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Molecular characterization of humic substances and regulatory processes activated in plants, 2nd Edition

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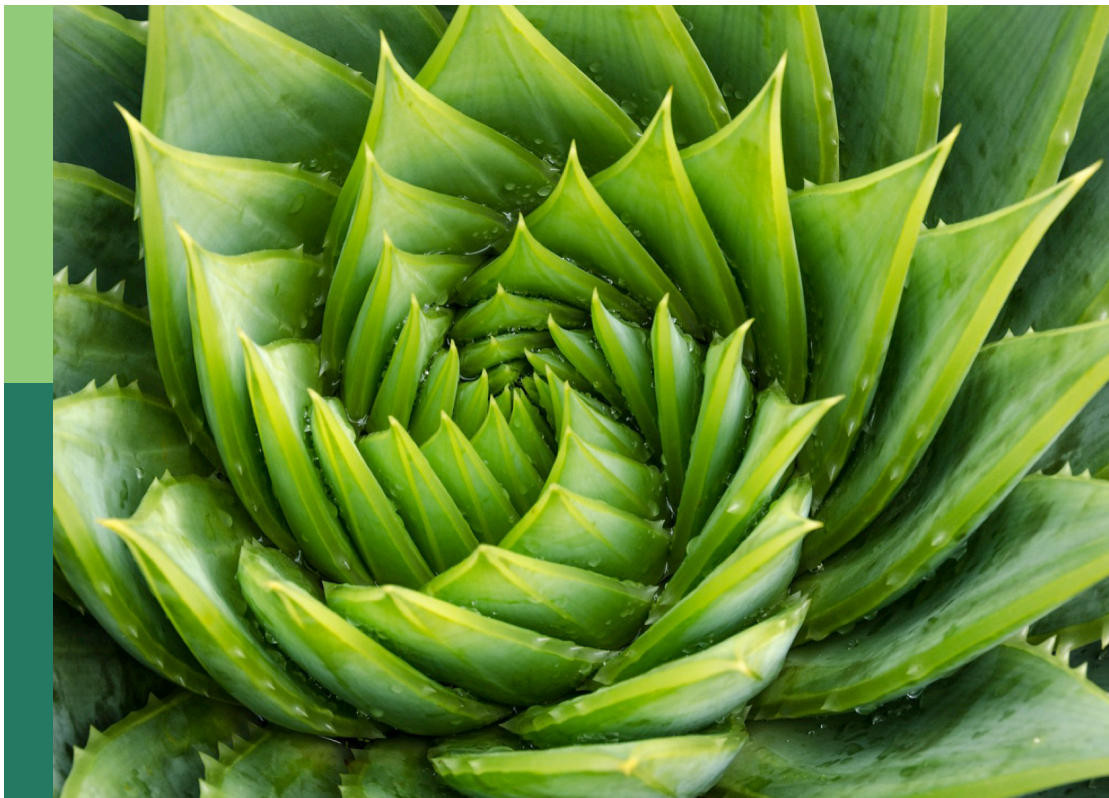

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Molecular characterization of humic substances and regulatory processes activated in plants, 2nd Edition

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EDITORIAL
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Editorial: Molecular Characterization of Humic Substances and Regulatory Processes Activated in Plants

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Keywords: humic substances (HS), biostimulants, microbiome, soil quality (SQ), plant nutrition and metabolism

Editorial on the Research Topic

Molecular Characterization of Humic Substances and Regulatory Processes Activated in Plants

The intense crop fertilization along with to climate change have a negative synergistic impact on soil fertility by decreasing the soil organic matter content and biological activity. As a result of these processes, plant productivity, and quality could be harshly impaired in agroecosystems. This is of global concern, particularly considering that a world with zero hunger is a challenge set in the 2030 Agenda for Sustainable Development, and that the COVID-19 pandemic has aggravated the condition of vulnerable populations. In this context, green and sustainable strategies could be endorsed to boost crop productivity and improve soil fertility. In particular, the role of humic substances (HS) as activators of plant development and metabolism, as well as stimulators of beneficial rhizosphere microorganisms attains great attention. Thus, the present topic collection encompasses studies that investigate HS action in either small-scale or agronomic trials.

One area of research is the link between structure of HS and their activity. Monda et al. studied the biostimulant properties of a sedimentary shale ore-extracted humic acid (HA) on tomato plants grown under increasing nutritional stress. The authors investigated the chemical features of HA and found that HA alleviated the nutritional stress of plants by improving their nutrient use efficiency more than plants fertilized with high NPK. Yield and fruit quality were enhanced by HA. Pizzeghello et al. showed that treating HS with a weak acid generated low and high sized-fractionated HS with greater bioactivity, because of novel molecular arrangements of HS components that better interacted with roots. Also, the the cover vegetation of the soils from where HS were obtained affected their bioactivity. HS were applied to garlic and stimulated plant growth and nutrition. Lamar et al. studied the effects of seven ore-derived HA on maize. Chemometric analyses evidenced that the primary driver of plant biomass and morphology was the ratio between HA electron accepting capacity (EAC) and electron donating capacity (EDC). The HA EAC was due to quinones and semiquinone free radicals, while the HA EDC was ascribed to polyphenolics and glycosylated polyphenolics. From this manuscript emerges a mechanism of action for ore-derived HA biostimulation that involves the interplay of pro-oxidants and antioxidants. This manuscript also indicates how the EAC/EDC ratio can be adjusted to a proportion to produce seedlings with desirable qualities, providing evidence of methods to produce more efficient HAs.

Several studies have shown the role of the endophytic microbiome in the evolvement of relevant metabolic effects within the plant. In this framework is plausible that the mechanisms responsible for the beneficial action of HS on plant growth involve some previous action on the endophytic

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microbiome. The results presented by Della Lucia, Bertoldo, Broccanello et al. strongly support this hypothesis. The authors observed that the foliar application of a formulation obtained from leonardite on sugar beet plants cultivated either in hydroponics or open field, caused the increase of a specific family of endophytes, *Oxalicibacterium* spp., that has a known plant growth promoting effect. These results were associated with the upregulation of genes involved in the auxin-dependent signaling pathways and yield increases. The results reported by De Hita, Fuentes, Fernández et al. also support a role of endophytic microbiome in the mechanisms responsible for the humic acid beneficial action on plant growth, since many of the culturable endophytes activated by the application of a leonardite humic acid in cucumber have relevant plant biostimulant traits such as the biosynthesis of cytokinins and auxins, the production of organic acids and siderophores that are involved in iron and phosphorous mobilization in soil, as well as the ability to grow without nitrogen available in the nutrient media.

Galambos et al. investigated the growth-related processes, bacterial colonization, and transcriptional responses activated by the combined applications of endophytic bacterial strains and HA in tomato roots and shoots, and indicates the optimization of dosages, complementation properties, and gene markers for the production of efficient PGPB- and HA-based biostimulants.

Della Lucia, Baghdadi, Mangione et al. described the effects of a biostimulant on tomato plants grown in well-watered and drought conditions. The biostimulant applied to roots increased the photosynthetic rate and the chlorophyll content of plants under drought, compared to the standard fertilizer, led to higher fruit dry matter and reduction in the number of cracked fruits, and improved the resistance of tomato to drought. De Hita, Fuentes, Zamarreño et al. evaluated the mechanisms of action of foliar application vs. root application of a sedimentary humic acid (SHA). Six markers related to plant phenotype, plant morphology, hormonal balance and root-plasma membrane H^+- ATPase were studied. Both application methods improved the plant growth, the concentrations of jasmonic acid and jasmonoyl-isoleucine and indole-3-acetic acid in roots and cytokinins in shoots. Foliar application did not lead to short-term increases in abscisic acid root-concentration and root plasma membrane H^+-ATPase activity, which were instead triggered by SHA root-application. This study suggest that the beneficial effects of SHA may result from plant adaptation to a mild transient stress caused by SHA application.

Different studies also evidenced the efficacy of HS as possible amendments, but few field researches considered the environmental factors of HS efficacy. Olk, Dinnes, Scoresby et al. evaluated the spatial and temporal variability in the efficacy of a micronized humic product on maize growth and grain yield in two rainfed fields supporting a maize–soybean rotation. Application of the humic product during four maize seasons evidenced that grain quality remained unchanged. Protein, starch, nutrient, and oil content showed only few significant responses to humic product application, while increases in agronomical maize traits were observed.

Field evaluations of commercial humic products have seldom involved replication across location or year. To evaluate the consistency of HS efficacy in field conditions, by Olk, Dinnes, Callaway et al. determined the effects of a humic product on maize growth in high-yielding Midwestern fields through replicated strip plots in five site-year combinations, and through demonstration strips in 30–35 production fields annually for 2009–2011 that covered major areas of Iowa. Olk, Dinnes, Callaway et al. demonstrated the capability of a humic product to improve maize growth in high-yielding conditions. Humic product application increased total leaf area in all field treatments at three site-year combinations.

In another field study, Vujinovic et al. studied the bioactivity of dissolved HS (DHS), isolated from the conversion of conventional (CF) farming and organic (OF) farming soil leachates, in maize. DHS were collected from bare and planted soils and stimulated lateral roots proliferation, nitrate uptake, and modulated genes involved in nitrogen acquisition. Wheat roots in soil, in particular, boosted the rhizosphere biological activity, and mineralization processes. The authors demonstrated that OF and CF managements of soil influenced the characteristics of DHS and that plant roots can interact with the active molecules in the soil solution.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Biostimulant Action of Dissolved Humic Substances From a Conventionally and an Organically Managed Soil on Nitrate Acquisition in Maize Plants

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Conversion of conventional farming (CF) to organic farming (OF) is claimed to allow a sustainable management of soil resources, but information on changes induced on dissolved organic matter (DOM) are scarce. Among DOM components, dissolved humic substances (DHS) were shown to possess stimulatory effects on plant growth. DHS were isolated from CF and OF soil leacheates collected from soil monolith columns: first in November (bare soils) and then in April and June (bare and planted soils). DHS caused an enhancement of nitrate uptake rates in maize roots and modulated several genes involved in nitrogen acquisition. The DHS from OF soil exerted a stronger biostimulant action on the nitrate uptake system, but the first assimilatory step of nitrate was mainly activated by DHS derived from CF soil. To validate the physiological response of plants to DHS exposure, real-time RT-PCR analyses were performed on those genes most involved in nitrate acquisition, such as *ZmNRT2.1*, *ZmNRT2.2*, *ZmMHA2* (coding for two high-affinity nitrate transporters and a PM H^+-proton pump), *ZmNADH:NR*, *ZmNADPH:NR*, and *ZmNiR* (coding for nitrate reductases and nitrite reductase). All tested DHS fractions induced the upregulation of nitrate reductase (NR), and in particular the OF2 DHS stimulated the expression of both tested transcripts encoding for two NR isoforms. Characteristics of DHS varied during the experiment in both OF and CF soils: a decrease of high molecular weight fractions in the OF soil, a general increase in the carboxylic groups content, as well as diverse structural modifications in OF vs. CF soils were observed. These changes were accelerated in planted soils. Similarity of chemical properties of DHS with the more easily obtainable water-soluble humic substance extracted from peat (WEHS) and the correspondence of their biostimulant actions confirm the validity of studies which employ WEHS as an easily available source of DHS to investigate biostimulant actions on agricultural crops.

Keywords: dissolved organic matter, nitrate uptake, organic farming, root gene expression, soil organic matter

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INTRODUCTION

Organic farming (OF) is claimed to mitigate the impact of agricultural practices on ecosystems while satisfactorily sustaining crop yields; in this framework, the crucial role of soil organic matter (SOM) has been thoroughly investigated (Schrama et al., 2018).

The meta-analysis carried out by Bai et al. (2018) on several long-term experiments confirms that SOM content is larger in soils managed according to OF principles rather than to conventional farming (CF). However, the authors suggested that quantitative differences alone might not provide full reason for the several benefits induced by organic farming on soil resilience and on the sustainability of soil biological fertility.

Conventional farming, on the other hand, often results in reduced biological fertility with a decreased capacity of soils to support healthy crop growth. Reasons for this are still poorly understood: loss of SOM, nutrient imbalance, and massive use of agrochemicals are proven to contribute, but do not fully explain the observed outcomes. Climate change is expected to exacerbate abiotic stresses, so there is a pressing need to better understand the mechanisms of soil–plant–microorganism interactions that support the resilience of not cultivated and organically managed soils and crops (Clair and Lynch, 2010).

Dissolved organic matter (DOM) is defined as the fraction of SOM dissolved in the soil liquid phase, therefore representing the most mobile and bioavailable pool of soil organic matter. DOM includes molecules with diverse degrees of biological recalcitrance, from simple labile plant and microbial metabolites (amino acids and sugars) to more persistent compounds that have undergone biotic or abiotic transformation (humic substances). Although representing a small and variable, in time and space, fraction of SOM, DOM plays an integral role in the soil C cycle since it is claimed to regulate the mineralization of SOM and plant residues by co-metabolism and/or by triggering soil microbial biomass (SMB) into activity (Kuzyakov et al., 2000; De Nobili et al., 2001; Kemmitt et al., 2008). In addition, DOM can modulate soil nutrient cycles as it affects both the transport and microbial transformation of nitrogen (N), phosphorus, and sulphur (Zsolnay, 2003) as well as the availability of micronutrients, such as Fe and Zn (Cesco et al., 2000; Chen et al., 2004). From an environmental point of view, DOM represents a major source of dissolved C and nutrient losses in surface and subsurface waters: van Kessel et al. (2009) showed that up to 216 kg ha^{-1} $year^{-1}$ of dissolved organic carbon (DOC) and 127 kg ha^{-1} $year^{-1}$ of dissolved organic nitrogen (DON) can leach out of agricultural systems.

Abbreviations: AC, active carbonates; BD, bulk density; CEC, cation exchange capacity; CF, conventional farming; C_{org}, organic carbon; DHS, dissolved humic substances; DOC, dissolved organic carbon; DOM, dissolved organic matter; DON, dissolved organic nitrogen; FA, fulvic acids; FC, field capacity; HA, humic acids; N, nitrogen; NR, nitrate reductase; NiR, nitrite reductase; OF, organic farming; PWP, permanent wilting point; SMB, soil microbial biomass; SOM, soil organic matter; SWC, saturated water content; WEHS, water-extractable humic substances.

As the role of DOM is strictly regulated by its concentration and composition, the collection and sampling of undisturbed DOM is essential to obtain meaningful information. Chantigny et al. (2006) carried out a thorough review of methods, emphasizing that different approaches may result in the collection of different amounts and fractions of the soil solution.

Among DOM components, dissolved humic substances (DHS) have well-documented stimulatory effects on plant growth (Chen et al., 2004). The natural occurrence and role of humic substances in soils was questioned (Lehmann and Kleber, 2015) because of the harsh alkali-based procedures used for their extraction. However, the usefulness of the humic substances-based approach to understand natural organic matter processes has been recently confirmed (Olk et al., 2019). Furthermore, DHS can be obtained without the use of alkaline extractants by simply leaching soil with water. Treatment of plants with water-extractable humic substances from peat and vermicompost was shown to induce changes in root morphology and modulate nutrient acquisition, pathways of primary and secondary metabolism, and hormonal and reactive oxygen balance (Varanini and Pinton, 2001; Nardi et al., 2002; Zanin et al., 2019).

Numerous studies have been performed to understand the molecular mechanisms activated by plant exposure to humic substances. Varanini and Pinton (2001) distinguished between indirect effects (such as improved nutrient availability through metal binding) and direct effects. Among the latter, the improvement of root ion uptake capacity, and rhizosphere acidification *via* stimulation of plasma membrane H^+-ATPase, and root proliferation involving hormone-like activity have been reported for humic substances (Varanini et al., 1993; Pinton et al., 1999; Canellas et al., 2002; Nardi et al., 2002; Zanin et al., 2015a; Zanin et al., 2015b; Zamboni et al., 2016). Transcriptomic studies indicated that root exposure to humic substances induced also changes in the expression profile of genes involved in the acquisition and assimilation of several nutrients, as shown in *Arabidopsis*, rapeseed, and maize (Trevisan et al., 2011; Jannin et al., 2012; Zanin et al., 2018). These effects depend on the origin, molecular size, and chemical characteristics of humic substances (Zandonadi et al., 2013; Olaetxea et al., 2018).

A frequent criticism raised by studies on the stimulatory activity of humic substances is that the investigations carried out so far have been implemented with humic substances extracted from organic-rich substrates (e.g., sphagnum peat, vermicompost, leonardite; Aguirre et al., 2009; Zanin et al., 2018) and none has actually employed DHS from cultivated mineral soils.

Poor information is also available on the chemical properties of DOM in soils under OF vs. CF and on the relationships between SOM and DOM in calcareous soils.

The aim of the present work was to investigate the biological properties and characteristics of DHS isolated from water leached from undisturbed soil monoliths of arable mineral soils. This approach allows avoiding any potential interference of the extraction procedure (Zsolnay, 2003).

While the conversion to OF can activate a positive trend towards the increase of SOM (Gattinger et al., 2012), no evidence

for a similar trend has been noticed for DOM (Hu et al., 2018). Although OF is claimed to improve organic matter-related soil quality, evidence of the effects of OF on the amount and biological activity of humic substances is still lacking. In addition, while soil use and management have been recognized to have a significant impact on humic substances' complexity and activities (Nardi et al., 2004; Olk et al., 2019), it is not known whether the presence of a crop can affect in itself the quality and quantity of DHS.

In this work, we investigated the biostimulant actions, on root development and nitrate acquisition by maize plants, of DHS isolated at different times of the year, from a CF and an OF soil, with and without the presence of plants.

To allow comparison with previous scientific literature and eventually validate the integrity of the use of water-extractable humic substances from organic soils, biological activities and chemical properties of DHS from the examined agricultural soils were compared with those of water-soluble humic substances extracted from peat (WEHS).

MATERIALS AND METHODS

Soil Sampling and Monolith Column Setup

Soil samples were collected from two adjacent arable soils in Friuli Venezia Giulia Region (NE Italy). One site had been managed for 10 years according to OF (CE 2092/91, 834/07), while the other had been continuously managed with CF practices.

The soils examined were silty-loam Fluvisols with similar granulometric composition. Chemical and physical characteristics of the soils are given in **Table 1**. The pH (measured in water) of the two soils was alkaline and was even more alkaline in the OF soil, in agreement with its larger amount of active carbonate [7 vs. 2 g 100 g^{-1} dry weight (d.w.) in CF soil]. Both soils are characterized by low organic carbon (C_{org}) and medium cation exchange capacity (CEC).

Undisturbed soil monolith columns were collected by gently driving polyvinyl chloride (PVC) pipes (30 cm internal diameter, 70 cm long) into the soil using a hydraulic press in order to reduce the impact on soil structure; soil water potential at sampling was about 0.6 ± 0.15 MPa. A trench was dug on one side to allow cutting the soil at the bottom of pipes and placing a nylon mesh to retain the soil; a perforated lid filled with coarse sand was finally welded before removal. Monolith columns were then arranged in a greenhouse following a completely randomized scheme and placed over concrete plinths, allowing the collection of leachates in PVC vessels (**Figure 1**). The experiment featured 200 monolith columns (100 with OF and 100 with CF soil) divided into two treatments: bare or planted with *Triticum aestivum* L., cv. Capo.

To collect DOM, monolith columns were subjected to three controlled drainage events: the first one was carried out in November, on bare soils, before seeding. The following events were carried out in April and June of the following year, corresponding, respectively, to the stem elongation (stage 3) and milk development (stage 7) of wheat plants (Zadoks et al., 1974). Each lysimeter was irrigated by suspended sprinklers providing about 15 mm/h, with a total of 1.4–1.7 L of water. Leachates were collected within 36 h and corresponding treatments were pooled together. The leachates collected from organic farming soils were called OF1, OF2, and OF3, while those collected from conventional farming soils were called CF1, CF2, and CF3. The leachates OF1 and CF1 were collected in November, OF2, OF-P2, CF2, and CF-P2 were sampled in April, and OF3, OF-P3, CF3, and CF-P3 were sampled in June. OF1, OF2, OF3 and CF1, CF2, CF3 refer to leachates collected from bare soil columns; OF-P2, OF-P3 and CF-P2, CF-P3 refer to leachates collected from planted soil columns (**Figure 1**).

The concentrations at field capacity (0.33 MPa) and wilting point (1.5 MPa) of soluble humic fractions in the soil solution were then calculated taking into account the hydrological properties of the soils and the recovered weight of DHA. In the sampling in November, the nitrate concentration in the leachates (before the DHS extraction) was about 14.9 mg L^{-1} in the OF and 24.5 mg L^{-1} in the CF soil.

Isolation of DHS From Leachates

In order to isolate a sufficient amount of humic substances to carry out the plant growth and nitrate uptake experiments, leachates from replicate monoliths were pooled and 80 L of leachate was processed for each treatment. Leachates were, first of all, filtered on Whatman WCN 0.2-µm nitrocellulose membrane filters and then acidified to pH 1–2 with H_2SO_4

TABLE 1 | Main physical and chemical traits of the soils under organic farming (OF) and conventional farming (CF).

Trait and method	Unit	OF	CF
Sand (>0.02 mm)	g 100 g^{-1} d.w.	22	18
Silt (0.02 ÷ 0.002 mm)	g 100 g^{-1} d.w.	62	58
Clay (<0.002 mm)	g 100 g^{-1} d.w.	16	24
Bulk density (excavation method)	Mg m^{-3}	1.41	1.34
Saturated water content (0 MPa)	g 100 g^{-1} d.w.	37.5	40.4
Field capacity (–0.03 MPa)	g 100 g^{-1} d.w.	32.0	35.2
Permanent wilting point (–1.5 MPa)	g 100 g^{-1} d.w.	14.9	18.5
pH (H_2O)		8.5	7.6
CEC ($BaCl_2$, pH 8.2)	$cmol^+$/kg d.w.	13.0	15.3
C_{org} (Walkley-Black)	g 100 g^{-1} d.w.	0.6	1.0
Active carbonates (Drouineau)	g 100 g^{-1} d.w.	7	2

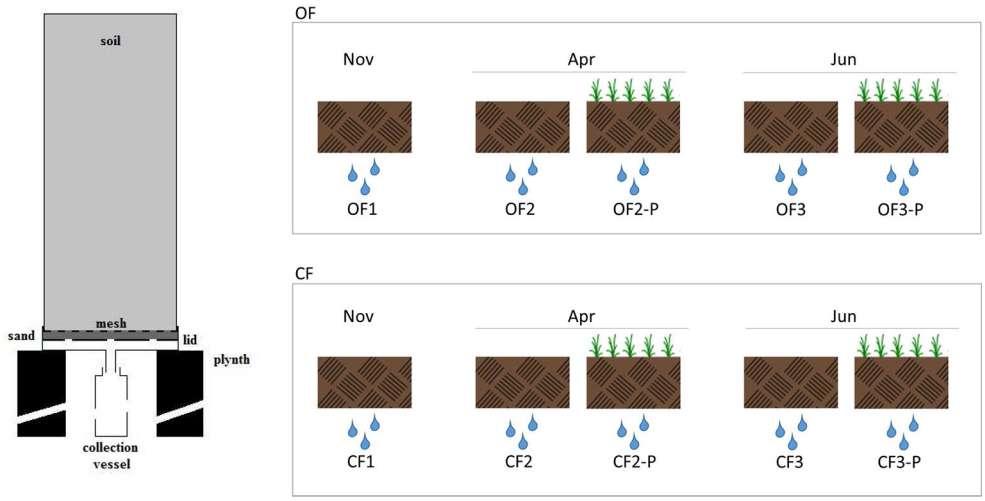

FIGURE 1 | Cross-section of the soil column and leachate collection apparatus and experimental setup used in this study. The leachates were collected from organic farming soils (OF1, OF2, and OF3) or from conventional farming soils (CF1, CF2, and CF3). OF1 and CF1 were sampled in November; OF2, OF-P2, CF2, and CF-P2 were sampled in April; OF3, OF-P3, CF3, and CF-P3 were sampled in June. OF1, OF2, OF3 and CF1, CF2, CF3 refer to leachates collected from bare soil columns. OF-P2, OF-P3 and CF-P2, CF-3 refer to leachates collected from planted soil columns.

before being loaded onto SPE columns (400 mm × 30 mm) of cross-linked polyvinylpyrrolidone. Each column was washed with double-distilled water. Adsorbed DHS were then eluted with NaOH 0.1 M. The eluates were treated with Amberlite IR-120 (H^+ from Sigma-Aldrich, Milan, Italy) to reduce ash content, adjusted to neutrality with KOH 0.1 M, and freeze-dried for storage before further analyses.

Isolation of Humic Substances From Sphagnum Peat (WEHS)

The WEHS were obtained as previously reported by Tomasi et al. (2009). Briefly, 50 ml of distilled water was added to 2.5 g of sphagnum peat (Novobalt, Lithuania) and shaken for 15 h at room temperature. The solution was filtered through a Whatman WCN 0.2-µm membrane filter and acidified to pH 1–2 with H_2SO_4. To concentrate and purify humic substances, the solution was loaded onto an Amberlite XAD-8 column (Ø 20 mm, height 200 mm; Sigma-Aldrich, Milan, Italy; Aiken et al., 1979). The column was washed with 100 ml of distilled water and the adsorbed humic substances eluted with 0.1 M NaOH. To remove exchangeable metals, WEHS were treated with Amberlite IR-120 H^+ from Sigma-Aldrich (Milan, Italy) and then adjusted to neutrality with 0.1 M NaOH. WEHS were stored as freeze-dried powder and redissolved in distilled water before use. Characterization of WEHS was reported by Tomasi et al. (2013).

Chemical Characterization of DHS

Molecular weight (MW) distributions were determined by high-performance liquid–size exclusion chromatography (HPLC-SEC) with a Bio-Rad Bio-Sil SEC 250-5 column (300 mm × 7.8 mm) and a Waters 484 Millipore UV–visible detector. The elution was performed with a 75-mM TRIS-phosphate buffer at pH 7.5 and column calibrated with a set of polystyrene sulfonate standards. Freeze-dried DHS samples were first dissolved into the TRIS-phosphate buffer at a concentration of 2 mg/ml and filtered with Minisart filters (0.20 µm). Afterwards, 20 µl of each sample was injected through a loop system into the flux of the eluting solution. The elaboration of the chromatogram obtained by recording absorbance at 400 nm allowed calculation of their molecular weight distribution.

E_{465}/E_{665} ratios were calculated from absorbances measured at 465 and 665 nm on 2 mg ml^{-1} DHS in 75 mM sodium bicarbonate buffer (pH 7.5).

Estimation of the number of carboxylic functional groups was performed using a Mettler Toledo titrator DL50 version 2.4. Freeze-dried DHS samples were dissolved in ultra-deionized deaerated Milli Q water to obtain a sample concentration of 4 mg/ml. Solutions were acidified to about pH 2 with Amberlite IR-120$^+$ and 4 ml aliquots were titrated under N_2 by addition of 0.05 ml of NaOH 0.1 M with an equilibration time of 2 min up to a maximum volume of 1.5 ml of the titrant.

Fourier transform infrared (FTIR) spectra of freeze-dried DHS (pH 7) were recorded from 4,000 to 700 cm^{-1} at a resolution of 4 cm^{-1} on KBr pellets. About 2–3 mg of oven-dried humic sample and anhydrous KBr powder (both dried for 24 h at 105°C) were mixed together, ground, and hydraulically pressed into 1-mm-thick pellets.

Plant Growth for Experiments With DHS

Maize plants (*Zea mays* L., PR33T56, Pioneer Hybrid Italia S.p.A.) were hydroponically grown as previously described by Zanin et al. (2018). Therefore, after germination over aerated 0.5 mM $CaSO_4$ solution, maize seedlings (3 days old) were transferred into an aerated hydroponic system under controlled conditions (16/8-h light/dark cycle, 220 µmol m^{-2} s^{-1} light intensity, 25/20°C temperature, 70–80% relative humidity). After 2 days, maize

plants (5 days old) were transferred to a N-free nutrient solution (in μM: $CaSO_4$, 500; KH_2PO_4, 175; $MgSO_4$, 100; NaFe-EDTA, 20; KCl, 5; H_3BO_3, 2.5; $MnSO_4$, 0.2; $ZnSO_4$, 0.2; $CuSO_4$, 0.05; Na_2MoO_4, 0.05).

After 1 h from the beginning of the light phase, nitrogen was added to nutrient solution in the form of calcium nitrate, 0.5 mM $Ca(NO_3)_2$, with or without 5 mg C_{org} L^{-1} of isolated humic substances (DHS or WEHS) as described by Pinton et al. (1999). The pH of solution was adjusted to pH 6.0 using potassium hydroxide.

The treatments lasted up to 24 h (for physiological and molecular analyses); during this time, plants were harvested and used for the analyses described below.

Measurement of Net High-Affinity Nitrate Uptake

The net influx of nitrate into roots of maize seedling was evaluated by depletion from an assay solution containing 0.2 mM KNO_3 and 0.5 mM $CaSO_4$, as described by Pinton et al. (1999). Briefly, maize seedlings were washed in 0.5 mM $CaSO_4$ and roots were incubated for 10 min in the assay solution. The assay solution was sampled (0.2 ml) every 2 min and mixed thoroughly with 0.8 ml of 5% (*w*/*v*) salicylic acid in concentrated H_2SO_4. After 20 min incubation at room temperature, 19 ml of 2 M NaOH was added to each sample. Samples were cooled to room temperature and nitrate concentrations were determined spectrophotometrically at 410 nm, as described by Cataldo et al. (1975). The net uptake rate was expressed as micromoles of nitrate per gram of root fresh weight (FW) per hour.

Real-Time RT-PCR Analyses

Real-time reverse transcription PCR (RT-PCR) analyses were performed as described by Zanin et al. (2016). Using Primer3 software (Koressaar and Remm, 2007; Untergrasser et al., 2012), primers were designed and synthesized by Sigma-Aldrich (**Supplementary Table S1**). The analyses were performed using the Opticon Monitor 2 software (Bio-Rad) and qPCR package for statistical R software (R version 2.9.0; www.dr-spiess.de/qpcR.html). For each primer, efficiencies of amplification were determined as indicated by Spiess et al. (2008). Three reference genes (*ZmRPL17*, *ZmGADPH*, and *ZmTUA*) were used to normalize the real-time RT-PCR data. Data were normalized using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analyses

Physiological and transcriptional analyses were performed on three independent biological replicates obtained from independent experiments ($N = 3$); a pool of six plants was used for each sample. Statistical significance was determined by one-way analysis of variance (ANOVA) using Holm–Sidak test ($p < 0.05$, $N = 3$). Statistical analyses were performed using SigmaPlot version 12.0 software.

DHS were isolated from pooled leachates of 50 monolith columns (80 L of pooled leachate for each treatment). Therefore, no statistical treatment of results was carried out and the reported standard deviation refers to the analytical variability of each measurement.

RESULTS

Biological Action of DHS

The biological activity of DHS isolated from organic farming soil or from conventional farming soils (OF or CF soils, respectively) were tested on maize plants after adding 5 mg C_{org} L^{-1} DHS to nutrient solution. As positive control, WEHS were used adding 5 mg C_{org} L^{-1} to nutrient solution. After 24 h, no significant changes in root growth were visible in WEHS-treated plants (N+WEHS; **Figure 2**) in comparison to control plants (N). On the contrary, DHS promoted visible root elongation and proliferation already after 24 h of treatment. Depending on their origin, some differences on the elongation and number of secondary roots were observed in the stimulatory effect of DHS. Plants treated with DHS isolated from OF soils induced a larger proliferation of secondary roots (N+OF1, N+OF2, and N+OF3; **Figure 2**). Moreover, the stimulatory action varied with sampling time as plants treated with DHS leached in June and particularly those leached from the CF soil (N+CF3) showed a lower capability to stimulate proliferation of secondary roots.

Net uptake rates of nitrate were measured on whole root systems of maize plants. After 4 h of treatment, WEHS (N+WEHS plants; **Figure 3**) promoted nitrate acquisition, doubling the capability of maize roots to take up nitrate in comparison to nitrate-treated control plants (N plants). Also, DHS isolated in autumn and spring from bare soil leachates of OF and CF soils were able to enhance the net nitrate uptake rates after 4 h (N+OF1, N+OF2, N+CF1, and N+CF2; **Figure 3**). The stimulatory effect on root nitrate uptake was also evident following application of DHS collected in June from OF bare soils (N+OF3), but DHS collected in June from CF soils did not increase the capability of plants to take up nitrate (N+CF3). DHS extracted from planted soils, irrespectively to soil management, had no stimulatory effect on root nitrate uptake (N+OF-P2, N+OFP3, N+CF-P2, and N+CFP3; **Figure 3**).

To validate the physiological response of plants to DHS exposure, real-time RT-PCR analyses were performed on those genes most involved in nitrate acquisition, as *ZmNRT2.1*, *ZmNRT2.2*, and *ZmMHA2* (coding for two high-affinity nitrate transporters and a PM H^+-proton pump) and *ZmNADH:NR*, *ZmNADPH:NR*, and *ZmNiR* (coding for assimilatory enzymes, as two isoforms of nitrate reductase and nitrite reductase; **Figure 4**). The analyses were performed on maize roots treated with DHS isolated in April (OF2, OF-P2, CF2, and CF-P2), which induced the maximum uptake rate of nitrate. After 2 h of treatment, the expression in maize roots of *ZmNRT2.1*, *ZmNRT2.2*, and *ZmMHA2* did not respond to treatment with WEHS. On the other hand, all DHS induced the upregulation of *ZmNRT2.2*, and DHS isolated in April from not planted CF soil (N+CF2; **Figure 4**) induced also the upregulation of *ZmNRT2.1* in comparison to control plants (N). Plants treated with DHS did

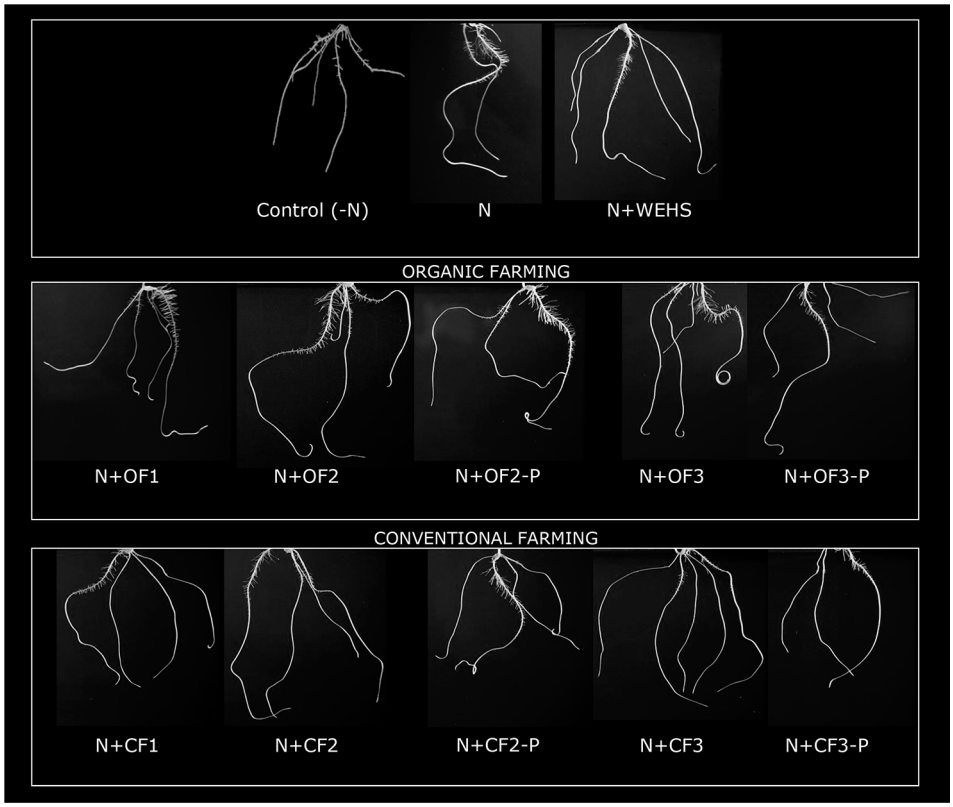

FIGURE 2 | Representative pictures of whole root system of maize plants (5 days old) after 24 h of treatment (in nutrient solution): N-free nutrient solution [*Control (-N)*], nutrient solution containing nitrate (0.5 mM $Ca(NO_3)_2$) with or without 5 mg C_{org} L^{-1} water-extractable humic substances (WEHS) (humic substances isolated from sphagnum peat; *N+WEHS* or *N*, respectively), nitrate (0.5 mM $Ca(NO_3)_2$) with dissolved humic substances (DHS) (5 mg C_{org} L^{-1}) isolated from organic farming (OF) soils (*N+OF1*, *N+OF2*, *N+OF-P2*, *N+OF3*, and *N+OF-P3*) or isolated from conventional farming (CF) soils (*N+CF1*, *N+CF2*, *N+CF-P2*, *N+CF3*, and *N+CF-P3*). The code name of samples is reported in **Figure 1**.

not alter significantly the expression of *ZmMHA2*, although a slight reduction of its expression occurred in the presence of CF DHS (CF2 and CF-P2; **Figure 4**).

