.22^{b}	140.67 ± 2.44^{b}
Bacillus subtilis ABW-30	20.00 ± 0.38^{b}	216.22 ± 6.32^{b}	20 ± 0.51^{c}	5.2 ± 0.17^{d}	76.62 ± 5.85^{e}
Bacillus megaterium CHW-2	22.00 ± 0.49^{ab}	244.44 ± 13.5^{a}	24 ± 0.63^{a}	4.3 ± 0.19^{e}	153.17 ± 3.27^{a}
Bacillus licheniformis MJW-22	16.00 ± 0.23^{cd}	177.78 ± 8.75^{c}	14 ± 0.88^{d}	5.5 ± 0.28^{bc}	144.40 ± 4.23^{ab}
Brevibacillus borstelensis CHW-38	24.00 ± 0.75^{a}	240.00 ± 9.32^{a}	22 ± 1.08^{ab}	4.6 ± 0.13^{ef}	101.78 ± 3.57^{d}
Bacillus xiamenensis BLW-7	15.00 ± 0.63^{d}	214.29 ± 15.7^{b}	19 ± 0.62^{c}	5.4 ± 0.35^{bc}	132.09 ± 4.62^{c}

Data are mean values ± SD of three replicates; means with different letters in the same column differ significantly at $p < 0.05$ according to Fisher's LSD. ND, means not detected. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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TABLE 2 Effect of zinc-solubilizing plant growth promoting rhizobacteria on growth, yield attributes, and yield of wheat under pot experiment.

Without Zn															
Control ($N_0P_0K_0$)	18.23^{e}	32.51^{e}	63.61^{e}	0.27^{e}	1.45^{d}	4.52^{e}	3.33^{e}	8.04^{e}	17.42^{e}	28.32^{b}	37.82^{a}	3.16^{e}	5.01^{e}	8.17^{e}	39.31^{a}
RDF ($N_{120}P_{60}K_{60}$)	20.45^{d}	35.61^{d}	70.40^{d}	0.32^{d}	1.52^{c}	4.78^{d}	3.54^{d}	8.78^{d}	18.19^{d}	31.84^{a}	38.33^{a}	3.96^{d}	6.10^{d}	10.06^{d}	39.36^{a}
RDF + *Bacillus altitudinis* AJW-3	21.64^{abc}	36.73^{abc}	74.59^{bc}	0.34^{b}	1.61^{ab}	5.04^{bc}	3.76^{ab}	9.15^{ab}	19.08^{b}	32.32^{a}	38.37^{a}	4.23^{ab}	6.41^{ab}	10.64^{ab}	39.70^{a}
RDF + *Bacillus subtilis* ABW-30	21.40^{c}	36.08^{c}	72.39^{cd}	0.34^{b}	1.58^{b}	4.88^{c}	3.68^{bc}	8.99^{bc}	18.78^{bc}	32.11^{a}	38.28^{a}	4.14^{c}	6.31^{c}	10.45^{c}	39.63^{a}
RDF + *Bacillus megaterium* CHW-22	21.94^{a}	37.58^{a}	78.02^{a}	0.35^{a}	1.68^{a}	5.27^{a}	3.84^{a}	9.32^{a}	19.87^{a}	32.55^{a}	38.48^{a}	4.32^{a}	6.49^{a}	10.81^{a}	39.95^{a}
RDF + *Bacillus licheniformis* MJW-38	21.81^{ab}	37.14^{ab}	76.47^{ab}	0.35^{a}	1.64^{ab}	5.18^{ab}	3.82^{a}	9.22^{ab}	19.43^{ab}	32.43^{a}	38.41^{a}	4.28^{a}	6.49^{a}	10.77^{a}	39.73^{a}
RDF + *Brevibacillus borstelensis* CHW-2	21.31^{c}	35.94^{c}	71.96^{cd}	0.33^{c}	1.56^{bc}	4.80^{cd}	3.64^{c}	8.90^{c}	18.68^{c}	32.04^{a}	38.36^{a}	4.09^{c}	6.34^{c}	10.43^{c}	39.41^{a}
RDF + *Bacillus xiamenensis* BLW-7	21.54^{bc}	36.25^{bc}	73.39^{c}	0.34^{b}	1.60^{ab}	4.98^{bc}	3.72^{b}	9.06^{bc}	18.89^{bc}	32.18^{a}	38.33^{a}	4.18^{ab}	6.35^{ab}	10.53^{ab}	39.70^{a}
Zn Applied															
Control ($N_0P_0K_0$)	19.46^{d}	34.2^{d}	66.41^{f}	0.28^{d}	1.53^{b}	4.65^{e}	3.50^{e}	8.41^{e}	18.26^{d}	29.51^{b}	39.60^{a}	3.35^{e}	4.99^{e}	8.34^{e}	40.16^{a}
RDF ($N_{120}P_{60}K_{60}$)	21.78^{c}	37.6^{c}	73.71^{e}	0.33^{c}	1.63^{ab}	4.90^{d}	3.70^{d}	9.20^{d}	19.08^{cd}	33.27^{a}	40.21^{a}	4.19^{d}	6.14^{d}	10.33^{d}	40.54^{a}
RDF + *Bacillus altitudinis* AJW-3	22.88^{ab}	38.9^{ab}	78.47^{bc}	0.36^{ab}	1.71^{ab}	5.27^{ab}	4.00^{ab}	9.66^{ab}	20.15^{abc}	34.00^{a}	40.48^{a}	4.49^{ab}	6.51^{ab}	11.00^{ab}	40.82^{a}
RDF + *Bacillus subtilis* ABW-30	22.72^{b}	38.1^{b}	75.94^{cde}	0.35^{b}	1.68^{ab}	5.09^{ab}	3.90^{bc}	9.47^{bcd}	19.74^{bc}	33.68^{a}	40.27^{a}	4.38^{c}	6.38^{c}	10.76^{c}	40.74^{a}
RDF + *Bacillus megaterium* CHW-22	23.12^{a}	39.9^{a}	82.23^{a}	0.37^{a}	1.80^{a}	5.53^{a}	4.10^{a}	9.87^{a}	21.04^{a}	34.41^{a}	40.75^{a}	4.60^{a}	6.61^{a}	11.21^{a}	41.04^{a}
RDF + *Bacillus licheniformis* MJW-38	23.03^{ab}	39.4^{ab}	80.52^{ab}	0.36^{ab}	1.75^{a}	5.42^{a}	4.00^{b}	9.75^{ab}	20.54^{ab}	34.21^{a}	40.60^{a}	4.57^{a}	6.57^{a}	11.14^{a}	41.04^{a}
RDF + *Brevibacillus borstelensis* CHW-2	22.67^{b}	38.0^{bc}	75.49^{de}	0.35^{b}	1.66^{c}	5.00^{c}	3.80^{c}	9.35^{cd}	19.61^{bc}	33.55^{a}	40.28^{a}	4.38^{c}	6.32^{c}	10.70^{c}	40.72^{a}
RDF + *Bacillus xiamenensis* BLW-7	22.84^{ab}	38.3^{b}	77.06^{cd}	0.35^{b}	1.69^{ab}	5.21^{ab}	3.90^{bc}	9.55^{bc}	19.89^{abc}	33.82^{a}	40.36^{a}	4.43^{ab}	6.44^{ab}	10.87^{ab}	40.77^{a}

RDF: recommended dose of fertilizers: 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 60 kg K ha^{-1}, DMA: dry matter accumulation, GY: grain yield, SY: straw yield, and BY: biological yield: HI: Harvesting Index, ET: effective tiller, SL: spike length, and DAS: days after sowing. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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phosphate followed by *B. altitudinis* AJW-3 (grain yield-−4.23 g pot^{-1}, straw yield-−6.41 g pot^{-1}, biological yield-−10.64 g pot^{-1}, and harvest index 39.70%) under nethouse conditions (Table 2). A more or less similar trend was recorded in the plant grown with zinc phosphate. However, these values were slightly higher in the case of zinc phosphate-amended plants (Table 2).

Effect of microbial inoculation on SOC, available nutrients, and microbial activity in rhizosphere soil

Unlike plant growth and yield attributes, microbial inoculation has a positive impact on SOC, available nutrients, and microbial activity in rhizosphere soil, which is further increased after adding zinc phosphate in general. When bioprimed seeds were sown in the pots, potential zinc-solubilizing plant growth-promoting bacteria reached the rhizosphere soil. The results obtained from the nethouse experiments showed that inoculation of *B. megaterium* CHW-22 has an impact on the percent soil organic carbon in the rhizosphere soil as compared to other inoculants. However, the differences were not significant. Similarly, these values were slightly higher in the soil amended with zinc phosphate and inoculated with rhizobacterial inoculants, RDF, and absolute control as compared to unamended soil (without zinc phosphate). Moreover, the trend was more or less similar (Table 3). Furthermore, inoculation of these bacteria significantly increased the available N, P, and K content in the rhizosphere soil amended with and without zinc phosphate under the nethouse conditions at harvest. As reported earlier, maximum available N (159.80 kg ha^{-1}), P (16.32 kg ha^{-1}), and K (222.60 kg ha^{-1}) content was reported in the rhizosphere soil of plants inoculated with *B. megaterium* CHW-22 without zinc phosphate followed by *B. licheniformis* MJW-38 (N: 157.60 kg ha^{-1}, P: 16.21 kg ha^{-1} and K: 220.40 kg ha^{-1}) and *B. altitudinis* AJW-3 (N: 155.30 kg ha^{-1}, P: 16.14 kg ha^{-1} and K: 219.20 kg ha^{-1}). However, the least content was reported in the absolute control (N: 141.80 kg ha^{-1}, P: 13.92 kg ha^{-1} and K: 204.30 kg ha^{-1}) and RDF (N: 150.40 kg ha^{-1}, P: 15.32 kg ha^{-1} and K: 208.30 kg ha^{-1}) under the nethouse conditions (Table 3). A more or less similar pattern was recorded in the rhizosphere soil amended with zinc phosphate with higher values (Table 3).

When comparing the effect of inoculation on the availability of micronutrients, i.e., Fe, Zn, Cu, and Mn, maximum content was reported in the plant rhizosphere inoculated with *B. megaterium* CHW-22 (4.56, 0.92, 1.86, and 5.46 μg g^{-1}, respectively) without zinc phosphate followed by *B. licheniformis* (4.52, 0.88, 1.85, and 5.42 μg g^{-1}, respectively) and *B. altitudinis* (4.48, 0.85, 1.83, and 5.39 μg g^{-1}, respectively). A similar trend was observed in the soil amended with zinc phosphate (Table 3). The results clearly revealed that maximum DHA (134.80 μg TPF g^{-1} soil 24 h^{-1}), APA (98.48 μg pNPg^{-1} soil h^{-1}), FDA (21.46 μg FLR g^{-1} soil hr^{-1}), and SMBC (132.70 μg g^{-1} soil) were recorded in the rhizosphere of plants inoculated with *B. megaterium* CHW-22 without zinc phosphate followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other inoculants and absolute control plants. Moreover, a similar pattern with slightly higher values was recorded in the rhizosphere of plants inoculated with rhizobacteria and amended with zinc phosphate at harvest (Table 3).

Effect of microbial inoculation on nutritional content in wheat

Microbial inoculation significantly affects nutritional content in wheat amended with and without zinc phosphate under pot experiments. The results clearly revealed that *B. megaterium* CHW-22 was the most potential inoculant, in general, and it has a significant impact on the nutritional biofortification in wheat under nethouse conditions. It was further observed that maximum N, P, and K content in the wheat grain and straw was recorded in the treatment RDF + *B. megaterium* CHW-22 and unamended zinc phosphate followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other inoculants and untreated control plants. When evaluating the micronutrients, especially Zn, maximum accumulation of Zn was reported in the grain and straw obtained from the wheat plants bioprimed with *B. megaterium* CHW-22 (46.44 and 33.64 μg g^{-1}, respectively) followed by *B. licheniformis* MJW-38 (45.28 and 32.98 μg g^{-1}, respectively) and *B. altitudinis* AJW-3 (43.11 and 31.38 μg g^{-1}, respectively) as compared to other treatments. Similarly, maximum Fe, Cu, and Mn were reported in the grain and straw obtained from the wheat plants bioprimed with *B. megaterium* CHW-22 followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other treatments. However, the least concentration of macronutrients (N, P, and K) and micronutrients including Zn (Zn, Fe, Cu, and Mn) was reported in the untreated absolute control and RDF alone amended plants without zinc phosphate (Table 4). A more or less similar pattern was recorded in the case of zinc phosphate-amended plants inoculated with selected strains of rhizobacterial inoculants (Table 4).

Evaluation of microbial inoculants under field conditions

Plant growth and yield attributes

Inoculation of selected potential strains of zinc-solubilizing rhizobacteria significantly increased the plant height, dry matter accumulation, yield and yield attributes, and harvest index as compared to the absolute control plants and RDF (alone) applied plants under field conditions. Similar to the observations under the pot experiment, *B. megaterium* CHW-22 was the best-performing strain followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 in general. From the results of the present investigation, it was clearly noticed that maximum plant height was recorded for *B. megaterium* CHW-22-inoculated plants followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3-inoculated plants at 30, 60, and 90 DAS as compared to other treatments under field conditions. *B. subtilis* ABW-30, *B. borstelensis* CHW-2, and *B. xiamenensis* BLW-7 showed minor differences (Table 5). The seed biopriming with *B. megaterium* CHW-22 significantly increased the dry matter accumulation followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to absolute control plants

TABLE 3 Effect of zinc-solubilizing plant growth-promoting rhizobacteria on organic carbon, available nutrients, and microbial activity in rhizosphere soil of wheat under pot experiment.

Treatment	SOC	N	P	K	Fe	Zn	Cu	Mn	DHA	APA	FDA	SMBC
	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	μg g^{-1}	μg g^{-1}	μg g^{-1}	μg g^{-1}	μg TPF g^{-1} soil 24 h^{-1}	μg pNPg^{-1} soil h^{-1}	μg FLR g^{-1} soil hr^{-1}	μg g^{-1} soil
Without Zn												
Control ($N_0P_0K_0$)	0.394^{a}	141.8^{e}	13.92^{f}	204.30^{f}	4.21^{e}	0.57^{f}	1.72^{e}	5.12^{f}	112.80^{e}	78.34^{f}	14.32^{e}	104.4^{e}
RDF ($N_{120}P_{60}K_{60}$)	0.404^{a}	150.4^{d}	15.32^{e}	208.30^{e}	4.34^{d}	0.61^{e}	1.77^{d}	5.19^{e}	119.60^{d}	82.45^{e}	15.48^{d}	109.2^{d}
RDF + *Bacillus altitudinis* AJW-3	0.419^{a}	155.3^{bc}	16.14^{ab}	219.20^{abc}	4.48^{b}	0.85^{bc}	1.83^{ab}	5.39^{abc}	129.50^{abc}	94.88^{bc}	18.32^{bc}	128.4^{ab}
RDF + *Bacillus subtilis* ABW-30	0.412^{a}	152.7^{c}	15.88^{d}	215.60^{cd}	4.39^{c}	0.78^{d}	1.80^{c}	5.32^{c}	126.20^{c}	92.32^{cd}	17.58^{cd}	123.8^{bc}
RDF + *Bacillus megaterium* CHW-22	0.424^{a}	159.8^{a}	16.32^{a}	222.60^{a}	4.56^{a}	0.92^{a}	1.86^{a}	5.46^{a}	134.80^{a}	98.48^{a}	21.46^{a}	132.7^{a}
RDF + *Bacillus licheniformis* MJW-38	0.421^{a}	157.6^{ab}	16.21^{ab}	220.40^{ab}	4.52^{ab}	0.88^{ab}	1.85^{a}	5.42^{ab}	132.30^{ab}	96.36^{ab}	20.32^{ab}	129.6^{ab}
RDF + *Brevibacillus borstelensis* CHW-2	0.409^{a}	151.5^{cd}	15.78^{ed}	213.40^{d}	4.36^{cd}	0.76^{d}	1.80^{c}	5.25^{d}	125.10^{c}	89.85^{d}	16.48^{cde}	118.8^{c}
RDF + *Bacillus xiamenensis* BLW-7	0.415^{a}	154.9^{bc}	16.08^{c}	217.80^{bc}	4.44^{bc}	0.82^{c}	1.82^{ab}	5.34^{bc}	127.90^{bc}	93.52^{cd}	17.56^{cd}	125.6^{b}
Zn Applied												
Control ($N_0P_0K_0$)	0.412^{a}	150.3^{e}	12.64^{e}	206.82^{f}	4.46^{e}	0.68^{f}	1.78^{d}	5.38^{f}	120.20^{d}	83.04^{e}	15.04^{g}	109.62^{f}
RDF ($N_{120}P_{60}K_{60}$)	0.423^{a}	160.8^{d}	14.83^{d}	214.13^{e}	4.60^{d}	0.74^{e}	1.84^{cd}	5.42^{e}	126.30^{c}	87.88^{d}	16.56^{f}	115.75^{e}
RDF + *Bacillus altitudinis* AJW-3	0.438^{a}	164.3^{bc}	15.62^{ab}	226.87^{abc}	4.70^{bc}	1.11^{c}	1.90^{ab}	5.68^{ab}	136.13^{ab}	100.57^{bc}	19.42^{bc}	134.82^{bc}
RDF + *Bacillus subtilis* ABW-30	0.427^{a}	162.5^{bc}	15.08^{c}	222.50^{cd}	4.61^{c}	1.00^{d}	1.88^{bc}	5.58^{c}	132.84^{bc}	97.58^{c}	18.72^{d}	131.23^{cd}
RDF + *Bacillus megaterium* CHW-22	0.444^{a}	170.7^{a}	16.02^{a}	230.84^{a}	4.83^{a}	1.25^{a}	1.95^{a}	5.73^{a}	142.35^{a}	105.87^{a}	23.28^{a}	143.32^{a}
RDF + *Bacillus licheniformis* MJW-38	0.441^{a}	166.7^{ab}	15.71^{ab}	228.33^{ab}	4.79^{ab}	1.17^{b}	1.94^{a}	5.72^{a}	138.91^{ab}	103.88^{ab}	21.95^{ab}	138.67^{ab}
RDF + *Brevibacillus borstelensis* CHW-2	0.429^{a}	$160.^{4c}$	15.03^{c}	219.59^{d}	4.60^{d}	0.99^{ed}	1.86^{c}	5.52^{d}	132.11^{bc}	95.96^{c}	17.80^{e}	127.12^{d}
RDF + *Bacillus xiamenensis* BLW-7	0.431^{a}	163.6^{bc}	15.09^{c}	225.21^{bc}	4.68^{bc}	1.07^{cd}	1.90^{ab}	5.64^{bc}	133.83^{bc}	100.41^{bc}	19.03^{c}	135.65^{bc}

RDF, recommended dose of fertilizers; 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 60 kg K ha^{-1}; SOC, soil organic carbon; DHA, dehydrogenase activity; APA, alkaline phosphatase activity; FDA, fluorescein diacetate activity; SMBC, soil microbial biomass carbon; TPF, triphenylformazan; pNP, p-nitrophenol phosphatase; FLR, fluorescein. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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TABLE 4 Effect of zinc-solubilizing plant growth-promoting rhizobacteria on nutrition concentration in wheat under pot experiment.

Treatment	N (%)		P (%)		K (%)		Zn (μg g^{-1})		Fe (μg g^{-1})		Cu (μg g^{-1})		Mn (μg g^{-1})	
	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw
Without Zn														
Control ($N_0P_0K_0$)	1.76^{e}	0.46^{d}	0.31^{e}	0.11^{b}	0.44^{d}	1.47^{d}	34.12^{g}	25.32^{e}	76.93^{e}	245.43^{f}	10.71^{d}	7.48^{f}	25.04^{f}	33.80^{g}
RDF ($N_{120}P_{60}K_{60}$)	1.84^{d}	0.50^{c}	0.33^{d}	0.11^{b}	0.49^{c}	1.53^{c}	36.23^{f}	26.88^{d}	80.46^{de}	255.37^{e}	10.79^{c}	7.54^{e}	25.28^{e}	34.18^{f}
RDF + *Bacillus altitudinis* AJW-3	1.94^{ab}	0.54^{ab}	0.38^{ab}	0.12^{a}	0.54^{ab}	1.60^{ab}	43.11^{bc}	31.38^{ab}	88.94^{abc}	275.96^{c}	11.02^{b}	7.73^{c}	26.12^{ab}	35.38^{bc}
RDF + *Bacillus subtilis* ABW-30	1.87^{bc}	0.52^{bc}	0.36^{bc}	0.12^{a}	0.53^{abc}	1.58^{abc}	40.45^{de}	29.19^{bc}	85.36^{cd}	271.67^{cd}	10.88^{bc}	7.61^{d}	25.84^{c}	34.98^{d}
RDF + *Bacillus megaterium* CHW-22	1.98^{a}	0.57^{a}	0.39^{a}	0.12^{a}	0.55^{a}	1.62^{a}	46.44^{a}	33.64^{a}	93.27^{a}	287.51^{a}	11.20^{a}	8.45^{a}	26.36^{a}	35.72^{a}
RDF + *Bacillus licheniformis* MJW-38	1.96^{ab}	0.56^{a}	0.38^{ab}	0.12^{a}	0.54^{ab}	1.62^{a}	45.28^{ab}	32.98^{a}	91.92^{ab}	280.33^{b}	11.17^{a}	7.93^{b}	26.28^{a}	35.54^{ab}
RDF + *Brevibacillus borstelensis* CHW-2	1.86^{cd}	0.52^{bc}	0.35^{c}	0.12^{a}	0.52^{bc}	1.57^{bc}	40.28^{e}	29.00^{c}	85.03^{cd}	268.66^{d}	10.88^{bc}	7.60^{d}	25.45^{d}	34.62^{e}
RDF + *Bacillus xiamenensis* BLW-7	1.89^{bc}	0.54^{ab}	0.37^{abc}	0.12^{a}	0.54^{ab}	1.60^{ab}	42.92^{cd}	31.08^{abc}	86.84^{bc}	274.25^{c}	10.97^{b}	7.61^{d}	25.98^{bc}	35.22^{c}
Zn Applied														
Control ($N_0P_0K_0$)	1.83^{c}	0.49^{d}	0.33^{e}	0.12^{b}	0.48^{d}	1.55^{d}	37.87^{g}	27.60^{e}	80.77^{e}	258.94^{g}	11.46^{e}	8.03^{d}	27.04^{f}	35.85^{e}
RDF ($N_{120}P_{60}K_{60}$)	1.91^{bc}	0.54^{c}	0.36^{d}	0.12^{b}	0.53^{c}	1.64^{c}	40.58^{f}	29.57^{d}	84.64^{d}	267.63^{f}	11.65^{d}	8.11^{c}	27.05^{e}	36.57^{d}
RDF + *Bacillus altitudinis* AJW-3	2.02^{ab}	0.58^{ab}	0.40^{abc}	0.13^{a}	0.57^{abc}	1.70^{ab}	50.87^{bc}	36.71^{b}	94.10^{abc}	290.59^{c}	11.79^{c}	8.27^{bc}	28.17^{bc}	38.21^{bc}
RDF + *Bacillus subtilis* ABW-30	1.97^{abc}	0.57^{abc}	0.39^{bc}	0.13^{a}	0.57^{abc}	1.68^{abc}	47.33^{de}	33.86^{bc}	91.32^{bc}	286.07^{de}	11.69^{d}	8.21^{bc}	27.61^{d}	38.03^{c}
RDF + *Bacillus megaterium* CHW-22	2.07^{a}	0.60^{a}	0.43^{a}	0.14^{a}	0.61^{a}	1.73^{a}	55.73^{a}	40.70^{a}	97.75^{a}	302.46^{a}	12.15^{a}	8.52^{a}	28.73^{a}	39.29^{a}
RDF + *Bacillus licheniformis* MJW-38	2.03^{ab}	0.60^{a}	0.41^{ab}	0.13^{a}	0.58^{ab}	1.71^{a}	53.88^{ab}	39.88^{a}	96.42^{ab}	295.47^{b}	11.95^{b}	8.36^{ab}	28.38^{ab}	38.56^{b}
RDF + *Brevibacillus borstelensis* CHW-2	1.95^{abc}	0.55^{bc}	0.38^{c}	0.13^{a}	0.57^{abc}	1.68^{abc}	46.72^{e}	32.93^{c}	91.20^{bc}	285.58^{e}	11.65^{d}	8.13^{c}	27.44^{d}	38.01^{c}
RDF + *Bacillus xiamenensis* BLW-7	1.99^{ab}	0.58^{ab}	0.40^{abc}	0.13^{a}	0.57^{abc}	1.70^{ab}	50.43^{cd}	36.67^{b}	91.36^{bc}	288.51^{cd}	11.74^{cd}	8.21^{bc}	27.80^{c}	38.04^{c}

RDF: recommended dose of fertilizers: 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 60 kg K ha^{-1}. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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TABLE 5 Effect of zinc-solubilizing plant growth-promoting rhizobacteria on growth, yield attributes, and yield of wheat under field experiment.

Treatments	Plant height (cm)			DMA (g m^{-2})			Yield attribute					GY (q ha^{-1})	SY (q ha^{-1})	BY (q ha^{-1})	HI (%)
	30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS	ET (M^{-2})	SL (cm)	Spikelet $spike^{-1}$	Grain $spike^{-1}$	Test weight				
Without Zn															
Control ($N_0P_0K_0$)	21.09^{d}	39.12^{e}	77.48^{e}	17.66^{d}	116.4^{c}	390.3^{d}	391.2^{c}	9.88^{e}	21.32^{e}	39.78^{e}	39.43^{b}	34.87^{e}	53.23^{d}	88.10^{c}	39.58^{a}
RDF ($N_{120}P_{60}K_{60}$)	24.78^{c}	42.40^{d}	85.44^{d}	21.12^{c}	124.5^{b}	428.8^{c}	425.9^{b}	10.77^{d}	22.66^{d}	42.12^{d}	39.84^{a}	44.78^{d}	67.23^{c}	112.01^{b}	39.98^{a}
RDF + *Bacillus altitudinis* AJW-3	26.60^{ab}	43.42^{b}	89.44^{b}	22.78^{a}	129.8^{ab}	454.8^{ab}	430.7^{ab}	11.12^{ab}	23.89^{ab}	43.46^{bc}	39.98^{a}	46.18^{ab}	68.64^{ab}	114.82^{a}	40.22^{a}
RDF + *Bacillus subtilis* ABW-30	25.94^{b}	42.80^{c}	87.32^{c}	22.32^{ab}	127.0^{b}	439.7^{bc}	429.7^{ab}	10.98^{bc}	23.20^{bc}	42.88^{c}	39.89^{a}	45.94^{bc}	68.42^{b}	114.36^{a}	40.17^{a}
RDF + *Bacillus megaterium* CHW-22	27.52^{a}	44.26^{a}	92.99^{a}	22.96^{a}	132.4^{a}	472.4^{a}	442.6^{a}	11.24^{a}	24.69^{a}	44.98^{a}	40.04^{a}	46.42^{a}	68.71^{a}	115.13^{a}	40.32^{a}
RDF + *Bacillus licheniformis* MJW-38	27.27^{a}	43.79^{ab}	91.36^{ab}	22.82^{a}	131.6^{a}	465.6^{a}	438.5^{a}	11.20^{a}	23.97^{ab}	43.82^{b}	39.99^{a}	46.24^{ab}	68.56^{ab}	114.80^{a}	40.28^{a}
RDF + *Brevibacillus borstelensis* CHW-2	25.73^{bc}	42.74^{c}	87.12^{c}	22.08^{b}	126.5^{ab}	437.3^{bc}	424.4^{b}	10.89^{c}	23.01^{c}	42.69^{cd}	39.87^{a}	45.72^{c}	68.27^{bc}	113.99^{ab}	40.11^{a}
RDF + *Bacillus xiamenensis* BLW-7	26.52^{ab}	42.89^{c}	88.32^{bc}	22.45^{ab}	128.4^{ab}	446.2^{b}	430.8^{ab}	11.04^{b}	23.43^{b}	43.35^{bc}	39.92^{a}	46.08^{b}	68.58^{ab}	114.66^{a}	40.19^{a}
Zn Applied															
Control ($N_0P_0K_0$)	22.3^{e}	40.8^{c}	80.66^{e}	18.51^{d}	123.4^{c}	405.12^{d}	409.6^{d}	10.32^{e}	22.30^{e}	41.29^{e}	40.18^{b}	36.13^{e}	54.51^{c}	90.64^{d}	39.86^{a}
RDF ($N_{120}P_{60}K_{60}$)	25.6^{d}	44.6^{b}	89.03^{d}	22.18^{c}	130.1^{bc}	440.95^{c}	440.7^{c}	11.35^{d}	23.65^{d}	43.80^{d}	40.63^{a}	46.48^{d}	68.53^{bc}	115.01^{c}	40.42^{a}
RDF + *Bacillus altitudinis* AJW-3	27.8^{b}	46.0^{ab}	94.81^{b}	23.95^{ab}	137.6^{ab}	475.76^{ab}	458.8^{ab}	11.80^{ab}	24.59^{bc}	45.85^{ab}	41.02^{a}	48.30^{ab}	70.25^{a}	118.56^{a}	40.74^{a}
RDF + *Bacillus subtilis* ABW-30	26.9^{c}	45.1^{ab}	91.08^{c}	23.50^{b}	135.3^{ab}	454.05^{b}	450.1^{b}	11.65^{bc}	24.13^{cd}	44.68^{bc}	40.80^{a}	47.87^{bc}	70.00^{b}	117.87^{ab}	40.61^{a}
RDF + *Bacillus megaterium* CHW-22	28.9^{a}	46.9^{a}	97.55^{a}	24.31^{a}	140.4^{a}	495.55^{a}	467.9^{a}	11.90^{a}	25.65^{a}	47.50^{a}	41.20^{a}	48.79^{a}	70.19^{a}	118.98^{a}	41.01^{a}
RDF + *Bacillus licheniformis* MJW-38	28.6^{a}	46.9^{a}	97.46^{a}	24.22^{a}	139.5^{a}	487.48^{a}	462.6^{a}	11.83^{ab}	24.89^{b}	46.10^{ab}	41.07^{a}	48.51^{ab}	70.14^{a}	118.64^{a}	40.88^{a}
RDF + *Brevibacillus borstelensis* CHW-2	26.7^{cd}	44.9^{ab}	90.87^{cd}	23.43^{b}	134.1^{b}	450.46^{b}	449.2^{b}	11.50^{c}	24.02^{cd}	44.48^{c}	40.74^{a}	47.50^{c}	69.76^{b}	117.26^{b}	40.51^{a}
RDF + *Bacillus xiamenensis* BLW-7	27.0^{c}	45.2^{ab}	92.21^{c}	23.86^{ab}	136.2^{ab}	469.28^{ab}	456.2^{ab}	11.75^{b}	24.37^{c}	45.13^{b}	40.91^{a}	48.11^{b}	70.17^{ab}	118.28^{a}	40.67^{a}

RDF: recommended dose of fertilizers: 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 60 kg K ha-1. DMA, dry matter accumulation; GY, grain yield; SY, straw yield; and BY, biological yield; HI, Harvesting index; ET, effective tiller; SL, spike length; and DAS, days after sowing. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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and RDF-applied plants. All the other strains significantly increased dry matter as compared to the absolute control, but this increase was significantly less when compared to *B. megaterium* CHW-22 treated plants at 30, 60, and 90 DAS under field conditions (Table 5). The results obtained from field experiments include the maximum number of effective tiller m^{-2} (442.6), spike length (11.24 cm), spikelet $spike^{-1}$ (24.69), number of grain $spike^{-1}$ (44.98), and test weight (40.04 g) being recorded in the plants bioprimed with *B. megaterium* CHW-22 without zinc phosphate followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other inoculants, RDF alone, and untreated absolute control. A more or less similar trend was reported in the plants amended with zinc phosphate and inoculated with microbial inoculants. However, these values are slightly higher as compared to the treatments without zinc phosphate (Table 5). Similarly, seed biopriming has a positive impact on grain yield (q ha^{-1}), straw yield (q ha^{-1}), biological yield (q ha^{-1}), and harvest index (%) under field conditions. Maximum grain yield (46.42 q ha^{-1}), straw yield (68.71 q ha^{-1}), biological yield (115.13 q ha^{-1}), and harvest index (40.32 %) were recorded in the plants inoculated with *B. megaterium* CHW-22 without zinc phosphate as compared to other inoculants under field conditions. There was a minor difference in the harvest index but a non-significant increase was observed in the harvest index (%) of plants inoculated with either the inoculants or the absolute control and RDF alone applied plants. A more or less similar trend was recorded in the grain yield (q ha^{-1}), straw yield (q ha^{-1}), biological yield (q ha^{-1}), and harvest index (%) in the plants bioprimed with selected rhizobacterial strains and amended with zinc phosphate. However, these values were slightly higher in the case of zinc phosphate-amended plants under field conditions (Table 5).

Effect of microbial inoculation on SOC, available nutrients, and microbial activity in rhizosphere soil

In line with the results obtained under nethouse conditions, rhizobacterial inoculation was observed to have a significant impact on SOC, available nutrients, and microbial activity in rhizosphere soil under field conditions too. These properties were further increased after adding zinc phosphate in general. Similar to the nethouse results, it was observed that seed bioprimed with *B. megaterium* CHW-22 has an impact on soil organic carbon (%) in the rhizosphere soil as compared to other treatments. However, the differences were not significant statistically. Similarly, these values were slightly higher in the soil amended with zinc phosphate and inoculated with rhizobacterial strains, RDF alone, and absolute control as compared to unamended soil (without zinc phosphate) under field conditions. However, the trend was more or less similar (Table 6). It was further observed that inoculation of rhizobacterial strains significantly increased the availability of macronutrients (N, P, and K) and micronutrients (Fe, Zn, Cu, and Mn) in the wheat rhizosphere soil amended with and without zinc phosphate under field conditions at harvest. Among the different inoculants, the maximum availability of macronutrients (N, P, and K) and micronutrients (Fe, Zn, Cu, and Mn) was recorded in the rhizosphere soil of wheat inoculated with *B. megaterium* CHW-22 amended with and without zinc phosphate under field conditions followed by *B. licheniformis* MJW-38- and *B. altitudinis* AJW-3-inoculated plant rhizosphere soil as compared to other inoculants, RDF alone, and untreated absolute control plants. However, the least available nutrient was reported in the rhizosphere of absolute control (Table 6). The results showed that maximum DHA (141.81 μg TPF g^{-1} soil 24 h^{-1}), APA (104.19 μg $pNPg^{-1}$ soil h^{-1}), FDA (22.88 μg FLR g^{-1} soil hr^{-1}), and SMBC (140.66 μg g^{-1} soil) were reported in the rhizosphere of plants inoculated with *B. megaterium* CHW-22 without zinc phosphate followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other treatments. Moreover, a similar pattern with slightly higher values was recorded for DHA, APA, FDA, and SMBC in the rhizosphere of plants inoculated with rhizobacteria and amended with zinc phosphate at harvest (Table 6).

Effect of microbial inoculation on nutritional content in wheat

Similar to the nethouse experiments, seed inoculation significantly increased the accumulation and content of macronutrients (N, P, and K) and micronutrients (Fe, Zn, Cu, and Mn) in the wheat amended with and without zinc phosphate under field conditions. The results showed that *B. megaterium* CHW-22 was found to be the most potential inoculant followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3, as compared to other inoculants. The results revealed that maximum N, P, and K content in the wheat straw and grain was recorded in the plants treated with RDF + *B. megaterium* CHW-22 without zinc phosphate followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other inoculants and untreated control plants. It was also observed that maximum accumulation of Zn was reported in the grain and straw obtained from the wheat plants bioprimed with *B. megaterium* CHW-22 (49.48 and 37.57 μg g^{-1}, respectively) followed by *B. licheniformis* MJW-38 (48.54 and 36.84 μg g^{-1}, respectively) and *B. altitudinis* AJW-3 (46.68 and 35.52 μg g^{-1}, respectively) as compared to other treatments under field conditions. Similarly, maximum Fe, Cu, and Mn were reported in the grain and straw obtained from the wheat plants bioprimed with *B. megaterium* CHW-22 followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 as compared to other inoculants. However, the least concentration of macronutrients (N, P, and K) and micronutrients including Zn (Zn, Fe, Cu, and Mn) was reported in the untreated absolute control and RDF alone amended plants without zinc phosphate (Table 7). A more or less similar pattern was recorded in the case of zinc phosphate-amended plants inoculated with selected strains of rhizobacterial inoculants (Table 7).

Effect of microbial inoculation on NUE of wheat

NUE is an important aspect of field experiments. It was clearly observed that microbial inoculation significantly influenced the

TABLE 6 Effect of zinc-solubilizing plant growth-promoting rhizobacteria on organic carbon, available nutrients, and microbial activity in rhizosphere soil of wheat under field experiment.