Concerning the nitrate reductive pathway, the treatment with WEHS upregulated the transcripts encoding nitrate and nitrite reductases; in particular, the expressions of *ZmNADH:NR*, *ZmNADPH:NR*, and *ZmNiR* were at least five times higher than the expression levels induced by nitrate alone. A significant upregulation of *ZmNADPH:NR* was induced also by all tested DHS fractions in comparison to nitrate only, while the upregulation of *ZmNADPH:NR* occurred only with the N+OF2 treatment. In comparison to the control (N treatment), no significant changes in the expression of *ZmNiR* were caused by the treatment with DHS (N+OF2, N+OF-P2, N+CF2, and N+CF-P2).

Quantitative and Chemical Characteristics of DHS in CF and OF Leachates

The concentration range of soluble humic carbon in the soil solution of the two soils, calculated by dividing the total DHS carbon of leachates by the water content of soil monoliths at field capacity and wilting point, ranged between 5.3 and 27.1 mg C_{org} L^{-1} (**Table 2**).

At field capacity, DHS concentrations were slightly higher in CF with respect to OF soils (mean values: 10.2 vs. 9.1 mg C_{org} L^{-1}) and in bare compared to planted soils (mean values: 10.4 vs. 8.4 mg C_{org} L^{-1}).

DHS are expected to be mostly composed of small molecular size components. Size exclusion chromatography of DHS (**Figure 5**) confirmed this assumption, but showed that a fraction of relatively large molecules (e.g., apparent MW > 1,000 Da) was present at the beginning of the experiment (November) and particularly in the OF soil (18% of large molecules in OF1). The molecular size distribution of DHS isolated in November from the OF soil was the most similar to that of the WHSH from peat.

However, in leachates collected from the same soil in April (OF2) and June (OF3), only small amounts of high apparent MW components occurred and the percentage of DHS with an apparent MW < 1,000 Da increased. Fractions of apparent MW between 1,000 and 300 Da were more abundant in planted soils (OF-P2 and OF-P3).

In the DHS from the CF soil, apparent MW fractions between 1,000 and 300 Da accounted for 45–60%, and the smallest molecules (apparent MW < 300 Da) accounted for 40–50% of the total DHS. In particular, the percentage of substances with

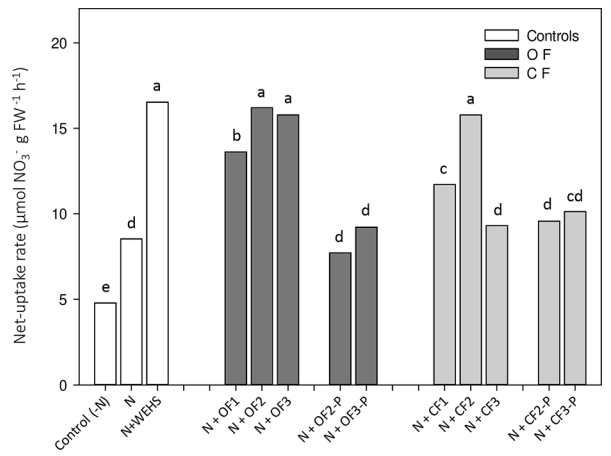

FIGURE 3 | Net uptake rates of nitrate by roots of maize plants exposed for 4 h to humic substances of different origin. *White bars*, As controls, plants exposed to N-free nutrient solution [*Control (-N)*, or nutrient solution containing nitrate (0.5 mM $Ca(NO_3)_2$) with or without water-extractable humic substances (WEHS) (5 mg C_{org} L^{-1}; *N+WEHS* or *N*, respectively). *Dark gray bars*, Plants exposed to nutrient solution with nitrate and dissolved humic substances (DHS) (5 mg C_{org} L^{-1}) isolated from organic farming (OF) soils. *Light gray bars*, Plants exposed to nutrient solution with nitrate and DHS (5 mg C_{org} L^{-1}) isolated from conventional farming (CF) soils. The code name of samples is reported in **Figure 1**. Bars with the same letters are not significantly different at $p \leq 0.05$.

apparent size between 1,000 and 300 Da was largest in bare soil leachates collected in April (CF2) and lowest in planted soil leachates collected in June (CF-P3); the latter also showed the highest enrichment of very low MW fraction (< 300 Da apparent MW).

Trend observed by size exclusion chromatography were confirmed by E_{465}/E_{665} absorption ratios which are inversely related to the molecular size of HS. The E_{465}/E_{665} values were typical of small-sized HS, i.e., fulvic acids, and relatively lower in OF soils in November (8.9, OF1). E_{465}/E_{665} ratios increased during the experiment (OF2 and OF3), especially in planted soils (OF-P3; **Figure 6A**). In June, the E_{465}/E_{665} of bare OF soils was 12.7 (OF3), while in planted OF soils it reached a value of 14 (OF-P3). In the CF soil, DHS had larger and more constant E_{465}/E_{665} ratios, namely, 11.8 in November (CF1) and respectively 12.2 and 13.1 in June in bare and planted soils (CF3 and CF-P3).

The content of carboxylic groups (**Figure 6B**) increased steadily throughout the experiment in the DHS of the OF soil, whereas it decreased in DHS leached from the CF soil. In November, the total density of carboxylic groups in DHS of the OF soil was about half than that observed in the CF soil (9.3 mmol g^{-1} in OF1 vs. 20.7 mmol g^{-1} in CF1), but during the experiment the amount of carboxylic groups increased in the OF DHS. In June, the amount of carboxylic groups in OF soils was around 20 mmol g^{-1} of DHS independently of the plant presence (OF3 and OF-P3). In CF soil, DHS showed a slight decrease in carboxyl content during the time of the experiment for both bare and planted soils, as in summer the amounts of carboxylic groups were 16.3 mmol g^{-1} (in CF3) and 18.4 mmol g^{-1} (in CF-P3), respectively.

The characterization of DHS was further achieved by FTIR spectroscopy (**Figure 7**). Compared with most FTIR spectra of HS, DHS and WEHS spectra are relatively simple and characterized by four main bands. All spectra displayed intense very broad absorption in the region between 3,440 and 3,380 cm^{-1}. This band corresponds to O–H stretching vibrations of phenolic groups overimposed on O–H stretching of carbohydrates (Coates, 2006). The intensity of this band was more pronounced in DHS of both soils at the beginning of the experiment (OF1 and CF1). In the organically managed soil, it shifted to lower wavelengths (3,440–3,400 cm^{-1}) and became

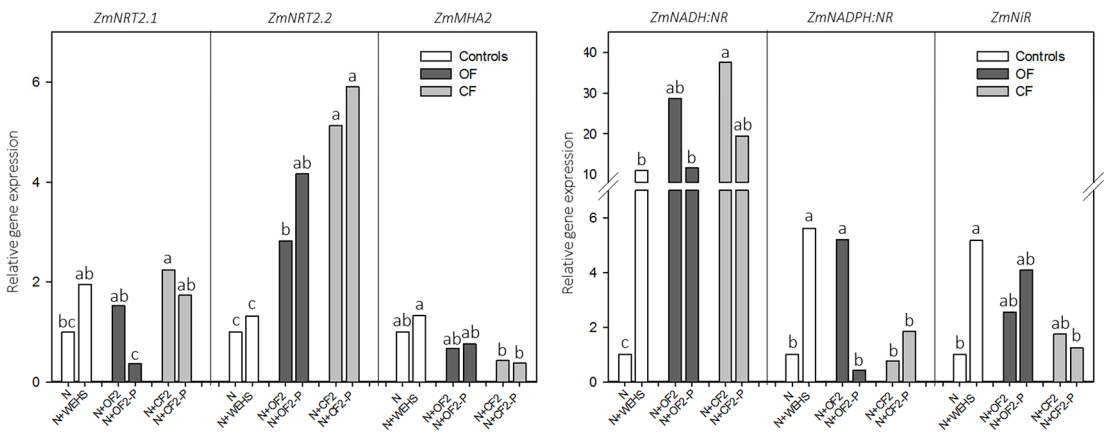

FIGURE 4 | Real-time RT-PCR analyses of the main genes involved in the nitrate acquisition—*ZmNRT2.1* and *ZmNRT2.2* (coding for two high-affinity nitrate transporters), *ZmMHA2* (coding for a PM H^+-proton pump), *ZmNADPH:NR* and *ZmNADPH:NR* (coding for two isoforms of nitrate reductase), and *ZmNiR* (coding for nitrite reductase)—and performed on roots of maize plants exposed for 2 h to humic substances of different origin isolated in April. *White bars*, As controls, plants exposed to nutrient solution containing nitrate (0.5 mM $Ca(NO_3)_2$) with or without water-extractable humic substances (WEHS) (5 mg C_{org} L^{-1}) (*N+WEHS* or *N*, respectively). *Dark gray bars*, Plants exposed to nutrient solution with nitrate and dissolved humic substances (DHS) (5 mg C_{org} L^{-1}) isolated from organic farming (OF) soils. *Light gray bars*, Plants exposed to nutrient solution with nitrate and DHS (5 mg C_{org} L^{-1}) isolated from conventional farming (CF) soils. The code name of samples is reported in **Figure 1**. Bars with the same letters are not significantly different at $p \leq 0.05$.

TABLE 2 | Estimated concentration ranges (mg C_{org} L^{-1}) of soluble humic fractions (dissolved humic substances, DHS) in the soil solution of bare soils (OF and CF) and planted soils (OF-P and CF-P) at field capacity and wilting point.

DHS at field capacity (mg C_{org} L^{-1})	**OF**	**OF-P**	**CF**	**CF-P**
November	10.5		10.8	
April	11.9	8.3	8.8	5.3
June	6.3	8.5	14.3	11.7
*Mean value	9.1		10.2	
DHS at wilting point (mg C_{org} L^{-1})	**OF**	**OF-P**	**CF**	**CF-P**
November	22.3		20.4	
April	25.4	17.7	16.7	10.0
June	13.4	18.1	27.1	22.1
*Mean value	19.4		19.3	

**Mean values refer to all soil treatments and sampling times (N = 5).*

broader with time, indicating stronger hydration and H bonding, but also an increasing contribution from the stretching vibration of O–H in phenols. The progressively lower presence of carbohydrate moieties in DHS molecules from the OF soil is confirmed by the decrease of absorbance around 1,080–1,040 cm^{-1}. The weak broad absorption around 1,080–1,040 cm^{-1} may, in fact, be assigned to C–O and C–C stretching vibrations of carbohydrate rings. In OF-leached DHS, this band decreased during the experiment, whereas it remained stable till June in CF-leached DHS.

All spectra also exhibited very weak absorption due to aliphatic C–H stretching at about 2,920 cm^{-1}.

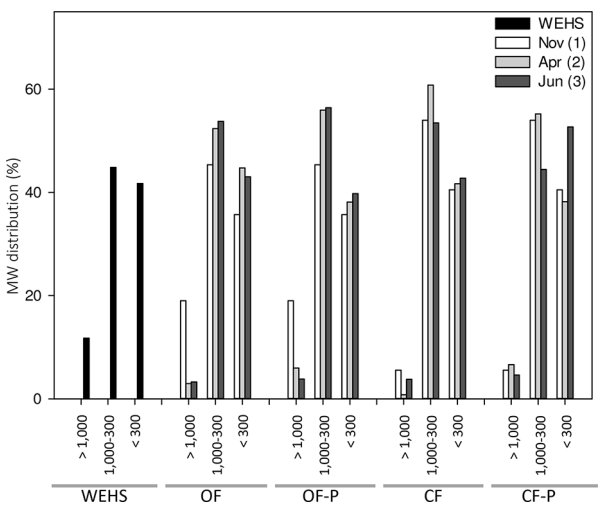

FIGURE 5 | Molecular weight (MW expressed in dalton) distribution of humic substances isolated from leachates collected from bare and planted soils at different sampling times. As control, MW distribution of water-extractable humic substances (WEHS) isolated from sphagnum peat (Novobalt, Lithuania) are also shown. *Nov (1)* refers to leachates sampled in November; *Apr (2)* refers to leachates sampled in April; *Jun (3)* refers to leachates sampled in June. *OF* and *CF* refer to leachates collected from bare soil columns; *OF-P* and *CF-P* refer to leachates collected from planted soil columns.

Strong asymmetric and symmetric COO^- stretching bands, coherent with the fact that DHS were freeze-dried at pH 7, are present in all spectra. These bands are located around 1,570 and 1,440 cm^{-1} in the DHS of organically managed soil at the beginning of the experiment (OF1). The first band shifts to longer wavelengths (1,590 cm^{-1}) towards the end of the experiment (OF3 and OF3-P), which may indicate loss of double bonds conjugated to carboxyl groups. Conversely, in all DHS from CF soil, the band remains centered at 1,580 cm^{-1}.

Besides symmetric COO^- stretching, bands in the 1,440–1,380 region can also be attributed to the absorption of C–O groups in phenols and tertiary alcohols. The shift of maximum intensity from the 1,440 to the 1,380 band, which contributes to absorption in this region, is related to a stronger contribution of this type of structures.

In fact, the ratio between the absorbance intensity of the two main peaks is related to asymmetric and symmetric COO^- stretching at respectively 1,590–1,570 cm^{-1} and 1,440–1,380 cm^{-1}. If all absorption in this region was due to carboxylate moieties, the value of this ratio would be about 1.4 (Max and Chapados, 2004). Under both types of soil management, DHS collected in November showed ratios (1.34 in OF1 soil and 1.38 in CF1 soil) compatible with a nearly exclusive contribution from carboxyls. In April, however, all samples showed a ratio lower than 1, with the only exception of the bare CF soil that maintained a ratio of 1.29 (CF2). Independently from management, DHS collected in June from planted soils exhibited again high values of the ratio (1.41 in OF-P3 soil and 1.50 in CF-P3 soil), indicating release of carboxylic and polycarboxylic substances. Conversely, in the absence of plants, ratios remained lower than 1 in OF3 and CF3.

DISCUSSION

In the present work, DHS were extracted from leachates of soil monoliths through a procedure that mimics the natural process of "extraction" of humic substances by rainwater percolating through soil horizons under field conditions (Olaetxea et al., 2018). The two soils selected were cultivated soils of low and

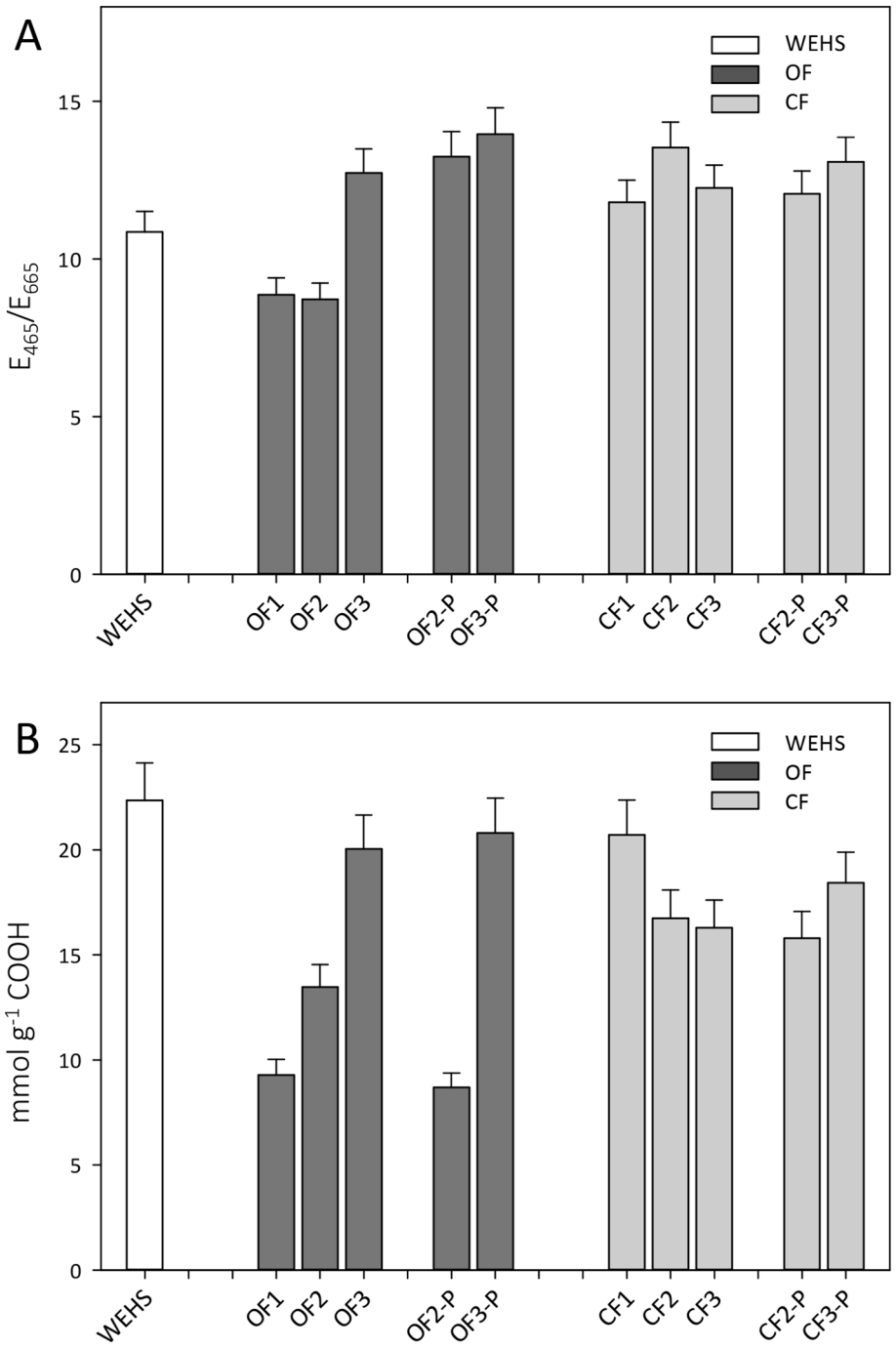

FIGURE 6 | E_{465}/E_{665} ratios of organic farming (OF) and conventional farming (CF) fractions **(A)** and density of carboxylic groups in dissolved humic substances (DHS) obtained by titration with NaOH 0.1 M **(B)**. *White bar* refers to water-extractable humic substances (WEHS); *dark gray bars* refer to DHS isolated from OF soils; *light gray bars* refer to DHS isolated from CF soils (data shown are means plus standard deviation). OF1 and CF1 were sampled in November; OF2, OF-P2, CF2, and CF-P2 were sampled in April; OF3, OF-P3, CF3, and CF-P3 were sampled in June. OF1, OF2, OF3 and CF1, CF2, CF3 refer to leachates collected from bare soil columns. OF-P2, OF-P3 and CF-P2, CF-P3 refer to leachates collected from planted soil columns.

comparable organic matter content, which are highly representative of real agricultural field conditions. These soils are sub-alkaline soils rich in calcium carbonate, which strongly suppresses solubility of HS. Our results therefore demonstrate, first of all, that even in arable calcareous soils of low organic matter content some humic substances are dissolved in the soil solution and can therefore act in agricultural soils as they do in hydroponic experiments. Another important issue is the concentration of DHS: the minimum calculated DHS concentration (field capacity) was about 10 mg C_{org} L^{-1}

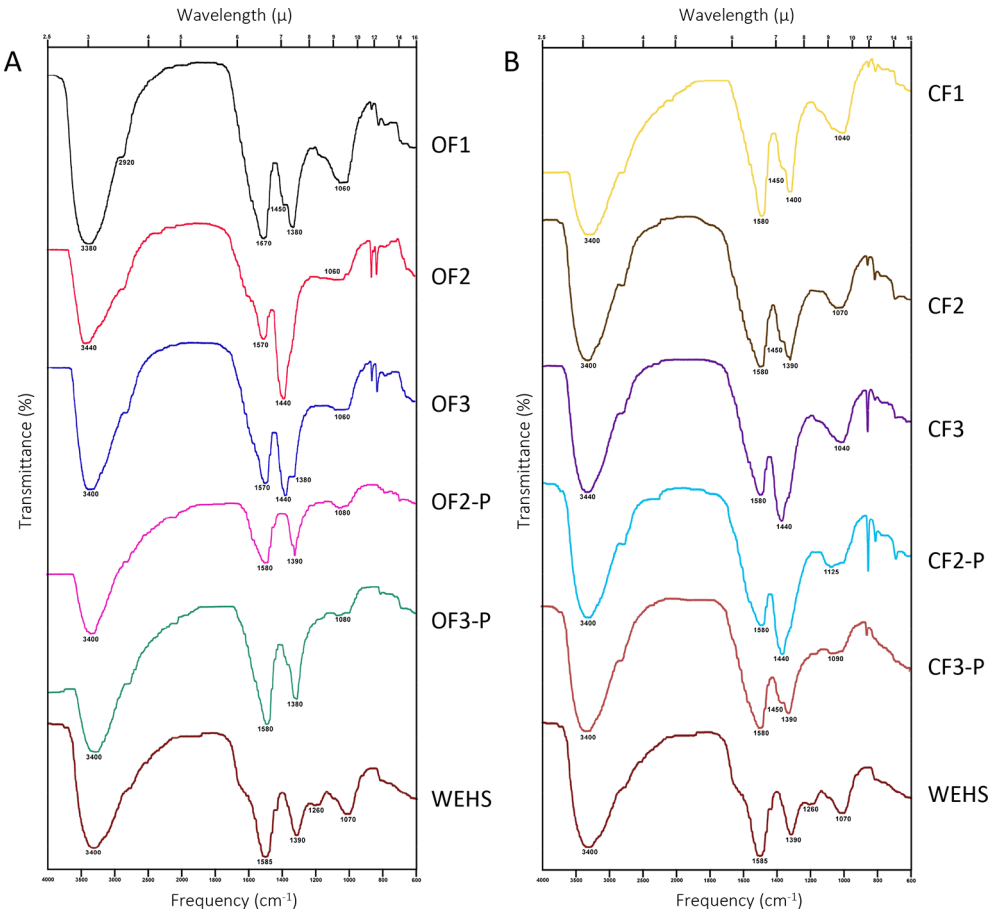

FIGURE 7 | FTIR spectra of dissolved humic substances (DHS) fractions isolated from organic farming (OF) soils **(A)** and from conventional farming (CF) soils **(B)**. OF1 and CF1 were sampled in November; OF2, OF-P2, CF2, and CF-P2 were sampled in April; OF3, OF-P3, CF3, and CF-P3 were sampled in June. OF1, OF2, OF3 and CF1, CF2, CF3 refer to leachates collected from bare soil columns. OF-P2, OF-P3 and CF-P2, CF-P3 refer to leachates collected from planted soil columns.

in both soils and slightly larger in bare than in planted soils (mean values: 10.4 mg C_{org} L^{-1} vs. 8.4 mg C_{org} L^{-1}). These concentration ranges represent a good approximation of the actual concentration of DHS in the solution of cultivated soils. It is important to underline that these concentrations are even larger than those usually applied in biological tests (**Table 2**) (Pinton et al., 1999; Cesco et al., 2000; Zanin et al., 2019), which are therefore validated by our results from a quantitative point of view.

Over time, leachates of OF soil showed an overall reduction in DHS content (**Table 2**). In the bare OF soil, the stronger decrease was observed between April and June (OF2 and OF3), while the presence of plants stabilized DHS concentrations in leachates (OF-P2 and OF-P3). A different behavior was observed in CF soil leachates since in this soil DHS concentrations were smallest in April (CF2 and CF-P2) and increased in June (CF3 and CF-P3), irrespectively of the presence of plants.

At the morphological level, the presence of DHS induced an overall higher development and proliferation of secondary and lateral roots in maize plants, confirming the biostimulant action of humic substances on plant growth (Canellas et al., 2002; Nardi et al., 2002).

Previous works reported a direct effect of WEHS on roots promoting nitrate acquisition. Like WEHS (Zanin et al., 2018), DHS also promoted nitrate uptake in roots. However, their action changed depending on time of the year and type of soil management and was nullified in the presence of growing plants. After 4 h of root contact with nitrate and DHS, an overall larger and more stable biostimulatory effect was observed with DHS from OF soils (N+OF1, N+OF2, and N+OF3). When maize plants were treated with DHS deriving from CF soils, nitrate uptake rates were highly variable, and the biostimulant effect occurred only with DHS collected in April, N+CF2 (a significant but mild effect was observed in November, N+CF1).

Transcriptional analyses of the genes most involved in N acquisition highlighted changes in their expression patterns which depended on the nature of DHS.

Confirming previous results reported in literature (Zanin et al., 2018), WEHS stimulated root expression of transcripts coding for N assimilatory enzymes more than for N transporters.

Indeed, no significant changes in the expression levels of *ZmNRT2.1*, *ZmNRT2.2*, and *ZmMHA2* occurred between nitrate-treated plants and those treated with nitrate plus WEHS (N vs. N+WEHS). In contrast, our results indicate that the addition of DHS to nitrate-containing nutrient solution significantly promoted the expression of *ZmNRT2s* high-affinity nitrate transporters. In particular, in comparison to OF, the CF soil management results in the production of DHS that enhance the expression of both nitrate transporters, *ZmNRT2.1* and *ZmNRT2.2*. Besides transcriptional regulation, it has been reported that, in maize, the uptake rate of nitrate is also regulated at translational level, based on protein–protein interactions of NRTs and accessory protein (NRT3.1; Pii et al., 2016). Therefore, it is plausible to suppose that the biostimulant action exerted by humic substances might be ascribed to a stimulation at transcriptional level in root cells and also to a modulation of the interactions between proteins on the plasma membrane of root cells (e.g., nitrate transporters and proton pumps).

Concerning nitrate assimilation, initial reductive reactions are key points of this pathway and are mediated by nitrate and nitrite reductases (Nacry et al., 2013). Two isoforms of this enzyme are ubiquitously expressed in maize roots (Pandey et al., 1997): one is NADH-dependent (E.C. 1.6.6.1) and the other NAD(P)H-dependent (E.C. 1.6.6.2). The bispecific NAD(P)H:NR isoform occurs in many species (Srivastava, 1992), including roots and scutellum of maize seedlings, but not leaves (Redinbaugh and Campbell, 1981). However, their physiological role and their specific contribution to N assimilation are still unclear. As reported above, the treatment with WEHS induced the expression of transcripts encoding nitrate and nitrite reductases. Similarly, all tested DHS fractions also induced the upregulation of NR, and in particular the OF2 DHS stimulated the expression of both tested transcripts encoding for two NR isoforms.

This evidence might indicate that the isolated humic substances exerted the same stimulatory effect on nitrate acquisition but, depending on their origin (soil or peat), this physiological response might be acting on the expression of different molecular components. High-affinity nitrate transporters and nitrate reductase are activated by soil DHS, whereas peat WEHS act mainly on assimilatory enzymes.

Within the same agricultural management (CF or OF), gene expression analyses showed only slight variations among treatments. However, the stable stimulatory action of OF DHS on nitrate uptake rates (from November to June) might be a consequence of a wider and more active upregulation of molecular components involved in nitrate acquisition (nitrate transporters and reductive enzymes), while CF DHS induced the expression of only one isoform of nitrate reductase (*ZmNADH:NR*).

These differences can only in part be justified on the basis of changes in chemical characteristics recorded in the collected DHS. DHS leached from the two soils differ from peat WEHS. The apparent MW distribution of DHS showed that components with apparent MW > 1,000 Da (which are a sizeable fraction of WEHS) were present only in the OF soil. This fraction might be associated with abundance of organic C inputs relative to C mineralization, such as occurs in peat and in soils which receive organic amendments. Coherently with this hypothesis, this fraction strongly diminished during the experiment since no organic fertilizer or amendment was applied. This is also in agreement with the increasing oxidation observed in OF DHS (increased number of carboxyl groups and reduced structural contribution of carbohydrates).

High-MW components are obviously lacking in the CF soil that has not received organic amendments for a long time. At the beginning of the experiment in November, CF leachates already had a very low content of DHS with high apparent MW (CF1), and this fraction did not decrease in June.

The overall trend of molecular weight distributions is confirmed by the trend of E_{465}/E_{665} absorption ratios which are also linked to the average molecular size of humic substances (Chen et al., 1977).

Besides their low apparent molecular sizes and coherently with their solubility, DHS fractions were characterized also by a high content of carboxylic functional groups. During time, the CF DHS showed a large and stable density of COOH, which was altogether quite similar to that of the WEHS fraction. A wider variability was recorded in OF DHS since the COOH content reached values similar to those recorded for WEHS only at the end of the treatment (June, OF3), while at the beginning of the experiment (November, OF1) the COOH content was about 50% lower. During the experiment, the increase in the carboxylic group content, in the E_{465}/E_{665} ratio, and the prevalence of smaller molecules, as well as trends of absorption of oxygen containing functional groups in FTIR spectra, indicated that the DHS fractions underwent fragmentation and oxidation.

Before isolation of DHS, all leachates were analyzed also for their nitrate content. The nitrate leached from CF soil was twice that collected from the OF soil. This behavior might be a direct consequence of the agricultural management of soil and, indirectly, a consequence of a different rate of nitrification processes occurring thereafter in the OF and CF monolith columns. This latter hypothesis is in agreement with the FTIR spectra that displayed a decrease of the carbohydrate C–O stretching signal (1,040 cm^{-1}) in the OF DHS fractions in both planted and non-planted soils during the experiment, suggesting the occurrence of extensive organic matter mineralization from November to June. Decomposition of carbohydrates might have been accompanied by an overall immobilization of mineral N in microbial cells. In the non-planted CF soil (CF1, CF2, and CF3), the FTIR spectra showed a much lower decrease of the C–O stretching signal: it is likely that the microbial biomass was less active in this treatment than in the OF soil, and therefore more nitrogen (in the form of nitrate) was present in leacheates from the bare CF soil. The same happened in the planted CF soil (CF-P2 and CF-P3), but wheat plants appeared to support mineralization, as shown by the decrease of the C–O stretching signal, which became comparable to that observed in the OF soil.

It is interesting to observe that DHS from planted soils exerted a weak effect on maize, and in particular did not

display the capability to stimulate the nitrate acquisition. Two hypotheses can be formulated to explain this effect. In the first place, it is possible that the presence of wheat roots might have boosted biological activity and stimulated mineralization in the rhizosphere, leading to modification or decomposition of bioactive components of humic substances fraction. This hypothesis is supported by the FTIR spectra of planted CF and OF DHS, which showed distinct changes in absorbance intensity ratios between the two main peaks of carboxylate ions related to antisymmetric and symmetric COO^- stretching. This pattern, however, may also support the hypothesis that some aromatic compounds, such as phenols and flavonoids, may have been released by wheat roots. Flavonoids or similar compounds might have been sorbed on the PVP resin, together with humic substances. In literature, it is widely reported that phenolic compounds are the major secondary metabolites involved in plant allelopathy (Li et al., 2010) and might therefore impact nutrient acquisition in other crop species. On the other hand, the FTIR spectra and the analysis of carboxyl groups of DHS also bear evidence of extensive oxidation of organic matter in soil monoliths, which may have resulted in DHS with a low capability to stimulate nitrate acquisition.

CONCLUSION

This study showed that OF and CF managements of soil qualitatively modify the characteristics and biostimulant potential of DHS and that the presence of plant roots also resulted in a dynamic interaction with these active components of the soil solution.

Further studies will be necessary to find out whether modification of DHS composition or their enhanced decomposition fostered by root exudates can actually explain the observed behavior. The complexity of the structural trends highlighted by the chemical characterization of DHS collected from planted and non-planted soils suggests that they should be further fractionated in order to isolate active fractions and allow a better characterization of their structure.

Besides confirming activation of genes involved in nitrate acquisition, this study demonstrated not only that the range of concentrations generally employed to investigate actions of HS on plants are indeed representative of agricultural field conditions but also the integrity of the use of the easily available WEHS in this type of studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception, design, data collection, analyses, and manuscript preparation. TV, LZ, SV performed the experiments. TV and LZ wrote the article, MN, SC, NT, RP supervised and completed the writing. TV, LZ, PC, SC, RP contributed to the study design. TV, LZ, SV, MC, NT, SC, MN contributed to the data analysis. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01652/full#supplementary-material

TABLE S1 | List of primers used for real-time RT-PCR analyses.

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frontiers in Plant Science

ORIGINAL RESEARCH
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Discriminating the Short-Term Action of Root and Foliar Application of Humic Acids on Plant Growth: Emerging Role of Jasmonic Acid

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Humic substances (HS, fulvic and humic acids) are widely used as fertilizers or plant growth stimulants, although their mechanism of action still remains partially unknown. Humic substances may be applied either directly to the soil or as foliar sprays. Despite both kind of application are commonly used in agricultural practices, most of the studies regarding the elicited response in plants induced by HS are based on the root-application of these substances. The present work aimed at discriminating between the mechanisms of action of foliar application versus root application of a sedimentary humic acid (SHA) on plant development. For this purpose, six markers related to plant phenotype, plant morphology, hormonal balance and root-plasma membrane H^+-ATPase were selected. Both application strategies improved the shoot and root growth. Foliar applied- and root applied-SHA shared the capacity to increase the concentration of indole-3-acetic acid in roots and cytokinins in shoots. However, foliar application did not lead to short-term increases in either abscisic acid root-concentration or root-plasma membrane H^+-ATPase activity which are, however, two crucial effects triggered by SHA root-application. Both application modes increased the root concentrations of jasmonic acid and jasmonoyl-isoleucine. These hormonal changes caused by foliar application could be a stress-related symptom and connected to the loss of leaves trichomes and the diminution of chloroplasts size seen by scanning electron microscopy. These results support the hypothesis that the beneficial effects of SHA applied to roots or leaves may result from plant adaptation to a mild transient stress caused by SHA application.

Keywords: humic substances, humic acids, foliar application, root application, shoot growth, root growth, jasmonic acid, salicylic acid

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INTRODUCTION

There is a growing interest in the development and implementation of more sustainable land management practices, aiming to stop the progressive degradation of soils while maintaining or enhancing food production in a context of increasing demands. Among the different strategies, the use of humic-based soil amendments constitutes an environmentally friendly approach. Many

studies have shown that humic substances (HS) from different origins applied to plant roots can improve plant growth and mineral nutrition (see reviews by Chen et al., 2004; Rose et al., 2014; Olaetxea et al., 2018; and references therein). These positive effects involve various mechanisms, including the action of HS on soil and rhizosphere properties, as well as their interactions with plant roots.