Treatment	SOC	N	P	K	Fe	Zn	Cu	Mn	DHA	APA	FDA	SMBC
	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	μg g^{-1}	μg g^{-1}	μg g^{-1}	μg g^{-1}	μg TPF g^{-1} soil 24 h^{-1}	μg pNPg^{-1} soil h^{-1}	μg FLR g^{-1} soil hr^{-1}	μg g^{-1} soil
Without Zn												
Control ($N_0P_0K_0$)	0.387^{a}	134.68^{e}	13.99^{e}	198.40^{c}	4.14^{d}	0.55^{e}	1.69^{c}	5.04^{e}	116.86^{e}	81.71^{e}	15.11^{e}	109.41^{f}
RDF ($N_{120}P_{60}K_{60}$)	0.399^{a}	142.32^{d}	15.41^{d}	202.80^{b}	4.21^{c}	0.61^{d}	1.76^{b}	5.07^{d}	124.03^{d}	86.16^{d}	16.36^{d}	114.66^{e}
RDF + *Bacillus altitudinis* AJW-3	0.412^{a}	147.55^{b}	16.31^{ab}	212.80^{a}	4.38^{ab}	0.83^{b}	1.81^{ab}	5.16^{ab}	135.33^{b}	99.91^{b}	19.47^{b}	135.72^{b}
RDF + *Bacillus subtilis* ABW-30	0.407^{a}	145.66^{bc}	16.13^{bc}	209.30^{ab}	4.28^{bc}	0.78^{c}	1.79^{ab}	5.12^{b}	131.12^{c}	96.84^{c}	18.63^{bc}	130.49^{cd}
RDF + *Bacillus megaterium* CHW-22	0.418^{a}	158.83^{a}	16.42^{a}	216.40^{a}	4.48^{a}	0.88^{a}	1.87^{a}	5.21^{a}	141.81^{a}	104.19^{a}	22.88^{a}	140.66^{a}
RDF + *Bacillus licheniformis* MJW-38	0.414^{a}	148.48^{b}	16.39^{a}	214.60^{a}	4.42^{a}	0.86^{ab}	1.84^{a}	5.18^{a}	141.56^{a}	101.66^{ab}	21.62^{ab}	137.12^{ab}
RDF + *Brevibacillus borstelensis* CHW-2	0.404^{a}	144.98^{c}	15.96^{c}	207.60^{ab}	4.26^{bc}	0.76^{cd}	1.78^{ab}	5.09^{c}	129.85^{cd}	94.07^{cd}	17.45^{c}	124.98^{d}
RDF + *Bacillus xiamenensis* BLW-7	0.409^{a}	146.32^{bc}	16.21^{b}	211.20^{a}	4.33^{b}	0.81^{bc}	1.80^{ab}	5.14^{ab}	133.27^{bc}	98.38^{bc}	18.54^{bc}	132.51^{c}
Zn Applied												
Control ($N_0P_0K_0$)	0.399^{a}	138.05^{e}	13.65^{e}	203.56^{d}	4.27^{d}	0.65^{f}	1.74^{c}	5.23^{d}	119.78^{e}	84.00^{f}	15.65^{e}	112.69^{f}
RDF ($N_{120}P_{60}K_{60}$)	0.412^{a}	146.30^{d}	14.99^{d}	208.48^{c}	4.38^{c}	0.73^{e}	1.82^{b}	5.27^{c}	127.75^{d}	88.66^{e}	16.98^{d}	118.33^{e}
RDF + *Bacillus altitudinis* AJW-3	0.425^{a}	152.27^{b}	15.78^{ab}	220.25^{a}	4.55^{ab}	1.05^{b}	1.88^{ab}	5.38^{ab}	140.06^{b}	103.41^{b}	20.33^{b}	140.88^{b}
RDF + *Bacillus subtilis* ABW-30	0.422^{a}	150.76^{bc}	15.58^{bc}	216.00^{ab}	4.43^{bc}	0.95^{d}	1.85^{ab}	5.32^{b}	135.45^{c}	99.85^{c}	19.40^{bc}	135.05^{c}
RDF + *Bacillus megaterium* CHW-22	0.434^{a}	164.87^{a}	15.94^{a}	224.62^{a}	4.66^{a}	1.14^{a}	1.95^{a}	5.45^{a}	146.78^{a}	108.36^{a}	23.97^{a}	146.29^{a}
RDF + *Bacillus licheniformis* MJW-38	0.427^{a}	153.97^{b}	15.88^{a}	222.54^{a}	4.59^{a}	1.10^{ab}	1.92^{a}	5.41^{a}	146.66^{a}	105.52^{ab}	22.62^{ab}	142.46^{ab}
RDF + *Brevibacillus borstelensis* CHW-2	0.417^{a}	149.91^{c}	15.44^{c}	213.83^{b}	4.41^{bc}	0.92^{de}	1.84^{ab}	5.29^{bc}	134.01^{cd}	96.90^{d}	18.13^{c}	129.23^{d}
RDF + *Bacillus xiamenensis* BLW-7	0.422^{a}	151.15^{bc}	15.69^{b}	218.17^{ab}	4.49^{b}	1.00^{c}	1.87^{ab}	5.36^{ab}	137.80^{bc}	101.63^{bc}	19.32^{bc}	137.41^{bc}

RDF: recommended dose of fertilizers: 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 60 kg K ha^{-1}. SOC, soil organic carbon; DHA, dehydrogenase activity; APA, alkaline phosphatase activity; FDA, fluorescein diacetate activity; SMBC, soil microbial biomass carbon; TPF, triphenylformazan; pNP, p-nitrophenol phosphatase; FLR, fluorescein. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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TABLE 7 Effect of zinc-solubilizing plant growth-promoting rhizobacteria on nutrition concentration in wheat under field experiment.

Treatment	N (%)		P (%)		K (%)		Zn ($\mu g\ g^{-1}$)		Fe ($\mu g\ g^{-1}$)		Cu ($\mu g\ g^{-1}$)		Mn ($\mu g\ g^{-1}$)	
	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw
Without Zn														
Control ($N_0P_0K_0$)	1.65^{d}	0.43^{c}	0.29^{e}	0.106^{a}	0.41^{d}	1.43^{d}	32.56^{e}	24.96^{e}	91.58^{e}	314.66^{e}	10.05^{e}	7.34^{d}	23.47^{e}	32.82^{d}
RDF ($N_{120}P_{60}K_{60}$)	1.74^{cd}	0.47^{bc}	0.31^{d}	0.112^{c}	0.45^{c}	1.50^{c}	34.32^{d}	26.12^{d}	95.78^{d}	327.40^{d}	10.29^{d}	7.56^{c}	23.74^{d}	33.28^{c}
RDF + *Bacillus altitudinis* AJW-3	1.82^{b}	0.52^{ab}	0.36^{ab}	0.118^{ab}	0.50^{a}	1.56^{ab}	46.68^{b}	35.52^{b}	105.88^{b}	353.80^{b}	10.55^{ab}	7.70^{ab}	24.52^{ab}	34.27^{ab}
RDF + *Bacillus subtilis* ABW-30	1.77^{c}	0.51^{ab}	0.34^{bc}	0.117^{b}	0.49^{ab}	1.54^{b}	43.82^{c}	33.34^{c}	101.62^{c}	348.30^{bc}	10.42^{bc}	7.61^{b}	24.18^{bc}	33.89^{b}
RDF + *Bacillus megaterium* CHW-22	1.89^{a}	0.55^{a}	0.38^{a}	0.122^{a}	0.51^{a}	1.59^{a}	49.48^{a}	37.57^{a}	111.04^{a}	368.60^{a}	10.68^{a}	7.81^{a}	24.82^{a}	34.68^{a}
RDF + *Bacillus licheniformis* MJW-38	1.86^{ab}	0.54^{a}	0.37^{a}	0.120^{a}	0.50^{a}	1.57^{ab}	48.54^{ab}	36.84^{ab}	109.43^{ab}	359.40^{ab}	10.65^{a}	7.77^{a}	24.78^{a}	34.51^{a}
RDF + *Brevibacillus borstelensis* CHW-2	1.76^{c}	0.49^{b}	0.33^{c}	0.115^{bc}	0.48^{b}	1.53^{b}	42.13^{cd}	32.06^{cd}	101.23^{cd}	344.43^{c}	10.38^{c}	7.63^{b}	23.88^{c}	33.54^{bc}
RDF + *Bacillus xiamenensis* BLW-7	1.79^{bc}	0.51^{ab}	0.35^{b}	0.117^{ab}	0.50^{a}	1.56^{ab}	45.66^{bc}	34.67^{bc}	103.38^{bc}	351.60^{b}	10.48^{b}	7.68^{ab}	24.36^{b}	34.12^{ab}
Zn Applied														
Control ($N_0P_0K_0$)	1.70^{d}	0.45^{d}	0.30^{e}	0.110^{e}	0.42^{d}	1.50^{e}	34.84^{e}	27.14^{e}	96.16^{e}	331.97^{d}	10.50^{f}	7.60^{e}	24.53^{e}	34.66^{e}
RDF ($N_{120}P_{60}K_{60}$)	1.80^{c}	0.49^{c}	0.32^{d}	0.116^{d}	0.47^{c}	1.57^{d}	37.95^{d}	29.64^{d}	100.76^{d}	343.12^{c}	10.79^{e}	7.85^{d}	24.74^{d}	35.08^{d}
RDF + *Bacillus altitudinis* AJW-3	1.88^{ab}	0.55^{ab}	0.38^{ab}	0.123^{ab}	0.53^{a}	1.63^{b}	51.81^{b}	40.44^{b}	112.02^{b}	372.55^{b}	11.13^{b}	8.00^{b}	25.65^{ab}	36.19^{ab}
RDF + *Bacillus subtilis* ABW-30	1.83^{b}	0.53^{b}	0.36^{bc}	0.121^{b}	0.51^{ab}	1.61^{bc}	48.42^{c}	37.84^{c}	110.62^{c}	366.76^{bc}	10.93^{d}	7.90^{c}	25.32^{b}	35.75^{bc}
RDF + *Bacillus megaterium* CHW-22	1.97^{a}	0.58^{a}	0.40^{a}	0.127a	0.54^{a}	1.68^{a}	54.43^{a}	42.48^{a}	116.37^{a}	387.77^{a}	11.28^{a}	8.16^{a}	26.04^{a}	36.66^{a}
RDF + *Bacillus licheniformis* MJW-38	1.95^{a}	0.57^{a}	0.38^{ab}	0.123^{ab}	0.53^{a}	1.65^{a}	53.39^{ab}	41.68^{ab}	114.79^{a}	378.81^{ab}	11.26^{a}	8.09^{ab}	25.99^{a}	36.51^{a}
RDF + *Brevibacillus borstelensis* CHW-2	1.82^{bc}	0.51^{bc}	0.34^{c}	0.119^{c}	0.50^{b}	1.60^{c}	46.55^{cd}	36.37^{cd}	109.90^{c}	366.13^{bc}	10.93^{d}	7.87^{cd}	24.93^{c}	35.38^{c}
RDF + *Bacillus xiamenensis* BLW-7	1.86^{ab}	0.53^{b}	0.37^{b}	0.121^{b}	0.52^{ab}	1.63^{b}	50.13^{bc}	39.14^{bc}	110.76^{c}	371.44^{b}	11.02^{c}	7.95^{bc}	25.38^{b}	35.93^{b}

RDF: Recommended dose of fertilizers: 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 60 kg K ha^{-1}. Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

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NUE of wheat in general. In the present investigation, partial factor productivity, agronomic efficiency, apparent nutrient recovery, and physiological efficiency were calculated and correlated with the microbial inoculation and zinc applied under field conditions. The results revealed that apparently higher partial factor productivity for N (38.68), P (77.37), K (77.37), and Zn (928.40) was reported in the plants bioprimed with *B. megaterium* CHW-22 followed by *B. licheniformis* MJW-38 (N: 38.53, P: 77.07, K: 77.07 and Zn: 924.80) and *B. altitudinis* AJW-3 (N: 38.48, P: 76.97, K: 76.97, and Zn: 923.60) as compared to other inoculants without zinc phosphate (Table 8). The partial factor productivity for N, P, K, and Zn was slightly higher in all the treatments amended with zinc phosphate under field conditions. Moreover, the pattern was more or less similar as recorded in the treatments without zinc phosphate (Table 8). Similarly, maximum agronomic efficiency for N, P, and K was also reported in the plants bioprimed with *B. megaterium* CHW-22 (1.37, 2.73, and 2.73, respectively) followed by *B. licheniformis* MJW-38 (1.22, 2.43, and 2.43, respectively) and *B. altitudinis* AJW-3 (1.17, 2.33, and 2.33, respectively) as compared to other inoculants without zinc phosphate (Table 8). Similarly, agronomic efficiency for N, P, K, and Zn was also calculated in the plants inoculated with selected strains of rhizobacteria and amended with zinc phosphate. These values were slightly higher in the case of zinc phosphate-amended plants, while the trend was more or less similar (Table 8). Table 8 clearly showed that maximum apparent nutrient recovery and physiological efficiency were observed in the plants bioprimed with *B. megaterium* CHW-22 followed by *B. licheniformis* MJW-38 and *B. altitudinis* AJW-3 with and without zinc phosphate as compared to other inoculants under field conditions. The significant increase in the partial factor productivity, agronomic efficiency, apparent nutrient recovery, and physiological efficiency of the inoculated plants showed the importance of rhizobacterial inoculants across the treatments. These results indicate the efficacy of the selected zinc-solubilizing rhizobacteria in enhancing the bioavailability of zinc and mobilizing it toward wheat grains.

Correlation and principal component analyses

The univariate Pearson's correlation coefficient (r) matrix (Figure 2) indicated that nutrient content in the grain and straw of the wheat obtained from microbial inoculated plants without zinc phosphate (Figure 2A) as well as soil amended with zinc phosphate (Figure 2B) were found to be significant and positively correlated among them as compared to absolute control under field conditions.

The generated heatmap (Figure 3) displayed the nutrient content vertically and treatments horizontally wherein different clusters were formed based on similarities. A positive interaction was observed with respect to the level of nutrients accumulated in the wheat grain and straw obtained from microbial inoculated plants as compared to absolute control without zinc phosphate (Figure 3A) as well as soil amended with zinc phosphate (Figure 3B) under field conditions.

The results of the principal component analysis indicated that the position of different macronutrients (NPK) and micronutrients (Zn, Fe, Cu, and Mn) contents in straw and grains of wheat influenced by rhizobacterial strains with or without zinc application are depicted in the four zones of biplot of PCA (Figure 4, Supplementary Table 5). In the absence of zinc application, the PCA comprising two principal components (PC1 70.69% and PC2 27.56%) accounted for 98.25% of the variance (Figure 4A). The N, P, K, and Fe content in straw and K content in grains were influenced by RDF + *B. subtilis* ABW-30 and RDF + *B. xiamenensis* BLW-7 as depicted in right upper side biplot having large positive loading for both PC1 and PC2, indicating that these two strains ABW-30 and BLW-7 have directly impacted the accumulation of nutrients in straw. In contrast, the N, P, Fe, Cu, Zn, and Mn content in grain and the Zn and Mn content in straw were influenced by RDF + *B. megaterium* CHW-22, RDF + *B. altitudinis* AJW-3 and RDF + *B. licheniformis* MJW-38 as depicted in the right lower side of biplot having positive loading PC 1 and negative loading for PC 2, indicating that strains CHW-22, AJW-3, and MJW-38 have influenced nutrient contents in grains.

In presence of zinc application, the PCA comprising two principal components (PC1 65.61% and PC2 32.43%) accounted for 98.04% of the variance (Figure 4B). The P, Zn, and Fe in straw and K, Zn, and Fe in grains were influenced by RDF + *B. altitudinis* AJW-3 and RDF + *B. xiamenensis* BLW-7 as depicted in the right upper side biplot having large positive loading for both PC1 and PC2. However, N, K, Mn, and Cu in straw and N, P, Mn, and Cu in grains were influenced by RDF + *B. megaterium* CHW-22 and RDF + *B. licheniformis* MJW-38 as depicted in the right lower side biplot having large positive loading for PC1 and negative loading for PC2. It was revealed from the above mentioned results that bacterial strains have differential behavior in the partitioning of nutrients in straw and grains in the presence and absence of zinc phosphate in conjunction with RDF. In the absence of zinc application, some bacteria partitioned nutrients in straw, whereas some of them were transferred to the grains, but in the presence of zinc application, the behavior of strains was found mixed. The possible reasons for such behavior need further investigation.

Discussion

Zinc availability in soil is decreasing day by day with the increasing salinization and land degradation over a period of time. Micronutrient deficiencies including Zn are often referred to as "hidden hunger" for plants as well as human beings (Cakmak, 2008; White and Broadley, 2011). It is very difficult to detect deficiencies over a short period of time. Zn is not only essential for plant growth and development but also plays a key role in the physico-biochemical process in plants. It provides the major dietary source of Zn for a large segment of the world population. The long-term deficiencies can have irreversible serious consequences for plant health (White and Broadley, 2011; Singh et al., 2021b; Yadav et al., 2022). It is therefore difficult to eliminate Zn deficiency through conventional agriculture practices including crop diversification and nutritional supplements through inorganic fertilizers (Ramesh et al., 2014; Khande et al., 2017; Singh et al., 2021b). Conversely, increasing intrinsic Zn content in food

TABLE 8 Effect of zinc-solubilizing plant growth-promoting rhizobacteria on nutrient use efficiency (NUE) of wheat.

Treatment	Partial Factor Productivity (PEP)				Agronomic Efficiency (AE)				Apparent Nutrient Recovery (ANR)				Physiological Efficiency (PE)			
	(N)	(P)	(K)	(Zn)	(N)	(P)	(K)	(Zn)	(N)	(P)	(K)	(Zn)	(N)	(P)	(K)	(Zn)
Without Zn																
Control (N0P0K0)	29.06	58.12	58.12	697.40	-	-	-	-	-	-	-	-	-	-	-	-
RDF (N120P60K60)	37.32	74.63	74.63	895.60	-	-	-	-	-	-	-	-	-	-	-	-
RDF + *Bacillus altitudinis* AJW-3	38.48	76.97	76.97	923.60	1.17	2.33	2.33	-	0.09	0.06	0.15	-	13.69	42.26	15.26	-
RDF + *Bacillus subtilis* ABW-30	38.28	76.57	76.57	918.80	0.97	1.93	1.93	-	0.06	0.04	0.11	-	17.33	52.41	16.86	-
RDF + *Bacillus megaterium* CHW-22	38.68	77.37	77.37	928.40	1.37	2.73	2.73	-	0.13	0.08	0.20	-	10.24	35.57	13.75	-
RDF + *Bacillus licheniformis* MJW-38	38.53	77.07	77.07	924.80	1.22	2.43	2.43	-	0.11	0.06	0.16	-	11.38	37.86	14.95	-
RDF + *Brevibacillus borstelensis* CHW-2	38.10	76.20	76.20	914.40	0.78	1.57	1.57	-	0.04	0.03	0.09	-	21.34	61.55	17.40	-
RDF + *Bacillus xiamenensis* BLW-7	38.40	76.80	76.80	921.60	1.08	2.17	2.17	-	0.07	0.05	0.15	-	16.37	47.44	14.40	-
Zn Applied																
Control (N0P0K0)	30.11	60.22	60.22	722.60	-	-	-	-	-	-	-	-	-	-	-	-
RDF (N120P60K60)	38.73	77.47	77.47	929.60	-	-	-	-	-	-	-	-	-	-	-	-
RDF + *Bacillus altitudinis* AJW-3	40.25	80.50	80.50	966.00	1.52	3.03	3.03	36.40	0.10	0.07	0.18	0.04	14.92	43.63	17.06	856.80
RDF + *Bacillus subtilis* ABW-30	39.89	79.78	79.78	957.40	1.16	2.32	2.32	27.80	0.06	0.05	0.13	0.03	18.64	47.12	18.11	865.91
RDF + *Bacillus megaterium* CHW-22	40.66	81.32	81.32	975.80	1.93	3.85	3.85	46.20	0.16	0.09	0.25	0.05	11.80	41.20	15.58	910.49
RDF + *Bacillus licheniformis* MJW-38	40.43	80.85	80.85	970.20	1.69	3.38	3.38	40.60	0.13	0.07	0.20	0.05	12.57	47.90	16.91	859.42
RDF + *Brevibacillus borstelensis* CHW-2	39.58	79.17	79.17	950.00	0.85	1.70	1.70	20.40	0.04	0.03	0.10	0.03	21.32	62.64	17.21	782.77
RDF + *Bacillus xiamenensis* BLW-7	40.09	80.18	80.18	962.20	1.36	2.72	2.72	32.60	0.08	0.06	0.17	0.04	17.28	47.00	16.37	872.39

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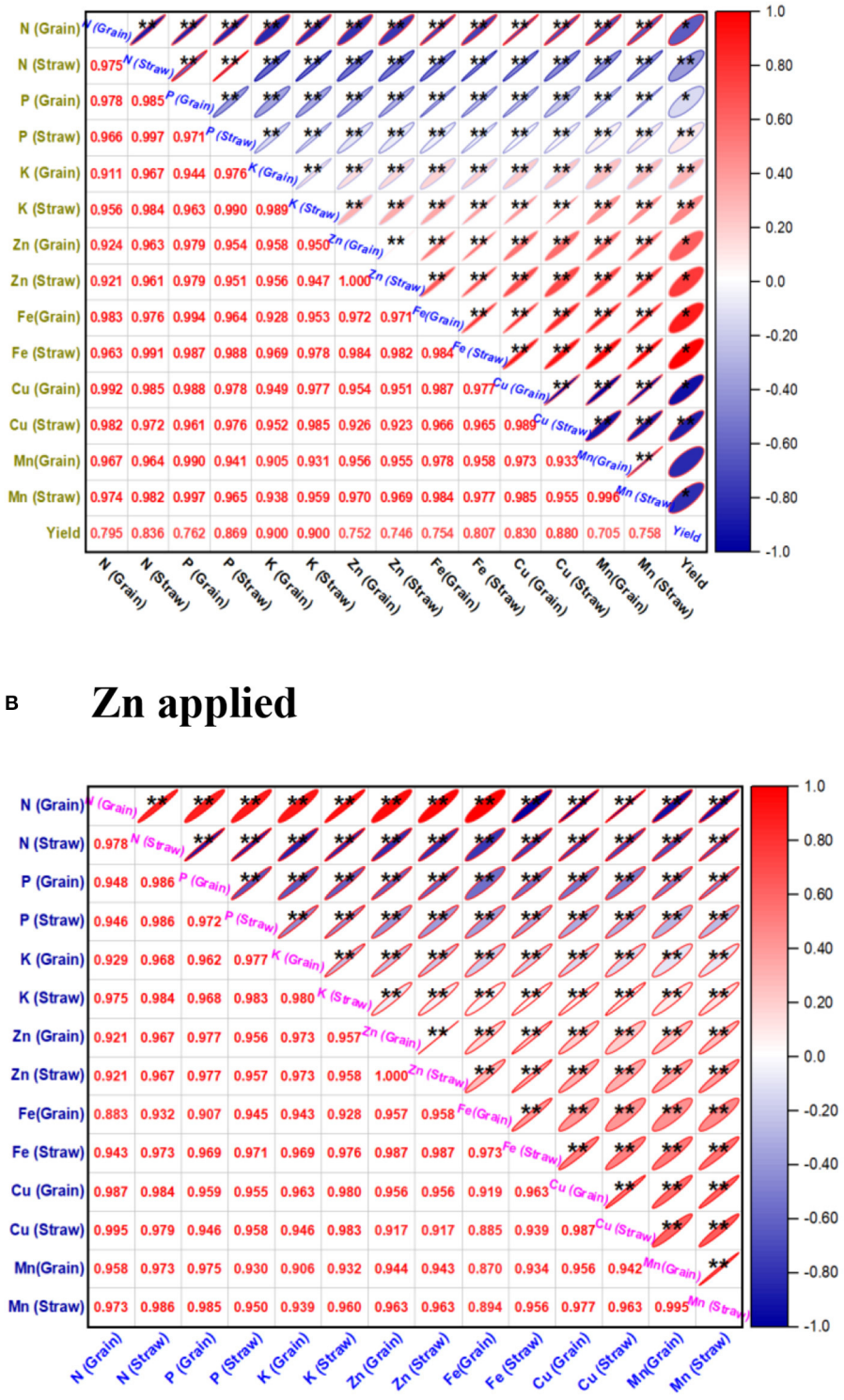

FIGURE 2
Correlation coefficient (r)* matrix showing the effect of rhizobacterial inoculants on nutrient content in the straw and grain of the wheat plants grown without zinc phosphate **(A)** and with zinc phosphate **(B)** and their interaction under field conditions. The correlation coefficient (r) values are significantly positive at $p < 0.01$, and they are indicated with ** in the matrix.

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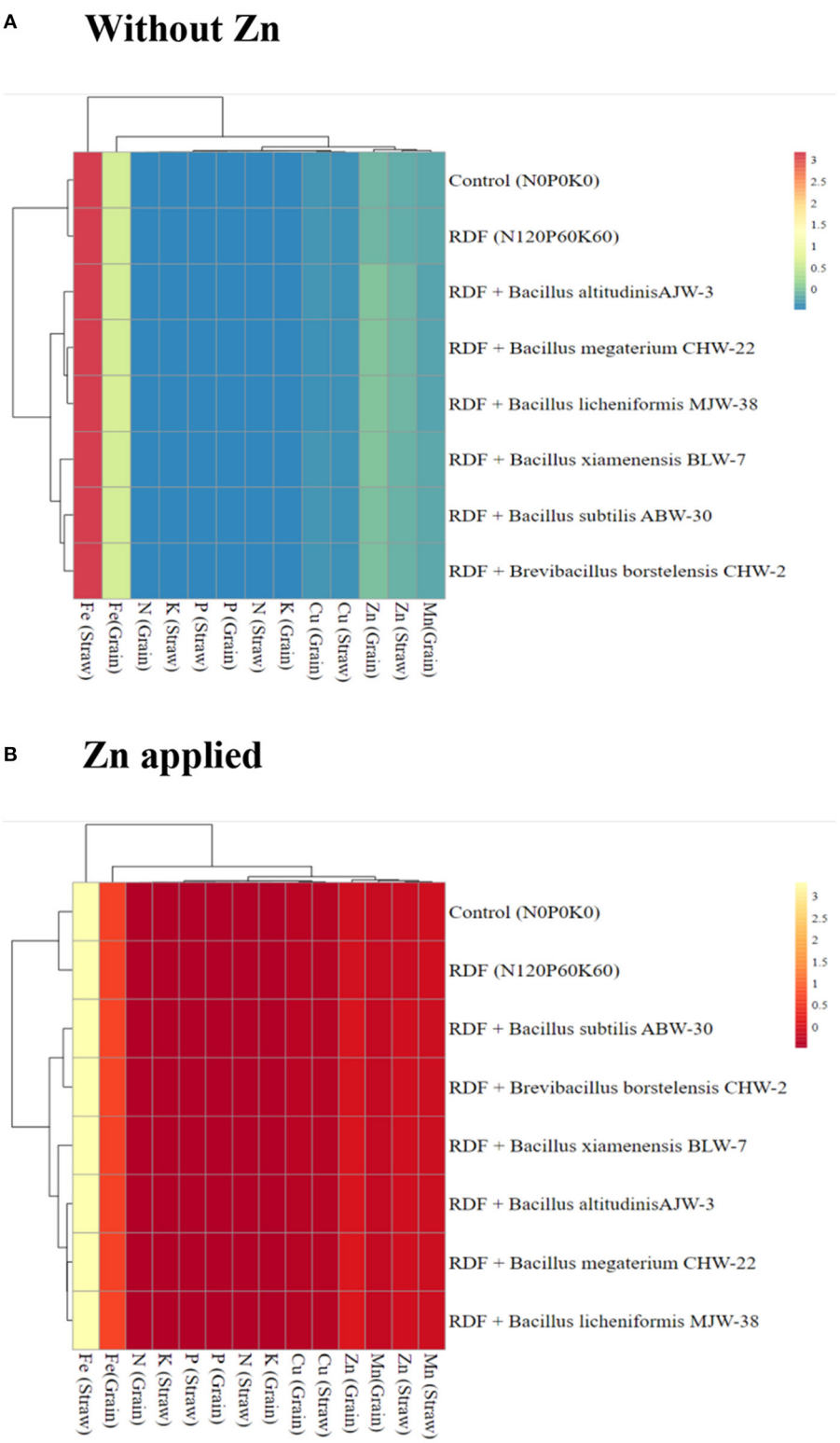

FIGURE 3
The heatmap depicting the interaction of rhizobacterial inoculants and nutrient content in the straw and grains of the wheat plants grown without zinc phosphate **(A)** and with zinc phosphate **(B)** and their interaction under field conditions. *Correlation coefficient (r) values correspond directly to the color code from (lowest to highest) blue to yellow and red, respectively.

crops, especially wheat, by breeding and microbial intervention is well documented as an environment-friendly, cost-effective, and sustainable strategy to tackle Zn deficiency (Cakmak, 2008; White and Broadley, 2011). Furthermore, a large amount of Zn is present in the soil in insoluble forms being unavailable to plants. There is an urgent need to search for potential Zn-solubilizing

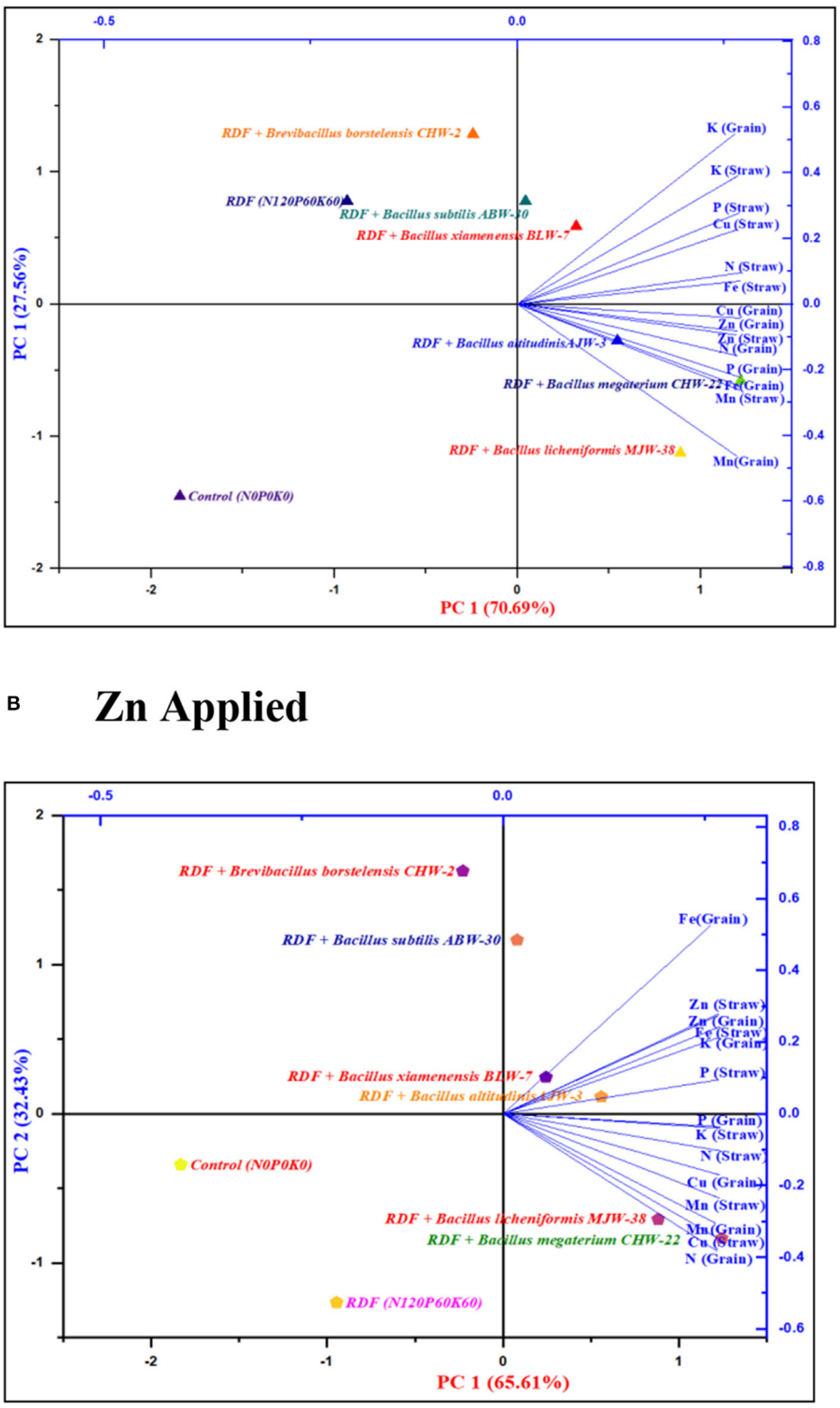

FIGURE 4
The two-dimensional graphical biplot showing the grouping of variables of wheat crops on principal component scores (PC1 and PC2) derived from variables (nutrient content in grain and straw along with bacterial treatments) of wheat crop grown without zinc phosphate **(A)** and with zinc phosphate **(B)** under field conditions.

microorganisms, which can solubilize insoluble Zn pool present in the soil and make it available to the plants without harming the environment (Sharma et al., 2010; Ramesh et al., 2014; Singh et al., 2021b). Thus, it plays as a bridge between the soil and plant roots. Among soil-dwelling microorganisms, harnessing the potential of native plant growth-promoting rhizobacteria (PGPR) including Zn-solubilizing rhizobacteria is an alternate strategy for enhancing Zn solubilization, uptake and translocation, and biofortification of Zn in plants including wheat (Sharma et al., 2011; Ramesh et al., 2014; Singh et al., 2021b). In general, PGPR have the ability to solubilize and mobilize unavailable zinc and increase the assimilation of zinc in grains. PGPR are an integral part of any ecological niche. They play a crucial role in nutrient geo-cycling including nutrient mineralization in the environment (Sharma et al., 2010; Rana et al., 2012; Ramesh et al., 2014). Zinc-solubilizing rhizobacteria secret a large amount of organic acids, protein extrusion, and chelating agents that enhance the overall availability of Zn in the rhizosphere ecosystem (Nahas, 1996; Seshadri et al., 2000), which can be substantially taken up by plants. Therefore, the key aim of the present study was to characterize the native potential zinc-solubilizing rhizobacterial strains and their application for overall plant growth with special reference to zinc biofertilization in wheat in conjunction with soil-applied fertilizers and zinc phosphate under pot and field conditions.

In the present investigation, a total of 175 different rhizobacterial strains were isolated from the wheat rhizospheric soils collected from different parts of the Indo-Gangetic plains of Northern India (Supplementary Table 1). The soils of the Indo-Gangetic plains of the Indian sub-continent are considered one of the mineral-rich soils in India. It harbors a rich microbial gene pool, which plays an important role in nutrient geo-cycling, nutrient mineralization, waste decomposition, and soil processes, and provides protection from several biotic and abiotic stresses including nutritional deficiencies (Malviya et al., 2011; Srivastava et al., 2014; Sharma et al., 2015; Kumari et al., 2016; Choudhary et al., 2018a,b; Singh et al., 2020). Of the 175 bacterial strains, 42 strains were found to solubilize either zinc carbonate ($ZnCO_3$), zinc oxide (ZnO), and zinc phosphate {$Zn_3(PO_4)_2$} or any two or all three compounds of insoluble Zn. These strains were found to produce organic acids such as gluconic acid, oxalic acid, citric acid, malic acid, lactic acid, and succinic acid (data not shown). The production of organic acids in the broth supplemented with $ZnCO_3$, ZnO, and $Zn_3(PO_4)_2$ and inoculated with bacterial strains indicated that 'organic acids' are essential for Zn solubilization as reported by earlier studies (Desai et al., 2012; Ramesh et al., 2014; Kumari et al., 2016; Yadav R. C. et al., 2021). However, gluconic acid is the key organic acid produced by the majority of Zn-solubilizing bacterial strains for the solubilization of insoluble minerals besides other organic acids. These results are in line with the observations made by many researchers (Zhao et al., 2011; Rashid et al., 2012; Yadav et al., 2022). These 42 strains were further identified on the basis of 16S rRNA gene sequences. The results of the present investigation clearly indicated that the rhizosphere of wheat grown in the IGP region harbor rich diversity of rhizobacteria. It was reported that 19 different species of rhizobacteria were isolated and characterized from the wheat rhizosphere. *Bacillus* and *Bacillus*-derived genera are ubiquitous in nature and possess multiple growth-promoting traits (Kohler et al., 2007; Ramirez et al., 2010; Zhao et al., 2011).

In general, bacilli are root-associated mutualistic plant symbionts widely present in the rhizosphere soil (Singh et al., 2016, 2021a). They have the vast capability to colonize plant roots, nourish the host, and protect plants from biotic and abiotic stresses (Singh et al., 2016, 2021a,b; Yadav et al., 2022). The variation in Zn solubilization may be found due to the type and amount of organic acid produced, culture conditions, pH of culture medium, nature of microbes, and gene induced (responsible for Zn mineralization) in response to the nature of Zn compounds used as the sole source of Zn (Ramesh et al., 2014; Khande et al., 2017). Based on the halo zone produced on plates containing tris-minimal media supplemented with 0.1% $ZnCO_3$, ZnO, and $Zn_3(PO_4)_2$, six potential zinc-solubilizing bacterial strains, i.e., *B. altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-2, *B. licheniformis* MJW-22, *B. borstelensis* CHW-38, and *B. xiamenensis* BLW-7, were selected for further *in vitro* and *in planta* assay. In the present investigation, the selected strains showed varying degrees of ZSE, amount of solubilized Zn, pH of culture medium, and gluconic acid produced. The organic acid specifically gluconic acid of microbial origin possibly works in a non-specific way during the Zn solubilization process and thereby influencing the bioavailability of zinc (Agusto da Costa and Duta, 2001). *In vitro* plate assay clearly showed that the selected strains *B. altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-2, *B. licheniformis* MJW-22, *B. borstelensis* CHW-38, *and B. xiamenensis* BLW-7 possess several other PGP traits such as P and K solubilization, IAA, siderophore, HCN, and ammonia production, thereby promoting plant growth and development directly and/or indirectly. These findings are in accordance with the observation of several other studies (Ramesh et al., 2014; Mumtaz et al., 2017; Dinesh et al., 2018; Singh et al., 2021a).

The effects of seed inoculation were studied in the wheat plants under the pot as well as field conditions. Seed biopriming with these strains significantly increases plant height, dry matter accumulation, yield and yield attributing characters (ET Plant^{-1}, spike length, spikelet spike^{-1}, grain spike^{-1}, and test weight), grain yield, straw yield, biological yield, and harvest index in the wheat supplemented with Zn and without Zn under nethouse conditions. The values are significantly higher in the plants supplemented with Zn and with the recommended dose of fertilizers as compared to the plants supplemented with the recommended dose of fertilizers and without Zn. When compared with the absolute control ($N_0P_0K_0$ and no inoculation), bacterial inoculation has a significant impact on plant growth parameters and yield-attributing traits in the plants supplemented with and without Zn. The application of PGPR under nethouse experiments significantly increases plant growth and development (Lwin et al., 2012; Ram et al., 2013; Porcel et al., 2014; Singh et al., 2021b; Yadav et al., 2022). These inoculants produced several growth hormones (Dodd et al., 2010; Jha and Saraf, 2011; Kang et al., 2012; Maheshwari et al., 2015; Tsukanova et al., 2017; Khan et al., 2020) and improved chlorophyll content (Heidari and Golpayegani, 2012; Vafadar et al., 2014; Mathivanan et al., 2017), induction of physiological processes (Meena et al., 2020; Singh et al., 2021a,b), mineralization, and solubilization of

mineral nutrient (Saravanan et al., 2007; Desai et al., 2012; Lucas et al., 2014; Wang et al., 2014; Kumari et al., 2016).

Application of microbial inoculants significantly increased the availability of macro (N, P, and K) and micro (Fe, Zn, Cu, and Mn) nutrients in the wheat rhizosphere soil (Singh et al., 2021b; Tirry et al., 2021). PGPR are known to break down the complex organic and inorganic compounds such as proteins, lignin, cellulose, hemicellulose, and lipids in the natural ecosystem. They mineralize/solubilize the mineral nutrients in the soil ecosystem and transform them into available states (Ren et al., 2021; Shabaan et al., 2022). These strains were previously known for their ability of P, K, and Zn solubilization, IAA production, and ACC deaminase activity, thereby enhancing P-uptake and plant biomass in many crops (Saravanan et al., 2004; Lucas et al., 2014; Pereira and Castro, 2014; Wang et al., 2014). Furthermore, inoculation of bacterial inoculants significantly also increased (1.25-1.67 fold) the activity of DHA, APA, and FDA in the rhizosphere soil along with SMBC under nethouse experiments with and without Zn. These results are in agreement with the other researchers (Kohler et al., 2007; Rana et al., 2012; Song et al., 2015; Arif et al., 2016; Islam et al., 2016; Sood et al., 2018; Maddhesiya et al., 2021; Ren et al., 2021; Shabaan et al., 2022).

Seed biopriming significantly enhanced the zinc content in grain and straw as compared to non-inoculated absolute control and plants supplemented with recommended doses of fertilizers and Zn. The amount of Zn accumulated in the wheat grain and straw of the plants inoculated with bacterial inoculants and applied with Zn was significantly higher as compared to plants without Zn application. Among the different bacterial inoculants, maximum Zn was recorded in the grain and straw of the plants inoculated with *B. megaterium* CHW-22 as compared to other inoculants. These results are in line with the previous reports wherein inoculation of plants with PGPR led to enhanced Zn content in wheat grain (Islam et al., 2014, 2016; Ramesh et al., 2014; Sirohi et al., 2015; Kamran et al., 2017; Singh et al., 2021b; Azmat et al., 2022; Yadav et al., 2022). The ZIP (Zn-regulated, iron-regulated transporter-like protein) transporter families are well-studied transporters in terrestrial plants. It is an important transporter present in the different cell organelles and regulates the uptake, transport, and accumulation of Fe and Zn in the plant's system (Singh et al., 2021b; Aloo et al., 2022; Yadav et al., 2022). Along with the uptake and translocation of Fe and Zn in the plants, they also play a key role in several other developmental processes, such as plant growth, uptake and translocation of other key microelements, tissues differentiation, and biofortification in the plant system (Velmourougane et al., 2017; Yadav et al., 2022). The ZIP protein family has been widely studied in *Arabidopsis thaliana,* a model plant. These transporters have also been mapped in several other plant species such as *Oryza sativa*, *Lycopersicon esculentum* Mill., and *Zea mays* in the past few years. However, the importance of the ZIP family in wheat is not well-studied at present and it needs in-depth investigation. Furthermore, it is the need of the hour to get the ZIP transporters mapped in wheat in relation to the microbial inoculants interaction which is in the infancy stage (Singh et al., 2021a,b; Yadav et al., 2022). Besides the increase in the Zn content in the plant, they also increase the uptake and content of other mineral nutrients (N, P, K, Fe, Cu, and Mn) in the grain and straw of the wheat plants bioprimed with selected strains of *B. altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-2, *B. licheniformis* MJW-22, *B. borstelensis* CHW-38, and *B. xiamenensis* BLW-7. Several studies reported increased mobilization of macro- and micronutrients by PGPR including zinc-solubilizing bacilli in many crops including wheat (Sharma et al., 2011; Rana et al., 2012; Kumar et al., 2014, 2021). It was clearly indicated that bacterial inoculation significantly affects the NUE of wheat in general. Inoculation of *B. megaterium* CHW-2 significantly increased the partial factor productivity, agronomic efficiency, apparent nutrient recovery, and physiological efficiency as compared to other inoculants under field conditions. These results are in agreement with the finding of several other studies which reported that PGPRs significantly increased the uptake and translocation of mineral nutrients from the soil and biofortified wheat and other crops (Adesemoye et al., 2008; Shaharoona et al., 2008; Arif et al., 2017; Çakmakçi et al., 2017; Di Benedetto et al., 2017; Pereira et al., 2020; Singh et al., 2020). Based on the results of the present investigation and the observations of the previous researchers, it can be summarized that zinc-solubilizing rhizobacteria can be used as an alternate strategy for *in vivo* Zn solubilization and enhance Zn uptake and translocation in the plants to cope with the Zn deficiency in wheat. In this way, these potential rhizobacteria mobilize unavailable zinc present in the soil and increase the assimilation of zinc, accelerating plant growth and enhancing the overall yield of plants. Furthermore, they play a crucial role in the environmental geo-cycling of mineral nutrients, which can be suitably taken up by plants in a sustainable way. Moreover, Zn solubilization by native PGPR is a comparatively new approach and not many strains have yet been characterized and reported so far. In the present investigation, a number of native potential zinc-solubilizing rhizobacterial isolates have been explored and characterized, which can be used as biofertilizers to overcome zinc deficiency in wheat crops.

Conclusion

Inoculation of PGPR linked with zinc-solubilizing ability effectively improved soil biological properties, growth, yield, and micronutrient-enriched seeds of wheat. Of the 175 rhizobacterial isolates recovered and screened, only 6 bacteria were found effective in the solubilization of insoluble zinc compounds. Of the six zinc-solubilizing bacteria, inoculation of *B. megaterium* CHW-22, *B. licheniformis* MJW-38, and *B. altitudinis* AJW-3 in conjunction with RDF and zinc phosphate enhanced wheat growth and grain yield and grain with zinc in the wheat crop in degraded soil with high pH of Eastern Uttar Pradesh of India. The results revealed that bacterial inoculation enhanced the NUE of the applied fertilizers and zinc phosphate in comparison with RDF alone. Such effective bacterial inoculants are beneficial for sustainable agriculture as they have the ability to combat abiotic stresses and facilitate functioning in degraded soil for ensuring food and nutritional security and, in turn, achieve the objectives of Sustainable Development Goals by the year 2030.

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

SS, AV, and RY conceptualized the idea for this research work and corrected the first draft of the manuscript. SS and RY designed the experiments. RY and US conducted experiments and developed the first draft of the manuscript. AK and IB performed the statistical analyses using different software and interpreted the data thereafter. SS, PS, and JR edited the final draft of the manuscript. The final draft of the manuscript was read by all the authors and all of them gave their consent for publication. 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/fmicb.2023.1210938/full#supplementary-material

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Endophytic fungi: hidden treasure chest of antimicrobial metabolites interrelationship of endophytes and metabolites

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Endophytic fungi comprise host-associated fungal communities which thrive within the tissues of host plants and produce a diverse range of secondary metabolites with various bioactive attributes. The metabolites such as phenols, polyketides, saponins, alkaloids help to mitigate biotic and abiotic stresses, fight against pathogen attacks and enhance the plant immune system. We present an overview of the association of endophytic fungal communities with a plant host and discuss molecular mechanisms induced during their symbiotic interaction. The overview focuses on the secondary metabolites (especially those of terpenoid nature) secreted by endophytic fungi and their respective function. The recent advancement in multi-omics approaches paved the way for identification of these metabolites and their characterization via comparative analysis of extensive omics datasets. This study also elaborates on the role of diverse endophytic fungi associated with key agricultural crops and hence important for sustainability of agriculture.

KEYWORDS

secondary metabolites, terpenoids, symbiosis, alkaloids, endophytic fungi

1. Introduction

Numerous microorganisms exist in mutualistic associations with plants, whereby their interaction results in a beneficial outcome for both entities. Microbes with such capabilities are ample in above-ground as well as in underground (edaphic) habitats. Epiphytes are microorganisms that are found on the external surface of plants, whereas endophytes are microorganisms that reside and establish themselves within plant tissues, specifically within the leaves or roots. Among these, the rhizosphere harbors a plethora of active microorganisms that enhance plant nutrition, growth, and development of plants. The rhizosphere can be defined as the soil zone in the proximity of plant roots that is affected by the presence of microorganisms and root exudates, including compounds secreted by the root system of the plants (Kanchan et al., 2020). The region is characterized by multifaceted plant-microbe interactions, encompassing mutualistic, symbiotic, or parasitic relationships. These interactions are capable of triggering the biosynthesis of secondary metabolites by the microbial cells, which can be advantageous for the plants by conferring tolerance to biotic and abiotic stresses contributing to the plant's overall fitness (Koza et al., 2022). Endosymbiotic microorganisms, which are

typically bacteria and/or fungi, establish mutually beneficial relationships with their plant host including improved host fitness. These organisms are considered to be non-pathogenic since they can complete a part of their life cycle or even the whole life cycle within their host without causing any symptoms of disease (Nair and Padmavathy, 2014). The endophytic community can be further divided into two subgroups which involve 'obligate endophytes' (endophytes that critically dependent on plant metabolism for their survival) and 'facultative endophytes' (endophytes that spend certain stages of their life cycle outside the host body and are mostly associated with plants present in their nearby soil environment) (Hardoim et al., 2008; Abreu-Tarazi et al., 2010).

Of all the microorganisms inhabiting the rhizosphere, endophytic fungi or fungal endophytes have garnered significant attention from researchers. This is owing to their ability to produce various bioactive molecules such as antibacterial compounds, and biostimulants, which can facilitate essential oil biosynthesis (Radic and Strukelj, 2012; Uzma et al., 2018; El Enshasy et al., 2019). Essential oils are the aromatic compounds that have high vapor pressure and low boiling point. Vanillin and 3-methoxy-4-hydroxytoluene was isolated from the roots of *Zingiber officinale* Rosc. infected with *Streptomyces aureofaciens* SMUAc130 (Taechowisan et al., 2005). The bacterial volatile compounds are significant in establishing interspecific and or intraspecific communication and role in defence mechanism against antagonistic microbes. Volatile organic compounds (VOCs) play extremely important role in antibiosis and signaling mechanism for microbes with symbiotic relation under competitive soil conditions (Polito et al., 2022). Endophytic fungi have been recognized for their multifaceted roles in plant-microbe interactions: nutrient solubilization in the plant rhizosphere, promoting plant growth, acting as biocontrol agents against pests and pathogens, inducing systemic resistance against biotic and abiotic stresses, and participating in the biosynthesis of secondary metabolites (Mehta et al., 2019; Poveda et al., 2020a; Cui et al., 2021; Poveda et al., 2021; Poveda and Baptista, 2021). Fungal secondary metabolites may act by causing alterations in the host plant's morphology and physiology (Alam et al., 2021). Endophytic fungi establish symbiotic associations with host plants through a process that involves the enzymatic degradation of the host plant's cell wall. To colonize the host plant tissue, endophytic fungi produce a range of cell wall-degrading enzymes including cellulase, laccase, pectinase, and xylanase. These enzymes facilitate the infiltration, colonization, and proliferation of endophytic fungi within the host plant tissue by altering the physical and structural properties of the host cell wall (Chow and Ting, 2019). Upon degradation of the host plant cell wall, host plant activates its defence mechanisms such as pattern-recognition receptors (PRRs) which provide innate immunity to the plant. In order to survive in plants, the endophytes require specific strategies to avoid being detected by the plant based immune system. One such strategies include, hiding of chitin by modifying it or oligomers derived from chitin employing chitin deacetylases (Cord-Landwehr et al., 2016). Apart from these, many fungal endophytes have been identified to secrete bioactive compounds known as secondary metabolites, which are helpful in coping with biotic and abiotic stresses. These secondary metabolites include mostly alkaloids, terpenoids, polyketides. Besides these major ones, certain secondary metabolites are also secreted such as flavonoids, saponins, phenols, and phenolic acids, aliphatic and chlorinated metabolites, peptides, and steroids. It was found that similar secondary metabolites are being produced by the fungal endophytes and even in larger concentrations as compared to the plants. Using gene clustering, transcription factors and horizontal gene transfer, we can study the biosynthesis pathways and genes encoding specific types of bioactive molecules.

Fungal endophytes are emerging source for terpenoids, an important class of secondary metabolites. Terpenoids are a diverse group of natural products, which consist of C5 isoprene units and are the largest class of natural products (Perveen, 2018). The structural diversity of terpenoids is substantial, with more than 80,000 terpenoids having been identified from both plant and microbial sources. The isoprene units required for terpenoid biosynthesis are typically synthesized through either the mevalonic acid pathway (MVA) or the methylerythritol phosphate pathway (MEP). Terpenoids exhibit a diverse range of pharmacological and nutritional activities, as well as utility in the food and cosmetic industries. Moreover, their therapeutic potential has been explored in the context of COVID-19 treatment due to their notable antiviral properties (Diniz et al., 2021). Due to these activities, there is increase in demand of terpenoids which imposes a strain on the terpenoid-producing plant species. A wide variety of terpenoids are produced by endophytic fungi but none of them is capable of producing terpenoids at commercial scale as their yield is low, especially after repeated subculturing.

Initially, with the advent of first generation sequencers alongwith amplicon based sequencing for variable regions of genomes was being employed for deciphering microbiome understanding. However, extensive studies on root morphology and root exudation in deciphering rhizobiomes can be done employing next generation sequencing (NGS). Metagenomics allows detailing of complete genomic information by assembling DNA sequences into genes. It also provides information about novel genes, bio based-products, biomolecules, interaction between microbial communities. Whereas, transcriptomics utilizes the NGS based information to identify and quantify the presence of the particular RNA molecule in biological samples. Metabolomics, on the other hand is a technique which is utilized to identify and analyze metabolite changes occurring due to overexpression or mutation in a desired gene (Chen et al., 2022). The combination of various omics-based approach can help in better understanding of plant-endophyte interaction mechanisms. This study focusses on the secondary metabolites (especially those of terpenoid nature) secreted by endophytic fungi. The study elaborates the role of endophytic fungi as potential source of terpenoid producer alongwith its host related molecular interactions. Concomitantly, a noteworthy discussion on recent multi-omics based approaches employed to have better understanding of host and endophytic fungi interaction for secondary metabolite production.

2. Endophytes: fungal association with plants

Endophytic fungi (EF), also known as fungal endophytes, are communities of fungi that form associations either inter- or intracellularly with host plant tissues while simultaneously providing benefits to the host and gaining benefits from it (Alam et al., 2021). These fungi establish a mutualistic or symbiotic relationship with the host plant, and are categorized into two groups based on this relationship. The first group is the Clavicipitaceous fungi or

Balansiaceous group, which includes class I endophytes. This group comprises free-living symbiotic species that survive in cool season grasses (Poaceae), infect the ovules of host plants, and are transmitted vertically to progenies through host seeds. The living rhizomes and shoots of the host plant are colonized by these endophytes. The second group is the Non-Clavicipitaceous fungi or Non-Balansiaceous group, which includes class II, class III, and class IV endophytes. The Class I endophyte species are considered as obligate endophytes that offer protection to their host plant under conditions of drought stress, by secreting bioactive metabolites that exhibit defensive or supportive properties (Roberts and Lindow, 2014; Poveda et al., 2021). Non-Clavicipitaceous fungi or Non-Balansiaceous group (includes class II, class III and class IV endophytes) are non-grass host related groups having extensive biodiversity and distribution. These endophytes reside within plant tissues in a dormant or quiescent state without causing any apparent harm to the host plant, that is, they are not closely associated with the host plant. However, as soon as the chemical changes in the host plant occurs either through injuries or environmental stresses, these EFs then enter the host plant intracellularly through roots shoots or rhizomes (Carroll, 1988; Rodriguez et al., 2008; Mishra Y. et al., 2021). In Class II endophytes colonization of the host tissue occurs through roots, shoots and rhizomes whereas colonization occurs through shoots only and roots only in class III and class IV endophytes, respectively. The transmission occurs both vertically and horizontally in class II endophytes whereas only horizontally in class III and class IV endophytes (Rodriguez et al., 2009). Brief list of important crops and beneficial endophytic fungi with their respective functions have been provided in Table 1.

2.1. Molecular mechanism of host-endophytic fungi interaction

Endophytic fungi interact with the host plant via three types of interactions, that are, mutualistic, commensalistic, and pathogenic interactions (Figure 1). In mutualistic symbiosis, both host plants and EFs benefit from each other leading to evolutionary and ecological success. The EFs on colonizing the host plant tissue alters their metabolism improving plant's tolerance to stresses such as heavy metal and drought, augmenting growth and development, nutrient acquisition. It can also protect the host plant from herbivore animals, pests, and pathogenic microorganisms whereas the host plant provides shelter and adequate amount of nutrients to the EFs for their proliferation and life cycle completion. In commensalistic or latent pathogenic relationship, EFs interact with the host plant and may or may not show any kind of beneficial effect on the plant physiology. According to various studies, EFs reside in the host plants as dormant or latent pathogen (Brown et al., 1998; Photita et al., 2004; Rodriguez and Redman, 2008; Gorzynska et al., 2018). In other words, during this stage the fungus is harmless and does not induce any symptom of the disease but as the environmental conditions become unfavorable, latent fungi becomes active and cause obvious pathogenic symptoms eventually leading to destruction of the host plant (Romero et al., 2001; Photita et al., 2004; Poveda et al., 2020b).

In the rhizosphere, plants engage in symbiotic interactions with a diverse range of microorganisms through pattern-recognition receptors (PRRs), which are cell surface proteins capable of detecting microbial- or pathogen-associated molecular patterns (MAMPs/PAMPs) produced by the interacting microbe. These PRRs are involved in triggering the first layer of plant innate immunity. Typically, during the formation of a mutualistic symbiotic relationship, the signaling pathways that hinder the expansion of endophytic proliferation, such as miRNA-mediated pathways involved in plant defence mechanisms, are suppressed (Plett and Martin, 2018). This extracellular recognition via MAMPs/PAMPs or damage-associated molecular patterns (DAMPs) has been identified to lead to first layer of innate immunity via triggered defences, and is coined as pattern-triggered immunity (PTI) (Tang et al., 2017; Saijo et al., 2018). Another PTI can be activated upon cellular disintegration through degradation of the plant cell wall compounds such as oligonucleotides, cellodextrins, and the compounds released under stress conditions (cutin monomers and small peptides), which in turn generates endogenous signals termed DAMPs that are also detected by PRRs. Upon infection of the host plant by a microorganism, the botrytis-induced kinase1 (BIK1) effector kinase of the pattern recognition receptors (PRRs) complex becomes activated. This activation leads to an increase in the cytosolic calcium (Ca^{2+}) level mediated by cyclic nucleotide-gated channels (CNGCs). This elevation of Ca^{2+} is recognized as a crucial signal for triggering the activation of pathogen-associated molecular pattern (PAMP) signals involved in plant immunity during pathogen-associated molecular pattern-triggered immunity (PTI) (Tian et al., 2019). Establishment of successful infection by the pathogen by overcoming PTI via effector-triggered susceptibility (ETS) leads to activation of plants' second immune system termed effector-triggered immunity (ETI) which is an amplified and robust defense system of the plant. Both Pathogen-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) are plant defence mechanisms that respond to microbe invasion. These mechanisms alter the ionic balance, leading to increased cytosolic Ca^{2+} and apoplastic reactive oxygen species (ROS) levels. Additionally, they activate the mitogen-activated protein kinase (MAPK) pathway and cause the accumulation of nitric oxide (NO). This cascade results in the production and release of phytohormones such as ethylene (ET), jasmonic acid (JA), and salicylic acid (SA). Furthermore, it induces stomata closure and callose deposition, and initiates transcriptional and metabolic reprogramming to facilitate the plant's defense response (Nguyen et al., 2021).

In the context of fungal endophytes, many plants have chitin-specific receptors (PR-3) on their surface that recognize the chitin oligomers found on the fungal cell wall, resulting in the activation of the plant's defence mechanism (Sanchez-Vallet et al., 2015). Besides these, plant pattern-triggered immune signaling, such as MIN7 and CAD1, are the important components in controlling the level and nurturing the endophytes present in the nearby soil environment so that host can survive easily in the microorganism rich environment (Chen et al., 2020). In addition to various pathways, plants utilize extracellular vesicles (EVs) to facilitate the transport of signaling lipids, proteins, RNA, and metabolites between cells. Research indicates that these EVs play a role in plant stress response by exhibiting antipathogenic activity. During stress, plant cells secrete EVs containing host-derived small interfering RNAs and microRNAs that are capable of silencing fungal genes and stress responses. This evidence suggests that plant EVs may mediate trans kingdom RNA interference (Rutter and Innes, 2018). Alternately, when fungal endophytes invades the host plant, they start producing chitin

TABLE 1 Important crops and beneficial endophytic fungi with their respective functions.

Agricultural Crop	Beneficial Endophytic Fungi	Function	Reference
Rice	*Phoma glomerata, Penicillium simplicissimum, Galactomyces geotrichum, Fusarium oxysporum, Phoma sp., Aspergillus ustus*	Growth promoting factors, Salt tolerance, improves mineral nutrition and quality especially, under low nitrogen content in soil, ameliorates crop production.	Tang et al. (2022) and Potshangbam et al. (2017)
Wheat	*G. etunicatum, G. intraradices, G. fasciculatum T. atroviride, Glomus spp., Trichoderma atroviride, Alternaria alternata*	Improves germination of seed, rate of the shoot and root growth, chlorophyll content, alleviates drought tolerance and ameliorates crop yield.	Qiang et al. (2019), Saxena et al. (2013), and Colla et al. (2015)
Maize	*Gibberella fujikuroi, Fusarium oxysporum, Fusarium sacchari, Gibberella intermedia, Trichoderma atroviride, Aspergillus awamori, Metarhizium robertsii, Sarocladium zeae*	Plant growth promoting factors, induces disease resistance by improving plant immune response, protects against black cutworm larvae, improves crop yield	Ahmad et al. (2020), Mehmood et al. (2019), Contreras-Cornejo et al. (2018), Potshangbam et al. (2017), and Renuka and Ramanujam (2016)
Citrus	*Alternaria alternata, Alternaria citri, Alternaria rosae, Alternaria sp., Aspergillus sp., Colleotrichum karstii, Diaporthe eres, Piriformospora indica, Cladosporium sp., Pseudozyma sp., Meyerozyma sp.*	Acts as a biocontrol agent against pathogenic bacteria and fungal species, improved soil quality by enhancing the total phosphatase activity, and better fruit quality due to enhanced macronutrient content.	Cheng et al. (2022), Nicoletti (2019), and Sadeghi et al. (2019)
Banana	*Fusarium sp., Phoma sp., Nigrospora sp., Penicillium sp., Colleotichum sp., Piriformospora indica*	Tolerance to cold stress, resistance to pathogenic fungi, protects from nematode infection, increases productivity.	Kisaakye et al. (2023), Li et al. (2021), and Zakaria and Aziz (2018)
Soybean	*Penicillium minioluteum, Porostereum spadiceum, Rhizopus oryzae, Paecilomyces formosus*	Protects from abiotic salinity and thermal stress, helps in promoting photosynthetic activity, improves plant growth by increased macronutrient uptake, disease resistance.	Bilal et al. (2020), Ismail et al. (2020), Bajaj et al. (2018), Hamayun et al. (2017), and Khan et al. (2012)
Tomato	*G. intraradices, T. atroviride, Fusarium solani, Pochinia sp., Pythium sp., Piriformospora sp.*	Acts as biofertilizers by improving the nitrogen content of the soil, Increases biomass production, counteracts bacterial pathogen attacking the plant	Sinno et al. (2020), Pappas et al. (2018), and Colla et al. (2015)
Potato	*Rhizophagus irregularis, Epicoccum nigrum, Curvularia lunata*	Mitigates oxidative stress in roots and shoots during plant growth, acts as a biocontrol agent against blackleg disease of potato and has antimicrobial properties.	Deja-Sikora et al. (2020), Bagy et al. (2019), and Avinash et al. (2015)
Sunflower	*Penicillium citrinum, Talaromyces assiutensis, Aspergillus terreus, Rhizophus oryzae, Brassica napus, Piriformospora indica*	Alleviates thermal stress, disease resistance, act as biocontrol agent against pathogenic fungi, mitigates cadmium toxicity and helps in improving chlorophyll content under stress	Farhat et al. (2023), Ismail et al. (2020), Shahabivand et al. (2017), and Waqas et al. (2015)
Cotton	*Penicillium simplicissimum, Leptosphaeria sp., Talaromyces flavus, Acremonium sp., Beauveria bassiana, Purpureocillium lilacinum*	Protects against cotton wilt against Verticillium sp., promotes plant growth, protects the host plant against aphid and control crop infestation.	Yuan et al. (2017) and Lopez and Sword (2015)

deacetylases, that leads to deacetylation of the chitosan oligomers, and these oligomers are not detected by the host plant chitin specific receptors (Cord-Landwehr et al., 2016). The formation of biofilms in endophytic bacteria aids in their ability to adhere to host plant tissues and facilitate communication among themselves, thereby enabling them to evade the host plant's defence system. Endophytic microorganisms have evolved mechanisms to evade the oxidative stress generated by the plant host in response to pathogenic invasion. The plant's defence system includes the production of reactive oxygen species (ROS) such as superoxide (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), which are toxic to invading pathogens. To counteract this, endophytes have developed an array of antioxidative enzymes, such as superoxide dismutases (SODs), catalases (CatAs), peroxidases (PODs), alkyl hydroperoxide reductases (AhpCs), and glutathione-S-transferases (GSTs). These enzymes play a crucial role in the elimination of ROS and the protection of the endophyte from oxidative damage, allowing it to establish and maintain a symbiotic relationship with the host plant (Zeidler et al.,

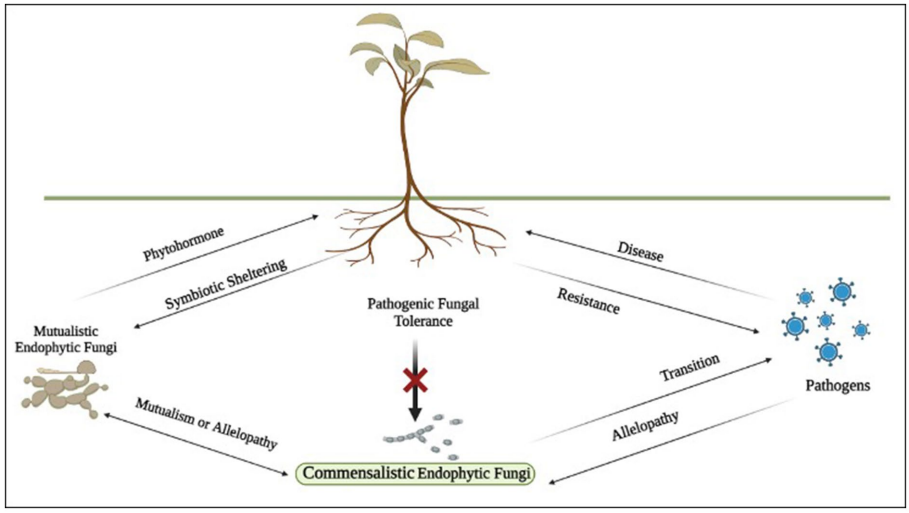

FIGURE 1
Schematic model of plant-fungi interactions.

2004). Endophytic microbes possess genes that encode for molecules known as Microbe-Associated Molecular Patterns (MAMPs) inhibitors. MAMPs are recognized by plants and trigger their immune response (Figure 2).

However, these inhibitors repress the MAMP-triggered immune response of the host plant. Endophytic communities can influence several physiological processes of the host plant, including the activation of silent gene clusters, leading to the synthesis of novel secondary metabolites. These secondary metabolites, which are also produced by the endophytes, play a crucial role in preventing the invasion of pathogenic microorganisms in the host plant, thereby preventing infections. The plant phenotype mostly depend upon the genetic arrangement within itself alongwith the biotic and abiotic stress and microbiome activity. The endophytic fungi stimulates the plant immune system through epigenetic events and hence, induce enhanced production of SMs leading to physiological and phenotypic change (Alam et al., 2021). Clustering of genes responsible for secondary metabolite production help in conserving the genetic arrangement. The encoding genes for these secondary metabolites are regulated by various factors such as horizontal gene transfer (HGT), transcription factors, the presence of effector molecules, and gene clustering.

2.1.1. Gene clustering and transcription factors

Gene clustering can be defined as the phenomenon where the genes responsible for coding the biosynthesis of certain secondary metabolites tend to cluster together in close proximity to the telomeric or dynamic regions of the fungal chromosome. These clusters of genes can vary in two significant ways. Firstly, different clusters of genes can work together, resulting in the synthesis of highly complex secondary metabolites. Secondly, in some cases, different pathways for secondary metabolite production have gene clusters located adjacent to each other in the fungal genome (Chiang et al., 2016; Henke et al., 2016; Nützmann et al., 2018). Nevertheless, because they are involved in gene expression control, the mechanism of coding of gene clusters of secondary metabolites in unstable DNA regions remains unexplained (Osbourn, 2010a). The spatial arrangement of clustered genes is believed to be a fundamental necessity for the biosynthesis of bioactive products that are linked to a pathway. This is due to the fact that genes involved in a pathway are generally maintained in close proximity through genomic rearrangements (Deepika et al., 2016). The resulting genetic structure may allow favorable allele combinations to co-inherit at these multigene loci (Chu et al., 2011; Field et al., 2011). This may also regulate chromatin synchronization by rearranging the chromatins (Field and Osbourn, 2008; Osbourn and Field, 2009; Osbourn, 2010b).

Essentially correlated genes interspersed in higher plant genomes can form transcriptional clusters via the helix–loop–helix domain. In this, the formation of DNA loops results in the co-localization of cis-regulatory elements, leading to increased local concentrations of transcription factors at the corresponding gene's transcription initiation sites, thereby initiating transcription. Any perturbations in the transcriptional regulation of clustered genes could result in the loss of their protein products and the accumulation of potentially harmful intermediates within the associated biochemical pathways. Many researchers showed that chromatin-level gene control is critical for the expression of secondary metabolic gene clusters (Osbourn, 2010a).

In the synthesis of secondary metabolites, clustered genes are regulated by two distinct groups of transcription factors, namely narrow domain transcription factors (NDTFs) and broad domain transcription factors (BDTFs). NDTFs exert their regulatory influence on the clustered genes directly, while BDTFs can act on the clustered genes at different gene locations than those of the NDTFs. This fact is exemplified by the renowned non-ribosomal peptide synthetase (NRPS) AflR, which is classified as a Zn_2Cys_6 transcription factor. AflR modulates the expression of the aflatoxin and sterigmatocystin gene clusters through its binding to the palindromic sequence 5′-TCG(N5)CGA-3′, an 11-bp motif that is present in the promoter regions of coding genes in selected *Aspergillus* species (Slot and Rokas, 2011). It also regulates three additional genes that are not associated with the aflatoxin metabolite gene cluster (Woloshuk et al., 1994; Yu et al., 1996; Cánovas et al., 2017). BDTFs, which are transcription factors

that regulate the expression of multiple genes, function as high-level regulatory systems that respond to external stimuli that are not directly related to secondary biochemical gene clusters. Recent studies have revealed that ethylene-forming (EF) signals specifically disrupt transcription factors that are targeted by ethylene, indicating that these signals can influence gene expression in a variety of biological processes (Camehl et al., 2010). Thus secondary metabolites are synthesized by an organism through the interplay of developmental competence and environmental factors. These factors include, but are not limited to, nutrient supply, artificial light, pH, injury, infection, and developmental changes that occur during different stages of the host's life cycle. It is widely accepted that a combination of these factors is essential for the efficient biosynthesis of secondary metabolites (Bayram and Braus, 2012; Xie et al., 2019). BDTFs are essential for transmitting environmental signals to the genome. They create and govern signaling transmission from environmental inputs to cellular responses during the creation of distinct SMs.

Several studies have investigated the molecular mechanisms underlying the relationship between Nucleosome-Dependent Transcription Factors (NDTFs) and Chromatin-Dependent Transcription Factors (BDTFs). These investigations aim to elucidate how BDTFs, or global transcription factors, perceive developmental or environmental cues, and subsequently transmit these signals to NDTFs through various biochemical pathways, such as chromatin and histone modifications, or through specific biochemical cascade reactions like methylation, phosphorylation, and acetylation. These responses are crucial in activating silent genes associated with specific secondary metabolites (SMs), which are required for specific cell metabolisms, growth stages, or environmental conditions. Furthermore, in addition to the plant's genetic makeup, plant phenotypes are also influenced by the activity of the microbiome and environmental factors. This can lead to genetic changes in the appearance of endophytic fungi associated with the plant (Jia et al., 2016). It has been seen that the presence of EFs helps to improve host plant resistance to biotic and abiotic stresses (Rodriguez et al., 2008; Xie et al., 2019; Yan et al., 2019). While the precise processes are unknown, evidence shows that the presence of EFs alters plant genetic expression patterns (Mejia et al., 2014; Xie et al., 2019). Epigenetic interactions between the host and endophyte can modulate the host's genomic expression. Endophyte-mediated alterations in DNA methylation and demethylation can enhance the host's defence mechanisms in epigenetic processes (Geng et al., 2019). EFs strengthen plant immune systems and increase the quantity of SMs, which may cause physiological changes in infected host plants (Xie et al., 2019). Bailey et al. (2006) reported that exposure to EFs leads to changes in gene expression patterns in both the endophytic microorganisms and host organisms. These changes occur due to a complex system of genetic interactions between the EFs and hosts, resulting in altered genomic expressions during the interference process.

2.1.2. Horizontal gene transfer

Horizontal gene transfer (HGT) is the process by which genetic elements are transferred between isolated lineages without sexual reproduction. This unique evolutionary mechanism is considered a novel adaptive trait of effector proteins, enabling them to invade, degrade, and manipulate host organisms (Soanes and Richards, 2014). HGT can facilitate the acquisition of a new and complete metabolic pathway by transferring the metabolic cluster of an organism to another (Slot and Rokas, 2011). The mechanism of HGT remains to be debated as to how the expression is initiated in the recipient organism. Slot and Rokas (2011) reported the successful functional transfer of a gene cluster responsible for the biosynthesis of sterigmatocystin, a toxic secondary metabolite, from *Aspergillus* species to *Podospora anserina*. The presence of certain metabolites, such as djalonensone in various *Alternaria* fungi, and aureonitol in *Chaetomium* sp. and extracts of *Helichrysum aureonitens*, which originated from horizontal gene transfer or genetic recombination during the coevolution of hosts and endophytes, provide evidence for the development of genetic regulatory mechanisms of secondary metabolite biosynthesis (Aly et al., 2010; Kozyrovska, 2013).

Through the application of the aforementioned techniques are applied by human beings for secondary metabolite production, fungal endophytes are capable of synthesizing a diverse range of secondary

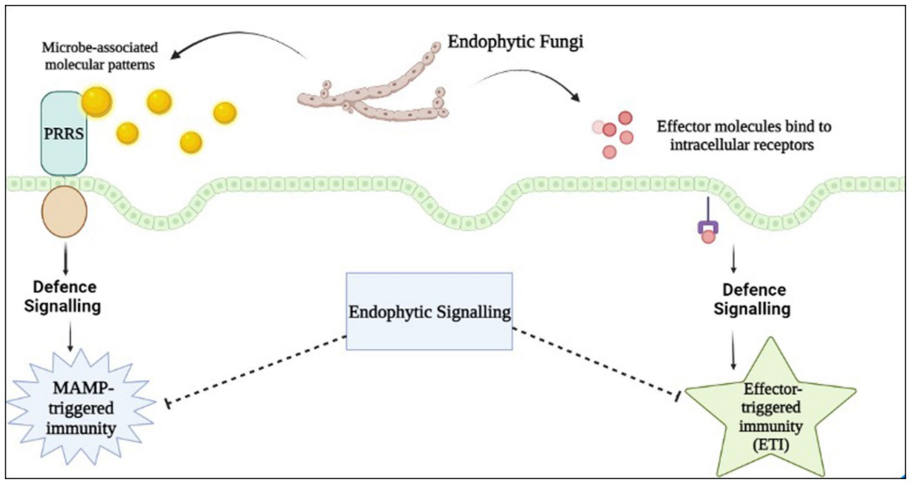

FIGURE 2
Schematic of plant immune system recognizes fungal signal molecules, resulting in two distinct defence mechanisms.

metabolites, including alkaloids, terpenoids, polyketides, phenylpropanoids, lignins, flavonoids, saponins, phenols and phenolic acids, aliphatic and chlorinated metabolites, peptides, and steroids. These secondary metabolites play a critical role in enabling the host plant to combat a range of stress conditions, such as drought, salinity, nutrient deficiency, metal toxicity, and biotic stress caused by pathogenic microorganisms present in the plant's immediate environment. The plant's capacity to endure stressors protects it from harm and prevents destruction. Elicitors or elicitor factors can even induce the synthesis of secondary metabolites with potential applications in various industries, including but not limited to pharmaceuticals, agriculture, and cosmetics. These secondary metabolites may have commercially valuable properties such as antibiotic, anti-carcinogenic, cytotoxic, insecticidal, and allelopathic activities. Some specific examples of important secondary metabolites synthesized by elicitor factors are outlined in the table below (Table 2).

3. Endophytic fungi as source of terpenoid production

Endophytic fungi are capable of producing a diverse range of bioactive compounds that exhibit a wide array of biological activities including, but not limited to, insecticidal, antioxidant, antifungal, antiviral, antibacterial, and cytotoxic properties. These compounds belong to various chemical classes such as terpenoids, phenols, alkaloids, polyketides, quinones, steroids, enzymes, and peptides. The secretion of these bioactive compounds by endophytic fungi is known to host plant defence response, enabling it to better cope with both biotic and abiotic stressors. Methods to enhance the potential of endophytic fungi for producing these secondary metabolites include the activation of silent biosynthetic pathways, epigenetic modifications, and other techniques. Among these secondary metabolites, terpenoids have emerged as a particularly important class of molecules that have significant applications in human health and agriculture. Thus, endophytic fungi represent a promising novel source for the bioproduction of these valuable terpenoid compounds. The isoprene units are typically obtained through two pathways, the mevalonic acid pathway (MVA) and the methylerythritol phosphate pathway (MEP). Based on the number of carbon atoms in their skeletal structure [$(C_5)_n$], terpenoids can be classified into various subgroups, including hemi terpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), and tetraterpenoids (C_{40}) (Gershenzon and Dudareva, 2007).