The capacity of HS to enhance plant growth has promoted the development of humic-based commercial products for plant production (Rose et al., 2014; Canellas et al., 2015; Olk et al., 2018). In general, commercial HS-based products can be applied not only to the soil (root area) but also as foliar sprays (Rose et al., 2014; Canellas et al., 2015). While the mechanisms of action involved in the plant growth promoting effect of soil-applied HS have been the subject of different studies (Pinton et al., 1999; Nardi et al., 2002; Chen et al., 2004; Berbara and García, 2013; Canellas et al., 2015; García et al., 2016b; Olaetxea et al., 2018), the beneficial action of foliar-applied HS remains unexplored to date. Indeed, it is assumed that foliar-applied HS promote plant growth by mechanisms similar to those involved in HS root application (Rose et al., 2014). However, there are many differences regarding the modes of absorption, transport and interaction of root- versus foliar-applied HS. For example, the range of concentration of HS that is needed to improve plant growth via foliar application is much lower compared to that for root HS application (Chen and Aviad, 1990). Likewise, HS applied to the leaves do not interact with the soil and rhizosphere, where important reactions and interactions that lead to an enhanced nutrient bioavailability take place (Baigorri et al., 2013; Urrutia et al., 2014; Olaetxea et al., 2018; Zanin et al., 2019). It is therefore plausible that the mechanisms underlying the response of plants to foliar-applied HS may involve nutritional, metabolic and physiological differences compared to the response to root-applied HS.

Hence, the aim of this study is to evaluate some of the mechanisms triggered after foliar application of a well-characterized sedimentary humic acid (SHA) previously found to improve plant growth when applied to roots (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). Our hypothesis is that the interaction of HS with plant leaves might induce some kind of mild stress signals that may activate hormonal and molecular pathways involved in the regulation of plant stress responses. As the nature of HS-leaf interactions in the phyllosphere may be quite different from that of HS-root/rhizosphere interactions, we hypothesize the occurrence of potentially different mechanisms responsible for the beneficial effects of both HS supply modes on plant growth.

MATERIALS AND METHODS

Extraction and Purification of a Leonardite HA (SHA)

Sedimentary humic acids (SHA) were obtained from a leonardite originated in the Danube basin (Czechia). The extraction and purification of SHA were performed according to the International Humic Substances Society methodology with some modifications, following the protocol described in detail in Aguirre et al. (2009; **Supplementary Information**). The main physico-chemical features of SHA are described in **Supplementary Table S1** and **Supplementary Figures S1, S2**.

Plant Growth and Experimental Design

Cucumber (*Cucumis sativus* L. var. Ashley) seeds were germinated in the dark, on perlite and filter paper moistened with a 1 mM $CaSO_4$ solution. The germination chamber conditions were 25°C and 75% relative humidity (RH). One week after, seedlings were transferred to a hydroponic system with vessels filled with 7 L of nutrient solution. This solution contained: 0.63 mM K_2SO_4, 0.5 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.30 mM $MgSO_4$, 0.25 mM KNO_3, 0.05 mM KCl, 0.87 mM $Mg(NO_3)_2$, 40 μM H_3BO_3, 27.3 μM $MnSO_4$, 2 μM $CuSO_4$, 2 μM $ZnSO_4$, and 1.4 μM Na_2MoO_4. The solution was supplemented with 80 μM iron as Fe-ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid) chelate (80% [w/w] ortho-ortho-isomer). The average value of the pH of the nutrient solution during the experiment was 6.7. The different experiments were performed in a growth chamber where the experimental conditions were set up to 25°C/21°C and 70%/75% RH in a day-night cycle and the photoperiod was 15 h/9 h (PAR of 250 $\mu mol\ m^{-2}s^{-1}$).

In order to assess the effects caused by the foliar application of SHA, several solutions with different SHA concentrations (in the range 20–100 mg C L^{-1}), at pH 6, were prepared by dissolving the required amount of SHA in water, with the addition of 0.1% Tween20 (vol/vol). The corresponding treatments were sprayed on both abaxial and adaxial sides of leaves of cucumber plants 10 days after transplantation. Leaves of control plants were treated with 0.1% Tween20 in water (vol/vol). All foliar treatments were always applied 2 h after the start of the diurnal period. Plants were always harvested at the same time of the day (6 h after the start of the light period) to avoid diurnal variations.

An additional experiment was performed in order to explore the effects caused by root-applied SHA on the concentration of jasmonic acid (JA), jasmonoyl-isoleucine (JAIle), and salicylic acid (SA) in plant tissues. In this experiment, plans were grown in the same conditions as described above, and 10 days old cucumber plants were treated with 100 mg C L^{-1} of SHA added to the nutrient solution (SHA.R100).

Measurement of Root and Shoot Dry Matter

Shoots and roots were sectioned with a scalpel and separated before fresh weight (FW) measurement. Five plants were harvested for each treatment and each harvest time. Root and shoot samples were then dried at 50°C for 3 days in a lab stove, and their dry weight (DW) was subsequently measured individually.

Mineral Nutrition Analysis

Dried samples (five shoots and five roots for each treatment and harvest time) were used to determine the concentration of the mineral nutrients in leaves. Leaf-samples (0.15 g dry sample) were subjected to acidic digestion (8 mL of 65% HNO_3 and 2 mL

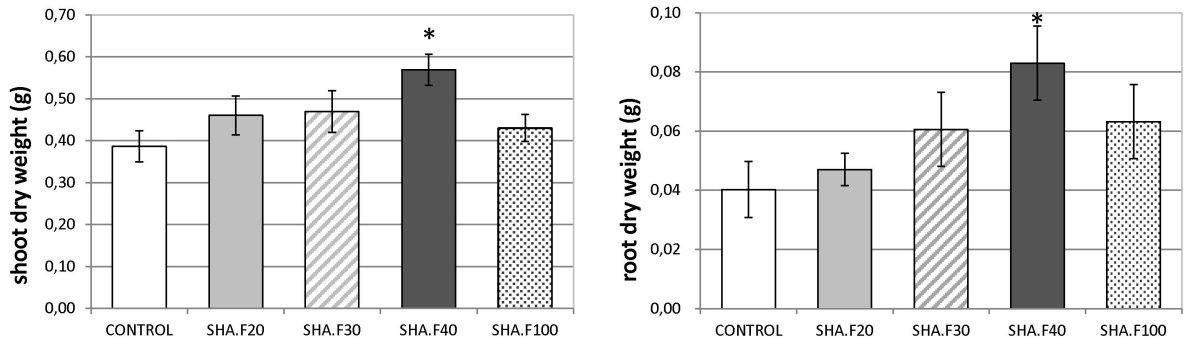

FIGURE 1 | Effects of foliar application of different SHA doses (20, 30, 40, and 100 mg organic C L^{-1}) on the shoot and root dry weight of cucumber plants after 72 h from the onset of treatments. The results are the mean ± *SE* (n = 5). Significant differences (Anova test; $p \leq 0.05$) between treatments and control plants are indicated by an asterisk.

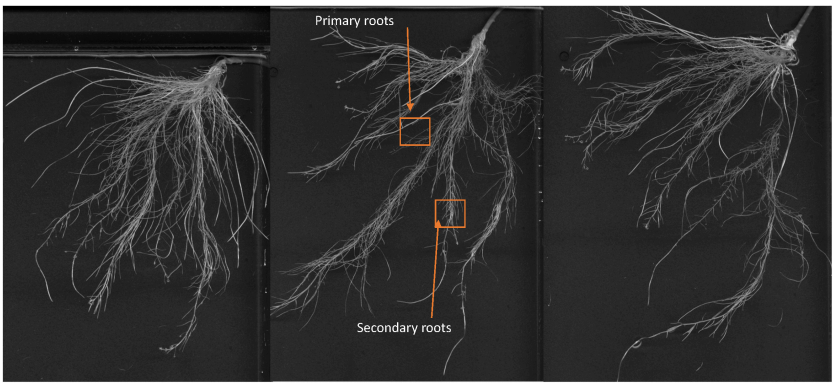

FIGURE 2 | Whole root of cucumber control plants after 72 h from the onset of the treatments.

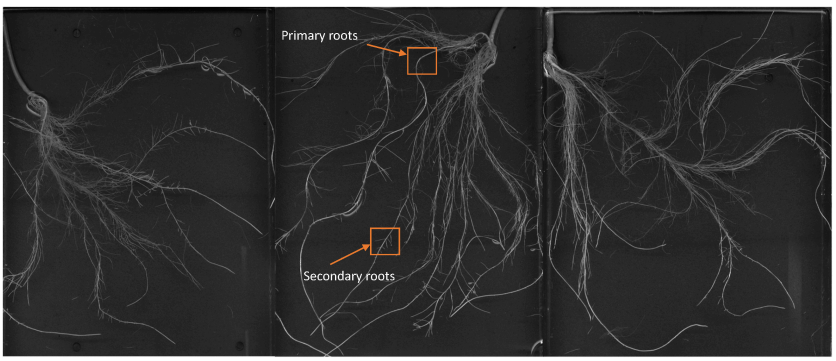

FIGURE 3 | Whole root of cucumber plants 72 h after the foliar treatment with 40 mg C L^{1} of SHA (SHA.F40).

of 33% H_2O_2) in a microwave at a controlled temperature of 200°C. Digested samples were then diluted with dH_2O in 25 mL volumetric flasks, and the nutrient concentrations were measured by ICP-OES (iCAP 7400 DUO, Thermo Scientific).

Root Morphology

Root morphology images were acquired with the software WINRHIZO (Regent Instruments Inc., Canada) implemented in a scanner (EPSON Perfection V700 Photo). In this study, three plants per treatment and harvest time were analyzed.

Leaf Morphology

Morphological features of leaves were analyzed by transmission (TEM) and scanning (SEM) electron microscopy. Second true leaves (fully expanded) were harvested after 7 days from the onset of the treatments. For both SEM and TEM, 4 mm^2 pieces

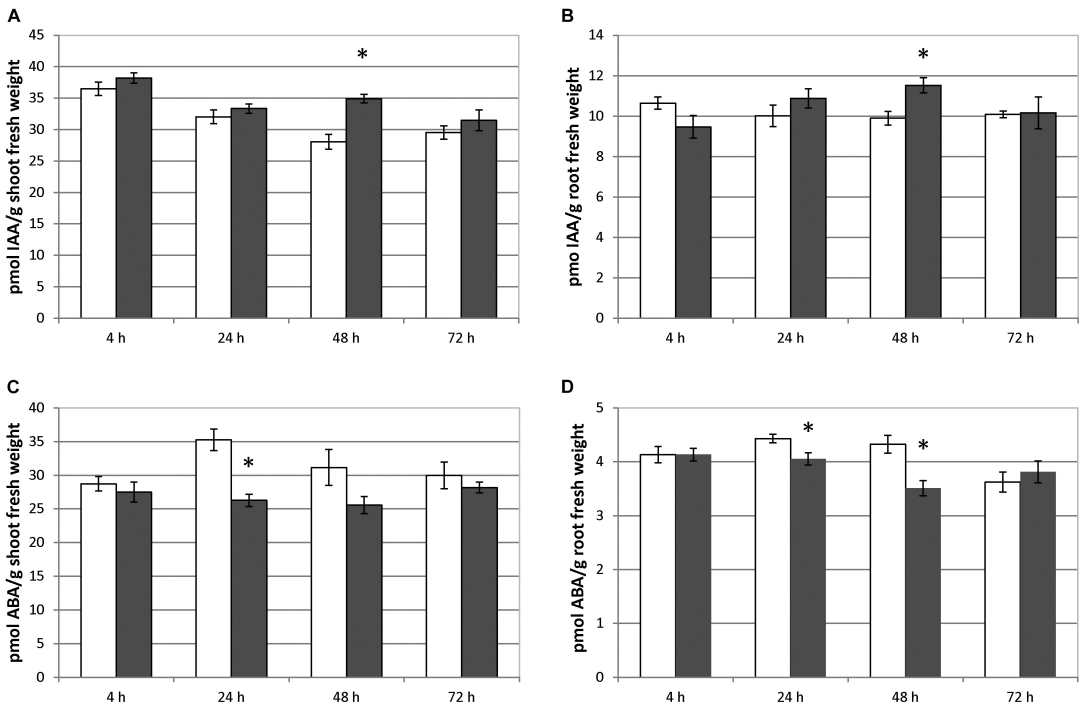

FIGURE 4 | Effect of foliar applied SHA (40 mg C L^{-1}, SHA.F40) on IAA concentration in shoots **(A)** and roots **(B)** and on ABA concentration in shoots **(C)** and roots **(D)** of cucumber plants (white bars: control; dark gray bars: SHA.F40). The results are the mean ± *SE* (n = 5). Significant differences (Anova test; $p \leq 0.05$) between treatments and control plants are indicated by an asterisk.

were cut and subsequently fixed in 2.5% glutaraldehyde-4% paraformaldehyde for 6 h at 4°C. Then they were rinsed in ice-cold phosphate buffer, pH 7.2, 4 times within a period of 6 h and left overnight.

For SEM, fixed leaf tissues were dehydrated in a series of absolute ethanol (i.e., 30, 50, 70, 80, 90 and 100%; ×3 times each concentration). They were subsequently subjected to critical point drying (Leica EM CPD300). Before observation, samples were gold-sputter and examined with a JEOL 6400 SEM.

For TEM, fixed and phosphate buffer rinsed cucumber leaf samples were post-fixed for 1.5 h in 1:1 water: 2% aqueous osmium tetroxide solution containing 3% potassium ferrocyanide. Tissue were consequently washed with distilled water (×3), dehydrated in a series of 30, 50, 70, 80, 90, 95, and 100% acetone (×2, 15 min each concentration) and embedded in acetone-Spurr's resin mixtures (3:1, 2 h; 1:1, 2 h; 1:3, 3 h) and kept in pure resin overnight (kept at 25°C). Pure resin sample embedding was carried out in blocks which were incubated at 70°C for 3 days. Semi-thin leaf sections were cut, mounted on nickel grids and post-stained with Reynolds lead citrate for 5 min, prior to TEM observation (Jeol 1010, equipped with a CCD megaview camera) at 80 kV.

Determination of Hormones in Roots and Shoots

Cucumber plants (five replicates per treatment and harvest time, with one plant per replicate) were harvested and separated into root and shoot prior to freezing in liquid nitrogen. Samples were reduced to a powder in a Freezer/Mill cryogenic grinder (SPEX SamplePrep) and stored at −80°C prior to analyses.

The content of indole-3-acetic acid (IAA), abscisic acid (ABA), SA, JA, and JA-Ile in plant tissues was analyzed by high-performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). These hormones were extracted and purified as described in Silva-Navas et al. (2019) from 0.25 g of ground frozen plant tissue, homogenized with 2.5 mL of precooled (−20°C) methanol:water:HCOOH (90:9:1, v/v/v, with 2.5 mM Na-diethyldithiocarbamate) and 25 μL of a stock solution of 1000 ng ml^{-1} of deuterium-labeled internal standards in methanol. Samples were shaked in a Multi Reax shaker at room temperature for 60 min at 2000 rpm. Immediately afterward, solids were separated by centrifugation at 20.000 × g for 10 min, and re-extracted with 1.25 mL of fresh extraction mixture by shaking for 20 min and subsequent centrifugation. Aliquots of 2 mL of the pooled supernatants were separated and evaporated in a RapidVap Evaporator operating at 40°C. The residue was re-dissolved in 500 μL of methanol/0.133% acetic acid (40:60, v/v) and centrifuged at 20.000 × g for 10 min before the injection in the HPLC-ESI-HRMS system. Detailed description of the quantification is reported in Silva-Navas et al. (2019).

The endogenous content of the following cytokinins was also analyzed: *trans*- and *cis*-zeatin (tZ and cZ), dihydrozeatin (DHZ), *trans*- and *cis*-zeatin riboside (tZR and cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP),

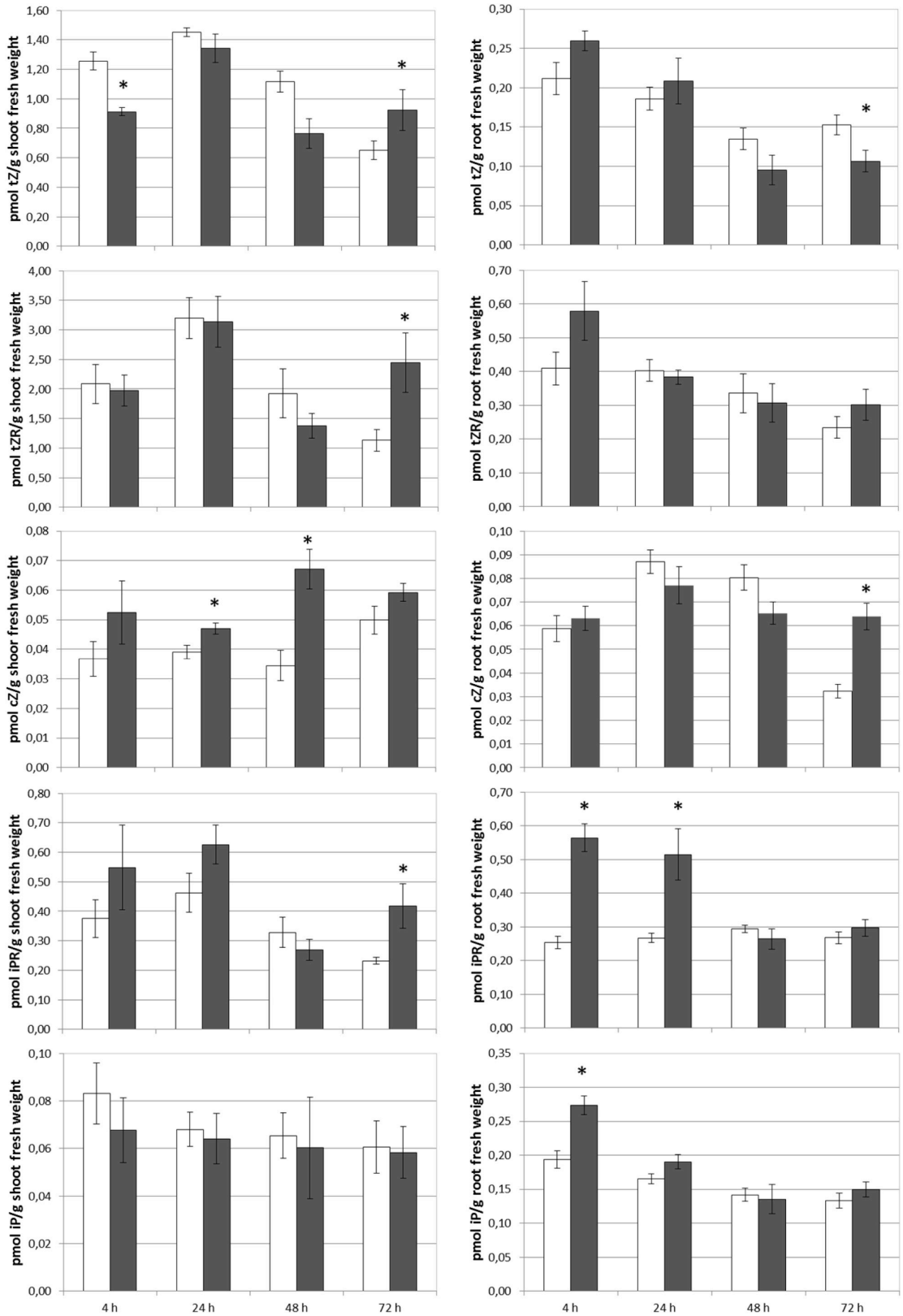

FIGURE 5 | Effect of foliar applied SHA (40 gm C L^{-1}, SHA.F40) on cytokinin concentration in shoots and roots (white bars: control; dark gray bars: SHA.F40). The results are the mean ± *SE* ($n = 5$). Significant differences (Anova test; $p < 0.05$) between treatments and control plants are indicated by an asterisk.

and isopentenyladenosine (iPR). Extraction process was carried out following the method described in Silva-Navas et al. (2019), using 0.25 g of frozen plant material previously ground with liquid nitrogen. Sample homogenization was made with 4 mL of precooled (−20°C) methanol-water-formic acid (15:4:1, v/v/v), and with 25 μL of a stock solution of 100 ng/mL of each deuterium-labeled standard (in methanol). An overnight extraction at −20°C was carried out, after which solids were separated (20.000 g, 10 min, 4°C). Then, they were re-extracted with 2 mL of extraction mixture and centrifuged again. Supernatants were passed through a Sep-Pak C18 cartridge preconditioned with 2 mL of methanol and 2 mL of extraction medium. Afterward, the eluted was evaporated near to dryness with a RapidVap Evaporator and the residue was re-dissolved in 2 mL of 1 M formic acid. This solution was applied to an Oasis MCX column preconditioned with 2 mL of methanol and 2 mL of 1M formic acid. Column was washed with 2 mL of 1 M formic acid, 2 mL of methanol, and 2 mL of 0.35 M NH_4OH, applied in succession. Finally, cytokinins bases and ribosides were eluted with 2 mL of 0.35M NH_4OH in 60% methanol (v/v). The eluted was evaporated to dryness in the RapidVap Evaporator and re-dissolved with 250 μL of methanol and 250 μL of 0.04% formic acid and centrifuged (20.000 × *g* and 10 min) before injection in HPLC-ESI-HRMS system. Description of the quantification and data processing was detailed in Silva-Navas et al. (2019).

Root PM H^+-ATPase Activity

Plasma membrane vesicles were extracted from the apical part of the roots (3–5 cm, 2 g (FW) from two plants per sample) using a sucrose-gradient technique as described in Mora et al. (2010). Extraction of vesicles (and subsequent enzymatic activity determination) was performed in quintuplicates (two plants per replicate) for each treatment and harvest time.

Briefly, apical roots were cut and ground in a mortar with a pestle in an ice cold extraction buffer containing: 250 mM sucrose, 10% (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EDTA, 2 mM dithiothreitol (DTT), 2 mM EGTA, 2 mM ATP, 1 mM PMFS, 20 mg mL-1 chymostatin, 5.7% (w/v) choline-iodine, and 25 mM BTP (1,3-bis [TRIS (hydroxymethyl) methylamino] propane) buffered to pH 6.7 with MES. The homogenate mix was filtered through four layers of sterile gauze and then centrifuged 3 min at 13.000 × *g* and 4°C. The supernatant was conserved and centrifuged 25 min again under the same conditions. The pellets were recovered and resuspended in extraction buffer; this solution was loaded onto 1.5 mL tubes with the sucrose density gradient which consisted in 700 mL of 1.17 g/cm^3 sucrose over 300 mL 1.13 g/cm^3.

Sucrose solutions were prepared in 5 mM BTP-MES (pH 7.4) with all the protectants present in the extraction buffer. The gradients were centrifuged for 1 h at 13000 × *g*, and the vesicles banding at the interface were collected, resuspended again in extraction buffer for cleaning the residuals of sucrose, and centrifuged for 30 min at 13000 × *g*. The resulting pellets were resuspended in 0.5 mL of conservation buffer (20% glycerol; 5 mM DTT; 0.5mM ATP; 50 μg/ml chymostatin; 2 mM EDTA; 2mM EGTA; 2 mM BTP buffered with MES; pH 7.0). Finally, the PM vesicles were frozen with liquid N_2 and stored at −80°C for enzyme activity measurements.

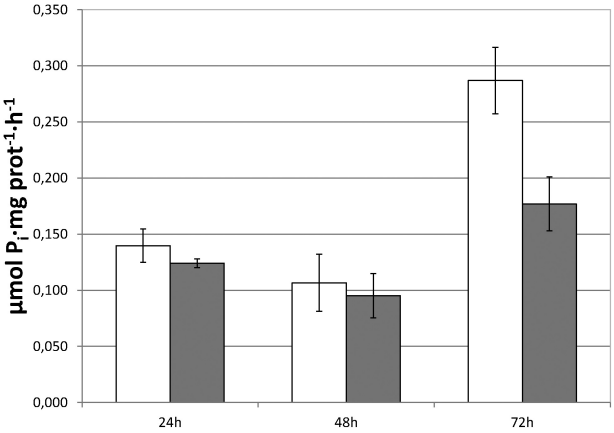

FIGURE 6 | Root plasma membrane H^+-ATPase activity of control and SHA foliar-treated plants. White bars: control; dark gray bars: 40 mg L^{-1} (SHA.40). The results are the mean ± *SE* (*n* = 5).

Enzyme activity was measured following the guidelines of ATPase/GTPase Assay Kit (DATG-200 kit, BioAssay Systems ATPase/GTPase – QuantiChromTM). Total protein quantification was based on the Bradford assay (Bradford, 1976).

Statistical Analysis

Significant differences ($p \leq 0.05$) among treatments were calculated by using one-way analysis of variance (ANOVA) and the LSD Fisher *post hoc* test. All statistical tests were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa, OK, United States).

RESULTS

Foliar-Applied SHA Led to Significant Shoot and Root Growth Increases but Did Not Induce Changes in Leaf Nutrient Concentrations

In the first set of experiments, we evaluated the dose-effect on plant growth. Leaves of cucumber plants were treated with four doses of SHA: 20, 30, 40 and 100 mg of organic C L^{-1} (SHA.F20, SHA.F30, SHA.F40, and SHA.F100). Seventy two hours from the onset of treatments, the only dose that showed significant increases in shoot and root dry matter was SHA.F40 (**Figure 1**). This dose (SHA.F40) was then selected for subsequent experiments. The foliar application of SHA did not cause any changes on the concentration of mineral nutrients in plant leaves (**Supplementary Figure S3**).

Foliar-Applied SHA Led to Noticeable Changes in Root Architecture

Images of the roots of cucumber plants corresponding to the control and foliar-applied SHA (SHA.F40) harvested 72 h from

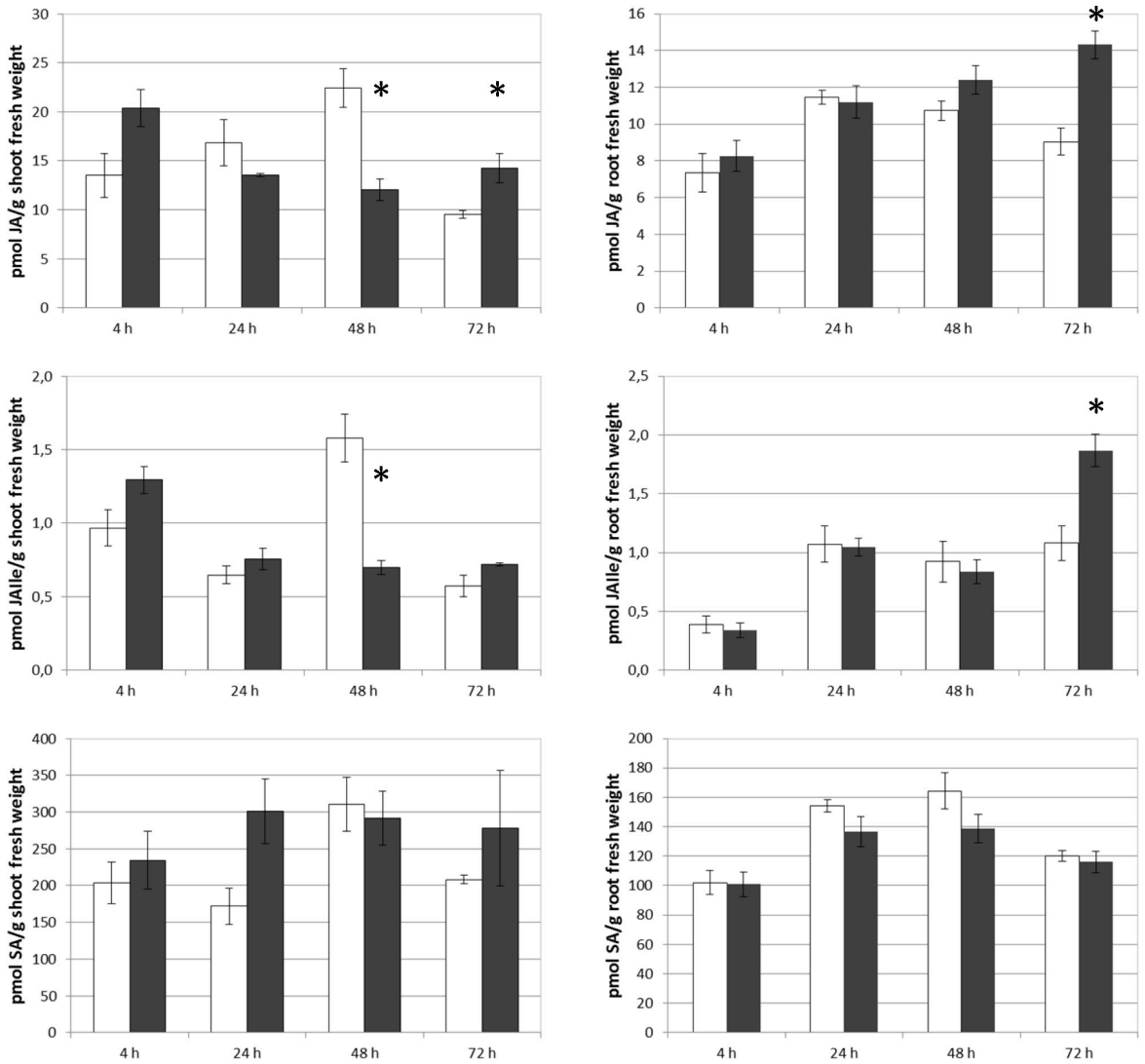

FIGURE 7 | Effect of foliar applied SHA (40 gm C L^{-1}, SHA.F40) on shoot and root concentration of JA, JAIle, and SA of cucumber plants (white bars: control; dark gray bars: SHA.F40). The results are the mean ± *SE* (n = 5). Significant differences (Anova test; $p < 0.05$) between treatments and control plants are indicated by an asterisk.

the onset of treatments are presented in **Figures 2**, **3**. Noticeable effects on root architecture were observed upon SHA foliar-treatment. The qualitative analyses of the results indicated that the roots of control plants presented shorter principal roots but higher proportion of secondary roots than plants treated with SHA, which had longer principal roots but less density of secondary roots, as well as higher volume and more dry matter production (**Figure 1**).

Foliar-Applied SHA Increased IAA but Decreased ABA, in Both the Root and the Shoot

Foliar-applied SHA.F40 caused a significant increase in IAA root concentration after 48 h from the onset of treatments (**Figure 4A**). This effect was accompanied by a concomitant increase in IAA concentration in the shoot also after 48 h from the treatment (**Figure 4B**). As for ABA, SHA.F40 decreased its concentration in both roots (after 48 and 72 h from the onset of treatment) and shoots (after 24 h from the onset of treatment) (**Figures 4C,D**).

Foliar-Applied SHA Increased the Concentration of Several Cytokinins in Both Roots and Shoots

The foliar application of SHA.F40 caused an increase in the shoot concentrations of tZR after 72 h, cZ after 24 and 48 h, and iPR after 72 h (**Figure 5**). In the case of tZ a slight increase was observed after 72 h that was not significant (p = 0.13)

(**Figure 5**). In the roots, SHA.F40 caused a significant increase in the concentration of iP after 4 h, iPR after 4 and 24 h, and cZ after 72 h (**Figure 5**). A slight increase in tZ after 4 h was also observed (p = 0.09) that was accompanied by a significant decrease after 72 h.

Foliar-Applied SHA Did Not Induce Short-Term Increases in PM H^+-ATPase-Activity in Plant Roots

The capacity of foliar-applied SHA (SHA.F40) to increase the activity of root PM H^+-ATPase activity was also studied. The results showed that SHA.F40 was not able to induce a short-term increase in the root PM H^+-ATPase activity (**Figure 6**).

Foliar-Applied SHA Led to Significant Increases in the Shoot- and Root- SA and JA/JAIle Concentrations

Considering that the deposition of SHA onto the leaves does not occur in nature and may present certain analogies with aggressions caused by external agents, the main plant hormones that are involved in the plant responses to this type of affection were also analyzed in roots and shoots: SA, JA, and JA-Ile.

The results obtained show that SHA.F40 caused a significant increase in the root concentration of JA and JA-Ile after 72 h from the onset of treatments, whereas SA concentration was not affected (**Figure 7**). In shoots, however, SHA.F40 caused an increase in JA after 72 h and tended to increase SA concentration after 24 h (p = 0.081) and JA-Ile concentration after 4 h (p = 0.065) (**Figure 7**).

In order to compare these results with those corresponding to SHA-root application, and considering that there were no previous experimental results regarding the effects of root-applied SHA on the root and shoot concentration of SA, JA, and JA-Ile, the effect of 100 mg L^{-1} root-applied SHA (SHA.R100) on the concentration of these plant hormones was also investigated in cucumber. The results obtained show that SHA.R100 did not have a significant effect on the shoot-concentration of SA and JA/JA-Ile for the considered sampling times (data not shown), whereas a significant increase in both JA and JA-Ile was observed in the roots (**Figure 8**).

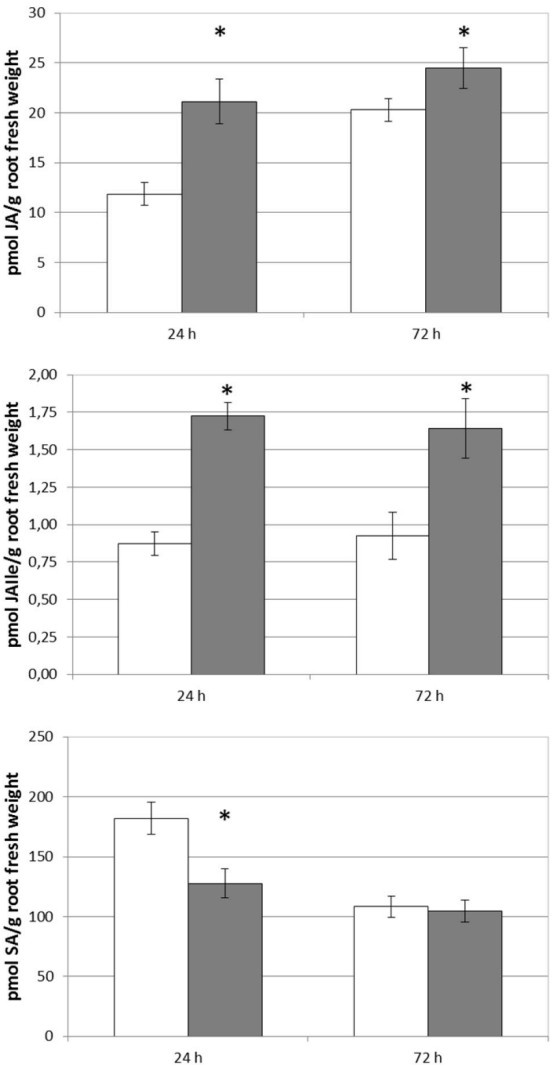

FIGURE 8 | Effect of root applied SHA (100 gm C L^{-1}, SHA.R100) on root concentration of JA, JAIle, and SA of cucumber plants (white bars: control; dark gray bars: SHA.R100). The results are the mean ± *SE* (n = 5). Significant differences (Anova test; $p < 0.05$) between treatments and control plants are indicated by an asterisk.

Foliar-Applied SHA Affected Leaf Surface Structure and Mesophyll Cell Starch

Images from both scanning (SEM) and transmission electron microscopy (TEM) revealed that the foliar application of SHA.F40 SHA affected some leaf structures, such as trichomes, cuticles, and starch granules. Images from SEM showed that the leaves of plants treated with foliar-applied SHA have undergone a loss of trichomes in both adaxial and abaxial leaf sides, compared to control plants (**Figure 9**), whereas there were no differences in the number of stomata or in the proportion of open/closed stomata (**Figure 10**). This result is in line with the values of stomatal conductance, which showed that there were no statistical differences between the stomatal conductance of control plants and SHA.F40 treated plants (data not shown).