The building blocks of terpenoids, which are a diverse group of natural products, are isopentenyl pyrophosphate and dimethylallyl pyrophosphate. These are synthesized through the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways, and are interconvertible by the action of isopentenyl pyrophosphate isomerase. Subsequently, prenyltransferases convert these shorter chain precursors to longer chain terpenoid skeletons, such as geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate, which serve as the C10, C15, and C20 backbones of monoterpenoids, sesquiterpenoids and triterpenoids, diterpenoids and tetraterpenoids, respectively. Further structural diversification of terpenoids can be achieved by the introduction of functional groups, such as glycosyl, hydroxyl, ketone, carbonyl, and aldehyde, or by rearrangement of the carbon skeleton. These modifications can lead to the expression of various bioactivities associated with terpenoids (Sun et al., 2019). Numerous plant species are recognized for their capacity to biosynthesize terpenoids, including citral, menthol, camphor, salvinorin A (in the case of the plant *Salvia divinorum*), ginkgolide, and bilobalide (in the case of the plant *Ginkgo biloba*), and cannabinoids (in the case of the cannabis plant). Terpenoids are also commonly synthesized by tea, thyme, Spanish sage, and citrus fruits (such as lemon, orange, and mandarin). These terpenoids exhibit diverse functions in the food and cosmetic industries, as well as various pharmaceutical and nutritional applications. Furthermore, terpenoids have demonstrated good antiviral activity, and have therefore been investigated for their potential clinical use in the treatment of COVID-19. Due to these activities, there is an increase in demand of terpenoids which possess stress on the terpenoids producing plant species. In order to fulfil the increasing demand of terpenoids, other sources which are environmentally friendly and commercially fulfilling need to be discovered. The endophytes have the potential of synthesizing these terpenoids which have gained the attention for using the endophytes for alternate source of terpenoid-producing strains or terpenoid synthetic genes (Chen et al., 2021).

Endophytic fungi and its host plant undergo various complex interactions which enhance the plants growth and nutrition, also helps the plant in combating various biotic and abiotic stress conditions. Endophytes are known to exhibit a high degree of variability that is dependent on several factors such as the genotype of the host plant, growth stage, physiological state, tissue type, soil environment, and various agricultural practices (Gupta et al., 2020). These microorganisms have been shown to not only produce terpenoids themselves, but also stimulate their production in host plants through mechanisms such as horizontal gene transfer, the heterologous expression of terpenoid biosynthetic genes sourced from endophytes, biotransformation of terpenoids, and various signaling pathways that include elicitor recognition, signal transduction, integration with transcription factors, and gene activation (Zhai et al., 2017). Numerous scientific methodologies have been created to advance terpenoid biosynthesis research. These include metabolic engineering and synthetic biology, system biology approaches, modern metagenomic sequencing methods, and the *de novo* assembly of microbial genomes from metagenome data. These techniques enable the expression of the terpenoid biosynthetic pathway in industrial microbes, increase the potential gene pool involved in terpenoid biosynthesis from various environments, and offer effective strategies for discovering novel microbes and genes related to terpenoid biosynthesis in the endosphere and rhizosphere microbiomes, regardless of their culturability (Carrión et al., 2019; Belcher et al., 2020). Huperzine A (HupA) is a naturally occurring sesquiterpene alkaloid that is synthesized by members of the Huperziaceae family, which includes species such as *Huperzia serrata*. HupA has been found to possess potent anti-acetylcholinesterase activity, making it an effective treatment option for Alzheimer's disease (AD). Furthermore, HupA's ability to effectively inhibit acetylcholinesterase has been associated with minimal side effects, highlighting its potential as a safe and effective treatment option for AD (Zhao et al., 2013). The enzymes lysine decarboxylase and copper amine oxidase catalyze the first two steps of HupA biosynthetic pathway in which L-lysine is converted to 5-aminopentanal which is the precursor of HupA. Researchers successfully isolated two strains of endophytic fungi, EFs *Shiraia* sp.

TABLE 2 Secondary metabolites produced by endophytic fungus with their respective structure and functions.

Secondary Metabolites	Endophytic Fungi	Function	Reference
Terpenoids	*Pestalotiopsis microspora*, *Penicillium brevicompactum, Aspergillus terreus, Fusarium oxysporum, Colletotrichum gloeosporioides*	Participates in moderating cross-talk between endophytic fungi-host plants and also intervenes in the endophytic fungi-microbiome interaction. Act as signaling molecules between fungal-bacterial interaction and at times also aids in plant-growth promotion. These have different bioactive attributes like anti-microbial, anti-viral, anti-parasitic, antioxidant, anti-inflammatory, anticancer, herbicidial, and hypoglycemic properties.	de la Porte et al. (2020), Schulz-Bohm et al. (2017), Ditengou et al. (2015), Kaddes et al. (2019), and Ancheeva et al. (2020)
Steroids	*Alternaria alternata, Aspergillus fumigatus, Colletotrichum gloeosporioides, Xylaria sp. Neosartorya sp., Nodulisporium sp.*	Lipid-based biologically active compound having a range of anti-inflammatory, anti-cancer, anti-parasitic properties. Few examples include ergosterol, stigmasterol, campesterol, 22-hydroxy-cholesterol, brassicasterol, aspergilolide	Alam et al. (2021), Zhang et al. (2019), Nowak et al. (2016), and Yang et al. (2015)
Xanthones	*Aspergillus fumigatus, Phomopsis sp., Pestalotiopsis sp., Diaporthe sp., Colletotrichum gloeosporioides, Exserohilum rostratum*	Polysubstituted polyketide derivatives with a wide spectrum of pharmacological activities. Xanthones and its derivatives have potential, phytotoxic, cytotoxic, anti-protozoal, antibacterial properties. Also few xanthones like vertixanthone, danthron, globosuxanthone have shown antifungal activity against phytopathogenic fungi.	Ming et al. (2022), Pina et al. (2021), Miao et al. (2020), and Kaddes et al. (2019)
Quinones	*Alternaria sp., Fusarium sp., Xylaria sp., Phoma sp., Pestalotiopsis sp., Aspergillus sp., Talaromyces assiutensis*	These have a wide spectrum of medicinal properties. Phomol and phomenone are types of quinone known for their antibacterial properties. Other derivatives Xylarione, Pestaloside, Fusaric Acid are reported to have anti-tumor activities and hence are being studied for being potential anti-cancer drugs. These also possess antioxidant and antiviral attributes.	Lou et al. (2013), Christiansen et al. (2021), Mishra R. C. et al. (2021), and Ancheeva et al. (2020)
Phenols	*Colletotrichum gloeosporioides, Taxomyces andreanae, Phomopsis sp., Curvularia lunata, Cryptosporiopsis sp., Aspergillus flavus, Pestalotiopsis mangiferae, Vernonia amygdalina*	There are a variety of properties of different phenolic compounds extracted from endophytic fungi.Taxol, for example, has found extensive use in chemotherapy for its anti-cancer property. Similarly, resveratrol, gallic acid, curcumin, catechin has been found to exhibit significant anti-inflammatory, anti-oxidant and anti-microbial attributes.	Ancheeva et al. (2020), Praptiwi et al. (2020), Yadav et al. (2014), and Subban et al. (2013)
Mycorrhizin	*Plectophomella sp., Pezicula sp.*	These secondary metabolites exhibit significant cytotoxic, nematicidal, antibacterial antifungal properties. Also having such distinct antimicrobial activities reflects potential usage in food industries.	Adeleke and Babalola (2021), Preethi et al. (2021), McMullin et al. (2017), Hussain et al. (2014), and Schulz et al. (2015)

(Continued)

Slf14 (Zhu et al., 2010) and *Cladosporium cladosporioides* LF70 (Zhang et al., 2019), from the leaves of *Huperzia serrata*. These strains were found to produce Huperzine A (HupA) and were able to yield 142.6 μg/g and 39.61 μg/g dry mycelium, respectively. On sequencing the whole genome of *Shiraia* sp. Slf14, HupA biosynthetic gene cluster was identified, which is then expressed into *Escherichia coli*, which

TABLE 2 (Continued)

Secondary Metabolites	Endophytic Fungi	Function	Reference
Furandiones	*Aspergillus fumigatus, Aspergillus terreus, Fusarium solani, Fusarium graminearum, Phoma sp.*	These secondary metabolites are characterized by the presence of a furan ring. It is suggested these metabolites have a role in host-fungus interaction by protecting the host plant against phytopathogenic invasion. Furandione like beauvericin, phomodine, terrein, camptothecin, fumitremorgin C, have antifungal, antiviral, antibacterial properties.	Wang et al. (2022), Pavithra et al. (2020), Garcia et al. (2012), Ding et al. (2019), and Rajashekar et al. (2016)
Isocoumarin	*Xylaria grammica, Xylaria mali, Xylaria cubensis, Ampelomyces sp., A. truncatum, Xylariaceae sp., Geotrichum sp., Aspergillus banksianus*	These are bioactive molecules having a vast arena of antimicrobial, algicidal, antimycobacterial, antiplasmodial, antiviral, antimalarial activities. Also these have plant growth regulatory functions along with protein kinase inhibitory acetylcholinesterase, and glucosidase activity. Different derivatives of isocoumarins have also been reported to have significant anti-inflammatory, anti-oxidant, and cytotoxic properties.	Lv et al. (2023), Cheng et al. (2020), and Noor et al. (2020)

showed that genetically modified *E. coli* strain was able to convert cadaverine to 5-aminopentanal (Yang et al., 2016). Afterwards, some other strains of EFs were isolated, such as *Colletotrichum gloeosporioides* ES026, which were then sequenced and expressed for its yield of 5-aminopentanal which is a precursor of HupA.

Traditionally utilized medicinal plants *Nothapodytes nimmoniana* and *Campotheca acuminata* contain campothecin which belongs to the class of monoterpene indole alkaloids and functions as potent topoisomerase inhibitor in DNA replication. Reports from various studies suggest that *Entrophospora infrequens* and *Neurospora crassa* isolated from *N. nommoniana* produce campothecin (Puri et al., 2005; Rehman et al., 2008). Kusari et al. (2009) suggested that *Fusarium solani* isolated from the barks of *C. acuminata* are potential source of campothecin production. The endophytic strain of *F. solani* utilizes enzymes encoded by its gene in presence of strictosidine synthase, one of the majorly important plant enzyme for the campothecin biosynthesis. Another report by Yan et al. (2019) suggested that ginsenosides Rh2 and Rg3 are produced by strain PDA-2 which is closely related to *Agrobacterium rhizogenes* which was isolated from *Panax ginseng*. The ginsenosides Rh2 and Rg3 have major roles in inhibiting tumor cell proliferation and induce apoptosis. In a study by Palem et al. (2015), authors have suggested biosynthesis of vincristine and vinblastine by endophytic fungus *Talaromyces radicus* isolated from various tissues of *Catharanthus roseus*. The study screened 22 endophytic fungi for presence of gene encoding tryptophan decarboxylase which could only be amplified in *T. radicus*. The gene tryptophan decarboxylase is one of the most important enzyme terpenoid indole alkaloid biosynthetic pathway. Maximum yield of 670 μg/L and 70 μg/L of vincristine and vinblastine from modified M2 medium and PDB medium was obtained, respectively. Parthasarathy et al. (2020) recorded the vinblastine production *Curvularia verruculosa* isolated from leaves of *C. roseus*. Maximum yield of 182 μg/L of vinblastine was obtained from endophytic fungi *C. verruculosa*.

4. Biotic stress regulation and terpenoids

Stress can be defined as any substance, microorganism or unfavorable condition which directly or indirectly affects the plant's growth and development, metabolism or nutrition. Stress on vegetation depends on factors such as, temperature, mineral deficiency, long rainy periods, desiccation problems, insects and pathogens, herbicides and pesticides, pollutants, climatic conditions like drought or floods, increased UV radiations and so on. Based on the LICHTENTHALER model of stress in plants (Lichtenthaler, 1998), stress can be divided into several distinct phases. The first phase is the response phase, which is characterized by an alarm reaction and marks the beginning of stress. This is followed by the restitution phase, where the plant enters a stage of resistance and continues to experience stress. The third phase is the terminal phase, where the plant enters a stage of exhaustion after long-term stress. Finally, the regeneration phase allows the plant to recover and rejuvenate after experiencing stress. These phases of stress describe in detail the changes that occur in the plant during the unfavorable or stress conditions. Initially, there is decline in physiological functions which leads to decrease in metabolic activities and decline in growth, development and vitality of the plant. In response, the plant's defence system gets activated leading to hardening of the plant by increasing the plant's resistance against stressors by reaching plant resistance maximum. If the stressors are removed before senescence the plant survives via regeneration but if the stressors are not being removed then the plant leads to cell death which ultimately causes plant death (Lichtenthaler, 1998). Plant stressors can be divided into three types, that is, natural or abiotic stressors such as high light, heat, drought, mineral deficiency, low temperature, chilling, wounding, UV-A and UV-B, biotic stressors such as insects, pathogens, elicitors, bacteria, fungi and virus, anthropogenic stressors such as, herbicides, air pollutants, peroxyacyl nitrates, free radicals, acid rain, acid fog and heavy metal load. Plants sense these stressors and act accordingly via activation of

signal transduction pathways after signal perception further leading to gene expression or metabolic responses in the plant. Under stress conditions, plants synthesize bioactive molecules such as phytohormones and secondary metabolites which not only have the tendency to overcome the stress conditions but also have antifungal, anticarcinogenic and various other medicinal properties which can be utilized to treat various diseases. EFs have the capability of producing the secondary metabolites (terpenoids, alkaloids, phenols, quinones) that would help in fighting the biotic and abiotic stress in plants as well as can be utilized in pharmaceutical industry due to its medicinal properties (Gershenzon and Dudareva, 2007; Christiansen et al., 2021).

When endophytic fungi interact with their host plant, the plant's defence system is activated to counteract the presence of the endophytic fungi which are perceived as potential pathogens. However, endophytic fungi are capable of evading these defence mechanisms and colonizing the host plant. Subsequently, endophytic fungi produce bioactive compounds, such as terpenoids, phenols, alkaloids, steroids, quinones, poly-ketones, and peptides, which exhibit inhibitory effects on the growth of pathogens and herbivores invading the host plant in the presence of endophytic fungi (Lu et al., 2021). This phenomenon provides a protective mechanism to the host plant from pathogenic microorganisms. Various terpenoids are synthesized by endophytes which will help host plants to combat biotic and abiotic stresses. Examples of these terpenoids include indole diterpenoids, synthesized in endophytic infected grasses, are neurotoxins, responsible for intoxication, helpful in protecting the grass from cattle grazing (Hardoim et al., 2015) multi-cyclic indolosesquiterpene synthesized by *Streptomyces* sp. HKI0595 in *Kandelia candel* has antibacterial activity (Maier et al., 2011), cadinane sesquiterpene derivatives synthesized by *Phomopsis cassia* in *Cassia spectabilis* has antifungal activity against phytopatogenic fungi *Cladosporium cladosporioids* and *C. sphaerospermum. Persicaria minor* also known as kesum is herbaceous plant widely found in south-east Asia and is mainly important for flavonoid and terpenoid synthesis. Study by Samad et al. (2019) reported that six miRNAs of *Persicaria minor* post-transcriptionally regulated the terpenoid biosynthesis induced by *Fusarium oxysporum*.

5. Multi omics technology to uncover secondary metabolites from endophytes

A large diversity of microorganisms is present in the soil that interacts with the plants growing in that soil. These interactions are known as plant-microbe interaction. These interactions play a very important role in making a sustainable balanced ecosystem. Plants synthesize various organic and inorganic nutrients which makes the soil nutrient enriched that is very beneficial for the microbial consortia growing in that soil. Plants house endophytes which are one of the examples of plant-microbe interaction. Such interactions between plants and various microbes such as bacteria and fungi are beneficial and attract the interest of various researchers due to their potential for combatting biotic and abiotic stresses along with its application in agriculture. Throughout their life cycle, plants engage in the synthesis of two distinct categories of metabolites: primary and secondary metabolites. Primary metabolites, which are essential for the plant's growth, development, and nourishment, remain constant across all plant species. In contrast, secondary metabolites function as signaling molecules that play a crucial role in signal transduction. These metabolites are typically secreted by plants in response to herbivore, pathogen, or environmental stress but in low quantities. However, research suggests that endophytes, particularly EFs, have the capability to produce these secondary metabolites in large quantities when compared to plants. There are several researchers working for the discovery of these secondary metabolites as these are potential source for depleting the use of chemical pesticides, insecticides or herbicides and these discoveries are categorized as: (a) metabolites that are unknown, (b) metabolites with known function but unknown structure, (c) metabolites with unknown function but known structures (Luo et al., 2022).

Researchers face several difficulties in the discovery of these secondary metabolites which include (1) metabolites are secreted by the plants in very low concentrations, (2) the metabolites that are being secreted and synthesized are being actively metabolized as well, (3) they have diverse physical and chemical properties which require specific assays to determine their functions and structures, (4) these metabolites are secreted at specific stages of the plant's life which makes it difficult for scientists to discover them (Luo et al., 2022). To surmount the hurdles in the study of plant secondary metabolites, it is imperative to adopt interdisciplinary methods. A multi-omics approach, for instance, has been instrumental in the identification of these metabolites via the methodical comparative analysis of extensive datasets by researchers. In a broader aspect, "omics" are the scientific fields that are related to measuring biological molecules in high throughput methods. This includes various biological fields such as genomics, proteomics, metagenomics, metabolomics, phenomics, epigenomics, transcriptomics and so on. When all these fields are studied in an integrated manner for discovery of some biological molecule, then it is called a multi-omics approach. This review utilizes a multi-omics approach to investigate a diverse range of secondary metabolites synthesized by endophytes. By employing one or multiple omics approaches, a scientific experiment can be designed to align with the function and structure of the metabolite. Each omics approach has its unique characteristics and can complement the limitations of the other omics approach, thereby providing a comprehensive understanding of the endophytic secondary metabolite's synthesis and its potential applications.

Genomics is the field of biology that deals with the study of the whole genome (including structure, function, evolution, mapping and editing of genome) of the organism. Genome is the whole sequence of the DNA set of the organism. The fundamental genetic material, DNA, contains essential information about the regulation of gene expression, including promoter regions, untranslated regulatory regions, and splicing sites, as well as the protein-coding sequence that determines the function of a given gene within an organism (Chu et al., 2020). The field of genomics is driven by advanced technologies such as high-throughput DNA sequencing techniques, such as Illumina HiSeq, PacBio, and Nanopore sequencing, as well as single nucleotide polymorphism (SNP) chips that enable the identification and analysis of genetic variations among individuals. The whole genome sequencing analysis would help us understand that which genes are responsible for growth and development, defence system, nutrient acquisition,

TABLE 3 Selected studies on secondary metabolites based on omics technology.

Host Plant	Endophyte	Technique	Platform used for the study	Metabolites analyzed	Reference
Arabidopsis thaliana	*P. variotii*	Transcriptomics	-	Salicylic acid	Lu et al. (2019)
Zea mays	*T. virens*	Transcriptomics	NovaSeq 6,000	Salicylic acid and Jasmonic Acid	Malinich et al. (2019)
Anoectochilus roxburghii	*Ceratobasidium* sp. AR2	Transcriptomics; Metabolomics	Illumina HiSeq 2000; HPLC–MS/MS	Flavonoid biosynthetic genes; Flavonol glycosides	Zhang et al. (2020)
Panax quinquefolius	*Conexibacter* sp.	16S rRNA sequencing; Metabolomics	Illumina NovaSeq platform; LC–MS/MS	Saponin biosynthetic genes	Li et al. (2023)
A. thaliana	*B. megaterium* (BT 22)	Transcriptomics; Metabolomics	DNBSEQ high-throughput platform; LC–MS/MS	Auxin response genes, flavonoid biosynthetic genes	Liu et al. (2023)

production of secondary metabolites, and various other processes of the organism which we are studying. This approach of whole genome sequencing would help in determining the genes that are directly or indirectly influencing the various bioactivities or metabolic activities of the endophyte or plant which is synthesizing the secondary metabolites.

In the context of the interactions between endophytes and plants, various biotic and abiotic stresses may occur during their interactions leading to the production of secondary metabolites as a defence mechanism. Genomics approaches utilizing high-throughput sequencing techniques offer a powerful tool for identifying the specific genes responsible for the activation of signaling pathways leading to the production of secondary metabolites (Alam et al., 2021). Additionally, such approaches can be used to investigate plant growth promotion, endophytic secretory systems, surface attachment and insertion elements, transport systems, and other related metabolic mechanisms. This detailed knowledge about the genes and its functions would provide us with better understanding about the ecology and evolution of the endophyte and we could accordingly extract the gene responsible for secondary metabolite production and amplify it using PCR for its further utilization. Compounds like alkaloids, flavonoids, minerals, polyphenols and vitamins are mostly synthesized by endophytes have positive influence on adjusting in adverse conditions and hence, promoting plant health (Balestrini et al., 2021). These molecules/compounds can be detected and analyzed employing techniques such as gas chromatography–mass spectroscopy (GC–MS), Fourier transform infrared (FT-IR), nuclear magnetic resonance (NMR) spectroscopy, metabolite fingerprinting, time-of-flight mass spectrometry (TOF-MS), Orbitrap Mass Spectrometer (Orbitrap-MS), flash chromatography. The signaling pathway initiation and colonization factors due to host cell and endophytic interactions can be evaluated on the basis of proteomics studies. However, there are new omics tools available for deciphering the fungal genome and the metabolites produced by them through epigenomics, ionomics, fluxomics, lipidomics, nutrigenomics, toxicogenomics. By integrating these various techniques, the secondary metabolite production can be verified and analyzed at every level (Table 3; Shankar and Sharma, 2022). The host endophyte interaction from recognition phase to stress resistance development can be thoroughly studied via transcriptomic analysis.

During the investigation of the metabolic output of the colocalization phenomenon of prenyltransferase (Polyprenyl synt (PT)) and terpene synthase (Terpene synth C (TPS)) domains in plant genomes, the biosynthesis of sesterterpenes, a rare type of terpenoid, was discovered to occur in both plants and fungi (Huang et al., 2017, 2018). *P. indica*, has the potential of acting as plant probiotic agent, that has been revealed during its genome sequence analysis (Qiang et al., 2012). EFs of order sebacinales helps the host plant in enhancing its growth, development and stress tolerance potential, is revealed by this approach (Weiss et al., 2011). Several bioinformatic tools, such as plantiSMASH (Kautsar et al., 2017), phyto cluster (Töpfer et al., 2017), and clusterfinder (Schläpfer et al., 2017; Chavali and Rhee, 2018), have been developed to predict plant biosynthetic gene clusters (BGCs) using plant genomic sequences, protein annotations, and gene expression profiles. This facilitates the identification of plant secondary metabolites, which can have important implications in fields such as drug discovery and agriculture.

6. Future prospect and conclusion

The development of a sustainable agriculture is of utter importance. Microorganisms like the endophytic fungi represent an intriguing area of study in the field of plant-microbe interactions in this context. The unique ability of these fungi to inhabit and colonize host plant tissues and thereby stimulate the production of wide range of secondary metabolites with diverse biological activities, including antifungal, antibacterial, anticancer, and immunomodulatory properties are of extreme importance. These attributes enhance the plants disease resistance ability, growth and nutrient uptake abilities. Most of these secondary metabolites produced by these fungi are valuable sources of antimicrobial, antitumor, and antiviral agents, among other bioactive compounds, making them a vital component in the development of new drugs. Multi-omics technologies can further help in deciphering the physiological development of endophytes in host plant. Concomitantly, generation of more high-throughput data will provide updated information on the core areas of study, thus discovering unknown genes, metabolites, and microbial species for better harnessing of beneficial aspects from endophytes. Thus, in conclusion it can be well

understood that the overall, investigation of endophytic fungi and their metabolic products has gained significant attention in recent years, and this approach holds great promise for sustainable agriculture. With the increasing demand for eco-friendly and non-toxic agricultural practices, endophytic fungi present an attractive alternative to chemical pesticides and fertilizers. Further studies on these fungi will not only provide a better understanding of their interactions with host plants but also contribute to the development of sustainable agricultural practices and the discovery of novel bioactive compounds for various applications.

Author contributions

PJ and TM: conceptualization, editing, and supervision. TK and IC: literature review and drafting of original manuscript. AP, SP, and VK: writing and editing. All the authors have read and approved the final version of the manuscript.

Conflict of interest

IC was employed by Metropolis Healthcare Ltd.

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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Glossary

VOCs	Volatile Organic Compounds
PRRs	Pattern-Recognition Receptors
MVA	Mevalonic Acid Pathway
MEP	Methyl Erythritol Phosphate
NGS	Next Generation Sequencing
EF	Endophytic Fungi
MAMPs/PAMPs	Microbial- or Pathogen-Associated Molecular Patterns
BIK1	Botrytis-Induced Kinase1
EVs	Extracellular Vesicle
DAMPs	Damage-Associated Molecular Patterns
CNGCs	Cyclic Nucleotide-gated Channels
PTI	Pattern-Triggered Immunity
HGT	Horizontal Gene Transfer
ETI	Effector-Triggered Immunity
NDTFs	Narrow Domain Transcription Factors
BDTFs	Broad Domain Transcription Factors
SNP	Single Nucleotide Polymorphism
BGCs	Biosynthetic Gene Clusters
AD	Alzheimer's disease
GC–MS	Gas Chromatography–Mass Spectroscopy
FT-IR	Fourier Transform Infrared
NMR	Nuclear Magnetic Resonance Spectroscopy
TOF-MS	Time-of-Flight Mass Spectrometry
Orbitrap-MS	Orbitrap Mass Spectrometer

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Phyto-microbiome to mitigate abiotic stress in crop plants

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Plant-associated microbes include taxonomically diverse communities of bacteria, archaebacteria, fungi, and viruses, which establish integral ecological relationships with the host plant and constitute the phyto-microbiome. The phyto-microbiome not only contributes in normal growth and development of plants but also plays a vital role in the maintenance of plant homeostasis during abiotic stress conditions. Owing to its immense metabolic potential, the phyto-microbiome provides the host plant with the capability to mitigate the abiotic stress through various mechanisms like production of antioxidants, plant growth hormones, bioactive compounds, detoxification of harmful chemicals and toxins, sequestration of reactive oxygen species and other free radicals. A deeper understanding of the structure and functions of the phyto-microbiome and the complex mechanisms of phyto-microbiome mediated abiotic stress mitigation would enable its utilization for abiotic stress alleviation of crop plants and development of stress-resistant crops. This review aims at exploring the potential of phyto-microbiome to alleviate drought, heat, salinity and heavy metal stress in crop plants and finding sustainable solutions to enhance the agricultural productivity. The mechanistic insights into the role of phytomicrobiome in imparting abiotic stress tolerance to plants have been summarized, that would be helpful in the development of novel bioinoculants. The high-throughput modern approaches involving candidate gene identification and target gene modification such as genomics, metagenomics, transcriptomics, metabolomics, and phyto-microbiome based genetic engineering have been discussed in wake of the ever-increasing demand of climate resilient crop plants.

KEYWORDS

phyto-microbiome, abiotic stress alleviation, bio-inoculants, multi-omics, plant microbiome engineering

1. Introduction

Climate change has lead to several perturbations in the environment such as extremes of heat and cold, drought, waterlogging, and changing weather patterns, which are responsible for adverse effects on crop production globally. Various environmental and anthropogenic factors pose abiotic stress on plants such as temperature, salinity, drought, heavy metals (Sandrini et al., 2022), UV radiation (Shourie et al., 2014), and pesticides (Yasmin and D'Souza, 2010). Temperature fluctuations, erratic rainfall and frequent droughts are also attributed to shifts in agricultural cycles. More than 50% of agricultural losses are caused due to heat, salinity, drought and heavy metal stresses, both qualitatively and quantitatively (Salam et al., 2023). It is worth

noting that a minor temperature increase of even 1°C can reduce the crop yield of various crops by 3–7% (Zhao et al., 2017). These aspects are also responsible for changes in the edaphic factors like pH, moisture, salinity, ion content, mineral availability and organic content, which directly affect the crop yield. Salinity and heavy metal accumulation in soil has significant impacts on plant health and crop productivity. Salt stress inhibits seed germination and disturbs the homeostasis at cellular and biochemical level. It affects water uptake, exerts osmotic stress and causes nutritional imbalance in plants. Similarly, heavy metals also cause detrimental effects on plants by imposing toxicity and hampering physiological processes that are vital for survival and growth of plants.

Plants adopt various strategies to survive under unfavorable environmental conditions and have remarkable capabilities of enduring and adapting to abiotic stresses through transient and stable gene expression mediated by stress signaling. The microorganisms present in the soil, rhizosphere, and phyllosphere of plants play a crucial role in the maintenance of environmental homeostasis and enable plants to survive under stress conditions (Barea, 2015; Ngumbi and Kloepper, 2016). A plant acquires its microbiome from the parent plant, the soil in which the seed is sown, and the environment to which it is exposed. Plants and their microbiome are in an exquisite symbiotic relationship and mutually promote growth, health, and development. Plant-associated microbes offer numerous benefits to plants such as fixing atmospheric nitrogen, enhancing the bioavailability of minerals, producing organic nutrients, detoxifying pesticides, harmful chemicals and toxins, mitigating plant diseases, and producing plant growth hormones and bioactive compounds (Sagar et al., 2021). The rhizospheric occupants belonging to the genera *Azotobacter* (Sahoo et al., 2014), *Azospirillum* (Omar et al., 2009), *Rhizobium*, *Pantoea*, *Bacillus, Pseudomonas* (Sorty et al., 2016), *Enterobacter* (Nadeem et al., 2007), *Bradyrhizobium* (Panlada et al., 2013), *Methylobacterium* (Meena et al., 2012), *Burkholderia* (Oliveira et al., 2009), *Trichoderma* (Ahmad P. et al., 2015) and *cyanobacteria* (Joshi et al., 2020) have been reported to contribute in growth promotion of several crop plants.

Plant microbiome offers an abiotic stress protection mechanism to the host as the metabolic potential of microbiome is immense and it supplements the metabolic capacity of the plants to acquire nutrition and develop tolerance against stress. The phyto-microbiome is dynamic and its organization is sculpted by the degree and duration of the abiotic stress. The plant and its associated microbiome synergistically respond to abiotic stress for mutual survival and growth (Javaid et al., 2022). Under unfavorable environmental conditions, soil-dwelling microorganisms from the genera *Achromobacter*, *Azospirillum*, *Variovorax*, *Bacillus*, *Enterobacter*, *Azotobacter*, *Aeromonas*, *Klebsiella*, and *Pseudomonas* have been demonstrated to promote plant growth (Dardanelli et al., 2008; Belimov et al., 2009; Ortiz et al., 2015; Kaushal and Wani, 2016; Sorty et al., 2016). *Burkholderia phytofirmans* strain *PsJN* has been found to reduce salt stress in *Arabidopsis* (Pinedo et al., 2015) and maize (Naveed et al., 2014b), as well as drought stress in wheat (Naveed et al., 2014a).

The response of the phyto-microbiome to the abiotic stress largely influences the growth, tolerance, adaptation, and evolution of the host plant and microbes both. There is now mounting evidence that plant-associated microbes may prove to be instrumental in the sustenance of agriculture in times of drastic impacts of climate change. The phyto-microbiome architecture could be better utilized for abiotic stress alleviation of plants and the development of stress-tolerant crop plants if the ecological relationships of the plant-associated microbial diversity and mechanisms of their interactions are deeply understood. The potential options for overcoming crop plants' productivity constraints in stress-prone environments include the selection, screening, and application of stress-tolerant microorganisms. The application of beneficial microorganisms as bioinoculants can be a good alternative for promoting plant growth under various types of abiotic stresses.

In this review paper, the ecology of phyto-microbiome is summarized, focusing on the beneficial microbes and their role during abiotic stress conditions. The physiological and molecular responses of phyto-microbiome against major stressors drought, heat, salinity and heavy metal toxicity are discussed to determine the role of the microbiome in the stress alleviation of plants. Ascertaining the potential of microbes in the development of stress-resistant plants, the paper further emphasizes modern strategies like introducing novel bio-inoculants, application of multi-omics technologies for gene modification, and phyto-microbiome-based genetic engineering as sustainable solutions to enhance agricultural productivity.

2. Ecological structure and function of phyto-microbiome

The phyto-microbiome is composed of taxonomically diverse communities including bacteria, archaebacteria, fungi, and viruses, which establish various ecological relationships with the host plant such as symbiosis, mutualism, and parasitism (Chialva et al., 2022). Microbiomes associated with plants are either epiphytic or endophytic, and colonize both niches–the phylloplane (above ground part) and rhizoplane (below ground part) (Santos and Olivares, 2021). Various plant compartments in which the microbes form their niche and colonize are depicted in Figure 1. Rhizospheric microbiome is selectively attracted and recruited by the host plant though rhizodeposition in which root exudates containing compounds like amino acids, carbohydrates, organic acids, fatty acids, siderophores and flavonoids, are secreted. These root exudates act as signals to establish communication between plant and specific microbes. The type of root exudates varies with plant's genotype, innate immunity, signaling pathways and response to environmental conditions. The root exudate composition is instrumental in shaping the structure of phyto-microbiome assembly in the rhizosphere. The epiphytic interactions in the rhizoplane and phylloplane provide oppuortunity to the microbes to enter the tissues, systematically spread through vascular system and colonize other compartments as endophytes. The endophytic community structure is mostly substrate-driven and depends upon the allocation of resources in different plant compartments. Besides plant-microbe interactions, the microbe-soil interactions and microbe-microbe interactions significantly affect the plant growth (Santoyo, 2022).

2.1. Rhizobacteria

The soil microenvironment of the root region is rich in microbes because it contains a wide range of nutrients, minerals, and

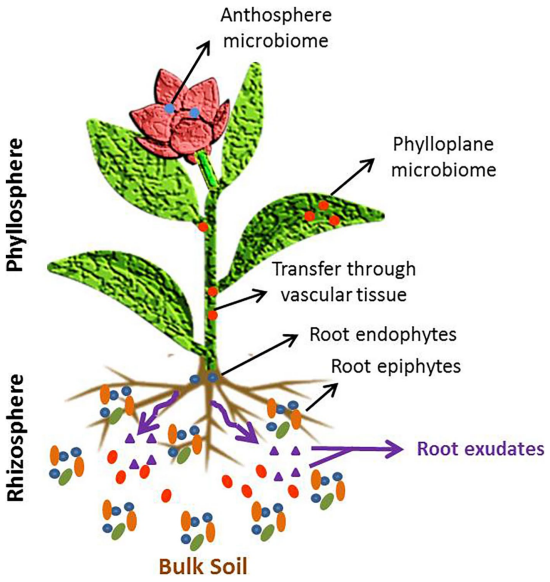

FIGURE 1
Structure of phyto-microbiome.

metabolites. The microbial colonization of the rhizosphere is significantly influenced by root exudates or substances that a plant root secretes (Kong and Six, 2012). Some microorganisms of the rhizosphere that aid in reducing abiotic stress include plant-growth-promoting rhizobacteria (PGPRs), act as beneficial microorganisms that adopt several strategies to reduce abiotic stress, including the production of phytohormones, a decrease in ethylene oxide levels, an increase in the dehydration response, and the induction of genes encoding antioxidant enzymes (Yang et al., 2009). Further, these bacteria contribute to the production of plant growth regulators like indole-3-acetic acid (IAA), deaminase, and 1-aminocyclopropane-1-carboxylic acid (ACC) that aid in enhancing plant growth (Glick, 2014). It was observed that the genera *Diazotrophicus*, *Bacillus*, *Pseudomonas*, *Azotobacter*, *Azospirillium*, *Rhizobium*, *Burkholderia*, *Gluconacetobacter* and *Serratia* are the most important rhizospheric inhabitants that help plants mitigate a variety of abiotic stresses (Backer et al., 2018). In order to reduce stress in rice, *Trichoderma harzianum* was used to increase aquaporin, dehydrin, and malondialdehyde (Pandey et al., 2016). Additionally, *T. harzianum* was used to increase the oil yield from salinity affected Indian mustard (*Brassica juncea*) which enhanced antioxidant synthesis, decreased Na + uptake, and improved nutrient uptake in plants (Cho et al., 2008). Rhizobacteria-induced drought endurance and resilience (RIDER) is defined as changes in the levels of phytohormones, defense-related proteins, enzymes, antioxidants, and epoxy polysaccharides on exposure to various stresses (Kaushal and Wani, 2016). These changes increase the plants' resistance to abiotic stress (Raymond et al., 2004). IAA and ACC-deaminase production in barley and oats appeared to be improved using *Pseudomonas* sp. and *Acinetobacter* sp. (Lu et al., 2013). *Pseudomonas* sp. improved its ability to inhabit roots sideways is due to its capacity to produce exo-polysaccharides (EPS) stimulus, and increased salinity resistance in rice during germination (Rojas-Tapias et al., 2012). Actinomycetes are known to promote plant growth and lessen the damage caused under abiotic stress. They are able to grow under harsh environment such as high salinity, drought and high temperature (Grover et al., 2016). In the rhizosphere the Actinomycetes utilizes the nutrient and water more efficiently in the stressed soil as they possess the ability to cleave the rhizospheric soil particles and hence form strong bonds with the plants (Sandrini et al., 2022). These bacteria follow several mechanisms such as changes in root and cell wall morphology, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, possess the ability to avoid oxidative damage, phytohormonal alterations, compatible solute production (glycine-betaine and proline) that promotes osmoregulation (Chukwuneme et al., 2020).

2.2. Phyllosphere bacteria

Phyllosphere is an ideal environment for microbes that harbors a huge variety of beneficial microbes belonging to bacteria, fungi and viruses. The performance of the plant is significantly influenced by the phyllospheric microbiome. These microbes also assist plants in purging contaminants. Additionally, they support the preservation of plant health and control the spread of plant pathogens. The long-distance transport process has a significant impact on the microbiota of plant parts that are distant from the soil or in other aerial parts of plants (Arun et al., 2020). When rice plants were stressed by drought, inoculating the plants with the plant growth-promoting, drought-tolerant *Bacillus altitudinis FD48* increased relative water content, chlorophyll stability index, and membrane stability index compared to control (uninoculated plants) (Awasthy et al., 2017).

2.3. Fungi

Many fungi inhabiting the rhizospheric soil have remarkable potential of degradation of various pollutants, thereby protecting the plants from abiotic stress (Shourie and Vijayalakshmi, 2022). Some fungi like Arbuscular mycorrhizal fungi (AMF) are obligate mycorrhizal fungi that form symbiotic relationships with vascular plants including halophytes. AMFs can sporulate in the rhizosphere as well as form vesicles and hyphae in roots. Plant growth is increased because of the excellent access to the soil surface area provided by the hyphal network that AMFs create. Through the effective translocation of nutrients, AMFs contribute to an improvement in plant nutrition. Additionally, they aid in enhancing the health of plants and the soil (Compant et al., 2010). Plant productivity is typically reduced by drought stress, in which AMFs assist plants in retaining growth and increasing yield. AMFs aid in increasing water uptake as part of the drought mitigation mechanism and aid the plant in enhancing nutrient uptake, which enables plants to withstand stresses (Jiang et al., 2016). It was found that the plant biomass, fruit yield, and shoot content of P, K, Cu, Fe, and Zn increased when a tomato plant was inoculated with *Funneliformis mosseae* under saline conditions (Chandrasekaran et al., 2021). Wheat plants inoculated with AMFs performed well under salt stress and the oxidative damage to the plants is significantly reduced (Hayat et al., 2010). Extremely low and high temperatures, however, were reported to inhibit the development of the extra radical hyphal network and AMF fungal activity, and

decrease AMF fungal growth (Mathur and Jajoo, 2020). AMF helps plants grow their root system for water absorption at high temperatures to ensure high photosynthetic capacity and prevent damage to the photosynthetic apparatus. The inoculation of barley (*Hordeum vulgare* L.) with AMF led to improved growth, photosynthesis, osmotic homeostasis, and potassium uptake under low-temperature conditions, and *Glomus versiforme* was frequently more successful than *Rhizophagus irregularis* at boosting survival rates (Hajiboland et al., 2019). Vesicular Arbuscular Mycorrhiza (VAM) also alters the physiological, functional, and biochemical makeup of plants in ways that increase their ability to withstand various abiotic stresses. AMF inoculation in vegetables has been shown to boost biomass production and increase yield (Haghighi et al., 2015; Duc et al., 2018). The uptake of greater amounts of nutrients, leaf water potential, and stomatal conductance are all significantly influenced by AMF inoculation (Khan et al., 2013). When lettuce plants were inoculated with AMF, their abscisic acid (ABA) levels decreased, indicating that they were less stressed than uninoculated plants. Therefore, AMF inoculation modified the plant's hormonal profile and physiology to make it more suited to saline conditions (Aroca et al., 2013).

2.4. Endophytes

Endophytes have symbiotic relationships with plants and live inside them for the entirety of their life cycles. Endophytes typically invade the seeds, roots, leaves, and stems of host plant, establish colonies in plant tissues and promote plant growth by enhancing nitrogen fixation, phytohormone secretion, and nutrient uptake. During periods of abiotic stress, endophytic microbes stimulate plant growth by various mechanisms such as osmolyte accumulation, induced systemic tolerance, production of phytohormones such as ABA, gibberellic acid (GA), cytokinins and IAA, ACC deaminase production for lowering ethylene. The endophytic *Arthrobacter* strains *EZB4*, *EZB18*, and *EZB20* inoculation increased the proline content in *Capsicum annum* L. exposed to abiotic stress (Sziderics et al., 2007). Salinity stress was alleviated by *Bacillus firmus* and *Bacillus* sp. in peanut, *Curtobacterium* sp. in soybean and rice, *Enterobacter ludwigii*, *Bacillus cereus* and *Micrococcus yunnanensis* in rice (Khan et al., 2019, 2020; Pal et al., 2021). The root fungal endophyte *Piriformospora indica* induced drought tolerance in Chinese cabbage (Sun et al., 2010) and salt tolerance in barley (Baltruschat et al., 2008), by boosting the levels of antioxidants.

3. Physiological and molecular response of phyto-microbiome against abiotic stress

The responses of crop plants against abiotic stresses are manifested as altered phenotypes at morphological, physiological, and biochemical levels. Plants have developed complex signaling mechanisms to counteract stress conditions and enable survival. The plant microbiome further supplements the metabolic capacity of the plants to combat stress conditions. Here, the major stress factors (drought, heat, salinity and heavy metal) are discussed for their impact, plant response, and the role of the microbiome in combating stress.

3.1. Drought stress

Severe drought stress leads to wilting, yellowing, discoloration, and leaf burning in plants. Plants have the inherent capability to respond to drought stress and they try to control the damage by complex mechanisms. They respond and regulate the drought stress by closing stomata, decreasing the surface area of succulent leaves, and increasing the roots. However, prolonged drought stress is known to stunt plant growth with a reduction in leaf size, and stems, production of a greater number of roots, decrease in RubisCO activity and photosynthetic pigments, reduction in seedling vigor, and decrease in seed germination. It reduces membrane potential and increases the concentration of reactive oxygen species (ROS) causing free radical damage and disruption of ATP synthesis (Shaffique et al., 2022).

3.1.1. Drought stress response and signaling

Water scarcity is detected by the leaves and roots. However, the signals are transmitted majorly from roots to shoots. Plants sense and transmit water deficit through signaling activated by osmotic pressure, ROS and mechanical stresses, involving numerous sensing molecules. ABA is an important phytohormone that is produced in response to drought stress and plays a crucial role in adaptation to drought stress. ABA is mostly produced in vascular tissues and is transported via a transporter to various tissues. It induces stomatal closure and activation of stress-related genes that increase drought resilience (Kuromori et al., 2022). Water deficit induces the expression of enzymes of ABA biosynthetic pathway such as ZEP/ABA1, AAO3, cis-epoxy carotenoid dioxygenase (NCED3), and molybdenum cofactor sulfurase (MCSU/LOS5/ABA3). The binding of ABA with ABA receptor proteins PYR/ PYL/ RCAR initiates the ABA-dependent stomatal regulation pathway leading to the activation of Protein Phosphatases 2C (PP2C) and SNF1-Related Protein Kinases 2 (SnRK2). The transcription factors such as ABF, MYC MYB, NAC, ERF, bZIP, and DREB/CBF are activated, and they bind to nuclear targets resulting in the expression of drought stress proteins (Ali S. et al., 2022; Aslam et al., 2022).

Drought stress causes a variety of biochemical changes inside the host, including an excessive build-up of reactive oxygen species (ROS), which can harm different tissues and cellular components like nucleic acids and other biomolecules, leading to programmed cell death (PCD) (Hasanuzzaman et al., 2020). In addition to altering biogeochemical cycles like the nitrogen and carbon cycles and slowing down the breakdown of organic matter, drought stress can also cause a considerable decrease in plant absorption and translocation of macronutrients (K, N, and P). Drought stress also reduces the absorption of cations (Ca^{2+}, K^{+}, and Mg^{2+}) leading to the inhibition of several vital enzymes (Farooq et al., 2009).

3.1.2. Role of phyto-microbiome in drought stress

It has been discovered that plant-associated microbiomes secrete a variety of chemicals during drought, including phytohormones, osmolytes, and antioxidants, which increase plant drought tolerance. Apart from facilitating plant growth, phytohormones such as IAA, cytokinin, and gibberellins, can assist plants in coping with abiotic stresses. Interestingly, the primary mechanism of mitigation of drought stress by plant microbiome is by inducing drought stress-responsive genes and regulating phytohormones (Iqbal et al., 2022).

3.2. Heat stress

Heat stress leads to a decrease in cell water content, cell size, plant size, growth, and biomass. Severe heat stress leads to scorching and discoloration of leaves, fruits, and other plant parts. Heat stress also leads to the alteration of biomolecular composition. It has been seen that heat stress increases the concentration of amino acids while decreasing the concentration of starch, sugars, and lipids. Maltose concentration has been seen to be elevated (Dastogeer et al., 2022). At the molecular level, heat stress leads to protein denaturation and misfolding. The cell membrane fluidity is increased while membrane integrity is compromised (Sehar et al., 2022). The indirect effects are complex to decipher. The increase in temperature can cause previously unknown infections due to the growth of microbial pathogens and the emergence of newer more pathogenic strains (Velásquez et al., 2018). Continuous thermal stress can increase the deposition of reactive oxygen species (ROS) resulting in membrane depolarization and initiation of programmed cell death (Katano et al., 2018).

3.2.1. Plant defense against heat stress

Plants possess inherent thermal tolerance known as basal heat tolerance while thermotolerance can also be acquired. Plants resort to short-lived or long-term adaptation strategies to combat heat stress. Some plants have leaf and bud shedding, annual flowering, or regenerative stage completion in winter as an adaptation to high-temperature habitats. Heat stress leads to the induction or activation of ion transporters, antioxidants, phytohormones and signal transduction elements. Late embryogenesis abundant (LEA) proteins are produced to protect against heat stress. To prevent acute heat injury and mortality, plants contain molecular stress memory states known as short-term acquired tolerance (SAT) and long-term acquired tolerance (LAT). Particularly, C4 and CAM plants adopt a variety of modifications to boost the process of photosynthesis - under heat stress. Intensive transpiration from leaves can prevent damage by lowering the temperature of the leaves by several degrees (Hasanuzzaman et al., 2013).

Heat stress leads to the production of reactive oxygen species and in response the production of antioxidants such as peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) is triggered in plants. The superoxide anion radical is changed by SOD into H_2O_2 and O_2, which are subsequently changed into water and oxygen by CAT and APX. In plant cells, GR plays a role in the regeneration of the reduced glutathione which is an essential antioxidant. These detoxification systems maintain cellular homeostasis and promote plant growth and development during heat stress (Zandi and Schnug, 2022). Plants activate a complex signaling system involving heat shock factors (HSFs) that control the transcription of heat shock genes, including the production of heat shock proteins (HSPs). HSPs help protect the plant by promoting the proper folding of proteins, preventing protein denaturation and aggregation, and facilitating the breakdown of defective proteins. Different types of HSPs are produced, such as Hsp60, Hsp70, Hsp90, Hsp100, and sHSPs, each has specific functions in maintaining cellular homeostasis and promoting thermotolerance. Overall, the synthesis and overexpression of HSFs and HSPs play a critical role in enabling plants to cope with high-temperature stress (Ul Haq et al., 2019).

3.2.2. Induction of thermotolerance

A brief pre-exposure to mild heat stress, also called priming, might cause plants to develop thermotolerance. This brief exposure builds a molecular stress memory which allows quicker and higher expression of heat stress transcription factors (HSFs) that control the production of heat shock proteins (HSPs) and antioxidant genes (Khan et al., 2022). Under heat stress, HSPs work as molecular chaperones to preserve the structure and function of proteins (Jacob et al., 2017). As a result, stress memory enables primed plants to respond swiftly to heat stress and recover from the adverse effects of heat. Four isomers of HSFA1A, B, D, and E, are known as master regulators of heat stress. They trigger the expression of HSFA2. A group of heat stress response genes, known as memory genes are in turn amplified by HSFA2 (Friedrich et al., 2021).

3.2.3. Phyto-microbiome in combating heat stress.

Plant growth-promoting microorganisms (PGPM) can induce thermotolerance in plants by the production of heat shock proteins and induction of structural changes in plants. Moreover, phytohormone production, nutrient mobilization, and nitrogen fixation are brought about by the PGPM. Rhizospheric microorganisms produce and secrete phytohormones like IAA, gibberellins, and cytokinins. Endophytic microorganisms modulate the levels of abscisic acid, salicylic acid, and jasmonic acid under multiple stresses. Auxins are required for cell division and differentiation, growth of root and shoot, and seed germination, gibberellins regulate embryogenesis, stem growth, flowering, and fruit ripening, and abscisic acid regulates cell division and fruit ripening. Cytokines are involved in seed germination, root and shoot development, while ethylene is involved in abscission, senescence, and reproductive development. PGPM which produce gibberellins stimulate plant growth and stress tolerance (Hakim et al., 2021). Plant-associated microorganisms known to secrete exopolysaccharide form a biofilm over the plant roots and make a protective barrier and facilitate nutrient supply. Exopolysaccharide-producing *Bacillus cereus* was found to increase root and shoot length, chlorophyll content, water-intake, flowering, and fruiting in tomatoes (Mukhtar T. et al., 2020).

3.3. Salinity stress

Salinity reduces nutrient and microbial diversity, organic matter, nitrogen, dissolved organic carbon, and microbial carbon biomass in soil. Additionally, it causes osmotic stress, disturbs the nutrient balance, reduces chlorophyll content, leaf area, and photosynthetic efficiency, and negatively impacts intracellular K^+ influx. Salinity stress also affects several cellular enzymes involved in nitrogen metabolism and synthesis of amino acids and indirectly induces the accumulation of ROS, which could damage the plant cells.

3.3.1. Salinity stress response and signaling

Salinity stress is perceived by cell surface receptors relaying the signals through secondary messengers like inositol phosphates and ROS, and the activation of proteins like calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) that

regulate the expression and function of numerous genes. Transcription factors play a pivotal role in imparting resilience towards salinity stress through modulation of expression of the salinity stress genes (Hasanuzzaman and Fujita, 2022).

3.3.2. Role of phyto-microbiome in salinity stress

Plant microbiome employs several strategies to survive salinity stress including production of osmolytes, synthesis of extracellular proteases, and activation of Na+/H+ antiporter. They induce the production of plant growth hormones auxins, cytokinins, and gibberellins. Under salinity stress, the hormone ABA production is stimulated which reduces salinity stress by promoting osmolyte build-up in root vacuoles and the uptake of Ca^{2+} and K^{+} (Chen et al., 2022). Cytokinins maintain plant totipotent cells in the shoot and root apical meristems. Under abiotic stress, ethylene is known to accumulate in plants. Ethylene is an essential hormone and signaling molecule which plays key role in growth, seed germination and ripening, root hair elongation, and leaf senescence. However, high ethylene concentration has a detrimental effect on plants. Plant growth promoting bacteria (PGPB) produce ACC deaminase which lowers ethylene by converting ethylene precursor to ammonia and ketobutyrate. In response to abiotic stress, microorganisms develop biofilms, which cover the roots and keep them from drying out. They also foster optimal microenvironments for interactions between plants and microbes (Hakim et al., 2021).

Highly soluble organic substances such as sugars, sugar alcohols, glucosyl glycerol, betaines, amino acids, and tetrahydropyrimidine are produced or accumulated by bacteria. These osmolytes help in maintaining the osmotic pressure of the cells under salinity. At the same time, plant cells also assimilate osmolytes such as disaccharides, oligosaccharides, sugar, alcohols, glycine, betaine, proline, and glutamate which in turn help in the survival of plant microbiome during salinity stress (Kumar et al., 2020).

Salinity stress impacts the uptake of Nitrogen (N), Phosphorus (P), Potassium (K), and water, leading to huge reduction in crop yields. PGPB improve nitrogen uptake and bioavailability of phosphorus by acidification and chelation. Similarly, the bioavailability of microelements such as Cu, Fe, Mn, Zn is also increased. Potassium-solubilizing bacteria such as *Burkholderia* convert potassium into a bioavailable form. Salinity reduces iron availability and exacerbates iron deficiency in plants. Iron is essential for the activity of several plant enzymes and for the synthesis of chlorophyll (Teo et al., 2022). Siderophore-producing PGPB contribute significantly to Fe accumulation in roots and to its transportation to leaves (Yasmin et al., 2020; Sultana et al., 2021). Endophytic *Streptomycetes* that produce siderophores have been shown to increase root and shoot biomass as a result of improved Fe supply. Siderophore-producing PGPB have been demonstrated to increase salt tolerance (Afzal et al., 2019; Saeed et al., 2021).

Trichoderma harzianum reduced salt stress in plants by upregulating monodehydroascorbate reductase generating ACC-deaminase, as supported by mutant experiments (Brotman et al., 2013). In salty soil, *Pseudomonas* sp. and *Acinetobacter* sp. were found to increase IAA and ACC-deaminase synthesis in barley and oats (Chang et al., 2014). *Streptomyces* sp. strain *PGPA39* was found to reduce salt stress and promote development in 'Micro-Tom' tomato plants (Palaniyandi et al., 2014). Tolerance in rice was increased against salt stress by inoculation of *Pseudomonas* sp. (Sen and Chandrasekhar, 2014) and against salt and high boron stress by inoculation of *Bacillus pumilus* (Khan et al., 2016).

3.4. Heavy metal stress in plants

Heavy metals pollutants such as Mercury (Hg), Arsenic (As), Cobalt (Co), Manganese (Mn), Iron (Fe), Cadmium (Cd), Nickel (Ni), Zinc (Zn), Copper (Cu), Chromium (Cr) and Lead (Pb) are released into the environment thorugh anthropogenic activities like growing industrialization, intensive agriculture, and urbanization (Ayangbenro and Babalola, 2017; Kurniawan et al., 2022). The uptake of an excessive amount of heavy metals by crop plants from the contaminated soil affects plant health due to toxicity and considerably reduces the yield.

Heavy metals impact the growth and physiological processes either directly by inhibiting cytoplasmic enzyme activity and inducing oxidative stress, or indirectly by altering the phyto-microbiome structure and functions (Dotaniya and Saha, 2016).

3.4.1. Heavy metal stress response and signaling

Plants have evolved various strategies to detect and respond to heavy metal stress in their environment through complex stress signaling processes that involve multiple pathways and mechanisms. The uptake and transportation of heavy metals in plants depend upon several transporters and proteins which help in their sequestration, intracellular or tissue compatrmentalization and detoxification. ATP-driven pumps HMA (Heavy Metal ATPases) are found on the plasma membrane and tonoplast (vacuolar membrane). They transport heavy metal ions such as such as Cu, Zn Cd and Pb across membranes and facilitate the sequestration of heavy metals into vacuoles or their extrusion from the cytoplasm. ZIP transporters (Zrt/Irt-like Protein) are involved in the uptake of essential metals such as Zn, Fe, Mn and Cu, but they can also transport toxic metals like Cd and Pb on exposure. ZIP transporters are located in the plasma membrane and are responsible for the uptake of these metals from the soil into the root cells. NRAMP transporters (Natural Resistance-Associated Macrophage Protein) are involved in the uptake and translocation of many divalent metal ions such as Fe, Mn, Zn and Cd. They are found in the plasma membrane and endomembranes of plant cells. NRAMP transporters have been shown to play a role in metal distribution within the plant and in metal detoxification processes. ABC transporters (ATP-Binding Cassette) constitute a large family of proteins involved in various cellular processes, including heavy metal transport. Some ABC transporters are known to transport heavy metals such as Fe, Cu and Zn. They are also present in the plasma membrane and other intracellular membranes.

It has been seen that initial abiotic stress signaling pathways are shared among the different types of abiotic stress. The heavy metal stress signaling involves production of Reactive Oxygen Species (ROS) such as superoxide radicals (O^{2-}) and hydrogen peroxide (H_2O_2), in response to heavy metal induced cellular damage in plants. They act as secondary messengers in the signaling pathways. Mitogen-Activated Protein Kinase (MAPK) signaling pathway is one of the major pathways activated by heavy metal stress. MAPKs modulate the expression of stress-responsive genes, including those involved in metal detoxification and ROS scavenging. Phytochelatins (PCs) are small peptides synthesized in response to heavy metal stress by the enzyme phytochelatin synthase (PCS), which conjugates glutathione molecules to form PC complexes. PCs play a crucial role in heavy metal detoxification by chelating heavy

metals and sequestering them into vacuoles, preventing their toxicity. Metallothioneins (MTs), low molecular weight, cysteine-rich proteins, are induced by heavy metal stress, which have a high affinity for heavy metals and can bind and sequester them, thereby reducing their toxicity. MTs are involved in metal homeostasis and play a protective role against heavy metal stress in plants. Heavy metal stress triggers changes in intracellular calcium (Ca^{2+}) concentrations, leading to calcium signaling. Calcium ions act as secondary messengers and regulate Calcium-dependent protein kinases (CDPKs) are activated by increased calcium levels. CDPKs modulate the expression of stress-responsive genes. Heavy metal stress can also activate the abscisic acid (ABA) signaling pathway in plants, which promotes the expression of stress-responsive genes, thereby improving plant tolerance to heavy metals. Several transcription factors specifically AP2/ERF, MYB, WRKY, and NAC families are involved in regulating the expression of stress-responsive genes under heavy metal stress and regulate the expression of heavy metal response genes (Tiwari and Lata, 2018; Keyster et al., 2020).

3.4.2. Role of phyto-microbiome in heavy metal stress

Phyto-microbiome plays a crucial role in heavy metal stress mitigation in plants. These microorganisms render the protection to plants from harmful effects of heavy metals through many ways, such as heavy metal sequestration or biosorption, nutrient mobilization and solubilization, heavy metal transformation and detoxification, induction of stress tolerance.

Microbes efficiently bind and sequester heavy metals, preventing their accumulation in plant tissues. They promote the immobilization and containment of heavy metals in the soil, reducing their bioavailability to plants. Microbial enzymes can mobilize and solubilize the essential nutrients in the soil, making them more accessible for plant uptake. They can convert insoluble compounds into soluble forms, increasing their bio- availability and reducing heavy metal toxicity. Microbes also transform and detoxify heavy metals through processes like reduction, oxidation, and methylation.

Plant-associated microorganisms induce systemic resistance and enhance the stress tolerance of plants. They can stimulate the production of plant growth-promoting hormones, antioxidants, and other protective compounds, which help plants to cope with heavy metal stress. It is important to note that the effectiveness of plant-associated microorganisms in heavy metal stress mitigation can vary depending on the specific microorganism, plant species, and environmental conditions. Syed et al. (2023) isolated strains of *Pseudomonas fluorescence* and *Trichoderma* spp. from heavy metal contaminated soil and improved the growth and yield of chickpea by lowering Cd uptake (; Oubohssaine et al., 2022; Syed et al., 2023) isolated rhizobacterial strains from heavy metal contaminated mining sites and studied their application on growth of *Sulla spinosissima* L. in a highly multi-polluted toxic soil. They observed that the strain LMR291 (*Pseudarthrobacter oxydans*), LMR340 (*Rhodococcus qingshengii*), LMR249 (*Pseudarthrobacter phenanthrenivorans*), and LMR283 (*Pseudomonas brassicacearum*) substantially improved all the growth parameters of *Sulla* plants, their photosynthetic pigments, and their antioxidative enzymatic activities (Oubohssaine et al., 2022).

4. Bioinoculants for alleviating abiotic stresses

Bioinoculants are formulations of microorganisms which can be inoculated in crop plants for facilitating growth and enhance production. These comprise of living or quiescent cells of specific microbial strains that benefit host plant by mechanisms such as facilitating nutrient acquisition, releasing plant growth hormones, and other biological activities like pest control. Additionally, bioinoculants may also be used to mitigate the harmful effects of abiotic stress (Benidire et al., 2020). Several theories have been proposed to elucidate the mechanisms of beneficial effects of bioinoculants including production of phytohormones, biofilm, EPS, and ACC deaminase (Tittabutr et al., 2013; Ansari et al., 2019; Goswami and Deka, 2020), production of antioxidants (Singh et al., 2019), cryoprotectants, heat shock proteins, solubilization of minerals such as phosphorus (P), potassium (K), and zinc (Zn), nitrogen (N) fixation, production of siderophores (Ferreira et al., 2019), antibiotics (Jin et al., 2021), hydrolytic enzymes such as proteases, cellulases, chitinases, and β-glucanases (Veliz et al., 2017), and volatile compounds (Harun-Or-Rashid and Chung, 2017). Some microbes also improve the induced systemic resistance (ISR) and systemic acquired resistance (SAR) and thereby help in alleviating multiple stresses in plants. Table 1 summarizes the effects of microbial inoculants on mitigation of abiotic stresses.

4.1. Bioinoculants for inducing salt stress tolerance in plants

In saline soils, plants experience two forms of stresses- nutrient stress and osmotic stress (Ashrafi et al., 2014). Salinity stress increases the production of ethylene hormone which is damaging and inhibits plant growth. Bioinoculants consist of microbes that produce ACC deaminase, which lowers ethylene concentration and maintain plant growth in saline conditions (Ansari et al., 2019). Numerous studies have shown that the ACC deaminase-producing microbes support plant growth in saline environments such as *Bacillus cereus* in *Vigna radiate* L. (mung bean) (Islam et al., 2016), *Bacillus pumilus* strain TUAT-1 in *Oryza sativa* L. (rice) (Win et al., 2022) and *Enterobacter* strain G in *Cajanus cajan* L. (Anand et al., 2021).

4.2. Bioinoculants for inducing drought stress tolerance in plants

Drought stress directly affects the water relations in plants and exters huge impacts on plant physiology. Bacterial IAA promotes the production of ACC deaminase, which dissociates one of the ethylene precursors and delays the onset of senescence in drought stressed plants (Uzma et al., 2022). Microbially produced ACC deaminase resists plant root drying by degrading ACC and reducing the level of ethylene in the plant cell (Ngumbi and Kloepper, 2016). Like auxin, cytokinin is another plant hormone that is important in preventing early leaf mortality during water shortages. Microbes also increase the synthesis of the endogenous stress hormone ABA, which is crucial for the plant's drought resistance. Higher levels of endogenous ABA increase root-water conductivity by upregulating the expression of

TABLE 1 Effects of various microbial inoculants in reducing abiotic stress and improving plant stress resistance.

Bioinoculants	Target plants	Effect on target plants	Effect on plant development	References
Salinity stress				
Microbacterium oleivorans, *Rhizobium massiliae*	*Capsicum annuum* L.	AA, ACC deaminase and siderophore production	Plant height, weight, and chlorophyll contents significantly enhanced	Hahm et al. (2017)
Bacillus pumilus	*Zea mays* L.	IAA, ACC deaminase activity, P-solubilization, EPS production and higher osmoprotectants and malondialdehyde production	Increased root and shoot dry weights	Mukhtar S. et al. (2020)
Azotobacter salinestris	*Sorghum bicolor* L.	increased ACC deaminase, salicylic acid, proline, and EPS production	Significant enhanced in growth parameters, chlorophyll, total carbohydrate, proline, and macro-elements content	Omer et al. (2016)
Bacillus pumilus	*Oryza sativa* L.	IAA, ACC deaminase, P-solubilization, proline aggregation, and EPS production	increased plant height, plant fresh, and dry weight, chlorophyll and carotenoids content	Ben Mahmoud et al. (2020)
Drought stress				
Trichoderma and *Pseudomonas*	*Oryza sativa* L.	the production of antioxidant enzymes such as peroxidase, glutathione peroxidase, ascorbate peroxidase, and glutathione	Promotes development of plants	Singh D. P. et al. (2020)
Bacillus amyloliquefaciens (MMR04)	*Pennisetum glaucum* L.	Reduced expressions of DREB-1E (drought–responsive) and ERF-1B (ethylene-responsive)	Promotes growth of the plants	Murali et al. (2021)
Temperature stress				
Pseudomonas vancouverensis and *Pseudomonas fredericksbergensis*	*Solanum lycopersicum* L.	Reduced ROS concentration, membrane damage and	Improved plant growth, and robustness in cold stress	Subramanian et al. (2015)
Bacillus amyloliquefaciens and *Brevibacillus laterosporus*	*Oryza sativa* L.	Increased proline, chlorophyll. Decreased leaf MDA content and electrolyte leakage	Increased overall plant growth in cold stress	Kakar et al. (2015)
Lysinibacillus fusiformis YJ4 and *Lysinibacillus sphaericus YJ5*	*Zea mays* L.	Raising the amount of total phenolic content, osmolytes, antioxidant enzyme, and phytohormones	Enhanced growth of plants	Jha and Mohamed (2022)
Heavy metals stress				
Enterobacter sp.	*Pisum sativum* L.	IAA, siderophore production	Increased growth parameters, xanthophyll, carotenoid, and chlorophyll content	Naveed et al. (2020)
Klebsiella sp	*Zea mays* L.	IAA, EPS, catalase, phosphate solubilization	Increased shoot and root growth	Ahmad I. et al. (2015)

aquaporins (Goswami and Deka, 2020). Microbial inoculation also increases the synthesis of antioxidant enzymes in the plant, which helps the plant to enhance drought tolerance by decreasing ROS and increasing the production of antioxidant enzymes. Batool et al. (2020) showed that inoculating potatoes with *Bacillus subtilis* HAS31 reduced ROS production and mono-dehydroascorbate (MDA) production while increasing catalase, peroxidase, superoxide dismutase, and total soluble sugar in drought-stressed environments.

4.3. Bioinoculants for inducing temperature stress tolerance in plants

Heat stress has an impact on plants at several growth stages, including seed germination and reproduction, on a physical, physiological, and biochemical level. During high-temperature stress, seed germination rate and stand establishment are reduced due to the disturbed activity of enzymes involved in the breakdown of starch and

synthesis of ABA & GA (Begcy et al., 2018). High temperatures have a significant impact on the photosynthetic process because they cause thylakoid disorganization, grana swelling and loss, a decrease in the activity of the electron acceptor and donor sites of photo-system (PS) II, and a decrease in the activity of enzymes such as RuBisCO (Hassan et al., 2020). Several microbes that are utilized as bioinoculants can withstand extremely high and extremely low temperatures. The temperature stress tolerance mechanisms include synthesis of heat and cold shock proteins, biofilm formation, and production of osmo-protective chemicals (Bruno et al., 2020). Inoculating *Solanum lycopersicum* L. (tomato) under chilling stress with *Trichoderma harzianum* was shown to increase photosynthesis and growth rate by lowering lipid peroxidation, electrolyte leakage, reducing ROS concentration, increasing leaf water and proline concentration (Ghorbanpour et al., 2018).

4.4. Bioinoculants for inducing heavy metal toxicity tolerance in plants

Heavy metals are absorbed by plants from contaminated soil through roots and are translocated to aerial parts through xylem, where they are bioaccumulated and impose considerable toxicity. Bioinoculants alleviate heavy metal toxicity by producing microbial siderophores for metal chelation, and phytohormones that boost the antioxidative enzymes in plants (Nazli et al., 2020). PGPB that are heavy metal tolerant (HMT) not only lessen the harmful effects of heavy metals but also encourage plant development in such conditions. Inoculation with the HMT-PGPB consortium increased the growth of *Sorghum bicolor* L. plants while also lowering the bioavailability of heavy metals Cu, Cd, Pb and Zn (El-Meihy et al., 2019). The inoculation of *Mucor* sp., *Klebsiella pneumoniae*, *Bacillus pumilus*, *Klebsiella* sp., and *Enterobacter* sp., also considerably reduced heavy metal contamination and improved plant growth (Ma et al., 2015; Karthik et al., 2016; Pramanik et al., 2017; Zahoor et al., 2017; Mitra et al., 2018).

5. Multi-omics approaches to mitigate abiotic stress in plants

Omics refers to the modern day technologies that give a deep insight into the metabolism, genomics and transcriptomics processes occurring in plants and hence the multi-omics approaches are advantageous in plant improvement against abiotic stresses. The understanding and sequencing of the whole plant genome in *Arabidopsis thaliana* L. have proved the potential benefits of omics tools. Various other plants such as rice, maize and soyabean possess a complicated genome, which have been fully sequenced by the use of omics technology (Ali A. et al., 2022). Numerous studies suggest that under abiotic stress not all genes are turned on or off at the same time, due to which the plant metabolism becomes complicated to understand and hence the phenotype cannot be determined by the genotype (Jha et al., 2019; Singh N. et al., 2020). Therefore, the amalgamation of proteomics, genomics transcriptomics, metabolomics, epigenomics, ionomics, interact omics, phenomics could help in identification of the candidate genes and improve the productivity of various crops under abiotic stress (Kumar et al., 2022).

The plant-microbe interactions are better understood due to the recent development in the various omics tools and sequencing technologies involving the regulation of gene expression and biodiversity (Sandrini et al., 2022). The characterization of beneficial microbes associated with plants and their functions along with the knowledge of rhizospheric science are possible due to the microbiome based multi-omics studies (White et al., 2017). The integrated omics approaches, computational and synthetic biology along with latest advances in high throughput culturing are providing significant knowledge about the structure and function of diverse natural microbiomes and providing avenues for artificially engineering the microbial communities and hence improving the crop growth, protection against pathogens and several abiotic stresses (Trivedi et al., 2021). Plants' regulatory networks function to induce protective genes while inhibiting the negative regulators activated by abiotic stress factors. These regulations are responsible for the restoration of cellular homeostasis during the stress phase of plant cells. Multi-omics approaches help to integrate cellular processes at different levels based on systems biology knowledge (Katam et al., 2022).

5.1. Transcriptomics in abiotic stress

Transcriptome studies are a novel approach to understand the response of plants to abiotic stresses. Next-generation sequencing (NGS) and parallel RNA sequencing (RNA-Seq) are the two most promising techniques that open a new dimension of biological research to identify the networks among genes that actually respond to stress (Janiak et al., 2016). The advent of high-throughput omics techniques and rapid advancement in post-genomic epoch, specifically the next-generation sequencing (NGS), molecular characterization and modeling have proved beneficial in improving the efficiency and resilience of crop plants under abiotic stress (Pandey et al., 2021). The benefits of plant associated microbes and their communities are well understood by the use of NGS on DNA extracted from soil and rhizosphere and hence lead to better knowledge of their diversity, structure, abundance and important microbes (Alawiye and Babalola, 2019). Also the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) is an important technology and serves to knockout non-transgenic plant and microbe mutants, characterize symbiosis-related protein, plant traits that sustain beneficial microbiome, various genetic factors and identification of candidate genes responsible stress tolerance and further assigning them specific functions (Levy et al., 2018; Khatabi et al., 2019). The overexpression of transcription factors increases the expression of genes that encodes enzymes and chaperones associated with endoplasmic reticulum stress response, resulting in an increase in rate of photosynthesis and tolerance to drought-like abiotic stress (Wang et al., 2018).

Expressed sequence tags (ESTs), microarray, Affymetrix GeneChip technology, and serial analysis of gene expression (SAGE) have been used to elucidate the function of various genes associated with abiotic stress (Varshney et al., 2009; Deokar et al., 2011; Le et al., 2012). Chickpea genotype microarray study shows 210 differentially expressed genes (DEGs) and numerous differentially expressed unigenes under drought stress (Wang et al., 2012). Global transcriptome profiling of the root tissues of drought-stressed lentils, chickpeas, and ground nuts identified differentially expressed genes (DEGs) involved in different energy metabolism pathways like TCA

cycle, glycolysis cycles mediated by transcription factors like WRKY, zinc finger family protein, bHLH, NAC, AP2/ERF and MYB protein domain family (Brasileiro et al., 2015; Singh et al., 2017). Genomics studies showed that ZmWRKY40 and ZmNF-YB2 genes encode a transcription factor that helps in resistance to drought in maize plants (Nelson et al., 2007; Zhang et al., 2008; Gangola and Ramadoss, 2020). In *Arabidospsis* bZIP was identified and it was associated with drought, salt, and cold tolerance by increasing oxidative enzyme level (Zong et al., 2018).

5.2. Meta-transcriptomics and metaproteomics

Meta-transcriptomics helps in the assessment of expressed genes (Nilsson et al., 2019). Studies related to ecology of microbial communities were possible by the sequencing of transcripts (RNA-seq) (Marcelino et al., 2019). However, interpretation of RNA-seq results is a tedious process but the advancement of databases and the increasing availability of annotated transcriptomes in curated databases as well as development of a robust *de novo* RNA-seq assembler can help in making the explanation of the result easier (Kuske et al., 2015). Metaproteomics provides functional data and suggests about the complex matrix such as specific soil sample. It emphasizes the study of proteins present in a biomass (Sandrini et al., 2022). It is an important phenomenon that helps in recognition of metabolic pathways, characterization of biological processes, plant-microbe interactions, their structure, function, significance, dynamics, and r egulation of symbiosis and molecular basis of cell communication (Khatabi et al., 2019).

5.3. MiRNA-omics in abiotic stress

Plant produces miRNAs (micro-RNAs) that are post-transcriptional gene-expression regulators, which help them to survive under stress conditions (Zhang and Wang, 2015). By using computational tools like screening of small RNAs library, different drought stress-responsive miRNAs were identified from *Arabidopsis*, rice, and sugarcane (Liu et al., 2008; Zhou et al., 2010; Gentile et al., 2015). Fourteen different stress-inducible miRNAs were identified from *Arabidopsis* and among them, miR168, miR171, and miR396 responded to all of the different types of stresses (Liu et al., 2008). In rice 18 cold-responsive miRNAs were identified by (Lv et al., 2010) most of which were downregulated by the cold stress (4°C) and it was supposed that miRNAs were ubiquitous regulators in rice. While on the other hand, due to cold stress sharp increase in the expression of *miR812q* in rice plants was observed in starting of the reproductive phase (Jeong et al., 2011). High-throughput sequencing revealed that 31 cold stress-induced genes were upregulated and 43 miRNAs were downregulated in tea (*Camellia sinensis* L.) (Zhang et al., 2014). In *Arabidopsis* roots enhanced expression of a few miRNAs like miR156g, miR157d, miR158a, miR159a, miR172a,b, miR391, and miR775 were observed, under low oxygen stress situations (Moldovan et al., 2010). In wheat *Tae*-miR6000, miR156, miR159, miR164, miR167a, miR171 and miR395 were identified as UV-B responsive microRNAs (Wang et al., 2013).

The functions of miRNAs in response to abiotic stresses as well as plants' development are determined by using artificial miRNAs (amiRNAs), which could be useful to design the strategies for silencing endogenous genes and inhibit the expression of target genes (Zhang F. et al., 2022). Technologies such as Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and microarrays suggested that abiotic stress conditions induce miRNA expression profiles and are diverse among plant species (Begum, 2022).