The foliar treatment with SHA.40 also caused a diminution of the size of starch granules present in the chloroplasts, in comparison with non-treated leaves from control plants (**Figure 11**).

DISCUSSION

Different Mechanisms Underlay the Plant Growth Promoting Action of Foliar- Versus Root-Applied HA

In agreement with previous results on the application of HS to plant leaves (Rose et al., 2014; Canellas et al., 2015), foliar-applied SHA was found to promote significant increases in both

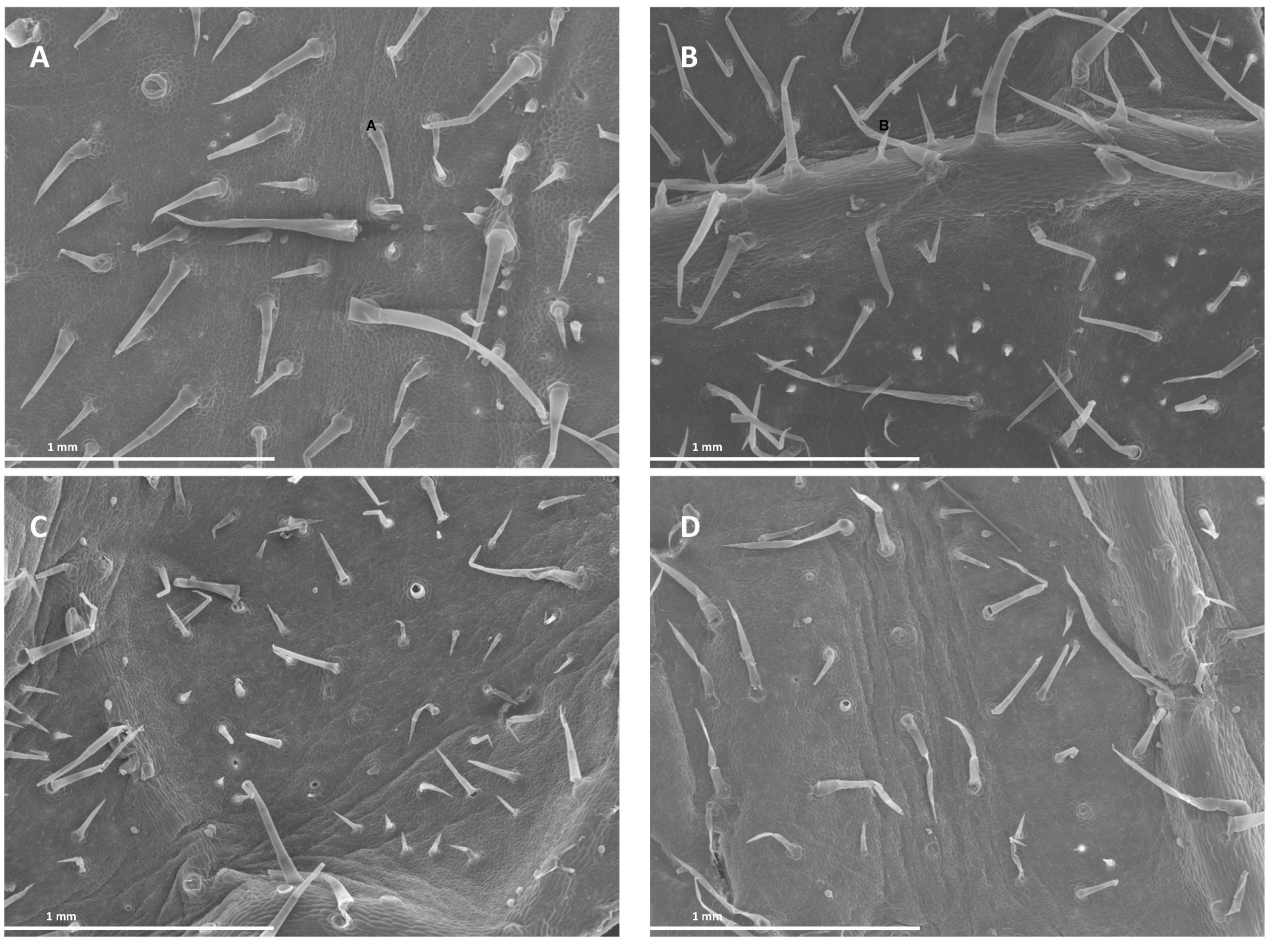

FIGURE 9 | Scanning electron micrographs of cucumber leaf surfaces 7 days after foliar application: adaxial **(A)** and abaxial **(C)** leaf side of control leaves, adaxial **(B)** and abaxial **(D)** leaf side of 40 mg L^{-1} SHA-sprayed leaves (SHA.F40).

shoot and root dry matter at the concentration of 40 mg C L^{-1} (SHA.F40) (**Figure 1**). These results are in line with the results obtained with root-applied SHA in cucumber plants cultivated in hydroponics under the same environmental and nutritional conditions as that used in the present study (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). In principle, these results indicate that SHA is able to promote plant growth regardless the mode of application. However, this fact does not mean that the mechanisms of action underlying these effects are similar to each other.

In fact, some differences were observed regarding the effects on root morphology and architecture. Many studies have reported the capacity of root-applied HA to promote the proliferation of secondary roots (Nardi et al., 2002; Zandonadi et al., 2010; Canellas et al., 2012; García et al., 2016a; Olaetxea et al., 2018). In the case of other studies involving cucumber plants cultivated in hydroponics under similar conditions as in the present study, Mora et al. (2012) reported that root-applied SHA promoted the number of secondary roots as well as root growth in short-term experiments. However, the short-term response to foliar-applied SHA showed that SHA tended to reduce the presence of secondary roots with respect to control plants and increase principal root length and root dry weight with respect to the control (**Figures 1–3**). This fact might be related to the different effect of foliar-applied SHA and root-applied SHA on the concentration in roots of two phytoregulators related to the regulation of root growth and architecture: IAA and ABA. Several studies have shown that the capacity of root-applied HA to enhance lateral root proliferation appears to be mediated by auxin and nitric oxide signaling pathways (Nardi et al., 2002; Zandonadi et al., 2010; Canellas et al., 2015; Olaetxea et al., 2018). Other studies in cucumber with a similar experimental design and conditions as reported here showed that SHA applied to the roots increased the root concentration of IAA and ABA (Mora et al., 2012; Olaetxea et al., 2015). However, whereas inhibitors of IAA biosynthesis and action affected secondary root development but not the SHA-mediated increase in root dry matter (Mora et al., 2012), the inhibition of ABA biosynthesis prevented the SHA effect on the whole root growth reflected in root dry matter production (Olaetxea et al., 2019). These results suggested a relevant role of ABA in the mechanisms underlying the action of root-applied SHA on the

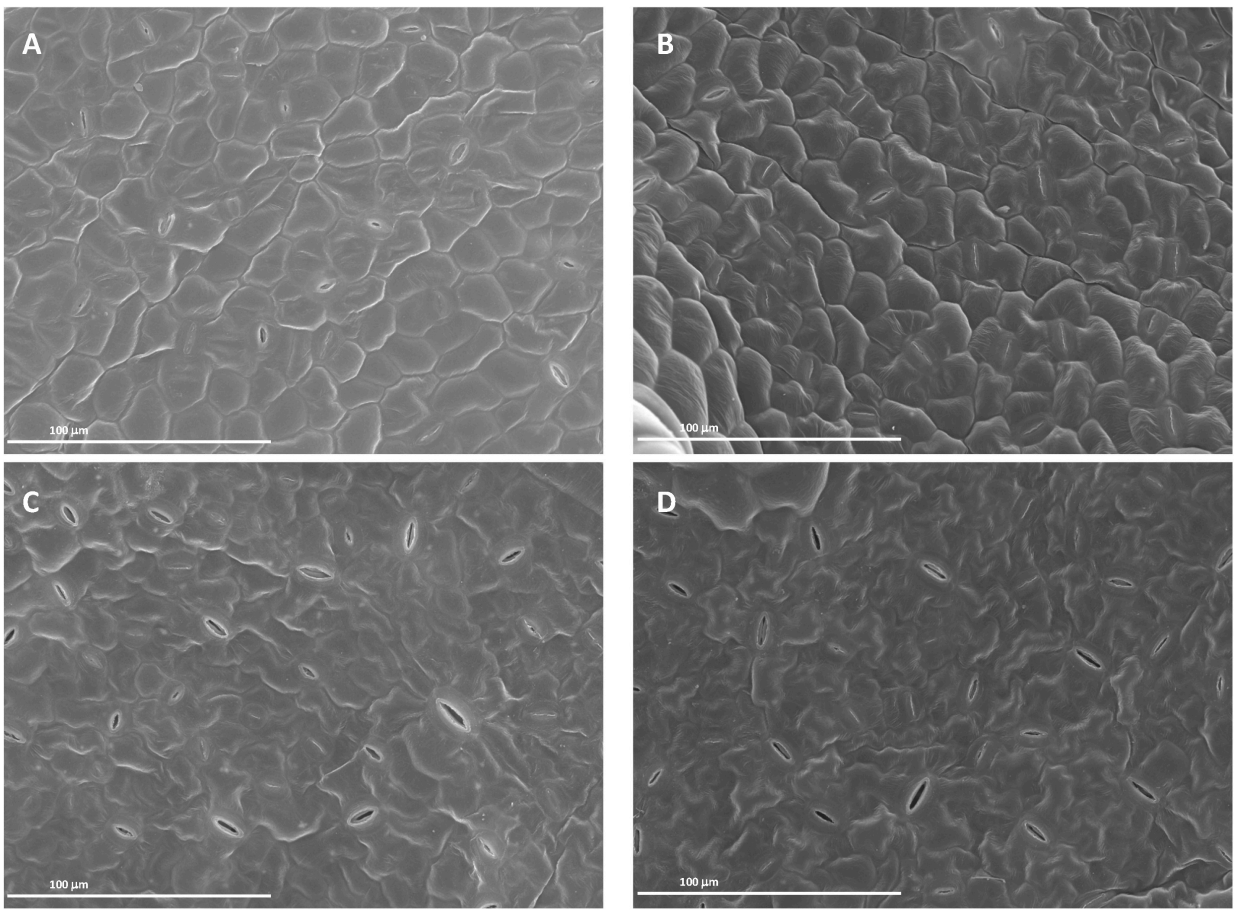

FIGURE 10 | Scanning electron micrographs of cucumber leaf surfaces 7 days after foliar application: adaxial **(A)** and abaxial **(C)** leaf side of control leaves, adaxial **(B)** and abaxial **(D)** leaf side of 40 mg L^{-1} SHA-sprayed leaves (SHA.F40).

root development (Olaetxea et al., 2019). However, the results obtained in our experiments indicate that some other factor (or factors) must be affecting the effect of foliar-applied SHA on root growth and architecture. This conclusion is supported by the fact that we do not observe any increase in secondary root proliferation linked to the increase in root IAA, but we do observe an increase in the global root growth as reflected by the dry matter production despite root ABA concentration did not increase. As it will be discussed later, these results might be associated with the crosstalk between the different hormones affected by foliar-applied SHA, rather than with an effect of a specific hormone. In any case, it is clear that the short-term effects of SHA applied to the leaves and those for SHA applied to the roots show different patterns regarding root development and architecture.

Previous studies reported that the improvement in cucumber shoot growth associated with root-applied SHA was linked to the increase in IAA caused by SHA in the roots (Mora et al., 2014). SHA applied to the leaves also caused an increase in the concentration of IAA in roots (**Figures 4A,B**). Interestingly, this effect was accompanied with an increase of IAA concentration in the shoot (**Figures 4A,B**), which might play a relevant role in the promotion of shoot growth associated with foliar-applied SHA since several studies have reported its role in the regulation of stem elongation and shoot growth (Gallavotti, 2013). In summary, these results support that IAA could also play a relevant role in the shoot growth promotion resulting from foliar-applied SHA.

The decrease in ABA in root and shoot linked to the foliar application of SHA may be relevant regarding shoot growth. It is well known that increases in ABA in the shoot are normally associated with a decrease in shoot growth (Vysotskaya et al., 2018) and leaf senescence promotion (Ghanem et al., 2008). It is therefore possible that the decrease in shoot ABA caused by foliar-applied SHA might have also contributed to the shoot growth.

Further studies in cucumber showed that root PM H^+-ATPase activity played a crucial role in the shoot growth-promoting action of root-applied SHA (Olaetxea et al., 2019). In fact, the use of inhibitors of the activity of this enzyme prevented the increase in shoot growth mediated by SHA applied to roots (Olaetxea et al., 2019). It is therefore plausible that this enzyme may also be involved in the increase in shoot growth caused by foliar-applied SHA. However, the results obtained in experiments with foliar-applied SHA.F40 associated with short-term increases in

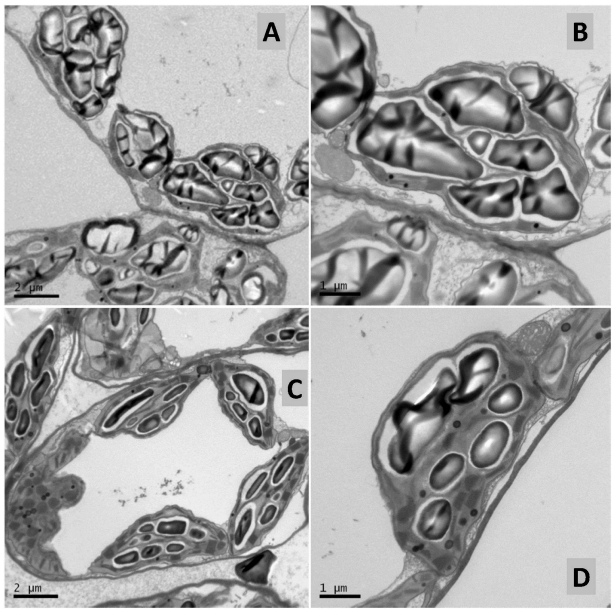

FIGURE 11 | Transmission electron micrographs of control **(A,B)** and 40 mg L^{-1} SHA-sprayed (SHA.F40) **(C,D)** cucumber leaves, 7 days after foliar treatment. Detail of chloroplasts containing starch in mesophyll cells.

shoot growth, did not show any noticeable short-term effect on root PM-H^+-ATPase activity (**Figure 6**). Therefore, although a medium- and/or long- term stimulation of root PM H^+-ATPase activity resulting from foliar-applied SHA cannot be ruled out, this action would not explain the short-term enhancement of shoot growth promoted by foliar SHA application (**Figure 1**). In addition, the lack of effects of foliar-applied SHA on the root PM H^+-ATPase activity may explain why foliar applied-SHA did not change the leaf concentration of the nutrients analyzed (**Supplementary Figure S3**) since this enzyme is directly involved in root nutrient uptake (Olaetxea et al., 2018).

Another event that played a relevant role in the mechanism underlying the shoot-growth promoting action of root-applied SHA was a short-term increase in the concentration of some cytokinins in the leaves and roots (Mora et al., 2010). In the case of foliar-applied SHA we also observed an increase in the root and shoot concentration of several cytokinins (**Figure 5**). This fact is in line with the enhancement of shoot growth observed in foliar-SHA treated plants. In the case of root-applied SHA the effect of cytokinin concentration in leaves was mediated by the stimulation in root-PM H^+-ATPase activity (Olaetxea et al., 2019). Nevertheless, for foliar-applied SHA this mechanism does not appear to be involved in the regulation of this process since this treatment did not have any short-term effect on root-PM H^+-ATPase activity.

Olaetxea et al. (2015, 2019) reported that root ABA also played an important role in the promotion of shoot growth after root SHA application (Olaetxea et al., 2015, 2019). However, foliar-applied SHA did not increase ABA concentrations in roots (**Figure 4**), thus suggesting that this event is not involved in its effect on shoot growth. Therefore, in addition to IAA, other signaling pathways different from root PM- H^+-ATPase and root ABA must be involved in the shoot growth promoting action of foliar-applied SHA and the increase in cytokinin leaf concentration resulting from this treatment.

SHA Applied on the Leaves, but Also to the Roots, Affects SA and JA Signaling Pathways

As described in the introduction, the interaction of HS with leaf surfaces does not occur in nature and can be sensed by plants as an external aggression. In such case, plants normally activate SA and JA/JA-Ile signaling pathways as a defensive and adaptive response (Wasternack and Hause, 2013; Nazar et al., 2017). It is therefore plausible that foliar-applied SHA may activate these signaling pathways. In this framework, the results obtained regarding the root- and shoot- concentration of SA and JA/JA-Ile are very relevant. Our results confirm this hypothesis since SHA applied to leaves clearly affected the concentration in roots and shoots of JA and JA-Ile that is the active form of the hormone (**Figure 7**).

These results suggest that foliar-applied SHA may cause some damage at a leaf surface level. Analysis of foliar-SHA treated leaves by SEM and TEM showed some anatomical changes associated with SHA application.

On the one hand, SHA treatment decreased trichome densities (**Figure 9**). A further interesting finding was the decrease in leaf mesophyll starch accumulation in the chloroplasts upon SHA foliar application (**Figure 11**). This effect was unexpected since the application of HA to plant roots is associated with an increase in chloroplast starch accumulation (Jannin et al., 2012). This effect may be potentially linked to a mobilization of carbohydrates associated with higher metabolic activity and regulated by cytokinin activity. However, the effect of foliar SHA supply of leaf starch concentrations should be studied more in depth in future investigations.

In order to compare the effects of SHA foliar application on JA, JA-Ile, and SA with those obtained with root-applied SHA, we carried out a new experiment exploring the action of SHA applied to the roots on the concentration of these hormones in roots and shoots. This experiment was performed in cucumber plants cultivated in hydroponics under the same environmental and nutritional conditions as that used in foliar SHA application and preliminary root SHA supply trials (Olaetxea et al., 2019). Surprisingly, SHA root application led to significant short-term increases in the root concentration of both JA and JA-Ile (**Figure 8**), whereas no clear effects were observed in shoots. As in the case of foliar-applied SHA, these results are consistent with some potential involvement of JA signaling pathway in the whole mechanism of action of root-applied SHA on plant growth.

Regarding the potential roles that SA and JA could play in the mechanisms responsible for the plant growth-promoting action of SHA applied to either roots or leaves, several studies reported negative cross-talk between SA and JA in the regulation of several processes related to plant development, such as plant defense mechanisms and root development (Traw and Bergelson, 2003).

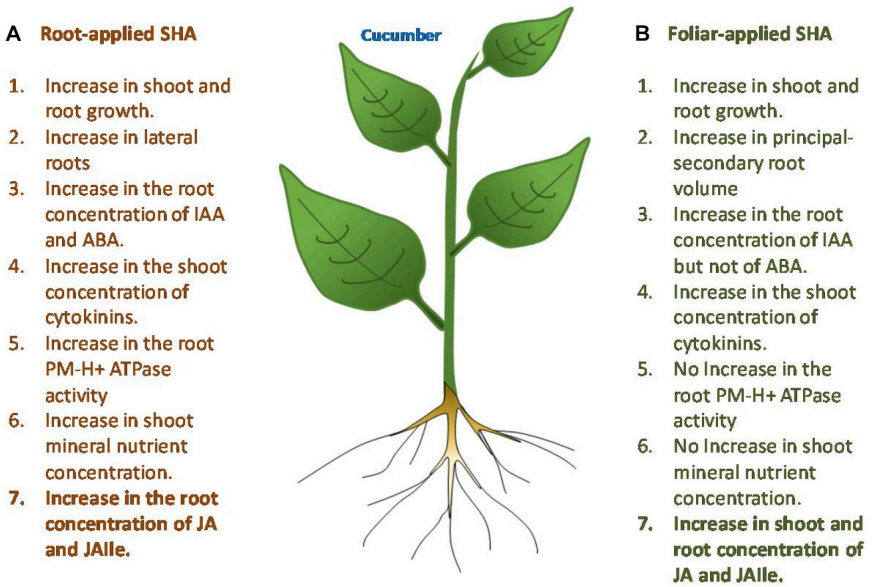

FIGURE 12 | Comparison of some short-term responses on cucumber plants to root-applied SHA and foliar-applied SHA.

Likewise, it is well known that SA is generally involved in the regulation of plant responses to biotrophic and hemi-biotrophic pathogens, whereas JA is involved in plant responses to necrotrophic pathogens and herbivorous (Wasternack and Hause, 2013; Nazar et al., 2017). In this context, it is therefore complicated to discuss the role of both SA and JA in the positive regulation of the same process.

Some studies described that the application of low concentrations of SA increased root growth and root dry matter production (Deef, 2007). Conversely, several studies reported that JA inhibited plant growth but promoted secondary root formation (Wasternack and Hause, 2013). In our experiments with foliar-applied SHA, we observed short-term increases in JA and JA-Ile root concentrations that were not accompanied by a reduction in root growth or increases in lateral root formation (**Figures 9A**, **3**, **4**, respectively). On the contrary, we observed an increase in root dry matter production and a reduction in secondary root formation (**Figures 3**, **4**). This fact suggests that JA signaling pathways do not play a dominant role in short-term effects of foliar-applied SHA on root development. As mentioned above, these results suggest that these processes might be regulated by the ratios, the relative proportion, between specific hormones involved in root development regulation such as IAA, ABA, cytokinins, SA and JA.

However, regarding root-applied SHA, the results obtained are compatible with a relevant role of JA in the SHA mediated effects on secondary root production along with other hormones such as IAA and ABA (Olaetxea et al., 2015, 2019).

Finally, the effects of foliar-applied SHA on JA signaling pathways are compatible with the induction of higher resistance of treated plants against eventual pathogen attacks. In any case, it becomes clear that more research is required in order to elucidate the role of JA in the whole mechanism underlying the beneficial action of SHA on plant development and, eventually, plant defense against pathogens.

Likewise, it is highly likely that additional biochemical and molecular processes may also be involved in the long-term response of plants sprayed with HS. However, in light of our findings, the short-term reaction of plants to HS application has great influence in the whole action of HS during the entire growing cycle (Olaetxea et al., 2018).

CONCLUSION

The results obtained are compatible with the hypothesis that the beneficial action of foliar-applied SHA or root-applied SHA on plant growth may result from molecular and biochemical events triggered by a transient mild stress associated with SHA application (**Figure 12**), although the mechanisms underlying these responses are different depending on the mode of application. Whereas the root application of SHA increases plasma membrane H^+-ATPase activity, shoot mineral nutrient concentration, and ABA concentrations in roots, among other effects, foliar-applied SHA did not induce those effects. However, both root-applied and foliar-applied SHA caused increases in IAA cytokinins, JA and JA-Ile. In this sense, further studies are needed in order to unveil the role of JA in the mechanisms of action of SHA.

DATA AVAILABILITY STATEMENT

All datasets generated in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DD, MF, VF, and JG-M conceptualized and designed the study. DD, MF, and MO performed the experimental work. AZ assessed the hormone detection. VF performed the microscopy studies. DD, MF, and JG-M analyzed the data. JG-M, DD, MF, VF, and AZ prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00493/full#supplementary-material

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Culturable Bacterial Endophytes From Sedimentary Humic Acid-Treated Plants

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The global decrease in soil fertility leads to a new agricultural scenario where eco-friendly solutions play an important role. The plant growth promotion through the use of microbes, especially endophytes and rhizosphere microbiota, has been proposed as a useful solution. Several studies have shown that humic substances are suitable vehicles for the inoculation of plant growth promoting bacteria, and that this combination has an enhanced effect on the stimulation of plant development. In this work, cucumber plants grown hydroponically have been pre-treated with a sedimentary humic acid (SHA) with known plant growth-enhancing effects, and culturable bacterial endophytes have been isolated from these plants. The hypothesis was that this pre-treatment with SHA could lead to the isolation of certain endophytic taxa whose proliferation within the plant could have been promoted as a result of the effects of the treatment with SHA, and that could eventually reinforce a potential synergistic effect of a combined application of those endophytic bacteria and SHA. The culturable endophytes that have been isolated from humic acid-treated cucumber plants have been identified as members of four main phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. Isolates were characterized according to the following plant growth-promoting traits: nitrogen fixation/scavenging, phosphate solubilization, siderophore production and plant hormone production. Most of the isolates were able to fix/scavenge nitrogen and to produce plant hormones (indole-3-acetic acid and several cytokinins), whereas few isolates were able to solubilize phosphate and/or produce siderophores. The most promising endophyte isolates for its use in futures investigations as plant growth-promoting bacterial inocula were *Pseudomonas* sp. strains (that showed all traits), *Sphingomonas* sp., *Stenotrophomonas* sp. strains, or some *Arthrobacter* sp. and *Microbacterium* sp. isolates.

Keywords: endophyte, plant growth promotion, microbiota, humic, phosphate solubilizing bacteria, cucumber

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INTRODUCTION

In the last decades the human population has grown exponentially, reaching 7,600 million people in 2018, and as the Food and Agriculture Organization (FAO) has predicted, in 2050 the world population will be near to 10,000 million[1]. This fact involves an increasing pressure over global food production and the land surface dedicated to that purpose.

[1] http://www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050_Global_Agriculture.pdf

However, ∼35% of the world surface is already dedicated to crop production, according to FAO database[2], and increasing the crop land surface is not an ecologically valid solution, being in fact very controversial in most of the developing countries where population demands new eco-friendly politics. Only increasing crop yields appears as a possible solution to prevent food shortage in this future scenario, although the excessive use of NPK chemical fertilizers is already negatively affecting soil fertility, soil microbial activity, and may cause the pollution or/and eutrophication of water reservoirs (Torrent et al., 2007; Ulén et al., 2007; Youssef and Eissa, 2014).

Therefore, more rational, environmentally friendly, and efficient agricultural practices are needed. One approach is the use of biofertilizers containing living microorganisms (Dastager et al., 2010; Bhardwaj et al., 2014; Canellas et al., 2015; Pérez et al., 2016; Suhag, 2016; Dias et al., 2017). This strategy has recently gained relevance with the development of a new generation of gene sequencing techniques, which have allowed the assessment of microbe-plant relationships and the development of a new evolutionary model, the holobiontic theory (Rosenberg and Zilber-Rosenberg, 2016). This model proposes that microbiota would evolve over time to improve the fitness of the plant under changing environmental conditions such as drought, salinity, nutrient deficiency, or soil contamination (Murphy et al., 2015; Fidalgo et al., 2016; Soussi et al., 2016; Kumar et al., 2017).

Among the different kinds of biofertilizers, those including plant growth-promoting rhizobacteria (PGPR) are frequent (Mahaffee and Kloepper, 1997; Bhattacharyya and Jha, 2012; Sarathambal et al., 2015; Kumar et al., 2017; Gouda et al., 2018). The main effect of PGPR in plants is the improvement of both nutrient availability in the rhizosphere and the plant resistance to biotic/abiotic stresses (Gouda et al., 2018). However, the application of these microorganisms has several efficiency limitations when applied to the soil under field conditions due to the competition with native soil microbiota and their low survival rate. These facts cause the poor reproducibility of the agronomical results of PGPR-based treatments in field crops (Oliveira et al., 2006). Recent studies have shown that a promising approach to overcome all these limitations in efficiency might be the application of PGPR directly on the leaves (Canellas et al., 2013, 2015; Olivares et al., 2017).

In contrast with PGPR, the endophytic microbiota has been only recently explored as a potential source of beneficial microorganisms for improving plant growth (Brader et al., 2013; Sessitsch et al., 2019). Endophytes are those microorganisms inhabiting inner plant tissues (Hallmann et al., 1997). Their main source is the rhizosphere so that they share the same advantages as those of PGPR but showing special characteristics that may overcome some of the limitations associated with the use of PGPR even when applied to the leaves. Endophytes are well adapted to living within the plant, thus favoring in some way their efficacy as plant growth promoters (Reiter and Sessitsch, 2006; Hardoim et al., 2008; Compant et al., 2010). Indeed, endophytes have evolved to transmit themselves to the next plant generation through seed colonization (Johnston-Monje and Raizada, 2011; Truyens et al., 2015; Nelson, 2018). In fact, this trait would justify the use of PGP endophytes instead of PGPR inoculums: the evolutionary selection of endophytes and the capability of being inherited through plant generations provide them with high biocompatibility with plant tissues thus increasing their possibilities to help plants to grow under normal conditions or under stress conditions (López et al., 2018).

On the other hand, several studies have shown the compatibility and synergistic beneficial action on plant growth of PGPR and humic substances (HS) when applied together (Olivares et al., 2017 and references therein), the most studied combination being with diazotrophic endophytic bacteria, especially *Herbaspirillum* spp. HS are a specific fraction of soil organic matter that can be extracted using alkaline solutions (Stevenson, 1994) and have been proven to promote the plant development by increasing nutrient availability in soils and activating plant metabolism (Mora et al., 2010, 2013; Zandonadi et al., 2013; García et al., 2014; Olaetxea et al., 2018; de Hita et al., 2019; Zanin et al., 2019).

The aim of the present work was to isolate the culturable endophytic bacteria from plants pre-treated with HS. The HS used was a sedimentary humic acid (SHA) with a known plant growth-enhancing effect on cucumber plants (Aguirre et al., 2009; Mora et al., 2010, 2013; Olaetxea et al., 2015). To the date of the preparation of this manuscript, there were no published papers about how HS affect the endophytic microbial populations, with the exception of de Hita et al. (2018). In that preliminary study, the data showed that the application of SHA can modulate the relative abundances of some bacterial (i.e., *Actinobacteria*) and fungal endophytic communities in cucumber plants, based on cultured-independent techniques (metagenomics sequencing). In this context, our approach has been to pre-treat the plants with SHA, and to isolate the culturable endophytes, whose proliferation (or at least the proliferation of some of them) within the plant might have been helped by the application of SHA. The PGP traits of each isolate have then been tested. The ultimate goal of this work was to identify potentially promising endophytic PGP bacterial candidates isolated from plants pre-treated with SHA, with the hypothesis that they could also show a synergistic effect when applied as inocula in combination with SHA, based on the review by Olivares et al. (2017).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cucumber seeds (*Cucumis sativus* L. var. Ashley) were sown in a bed of sterile perlite and wet filter paper, and placed in a germination chamber in darkness, at 25°C, and 75% relative humidity. One week later the seedlings were transferred to a hydroponic system in a growth chamber whose day/night conditions were: 16 h/9 h (irradiance of 250 μmol m^{-2} s^{-1}), 25°C/21°C and 70%/75% relative humidity. The nutrient solution utilized was previously described in Mora et al. (2010) and Olaetxea et al. (2015), with minimum changes in the final concentration of Fe-EDDHA and $MnSO_4$ (80 and 27.3 μM, respectively). After 10 days, plants were treated with a 100 mg L^{-1}

[2]http://www.fao.org/faostat/en/3data

C of a SHA obtained from leonardite as described in Mora et al. (2010) and characterized in Aguirre et al. (2009). The treatment was applied 2 h after the start of the diurnal period. Plants were harvested 7 days from the onset of the treatments.

Plant Surface Sterilization and Bacterial Endophyte Isolation

SHA pre-treated cucumber plants were surface-sterilized prior to the isolation of bacterial endophytes. Firstly, three different cucumber plants were rinsed, separately, with autoclaved deionized water (dH_2O) to wash the nutrient solution from the roots. Plant surfaces were afterward sterilized following a protocol based on the methods reported in Hardoim et al. (2012) and Reinhold-Hurek et al. (2015), with little modifications. Briefly, cucumber plants were rinsed with commercial bleach (<5% sodium hypochlorite) containing 0.1% Tween 20 for 3 min. The next steps consisted of three consecutive washes with autoclaved dH_2O for 5 min each one and stirring in an orbital shaker. Finally, roots, stem, and leaves were separated with a sterile scalpel and frozen with liquid N_2 for later use. To verify the surface sterilization, 1 mL of the last wash was plated and cultured on R2A agar medium. Plates were incubated at 27°C for 3 days. No growth was detected for any plant. All sterilization steps were carried out in a laminar flow cabinet in sterile conditions.

Plant organs were ground with sterile mortar and pestle using autoclaved peptone water (0.9 mL per tissue gram) to recover the microorganisms. The liquid was filtered through sterile gauze to eliminate plant debris. This filtrate was used (100 μL) for microbial culturing 10-fold serial dilutions in autoclaved peptone water (10^0–10^{-4}).

Microorganisms were isolated plating one milliliter from each dilution, by the pouring plate method, in a minimal medium (R2A agar) with the aim to favor the slow-growing bacteria from endosphere (Eevers et al., 2015, 2016). Plates were incubated for 7 days at 27°C. Morphologically single colonies from each plate were selected, picked, streaked, and re-streaked on new R2A agar plates to obtain axenic cultures of each isolate. Finally, each pure culture was inoculated in LB broth and incubated for 20–72 h, at 27°C, and 160 rpm in a microbiological incubator; then bacterial stocks in 25% glycerol were prepared and conserved at −80°C. A total of 72 isolates were successfully grown and conserved in glycerol stocks.

Isolate Identification

Partial PCR amplification of the 16S rRNA gene was performed directly from the glycerol stocks, using the universal primers F799 (5′-AACMGGATTAGATACCCKG-3′), and 1492R (5′-AAGGAGGTGATCCANCCRCA-3′) (Hogg and Lehane, 1999; Chelius and Triplett, 2001). The PCR mix contained: 1 μL of 10 μM F799 primer, 1 μL of 10 μM 1492R primer, 2.5 μL of bacterial glycerol stock, and 10.5 μL Premix Ex Taq RR003A. The PCR was performed in a iCycler iQ thermocycler, with the following protocol: an initial denaturation step at 98°C for 1 min; 30 PCR cycles at 98°C for 10 s, 57°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were purified with the NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel, following the manufacturer guidelines.

DNA concentration in each purified PCR product was measured in a Nanodrop ND-1000 spectrophotometer. Capillary sequencing was carried out by CIMA Lab Diagnostics. Sequencing reads were searched against RDP SeqMatch (Cole et al., 2014) and BLASTn databases using default parameters. For the majority of the isolates, both databases provided the same taxonomical assignment. If not, BLASTn[3] taxonomical assignment prevailed. The reference sequences selected belonged to GenBank 16S partial sequences and were used for building the phylogenetic tree. The reference sequences and the sequences of the isolates were aligned by Clustal Omega web service[4] (Madeira et al., 2019) with default parameters except for the number of combined iterations, max guide tree iterations, and max HMM iterations, that were shifted from default to five in all of them. The alignment tree distances resulting from Clustal Omega were used as basic data to create the circular cladogram tree in iTOL[5] (Letunic and Bork, 2019).

Bacterial Endophytes Characterization

Growth on Nitrogen-Free Medium

The endophyte isolates were tested for their capability to fix or scavenge nitrogen using NFC medium (10 g/L mannitol, 0.2 g/L $MgSO_4.7\ H_2O$, 0.2 g/L KH_2PO_4, 0.2 g/L NaCl, 0.2 g/L $CaSO_4.2\ H_2O$, 5 g/L $CaCO_3$, 15 g/L European bacteriological agar; pH 7.2), based in Ashby's mannitol agar (Liu et al., 2016; Li et al., 2018). Microorganisms were picked from the glycerol stocks (10 μL), streaked on NFC medium, and incubated at 30°C for 7 days. Those plates with positive growth were re-streaked over fresh NFC plates twice (each 7 days). Only the plates with consistent bacterial growth after 21 days were considered positive isolates for nitrogen-free medium growth trait. An *Azotobacter vinelandii* DSMZ 85 strain was used as a positive control microorganism.