5.4. Metabolomics in abiotic stress

Metabolomics technology provides a chemical profile of thousands of compounds and involves the use of high-pressure liquid chromatography along with high resolution mass spectrometry (LC–MS), gas chromatography and mass spectroscopy (GC–MS) and nuclear magnetic resonance (NMR) spectroscopy for characterization of stress induced metabolites (Crandall et al., 2020). The suitability of the selection of the technique depends on the speed, sensitivity, and accuracy of the method used (Ghatak et al., 2018). Due to stress conditions plants can adapt to the stress or tolerate the stress. Mostly metabolomics studies focused on the comparative analysis of stress-susceptible and stress-tolerant responses of plants. Along with amino acids other metabolites like sugar, phenolic compounds, and organic acid also play an important role in plant abiotic stress (Dawid and Hille, 2018; Ghatak et al., 2018). The metabolomics and other -omics technologies, allowed a detailed and in-depth analysis of plant stress as the result of the alteration of metabolites and gene expressions (Anzano et al., 2022). It was observed that under stress conditions plants activate high proline production but under the stress recovery phase, it undergoes proline catabolism (Krasensky and Jonak, 2012). Metabolomics was used to study phytohormone response against salt stress as a targeted approach in roots and shoots in *Arabidopsis* seedlings (Šimura et al., 2018) and non-targeted approach in maize (Richter et al., 2015). Metabolomics revealed the contribution of overexpression of GmGSTU4, responsible for production of glutathione transferases (GSTs) and increase the glutathione biosynthesis under salt stress in transgenic tobacco (Kissoudis et al., 2015). In mycorrhizal roots the variations in the metabolomics profile have been observed leading to the identification of potential primed compounds that are involved in improved stress tolerance in mycorrhizal plants (Rivero et al., 2018; Bernardo et al., 2019). Metabolomics play an important role in studying the exudates released by the roots in the rhizosphere which serve as a significant feed source for the microbes that are associated with the roots as compared to the surrounding soil that is poor in nutrients (Escudero-Martinez and Bulgarelli, 2019). A major issue in metabolomics is that a huge variety of potential metabolites are present in any given sample and due to the limited extent of public metabolite reference databases assigning a measured metabolite to a specific organism or condition, it becomes difficult to correlate the metabolite production to a particular stress (Khatabi et al., 2019).

5.5. Genomics in abiotic stress

Abiotic stress studies on plants include many cellular processes like sensing, signaling, transcription, transcript processing, translation,

and post-translational protein modifications. These studies ultimately boost crop productivity and agricultural sustainability through genetic, chemical, and microbial approaches (Zhang H. et al., 2022). The effect of different abiotic stress in plants was monitored quantitatively by using imaging technology along with the support of information technology. Various phenotypic expressions of plants are useful quantitative phenotypic tools, as genotypic changes lead to the expression of phenotypes. By using a combination of genomics and data science, we can analyze the plant stress responses under different combinations of environmental stress (Zandalinas and Mittler, 2022). In this method target sequences can be designed and introduced into the most appropriate vectors. DNA, RNA, or RNPs like genetic cargo is selected for further delivery by (i) modifying the targeted sequence, (ii) regenerating the edited calli, and (iii) producing the edited plants (Farooqi et al., 2022).

5.6. Metagenomics

Metagenomics identifies the genomic diversity and functions of microbial genes (Solden et al., 2016) and suggests about the relative abundance and taxonomic composition (Singer et al., 2016). The plant-microbe complexity can be studied through next-generation DNA sequencing methods like 454 pyrosequencing and second and third generation sequencing platforms such as PacBio RSII Sequel, GridION, Illumina MiSeq, NovaSeq, GeneStudio, Oxford Nanopore MinION, PrometION, Ion Torrent PGM (Nilsson et al., 2019). Two key methods such as shortgun metagenomics and metabarcoding are employed to identify the microbial communities and compare them on the basis of composition, richness, evenness and assembly (Sharma et al., 2020).

5.7. Phenomics-manipulating plant root-associated microbiomes

The plant root-associated microbiome influences numerous plant traits, primary and secondary metabolites that act as growth substrate for few microbes and have antagonistic effect on the others, they act as signals that regulate the plant microbe interactions. Although few rhizospheric microbial species act as symbionts or growth promoting rhizobacteria are beneficial for the plants and helps in enhancing the plant pathogen defense and nutrition, few microbes may be parasitic and commensal (Lareen et al., 2016; Pascale et al., 2020; Chen et al., 2021). Therefore the study and classification of the complex interaction of plant microbiome and soil rhizosphere is important for developing novel approaches towards crop resilence against pathogens and environmental stresses (Zenda et al., 2021).

6. Dynamic role of fauna in plant microbiome functions

Some of the useful fungi serve as plant parasitic nematode hunters such as *A. avenae* is effective against *Ditylenchus* (plant-parasitic nematode) propagation and is an active bio-controlling agent in many parasitic nematodes and pathogenic fungi (Haraguchi and Yoshiga, 2020). Members of Protista are abundantly present in the plant rhizosphere and regulates various mechanism such as nutrient recycling, and interactions in the food web, promotes productivity (Hünninghaus et al., 2017), and lowers the total bacterial biomass (Krome et al., 2010). It also suppresses plant pathogens and boosts immunity of plants against various pathogens. They represent diverse modes of nutrition, wide range of prey interactions, and chemical communication (Mahmud et al., 2021; Solanki et al., 2022). Due to their numerous interactions, the protists cause significant changes in the structure and function of the microbiome. Also regulates auxin and cytokinin levels and hence possesses a significant effect on plant microbiome linked to several hormonal fluctuations (Krome et al., 2010). Earthworms also possess a strong impact on the soil microbiome. It is present in the soil rhizosphere and significantly adds to about 80% of the biomass in the soil macrofauna (Yasmin and D'Souza, 2010). Depending on the earthworm type and the micro-habitat, its impact can be positive, negative, or neutral on the diversity and enrichment of the microbial population (Afridi et al., 2022b). The activities of microorganisms are activated due to the release of acutaneous mucus (glycoprotein) by the earthworm that enhances the interactions. Due to the significant interaction between the microorganisms and the earthworm, there is improved microbial activity in the soil, increased availability of nutrients and increased carbon turnover (Bedano et al., 2019).

7. Plant microbiome engineering to combat abiotic stress

Plant microbiome engineering (PME) is an important approach to promoting plant health, growth, and productivity under adverse environmental conditions (Afridi et al., 2022a). According to Parnell et al. (2016) soil microbiome is the next green revolution as it is serving as a promising tool that will meet the future global food demands. PME has been used to improve nitrogen use efficiency (NUE) in plants (Lau et al., 2022). Also, it is a beneficial technology in plants as it regulates the mechanism of hormones and specific antagonistic metabolite (rhizobitoxine) production that provides resistance against several pathogens, suppresses soil-borne diseases, and regulates nutrient availability in the rhizosphere (Rodríguez et al., 2020; Figure 2). Various approaches used for engineering the phyto-microbiome for developing abiotic stress tolerance in crop plants are summarized in (Table 2). The steps involved in engineering the plant microbiome include selection and engineering of the host-mediated multi-generation microbiome, inoculation of microbial communities as bioinoculants in the rhizosphere, soil, seedling/seeds, mixed strain inoculation, tissue atomization, and direct injection in the plant tissues.

7.1. Selection and engineering of the host-mediated multi-generation microbiome

The selection and engineering of the microbiome are important as it selects the microbial communities through the host and influences and modifies the traits of the host plant which further influences the microbiome (Mueller and Sachs, 2015). Hence there is a synergistic relationship between host-mediated microbial communities and contributes significantly to agricultural yield,

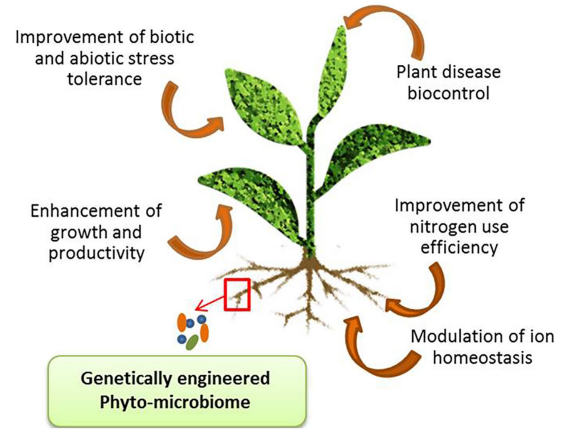

FIGURE 2
Major applications of plant microbiome engineering (PME).

biodiversity, and food security (Kaul et al., 2021). The "artificial selection of the ecosystem" was done to screen the plant biomass of *Arabidopsis thaliana* L.with the lowest (low selection lines) and highest (high selection lines) plant biomass which was earlier improved by the microbial community and their interactions with the plants for over 16 generations (Swenson et al., 2000). Different studies in *Arabidopsis thaliana* L. and *Brassica rapa* L. (Panke-Buisse et al., 2015) suggest a positive correlation between plant biomass and increase activity of microbial extracellular enzymes leading to soil nitrogen mineralization and suggesting the dynamic role of microbiomes to deal with numerous environmental and agronomic issues (Orozco-Mosqueda et al., 2018).

7.2. Inoculation of microbial communities as bioinoculants in the rhizosphere, soil, seedlings/seeds

Using microbial communities as bioinoculants finds wide applications such as plant growth, enhanced nutrient mobilization, stress resilience (Al Kahtani et al., 2020; Alok et al., 2020). The inoculation of different external strains from the rhizospheric soil can alter the structure of the microbiome. Different studies suggest their significant role. For example, the healthy oilseed crop was grown by using the bioinoculants on oil palm seedlings (*Elaeis guineensis* Jacq.), it modified the enzymatic and dynamic potential of rhizospheric microbes (Veeramachaneni and Ramachandrudu, 2020). Inoculation of *Agrobacterium* sp. *10C2* in *Phaseolus vulgaris* enhanced plant biomass and nodule formation by increasing antioxidant level, flavonoids, polyphenols, and phosphorus content in the beans, and also promoted colonization of beneficial rhizobacteria *Brevibacterium*, *Paenibacillus koreensis*, *Bacillus pumilus* and *Actinomyces* (Chihaoui et al., 2015). A group of biocompatible microbial communities enhanced the growth of maize seedlings under a greenhouse with low-phosphorus soil. They are the group of engineered bacteria that were studied for biofilm formation, phosphate solubilization, and root colonization (Magallon-Servin et al., 2020). In orchids, the inoculation of *Klebsiella oxytoca* and *Pseudomonas fluorescens* into *Dendrobium nobile* Lindl. promoted the vigor, growth, germination, and adaptability (Pavlova et al., 2017). Growth of tomato seedlings (*Lycopersicon esculentum* L. cv. Saladette) was significantly enhanced with the co-inoculation of two endophytic strains *Pseudomonas stutzeri E25* and *Stenotrophomonas maltophilia CR71* in the rhizosphere as compared to single inoculation (Rojas-Solís et al., 2018).

7.3. Mixed strain inoculation and tissue atomization

The significant effect of the microbiome depends upon its interaction, multiple mechanisms, and functions carried out by the microbial community. Mixed strain inoculation proved beneficial compared to single or no inoculation in Populus plants where additive incorporation of strains of *Pseudomonas* and *Burkholderia* isolated from *Populus deltoids* L. significantly improved the plant's photosynthetic capacity and root biomass (Timm et al., 2016). Also, the response was analyzed by transcriptomics, and specific genes for *Pseudomonas* and *Burkholderia* were turned on through inoculation of each strain and mixture including genes that encode for stress (temperature & salinity) and regulate plant hormone (ethylene). The mixed inoculation was studied in various other genes involved in the synthesis of lipids, sulfate, and thiamine and also the comparison of mixed and single inoculation was studied on the metabolic profiling of the leaf (Timm et al., 2016). The tissue atomization technique proved successful in improving plant development through the bioengineering of plant microbiomes without any genetic manipulation. This technique was exploited by using an endophytic bacterium *Paraburkholderia phytofirmans PsJN* in the flowers of dicot and monocot plants and significant improvements were observed in the seed microbiome by vertical inheritance as well as growth parameters (Mitter et al., 2017).

7.4. Direct injection in the plant tissues

This technique helped in incorporating the antimicrobial properties in the plants susceptible to attack by pathogens. For example, Manuka (*Leptospermum scoparium* L.), a medicinal plant produces anti-microbial oil that is having a potential effect against pathogenic bacteria (*Pseudomonas syringae*; Orozco-Mosqueda et al., 2018). Bacteria were able to colonize and survive in the plant through direct inoculation of biocontrol agent and PGPB (*Arthrobacter agilis UMCV2*) which is given a direct injection in the stem of *Medicago trancatula* L. plant (Avilés-García et al., 2016). More efficient colonization will occur depending on the bioavailability of nutrients (Avilés-García et al., 2016). Certain plants for example *Zea mays* L., and teocinte have shown direct injection techniques using bacterial endophytes (Johnston-Monje and Raizada, 2011).

8. Conclusion

Microbial interactions with plants have multifaceted functions; on one hand, microbes help plants to maintain their growth and

TABLE 2 Phyto-microbiome engineering approaches for abiotic stress tolerance in crop plants.

Plant growth promoting microbes	Host plant	Microbiome engineering approaches	References
Brucella sp. PS4	*Gossypium hirsutum* L.	Promotes pesticide degradation	Ahmad et al. (2022)
B. subtilis PM32	*Solanum tuberosum* L.	Biocontrol of fungal diseases	Mehmood et al. (2021)
G. intraradices	*Cucumis sativus* L.	Improves biomass, regulates salinity stress, enhances the production of antioxidant enzymes	Hashem et al. (2018)
B. firmus SW5	*Glycine max* L.	Regulates production of antioxidant enzymes, salinity tolerance	El-Esawi et al. (2018)
B. subtilis GB03	*Arabidopsis thaliana* L.	Monitors the import of sodium ions in root	Wang et al. (2016)
Beauveria bassiana and *Metarhizium brunneum* BIPESCO5	*Capsicum annuum* L.	Inhibit pathogenic *Fusarium* sp.	Jaber and Alananbeh (2018)
A. pullulans 490	*Solanum lycopersicum* L.	Biocontrol activity and helps in the production of biosurfactants	Köhl et al. (2020)
B. pumilus JPVS11	*Oryza sativa* L.	Regulates salt tolerance	Kumar and Sharma (2020)
Bacterial endophytes (*Bacillus* and *Brevibacillus*)	*Zea mays* L.	Enhances plant growth and development	Al Kahtani et al. (2020)
Glomus mosseae	*Triticum aestivum* L.	Controls osmotic potential and drought stress, regulates production of antioxidant enzymes	Rani et al. (2018)
B. xiamenensis	*Saccharum officinarum* L.	Improves the phytoremediation capacity	Zainab et al. (2021)
P. geniculata, *B. subtilis*, *B. siamensis*, *B. gelatini*, *B. ubonensis*, *B. territorii*	*Piper nigrum* L.	Antagonistic to soil-borne *Fusarium solani*	Lau et al. (2020)
R. irregularis	*Triticum aestivum* L.	Regulates heat stress, allocation of nutrient and nutrient composition in roots	Cabral et al. (2016)
T. harzianum Thar DOB-31	*Curcuma longa* L	Regulates production of Indole-3-acetic acid (IAA) and hydrogen cyanide	Vinayarani and Prakash (2018)
Halomonas sp.	*Avicennia marina* L.	Regulates heavy metal stress	Mukherjee et al. (2019)
Endophytic diazotrophic bacteria	*Sarracenia species* L.	Helps in nitrogen fixation	Sexton et al. (2019)
B. safensis SCAL1	*Solanum lycopersicum* L.	Regulates heat stress	Mukhtar et al. (2022)

development by fixing, mobilizing, and producing nutrients, hormones, and organic phyto-stimulant compounds, while on the other hand, they induce local or systemic stress alleviation response mechanisms in plants to survive under abiotic stress conditions. Phyto-microbiome essentially helps crop plants in their adaptation and survival on exposure to abiotic stress via induced systemic tolerance. It plays a key role in determining the varying levels of phytohormones, defense-related proteins, enzymes, antioxidants, and secondary metabolites, which mediate the stress-signaling processes. Growth-promoting microbes use various mechanisms to enhance plant growth under stress conditions, which include the production of plant growth regulators, iron and zinc sequestration, phosphorus and potassium solubilization, siderophore production, atmospheric nitrogen fixation, secondary metabolite production, as well as facilitation of uptake of other essential macro- and micronutrients from the soil. Microbial communities exhibit excellent resilience towards environmental challenges. The microbiome also displays functional redundancy by which, in the wake of environmental stresses, one microbial taxon can be replaced by another that can survive the stress. In addition to enhancing the microbial community structure, the introduction of advantageous stress-tolerant microorganisms can help improve plant and soil health when exposed to abiotic stress.

Recent agricultural practices have provided evidence that microbial bio-inoculants such as PGPRs not only aid in reducing environmental stresses but also increase the production of a variety of crop plants including rice, maize, barley, and soybean. The bio-inoculants not only enhance crop yield by bolstering the plant's defense mechanism and protecting it from abiotic stress such as drought, and salinity, but they also improve soil health. Recently, the use of a consortium of microorganisms in crop production has been promoted because a single bioinoculant might not be sufficient to protect plants from various stresses. The development of a novel and effective bio-inoculant formulation must be centered on the selection of effective native strains for improved outcomes and the bioinoculants must be tested at multiple sites prior to commercialization to avoid failure at field level.

Multi-omics tools and technologies have revolutionized crop improvement research for the development of abiotic stress-tolerant varieties. The potential of multi-omics approaches can be utilized to decipher the stress tolerance mechanisms governed by phyto-microbiome. Metagenomics and meta-transcriptomics offer huge potential to identify the complex microbial networks implicated in stress signaling and tolerance development. Genomics technologies enable high-throughput screening of beneficial microbes, leveraging gene modification and genetic engineering approaches for introducing

abiotic stress resistance in plants. Plant microbiome engineering could be immensely beneficial in the development of strategies to improve plant health, enhance crop productivity, improve resistance against abiotic and biotic stresses, and achieve sustainable agriculture in an eco-friendly manner.

Author contributions

AbS did the conception, design, editing, finalization, and submission of manuscript. AnS, SM, SC, PG, and AbS did literature survey, analysis and preparation of draft of the manuscript. AnS, SM, SC, and PG did revision and redrafting. All authors contributed to the article and approved the submitted version.

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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Genome analysis of a halophilic *Virgibacillus halodenitrificans* ASH15 revealed salt adaptation, plant growth promotion, and isoprenoid biosynthetic machinery

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Globally, due to widespread dispersion, intraspecific diversity, and crucial ecological components of halophilic ecosystems, *halophilic* bacteria is considered one of the key models for ecological, adaptative, and biotechnological applications research in saline environments. With this aim, the present study was to enlighten the plant growth-promoting features and investigate the systematic genome of a halophilic bacteria, *Virgibacillus halodenitrificans* ASH15, through single-molecule real-time (SMRT) sequencing technology. Results showed that strain ASH15 could survive in high salinity up to 25% (w/v) NaCl concentration and express plant growth-promoting traits such as nitrogen fixation, plant growth hormones, and hydrolytic enzymes, which sustain salt stress. The results of pot experiment revealed that strain ASH15 significantly enhanced sugarcane plant growth (root shoot length and weight) under salt stress conditions. Moreover, the sequencing analysis of the strain ASH15 genome exhibited that this strain contained a circular chromosome of 3,832,903 bp with an average G+C content of 37.54%: 3721 predicted protein-coding sequences (CDSs), 24 rRNA genes, and 62 tRNA genes. Genome analysis revealed that the genes related to the synthesis and transport of compatible solutes (glycine, betaine, ectoine, hydroxyectoine, and glutamate) confirm salt stress as well as heavy metal resistance. Furthermore, functional annotation showed that the strain ASH15 encodes genes for root colonization, biofilm formation, phytohormone IAA production, nitrogen fixation, phosphate metabolism, and siderophore production, which are beneficial for plant growth promotion. Strain ASH15 also has a gene resistance to antibiotics and pathogens. In addition, analysis also revealed that the genome strain ASH15 has insertion sequences and CRISPRs, which suggest its ability to acquire new genes through horizontal gene transfer and acquire immunity to the attack of viruses. This work provides knowledge of the mechanism through which *V. halodenitrificans* ASH15 tolerates salt stress. Deep genome analysis, identified MVA pathway involved in biosynthesis of isoprenoids, more precisely "Squalene." Squalene has various applications, such as an

antioxidant, anti-cancer agent, anti-aging agent, hemopreventive agent, anti-bacterial agent, adjuvant for vaccines and drug carriers, and detoxifier. Our findings indicated that strain ASH15 has enormous potential in industries such as in agriculture, pharmaceuticals, cosmetics, and food.

KEYWORDS

***Virgibacillus halodenitrificans*, whole genome, salt-tolerant, plant growth promoting traits, CRISPRs, isoprenoids, squalene**

Introduction

With the ever-changing climate and global warming, rising sea level are a growing concern which increase soil salinity across the coastal areas, thereby globally imposing a detrimental effect on soil quality, decreasing land areas, and reducing agricultural crop production, resulting in an instable national economy (Corwin, 2021; Godde et al., 2021; Ullah et al., 2021). Salinity stress causes changes in various physiological and metabolic processes of the plant, ultimately inhibiting the quality and productivity of agriculturally important crops (Sharma et al., 2021). In the current scenario, with limited cultivated land resources, growing crops in saline soil may be a feasible opportunity (Xiaoqin et al., 2021). In light of the rising global need for food and agricultural production, scientists are looking for new, more eco-friendly, greener, and more sustainable alternatives pesticides and chemical fertilizers (Ullah et al., 2021). In this framework, the unusual halophilic bacteria thriving in saline environments have been a subject of study for the last few years due to their interesting physiological and metabolic adaptation properties to their extreme environmental conditions (Dutta and Bandopadhyay, 2022). These incredibly resilient microorganisms can thrive at 0% to saturation salt concentrations. The basic mechanisms of these extremophiles microbes for halo-adaptation and surviving in saline habitats are based on two strategies to regulate intracellular osmolarity and evade water loss (Zhou et al., 2017). The first is the "salt-in" approach, where KCl inorganic salt is stored to provide the cellular osmotic pressure and balance the external osmotic pressure. The other is known as the "salt-out" (compatible solutes) strategy, in which low-molecular-weight, highly soluble organic molecules are cumulated to ensure cellular osmotic balance without hindering crucial cellular processes even when they are occurring at high levels (Sharma et al., 2016). Extremely halophilic and anaerobic moderately halophilic bacteria adopted the "salt-in" strategy, while the majority of bacteria used the "salt-out" (compatible solutes) approach (Zhou et al., 2017). There are numerous reports of new halophilic bacteria and archaea species available from diverse hypersaline locations in various nations, primarily from USA, Australia, Korea, China, India, Thailand, Taiwan, Russia, France, Austria, Spain, Japan, Egypt, Iran, Indonesia, Mexico, the Philippines, Poland, and Romania (Naghoni et al., 2017; Corral et al., 2019; Reang et al., 2022).

The exploitation of new halophilic microorganisms is always of special importance and interest in the current era for the evaluation and development of new biomolecules with potential applications in agriculture and several industries. Halophilic microorganisms help in the improvement of soil structure, plant salt tolerance, and growth through various mechanisms such as phytohormones production (IAA and ABA), solubilizing the essential nutrients (P, K, and Zn, etc.), regulating the ethylene level, and inducing systemic resistance (ISR) against the harmful plant pathogens through the production of secondary metabolite/antimicrobial peptides (Arora et al., 2020; Masmoudi et al., 2023). Additionally, halophilic microorganisms mitigate the salt stress in plants by maintaining high K^+/Na^+ and Ca^{2+} and scavenging ROS by regulating the expression of antioxidant enzymes and stress-responsive genes (Sharma et al., 2021). In addition, low nutritional requirements, genetic pieces of machinery, and great metabolic versatility for adaptation to harsh environmental environments make halophilic microorganisms a promising candidate and a hope for new sources of enzymes, drug discovery, and other biological materials with applications in various human welfare fields (Vaidya et al., 2018). Several extremely halophilic and halotolerant bacteria, such as *Bacillus*, *Haloferax, Micrococcus, Salinibacter Halobacterium, Halobacillus, Virgibacillus,* and *Haloarcula,* were reported from various saline environments (Gupta et al., 2015; Satari et al., 2021). Also, the plant growth-promoting effectiveness of so many halophilic bacteria was investigated in seed germination and growth promotion of several agriculturally important crops such as rice (Abbas et al., 2019; Suriani et al., 2020), tomato, cotton, maize (Anbumalar and Ashokumar, 2016), and sugarcane (Sharma et al., 2021).

Among the various bacterial genera inhabiting various extreme environments, the morphologically, biochemically, and genetically diverse genus *Virgibacillus* is widely recognized as an important model group for agriculture and industrial applications (Sánchez-Porro et al., 2014; Fayez et al., 2022). A wide range of different species of *Virgibacillus* have been reported globally from various saline environments, such as seawater, marine sediments, lakes, soil, and fermented seafoods (Montriwong et al., 2012; Amziane et al., 2013; Xu et al., 2018; Mechri et al., 2019; Bhatt and Singh, 2022). To date, 39 validated and 381 non-validated species in the *Virgibacillus* genus have been published in the NCBI database (Chen et al., 2018), whereas six complete genomic sequences of *Virgibacillus* species are available in the NCBI database (2017) (Chen et al., 2018). *Virgibacillus halodenitrificans* is one of such bacteria whose potential is less explored (Lee et al., 2012; Kumaunang et al., 2019; Fayez et al., 2022). In 1989, the first report of a halophilic denitrifier, *Bacillus halodenitrificans,* from a solar saltern was reported by Denariaz et al. (1989). The isolates grew and survived well in various NaCl (0.35 to 4.25 M NaCl) supplement mediums, with optimum growth was considered between 0.5 and 1.35 M (3 to 8%)

NaCl. Later on, in 2004, *Bacillus halodenitrificans* was transferred into the genus *Virgibacillus* as *Virgibacillus halodenitrificans* (Yoon et al., 2004). Although numerous halophiles have been thoroughly defined to date, *V. halodenitrificans* is still one of the least explored organisms in terms of the number of published research studies, strain characterizations, and whole genome sequence analysis (Lee et al., 2012). Thus, in order to explore its capabilities to be commercialized, it is required to understand its genetic structure and metabolic mechanisms involved in the biosynthesis of beneficial biomolecules.

With the advancement of powerful tools and "omics" approaches such as whole-genome sequencing analysis, deciphering new insights into halophilic microorganisms (Durán-Viseras et al., 2021; Lam et al., 2021). Also, in response to the extreme environments, the concomitant advances in genomics are disclosing uncountable encoding genes for understanding the adaptation strategies, physiological attributes, and metabolic features of the halophilic bacteria (Corral et al., 2019). This resulted in a plethora of genomic information that was mined for potential agricultural and industrial applications (Ziemert et al., 2016; Othoum et al., 2019; Passari et al., 2019). Draft genomes with inaccurate or incomplete genomic data and low completeness are not fully reliable for phylogenomics, genome structure, genome synteny, and pan-genomic investigations (Denton et al., 2014). Thus, to know more about physiological, metabolic, and functional mechanisms, there is a need to generate high-quality whole genome sequences of halophilic microorganisms on a large scale to further understand the complete role of genes and their proteins in various extreme environments. The information from whole genome sequence analysis will constitute an exciting period for microbiology and the allied sector in the near future, which is generally not fully explored in the draft genome sequencing analysis. Since the first report of *Virgibacillus halodenitrificans* (Denariaz et al., 1989; Yoon et al., 2004), only one draft genome (Lee et al., 2012) and one complete genome (Zhou et al., 2017) have been published.

Therefore, in this study, we isolated and characterized a halophilic bacterium *Virgibacillus halodenitrificans* strain ASH15, from sugarcane-grown field in the coastal regions of Beihai, China and sequenced its entire genome. In addition, the plant growth-promoting efficiency of *V. halodenitrificans* ASH15 was evaluated for sugarcane plant growth under greenhouse conditions. Furthermore, systematic analysis of whole genome sequence data and the identification of genes will aid our understanding of the molecular mechanisms of osmoadaptation and the metabolic activities of the strain. Moreover, the obtained genome information will help to develop the strain ASH15 as an eco-friendly model for industrially important biomolecules and sustainable agriculture production.

Materials and methods

Sampling and isolation of halophilic bacteria

The soil sample was collected from the sugarcane-growing field of the sea city of Beihai, China (Latitude 21.4811° N, Longitude 109.1201° E). For the isolation of specific halophilic bacteria, the collected soil sample was enriched in a nutrient broth (NB) medium containing 10% NaCl at 37°C for 72 h. After the enrichment, the enriched soil sample was heat-treated at 80°C for 15 minutes to kill vegetative cells (Sharma et al., 2015). Spore-forming halophilic bacteria were isolated through the standard serial dilution method by spreading the diluted soil sample (10^{-4}) over a nutrient agar (NA) growth medium plate supplemented with 10% NaCl. Plates were incubated at 37°C for 72 h. After the incubation, a dominant bacterial isolate designated as ASH15 was recovered and purified by sub-culturing on the same NaCl-amended growth medium. The purified culture was preserved in 50% glycerol stock at −80°C for further study.

Growth kinetics studies under different levels of NaCl stress

Bacterial growth kinetics under different NaCl concentrations was spectroscopically determined in a 96-well microplate at 37°C. In brief, 1% of pure bacterial culture was transferred to an individual well containing 200 µl of NB broth with different NaCl concentrations, *viz*., 0, 5, 10, 15, 20, and 25%, and incubated to grow at 37°C under shaking condition at 150 rpm. The growth was spectroscopically monitored in a microplate reader by taking absorbance at 600 nm at every 12 h time interval.

Scanning electron microscopic analysis

A pure single bacterial colony was inoculated in NB and incubated for 48 h at 37°C under shaking conditions. After incubation, the cell pellet of bacterial culture was collected via centrifugation at 10,000 rpm at room temperature for 10 min. The collected pellet was washed 2–3 times with 100 mM phosphate buffer and then fixed with a 2.5% glutaraldehyde solution and incubated at 4°C for 10–12 h. After fixation, treated cells were further washed with phosphate buffer and with a gradient concentration of ethanol (10 to 100%) every 10 min. Following by, sample was dried in desiccators, mounted onto SEM stubs, and coated thinly with gold and palladium (60:40), and then sample was examined using the SEM machine.

Metabolic characterization through BIOLOG

The metabolic potentiality of the strain ASH15 was tested based on the carbon (C) utilization pattern in the BIOLOG Micro-Array TM GENIII plate (Biolog Inc., Hayward, CA) which contained 95 different carbon sources. Briefly, a single pure colony of strain ASH15 was streaked on NA plates and incubated at 30°C for 24 h. After the incubation, the bacterial culture mass was scraped from the surface of the plate and transferred into a sterile 2-ml centrifuge tube. Collected cells were washed with phosphate buffer and suspended in 20 ml of inoculation fluid (IF) (Biolog Inc., Hayward, CA) to reach a transmittance of 81–85% as per the

manufacturer's instructions. A 100 μl of suspension was inoculated in each well of the GNIII plate, which contained 95 different C sources. After incubation to record the results, the plate was read in an automated BIOLOG(R) Micro-Station Reader according to the manufacturer's instructions.

Biofilm formation and motility assay

The biofilm formation capacity of the strain ASH15 was assayed according to the method of Qurashi and Sabri (2012). 100 μl of pure bacterial culture suspension (10^8 CFU/ml) was transferred into a well of a microtiter plate which contained 200 μl NB of different NaCl concentrations. The plate was airtight packed and incubated at 37°C for 72 h. The medium from each well of the plate was thrown out, and the well was washed 2–3 times using distilled water. A 0.01% solution of crystal violet dye was added to each dried well. After 10 min of incubation, the dye was drained, and the plate was rinsed 2–3 times with sterile distilled water (D/W). Following that, in a rinsed and dried plate, 100 μL of acetic acid (30%) was added to solubilize the cell's bound remaining dye. Absorbance at 570 nm was taken to quantify the biofilm formation.

Swarming motility was detected by adopting the method of Connelly et al. (2004). Freshly grown bacterial cultures was point-inoculated on swarm plates consisted of 0.5% Bacto-agar (w/v) and 8 g l^{-1} of NB supplemented with 5 g l^{-1} of dextrose. After 24 h of incubation at 37°C, positive results as a swarming zone was recorded.

Characterization of different plant growth-promoting traits

The indole acetic acid (IAA) production ability of the test strain ASH15 was determined by following the standard protocol of Brick et al. (1991) using the Salkowski reagent. The phosphate solubilizing ability of the strain was analyzed by spot inoculation of the bacterial culture on the National Botanical Research Institute's Phosphate Medium agar plate (NBRIP) (Mehta and Nautiyal, 2001). The in-vitro zinc solubilization ability of the strain was carried out by employing plate assay of Sharma et al. (2012). The siderophore production ability of the test strain was assayed on a chrome azurol S (CAS) agar plate by adopting the standard method of Schwyn and Neilands (1987). The qualitative nitrogen (N) fixation capacity of the strain ASH15 was tested on an Ashby's Mannitol agar medium (Ashby, 1907). Exopolysaccharide (EPS) production was determined according to the method of Kumari et al. (2015). All the abovementioned plant growth-promoting traits were assayed under normal and different NaCl concentrations.

Effect of strain ASH15 on sugarcane growth under greenhouse condition

To evaluate the plant growth-promoting activity of the strain ASH15, a greenhouse experiment with sugarcane under salt stress (NaCl) and non-stressed conditions was performed at the Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China. The bacterial inoculum was prepared by centrifugation of freshly grown bacterial cultures. The collected bacterial cell pellet was washed 2–3 times with 0.1 M phosphate buffer and resuspended in the same buffer to make the bacterial suspension. Sugarcane seedlings GT42 (15 days old), were washed 3–4 times with sterilized distilled water (D/W) and treated with bacterial suspension (CFU 10^8) for 3 h. For control treatment, D/W was used in place of bacterial suspension. All the treated and non-treated sugarcane plants were planted in plastic pots that contained sterilized soil: sand mixture (3:1). A salt stress treatment of 200 mM NaCl was given after 10 days of plant establishment in the pot. The following treatments (T) were applied in the greenhouse experiment: T-1 (control: un-inoculated, no stress), T-2 (bacterial treatment), T-3 (200 mM salt stress treatment), and T-4 (200 mM salt stress + bacterial treatment). The experiment was conducted in a completely randomized manner in triplicate under a 16/8 h light/dark cycle with 80% field water capacity (FWC) moisture at 28 ± 2°C temperature. After 30 days of stress treatment, the plants from all treatments were uprooted, cleaned, and vegetative growth parameters, such as shoot length (SL), root length (RL), shoot fresh weight (SFW), and shoot dry weight (SDW), root fresh weight (RFW), and root dry weight (RDW), were taken.

DNA extraction, library construction and whole genome sequencing analysis

For complete genome sequencing, genomic DNA was extracted from a full-grown culture of strain ASH15 using the DNA extraction kit (CWBIO, Beijing, China) according to the manufacturer's instructions. DNA quality and quantity were assessed using the TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA, United States), and high-quality genomic DNA (OD260/280 = 1.8–2.0, >20 μg) was used for further processing. The genome sequencing was performed using single-molecule real-time (SMRT) Oxford-Nanopore and Pacbio (third generation) sequencing technology. Moreover, 15 μg of high-quality DNA was processed for fragmentation using Covaris G-TUBE (Covaris, MA, United States) for 60 s at 6,000 rpm. Genomic DNA fragments were purified, end-repaired, and ligated via SMRTbell sequencing adapters according to the manufacturer's protocol (Pacific Biosciences, CA, United States) and purified using AMPureXP beads (Beckman Coulter Genomics, MA, United States). Further, approximately 10 kb insert library was sequenced on one SMRT cell by standard procedures. For Nanopore sequencing, high-quality genomic DNA with a large fraction was selected using Blue Pippin (Sage Science, USA), followed by end-repair/dA tailing. End-repaired DNA fragments are processed for adapter ligation using a ligation sequencing kit (NBD103 and NBD114, Oxford Nanopore Technologies USA). Finally, the DNA library was quantified through Qubit 3.0 (Thermo Fisher Technologies USA). Afterward, 11 μL of DNA library was loaded into a 1 flow cell and sequenced on a PromethION sequencer (Oxford Nanopore Technologies USA).

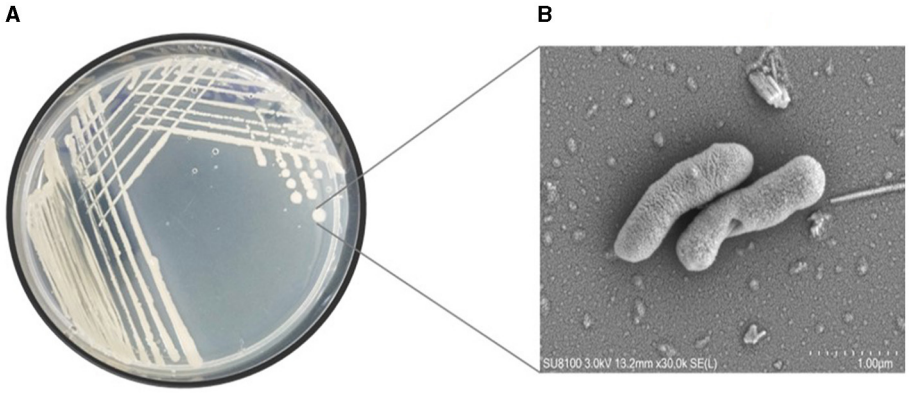

FIGURE 1
(A) Pure culture and **(B)** SEM micrograph of the halophilic strain *Virgibacillus halodenitrificans* ASH15 cell.

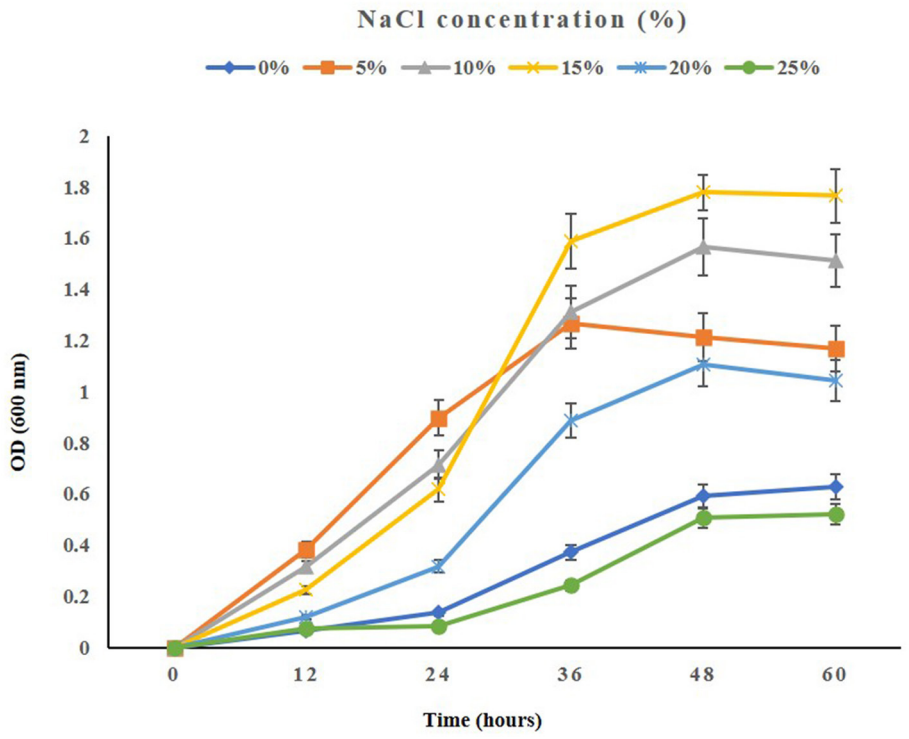

FIGURE 2
The growth pattern of the halophilic strain *Virgibacillus halodenitrificans* ASH15 at different NaCl concentrations.