Inorganic Phosphate Solubilization

For the detection of mineral phosphate solubilizer microorganisms, NBRIP agar was used as the culture medium: 10 g/L glucose, 5 g/L $Ca_3(PO_4)_2$, 5 g/L $MgCl_2{\cdot}6\ H_2O$, 0.25 g/L $MgSO_4{\cdot}7\ H_2O$, 0.2 g/L KCl, 0.1 g/L $(NH4)_2SO_4$, 15 g/L European bacteriological agar, pH 7–7.2 (Nautiyal, 1999; Truyens et al., 2013). Glucose was dissolved in a small volume of sterilized dH_2O, filter-sterilized (0.45 μm), and then added to the sterilized medium.

Each isolate was tested in two different plates, placing in each of them five 10 μL-drops from the corresponding bacterial glycerol stock. After 7 days at 27°C in darkness, clear halos around positive isolates were measured. These isolates were classified as fast solubilizers. Seven days later, solubilization halos were measured again, and those isolates with new clear halos were classified as slow solubilizers. The IPS ratio (Inorganic Phosphate Solubilization ratio) between the halo diameter and the colony diameter was also used as a classification parameter

[3]https://blast.ncbi.nih.gov

[4]EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/clattalo

[5]Interactive Tree of Life, https://itol.embl.de

(Batista et al., 2018). A *Bacillus megaterium* var. *phosphaticum* DSMZ 3228 strain was used as a positive control for inorganic phosphate solubilization.

Siderophore Production

Isolates were tested as siderophore producers with the CAS-agar protocol developed by Schwyn and Neilands (1987) and modified by Cordero et al. (2012). Firstly, all the PYREX glasswares were deferrated, rinsing with 10% HCl (vol/vol) overnight and five consecutive washes with dH2O. Then, the CAS-Fe-HDTMA dye was prepared (1 L): 10 mL $FeCl_3$ 10 mM dissolved previously in 100 mM HCl, 590 mL 1 mM Chrome azurol sulfonate, and 400 mL 2 mM HDTMA. The solution was autoclaved (25 min, 121°C) in an opaque PYREX bottle and stored at room temperature. After that, the CAS-agar was prepared, containing 30.24 g PIPES, 1 g/L NH_4Cl, 3 g/L KH_2PO_4, 20 g/L NaCl, adjusting at a final pH of 6.8, and finally adding 9 g/L agar noble. After autoclaving (20 min, 121°C), 30 mL of filter-sterilized (0.45 μm) 10% (w/v) casamino acids, 10 mL of filter sterilized (0.45 μm) 20% glucose (w/v) and 100 mL of previously prepared CAS-Fe-HDTMA solution were added to the medium and dispensed in plates. Each isolate was tested in the same way as in the phosphate solubilization assay. After 7 days at 27°C in darkness, yellow-orange halos around positive isolates were measured. These isolates were classified as siderophore producers. The MCI ratio (Metal Chelation Index ratio) between the halo diameter and the colony diameter was also used as a parameter to evaluate the siderophore production (Batista et al., 2018). A *Pseudomonas* sp. DSMZ 25842 strain was used as a positive control for siderophore production.

Plant Hormones Production

The production of plant hormones by bacterial isolates was tested by growing each isolate in 5 mL of LB broth supplemented with filter-sterilized (0.45 μm) 5 mM L-Tryptophan, for IAA production (Lin et al., 2015; Gilbert et al., 2018). Isolates were grown in triplicates for 20 h at 28°C with 250 rpm shaking in 50 mL sterile centrifuge tubes. OD at 600 nm of all isolates was measured, but those that did not reach a minimum OD_{600} value of 0.6 were not considered for hormone concentration measurements. In resume, only 55 isolates were considered for hormone production analyses. The cultures were centrifuged at 5,200 rpm for 10 min, and supernatants were transferred to clean 12 mL tubes and stored at −80°C until hormone quantification. A *Pseudomonas* sp. DSMZ 25842 strain was used as a previously known IAA producer. Final concentration for each replicate was calculated after subtracting the control (LB medium) hormonal concentration and dividing by the OD_{600} value.

The content of acidic hormones (IAA; jasmonic acid, JA; jasmonoyl isoleucine, JA-Ile; abscisic acid, ABA; and salicylic acid, SA) and CKs (isopentenyladenine, iP; isopentenyladenosine, iPR; trans- and cis-zeatin, tZ and cZ; trans- and cis-zeatin riboside, tZR and cZR; dihydrozeatin, DZ; dihydrozeatin riboside, DZ) were analyzed by high performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (UHPLC-ESI-HRMS).

The procedures for the determination of acidic hormones and CKs are different and were performed separately using two different aliquots from the same sample/culture. The quantification was carried out in a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific), equipped with an ESI source, a quadrupole mass filter, a C-Trap, a HCD collision cell, and an Orbitrap mass analyzer, following the methodology elaborately described in Silva-Navas et al. (2019).

The content of IAA, JA, JA-Ile, ABA, and SA was analyzed as follows: for each triplicate of every bacterial culture, and for three replicates of the pure culture medium (without bacteria, as a blank), aliquots of 90 μL of culture broth were added to 10 μL of internal standard (1000 ng ml^{-1} of deuterium-labeled internal standards in metanol), 150 μL of MeOH, and 150 μL of acetic acid 0.133%, and centrifuged at 20,000 *g* (Sigma 4–16 K Centrifuge) for 10 min before the injection in the UHPLC-ESI-HRMS system, using exactly the same conditions detailed in Silva-Navas et al. (2019), and the same identification and quantification procedure.

For the analysis of the production of CKs by endophytic bacterial isolates, 100 μL of the culture medium for each bacteria and replicate were mixed with 25 μL of internal standard (100 ng/mL of each standard in methanol), 225 μL of methanol and 150 μL of formic acid 0.04% and centrifuged at 20.000 *g* for 10 min before the injection of the sample. Three aliquots of 100 μL of the pure culture medium without bacteria were subjected to the same procedure, with the purpose of serving as a blank for the determination of the concentration of hormones produced by the bacteria. The measurement conditions, detection, and quantification have already been described in Silva-Navas et al. (2019).

Statistical Analysis

For comparison between hormonal productions by genus, ANOVA signification tests were carried out followed by HSD Tukey *post-hoc* tests. The statistical tests were performed with the stats package in R (RStudio Team, 2016). The $p \leq 0.05$ was used as statistically significant threshold.

RESULTS

Taxonomic Diversity of SHA Pre-treated Cucumber Culturable Endophytic Microbiota

The number of viable endophytic bacterial isolates obtained from three different plants of cucumber previously grown in the presence of 100 ppm C SHA in the nutrient solution was 72. For the taxonomic identification of the isolates, BLASTn and RDP SeqMatch databases were utilized. Both identifications were similar, with only a misleading identification in one isolate (CR329.a, **Supplementary Table S1**). Most of the microorganisms identified (97% of the isolates) showed ≥ 97% of similarity with reference sequences in BLASTn database.

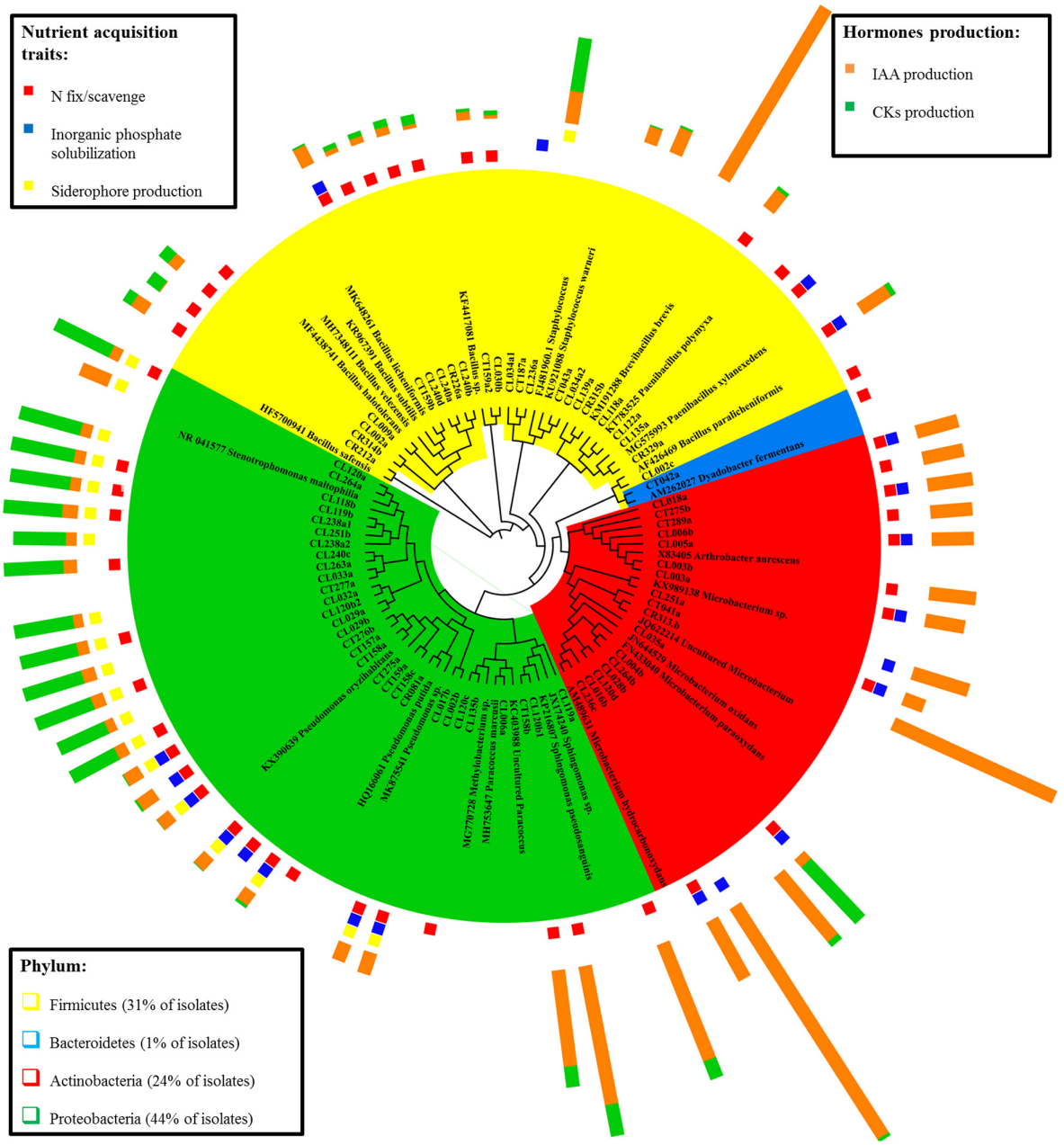

FIGURE 1 | Cladogram representing the identification of the endophytic bacterial isolates and the corresponding plant growth promotion traits. Isolates are grouped by their sequence similarity to the BLASTn reference sequence. Phylum taxonomic level is showed by color: *Firmicutes* (yellow), *Bacteroidetes* (blue), *Actinobacteria* (red), and *Proteobacteria* (green). Plant growth promotion traits are separated by nutrient acquisition traits (presence or absence) and hormone production.

The cladogram tree represents the closest classification of isolates to reference sequences in BLAST (**Figure 1**). Most of the isolates belonged to the phylum *Proteobacteria* (44%), followed by *Actinobacteria* (24%), *Firmicutes* (31%), and *Bacteroidetes* (1%) (class, order, and family distributions of isolates are shown in **Supplementary Figure S1**). *Proteobacteria* isolates were the most diverse group, with five different families (*Xanthomonadaceae*, *Pseudomonadaceae*, *Sphingomonadaceae*, *Rhodobacteraceae*, and *Methilobacteraceae*) representing two different classes (α- and γ-*proteobacteria*). Both *Actinobacteria* and *Firmicutes* were represented by only one phylogenetic class: *Actinobacteriia* and *Bacilli*, respectively. In each class, bacteria from two (*Micrococcaceae* and *Microbacteriaceae*) and three (*Staphylococcaceae*, *Paenibacillaceae*, and *Bacillaceae*) different families were identified. *Bacteroidetes* phylum had only one isolate belonging to *Cytophagaceae* family. The most represented isolated species was *Stenetrophomonas maltophilia* (15 isolates), followed by *Arthrobacter* aurescens (seven strains)

and *Pseudomonas oryzihabitans* (seven strains). The cladogram confirmed the identification by BLASTn and RDP SeqMatch.

Plant Growth-Promotion Traits in Endophyte Isolates

Endophytic isolates were screened for their *in vitro* plant growth-promoting traits (PGP). The traits selected were those related to the mineral nutrient acquisition (nitrogen fixation/scavenging, inorganic phosphate solubilization, and siderophore production) and those associated with plant growth regulators (IAA and CKs plant hormones) production. Isolates clustering together (**Figure 1**) showed similar PGP performance according to the studied traits, but the functional strain diversity was highlighted as well.

The biological nitrogen fixation/scavenging was the most prevalent PGP trait within the nutrient acquisition features studied, with 68% of isolates being able to grow in an N free medium (**Figure 2**). This trait is showed by diverse phylogenetic groups (**Figure 1**).

Regarding inorganic phosphate solubilization, it was performed by only 28% of isolates. They were classified as: fast solubilizers, which solubilized phosphate after 7 days and had the greatest IPS ratios after 14 days; or slow solubilizers, whose halos of solubilization were visible not at 7 days but after 14 days, or showed small IPS ratios. All the fast solubilizers (9 isolates) were identified as *Pseudomonas* genus. The slow phosphate solubilizers were more diverse, and there were 11 isolates from six different genera: *Arthrobacter*, *Paenibacillus*, *Microbacterium*, *Stenotrophomonas*, and *Staphylococcus* (**Supplementary Table S2**).

Most of the isolates producing siderophores able to chelate iron (29% of isolates) belong to *Pseudomonas* and *Stenetrophomonas* genera (**Figure 2** and **Supplementary Table S3**). Those isolates producing a MCI ratio higher than 1.5 were classified as great siderophore producers, all the identified *Pseudomonas* strains pertained to that group.

Although the production of a wide group of plant hormones (listed section "Materials and Methods") has been tested, only IAA, cZ, cZR, iP, and iPR were detected in our cultured endophytic isolates.

There were 16 isolates that did not grow appropriately in LB broth supplemented with 5 mM Trp, so the hormonal production was not measured for those bacteria (**Supplementary Table S1**). The rest of the isolates were able to produce IAA and CKs. IAA production ranged between 1 and 245 ng/mL (**Figure 3**), and the isolates were classified according to their IAA production in three levels: low producers (<50 ng/mL), medium producers (50–99 ng/mL) and great producers (>100 ng/mL). Most of the isolates were low producers (65% of all isolates), but 7% of isolates (5 strains) were great IAA producers and identified by BLASTn as *Microbacterium paraoxydans*, *Sphingomonas pseudosanguinis*, *Sphingomonas* sp., an uncultured *Microbacterium*, and *Brevibacillus brevis*.

The four different CKs detected (cZ, cZR, iP, and iPR) showed different dynamics in the isolates (**Figure 4**) and we have classified the CKs producers according to net CKs production. This net production was categorized as highly positive (>30 pmol/mL, CKs great producers), positive or zero (0–30 pmol/mL, CKs low or no producers), or negative (<0 pmol/mL, CKs consumers). Most of the isolates consumed part of the iPR initially present in the culture broth, and some of them produced larger amounts of iP (**Figure 4**). On the other hand, with the exception of *Arthrobacter*, all isolates produced smaller quantities of cZR than cZ. CKs net production appears as a diverse and essential trait for different endophytic bacterial taxa. There were 16 isolates with a high net production, ranging between 45 and 72 pmol/mL of total CKs. The most represented genera among these CKs producers were *Stenotrophomonas maltophilia* (14 isolates) strains (**Supplementary Table S1**). In general, high CKs net production was accompanied by low levels of IAA production.

DISCUSSION

Plant Growth Promotion Traits of Cultured Endophyte Taxa From SHA-Treated Plants

Endophytic communities are commonly shaped by the soil, being the roots the main entrance door (Hardoim et al., 2008, 2015; Reinhold-Hurek et al., 2015). Another important source of bacterial endophytes is the vertical inheritance of endophytes through the seeds (Johnston-Monje and Raizada, 2011; Hardoim et al., 2012; Truyens et al., 2013, 2015; Khalaf and Raizada, 2016, 2018; Nelson, 2018). These vertically transmitted endophytes have been specially selected by evolutionary forces; therefore conforming an interesting option for applications in agriculture. In our experimental design, in which cucumber plants have grown in hydroponics, presumably most of the culturable endophytes isolated are inherited from the seeds of *Cucumis sativus* var. *Ashley*.

Despite only being a fraction of the total number of bacteria living within the plant, the distribution of the cultured endophytes among different phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*) was similar to the predominant composition of bacterial communities in rhizospheres and endospheres of angiosperms plants (Hardoim et al., 2015), such as barley (Bulgarelli et al., 2015), rice (Hameed et al., 2015), wheat (Liu et al., 2017), the plant model *Arabidopsis thaliana* (Lundberg et al., 2012), and other plants (Fitzpatrick et al., 2018).

In this work, PGP traits related to the improvement of nutrient acquisition or to the production of plant growth hormonal regulators have been assessed. In the case of the nutrition-related traits, most of the isolates (68%) were able to grow in N-free medium. This trait implies the potential capability of these microorganisms to fix atmospheric nitrogen or the scavenging of trace N-compounds from the atmosphere (such as NH_3 or N_2O) (Zuluaga et al., 2020). Juraeva et al. (2006) already reported a correlation of N content and endophytic nitrogen fixation in cucumber plants, especially in roots, based on the analysis of nifH gene copy numbers, indicating the relevance of this trait in

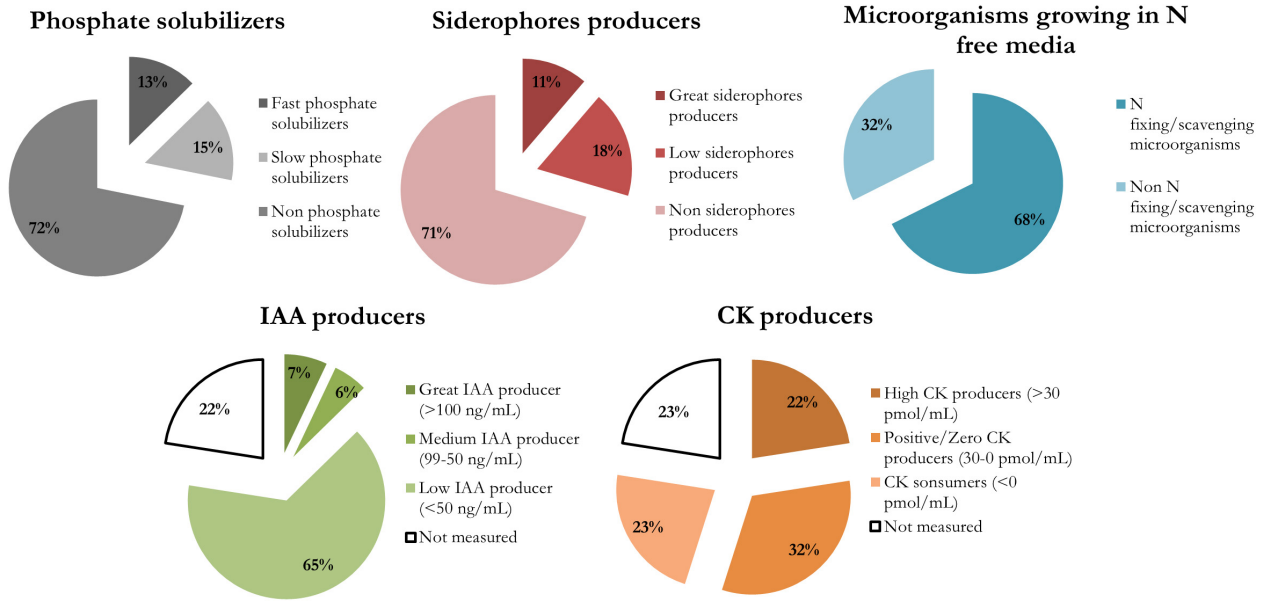

FIGURE 2 | Prevalence of each plant growth promotion trait in the endophytes isolated from cucumber plants pre-treated with a sedimentary humic acid. The traits evaluated were: inorganic phosphate solubilization at 14 days, siderophore production at 7 days, capability of growing in a N-free medium, and phytohormones production (IAA and CKs).

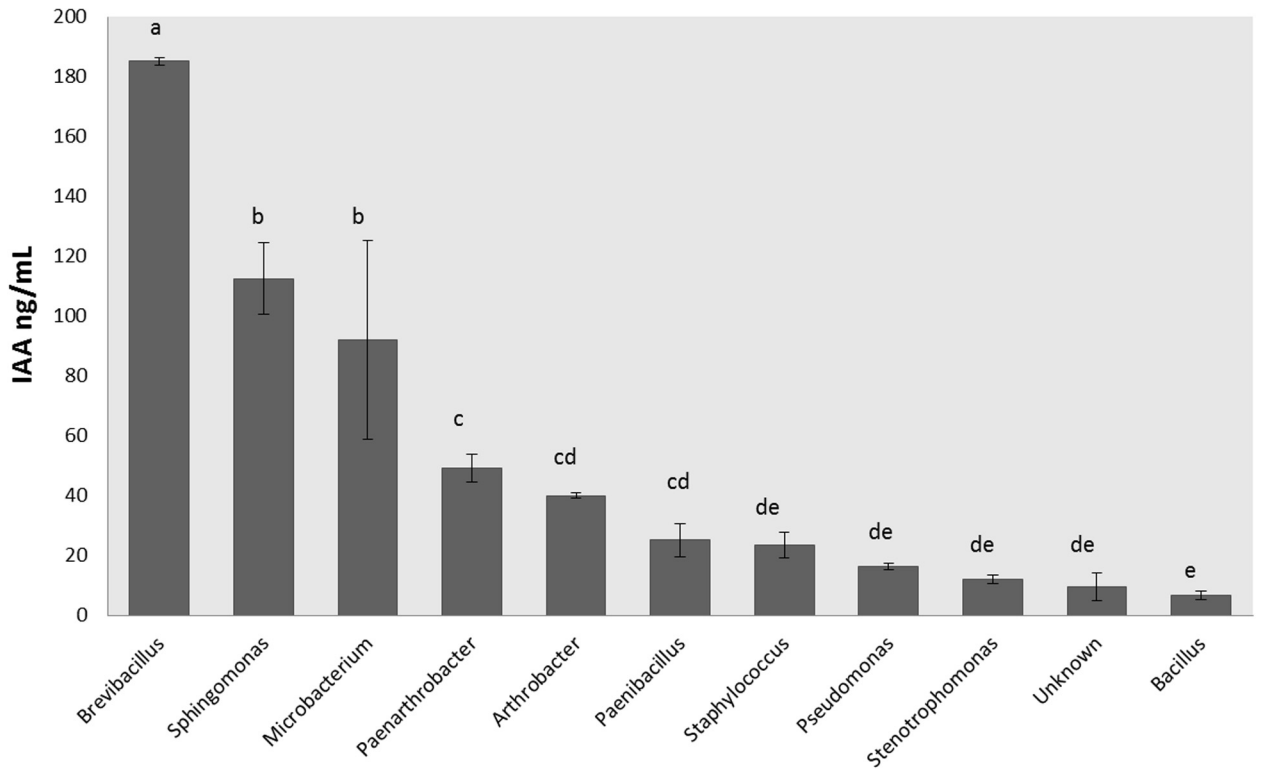

FIGURE 3 | Production of indole-3-acetic acid by endophytic isolates according the genus. Indole-3-acetic acid production was measured in ng per mL after 20 h of growth at 28°C in LB medium supplemented with Trp 5 mM. Production was measured in triplicates for each isolate, and the final concentration was obtained after standardization. Bar errors represent the standard error. Letters represent the significant groups after ANOVA and HSD Tukey *post-hoc* tests. Signification threshold: $p \leq 0.05$.

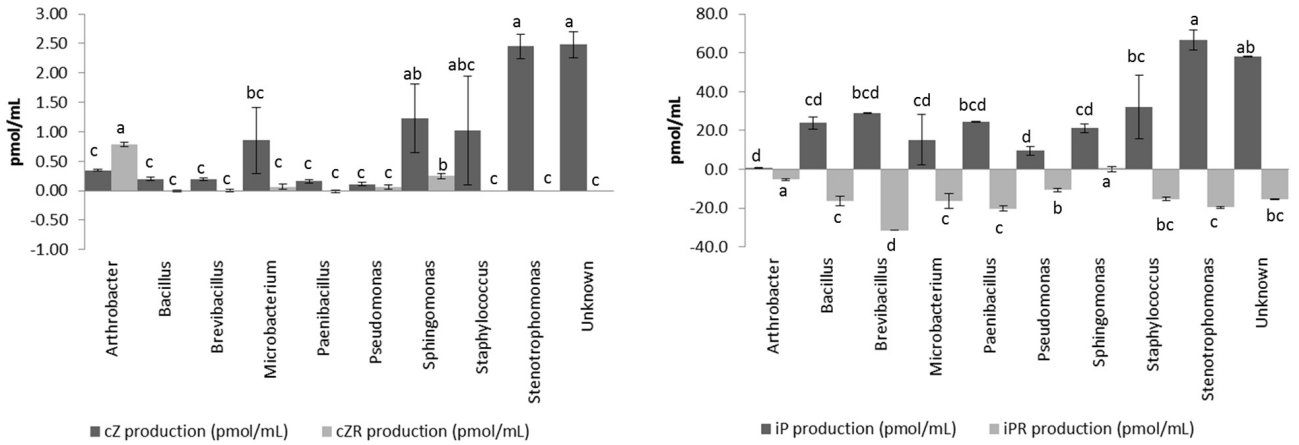

FIGURE 4 | Production of cytokinins (cZ, cZR, iP, and iPR) by endophytic isolates according the genus. Cytokinin production was measured in pmol per mL after 20 h of growth at 28°C in LB medium supplemented with 5 mM L-Tryptophan. Production was measured in triplicates for each isolate, and the final concentration was obtained after standardization. Bar errors represents the standard error. Letters represents the significant groups after ANOVA and HSD Tukey *post-hoc* tests. Signification threshold: $p \leq 0.05$.

cucumber plants, although further assays should be carried out to confirm the actual biological nitrogen fixation or scavenging activity of our isolates.

Siderophore production and phosphate solubilization were the less common traits in our isolates. Bacterial siderophore iron complexes can contribute to iron uptake in plants, and they might confer a certain level plant defense induction through limiting the availability of Fe for pathogens (Ahmed and Holmström, 2014). Phosphate solubilization is an ecologically important trait because P is the second major limiting nutrient for plant growth despite its abundance in soils (Weyens et al., 2009; Khalaf and Raizada, 2016). Phosphorus is habitually found in non-plant-available forms, such as tricalcium phosphate or phytate, so microbial solubilization of inorganic phosphate or mineralization of organic phosphorus would enhance its bioavailability for plants (Mahanty et al., 2016). Within our endophytic isolates, only *Pseudomonas* sp. showed both siderophore production and phosphate solubilization traits, producing the greatest MCI and IPS ratios (**Supplementary Tables S2, S3**). *Pseudomonas* genus is a well-known siderophore producer, especially by the synthesis of pyoverdine (Kloepper et al., 1980; Gamalero and Glick, 2011), as well as an inorganic P solulibilizer (Browne et al., 2009). *Stenotrophomonas maltophilia* isolates were good siderophore producers (Egamberdieva et al., 2016; Singh and Jha, 2017), while *Arthrobacter* strains were promising inorganic phosphate solubilizers.

On the other hand, the plant growth hormonal regulators produced by the isolated bacterial endophytes were indole-3-acetic acid (IAA) and several cytokinins (cZ, cZR, iP, an iPR). Bacterial production of IAA has been extensively studied (Dodd et al., 2010; Hameed et al., 2015; Fidalgo et al., 2016; Gilbert et al., 2018; López et al., 2018; Afzal et al., 2019; Zuluaga et al., 2020) but, in contrast with most of the works previously referenced, in our study the IAA production has been analyzed by means of UHPLC-ESI-HRMS. This technique has lower detection limits than the Salkowski reagent method (Gordon and Weber, 1951).

The increment in IAA plant concentration promotes the cell proliferation, enlarges the root system, increases the root biomass, changes the root architecture, and enhances nutrient and water uptake efficiency (Dodd et al., 2010; Egamberdieva et al., 2016; Gilbert et al., 2018). The highest production of these plant hormones in our isolates was found in *Brevibacillus*, *Microbacterium*, and *Sphingomonas* genera (**Figure 3**). The bacterial production of plant-like growth regulators has been commonly associated with host-microbe cross-talk and plant colonization (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011; Carvalho et al., 2014; Koul et al., 2015). Defez et al. (2017) also reported that IAA-overproducing transformed endophytic diazotrophs improved their nitrogen-fixing capacity both *in vitro* and in inoculated rice-roots.

The production of cytokinins by bacteria, although widely known, is rarely taken into account in PGP bacterial characterization, and it only has been measured in a few works (Timmusk et al., 1999; Dodd et al., 2010 and references therein). Our results showed that the isolated endophytic bacteria produced mainly iP, and also small amounts of cZ, while iPR was transformed/consumed from the medium. The high production of iP by some of these bacterial isolates could have important effects on plant development since iP is considered one of the most active CKs in the plant (Osugi and Sakakibara, 2015). In general, the action of cytokinins in plants is related to the formation of shoots, chloroplastic maturation, cell expansion, stomatic conductance, and meristematic tissue differentiation (Cassán et al., 2014; Osugi and Sakakibara, 2015). Recently, new effects have been found for this family of hormones, such as the role of cZ in biotic and abiotic response, or nutritional status (Großkinsky et al., 2013, 2016; Schäfer et al., 2015; Silva-Navas et al., 2019).

Relationships Between the PGP Traits of Culturable Endophytes in SHA-Treated Plants and the Mechanism of Action of SHA in Plants

Mora et al. (2010) reported that SHA is able to promote nitrate root uptake and nitrate reductase activity in cucumber plants. Other studies showed that humic acids extracted from different sources were able to induce the expression of plant genes directly involved in nitrate transport and further assimilation, as well as to enhance the root H^+-ATPase activity (Trevisan et al., 2010; Jannin et al., 2012; Olaetxea et al., 2018). But the detailed mechanisms through which HS enhance the uptake of different nutrients are still unveiled and, with the exception of the work by de Hita et al. (2018), there are no studies exploring the effects of HS on endophytic microbiomes. It might be possible that the thriving of certain endophytic bacteria in roots could lead to an acidification of the external pH, which could concomitantly contribute to an enhanced assimilation or bioavailability of several nutrients (i.e., nitrate or Fe). Therefore, the effects observed upon the treatment with HS could be additional to (or mediated by) the effects corresponding to bacterial endophytes.

Other studies have shown that HA-metal-phosphate complexes led to an increase in the internal utilization of P by plants (Urrutia et al., 2014; Jindo et al., 2016), with higher concentrations of soluble phosphate in plant tissues. The fact that several families of endophytes isolated from SHA pre-treated plants were able to mobilize inorganic P is also compatible with a possible P solubilization from those fractions precipitated with Fe or Ca in the apoplast (Snowden and Wheeler, 1995). Therefore, both SHA and endophytes could contribute to the mobilization of internal fractions of precipitated P.

A similar reasoning might be applied to Fe plant nutrition. Various studies have shown the ability of HS to improve Fe root uptake and further assimilation in cucumber (Pinton et al., 1999; Aguirre et al., 2009; Zanin et al., 2019), with a significant activation of Fe-deficiency root responses even under Fe sufficient conditions (Aguirre et al., 2009), and an increase in the physiologically active Fe fraction (1N HCl-extractable Fe, related to the chlorophyll content). In this framework, those endophytes producing siderophores could also contribute to this process through the solubilization of Fe precipitated in apoplast. Thus, as in this case of P, the effects observed upon SHA treatment improving Fe plant nutrition are compatible with a positive action of specific endophytic groups.

This hypothetical synergistic action of SHA and endophytic microbiota in the whole mechanism responsible for the growth enhancing effect of SHA in cucumber plants could also be extended to the case of plant hormone action. Several studies have reported that the shoot- and root-growth promoting action of SHA in cucumber is regulated by IAA, ABA, and some families of CKs, principally trans-zeatin (tZ) and adenine-based CKs (Mora et al., 2010; Olaetxea et al., 2015, 2018). Most of the endophytic bacteria isolated from SHA pre-treated cucumber plants were able to produce significant amounts of IAA abd CKs (**Figures 3**, **4**), what is also compatible with a potential cooperation between the biochemical action of SHA and bacterial endophytic activity in plant tissues. Regarding CKs, the cultured endophytes promoted the synthesis of cZ and not tZ, which is the main CK involved in the SHA shoot growth promoting effect. However, a recent study has shown that the cZ:tZ ratio plays a very relevant role in the regulation of plant responses to P deficiency (Silva-Navas et al., 2019). It could therefore be possible that the ability of endophytes to produce cZ has some influence in the improvement of the adaptation of SHA-treated cucumber plants to low concentrations of available P in the nutrient solution.

CONCLUSION

Endophytic microorganisms with PGP traits are a promising tool to improve the crop production due to its natural presence in plants tissues, which confer them an ecological advantage against rhizosphere microorganisms. This study also highlights the importance of seed microbiome, as the bacterial endophytes have been isolated from cucumber plants grown in a hydroponic system with a minimized entrance of microorganisms, compared to the size and variety of microbial communities present in soils.

The cultivable endophytes isolated from plants treated with a SHA present a relevant capacity to affect some processes related to plant mineral nutrition and hormonal signaling pathways. In addition to that, all these plant growth promotion traits can be evolved in a complementary, additive or synergistic way with the main mechanisms activated upon SHA application. One of the perspectives to explore in depth in future works would be the actual PGP activity of these isolated endophytic bacteria, applied either alone, as a consortium, or using SHA as a carrier.

DATA AVAILABILITY STATEMENT

All datasets generated in this study are included in the article and the **Supplementary Material**. Isolate sequencing reads were deposited in GenBank under accession numbers MN512151 to MN512214.

AUTHOR CONTRIBUTIONS

DD, MF, and JG-M conceptualized and designed the study and carried out the data analysis. DD and YR performed the experimental work. AZ assessed the hormone detection. DD did statistical analysis. DD, MF, JG-M, and AZ prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00837/full#supplementary-material

FIGURE S1 | Isolates distribution by phylum, class, order, and family.

TABLE S1 | Identification and PGP characterization of endophytes extracted from SHA pre-treated cucumber plants. The identification used as reference databases the BLASTn and RDP Seq-Match assignment of 16S rRNA sequences.

TABLE S2 | Inorganic phosphate solubilization (IPS) ratios for those isolates able to solubilize the inorganic phosphate after 7 or 14 days after incubation.

TABLE S3 | Metallic chelation index (MCI) ratios for those isolates producing siderophores after 7 days of incubation.

FILE S1 | 16S partial sequences of the isolates.

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frontiers in Plant Science

ORIGINAL RESEARCH
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Bioactivity of Size-Fractionated and Unfractionated Humic Substances From Two Forest Soils and Comparative Effects on N and S Metabolism, Nutrition, and Root Anatomy of *Allium sativum* L

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Humic substances (HS) are powerful natural plant biostimulants. However, there is still a lack of knowledge about the relationship between their structure and bioactivity in plants. We extracted HS (THE1-2) from two forest soils covered with *Pinus mugo* (1) or *Pinus sylvestris* (2). The extracts were subjected to weak acid treatment to produce size-fractionated HS (high molecular size, HMS1-2; low molecular size, LMS1-2). HS were characterized for total acidity, functional groups, element and auxin (IAA) contents, and hormone-like activity. HS concentrations ranging from 0 to 5 mg C L^{-1} were applied to garlic (*Allium sativum* L.) plantlets in hydroponics to ascertain differences between unfractionated and size-fractionated HS in the capacity to promote mineral nutrition, root growth and cell differentiation, activity of enzymes related to plant development (invertase, peroxidase, and esterase), and N (nitrate reductase, glutamine synthetase) and S (O-acetylserine sulphydrylase) assimilation into amino acids. A positive linear dose-response relationship was determined for all HS in the range 0–1 mg C L^{-1}, while higher HS doses were less effective or ineffective in promoting physiological-biochemical attributes of garlic. Bioactivity was higher for size-fractionated HS according to the trend LMS1-2>HMS1-2>THE1-2, with LMS2 and HMS2 being overall more bioactive than LMS1 and HMS1, respectively. LMS1-2 contained more N, oxygenated functional groups and IAA compared to THE1-2 and HMS1-2. Also, they exhibited higher hormone-like activities. Such chemical properties likely accounted for the greater biostimulant action of LMS1-2. Beside plant growth, nutrition and N metabolism, HS stimulated S assimilation by promoting the enrichment of garlic plantlets with the S amino acid alliin, which has

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recognized beneficial properties in human health. Concluding, this study endorses that i) treating THE with a weak acid produced sized-fractionated HS with higher bioactivity and differing in properties, perhaps because of novel molecular arrangements of HS components that better interacted with garlic roots; ii) LMS from forest soils covered with *P. mugo* or *P. sylvestris* were the most bioactive; iii) the cover vegetation affected HS bioactivity iv); HS stimulated N and S metabolism with relevant benefits to crop nutritional quality.

Keywords: humic substances, molecular size, biostimulants, growth, sulfur, nitrogen, amino acids, root cell differentiation

INTRODUCTION

A great deal of literature has long corroborated a key role of soil organic matter (SOM) in soil preservation and fertility (Nardi et al., 2004; Johnston et al., 2009). However, a number of factors, primarily ongoing climate changes, extensive farming and industrial agriculture, have progressively led to a dramatic decrease of SOM content, thus hampering soil fertility and crop yield goals (Kopittke et al., 2019).

To overcome the issue of soil fertility decline, innovative technologies have been proposed that make use of substances with biostimulant properties (Ertani et al., 2009; du Jardin, 2015; Colla et al., 2016; Nardi et al., 2016; Canellas et al., 2019). According to the definition formulated by Yakhin et al. (2017), biostimulants must be intended as products of biological origin able to afford benefits to plants, by stimulating their productivity and inducing positive physiological responses under sub-optimal nutritional inputs and environmental stresses. The effects of biostimulants in plants are not due to the sole occurrence of endogenous plant growth promoters or stress signaling and protective compounds, but are the result of joined properties of the mixture constituents (Nardi et al., 2016; Yakhin et al., 2017; Rouphael and Colla, 2018). Biostimulants can be grouped in two main categories, microbial and non-microbial (Regulation EU, 2019/1009). The former group includes beneficial fungi and bacteria, the latter humic substances, protein hydrolysates and various N-compounds, seaweed extracts and botanicals, beneficial elements, chitosan and other biopolymers.

Humic substances (HS) in particular, represent pivotal and abundant components of SOM (until 80%), capable of coordinating physical, chemical and biological processes in soil through the control of ion bioavailability (Zanin et al., 2019). Their nature is contentious, but the hypothesis that HS could be artifacts resulting from alkaline extraction procedures has been definitely rejected (Nebbioso and Piccolo, 2012). Early theories asserted that HS possess a macromolecular structure producing random coil conformations (Swift, 1999) or micelles or "pseudo micellar" structures (Wershaw, 1999) in solution. Later, other authors argued that HS are a buildup of small heterogenous organic molecules, such as sugars, fatty acids, polypeptides, aliphatic chains, and aromatic rings, hold together and stabilized by intermolecular hydrophobic interactions and hydrogen bonds (Piccolo, 2001; Piccolo, 2002; Sutton and Sposito, 2005; Šmejkalová and Piccolo, 2008).

Some constituents of HS and their arrangements can dictate the establishment of biotic rhizosphere associations and provoke plant responses *via* interaction with receptors localized at the root cell membranes (Shah et al., 2018). HS act as biostimulants owing their capacity to prompt plant growth and provide benefits to the primary metabolism and biochemical pathways involved in the synthesis of secondary compounds, primarily phenolics (Nardi et al., 2017; Nardi et al., 2018; Nunes et al., 2019; Zanin et al., 2019). The effects of HS on plant growth are mainly due to their capacity to increase soil micro- and macro-element availability for plant uptake by forming soluble complexes with several ions (Garcia-Mina et al., 2004; Varanini and Pinton, 2006). Furthermore, HS can induce changes in the perception of the plant nutrient status and in the signaling pathways implied in nutrient sensing, thus speeding up nutrient transport processes in the roots through increased synthesis and induced activity of plasma membrane localized-nutrient transporters and H^+-ATPases (Nardi et al., 1991; Chen et al., 2004; Zandonadi et al., 2007; Canellas and Olivares, 2014; Zanin et al., 2018; Canellas et al., 2019; Olaetxea et al., 2019). The activation of H^+-ATPase by HS seems to depend on mechanisms that use nitric oxide (NO) as a messenger during the early stages of lateral root emergence (Zandonadi et al., 2010).

Some authors ascribe part of HS bioactivity to the content in hormones and/or to the hormone-like activity displayed by certain HS functional constituents, such as aliphatic-C, carboxyl-C, and phenol-C (aromatic) groups (Schiavon et al., 2010; Canellas et al., 2011; Pizzeghello et al., 2013; Nardi et al., 2018). The HS biological activity was also ascertained to rely on HS hydrophobic features (Canellas et al., 2009; Martínez Balmori et al., 2014) and molecular distribution (Piccolo et al., 1992; Muscolo et al., 2007; Nardi et al., 2007; Zandonadi et al., 2007; Canellas et al., 2010).

The linkage between HS stimulation effects in plants and HS molecular dimension is however under debate, as contrasting results have been produced so far. Piccolo (2001), based on early evidence (Dell'Agnola and Nardi, 1987; Nardi et al., 1988; Nardi et al., 1991), stated that HS properties result from HS treatment with organic acids. Such assumption was explained in terms of permanent alteration of the hydrophobic domains in the micelle-like aggregations, with the shift from high molecular size (HMS)

to small molecular size (LMS) due to disaggregation effects caused by organic acids (Piccolo et al., 1996a; Piccolo et al., 1996b). Under this condition, the carboxyl groups of organic acids are oriented between the micelles and the water interface.

In many studies, the LMS fraction of HS is reported to be the most effective in inducing stimulatory responses in plants (Nardi et al., 1988; Piccolo et al., 1992; Muscolo et al., 2007; Nardi et al., 2007). Nevertheless, the HMS fraction proved to behave as a very positive root growth regulator in other works (Zandonadi et al., 2007; Dobbss et al., 2007; Canellas et al., 2009; Schiavon et al., 2010). The hypothesized mechanisms through which the two fractions induce plant physiological responses sturdily differ. While the LMS fraction might be able to enter the root cells and elicit intracellular molecular signals (Muscolo et al., 2007), the HMS fraction is believed to trigger hormone signaling pathways inside cells after binding to root cell membrane external protein receptors (Dobbss et al., 2007; Muscolo et al., 2007; Schiavon et al., 2010).

The HMS/LMS ratio varies based on soil type. In forest soils, the biological activity persists at minimum and a thick humus layer is formed as a result of SOM accumulation. More than 90% of this SOM consists of humus derived from intense decomposition of plant and animal litters, with rates varying for the different litter types according to the substrate quality. Fine roots and deciduous leaves that are high in nutrients and possess fungal and bacterial necromass can be decomposed in one year, while most coniferous leaves (needles) require some years to decay, and decades (branches) to centuries (tree trunks) to disappear, depending on pedoclimatic conditions (Breemen and Buurman, 2002). The small molecules derived from organic matter degradation become self-reassembled in molecular clouds, with properties that reflect those of their parental materials (Ponge, 2015). Studies conducted on bulk forest soil samples and HS fractions indicated that different forest humus types result from different rates, but common pathways, of litter decomposition (Ziegler et al., 1992). This humus is recognized as a rich source of HS that may be used as valuable biostimulants in agriculture.

To date, differences in biological activity of HS derived from forest soils have not been largely studied (Nardi et al., 2000; Pizzeghello et al., 2001; Pizzeghello et al., 2002). Also, despite all relevant findings on HS reported so far, the nexus between of HS bioactivity and chemical structure still represents a complex, controversial and partially unknown phenomenon that deserves more investigation (Calderín García et al., 2016; Calderín García et al., 2019). Therefore, this study is aimed at: i) assaying differences in chemical composition and biological activity between unfractionated and fractionated HS extracted from two forest soils subjected to different vegetation cover; ii) appraising differential effects of these HS in altering root nutrient content status, primary metabolism and ultrastructure of garlic (*Allium sativum* L.) over a short-time; iii) determining a dose-response relationship for both unfractionated and fractionated HS.

Garlic was used in this study because is a common and relevant horticultural crop worldwide, rich in healthy phytochemicals, including certain sulfur (S)-compounds. To date, the effect of HS on S metabolism has been poorly investigated and we aimed to assay whether short-term HS application to garlic plantlets could positively impact on it.

MATERIALS AND METHODS

Extraction and Fractionation of Humic Substances

Humic substances (HS) appraised in this study were extracted from two forest soils (Rendzic Leptosols) (IUSS WRB, 2015) derived from our Pedoteca and originally collected at Cortina d'Ampezzo (NE Italy 46°83' N, 12°80' E). The two soils were designed with (1) and (2) depending on the main vegetation cover, consisting of *Pinus mugo* and *Pinus sylvestris*, respectively. HS extraction and purification procedures were performed as previously described by Nardi et al. (2000). Briefly, HS were extracted in 0.1 M KOH (1:20 w/v) at room temperature for 16 h under a N_2 atmosphere. Alkaline extracts were dialyzed against double-distilled water using a dialysis membrane tubing with a molecular weight cut-off (MWCO) of 18,000 Da (Visking, London), and then completely desalted through an Amberlite IR 120 [H^+] cation exchange resin (Merck, Milan, Italy).

To fractionate HS, aliquots of each total humic extract (THE) (>18,000 MWCO) were treated with glacial acetic acid (99%) (Merck) until pH 2.1 was achieved, and further dialyzed against deionized distilled water by MWCO 3,500 Spectrapore 3 tubing (Spectrum, Gardena, CA). Two different nominal molecular size fractions, high (>3,500, HMS) and low (<3,500, LMS), were obtained (Nardi et al., 2000) from each THE. The two total humic extracts and their relative high and low size fractions were named as THE1, HMS1, LMS1 when deriving from the soil covered by *P. mugo*, and as THE2, HMS2, LMS2 when obtained from the soil covered by *P. sylvestris*.

Chemical, Spectroscopic and Biological Characterization of Humic Substances

The elemental composition (C, H, N, and S) of total humic extracts (THE) and their fractions (HMS and LMS) was determined using an elemental analyzer (Thermo Electron model EA 1110 Waltham, MA, USA), while oxygen content was computed by subtraction. The total acidity and the content of carboxylic groups were determined according to the procedure proposed by Swift (1996), while the content of phenol-OH groups was calculated by difference. The 13C NMR (Nuclear Magnetic Resonance) spectra were recorded by a Bruker AMX-500 spectrometer (Bruker, Kaarlsruhe, Germany), using inverse-gated decoupling experiments for quantitative intensity distribution. Further details regarding this analysis are reported by Nardi et al. (2000). The degree of aromaticity (AD), and HB (hydrophobic C content):HI (hydrophilic C content) ratio (hydrophobicity index) were used as variables to describe the HS in the multivariate analysis. AD was calculated using the formula: (105–165)/(105–0). HI/HB was computed based on the formula: [(48–105) + (165–190)]/[(0–48) + (105–165)]. The areas of the 0–48 and 105–165 ppm regions were used to calculate the

hydrophobicity (HB) of the HS, whereas those of the 48–105 and 160–190 ppm regions were used to obtain the hydrophilicity (HI) of the HS. The contributions of C functional groups were divided into different chemical-shift areas: 165–190 ppm (carboxyl-C), 145–165 ppm phenolic C, 110–165 ppm (aromatic-C), 48–105 ppm (protein and anomeric C) and 0–48 ppm (alkyl-C) (Nardi et al., 2000). The integration of the peaks within each of the chemical shift regions allowed the evaluation of the relative C contents expressed as percentages of the total area.

The amount of indole-3-acetic acid (IAA) in HS (total humic extracts and relative HMS and LMS fractions) was estimated by enzyme linked immunosorbent assay (ELISA) (Phytodetek-IAA, Merck). HS were then examined for auxin-, gibberellin - and cytokinin-like activity (Audus, 1972; Pizzeghello et al., 2001; Pizzeghello et al., 2013). The IAA-like activity in particular, was estimated by measuring the reduction of watercress (*Lepidium sativum* L.) root length after treatment with either IAA or HS. Conversely, the gibberellin-like (GA-like) activity was determined by evaluating increases in length of lettuce (*Lactuca sativa* L.) epicotyls following application of GA and HS (Audus, 1972). Specifically, watercress and lettuce seeds were surface-sterilized by soaking in 8% (v/v) hydrogen peroxide for 15 min. After rinsing five times with sterile distilled water, seeds were placed on sterile filter papers inside sterile Petri dishes (10 seeds per dish). For watercress, the filter paper was wetted with 1.2 mL of 1 mM $CaSO_4$ (control), or 1.2 mL of 0.1, 1, 10, 20 mg L^{-1} IAA solution (Merck, Milan, Italy) for the calibration curve, or 1.2 mL of a serial dilution of HS. For lettuce, the experimental design was the same as described for watercress except that the sterile filter paper was wetted with 1.4 mL, and the calibration curve was a progression of 0.1, 1, 10, 100 mg L^{-1} GA solution (Merck, Milan, Italy). The seeds were hold inside a germination room in the dark at 25°C. After 48 h for watercress and 72 h for lettuce, seedlings were removed and the root or epicotyl lengths were measured using a TESA-CAL IP67 electronic calibre (TESA, Renens, Switzerland) and Data Direct software, version 1 (ArtWare, Asti, Italy). Data were transformed on natural logarithmic scale to obtain the best linear fitting. The cytokinin-like activity was evaluated by weighing the cotyledons of radish (*Raphanus sativus* L.) seedlings (Pizzeghello et al., 2013). Seeds of *R. sativus* were germinated on wet paper towels hold for 2 to 3 days in the dark at 22 to 26°C. From each seedling, the smaller cotyledon was excised carefully removing all tissues of petiole, and then placed onto a Whatman No. 1 filter paper settled at the bottom of a 9-cm diameter Petri dish (density = 15 cotyledons per petri dish). The filter paper was imbibed with: i) cytokinin isopentenyladenosine (IPA) (20, 40, 60, and 80 μM) dissolved in 95% ethanol or ii) 95% ethanol only (controls), followed by evaporation of the ethanol under an IR lamp, or iii) with HS (0.1, 0.5, 1.0, 5.0, and 10 mg C L^{-1}). Three mL of 2 mM K-phosphate (pH 6.4) were added to each Petri dish to provide a growth medium. Cotyledons in Petri dishes were incubated at 27°C under continuous cool-white fluorescent light (10 μE m^{-2} s^{-1}). Fresh weight measurements, for groups of five cotyledons, were made after blotting excess water. Percentage increases in fresh weight are expressed relative to initial fresh weight.

Plant Material and Experimental Design

Garlic (*A. sativum* L., cv. Aglio Bianco Polesano) cloves were peeled and surface sterilized in 2–3% (v/v) H_2O_2 for 10 min. After being rinsed in deionized water, they were germinated in Petri dishes containing 1 mM $CaSO_4$ solution, covered with aluminum foil and placed in the dark at 25°C for 4 d. Rooted cloves (plantlets) were then transferred inside glass cups (10 cloves per cup) containing 50 g of glass beads and 18 ml of Hoagland n. 2 solution (Hoagland and Arnon, 1950), and grown for 4 d with a 16 h light at 25°C and 60% relative humidity, 8 h of dark at 18°C and 80% relative humidity. At the end of this period, the plantlets were supplied for 48 h with a Hoagland n. 2 solution supplemented with HS (THE, HMS or LMS) at different concentrations (0.1, 0.25, 0.5, 1.0, 2.0, and 5.0 mg C L^{-1}), or without HS (control). Plantlets were hence harvested and then carefully washed and dried with blotting paper. A sub-sample of the root material was immediately frozen with liquid nitrogen and kept at −80°C to be used for biochemical analyses. For dry weight measurement, 10 plants per treatment from each pot were randomly harvested. The samples were placed in a drying oven for 2 d at 70°C and allowed to cool for 2 h inside a closed bell jar.

Nitrate and Sulfate Quantification

Roots (1 g) developed from garlic plantlets were immersed in liquid N_2 and homogenized in 10 mM HCl (1:5 w/v). The extract was filtered through two layers of muslin and clarified by centrifugation at 35,000 g for 15 min at 4°C. The supernatant was further filtered at 0.22 μm (Millipore), and the concentration of NO_3^- and SO_4^{2-} ions was determined using a High-Performance Liquid Chromatography (HPLC) system, through an AS 4S-SC anionic-exchange column (Dionex, Sunnyvale, CA), equipped with a Dionex suppressor and a 431 conductivity detector (Waters-Millipore, Milford, MA). A solution of sodium bicarbonate and sodium carbonate (1.7 mM $NaHCO_3$/1.8 mM Na_2CO_3) was used as eluent at a flow rate of 2 mL min^{-1}. Sodium nitrate and potassium sulfate were used as reference standards (Fluka, Buchs, Switzerland).

Elemental Composition

Roots (0.5 g) of garlic plantlets were added with 9 mL of HNO_3 (30% v/v) and H_2O_2 30% (7:2), and placed in closed Teflon vessels of 100 mL volume. The digestion of root samples was carried out in a microwave system (Millestone Start-D 1200W). Mineralized samples were then diluted in 25 mL ultrapure water and the concentrations of Fe, K, Mg, and Ca were determined *via* inductively coupled plasma–atomic emission spectroscopy ICP-OES (Optima 2000 DV, Perkin Elmer Instruments Germany). Elements were quantified using certified multi-element standards.

Enzyme Activities

Roots (1 g) of garlic plantlets were immersed in liquid N_2 and homogenized (1:10 w/v) in 0.1 M potassium acetate buffer (pH 4.0) containing 0.1 M sucrose to determine invertase activity, or in 0.1 M phosphate buffer (pH 7.0) to test for peroxidase activity, or in 0.2 M Tris-HCl buffer (pH 7.0) to measure esterase activity. The extracts were centrifuged at 15,000 *g* for 15 min at 4°C and

the supernatants were used as the enzyme source. Invertase activity was evaluated according to Arnold (1965), peroxidase activity as described by Putter (1974), esterase activity was determined as described by Junge and Klees (1984).

For nitrate reductase (NR), glutamine synthetase (GS) and O-acetylserine sulfhydrylase (OAS-s) measurements, enzymes were extracted from roots by manually crushing plant material in a mortar with a solution containing 100 mM HEPES (acido 4-2-idrossietil-1-piperazinil-etansolfonico)-NaOH (pH 7.5), 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). The ratio of plant material to mixture solution was 1:3 (w/v). The extracts were filtered through two layers of muslin and clarified by centrifugation at 20,000 *g* for 15 min. The supernatants were used as the enzyme sources. All steps were performed at 4°C. Nitrate reductase activity was assayed in a solution containing 100 mM KH_2PO_4, 100 mM KNO_3, and the enzyme extract. The activity was measured spectrophotometrically at λ = 540 nm, and the calibration curve was plotted with known concentrations of $NaNO_2$ (Lewis et al., 1982). With respect to the glutamine synthetase assay, the mixture contained 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM $KAsO_4$, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine, and the enzyme extract. The assay was performed in a final volume of 750 µL. The enzymatic reaction was developed for 15 min at 37°C. The γ-glutamyl hydroxamate was determined by the addition of 250 µL of a mixture (1:1:1) of 10% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid, and 50% (w/v) HCl. The optical density was recorded at λ =540 nm (Schiavon et al., 2008). O-acetylserine sulfhydrylase (OAS-s) activity was determined by measuring the production of L-cysteine, according to the procedure described by Kuske et al. (1994). Briefly, 1 mL reaction mixtures containing 100 mM Tris, 20 mM 0-acetylserine, 1 mM Na_2S (pH 7.6) were initiated by addition of 1–10 µL of protein sample and further stopped by addition of 1.5 M trichloroacetic acid. Samples were centrifuged at 10,000 g for 5 min, and the amount of cysteine in the supernatants was estimated spectrophotometrically (λ =546 nm) using the ninhydrin reagent.

Free Amino Acid Quantification

Free amino acids in roots were quantified according to Seebauer et al. (2004). Fifty mg of homogenous dry powder was extracted for 1 h at room temperature with 1.5 mL of a 5% (w/v) trichloroacetic acid (TCA) solution. The sample was clarified by centrifugation, and 1.5 mL of the supernatant was analyzed for free amino acids. The analysis of amino acid was realized using a precolumn OPA derivatization of the sample followed by reverse phase separation, through an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a thermo-controlled auto-sampler, fluorescence detector and an Agilent HP Chemstation for data elaboration. The chromatographic conditions were described by Henderson et al. (2000). Aliin was extracted from roots of garlic and determined according to Hoppe et al. (1996).

Light Microscopy

Two cm-long root tip of garlic plantlets grown with unfractionated (THE1-2) or size-fractionated (HMS1-2, LMS1-2) HS or not (control), were fixed with glutaraldehyde (6%) and processed for light microscopy as previously described (Bonghi et al., 1993). Cross thin sections (1 µm thick) of roots were cut with an Ultracut Reichert-Jung ultramicrotome, stained with 1% toluidine blue and 1% tetraborate (1:1, v/v), and observed and photographed under a Leitz Ortholux microscope.

Statistical Analyses

Differences among mean values of chemical characteristics of HS were determined with the Student–Newman–Keuls test ($p \leq 0.05$). The linear regression analysis (Y= a + bX) was used to verify the relationship between several chemical and biochemical parameters of garlic at different doses of unfractionated and size-fractionated HS. Prior to the regression analysis, the database was divided into two sub-samples based on HS concentration (0–1 mg C L^{-1} and 2-5 mg C L^{-1}). Correlations between variables were determined using the Pearson's coefficient.

Structure of the interdependences between unfractionated and size-fractionated HS chemical and biochemical parameters (C, H, N, O, S, COOH, phenolic-OH, aliphatic-C, peptidic and carbohydratic-C, aromatic-C, phenolic-C, carboxyl-C, IAA, IAA-like, GA-like, IPA-like) and plant physiological and biochemical attributes (root biomass, nitrate, sulfate, Ca, Mg, K and Fe content, nitrate reductase, glutamate synthetase, O-acetylserine sulphydrylase, invertase, peroxidase, esterase enzyme activities, and aspartate, threonine, isoleucine, lysine, asparagine, glutamate, serine, methionine, cysteine, aliin content) was performed using a joint Principal Components Analysis (PCA). The standardized variables were submitted to PCA; rotated orthogonal components (varimax rotation method) were extracted and the relative scores were determined. Only PCs with eigenvalue >1 were considered for the discussion. The object points were labeled by principal vegetation cover (1) *P. mugo* and (2) *P. sylvestris*, and molecular size of humic substances (total humic extracts, THE, and relative high, HMS, and low, LMS, molecular size fractions).

The Automatic Linear Modelling (ALM) was used to determine the factors which best influenced the hormone-like activities of unfractionated and size-fractionated HS. ALM was performed at the confidence level of 95%. All statistics were conducted using IBM SPSS Statistics for Windows version 25.

RESULTS

Chemical-Spectroscopic Features, Hormone Content and Hormone-Like Activity of HS

The analysis of elemental composition and the spectroscopic characterization of total humic extracts (THE1-2) and relative

TABLE 1 | Elemental composition, carboxylic and phenolic acidity and carbon distribution in 13C NMR spectra (ppm) of total humic extracts (THE) and its size-fractions (high molecular size, HMS and low molecular size, LMS) separated during acetic acid treatment and dialysis.

	Element					Acidity		13C NMR						
HS	**C**	**H**	**N**	**O**	**S**	**COOH**	**Phenolic-OH**	**0-48**	**48-105**	**105-145**	**145-165**	**165-190**	**HI/HB[b]**	**AD[c]**
			%			meq g^{-1}		% Carbon distribution						
THE1[a]	58.1b*	4.2a	3.6c	36.1a	0.31a	5.2b	5.0b	27.3a	42.5a	15.6c	7.2a	7.4d	0.99	45.5
HMS1	56.2c	4.1a	2.8d	35.5a	0.15b	4.5b	5.5b	18.8b	44.1a	18.7b	7.4a	11.0c	1.22	58.1
LMS1	54.5d	4.5a	5.5b	32.2a	0.13b	6.2ab	5.5b	18.1b	20.2b	32.1a	5.2b	24.4b	0.80	67.3
THE2	59.3a	4.0a	3.8c	38.4a	0.28a	5.5b	6.0b	21.2b	42.4a	19.4b	7.0a	10.0c	1.10	55.4
HMS2	55.5d	3.9a	2.9d	34.6a	0.20ab	5.0b	5.8b	20.9b	43.2a	18.7b	6.8a	10.4c	1.15	54.9
LMS2	52.4e	4.2a	7.8a	33.1a	0.15b	7.5a	8.8a	14.8c	15.2c	33.7a	6.5a	29.8a	0.82	73.1

[a](1) soil with Pinus mugo cover and (2) soil with Pinus sylvestris cover.
[b]HI/HB = hydrophobic index = [(48–105) + (165–190)]/[(0–48) + (105–165)].
[c]AD = aromaticity degree = (105–165)/(105–0)* Values within column followed by the same label are not statistically different at p = 0.05 by Student-Newman-Keuls (Sokal and Rohlf, 1969).

high (HMS1-2) and low (LMS1-2) molecular size fractions indicated that some elements (C and N) and functional groups were unevenly distributed when THE1-2 were separated in size fractions during acetic acid treatment and dialysis procedure (**Table 1**). Precisely, THE1-2 were more enriched in C compared to HS fractions, but LMS1-2 were the highest in N. No significant differences were evident in O, H and S contents between HS. Oxygenated (carboxylic and phenolic-OH) groups were more abundantly present in LMS than in THE and HMS, with the maximum value reported for LMS2. Consistently, the 13C NMR integration areas (**Table 1**) showed greater occurrence of aromatic (105–145 ppm) and carboxylic (165-190 ppm) C in LMS1-2 (**Figure S1**), which were though the least abundant in protein and anomeric C (48–105 ppm) although this region may also include some signals of lignin and phenolic moieties (45–60 ppm) (Monda et al., 2017). The phenolic (145–165 ppm) C content, instead, did not vary significantly between THE and size-fractionated HS, except it was lower in LMS1. The hydrophobicity index and degree of aromaticity are usually used to indicate the biochemical and chemical stability of HS (Piccolo et al., 2005). The aromaticity of LMS1-2 was confirmed by the aromaticity degree (AD) values, which followed the trend THE1-2<HMS1-2<LMS1-2 (**Table 1**). Negative correlations (**Table S1**, Supplementary) were ascertained between the nominal size of HS and their aromatic degree (r = −0.72, $p < 0.001$), aromatic C (r = −0.65, $p < 0.003$), and carboxyl C (r = −0.65 $p < 0.003$). In contrast, positive correlations (**Table S1**, Supplementary) were found between the nominal size of HS and their carbohydratic-C (r = 0.54, $p < 0.020$), and aliphatic C (r = 0.77, $p < 0.001$). Evaluation of HS hydrophobicity based on HI/HB ratios (**Table 1**) indicated the elevated amount of aromatic C in LMS1-2, while the hydrophilic C was dominant in THE and HMS.

The content of IAA (**Table 2**) varied significantly ($p = 0.05$) between THE, HMS and LMS, with a trend that was similar between HS from soils (1) and (2). LMS1-2 in particular, contained more IAA than THE1-2 and HMS1-2, with maximum values recorded in LMS2. Consistently with the trend of IAA content, the IAA-like activity (**Table 2**) was significantly higher ($p \leq 0.05$) in LMS1-2, especially in LMS2. Similarly, the GA-like and IPA-like activities (**Table 2**) were

TABLE 2 | Indoleacetic acid (IAA) content and hormone-like activity ([†]) of total humic extracts (THE), high molecular size (HMS) and low molecular size (LMS) humic substances extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *Pinus sylvestris* cover).

HS	IAA	IAA-like	GA-like	IPA-like
	% (w/w)	mg L^{-1} ≅ 1 mg C L^{-1}		
THE1	0.01e*	0.14d	0.01e	0.25d
HMS1	0.03d	0.25d	0.04d	0.45b
LMS1	0.12b	1.67b	0.15b	0.84a
THE2	0.03d	0.72c	0.01e	0.12e
HMS2	0.05c	0.91c	0.07c	0.35c
LMS2	0.35a	5.25a	0.21a	0.79a

[†]Concentration (mg L^{-1}) of indoleacetic acid (IAA) or gibberellic acid (GA) or isopentenyladenosine (IPA) of equivalent activity as 1 mg C L^{-1} humic substances.
*Values within column followed by the same label are not statistically different at p = 0.05 by Student-Newman-Keuls (Sokal and Rohlf, 1969).

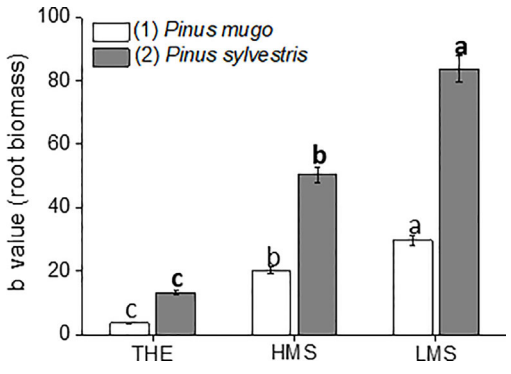

FIGURE 1 | Root dry biomass of garlic plantlets grown in hydroponics and treated for 48 h with total humic extracts (THE) and size-fractionated HS (high molecular size, HMS and low molecular size, LMS) extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *Pinus sylvestris* cover). The b values of the regression curve in the range 0–1mg C L^{-1} are reported. Different letters represent significant differences at $p < 0.05$ (n = 10). Unbolded letters compare HS from soil (1), letters in bold compare HS from soil (2).

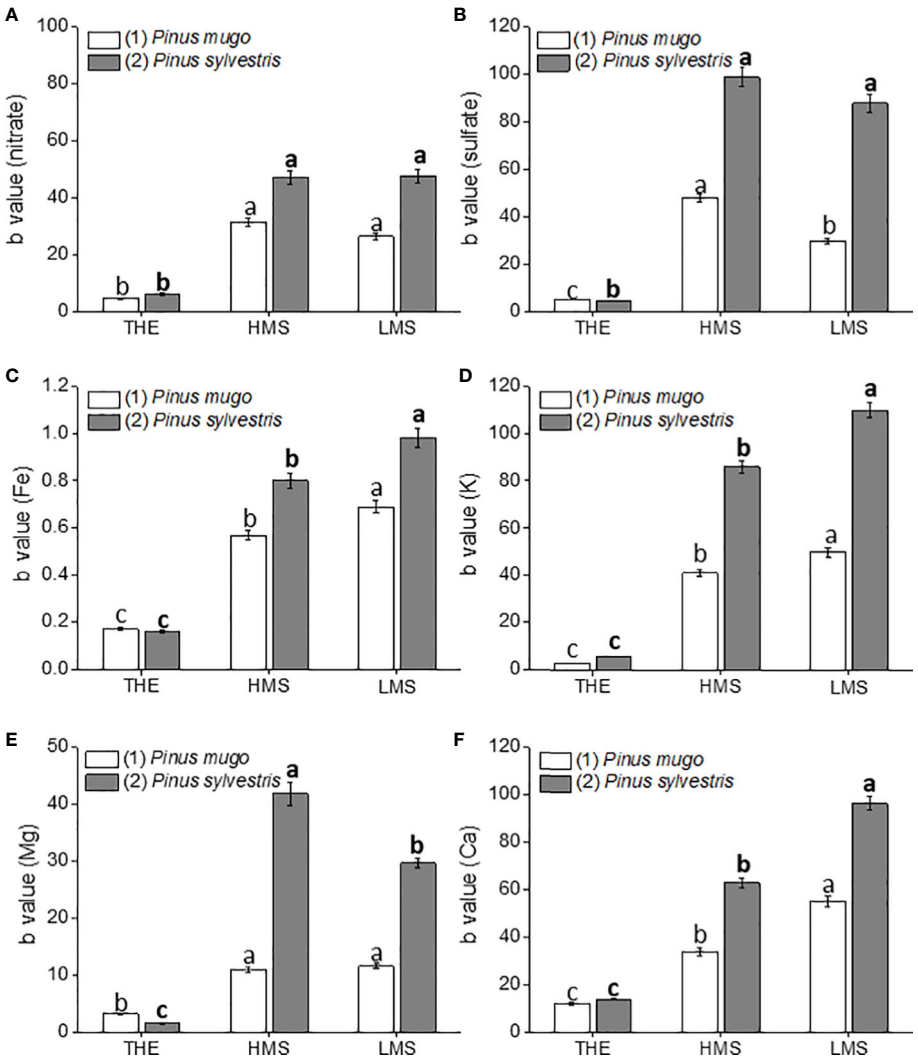

FIGURE 2 | Root nutrient [nitrate **(A)**, sulfate **(B)**, Fe **(C)**, K **(D)**, Mg **(E)**, Ca **(F)**] content of garlic plantlets grown in hydroponics and treated for 48 h with total humic extracts (THE) and size-fractionated HS (high molecular size, HMS and low molecular size, LMS) extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *Pinus sylvestris* cover). The b values of the regression curve in the range 0 –1mg C L^{-1} are reported. Different letters represent significant differences at $p < 0.05$ (n = 5). Letters not in bold compare HS from soil (1), letters in bold compare HS from soil (2).

prevalent in LMS1-2, mainly in LMS2, and were minimum in THE1-2.

Effects of THE and Fractionated HS (HMS and LMS) on Garlic Plantlets

To assay the dose-dependent effect of HS on garlic plantlet physiology and biochemical traits, a linear regression analysis of the whole data set was performed. Linear regression analyses were all significant (R^2 ranging from 0.60 to 0.99, $p \leq 0.01$) in terms of independent variables (**Supplementary Tables S2**-**S4**). Despite a big amount of data was generated from this analysis, we focused on the description of the most relevant findings. Thus, the regression coefficient (b) ± SE values of each curve, calculated in the HS concentration range of 0.1–1.0 mg C L^{-1} (linear positive range, first trait), were shown in **Figures 1–4**. The b value was indicative of how the HS dose and type (THE, HMS, and LMS) impacted on plant growth and biochemical attributes. Comparisons for statistical differences were accomplished within the same group of HS, i.e. HS derived from soil (1) or soil (2). Data referred to higher doses of HS (2 and 5 mg C L^{-1}, second trait) are reported in **Tables S2**-**S4** (Supplementary). In this case, less effect or no effect of HS on all plant traits subjected to evaluation was observed.