TABLE 1 Plant growth promoting attributes of the strain *Virgibacillus halodenitrificans* ASH15.

NaCl (%)	IAA (μg/ml)	P-solubilization.	Zn-solubilization.	Siderophore	N fixation	EPS (g/ml)
0%	54.76 ± 1.8	++	+	++	+	1.66 ± 0.11
5%	76.19 ± 2.4	+++	++	+++	++	2.40 ± 0.16
10%	82.86 ± 1.5	+++	+++	++	++	2.88 ± 0.15
15%	80.43 ± 2.0	+++	+	+	++	3.29 ± 0.17

(+) production in normal level (++) production in medium level, (+ + +) production in high level, (-) negative for test. Values are expressed as Mean ± Standard Error.

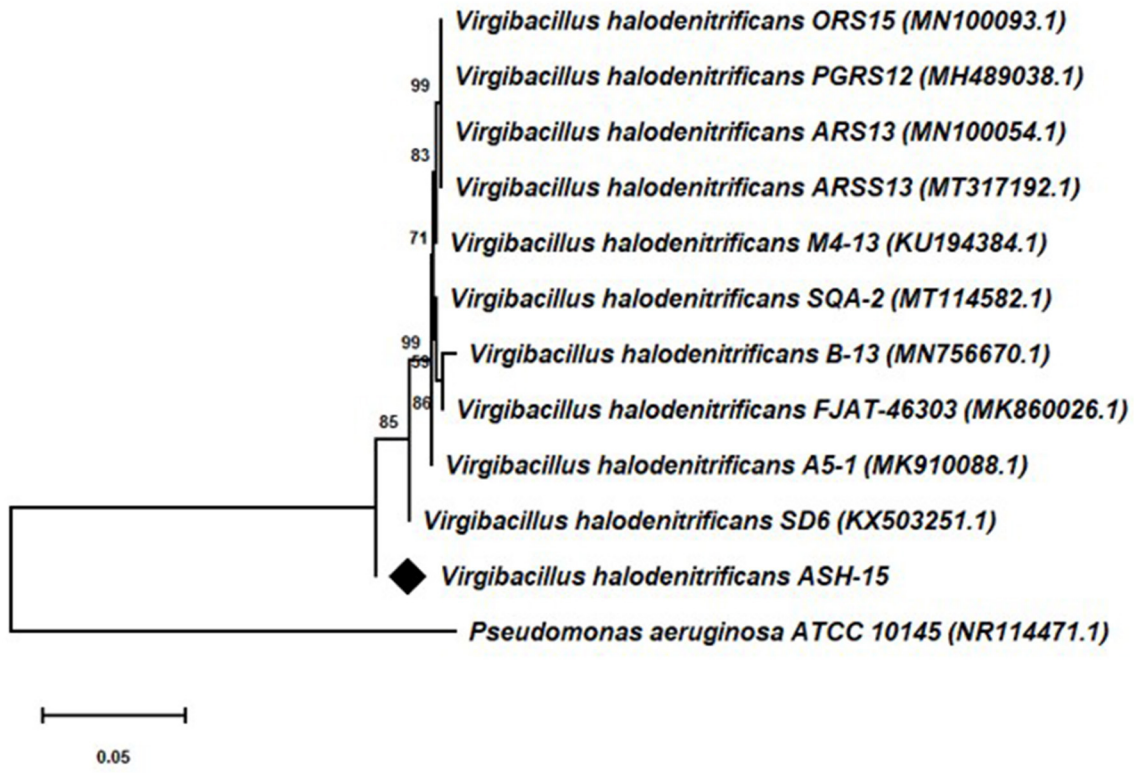

FIGURE 3
16S rRNA gene sequencing-based phylogenetic tree of the *Virgibacillus halodenitrificans* ASH15 with its nearest member of the NCBI GenBank database.

Genome assembly, gene prediction, and functional annotation

The raw sequence data was processed for quality checks by employing Majorbio Cloud Platform[1] (Shanghai Majorbio Co., Ltd.). Quality-passed raw sequence data reads were then assembled into contigs using the hierarchical genome assembly method (HGAP) (Chin et al., 2013). The final genome assembly was finished using Pilon. The assembled genome was further processed for gene prediction and annotations. Prediction of coding sequence (CDS) was conducted with Glimmer version 3.02, followed by annotation using multiple databases, i.e., Pfam, Swiss-Prot, NR, Clusters of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), and Gene Ontology (GO) (Delcher et al., 2007) with Basic Local Alignment Search Tool (BLAST), DIAMOND sequence alignment and HMMER, and other tools. tRNAs and rRNAs were predicted using tRNA-scan-SE (v1.2.1) (Borodovsky and Mcininch, 1993) and Barrnap. A circular genome map of strain ASH15 was constructed using genome annotation files on the CGviewer server (Grant and Stothard, 2008).

Taxonomic identification and phylogenetic analysis

The PCR 16S rRNA gene amplification was carried out using the universal primer pairs pA_F and pH_R (Sharma et al., 2022). The purified PCR product was sequenced using Sanger dideoxy-chain termination chemistry. The obtained sequence was assembled to make a consensus sequence by converting one strand to a reverse complement. For strain ASH15 identification, the assembled consensus 16S rRNA gene sequence was used for a BLAST (BLASTn) search against the available bacterial 16S rRNA gene sequences in the NCBI GenBank database. Further, a neighbor-joining (NJ)-based phylogenetic tree of 16S rRNA gene sequence of the strain ASH15 with 11 reference 16S rRNA gene sequences was constructed through MEGA-X. The bootstrap analysis was conducted using 1,000 replications by the Felsenstein method (Felsenstein, 1985). The evolutionary distances were calculated by the Jukes–Cantor coefficient procedure (Tamura et al., 2004).

Identification of biosynthetic gene clusters and metabolic system analysis

The genome sequence of strain ASH15 was analyzed using antiSMASH (Blin et al., 2019) software for predicting biosynthetic gene clusters (BCGs), such as non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), post-translationally modified peptides (RiPPs), hybrid lipopeptides (NRPS-PKS), and bacteriocins. Less than 70% of the amino acid identity shared by the biosynthetic Gene Clusters compared to the known clusters was considered novel. The Carbohydrate Active

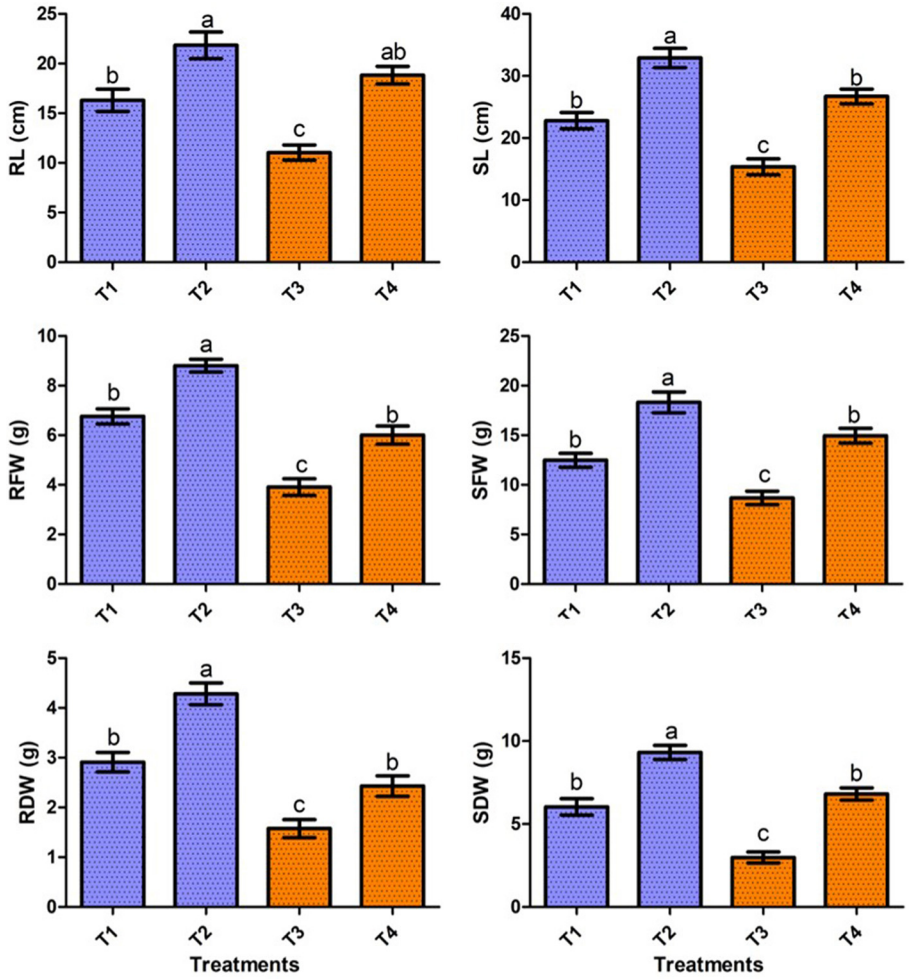

FIGURE 4
Effect of strain ASH15 on sugarcane growth under normal and NaCl (200 mM) stressed conditions. (RL, root length; RFW, root fresh weight; RDW, root dry weight; SL, shoot length; SFW, shoot fresh weight; and SDW, shoot dry weight). The figures represent the mean values ± standard error of three independent experiments. Bars indicate ± SE (standard error of mean). The letters on the bars indicate DMRT at a p-value of <0.05.

Enzyme Database (CAZy, http://www.cazy.org/) is a professional database for enzymes synthesizing or decomposing complex carbohydrates and sugar complexes. Carbohydrate activity enzymes derived from different species are divided into glycoside hydrolases (GHs), polysaccharide lyases (PLs), glycosyltransferases (GTs), carbohydrate esterases (CEs), auxiliary activities (AAs), carbohydrate-binding modules (CBMs), and other six major protein families.

Additional genome analysis (sRNA prediction, repeat sequence prediction, tandem repeat prediction, and scattered repetitive sequence prediction)

Bacterial sRNA is a type of non-coding RNA with a length of 50 to 500 nt. They are located in the intergenic region of genomes, and some are in the 5′and 3′UTR regions of coding genes. We used Infernal software (http://eddylab.org/infernal/) and the Rfam database (https://rfam.xfam.org/) to predict and annotate the sRNA from the genome of strain ASH15. Tandem Repeats Finder software (Benson, 1999) was used to predict the tandem repeat sequences. Interspersed repeat, also known as a transposon element, includes DNA transposons and retrotransposons transposed by DNA-DNA. RepeatMasker software (Tarailo-Graovac and Chen, 2009) was used to identify these sequences as similar to known repetitive sequences and classify them. IslandViewer (Bertelli et al., 2017) was used to identify genomic islands in strain ASH15.

Statistical analysis

The experimental data of the present study were subjected to analysis of variance (ANOVA) followed by DMRT (Duncan's multiple range test) with a significance level of p of $\leq$0.05 (Duncan, 1955). Bioinformatics analysis of the strain ASH15 genome was carried out through Majorbio I-Sanger (www.i-sanger.com).

Results and discussion

Isolation and characterization of halophilic bacteria

This study undertook the isolation, characterization and systematic genome analysis of a halophilic bacterial strain ASH15 and identification of their key genes that contribute in osmoadaptation (stress tolerance), plant growth-promoting (PGP) traits and other industrially important biomolecules production. A Gram-positive, spore-forming, rod-shaped, and motile halophilic bacteria strain ASH15 was isolated from the collected soil sample (Figure 1). Strain ASH15 showed medium-sized, round colonies with a ceramic-white, opaque appearance and smooth margins. Further, the carbon source utilization on GNIII MicroPlate™ (Biolog Inc., Hayward, CA), was used to extricate the metabolic sensitivity of the isolated halophilic bacterial strain ASH15 (Zhao et al., 2019). Results showed that strain ASH15 was able to metabolize a wide range of carbon sources. Strain ASH15 was positive for 24 sugars and chemically sensitive to 16 substrates, 2 hexose-PO4, 8 amino acids, 8 hexose acids, and 13 carboxylic acids, esters, and fatty acids (Supplementary Table 1). The utilization of different carbon sources by the bacterial cell assists as components of the metabolic network, whereby they are broken down to facilitate the source of amino acids and other building blocks to make up a cell (Wang et al., 2019). These metabolic properties of halophilic bacteria might lead to their response and adaptation in specific extreme environments (Sharma et al., 2021).

When testing the growth of the strain ASH15 under various NaCl concentrations, we observed that supplementing nutrient broth with 0.5–25% NaCl had a positive effect on bacterial growth. The maximum growth (OD at 600 nm) of the strain ASH15 was observed at 15% as compared to NB with 0.5% NaCl. Further results showed that the growth of ASH15 was in contrast, slightly delayed when NaCl concentration was increased up to 25% (Figure 2). These findings demonstrate that strain ASH15 is moderately halophilic and is able to withstand up to 25% NaCl concentrations. In accordance with our findings recently, Srivastava et al. (2022) reported that *Chromohalobacter salexigens* ANJ207 was able to grow up to 30% NaCl concentration. The capacity to withstand moderate salt stress might help ASH15 survive as a free-living bacterium in saline soils.

The qualitative evaluation of plant growth-promoting attributes revealed that strain ASH15 was able to fix nitrogen, solubilize phosphate and zinc, and produce siderophore and ammonia. In addition, quantitative estimation of IAA revealed that strain ASH15 produced 54.7, 76.1, 82.8, and 80.3 μg/ml IAA at 0, 5, 10, and 15% NaCl concentrations respectively. Interestingly, strain ASH15 has all the PGP activity up to higher NaCl concentrations. Furthermore, strain ASH15 produces EPS at all the tested NaCl concentrations (Table 1). Results of the biofilm formation assay showed that strain ASH15 was able to produced biofilm in all the tested NaCl concentrations with maximum at 15% NaCl. These results showed the possible strategies that the strain ASH15 might employ on its host plant for salt stress alleviation and plant growth promotion. These findings are consistent with previous research studies, where halophilic bacteria, such as *Bacillus halophilus, Marinococcus halophilus, Halobacillus litoralis, Saliiococcus hispanicus,* and *Halobacillus halophilus,* were recognized to grow optimally between 10 and 15% NaCl concentrations (Sarwar et al., 2015; Reang et al., 2022; Srivastava et al., 2022).

Phylogeny of strain ASH15

Based on the 16S rRNA gene sequencing analysis and BLASTn search, strain ASH15 showed 100% similarity with *Virgibacillus halodenitrificans* of the NCBI database. A neighbor-joining (NJ) phylogenetic tree was constructed with the similar bacterial sequences of the NCBI GenBank database (Figure 3). The phylogenetic analysis provides an important depiction of the evolutionary relationship between different strains (Srivastava et al., 2022).

Effect of strain ASH15 on sugarcane growth under greenhouse conditions

In the present study, the positive effect of *V. halodenitrificans* strain ASH15 on sugarcane growth under salt stress conditions was assessed under normal and salt stress (200 mM NaCl) conditions (Figure 4). Results of the greenhouse study showed that salinity stress imposes adverse effects on sugarcane vegetative growth. However, strain ASH15 application significantly ($p < 0.05$) enhanced the growth of sugarcane plants under normal as well as NaCl stress conditions (Figure 4). The results of greenhouse pot experiments showed that salt stress treatment (T-3, 200 mM NaCl) decreased root length (RL) and shoot length (SL) growth by 32.3% and 25.8%, respectively, as compared to uninoculated non-stressed (T1). Whereas, application of strain ASH15 (T-4) treatment remarkably ($p < 0.05$) enhanced the root length and shoot length by 71.2% and 64.4%, respectively, over un-inoculated NaCl-stressed plants (Figure 4). Similarly, salt stress (T-3) treatment decreases root fresh weight (RFW) and shoot fresh weight (SFW) by 42.1 % and 30.9%, respectively, over uninoculated non-stress plants (T-1). In contrast, strain ASH15 (T-4) boost up the RFW and SFW by 53.5% and 72.0%, respectively, as compared to uninoculated salt-stressed plants (T-3). Moreover, similar trends were observed in the case of root dry weight (RDW) and shoot dry weight (SDW), where salt stress (T-3) reduced the RDW and SDW by 45.8% and 57.6%, respectively, over their uninoculated control (T-1). However, strain ASH15 (T-4) increased the RDW and SDW by 54.1% and 109.1%, respectively, compared to the uninoculated salt-stressed control (T-3) (Figure 4). The results of the pot experiment demonstrated that the sugarcane plant's overall health, growth, and development were reliant on the presence of strain ASH15, which regulated an adequate amount of multiple plant nutrient levels (Alishahi et al., 2020; Khumairah et al., 2022). Therefore, in this study, we explored the strain ASH15 genome and mined the gene codes for almost all PGP traits like IAA, nitrogen fixation, phosphate solubilization, and siderophore production. Sultana et al. (2020) recently reported that salt-tolerant bacteria significantly increased

rice plant growth. The results are also in accordance with those reported by Bhattacharyya et al. (2017), Asaf et al. (2018), and Abdullahi et al. (2021), where they analyzed the presence of multiple genes encoding for PGP mechanisms in plant growth-promoting bacteria.

Genome analysis of *V. halodenitrificans* ASH15

The genome assembly details of the strain ASH15 are given in Table 2. The high-quality raw sequence data was assembled with a hybrid genome assembly, and a single scaffold was achieved. The genome of *V. halodenitrificans* strain ASH15 is composed of a circular chromosome (Figure 5) of 3,832,903 base pairs with an average G+C content of 37.54%. There was no plasmid identified in the genome assembly. The genome was processed for gene prediction, and the total predicted genes were 3,807, which included ~3,721 protein-coding genes (CDS), 62 tRNAs, and 24 rRNA genes (Table 2). CDS constitute 3,207,696 bases (83.69%) of the genome, with an average gene length of 862.05 bases. Approximately 10.78% (803483 bases) of the genome was found to be intergenic. Furthermore, predicted genes against various databases were characterized. The number of COG genes, Gene Ontology (GO), KEGG, NR (Non-redundant Protein Database), and SwissProt were 3,268, 2,575, 2,008, 3,644, and 2,683 respectively (Table 2 and Figures 6A–C). The complete genome sequence of the strain *V. halodenitrificans* ASH15 has been deposited at the NCBI/GeneBank with accession number CP090006.

TABLE 2 Genome characteristics of *Virgibacillus halodenitrificans* strain ASH15.

Characteristics	Value
Genome size (bp)	3,832,903
Chromosome	1
Chromosome size (bp)	3,832,903
GC content (%)	37.54
Topology	Circular
tRNA	62
rRNAs (5S, 16S, 23S)	24
CDS	3,721
CDS (bp)	3,207,696
CDS (% of genome)	83.69
Average gene length (bp)	862.05
Intergenic region (bp)	803,483
Intergenic region (%)	10.78
Genomic islands	7
CRISPR	36
Insertion sequences	9
Genes annotated with COG	3,326
Genes annotated with GO	2,575
Genes annotated with KEGG	2,008
Genes annotated with NR	3,644
Genes annotated with Swiss-Prot	2,683

Genetic potential of various stress tolerance in the ASH15 genome

Halophilic bacteria have unique inherent osmoadaptation mechanisms for stress adaptation, which could be used for agriculture, food, and fermentation industries (Gunde-Cimerman et al., 2018; Vaidya et al., 2018). Genome analysis of the strain ASH15 confirmed the presence of several key genes responsible for different abiotic stress tolerances, mainly osmotic stress (*proVWXSBA*, *fadANM, betBA, trkAH*, *opuBDCA*, *opcR, putP*, *yrgG,* kch, and *nhaC*), ectoine biosynthesis (*ectCBAD*), and oxidative stress *(hmp*, *pfpI*, Usp, *katE,* and *osmC*) (Table 3). These osmolytes, or compatible solutes, provide osmotic balance to the bacteria without disturbing their cell functions (Roberts, 2005; León et al., 2018).

In addition, further analysis revealed that the strain ASH15 genome has various other abiotic stress tolerance genes such as cold-shock protein (*cspA*), heat shock proteins (*hrcA*, *dnaK*, hsp20, *htpX*, *htpG*, and *ctsR*), heavy metals such as arsenic (*arsRBC*), cobalt (*czcD, ecfT, ecfA1, ecfA2*), zinc (*zupT, yqgT, rseP, czrA, znuACB, zurR, nprE, qor, sprL*), cadmium (*zntA*), magnesium (*corA*), molybdenum (*modABC*), copper (*copZA, csoR, copB, cutC, ycnK*), and manganese (*mntCBA*), antibiotics (*norM*, *bacA, lmrB, fsr, pbp1b, ykkDC,* and *yitG*), and fluoride resistance (*crcB*) (Table 3). These groups of genes provide stress-tolerant capabilities to strain ASH15 and enable it to survive in extreme conditions. Fluoride exporter genes, *crcB,* were involved in multilevel stress responses (Calero et al., 2022). Several PGPR genera have been described to succeed in heavy metal stresses to improve plant tolerance, especially abiotic stresses, and crop yields (Tiwari and Lata, 2018). These proteins are linked to tolerance to cobalt, zinc, copper, arsenic and cadmium (Kang et al., 2020).

Genes related to plant growth promotion in the strain ASH15

Sugarcane is a long-term economically important crop, and for its growth, various types of plant nutrients such as N, P, K, and phytohormones are required. Thus, to decrease the application of chemical fertilizers in the current era, PGPR with various PGP attributes promises an alternative approach to plant nutrient requirements (Sharma et al., 2021). Therefore, in the present study, we revealed the plant growth promotion potential of *V. halodenitrificans* ASH15. The genome of the strain covers so many genes that encode various PGP traits, such as phytohormone IAA production, nitrogen fixation, phosphate solubilization, ammonia assimilation, and siderophore production (Table 4, Guo et al., 2020).

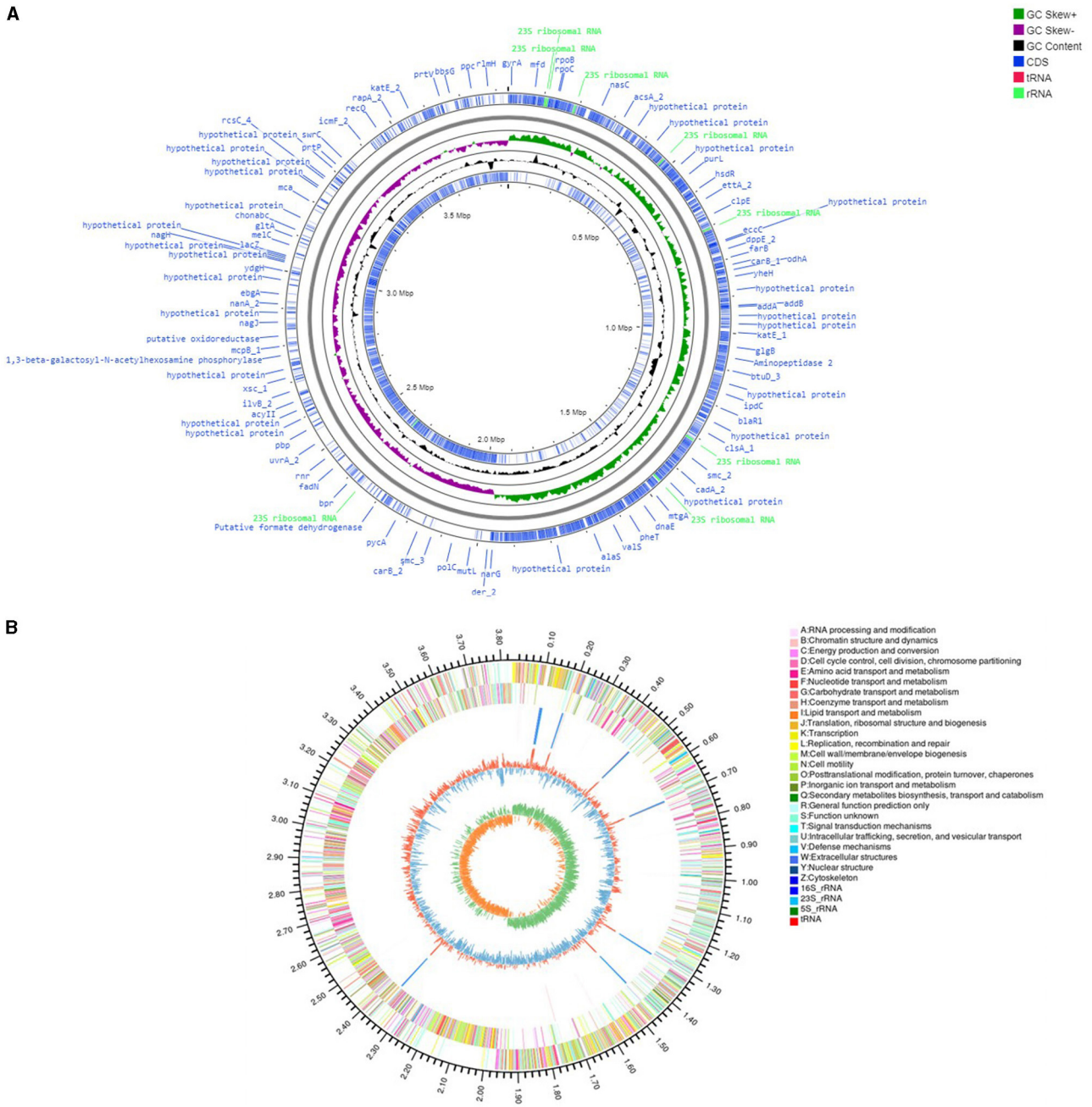

FIGURE 5
(A) Circular representation of the chromosome/genome of *Virgibacillus halodenitrificans* strain ASH15. The Outermost and innermost rings represent two strands of the genome. The outermost ring displays the functional annotation of genes (CDS), tRNA, and rRNA distribution in the genome. The third ring (black in color) shows the GC content of the genome, while the fourth ring (green in color) represents the GC skewness throughout the genome. **(B)** A–Z, the outer two circles, show the functional classification of the CDS genes in the chromosome with the colors of the COG. The inner circle shows rRNAs and tRNAs.

Indole-3-acetic acid (IAA) is an important phytohormone involved in various physiological processes, including cell enlargement and division, tissue differentiation, and responses to light and gravity. The ability to synthesize IAA is a well-characterized trait in halophilic PGPR (Pérez-Inocencio et al., 2022). Bacterial IAA is involved in overcoming stress, serving as a C/N source, and playing a role in plant–microbe interactions (Defez et al., 2019). In the current study, we observed that strain ASH15 was capable of synthesizing IAA, and its genome consists of *trp*A, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *trpG*, and *trpS* genes, which code for enzymes of the IAA biosynthesis pathway (Table 4). Similar to our findings, tryptophan biosynthesis genes (*trpABD*) are involved in IAA production in *Sphingomonas* sp. LK11 (Asaf et al., 2018).

Another strategy of PGPR to enhance plant growth is to fix atmospheric nitrogen. Nitrogen is an essential nutrient element for soil fertility, sugarcane plant growth and development,

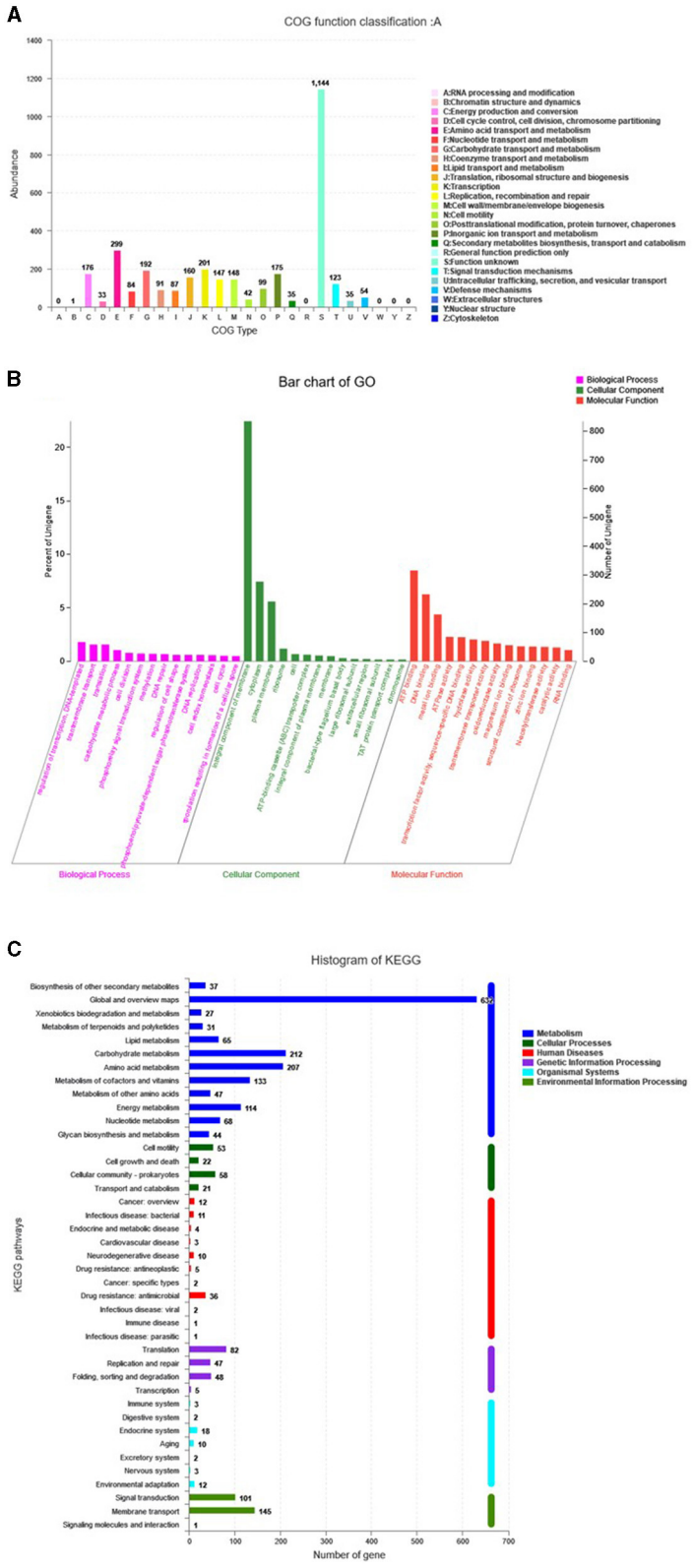

FIGURE 6
Predicted genes of the *Virgibacillus halodenitrificans* strain ASH15 genome through **(A)** COG, **(B)** GO, and **(C)** KEGG databases.

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TABLE 3 Genes associated with abiotic stress responses in *Virgibacillus halodenitrificans* strain ASH15.

Stress	Gene IDs	Gene annotations	GO IDs	Chromosome location
Osmotic stress				
Proline	*proV*	Glycine betaine/proline transport system ATP-binding protein	GO:0031460	891274-892473
	proW	Glycine betaine/proline transport system permease protein	GO:0055085	892463-893317
	proX	Glycine betaine/proline transport system substrate-binding protein	GO:0043190	893428-894330
	proX	Glycine betaine/proline transport system substrate-binding protein	GO:0043190	894427-895362
	proB	Glutamate 5-kinase	GO:0055129	768476-769576
	proA	Glutamate-5-semialdehyde dehydrogenase	-	769590-770837
	proX	Glycine betaine/proline transport system substrate-binding protein	-	3749454-3748555
	betB	Betaine-aldehyde dehydrogenase	GO:0019285	3749899-3751371
	betA	Choline dehydrogenase	GO:0019285	3751639-3753324
	proS	Prolyl-tRNA synthetase	GO:0006433	403135-404574
	putP	Sodium/proline symporter	GO:0015824	573152-574672
	putP	Sodium/proline symporter	GO:0015824	2279082-2280548
	yrbG	Sodium/calcium exchanger protein;	GO:0055085	284640-285590
	panF	Sodium/panthothenate symporter	GO:0036376	318578-320014
	cvrA	K(+)/H(+) antiporter NhaP2	GO:0006813	3592714-3594207
	kch	Potassium channel family protein	GO:0008076	195811-195101
	lctB	Two pore domain potassium channel family protein	GO:0016021	695254-695685
	trkA	trk system potassium uptake protein C	GO:0006813	2255550-2254885
	trkH	trk system potassium uptake protein D	GO:0016021	2282378-2281014
	nhaC	Na(+)/H(+) antiporter NhaC	GO:0016021	2821802-2820342
	fadA	Acetyl-CoA acyltransferase	GO:0016747	2442609-2441434
	fadN	3-hydroxyacyl-CoA dehydrogenase	-	2445023-2442624
	fadM	Proline dehydrogenase	GO:0010133	2446131-2445217
Ectoine	*ectC*	L-ectoine synthase	GO:0019491	2330170-2329784
	ectB	Diaminobutyrate-2-oxoglutarate transaminase	GO:0019491	2331484-2330207
	ectA	L-2,4-diaminobutyric acid acetyltransferase	GO:0019491	2332015-2331503
	ectD	Ectoine dioxygenase	GO:0016706	3474559-3473663
Glycine/betaine	*opuD*	Glycine betaine transporter	GO:0071705	323757-325247
	opuD	Glycine betaine transporter	GO:0071705	505199-506722
	opuC	Osmoprotectant transport system substrate-binding protein	GO:0043190	1990166-1988640
	opuA	Osmoprotectant transport system ATP-binding protein	GO:0005524	1991224-1990163
	opuBD	Osmoprotectant transport system permease protein	GO:0055085	2334871-2334209
	opuC	Osmoprotectant transport system substrate-binding protein	GO:0043190	2335802-2334873
	opuBD	Osmoprotectant transport system permease protein	GO:0055085	2336463-2335819
	opuA	Osmoprotectant transport system ATP-binding protein	GO:0031460	2337636-2336476
	opcR	HTH-type transcriptional regulator, osmoprotectant uptake regulator	GO:0003677	2337857-2338429
	opuD	Glycine betaine transporter	GO:0071705	2727709-2726204
Oxidative stress	*hmp*	Nitric oxide dioxygenase	GO:0009636	37386-36160
	pfpI	General stress protein 18	GO:0008233	38034-38549
	Usp	Universal stress protein	-	309979-309560
	katE	Catalase	GO:0006979	470556-469066

(Continued)

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TABLE 3 (Continued)

Stress	Gene IDs	Gene annotations	GO IDs	Chromosome location
	osmC	Response to oxidative stress	GO:0006979	2158630-2158211
Cold/heat stress				
	cspA	Cold shock-like protein CspC	GO:0005737	417715-417912
	cspA	Cold shock-like protein CspLA	GO:0005737	616606-616806
	cspA	Cold shock-like protein CspD	GO:0005737	1943998-1943798
	hrcA	Heat-inducible transcription repressor HrcA	GO:0045892	1716523-1717557
	-	Participates actively in the response to hyperosmotic and heat shock	GO:0006457	1717638-1718198
	dnaK	Heat shock protein	GO:0006457	1718231-1720072
	-	Heat induced stress protein YflT	-	2302479-2302829
	HSP20;	Small heat shock protein C4	-	2356669-2357109
	htpX	Protease HtpX	GO:0016021	3565876-3566754
	htpG	Chaperone protein HtpG	GO:0006457	80666-78786
	ctsR	Transcriptional regulator CtsR	GO:0006355	113128-113595
	-	Hsp20/alpha crystallin family	-	543367-542930
Antibiotic stress				
	-	Tetracycline resistance protein	-	126052-126561
	norM	Probable multidrug resistance protein NorM	-	838660-837296
	bacA	Bacitracin resistance protein BacA	GO:0008360	957794-956973
	lmrB	Lincomycin resistance protein LmrB	GO:0016021	1117314-1115830
	fsr	Fosmidomycin resistance protein	GO:0016021	2811379-2810162
	ykkD	Probable guanidinium efflux system subunit GdnD	GO:0016021	3328577-3328263
	ykkC	Probable guanidinium efflux system subunit GdnC	GO:0016021	3328918-3328577
	yitG	MFS transporter, ACDE family, multidrug resistance protein	-	3597908-3599146
	pbp1b	Penicillin-binding protein	-	1491396-1488400
Heavy metals				
	czcD	Cobalt-zinc-cadmium efflux system protein	GO:0016021	2264058-2264960
	ecfA1	ABC transporter	GO:0055085	161202-162041
	ecfA2	ABC transporter;AAA domain, putative AbiEii toxin, Type IV TA system	-	162017-162889
	ecfT	Cobalt transport protein	GO:0016021	162882-163682
	zupT	Zinc transporter, ZIP family	GO:0016021	1393351-1394172
	corA	Magnesium transporter	GO:0016021	1387297-1386344
	yqgT	Zinc carboxypeptidase	GO:0008270	1955473-1954283
	rseP	Zinc metalloprotease RasP	GO:0016021	2071722-2070457
	czrA	Zinc-responsive transcriptional repressor	GO:0003700	2263738-2264040
	czcD	Cobalt-zinc-cadmium efflux system protein	GO:0016021	2264058-2264960
	yogA	Zinc-binding alcohol dehydrogenase/oxidoreductase	-	2292089-2291109
	ftsH	ATP-dependent zinc metalloprotease FtsH	GO:0051301	90304-92340
	arsR	Transcriptional regulator	GO:0003700	1378392-1378730
	arsB	Arsenic transporter	GO:0046685	1378746-1380044
	arsC	Arsenate reductase (glutaredoxin)	GO:0046685	1380063-1380482
	znuA	Zinc transport system substrate-binding protein	GO:0030001	2449475-2450401
	znuC	Zinc transport system ATP-binding protein	GO:0005524	2450418-2452053

(Continued)

TABLE 3 (Continued)

Stress	Gene IDs	Gene annotations	GO IDs	Chromosome location
	znuB	Zinc transport system permease protein	GO:0043190	2451190-2452456
	zurR	Fur family transcriptional regulator, zinc uptake regulator	GO:0003677	2452043-2452827
	nprE	Zinc metalloprotease	GO:0005576	3186475-3184820
	qor	Zinc-binding dehydrogenase	GO:0016491	3671355-3670381
	sprL	Zinc ion binding	GO:0005737	470890-471348
	zntA	Zn2+/Cd2+-exporting ATPase	GO:0016021	661474-659534
Manganese	*mntC*	Manganese transport system substrate-binding protein	GO:0030001	24740200-2473076
	mntB	Manganese transport system permease protein	GO:0043190	2474910-2474053
	mntA	Manganese transport system ATP-binding protein	GO:0005524	2475650-2474907
Copper	*copZ*	Copper chaperone	GO:0030001	2446469-2446263
	copA	P-type Cu+ transporter	GO:0030001	2448887-2446497
	csoR	CsoR family transcriptional regulator, copper-sensing transcriptional repressor	GO:0006355	2449203-2448901
	copB	Copper-exporting P-type ATPase B	GO:0016021	1405364-1407472
	cutC	Copper homeostasis protein cutC	-	3246050-3245355
	ycnK	Copper-sensing transcriptional repressor	GO:0003677	3641001-3640405
Molybdenum	*modA*	Molybdate transport system substrate-binding protein	GO:0015689	3628744-3629541
	modB	Molybdate transport system permease protein	GO:0016021	3629557-3630246
	modC	Molybdate transport system ATP-binding protein	GO:0005524	3630272-3630889
Fluoride stress				
	crcB	Fluoride exporter	-	2452441-2452827
	crcB	Fluoride exporter	GO:0005887	2452824-2453159

physiological and metabolic activities, and sustainable sugarcane crop production (Singh et al., 2022). PGPR catalyzes nitrogen fixation through the *nif* (nitrogenase complex) gene-coded nitrogenase enzyme. In this study, the strain ASH15 genome lacks genes (*nifDHK*) coding the nitrogenase enzyme, but contains genes related to dissimilatory nitrate reduction (Table 4). These include narGHIJ, a nitrate/nitrite ABC transporter (narK), a putative nitrogen fixation protein (nifU), and various other genes associated with nitrogen metabolism and transport (*iscU*, *norG*, *nreBCA,* and nos). Strain ASH15 also has genes coding for ammonia assimilation, such as *gltXASDBC*, *glnQPHRA*, *gdhA*, *asnB,* and *pyrG* (Table 4). These results showed that strain ASH15 is able to incorporate nitrate and nitrite for assimilation into ammonia and can incorporate ammonia directly.