Effects of HS on Root Growth and Nutrient Content of Garlic Plantlets

The root biomass showed considerable variation depending on HS treatment (**Figure 1**, **Supplementary Table S2**). The trends

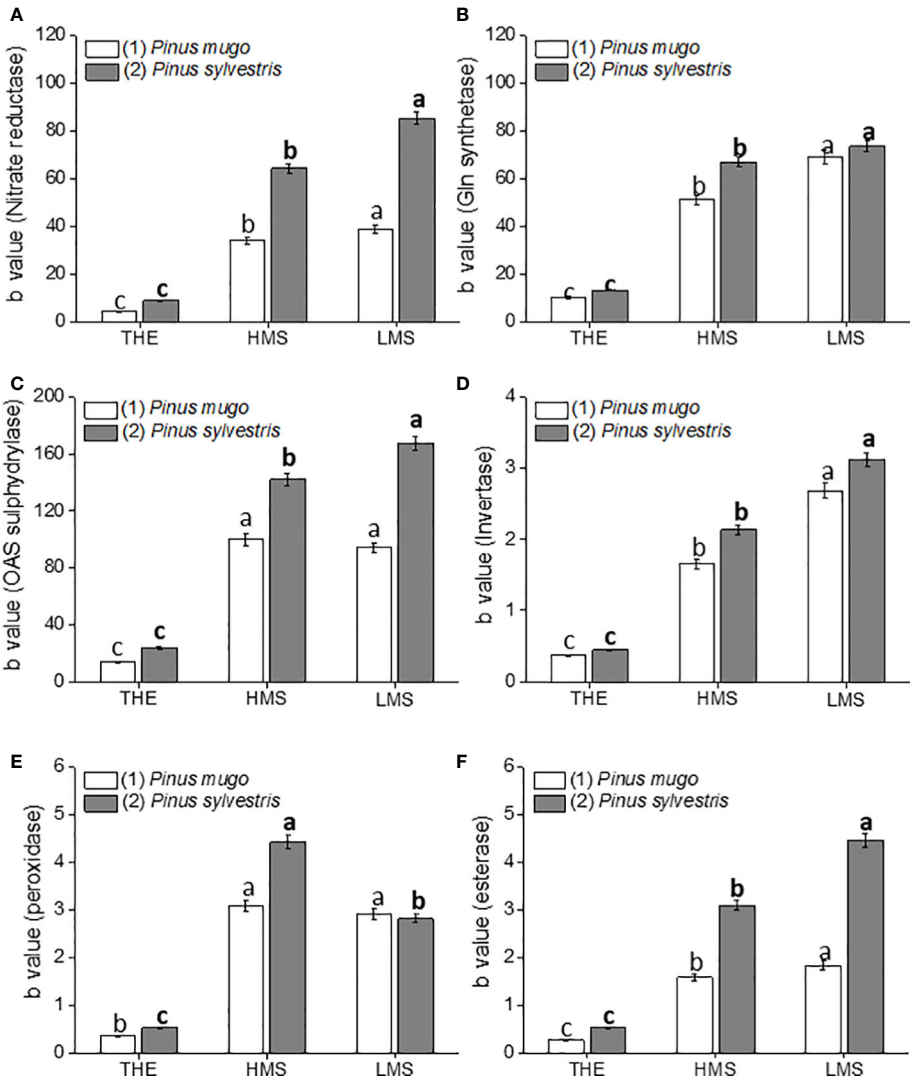

FIGURE 3 | Activity of nitrate reductase **(A)**, glutamine synthetase **(B)**, O-acetylserine sulphydrylase **(C)**, invertase **(D)**, peroxidase **(E)**, esterase **(F)** enzymes in roots of garlic plantlets grown in hydroponics and treated for 48 h with total humic extracts (THE) and size-fractionated HS (high molecular size, HMS and low molecular size, LMS) extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *Pinus sylvestris* cover). The b values of the regression curve in the range 0 –1mg C L^{-1} are reported. Different letters represent significant differences at $p < 0.05$ (n = 5). Letters not in bold compare HS from soil (1), letters in bold compare HS from soil (2).

observed for root biomass of plantlets treated with HS from either soil (1) or (2) were similar, although values were generally higher when HS derived from soil (2). Maximum and minimum root growth was ascribed to plantlets receiving LMS1-2 and THE1-2, respectively, with differences becoming more evident when HS fractions were extracted from soil (2). Indeed, LMS2-treated plantlets produced more root biomass than LMS1-treated plants (+66%) when compared to plantlets supplemented with HMS2 or HMS1 (+46%).

Garlic plantlets accumulated more nutrients (nitrate, sulfate, Fe, K, Mg, and Ca) when treated with HS fractions from soil (2) (**Figures 2A–F**). HMS2 and LMS2 induced similar accumulation of nitrate (NO_3^-) and sulfate (SO_4^{2-}) ions, while THE2 determined the lowest increments (**Figures 2A, B**). Similar findings were reported in plantlets supplemented with HS from soil (1). In this case, however, the sulfate content in plantlets subjected to HMS1 treatment was higher than in plantlets added with LMS1. The b values of the regression curves that refer to the root content of other mineral nutrients (Fe, K, and Ca) revealed a similar trend for the effects of HS from the two soils: THE<HMS<LMS (**Figures 2C, D, F**). Unlike Fe, K, and Ca, Mg accumulated more in HMS2- than in LMS2-treated plantlets,

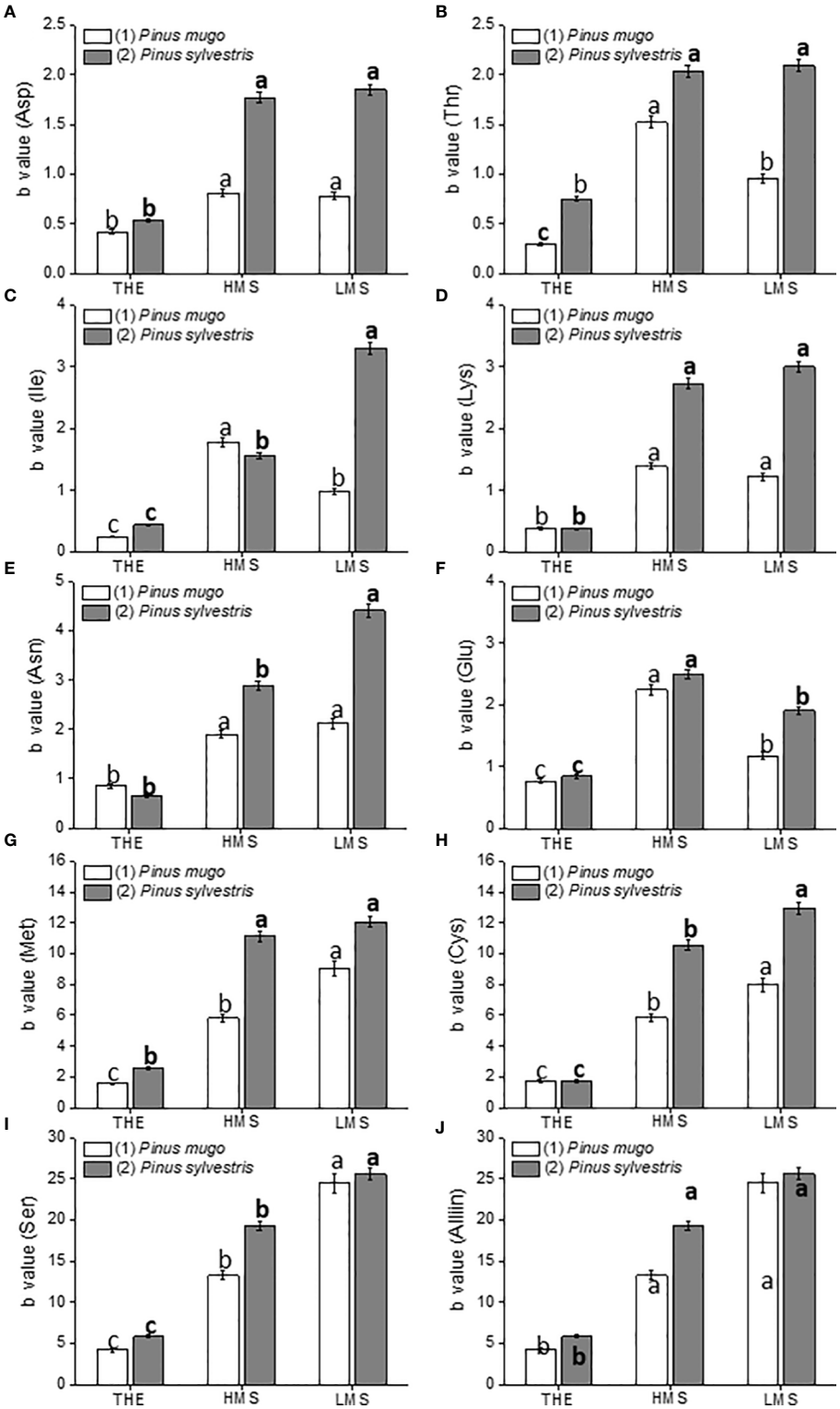

FIGURE 4 | Amino acid content in roots of garlic plantlets grown in hydroponics and treated for 48 h with total humic extracts (THE) and size-fractionated HS (high molecular size, HMS and low molecular size, LMS) extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *Pinus sylvestris* cover). The b values of the regression curve in the range 0 –1mg C L^{-1} are reported. Panels are: **(A)** Asp, asparagine; **(B)** Thr, threonine; **(C)** Ile, isoleucine; **(D)** Lys, lysine; **(E)** Asn, asparagine; **(F)** Glu, glutamate; **(G)** Met, methionine; **(H)** Cys, cysteine; **(I)** Ser, serine; **(J)** Aliin. Different letters represent significant differences at $p < 0.05$ (n = 5). Letters not in bold compare HS from soil (1), letters in bold compare HS from soil (2).

while no differences were evident between plantlets supplied with HS fractions from soil (1) (**Figure 2E**). As for the other elements, Mg accumulated less in roots of plantlets treated with THE.

Effects of HS on Plant Metabolism and Amino Acid Content at the Root Level

Treating garlic plantlets with HS prompted differential effects on enzymes involved in N metabolism (nitrate reductase and glutamine synthetase), S assimilation (OAS-sulphydrylase), and developmental processes (invertase, peroxidase and esterase) (**Figures 3A–F**). Such distinct effects mainly relied on HS type and soil of origin. HMS1-2 and LMS1-2 stimulated the activity of tested enzymes more than THE1-2, thus suggesting that THE1-2 was less effective than size-fractionated HS to enhance the primary metabolism and growth of garlic. The activities of all enzymes, except peroxidase, behaved similarly when plantlets were supplied with HS fractions from soil (2), as higher stimulation was generally induced by LMS2 over HMS2. HS fractions from soil (1) triggered similar increments in activity of nitrate reductase, OAS-sulphydrylase, peroxidase and esterase enzymes, but LMS1 intensified the activity of glutamine synthetase and invertase enzymes more than HMS1. The activity of the enzymes nitrate reductase, OAS-sulphydrylase and esterase was more increased in garlic roots after treatment with HS from soil (2). For the other enzymes, the stimulatory effect of their activity was comparable between HS from soil (1) and soil (2).

Maximum accumulation of amino acids, with the exception of glutamate (Glu), was evident in garlic roots treated with LMS2 (**Figures 4 A–J**). THE1-2 instead, was the least effective in increasing the content of all amino acids, while the effect of HS fractions from soil (1) varied depending on the target amino acid. HMS1 and LMS1 induced similar accumulation of aspartate (Asp), lysine (Lys), asparagine (Asn) and Alliin. HMS1, though, triggered higher accumulation of threonine (Thr), isoleucine (Ile) and Glu. Conversely, S amino acids (methionine, Met, cysteine, Cys), and serine (Ser) accumulated more under LMS1 treatment. HS fractions from soil (2) were however more effective than HS fractions from soil (1) in promoting the accumulation of several amino acids (Asp, Thr, Lys, Asn, Met, Cys, Alliin). Also, HMS2 and LMS2 provoked comparable incremental accumulation of Asp, Thr, Lys, Met and Alliin, while LMS2 induced higher accumulation of Ile, Asn, Cys and Ser. Glu was the only amino acid that accumulated more under HMS2 treatment.

Effects of HS on Root Anatomy

The analysis of root anatomy emphasized differences in cell differentiation between garlic plantlets grown in nutrient solution without HS (control) and plantlets treated with either HMS1-2 or LMS1-2 (**Figures 5A–E**). LMS1-2 caused a more evident and earlier differentiation pattern in the central cylinder (**Figures 5C, E**), especially with respect to protoxylem vessel formation, compared to HMS1-2 (**Figures 5B, D**) and the nutrient solution solely (**Figure 5A**). The differentiation process was more pronounced in the root central cylinder of garlic plantlets treated with LMS derived from soil (2). However,

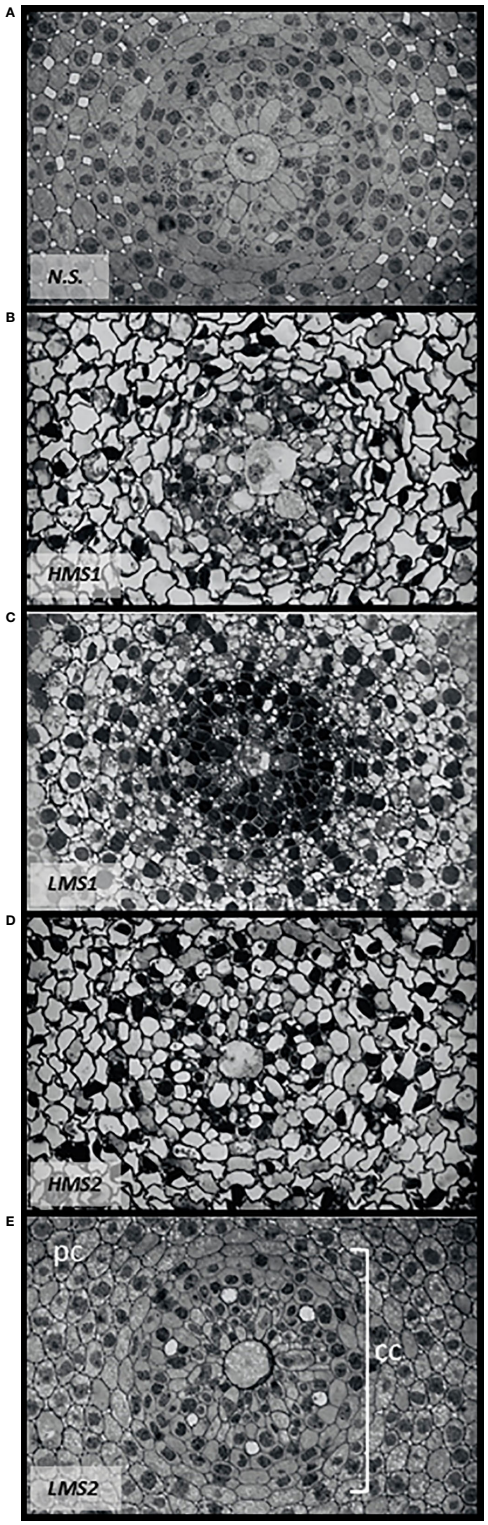

FIGURE 5 | Root anatomy of garlic plantlets grown in hydroponics with nutrient solution **(A)** (SN) without HS, or treated for 48 h with size-fractionated HS **(B, D)** high molecular size, HMS and **(C**, **E)** low molecular size, LMS) extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *Pinus sylvestris* cover). CC, central cylinder; PC, parenchyma cells.

HMS1-2 induced higher differentiation of cortical cells, which appeared bigger in size and contained a unique vacuole per cell. The root parenchyma of control plantlets was formed by cells with no vacuoles, therefore late in differentiation. In contrast, plantlets supplied with LMS showed a differentiation process in progress, as cells showed substantial vacuolization.

Correlation Analysis

The Pearson's correlation analysis (**Table S1**, Supplementary) highlighted significant relationships between quantitative variables of HS, as well as between metabolic traits of garlic plantlets after treatment with HS. In most cases, the r value ranged from 0.77 to 0.77–0.99 ($p \leq 0.01$). As an example, Alliin greatly correlated with Cys (r = 0.97, $p \leq 0.01$) and Lys (r = 0.96, $p \leq 0.01$), while the IPA-like activity negatively correlated with O (r = −0.93, $p \leq 0.01$).

Relationship Between Hormone-Like Activity of HS and Plant Biochemical Attributes Assayed by Automatic Linear Modeling

The ALM allowed to get the outcome of three linear models explaining the relationship between the hormone-like activity of HS and the biochemical attributes of garlic plantlets (**Table 3**). All calculated models provided high accuracy, which was equal to 99.7, 99.85, and 100% for GA-like, IPA-like and IAA-like activity, respectively. The IAA-like activity was explained by 7 variables over 21 assayed variables. The relative importance (i) and significance of the obtained predictive variables followed the order N (i = 0.442, $p \leq 0.000$) > OAS-sulfhydrylase activity (i = 0.292, $p \leq 0.000$) > S (i = 0.175, $p \leq 0.000$) > IAA (i = 0.042, $p \leq 0.002$) > phenolic-OH (i = 0.020, $p \leq 0.016$) > ISO (i = 0.016, $p \leq 0.027$) > O (i = 0.013, $p \leq 0.045$). All the 7 variables entering the equation positively influenced the IAA-like activity target variable. The GA-like activity model was explained by such variables as sulfate (i = 0.475, $p \leq 0.000$), carboxyl-C (i = 0.339, $p \leq 0.000$), OAS-sulphydrylase activity (i = −0.163, $p \leq 0.001$) and peptidic-C (i = −0.024, $p \leq 0.109$). The first two predictive variables entered the equation with a positive effect on GA-like activity, while the other two predictive variables exerted a negative effect. For the IPA-like activity, the model was explained by Mg (i = −0.368, $p \leq 0.000$), peptidic-C (i = −0.284, $p \leq 0.000$) and O (i = −0.251, $p \leq 0.000$), which displayed a negative effect, and by Cys (i = 0.079, $p \leq 0.000$), Fe (i = 0.009, $p \leq 0.017$) and phenolic-C (i = 0.009, $p \leq 0.021$) which conversely determined a positive effect.

Principal Component Analysis

The PCA analysis revealed that three factors accounted for 95.2% of the total variance. Principal component 1 (PC1) explained 53.9% of the variance, and positively correlated with OAS-sulphydrylase activity, Cys, Lys, Mg, sulfate, esterase activity, glutamate synthetase, nitrate, invertase, Alliin, Ca, K, Met, Ser, peroxidase activity, nitrate reductase activity, Glu, Fe, Asp, Thr, Asn, Ile, and root biomass (**Table S5**, Supplementary). Principal component 2 (PC2) explained 31.2% of the total variance and was positively correlated with carboxyl-C, N, IAA-like, IAA, phenolic-OH, GA-like, COOH, aromatic-C, and negatively with aliphatic-C, C and peptidic-C (**Supplementary Table S5**). The remaining 10% of the total variance was explained by Principal component 3 (PC3), which mostly correlated with phenolic-C, H, S, and O (**Supplementary Table S4**).

Plotting data according to PC1 and PC2, treatments resulted in well separated HS of soil (1) from HS of soil (2), and HMS versus LMS, while THE scattered around the origin (**Figure 6A**). In particular (**Figure 6B**), variables related to plant biochemical traits distinguished HMS2 from HMS1, while variables related to HS characteristics differentiated LMS2 from LMS1. Notably, HMS2 was characterized by the highest values in OAS-sulphydrylase activity, Cys, Lys, Mg, sulfate, esterase activity, glutamate synthetase activity, nitrate, invertase activity, Alliin, Ca, K, and Met, while LMS2 by the highest values in carboxyl-C, N, IAA-like, IAA, GA-like, IPA-like and phenolic-OH.

DISCUSSION

Plant responses to humic substances depend on HS nature, molecular size, chemical properties and concentration

TABLE 3 | Predictive importance (i) of chemical and biochemical variables to hormone-like (IAA-like, GA-like, IPA-like) activity of humic substances by Automatic Linear Modeling.

	Target variable							
	IAA-like		GA-like			IPA-like		
Predictor	i	*p* value	Predictor	i	*p* value	Predictor	i	*p* value
N	0.442	0.000	**SO_4^{2-}**	0.475	0.000	**Mg**	–0.368	0.000
OAS-s	0.292	0.000	**Carboxyl-C**	0.339	0.000	**Peptidic-C**	–0.284	0.000
S	0.175	0.000	**OAS-s**	–0.163	0.001	**O**	–0.251	0.000
IAA	0.042	0.002	**Peptidic-C**	–0.024	0.109	**Cys**	0.079	0.000
Phenolic-OH	0.020	0.016				**Fe**	0.009	0.017
Ile	0.016	0.027				**Phenolic-C**	0.009	0.021
O	0.013	0.045						

Carboxyl-C, Carboxyl-C in HS by NMR; Cys, cysteine content in plants roots; Fe, iron content in plants roots; IAA, indoleacetic acid content in HS by ELISA; Ile, isoleucine content in plants roots; Mg, magnesium content in plants roots; N, nitrogen in HS by elemental composition; O, oxygen in HS by elemental composition; OAS-s, O-acetylserine sulphydrylase activity in plants roots; Peptidic-C, Peptidic-C in HS by NMR; Phenolic-C, Phenolic-C in HS by NMR; Phenolic-OH, Phenolic-OH acidity in HS by titration; S, sulfur in HS by elemental composition; SO_4^{2-}, sulfate content in plants root.

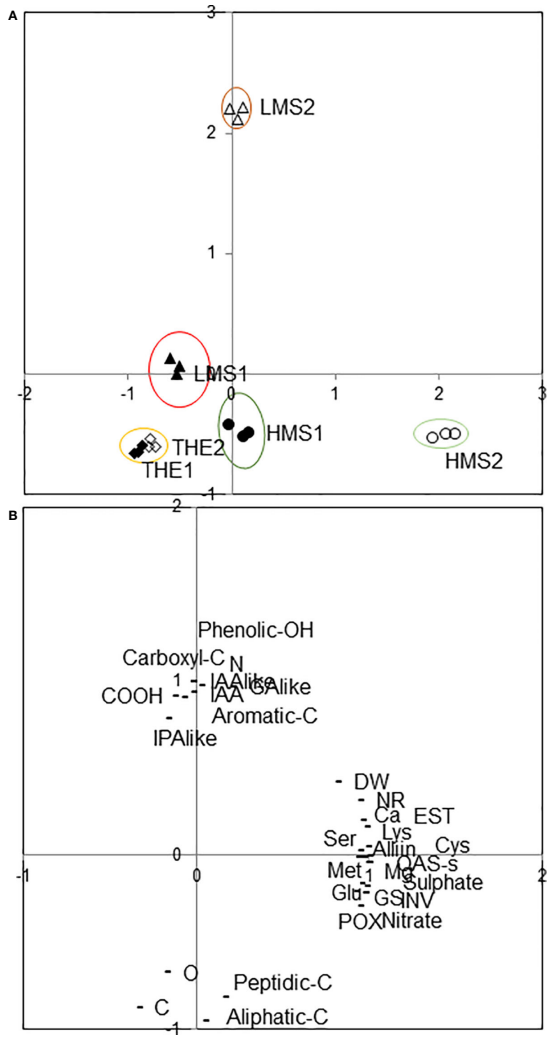

FIGURE 6 | Positions of the unfractionated (THE, total humic extract) and fractionated humic substances (HMS, high molecular size; LMS, low molecular size) (1) with *Pinus mugo* cover, (2) with *Pinus sylvestris* cover in the reduced space of the first two principal components after principal components analysis **(A)**. Variables projected in the plane determined by the first two principal axes **(B)**: HS chemical and biochemical parameters (aliphatic-C, aromatic-C, C, carbon, carboxyl-C, COOH, GA-like, gibberellin-like activity, IAA, indoleacetic acid content, IAA-like, auxin-like activity, IPA-like, cytokinin-like activity, N, nitrogen, peptidic and carbohydratic-C, phenolic-OH, O, oxigen), and plant physiological and biochemical attributes (Alliin; Ca, calcium; Cys, cysteine; DW, root dry weight; EST, esterase activity; Glu, glutamate; GS, glutamine synthetase activity; INV, invertase activity; Lys, lysine; Mg, magnesium; Met, methionine; NR, nitrate reductase activity; OAS-s, O-acetylserine sulphydrilase activity; POX, peroxidase activity; Ser, serine).

(Canellas and Olivares, 2014). Our results proved the impact of the plant cover at the sampling soil on the formation of bioactive HS with distinctive chemical-structural features. The two sampled soils were similar in properties, but differed in plant cover. From a previous study (Nardi et al., 2000) we know that *P. mugo* litter on soil (1) was characterized by a high content of lignin, proteins and ash, while *P. sylvestris* litter on soil (2) contained a large amount of hemicellulose, cellulose, fats, waxes and oils. The effect of the litter composition on HS chemistry and bioactivity was more evident for LMS1-2, as these fractions hold a higher content of N and oxygenated (-COOH and OH-phenol) functional groups, and displayed greater aromaticity compared to THE1-2 and HMS1-2. Interestingly, such parameters were all maximally intensified in LMS2.

The molecular fractionation method used in this study was based on HS treatment with a weak monocarboxylic acid, namely acetic acid. Such a treatment reduced the complexity of HS (Nardi et al., 1988) by leading to a progressive disturbance of the supramolecular humic structures resulting from the association of different chemical species (Piccolo, 2001; Piccolo, 2002). Although the disassociation induced by acetic acid relies on the nature of the associated species, it might overcome the properties of the isolated components, as a result of the specific molecular interactions controlling the affinity, organization and cooperation between aggregates. In this context, new molecular rearrangements could be responsible for diverse pathways of HS molecular communication with plants (Canellas et al., 2019). In general, the action exerted by acetic acid is comparable to that produced by root exudation or microbial transformation of organic matter (Nardi et al., 1997). Both these processes can promote the release of bioactive molecules that trigger positive effects in plants and the rhizosphere (Nardi et al., 2002; Nardi et al., 2005; Nardi et al., 2017).

The formation of simpler aggregates differing in size (HMS and LMS) defines differences in bioactivity between them and compared to unfractionated HS, because of their typical chemical composition and spatial distribution of hydrophilic and hydrophobic domains (Nebbioso and Piccolo, 2012). Previously, Nardi et al. (2007) showed that LMS was endowed with high hydrophilic degree due to the predominant carbohydrate component, which accounted for the greater bioactivity of this fraction. In this respect, LMS has been hypothesized to assume a specific spatial arrangement responsible for better interaction with the plant roots. However, other studies (Canellas et al., 2008; Canellas et al., 2009; Canellas et al., 2010) highlighted a strong relationship between the hydrophobic domain of HS and the H^+-ATPase activity of plant roots. In that case, HS bioactivity was ascribed to the capacity of the hydrophobic domain to promote the release of biologically active molecules, such as auxin-like substances, that target the plasma membrane H^+-ATPases (Canellas et al., 2010). Such effect was reported to be sparked by the action of root exudates (Canellas et al., 2019). Our results clearly show that the elevate bioactivity of LMS1-2 was heavily dependent on their high aromaticity degree and content of polar residues, while the hydrophobic domain likely contributed to the stability and functionality of LMS aggregates (Monda et al., 2018). This rationale is supported by the highest values of IAA content and hormone-like activities measured in LMS1-2. The hydrophilic domain was apparently less important in dictating the structure of THE and HMS because it did not contribute to the activity of HS appreciably. In this respect,

the hydrophobic/hydrophilic ratio of a humic aggregate may be indicative of its bioactivity potential.

A clear relationship between HS dose and early effects on biochemical attributes of garlic plantlets was also determined in this study within a short-period (48 h) of plantlet treatment with HS. This short period has long been used to evaluate the biostimulant activity of HS and other products on hasty changes in plant metabolism (Schiavon et al., 2008; Schiavon et al., 2010; Ertani et al., 2018; Schiavon et al., 2019). To estimate the dose-response function, several concentrations of unfractionated and size-fractionated HS were tested, and fitting curves were build-up. A linear regression model described the behavior of HS, which was positive at low HS doses, while negative at high HS doses. More specifically, the beneficial effects of HS on plant growth, nutrition and metabolism were evident in a close range of HS concentration, i.e., from 0.1 to 1 mg C L^{-1}, regardless of HS type (unfractionated or size-fractionated). Higher HS doses produced less or no effect.

Within the positive linear regression range, the most remarkable effects on garlic root growth and physiology were elicited by LMS1-2, thereby confirming the high bioactivity of these HS fractions inferred by the primary chemical-structural analyses. The elevate IAA content and hormone-like activity of LMS1-2 conceivably justified the capacity of these two fractions to induce the highest increase in root biomass and earliest root cell differentiation patterns in the central cylinder. The IAA-like activity strongly correlated with the IAA content, but could additionally be ascribed to other auxins (e.g., phenylacetic acid and indole butyric acid) (Russell et al., 2006) and aromatic biologically active compounds, such as phenol-C groups (Muscolo et al., 2007). The phenol-C groups can also be responsible for the GA-like and IPA-like activities of HS (**Table 3**). Auxins and auxin-like substances are tightly connected with the root development by controlling the increase in length of root hairs, the primary root length, and the number of lateral root primordia (Overvoorde et al., 2010), while the activity of GA principally targets the endodermis to regulate root growth and cell elongation (Ubeda-Tomás et al., 2008). Consistently with our findings, previous studies reported that root development is positively affected by the activity of hormones and hormone-like substances that are mediated or released by HS nearby the root system (Mora et al., 2010; Zanin et al., 2019). With respect to the IPA-like activity, it must be noted that cytokinins are positive plant growth regulators (Müller and Sheen, 2007; Müller and Sheen, 2008). However, at root level they can exert antagonistic effects on auxin-mediated responses by altering the expression of IAA transporters (PIN) in lateral root founder cells, thus preventing the formation of the auxin gradient required to shape lateral root primordia (Laplaze et al., 2007). Interestingly, the ratio IAA-like to IPA-like activity of LMS1-2 was about 3.6 fold higher compared to that of THE1-2 and HMS1-2, which may suggest that the maximum increase of root biomass observed in LMS-treated plantlets was at least in part due to less antagonistic inhibition exerted by IPA-like activity. Also, when the ratios IAA-like to IPA-like activity were compared between HS derived from the two soils, values were higher for HS from soil (2). These ratios are coherent with the observation that HS derived from soil (2) stimulated root growth more than HS from soil (1) (Nardi et al., 2000), as indicated by the higher b values of the regression curve (**Table S1**, Supplementary).

The trend of root growth was wholly consistent with the activity of the enzymes invertase and esterase, which have established roles in plant development and proved to be activated by plant biostimulants (Ertani et al., 2018). The enzyme esterase is implied in organogenesis processes and represents an early gauge of somatic embryogenesis (Balen et al., 2004), while the enzyme invertase controls plant growth by regulating the availability of hexose substrates for cellular metabolism, especially in sinks experiencing cell expansion (Tauzin and Giardina, 2014). Peroxidase activity, which is also involved in cellular differentiation processes (Balen et al., 2004), performed differently from esterase and invertase activities being preferentially targeted by HMS2. However, it was similarly more induced by size-fractionated HS from soil (2).

The hexose sugars released by sucrose hydrolysis can also be used to drive other energy-dependent processes, including the active transport of mineral nutrients. In our study, we measured higher accumulation of nitrate and sulfate ions in roots of garlic plantlets when treated with sized-fractionated HS. Nevertheless, no differences in nitrate were observed between plantlets treated with LMS and HMS derived from the same soil, while sulfate accumulation tended to decrease in LMS treated-plantlets. A possible explanation is that nitrate and sulfate ions were more consumed in plantlets receiving LMS because of higher assimilation rates, as suggested by higher activity of N (nitrate reductase and glutamine synthetase) and S (OAS-sulphydrilase) assimilation enzymes, and increased content of certain amino acids (e.g., Ile, Asn, Ser, Cys). So far, many studies have reported the stimulation of N metabolism in plants by either HS or other biostimulants (Schiavon et al., 2008; Santi et al., 2017; Palumbo et al., 2018; Zanin et al., 2018; Schiavon et al., 2019), while scarce literature exists on the effects of HS on the S pathway. Jannin et al. (2012) in particular, showed that treating *Brassica napus* plants with HS positively impacted on C, N and S metabolism, as the expression of several genes implied in primary metabolic pathways and in N and S uptake was substantially upregulated. We found that cysteine and its precursor serine accumulated more in roots of LMS-treated garlic plantlets. Cysteine in turn serves as a substrate for the synthesis of other S amino acids, i.e. methionine and the non-proteinogenic amino alliin (S-allylcysteine sulfoxide). Accumulation of S amino acids was significantly higher when size-fractionated HS were applied to garlic, especially if they were derived from soil (2). The high increase of alliin content in LMS2- and HMS2-treated plantlets is a valuable finding that might have relevant implications for the nutritional quality of garlic. Indeed, upon garlic clove tissue damage, the compound allicin is produced from alliin in a reaction catalyzed by the enzyme alliinase. Allicin displays a number of health-promoting properties by acting as a powerful antimicrobial, antifungal and anticarcinogenic agent, and by lowering cholesterol and blood pressure with benefits for the cardio-vascular system (Borlinghaus et al., 2014). Intriguingly,

the hormonal-like activity of HS, either from soil (1) or soil (2), was toughly linked to S metabolism (**Table 3**), thus confirming the relationships between hormones and S nutrition described in other studies (Dan et al., 2007; Falkenberg et al., 2008; Masood et al., 2016; Hasanuzzaman et al., 2018).

Similarly to nitrate and sulfate, the elements K, Fe, Mg and Ca accumulated more in garlic roots when plantlets were treated with sized-fractionated HS than with total HS extracts (THE), which reflected the greater capacity of LMS and HMS to induce more positive effects on plant nutrition. With the exception of Mg, the other nutrient elements followed the same trend as root growth. The increased accumulation of nutrient elements by HS and other biostimulants has been widely reported in crops, including garlic (Denre et al., 2014). HS can improve plant nutrition through direct and indirect mechanisms (Zandonadi et al., 2010; Shah et al., 2018), including the enhancement of root plasma membrane H^+-ATPase activity *via* hormones and NO signaling pathways, the increase of micro- and macro- nutrient bioavailability *via* formation of soluble ions-HS complexes (Garcia-Mina et al., 2004; Olaetxea et al., 2019), targeted and non-targeted effects at the cells membranes that trigger biochemical and molecular cascade transcriptional and post-transcriptional events regulating the expression of nutrient transporters (Van Oosten et al., 2017).

Based on the PCA analysis, it seems evident that the physiological effects elicited by LMS and HMS differed depending on the chemical properties and origin of HS. Interestingly, THE1 and THE2 were comparable in features and displayed similar capacity to influence plant metabolism, thus confirming the efficacy of weak organic acids to produce size-fractionated HS more bioactive than unfractionated HS.

CONCLUSIONS

In conclusion, this study confirms that importance of the cover vegetation in determining the bioactivity of HS. Treating THE with a weak acid produced sized-fractionated HS that exhibited different chemical properties and bioactivity, likely because of novel molecular arrangements that better interacted with the plant roots. Size-fractionated HS from forest soils were more bioactive than unfractionated HS, but LMS1-2 were the most effective in improving the biochemical and physiological attributes of garlic over 48 h owing to their chemical-structural properties. We also show that the bioactivity of LMS1-2 was heavily influenced by the elevated aromaticity, the large content of polar residues and the more pronounced IAA content and hormone-like activity. Another important finding of this study is that size-fractionated HS greatly stimulated S metabolism beside N assimilation, with positive implications for the nutritional value of this crop and human health. Although treating plants with HS for a short period has long been used to test their biostimulant properties, to reinforce the results obtained in this study and to verify the effects of HS on garlic productivity, a pot study conducted for a longer period until garlic harvest would be needed in the future.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SN conceived and designed the experiments and contributed to data interpretation. DP contributed to data acquisition, analysis, and interpretation. OF and MS contributed to data analysis and interpretation. FDV contributed to data acquisition, analysis, and interpretation for the root ultrastructure. AE contributed to data interpretation. DP, SN, OF, MS, FDV, and AE drafted the manuscript. MS, DP, OF, and SN were the major contributor to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01203/full#supplementary-material

FIGURE S1 | 13C-NMR spectra of low molecular size LMS humic substances extracted from soil (1) (with *Pinus mugo* cover) and (2) (with *P. sylvestris* cover).