Together with N, phosphorus (P) is also an important nutrient required for plant growth (Bergkemper et al., 2016). PGPR plays a key role in plant growth by facilitating the conversion of the available insoluble inorganic phosphate to the soluble PO_4^{3-} (Bergkemper et al., 2016). In PGPR, a mineral's phosphate-dissolving ability has been directly related to the presence of various genes responsible for producing organic acids. In this study, the genome of ASH15 contains genes coding for inorganic pyrophosphatase (*ppaC*) and alkaline phosphatase (*phoA*). The two-component system CS PhoB1/PhoR is involved in the alkaline phosphatase, phosphate starvation response (*phoH*), and an ABC transporter for phosphate uptake (*pstSCAB*), which are responsible for solubilizing the inorganic phosphate (Table 4). Moreover, the presence of an effective system in the PGPR for absorbing iron can help to protect the host plant from pathogen infestations (Herlihy et al., 2020; Lahlali et al., 2022). In the strain ASH15 genome, we also detected the presence of several siderophore-related genes in the ASH15 genome, including several iron ABC transporters (*fhuBD, afuABC,* and *FecCD),* a ferric uptake regulator (*perR),* an iron export ABC transporter permease (*fetB*), and a ferric transport system and ions import (*fhuBCG*) (Table 4). Our findings are in line with the fact that PGPR with salt-tolerant properties provides a range of benefits such as phytohormones, nitrogen fixation, P solubilization, ammonia production, and siderophore production for plant's stress tolerance and growth promotion (Egamberdieva et al., 2019; Arora et al., 2020; Khumairah et al., 2022).

In addition, genes like antimicrobial peptides and hydrolase genes, such as GTP cyclohydrolase (*ribBA*), α-amylase (*treC*), α-glucosidase (*malZ*), and glutamate dehydrogenase are also involved in plant immune responses. Moreover, oxidoreductase genes such as glutathione hydrolase proenzyme (ggt), superoxide dismutase (SOD), glutathione transport system (*gsiDCB*), and peroxiredoxin (DOT5, tpx) have been categorized. Strain ASH15's genome predicted some key genes of volatile substances such as

TABLE 4 Genes associated with PGP traits in *Virgibacillus halodenitrificans* strain ASH15.

PGP traits	Gene IDs	Gene annotations	GO IDs	Chromosome location
Nitrogen metabolism				
	narK	MFS transporter, NNP family, nitrate/nitrite transporter	GO:0016021	259339-260835
	nreB	Two-component system, NarL family, sensor histidine kinase NreB	GO:0016021	260896-261615
	nreC	Two-component system, NarL family, response regulator NreC	GO:0006355	261612-262265
	nreA	Nitrogen regulatory protein A	-	262255-262725
	nos	Nitric oxide synthase oxygenase	GO:0006809	519663-520754
Nitrogen fixation	*nifU*	Putative nitrogen fixation protein	GO:0016226	2409694-2409918
	iscU	Nitrogen fixation protein NifU and related proteins	GO:0016226	2430245-2429811
	norG	GntR family transcriptional regulator, regulator for abcA and norABC	GO:0009058	618000-619412
	narG	Nitrate reductase, alpha subunit	GO:0042126	1957499-1961182
	narH	Nitrate reductase, beta subunit	GO:0042126	1961172-1962665
	narJ	Nitrate reductase molybdenum cofactor assembly chaperone NarJ/NarW	GO:0051131	1962670-1963272
	narI	Nitrate reductase gamma subunit	GO:0016021	1963286-1963975
Ammonia assimilation				
	gltX	Glutamate–tRNA ligase	GO:0006424	120934-122403
	gltA	Citrate synthase	GO:0005737	1541961-1543076
	gltS	Glutamate:Na+ symporter, ESS family	-	2322419-2320896
	glnQ	Glutamine transport system ATP-binding protein	GO:0005524	2721456-2720734
	glnP	Glutamine transport system permease protein	GO:0071705	2722112-2721453
	glnH	Glutamine transport system substrate-binding protein	GO:0016020	2723108-2722317
	glnR	MerR family transcriptional regulator, glutamine synthetase repressor	GO:0006355	2011319-2010936
	glnA	Glutamine synthetase	GO:0006542	3089088-3087751
	gltD	Glutamate synthase [NADPH] small chain	GO:0006537	3090618-3089122
	gltB	Glutamate synthase [NADPH] large chain	GO:0006537	3095251-3090698
	gltC	HTH-type transcriptional regulator GltC	GO:0003677	3095376-3096278
	gdhA	Glutamate dehydrogenase	GO:0006520	2300858-2299479
	asnB	Asparagine synthetase	GO:0006541	3096385-3098229
	pyrG	CTP synthase	GO:0006541	3404454-3402853
Phosphate metabolism				
	ppaC	Manganese-dependent inorganic pyrophosphatase	GO:0005737	727813-728745
	phoA	Alkaline phosphatase	GO:0016791	627314-625959
	phoB1	Two-component system, OmpR family, alkaline phosphatase synthesis response regulator PhoP	GO:0006355	1546461-1547156
	phoR	Two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR	GO:0006355	1547153-1548529
	phoH	Phosphate starvation-inducible protein PhoH and related proteins	GO:0005524	1730983-1731942
	pstS	PstS family phosphate ABC transporter substrate-binding protein	GO:0042301	3588504-3589496
	pstC	Phosphate ABC transporter permease subunit PstC	GO:0006817	3589588-3590541
	pstA	Phosphate ABC transporter permease PstA	GO:0035435	3590544-3591428
	pstB	Phosphate import ATP-binding protein PstB	GO:0005886	3591505-3592341
Potassium transport				
	cvrA	Potassium/proton antiporter	GO:0006813	3592714-3594207

(Continued)

TABLE 4 (Continued)

PGP traits	Gene IDs	Gene annotations	GO IDs	Chromosome location
Siderophore				
	-	ABC transporter permease	GO:0016021	375879-376832
	-	Iron chelate uptake ABC transporter family permease subunit	GO:0016021	376822-377772
	-	ABC transporter ATP-binding protein	GO:0005524	377766-378521
	-	ABC transporter substrate-binding protein	GO:0006826	378831-379808
	-	Iron export ABC transporter permease subunit fetb	GO:0016021	507556-506792
	perR	Ferric uptake regulator, Fur family	GO:0003677	698241-698684
	-	Iron complex transport system substrate-binding protein	-	1096047-1097045
	-	Iron complex transport system permease protein	-	1097017-1098075
	-	Iron complex transport system ATP-binding protein	-	1098075-1099562
	fhuB	Iron ABC transporter permease	GO:0016021	2308243-2306216
	fhuD	Iron-siderophore ABC transporter substrate-binding protein	-	2309249-2308248
	-	Iron export ABC transporter permease subunit FetB	GO:0016021	2748819-2749649
	FecCD	Iron ABC transporter permease	GO:0016021	3012286-3011234
	afuB	Ferric transport system permease protein	GO:0055085	2962193-2960523
	afuC	Fe(3+) ions import ATP-binding protein	-	2963319-2962156
	afuA	Extracellular solute-binding protein	-	2964360-2963344
	-	ABC transporter ATP-binding protein	GO:0005524	3011187-3010357
	-	Iron ABC transporter permease	GO:0016021	3012286-3011234
	-	Iron ABC transporter permease	GO:0016021	3013281-3012283
	-	ABC transporter substrate-binding protein	-	3013486-3014466
	afuB	Iron ABC transporter permease	GO:0055085	3191977-3190292
	afuC	ABC transporter ATP-binding protein	GO:0043190	3193081-3191978
	afuA	Iron ABC transporter substrate-binding protein	-	3194230-3193124
	-	Iron(3+)-hydroxamate import ATP-binding protein FhuC	GO:0005524	3687309-3688151
	-	Iron(3+)-hydroxamate-binding protein FhuD	-	3688105-3689034
	-	Iron(3+)-hydroxamate import system permease protein FhuB	GO:0016021	3689118-3690314
	-	Iron(3+)-hydroxamate import system permease protein FhuG	GO:0016021	3690311-3691318
Plant hormones				
IAA biosynthesis	*trpA*	Tryptophan synthase alpha chain	-	338845-338054
	trpB	Tryptophan synthase beta chain	GO:0004834	340051-338846
	trpF	Phosphoribosylanthranilate isomerase	-	340649-340020
	trpC	Indole-3-glycerol phosphate synthase	GO:0000162	341436-340639
	trpD	Anthranilate phosphoribosyltransferase	GO:0000162	342462-341437
	trpG	Anthranilate synthase component II	GO:0006541	343045-342446
	trpE	Anthranilate synthase component I	GO:0000162	344426-343026
	trpS	Tryptophanyl-tRNA synthetase	GO:0006436	965341-964346
Root colonization chemotaxis, motility, biofilm	*fliA*	RNA polymerase sigma factor for flagellar operon FliA	GO:0006352	2078804-2078028
	cheD	Chemotaxis protein CheD	GO:0006935	2079416-2078919
	cheC	Chemotaxis protein CheC	GO:0016787	2080044-2079409
	cheW	Purine-binding chemotaxis protein CheW	GO:0007165	2080510-2080049

(Continued)

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TABLE 4 (Continued)

PGP traits	Gene IDs	Gene annotations	GO IDs	Chromosome location
	cheB	Two-component system, chemotaxis family, protein-glutamate methylesterase/glutaminase	GO:0006935	2081589-2080549
	flhG	Flagellar biosynthesis protein flhG	-	2082459-2081596
	flhF	Flagellar biosynthesis protein flhF	GO:0044781	2083573-2082452
	flhA	Flagellar biosynthesis protein flhA	GO:0044780	2085603-2083570
	flhB	Flagellar biosynthetic protein flhB	GO:0044780	2086702-2085623
	fliR	Flagellar biosynthetic protein fliR	GO:0044780	2087483-2086704
	fliQ	Flagellar biosynthetic protein fliQ	GO:0044780	2087756-2087487
	fliP	Flagellar biosynthetic protein fliP	GO:0009306	2088457-2087792
	fliOZ	Flagellar protein fliO/fliZ	GO:0044781	2089091-2088450
	cheY	Two-component system, chemotaxis family, chemotaxis protein cheY	GO:0000160	2089468-2089106
	fliNY	Flagellar motor switch protein fliN/fliY	GO:0071973	2090624-2089488
	fliM	Flagellar motor switch protein fliM	GO:0071973	2091612-2090614
	fliL	Flagellar flil protein	GO:0071973	2092064-2091645
	flbD	Flagellar protein flbD	-	2092272-2092057
	flgE	Flagellar hook protein flgE	GO:0071973	2093179-2092319
	-	Flagellar protein	-	2093634-2093263
	flgD	Flagellar basal-body rod modification protein flgD	-	2094104-2093655
	fliK	Flagellar hook-length control protein fliK	-	2095376-2094114
	fliJ	Flagellar fliJ protein	GO:0071973	2096441-2095995
	fliI	Flagellum-specific ATP synthase	GO:0071973	2097760-2096447
	fliH	Flagellar assembly protein fliH	-	2098545-2097757
	fliG	Flagellar motor switch protein fliG	GO:0071973	2099524-2098511
	fliF	Flagellar M-ring protein fliF	GO:0071973	2101132-2099537
	fliE	Flagellar hook-basal body complex protein fliE	GO:0071973	2101495-2101190
	flgC	Flagellar basal-body rod protein flgC	GO:0071973	2101958-2101512
	flgB	Flagellar basal-body rod protein flgB	GO:0071973	2102351-2101962
	motA	Chemotaxis protein motA	GO:0016021	1483620-1484444
	motB	Chemotaxis protein motB	GO:0016021	1484434-1485222
	pilB	Type IV pilus assembly protein pilB	-	1517804-1519432
	pilT	Twitching motility protein pilT	GO:0005524	1519445-1520485
	pilC	Type IV pilus assembly protein pilC	GO:0009306	1520488-1521687
	pilA	Type IV pilus assembly protein pilA	-	1521849-1522235
	pilM	Type IV pilus assembly protein pilM	-	1523108-1524025
	hofN	Pilus assembly protein hofN	GO:0016021	1524038-1524589
	-	Pilus assembly protein, pilO	GO:0016021	1524570-1525112
	fliT	Flagellar protein fliT	-	2541582-2541229
	fliS	Flagellar protein fliS	GO:0044780	2541983-2541582
	flaG	Flagellar protein flaG	-	2542403-2542038
	fliW	Flagellar assembly factor fliW	GO:0044780	2543144-2542704
	flgL	Flagellar hook-associated protein 3 flgL	GO:0071973	2544659-2543790
	flgK	Flagellar hook-associated protein 1 flgK	GO:0071973	2546184-2544670
	fglN	Flagellar protein flgN	GO:0044780	2546703-2546209

(Continued)

TABLE 4 (Continued)

PGP traits	Gene IDs	Gene annotations	GO IDs	Chromosome location
	flgM	Negative regulator of flagellin synthesis flgM	GO:0045892	2546978-2546718
	cheY	Two-component system, chemotaxis family, chemotaxis protein cheY	GO:0000160	2590918-2590562
	cheW	Purine-binding chemotaxis protein cheW	GO:0007165	2591400-2590918
	cheA	Two-component system, chemotaxis family, sensor kinase cheA	GO:0006935	2593417-2591411
	cheX	Chemotaxis protein cheX	-	2593903-2593442
	motB	Chemotaxis protein motB	GO:0016020	2594695-2593919
	motA	Chemotaxis protein motA	GO:0016021	2595473-2594682
	fliD	Flagellar hook-associated protein 2	GO:0071973	2600255-2598189

keto-acid metabolism (*ilvABCDEH*), 2,3-butanediol catabolism (*acuABC*), and Isopentenyl-diphosphate delta-isomerase (*idi*), which may be related to the biocontrol mechanism of strain ASH15.

Biofilm-related genes in strain ASH15

The motility of bacteria is another important feature that enables them to move, colonize, and systematically spread in plants (Palma et al., 2022). The motility ability of strain ASH15 allows it to move through the soil matrix and into the plant, as confirmed by the genes involved in the flagella biosynthesis and assembly such as *flhA*, *flhB*, *flhF*, *flhG*; *fliAROPLJIHFTSW*, *flbD*; flagellar proteins *fliO/fliZ* and *flbD*; flagellar motor switch proteins, *fliN*, *fliY*, *fliM*, and *fliG*; and flagellar hook associated protein, *fliDEK* and *flgBCDEMN*, and two sets of genes coding for the flagellar motor proteins (*motA* and *motB*) (Table 4). Genome analysis of the strain ASH15 showed that two genes *hofN* and *pilBCAM* are involved in the biosynthesis and assembly of the type IV pilus system (T4PS): (Table 4).

Secretion systems of strain ASH15

Bacteria have a set of different protein secretion systems that are essential for their growth and plant interaction. Bacteria secrete secondary metabolites, peptides, antibiotics, enzymes, and toxins to compete with nearby microbes or interact with host plants (Netzker et al., 2015; Köhl et al., 2019). Among the bacterial secretion systems, to transport proteins across the plasma membrane, the twin-arginine translocation (Tat) and general secretion (Sec) pathways are most commonly in use (Natale et al., 2008). The Tat pathway is mostly used to secrete folded proteins, while the Sec pathway primarily secretes unfolded proteins (Natale et al., 2008; Green and Mecsas, 2016). In this study, the strain ASH15 genome demonstrated five types of secretion systems: Type II/Type IV, Type III, Type VII (*yukDC*; ESX secretion system), and twin-arginine translocase (*tatAEC*). The operon *acuABC* encodes proteins to utilize acetoin and butanediol as carbon sources for energy requirements (Thanh et al., 2010). The presence of operon *acuABC* confirms that strain ASH15 has the ability to metabolize complex cyclic organic compounds and could be utilized for bioremediation.

Genome mining for biosynthetic gene clusters and metabolic system analysis

The PGPR secretes bioactive secondary metabolites in the soil (rhizosphere-niche), which are related to plant-microbe interaction and root colonization as well as play a significant role in the plant immune response (Backer et al., 2018; Sharma et al., 2019; Bukhat et al., 2020; Jamali et al., 2020). The antimicrobial potential of strain ASH15 to produce hydrolytic enzymes and siderophores was confirmed by genome analysis (Tables 4, 5). The biosynthesis potential of the halophilic PGPR was assessed using antiSMASH 6.0.0 to predict both known and unidentified functional secondary metabolites in order to better understand its antagonistic action. Results showed (Figure 7) that five biosynthetic gene clusters were present in the genome: the first cluster of T3PKS (45 genes), the second cluster of terpenes (23 genes), the third cluster of T3PKS (48 genes), the fourth cluster of ectoine (9 genes), and the fifth cluster of terpenes (17 genes) (Figure 7).

Carbohydrates play several important roles in different biological functions. Carbohydrate activity enzymes derived from various species are divided into glycoside hydrolases (GHs), polysaccharide lyases (Ls), glycosyltransferases (GTs), auxiliary activities (AAs), carbohydrate-binding modules (CBMs), carbohydrate esterases (CEs), and other six major protein families. CAZyme analysis resulted in the identification of 80 genes in the genome ASH15 and annotated them (Figure 6, Supplementary Table 4) as 9 genes for AAs, 22 genes for CEs, 25 genes for GHs, and 24 genes for GTs.

Other important genes identified in the genome

V. halodenitrificans strain ASH15 is an endospore-forming halophilic bacterium. Table 5 shows the predicted and identified genes (gene IDs, annotations, GO IDs, and chromosome locations) related to spore formation and spore germination in the genome of strain ASH15.

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TABLE 5 Sporulation/germination genes in *Virgibacillus halodenitrificans* strain ASH15.

Characteristics	Gene IDs	Gene annotations	GO IDs	Chromosome location
Sporulation/ germination	*yaaH*	Spore germination protein YaaH	GO:0005975	39995-38697
	gerD	Spore gernimation protein GerD	-	183600-182965
	yndD	Spore germination protein GerLA	GO:0009847	521045-522601
	yndE	Spore germination protein GerLB	GO:0009847	522603-523715
	yndF	Spore germination protein GerLC	GO:0009847	523804-524892
	yfkR	Spore germination protein GerQC	GO:0009847	841055-839922
	GerAB	GerAB/ArcD/ProY family transporter	GO:0009847	842167-841052
	GerA	Spore germination protein	GO:0009847	843668-842175
	gerPF	Spore germination protein PF	-	935193-934975
	gerPE	Spore germination protein PE	-	935623-935255
	gerPD	Spore germination protein PD	-	935809-935639
	gerPC	Spore germination protein PC	-	936391-935825
	gerPB	Spore germination protein PB	-	936670-936470
	gerPA	Spore germination protein PA	-	936897-936685
	gerKA	Spore germination protein KA	GO:0009847	1041166-1042710
	-	Spore germination protein YndE	-	1042694-1043785
	-	Spore germination protein GerQC	GO:0009847	1043782-1044918
	-	Spore germination protein XB	GO:0009847	1447960-1449033
	-	Spore germination protein XA	GO:0009847	1449030-1450457
	-	Spore germination protein XC	-	1450468-1451592
	gerE	Spore germination protein GerE	GO:0006355	1598214-1597990
	gerM	Spore germination protein GerM	-	1600644-1601720
	gpr	Germination protease	GO:0009847	1710456-1711565
	spoVAF	Spore germination protein	GO:0009847	1767148-1768605
	spoIIAA	Stage II sporulation protein AA	GO:0030435	1850841-1851194
	spoIIAB	Stage II sporulation protein AB	GO:0030435	1851191-1851631
	sigH	RNA polymerase sporulation-specific sigma factor	GO:0006352	1851640-1852392
	spoVAA	Stage V sporulation protein AA	GO:0016021	1852857-1853471
	spoVAB	Stage V sporulation protein AB	GO:0016021	1853452-1853877
	spoVAF	Stage V sporulation protein AF	GO:0009847	1853898-1855379
	cwlJ	Cell Wall Hydrolase	GO:0009847	1880202- 1881020
	ypeB	YpeB sporulation	GO:0009847	1881035-1882378
	gerQ	Spore coat protein GerQ	-	3432264-3432716
	spoIIID	Stage III sporulation protein D	GO:0003700	3352132-3351839
	spoIIQ	Stage II sporulation protein Q	GO:0016021	3355008-3354121
	spoIID	Stage II sporulation protein D	-	3357042-3355873
	tasA	Spore coat-associated protein	-	2562595-2562038
	tasA	Spore coat-associated protein	GO:0051301	2563259-2562663
	tagT_U_V	Polyisoprenyl-teichoic acid–peptidoglycan teichoic acid transferase	GO:0070726	2564384-2565328
	tagT_U_V	Polyisoprenyl-teichoic acid–peptidoglycan teichoic acid transferase	GO:0016021	2786947-2786009
	ltaS	Lipoteichoic acid synthase	GO:0016021	2584822-2582792
	tagA	WecB/TagA/CpsF family glycosyltransferase	GO:0071555	3280584-3279856

(Continued)

TABLE 5 (Continued)

Characteristics	Gene IDs	Gene annotations	GO IDs	Chromosome location
	tagT_U_V	Polyisoprenyl-teichoic acid–peptidoglycan teichoic acid transferase	GO:0016021	3280791-3281831
	tagH	Teichoic acids export ATP-binding protein TagH	GO:0005886	3293390-3292077
	tagG	Teichoic acid translocation permease protein TagG	GO:0055085	3294228-3293407
	tagF	Teichoic acid poly(glycerol phosphate) polymerase	-	3296463-3294349
	inlB	SH3-like domain-containing protein	-	3300922-3298235
	lytD	SH3-like domain-containing protein	-	3304409-3301056
	tagD	Glycerol-3-phosphate cytidylyltransferase	-	3304845-3305243
	tagB	CDP-glycerol glycerophosphotransferase family protein	-	3305236-3306417
	tagD	Glycerol-3-phosphate cytidylyltransferase	-	3306522-3306920
	tagB	CDP-glycerol glycerophosphotransferase family protein	-	3306910-3308055

Additional findings in the genome

Small RNAs play a significant role in metabolic pathway regulation and are thus very important. In the present investigation genome-wide analysis predicted a total of 120 sRNAs, which constitute 16,299 bases and 0.4252% of the strain ASH15 genome (Supplementary Table 2). We also identified tandem repeat sequences in the genome of strain ASH15. A total of 220 repeats were identified, which constitute 55,602 bases and 1.73% of the genome. Interspersed repeats, also known as transposable elements (transposon), includes DNA transposons and retrotransposons transposed by DNA-RNA. Common retrotransposons (also called Class I transposable elements or transposons via RNA intermediates) are LTR, LINE, and SINE. Genome analysis found that the strain has 6-SINE, 16-LINE, and 5 transposons, constituting 1,733 bases and 0.05% of the genome. Bacterial sRNA is a type of non-coding RNA with a length of 50 to 500 nt. They are mostly found in the intergenic region, while some are found in the coding genes' 5′ and 3′UTR sections. Bacterial sRNA mainly performs a variety of biological functions by binding to target mRNA or target protein. For example, bacterial sRNA plays an important role in regulating outer membrane protein expression, iron ion balance, community sense, and bacterial pathogenicity. Tandem repeats refer to the occurrence of two or more repeats in adjacent positions of the genome.

Analysis of movable components of the genome

In the long evolutionary process, to adapt to changes in the environment or improve their own survival competitiveness, bacterial genomes often take in some foreign gene fragments and integrate them into their own genomes. These fragments generally contain some genes encoding specific functions, such as virulence genes, drug resistance genes, and metabolic genes, etc., which can change the phenotype of bacteria and help bacteria get through "difficulties" or occupy a dominant niche. These exogenous genome fragments are collectively called mobile elements; this phenomenon is called horizontal gene transfer (HGT). In the present genome analysis, three types of movable elements were predicted in strain ASH15: Genome Island, Prophage, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Prophage is a bacteriophage genome integrated into the circular genome of the host bacteria (Piligrimova et al., 2021). As a ubiquitous mobile genetic element, prophage plays a key role in bacterial genetics, evaluation, and increasing survival and virulence potential through multiple mechanisms (Ramisetty and Sudhakari, 2019).

Conversely, the CRISPR system provides inherited and acquired sequence-specific adaptive immunity against the phage and other horizontally acquired elements like plasmid. CRISPR-associated (Cas) proteins constitute an RNA-guided adaptive immune system found in several prokaryotes. It is not only a type of bacteriophage defense system but also a regulator of bacterial physiology (Newsom et al., 2021). In recent times, to fulfill the demand for the next generation of industrial biotechnology (NGIB), the CRISPR/Cas system has been evaluated as a potential editing tool for customizing and reprogramming the genome of many extremophilic bacterial species for the production of natural compounds (Cress et al., 2016; Qin et al., 2018; Singh et al., 2021).

Insertion sequences

The insertion sequence is a transposon encoding the enzyme required for transposition, and it is flanked by short, inverted terminal repeats. A total of 9 IS were identified in the genome of ASH15 using ISEScan software (Supplementary Figure 1).

Genome island analysis

Genome island (GI) is one of the most important forms of horizontal transfer elements. It contains genes related to

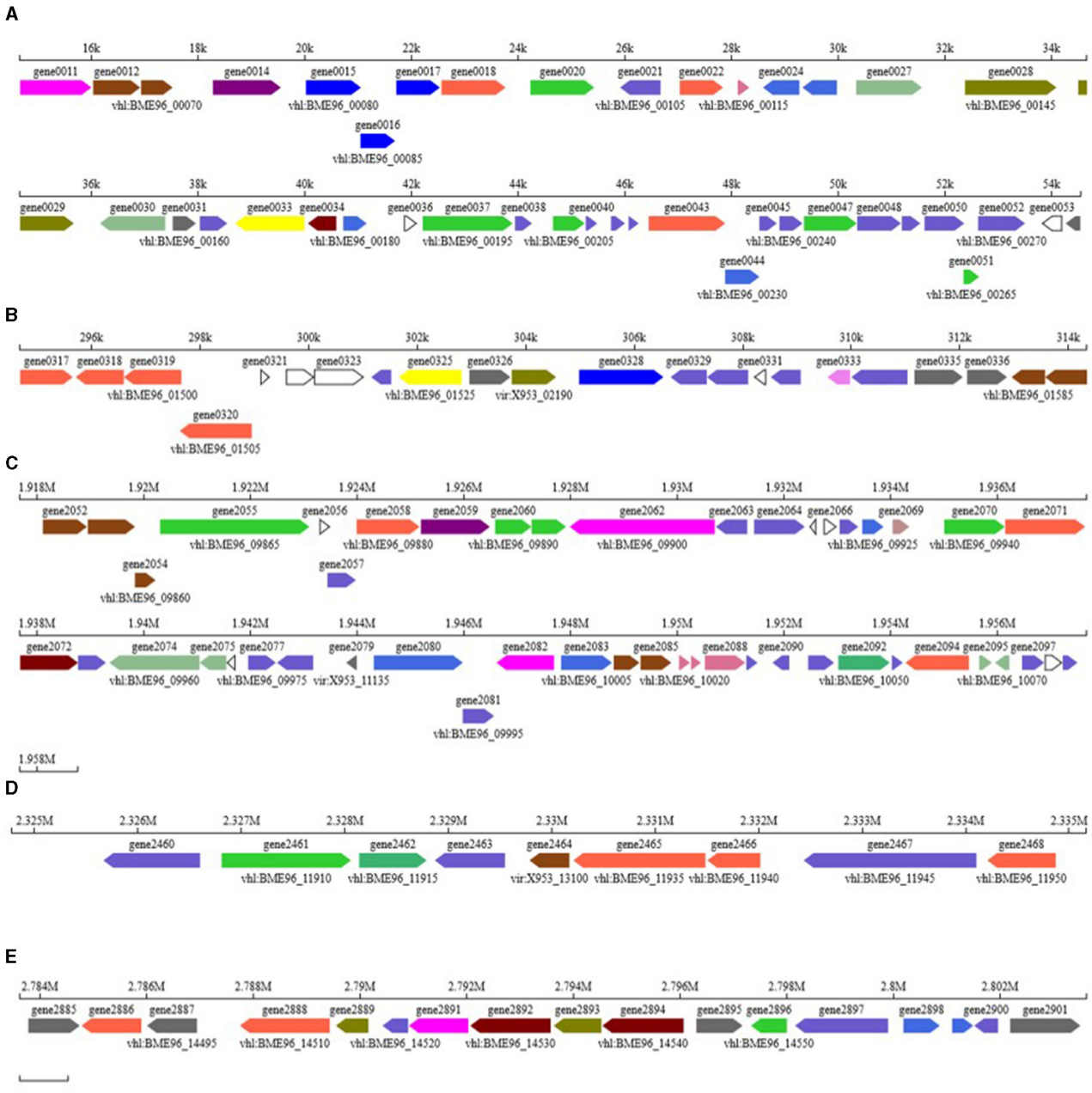

FIGURE 7
Biosynthesis gene clusters identified in the genome of *Virgibacillus halodenitrificans* strain ASH15. **(A)** T3PKS cluster with 45 genes; **(B)** terpene cluster with 23 genes **(C)** T3PKS cluster with 48 genes **(D)** ectoine cluster with 9 genes, and **(E)** terpene cluster with 17 genes.

a variety of biological functions. According to the different genes, genome islands can generally be divided into virulence, drug-resistant, metabolic, symbiotic islands, among others. Genome islands are usually large, ranging from 10 to 200 kb. A total of 7 GIs were predicted in the genome, which constituted 162,069 bases, where the smallest island was 8,201 bases and the largest was 82,517 bases. The island genome has coded for 167 CDS, mainly belonging to proton transporters, iron transport, sugar transporters, chaperons, and many hypothetical proteins (Supplementary Figure 2 and Supplementary Table 3).

Two-component systems identified in the genome of strain ASH15

Deep genome analysis revealed that in the strain ASH15 cell several two-component systems (nre*BC*, css*SR*, lia*SR*, pho*BR*, res*DE*, che*AY*, deg*US*, cit*TS*, yes*MN*, des*RKA*, wal*RK*, and vic*KR*) working, which play an important role in the modulation and regulation of the expression of critical proteins under stressful conditions. Specifically, nre*BC* is involved in nitrogen metabolisms, css*SR* is a sensory system, pho*BR* is associated with the phosphate metabolism mechanism, and histidine kinase sensor response

regulators (res*DE*, yes*MN*, and desRK) are responsible for various cellular processes. Additionally, vicKR is a regulator for cell wall metabolism.

LiaSR two-component system

Strain ASH15 has a *LiaIFRS* operon in the genome. The Lia*SR* is a two-component system widely found in Gram (+) bacteria. The Lia*SR* system is most studied in *Bacillus subtilis* as a part of the *LiaIHGFSR* operon. A striking characteristic of the Lia*SR* system is that its expression is induced upon exposure to antibiotics that target the cell envelope (Jordan et al., 2006; Suntharalingam et al., 2009; Shankar et al., 2015).

CheAY, two-component system

CheAY two-component system plays a regulatory role in the signal transduction of chemotaxis (Zschiedrich et al., 2016). The CheY and CheA system are well studied in *B. subtilis* and *E. coli* (Rao et al., 2004; Minato et al., 2017). In *B. subtilis*, the autophosphorylating activity of CheA increases through the binding of attractants to transmembrane receptors (Karatan et al., 2001). Phosphorylated CheA donates the phosphate to the response regulator CheY (Wang et al., 2014). The CheY that has been phosphorylated interacts with the flagellar motor switch complex to insist the flagella rotate counterclockwise (CCW), which induces a smooth swimming motion (Minamino et al., 2019; Mukherjee et al., 2019).

DegUS two-component system

DegUS two component system are identified in strain ASH15; it controls degradative enzyme synthesis. DegUS is involved in the complex network that mediates the regulation of transition state-specific processes. It contributes to controlling the development of natural competence for DNA uptake, motility, and degradative enzyme synthesis (Meliawati et al., 2022).

CitST two-component system

The CitST system [carbon catabolite repression (CCR)] is involved in citrate fermentation metabolism and citrate/succinate transport. This two component system has also been studied in other bacterial genomes (Repizo et al., 2006).

A unique finding in the genome of strain ASH15

Isoprenoid (squalene/phytoene) biosynthesis pathway

Genome analysis revealed that strain ASH15 has an MVA pathway to synthesize isoprenoids. The MVA pathway starts with the condensation of two acetyl-CoA molecules followed by a series of reduction (six) steps that produce IPP involving the expression (Figure 7) of genes: acs*A* (gene number 0346), HMG-CoA Synthase (gene number 00029; mva*S*), HMG-CoA reductase (gene number 0215; mvaE), mevalonate kinase (gene number 0216; mvaK1), phosphomevalonate kinase (gene number 0218; mva*K2*), and diphosphomevalonate decarboxylase (gene number 0217; mva*D*) (Figure 7). IPP and DMAPP are isomers, and inter-conversion is catalyzed by the enzyme isopentenyl-diphosphate Delta-isomerase (IdI). id*I* (gene number 0398) is the key regulatory gene that maintains the IPP to DMAPP ratio and has a significant impact on isoprenoid biosynthesis. The gene (gene number 1929), which codes for geranylgeranyl diphosphate synthase (GGPPS), converts farnesyl diphosphate (FPP) to geranylgeranyl diphosphate (GGPP). Farnesyl diphosphate (FPP) is an intermediate molecule that could be converted to many different terpenoids (Rinaldi et al., 2022), while GGPP could be further converted to diterpenoids and tetraterpenoids. Strain ASH15 has genes (SQS_PSY; 0327 and SQS_PSY; 2893) to produce triterpenoids and tetraterpenoids, Squalene (C30) and Phytoene (C40). Squalene synthase (gene number 0327) converts two molecules of FPP to squalene, and phytoene synthase (gene number 2893) could convert GGPP to Phytoene.

Squalene has been known for its various applications, such as an anti-cancer agent, an anti-oxidant agent, an anti-bacterial agent, a chemopreventive agent, an anti-aging agent, a detoxifier, and an adjuvant for drug carriers and vaccines (Kim and Karadeniz, 2012; Paramasivan and Mutturi, 2022). Therefore, it has enormous potential in the food, cosmetics, and pharmaceutical industries (Huang et al., 2009; Gohil et al., 2019). The global squalene market demand in 2014 was approximately 2.67 kilotons, and by 2022, it is estimated to reach a value of USD$ 241.9 million, with the majority of sales coming from personal care and cosmetic items (Rosales-Garcia et al., 2017). There is a pressing need to generate squalene in a renewable and sustainable way to meet this ever-increasing demand. The development of microbial cell factories could be a solution to fulfill this demand, and strain ASH15 is a natural bacterium that has all the genes for the biosynthesis of squalene.

Conclusion

According to the findings of the present study, the availability of *V. halodenitrificans* ASH15's entire genome will shed additional light on complex biological systems, which showed that strain ASH15 has open a number of opportunities to study this efficient plant growth-promoting bacterium. These results indicate that strain ASH15 may be used as a possible eco-friendly bioresource alternative for chemical fertilizers to promote plant growth in salt stressed agriculture area. However, the usability of *V. halodenitrificans* ASH15 under field trials is required for establishing it as a potential plant growth promoter for utilizing in sustainable agriculture under saline conditions.

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/nuccore/CP090006.1/.

Author contributions

AS, X-PS, and Y-RL conceived the idea and designed the experiments. AS performed the experiments and wrote the original draft of the manuscript. RNS assisted in analysis and software support. RS, PS, and D-JG assisted in the experiments. X-PS contributed to resource management. KV and Y-RL critically revised the manuscript. 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/fmicb.2023.1229955/full#supplementary-material

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