TABLE S1 | Correlation matrix (Pearson) for the chemical and biochemical variables.

TABLE S2 | Parameters of the regression curves (first and second traits) between concentration of humic substances (total humic extract, THE, high molecular size, HMS and low molecular size, LMS) and root biomass and macro and micro nutrients content in HS-treated garlic plantlets.

TABLE S3 | Parameters of the regression curves (first and second traits) between concentration of humic substances (total humic extract, THE, high molecular size, HMS and low molecular size, LMS) and enzyme activities in roots of HS-treated garlic plantlets.

TABLE S4 | Parameters of the regression curves (first and second traits) between concentration of humic substances (total humic extract, THE, high molecular size, HMS and low molecular size, LMS) and amino acid content in roots of HS-treated garlic plants.

TABLE S5 | Loadings values of chemical and biochemical variables on the axes identified by principal components analysis.

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Humic Acid Enhances the Growth of Tomato Promoted by Endophytic Bacterial Strains Through the Activation of Hormone-, Growth-, and Transcription-Related Processes

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Plant growth-promoting bacteria (PGPB) are promising alternatives in the reduction of the use of chemical fertilizers. Likewise, humic acid (HA) can improve plant growth and/or the establishment of endophytic PGPB. Although the effects of PGPB colonization or HA treatment have been studied separately, little information is available on plant response to the combined applications of PGPB and HA. Thus, the aim of this work was to understand the physiological effects, bacterial colonization and transcriptional responses activated by endophytic bacterial strains in tomato roots and shoots in the absence (control condition) and presence of HA (HA condition). Tomato shoot length was promoted by seed inoculation with *Paraburkholderia phytofirmans* PsJN, *Pantoea agglomerans* D7G, or *Enterobacter* sp. 32A in the presence of HA, indicating a possible complementation of PGPB and HA effects. Tomato colonization by endophytic bacterial strains was comparable in the control and HA condition. The main transcriptional regulations occurred in tomato roots and the majority of differentially expressed genes (DEGs) was upregulated by endophytic bacterial strains in the HA condition. Half of the DEGs was modulated by two or three strains as possible common reactions to endophytic bacterial strains, involving protein metabolism, transcription, transport, signal transduction, and defense. Moreover, strain-specific tomato responses included the upregulation of signal transduction, transcription, hormone metabolism, protein metabolism, secondary metabolism, and defense processes, highlighting specific traits of the endophyte-tomato interaction. The presence of HA enhanced the upregulation of genes related to signal transduction, hormone metabolism, transcription, protein metabolism, transport, defense, and growth-related processes in terms of number of involved genes and fold change values. This study provides detailed information on HA-dependent enhancement

of growth-related processes stimulated by endophytic bacterial strains in tomato plants and reports the optimized dosages, complementation properties and gene markers for the further development of efficient PGPB- and HA-based biostimulants.

Keywords: plant growth-promoting bacterial endophytes, humic acid, transcriptomic, RNA sequencing, tomato, endophytes, plant growth promoting rhizobacteria

INTRODUCTION

Conventional agriculture largely depends on chemical fertilizers (e.g., nitrogen-, phosphorus-, potassium-, and micro element-based fertilizers), which have numerous environmental drawbacks, such as surface and groundwater pollution and denitrification processes (Khan et al., 2018). Among crop plants, tomato (*Solanum lycopersicum*) is cultivated worldwide under field and greenhouse conditions (Hobson and Grierson, 1993) and requires an extensive use of chemical fertilizers that cause a significant negative environmental impact (Maham et al., 2020).

Plant growth-promoting bacteria (PGPB) can improve plant development and increase nutrient supply, such as nitrogen and iron (Ferreira et al., 2019). PGPB application has been considered as a promising alternative to maintain agroecosystem health and productivity (Gouda et al., 2018). Some PGPB can colonize the internal tissues of numerous plant species (endophytes) and can positively influence plant growth through various mechanisms, including the production of hormones, the improvement of nutrient uptake and protection against biotic or abiotic stresses (Gaiero et al., 2013). In particular, species of the bacterial genera *Bacillus*, *Enterobacter*, *Microbacterium*, *Pantoea*, *Paraburkholderia* and *Sphingomonas* are known to establish this type of association with plants (Sessitsch et al., 2005; Campisano et al., 2014; Hardoim et al., 2015). For example, bacterial endophytes isolated from grapevine, such as *Microbacterium* sp. C9D (C9D), *Pantoea agglomerans* D7G (D7G), *P. eucalypti* 727 (727), and *Sphingomonas* sp. 11E (11E), were able to increase the seed germination of *Arabidopsis thaliana* and exhibited beneficial traits *in vitro*, such as 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity (Campisano et al., 2014; Lòpez-Fernàndez et al., 2015a). Other endophytic bacteria, such as *Bacillus* sp. 54A (54A) and *Enterobacter* sp. 32A (32A), inhibited the growth of plant pathogens (e.g., *Botrytis cinerea*, *Botryosphaeria dothidea*, and *Botryosphaeria obtusa*) in dual-culture plate tests, suggesting that these strains can potentially protect plants against infections (Campisano et al., 2014; Lòpez-Fernàndez et al., 2015b). Among them, 32A affected the secondary metabolism and activated possible defense pathways in grapevine (Lòpez-Fernàndez et al., 2015a). A widely studied plant endophyte, *Paraburkholderia phytofirmans* PsJN (PsJN), previously classified as *Pseudomonas* and *Burkholderia* genus (Sessitsch et al., 2005; Sawana et al., 2014) is known to increase *A. thaliana* tolerance to salt stress through transcriptional and metabolic changes, such as proline accumulation, abscisic acid signaling and reactive oxygen species (ROS) scavenging (Pinedo et al., 2015). In particular, PsJN is able to improve the growth (Pillay and Nowak, 1997; Sharma and Nowak, 1998) and heat tolerance (Issa et al., 2018) of tomato plants, increasing net photosynthesis rate, stomatal conductance and chlorophyll content. For these reasons, the use of PGPB could be a promising approach in tomato production to improve plant growth and to reduce the use of chemical fertilizers. However, limitations to the wide use of beneficial endophytes were often encountered, for example because of the variable and/or inconsistent effect on the plant, especially under field conditions (Martínez-Viveros et al., 2010; Timmusk et al., 2017). Although they have been relatively well studied, a better understanding on the bacterial colonization (e.g., colonization rate and stability, competition with other microorganisms) and effects on tomato physiology (e.g., transcriptional response) is needed, in order to develop more efficient PGPB-based biofertilizers.

In addition to PGPB, organic humic substances present in the soil [e.g., humic acid (HA), humin and fulvic acid] can also improve plant growth and health and act as biostimulants (Olivares et al., 2017). Biostimulants are organic bioactive compounds that affect plant metabolism (Drobek et al., 2019). Among the natural biostimulants, HA is abundant in soil, peat or lignite and derives from the decay of organic materials (Drobek et al., 2019). HA improves nutrient uptake and the growth of tomato plants under hydroponic (Adani et al., 1998) and greenhouse conditions (Dursun et al., 2002), increasing electrolyte leakage, cell permeability, and nutrient accumulation (David et al., 1994). HA is a mixture of polymeric organic compounds, stabilized by weak forces (hydrophobic and hydrogen bonds) in a supramolecular arrangement that forms hydrophobic domains (Fischer, 2017). HA is refractory to degradation and its hydrophobic domains can provide protection for selected PGPB (Piccolo, 1996; Canellas and Olivares, 2014). The hydrophobic HA domain undergoes conformational changes in the presence of organic acids derived from root exudates and releases PGPB for the interaction with host plants (Nardi et al., 2009; Olivares et al., 2017). HA can also contribute to the endophytic establishment of PGPB (Olivares et al., 2017) and it has been suggested as a suitable carrier for PGPB formulation (Young et al., 2006; Olivares et al., 2017; Ma, 2019). For example, *Herbaspirillium seropedicae* Z67 inoculation in the presence of HA increased root surface area, enhanced grain production and altered carbohydrate and nitrogen metabolism in maize plants (Canellas et al., 2012). In particular, in low fertility soils, *H. seropedicae* Z67 and HA increased maize production compared to non-inoculated plants through PGPB-driven hormone production and HA-stimulated changes in phenolic metabolism (Canellas et al., 2015). Likewise, tomato fruit biomass was increased by *H. seropedicae* HRC54 and HA through the stimulation of nitrogen and secondary metabolism (Olivares et al., 2015). A mixed inoculum of *H. seropedicae* HRC54

and *Gluconacetobacter diazotrophicus* PAL 5 in combination with HA changed the metabolite fingerprints of amino acids, sugars and organic acids in maize and sugarcane seedlings, indicating that the activation of primary and secondary metabolism was partially responsible for the biostimulation effects (Aguiar et al., 2018; Canellas et al., 2019). Although considerable evidence of efficacy exist in literature, the molecular mechanisms of the combined applications of living PGPB and organic biostimulant on crops are less investigated (Bulgari et al., 2015). Our goal was to improve the understanding of the complementation effects and cellular pathways activated by endophytic bacterial strains and HA for the further development of sustainable biofertilizers for tomato production. More specifically, the present study aimed at understanding the colonization, growth promotion effects and transcriptional responses in tomato plants inoculated with bacterial endophytes in the absence (control condition) and presence of HA (HA condition).

MATERIAL AND METHODS

Growth of Bacterial Strains and Inoculum Preparation

The bacterial strains *Microbacterium* sp. C9D (C9D; isolate MiVv2), *Bacillus* sp. 54A (54A; isolate BaVs16), *Pantoea eucalypti* 727 (727; isolate PaVv9), *Pantoea agglomerans* D7G (D7G; isolate PaVv7), *Enterobacter* sp. 32A (32A; isolate EnVs6), and *Sphingomonas* sp. 11E (11E) were previously isolated from the grapevine endosphere (Campisano et al., 2014), while *Paraburkholderia phytofirmans* PsJN (PsJN) was isolated from surface-sterilized onion roots (Sessitsch et al., 2005). Bacterial strains were stored in 80% glycerol at −80°C and were grown in 5-ml nutrient broth (NB) in sterile 15-ml tubes at 25°C for 24 h under orbital shaking at 220 rpm.

For seed inoculation, bacterial cells were collected by centrifugation at 3,500 g for 10 min and washed twice with sterile 10 mM $MgSO_4$. Bacterial cells were then suspended in sterile 10 mM $MgSO_4$ and the bacterial suspension was adjusted to 1.0×10^7 colony forming units (CFU) per unit of volume (CFU ml^{-1}) based on an optical density conversion table at 600 nm (OD_{600}) optimized for each strain (**Table S1**).

Since HA is poorly soluble in water, a stock solution (1 g L^{-1}) of HA (Sigma-Aldrich, St. Louis, Missouri, USA; code 53680) was prepared in 0.1 M NaOH and the pH was then adjusted to 6.8 with 70% HNO_3 (HA stock solution) to avoid acidification of the NB and half-strength Hoagland. Since $NaNO_3$ was formed in the HA preparation, a water solution with NaOH and HNO_3 at an equivalent concentration to the HA stock solution was used as control in the bacterial compatibility, tomato seed inoculation, and transcriptomic analyses (control stock solution).

Bacterial Compatibility Assay With Humic Acid

To assess the bacterial compatibility with HA, 20 µl of each bacterial suspension (1.0×10^7 CFU ml^{-1}) was inoculated in 200 µl NB supplemented with 50 mg L^{-1} HA (10 µl HA stock solution) in a 96-well microplate (Thermo Fisher Scientific, Waltham, MA, USA). NB supplemented with 10 µl control stock solution was used as control (0 mg L^{-1} HA). Samples were incubated at 25°C for 72 h under orbital shaking programmed at medium shaking speed and bacterial growth was monitored by measuring the OD_{600} every 30 min using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA). Six replicates (wells) were used for each treatment and the experiment was carried out twice.

Tomato Seed Inoculation and Growth Conditions in Glass Tube and Square Dish

Seeds of *S. lycopersicum* L. cv. Moneymaker (Justseed, Wrexham, UK) were treated with 70% ethanol for 1 min and 2% sodium hypochlorite containing 0.02% Tween 20 for 5 min in a 50 ml tube (Subramanian et al., 2015) with vigorous shaking and washed three times with sterile distilled water (3 min each), in order to reduce the number of seed-associated microorganisms. Surface-sterilized seeds (50 seeds) were treated with 5 ml of sterile 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 5 ml of the bacterial suspension (bacterium-inoculated) of the respective endophytic strain (1×10^7 CFU ml^{-1}) by overnight incubation at 25 ± 1°C in a sterile 15-ml tube under orbital shaking at 40 rpm. Seeds were transferred to Petri dishes (20 seeds for each dish) containing 1% water agar (Thermo Fisher Scientific) and incubated for 48 h in a growth chamber (Binder KBWF 720, Bohemina, NY, USA) at 25 ± 1°C with a 16 h photoperiod (photon flux density of 0.033 mmol s^{-1} m^{-2}) to allow seed germination.

Germinated seeds with the same root length (1 mm) were selected and transferred to the growth medium in a glass tube or in a square dish as described below. To optimize the HA concentration for tomato plants, each germinated seed was transferred into a sterile 95 ml glass tube (Artiglass, Padova, Italy) containing 2.5 g sterile perlite and 10-ml half-strength Hoagland with 0, 25, 50, or 100 mg L^{-1} HA, and incubated in the growth chamber for six weeks. To assess the effect of HA on bacterium-inoculated plants, five seeds were transferred along a line in a central position of a 10 cm square dish (Sarstedt, Nümbrecht, Germany) containing 50 ml solid (14 g L^{-1} agar) half-strength Hoagland with 0 mg L^{-1} (control condition; 2.5 ml control stock solution for each dish) or 50 mg L^{-1} HA (HA condition; 2.5 ml HA stock solution for each dish), as optimized HA concentration. Dishes were incubated in vertical position in the growth chamber, shoot and root length was measured with a ruler and the fresh weight of the whole plant was assessed with a precision balance at three and six days after incubation (DAI). Four and five replicates were analyzed for each treatment in the experiment with glass tubes and square dishes, respectively, and each experiment was carried out twice.

Bacterial Re-Isolation From Tomato Plants

At the end of the incubation period, mock-inoculated and bacterium-inoculated plants were collected, and each plant was surface-sterilized in a 50 ml tube with 70% ethanol for 1 min, 2%

sodium hypochlorite for 1.5 min, followed by 70% ethanol for 1 min. Plants were washed three times with distilled water (2 min each), dried with a sterile filter paper before the assessment of the fresh weight. Plants were ground in a mixer-mill disruptor (MM 400, Retsch, Haan, Germany) at 25 Hz for 2 min in presence of 500 µl potassium phosphate buffer (1 mM, pH 7). Each suspension was serially diluted and 10 µl aliquots were plated in triplicates on nutrient agar (NA). Aliquots (10 µl) of the last washing solution were plated as the control of surface sterilization. After incubation at 25°C for 3 days, CFU values of endophytic bacterial strains per unit of plant fresh weight (CFU g^{-1}) were calculated. Five replicates (plants) were analyzed for each treatment and the experiment was carried out twice.

Fluorescence *In Situ* Hybridization Using Double Labeling of Oligonucleotide Probes

Double labeling of oligonucleotide probes for fluorescence *in situ* hybridization (DOPE-FISH) was performed on mock-inoculated plants and PsJN-, D7G-, or 32A-inoculated plants at 3 and 6 DAI in the control or HA condition in square dishes. Plants were aseptically cut into roots, stem, and leaves and were sectioned transversally using razor blades. Samples were then fixed in a 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) solution at 4°C for 5 h and were rinsed three times with 1× PBS as previously reported (Compant et al., 2011). Plants were dehydrated in increasing concentrations of ethanol solution (25%, 50%, 75%, and 99%; 20 min each step) and stored at 4°C. DOPE-FISH was carried out using probes from Eurofins (Germany) labeled at both the 5' and 3' positions. A probe mixture targeting eubacteria, composed of EUB338, EUB338II, EUB338III (EUBmix) coupled with a Cy3 fluorochrome and Bphyt probe targeting the 23S rRNA gene of PsJN coupled with Cy5 (Amann et al., 1990; Daims et al., 1999; Mitter et al., 2017). For D7G and 32A, EUBmix and Gam42a probe targeting the 23S rRNA gene of D7G and 32A coupled with Cy5 was used (Manz et al., 1992). NONEUB probe coupled with Cy3 or Cy5 was used independently as negative control (Wallner et al., 1993). Fluorescent *in situ* hybridization was carried out in sterile 1.5 ml tubes at 46°C for 2 h in the dark with 60 µl hybridization buffer for PsJN (containing 0.9 M NaCl; 0.02 M Tris HCl, 0.01% SDS, 10% formamide and 5 ng μl^{-1} of each probe) and with 60 µl hybridization buffer for D7G and 32A (containing 0.9 M NaCl; 0.02 M Tris HCl, 0.01% SDS, 35% formamide, and 5 ng μl^{-1} of each probe). Washing was conducted at 48°C for 30 min with a pre-warmed post-FISH solution containing 0.02 M Tris HCl, 0.01% SDS, NaCl and EDTA at a concentration corresponding to the formamide concentration. Samples were then rinsed with distilled water before overnight air-drying in the dark. Samples were observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 and HeNe (G) laser FV10-LAHEG230-2). Pictures were taken at 405, 488, 633 nm wavelengths with Cy3 assigned as green and Cy5 as red. Pictures were analyzed using Imaris 8 software (BITPLANE, UK). Z-stacks were used to generate whole-stack pictures. Five replicates (plants) were analyzed for each treatment and representative pictures were selected. Pictures were cropped and light/contrast balance improved in post process.

Sample Collection, RNA Extraction, and Illumina Sequencing

Mock-inoculated plants and PsJN-, D7G-, or 32A-inoculated plants in square dishes were collected at 3 DAI in the control and HA condition in square dishes. Five plants were randomly collected for each treatment (replicate), roots and shoots were cut, separately placed into 2 ml tubes, immediately frozen in liquid nitrogen and stored at −80°C. A metal bead was added to each tube and samples were ground in a mixer-mill disruptor (MM200, Retsch) at 25 Hz for 1 min. Total RNA was extracted from 0.1 g of ground sample using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) with an on-column DNase treatment with RNase-Free DNase Set (Qiagen, Hilden, Germany). Total RNA was quantified using a Qubit (Thermo Fisher Scientific) and RNA quality was checked using a Tapestation 2200 (Agilent Technologies, Santa Clara, CA, USA). For each treatment, three replicates (pool of five plants) were analyzed. RNA samples were subjected to RNA-Seq library construction, using the TruSeq SBS v3 protocol (Illumina, SanDiego, CA, USA) and rRNA depletion with the RiboZero rRNA Removal Kit for plant according to the manufacturer's instructions (Illumina). Paired-end reads of 150 nucleotides were obtained using a NovaSeq 6000 S2 instrument (Illumina) at the Institute of Applied Genomics (Udine, Italy) and sequences were deposited at the Sequence Read Archive of the National Center for Biotechnology (https://www.ncbi.nlm.nih.gov/sra) under the BioProject number PRJNA622763.

Bioinformatic Analysis and Identification of Differentially Expressed Genes

Raw reads were cleaned and filtered using the programme Trimmomatic version 0.36 (Bolger et al., 2014) and low-quality bases with an average Phred quality score lower than 15 in a sliding window of four base were removed. Any resultant reads shorter than 36 bp in length were removed from the analysis and the quality check of filtered reads was performed using Fast QC version 0.11.7. Filtered read pairs were aligned and counted using STAR 2.7 (Dobin et al., 2013) to the *S. lycopersicum* genome release ITAG3.2 and counts of unambiguously mapped read pairs was obtained during the alignment with the STAR 2.7 program. Differentially expressed genes (DEGs) were identified with the Limma-Voom package (Law et al., 2014), which estimates the mean-variance relationship of Log_2-transformed counts, generating a precision weight for each observation that is fed into the Limma empirical Bayes analysis pipeline (Smyth, 2006). A Volcano Plot was generated using the Python programming language and the matplotlib package (Hunter, 2007) and a double cut-off on *P*-value ($P \leq 0.01$) and minimum Log_2 fold change (FC) of one [Log_2 (FC) ≥ 1 or Log_2 (FC) ≤ -1] were imposed to identify DEGs through pairwise comparisons. Three pairwise comparisons were analyzed for shoots and roots: PsJN- vs. mock-inoculated, D7G- vs. mock-inoculated and 32A- vs. mock-inoculated plants. DEGs modulated by endophytic bacterial strains between the control and HA condition were compared in order to identify HA-dependent effects on processes activated by PsJN, D7G and 32A. Moreover, the pairwise comparison between the control and HA condition of mock-inoculated

plants was included, in order to analyze the effects caused by HA in the absence of endophytic bacterial strains. The distribution of DEGs was summarized using the Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) and DEGs were grouped in upregulated and downregulated genes by at least two endophytic bacterial strains (DEGs modulated by two or three strains), to highlight possible common reactions in response to endophytic bacterial strains, or specifically by only one endophytic bacterial strain (PsJN-, D7G-, or 32A-specific tomato DEGs) in the control and HA condition. The heat map diagram of fold change values of DEGs was visualized using the Java Treeview tool (Saldanha, 2004). Gene expression levels were then expressed as transcripts per million (TPM).

Gene Ontology (GO) terms and protein descriptions of tomato Heinz1706 genes (Sato et al., 2012) of the release ITAG3.2 were downloaded from the tomato genome browser (https://solgenomics.net/organism/Solanum_lycopersicum/genome). GO terms significantly overrepresented ($P \leq 0.05$, Benjamin and Hochberg FDR correction) in the DEG lists in comparison to the whole tomato transcriptome were identified using the Biological Networks Gene Ontology (BiNGO) tool (Maere et al., 2005) and the biological networks were visualized with Cytoscape version version 3.7.1 (Shannon et al., 2003). DEGs were further annotated on the basis of tomato protein description and grouped into 14 functional categories according to the previous literature. Genes that were not associated to any biological process were assigned to the unknown function category. Tomato cellular pathways were generated with Biorender (https://biorender.com/) according to literature search of functional annotation of DEGs.

Gene Expression Analysis by Quantitative Real-Time RT-PCR

Tomato gene markers were selected for quantitative real-time PCR (qPCR) analysis (**Table S2**). The first strand of cDNA was synthesized from 1 μg of DNase-treated RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and oligo-dT primer. qPCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) as previously described (Perazzolli et al., 2016). Briefly, the PCR conditions were: 50°C for 2 min and 95°C for 2 min as initial steps, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analyzed to verify the specificity of each amplification reaction. The Light Cycler 480 SV1.5.0 software (Roche) was used to extract Ct values based on the second derivative calculation and the LinReg software version 11.0 was used to calculate reaction efficiencies for each primer pair (Ruijter et al., 2009). For each gene, the relative expression level (fold change) was calculated according to the Pfaffl equation (Pfaffl, 2001) for each pairwise comparison between bacterium-inoculated and mock-inoculated samples in the control and HA condition. Five housekeeping genes were analyzed, such as genes encoding ankyrin repeat domain containing protein 2 (*ARD2*) (Pombo et al., 2017), kinesin light chain 2 isoform (*KLC*) (Pombo et al., 2017), vernalization insensitive 3 (*VIN3*) (Pombo et al., 2017), small nuclear ribonucleoprotein family protein (*LSM7*) (Müller et al., 2015), and ubiquitin carboxyl-terminal hydrolase (*UCH*) (Müller et al., 2015), and their stability was validated using the ΔCt method (Silver et al., 2006). *ARD2* was then selected as constitutive gene for normalization, because its expression was not affected by the different conditions (**Table S2**). Three replicates (pool of five plants) were analyzed for each condition.

Statistical Analysis

All functional experiments were carried out twice and data were analyzed with the Past 3.26 software (Hammer et al., 2001). After validating data for normal distribution (Shapiro-Wilk test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$), each experimental repetition was analyzed singularly and a two-way analysis of variance (ANOVA) was used to demonstrate non-significant differences between the two experiments ($P > 0.05$). Data from the two experimental repetitions were pooled and significant differences among treatments were assessed with the Student's t-test ($P \leq 0.05$) and the Tukey' test ($P \leq 0.05$) in case of pairwise and multiple comparisons, respectively. CFU values of bacterial resolution were Log_{10}-transformed and fold change values of gene expression analysis were Log_2-transformed. The Pearson's correlation coefficient between gene expression levels assessed by RNA-Seq and qPCR analysis was calculated with the Excel program.

RESULTS

Endophytic Bacterial Strains and Humic Acid Enhance Tomato Growth

HA improved tomato growth (**Figure S1**), the maximum growth promotion of shoot and root length was obtained with 50 mg L^{-1} HA and this dosage was selected as optimized HA concentration for the subsequent experiments (HA condition). All endophytic bacterial strains grew in presence of 50 mg L^{-1} HA (**Figure S2** and **Table S1**). The tomato shoot length was longer in PsJN-, D7G-, 32A-, and 11E-inoculated plants compared to mock-inoculated plants in the absence of HA (control condition; **Figure S3A**). Likewise, PsJN, D7G, and 32A improved tomato shoot length in the HA condition and these three strains were selected for the subsequent experiments. Plants were colonized by the endophytic bacterial strains tested and PsJN, D7G, and 32A were re-isolated from surface-sterilized tomato plants at 6 DAI at comparable levels in the control and HA condition (**Figure S3B**). Tomato shoot length was promoted by PsJN, D7G, and 32A at 3 DAI in the control condition and it was also stimulated by HA in mock-inoculated plants, through a possible complementation of the endophytic PGPB and the organic biostimulant (**Figure 1**). Moreover, PsJN, D7G, and 32A confirmed the promotion of tomato shoot length at 6 DAI in the HA condition and indicated different effects of growth promotion according to the incubation time (**Figure S4**).

To better characterize the colonization of tomato tissues by endophytic bacterial strains, the DOPE-FISH analysis was carried out in the control and HA condition using specific probes targeting the 23S rRNA gene and universal probes for bacteria. Yellow fluorescent PsJN (**Figures 2A, B**), D7G (**Figures 2C, D**) and 32A (**Figures 2E, F**) single cells, aggregates, and micro-colonies were found on the secondary root emergency site, root tip, root elongation zone, root hair, and xylem of tomato roots in the control and HA condition. PsJN, D7G, and 32A cells were also found on the tomato stem and xylem in the control and HA condition (**Figure S5**) and the colonization intensity of tomato roots among the tested strains were comparable in the control and HA condition at 3 DAI (**Figure 2**) and 6 DAI (**Figure S6**). In mock-inoculated plants only some native bacteria were present (**Figure S7**). The NONEUB probe was used as negative probe not targeting bacterial sequences and only a few green/blue-cyan/orange/reddish autofluorescent microbes could be seen in mock-, PsJN-, D7G-, and 32A-inoculated plants as indication of the rare presence of native autofluorescent microorganisms (**Figures S7, S8**).

Endophytic Bacterial Strains and Humic Acid Modulate Tomato Genes in Roots and Shoots

To further characterize the plant response to endophytic bacterial strains and HA, a transcriptomic analysis of tomato shoots and roots was carried out. From 11.7 to 23.8 million reads were obtained for each replicate of tomato shoots and roots collected from mock-inoculated plants and plants inoculated with PsJN, D7G, and 32A at 3 DAI in the control and HA condition (**Table S3**). More than 80.0% of tomato genes were expressed in at least one condition (**Table S4**). A total of 6,135 and 623 DEGs were identified in tomato roots and shoots respectively, according to the pairwise comparisons between bacterium-inoculated (PsJN-, D7G-, and 32A-inoculated) and mock-inoculated plants in the control and HA condition, while 4,227 and 422 genes were modulated by HA in mock-inoculated roots and shoots, with a *P*-value lower than 0.01 and minimum Log_2-transformed fold change of one (**Tables S5–S10**). The majority of DEGs was downregulated (79.4%) by endophytic bacterial strains in the control condition. Conversely, DEGs were mainly upregulated (80.0%) by endophytic bacterial strains in the HA condition, as a consequence of a possible HA-dependent enhancement of tomato reactions to endophytic bacterial strains (**Figure 3**). DEGs were grouped in genes modulated by at least two endophytic bacterial strains (DEGs modulated by two or three strains), to highlight possible common reactions to bacterial endophytes, or specifically by only one endophytic bacterial strain (PsJN-, D7G-, or 32A-specific DEGs), to highlight possible strain-specific reactions, in roots or shoots in the control and HA condition (**Figures 3**, **S9**). The RNA-Seq results were validated by a qPCR analysis of 10 tomato genes (**Table S2**) that were selected according to their expression profiles [five genes modulated in roots and five in shoots; five modulated only in the control condition and three modulated only in the HA condition and belonging to one of the four different clusters (modulated by two or three strains, PsJN-

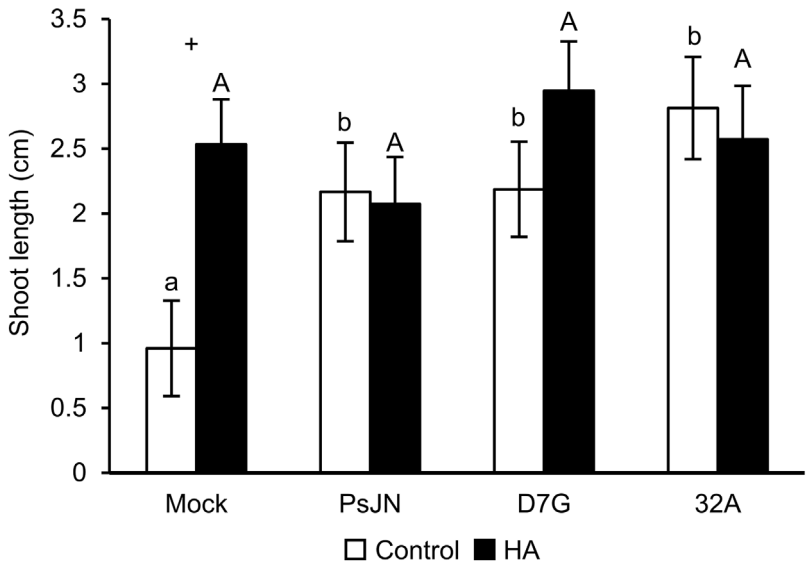

FIGURE 1 | Tomato growth promotion by endophytic bacterial strains. The shoot length (cm) of mock-inoculated plants (mock) and plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G), or *Enterobacter* sp. 32A (32A) was assessed 3 days after incubation in half-strength Hoagland with 0 mg L^{-1} (white, control) and 50 mg L^{-1} humic acid (black, HA) in square dishes. Mean and standard error values of nine replicates (plants) are presented for each treatment. Different lowercase and uppercase letters indicate significant differences among treatments in the control and HA condition according to Tukey's test ($P \leq 0.05$), respectively. For each treatment, plus symbols indicate significant differences in the pairwise comparisons between the control and HA condition according to Student's t test ($P \leq 0.05$).

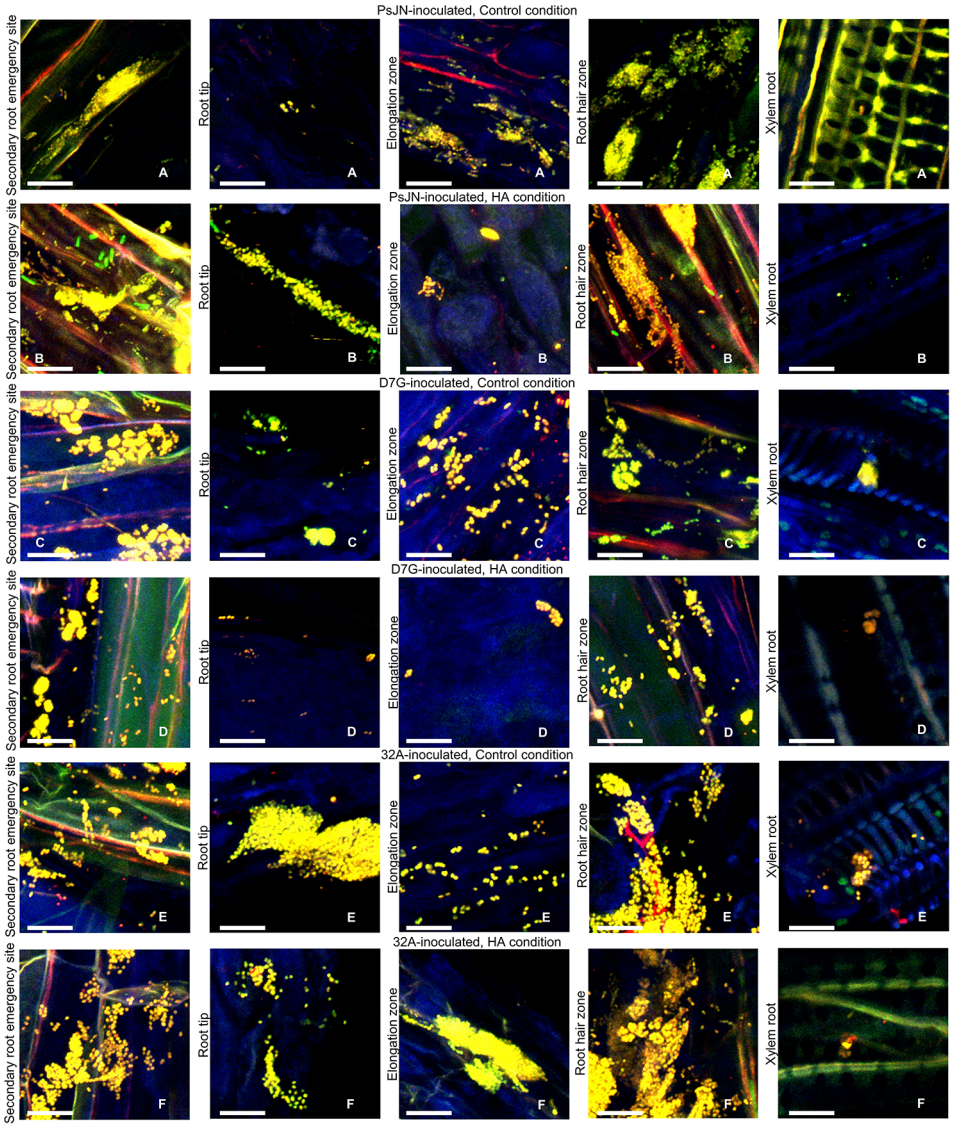

FIGURE 2 | Location of endophytic bacterial strains on and inside tomato roots. Bacterial cells of *Paraburkholderia phytofirmans* PsJN (PsJN) **(A, B)** were hybridized with the EUBmix and Bphyt probes, *Pantoea agglomerans* D7G (D7G) **(C, D)**, or *Enterobacter* sp. 32A (32A) **(E, F)** were hybridized with the EUBmix and Gam42a probes on secondary root emergency sites (a), root tip zone (b), root elongation zone (c), root hair zone (d), and xylem (e) 3 days after incubation (DAI) in half-strength Hoagland with 0 mg L^{-1} (Control condition; **A**, **C**, **E**) and 50 mg L^{-1} humic acid (HA condition; **B**, **D**, **F**) in square dishes. Five replicates (plants) were analyzed for each treatment and representative pictures were selected. Bars correspond to 10 μM.

specific, D7G-specific or 32A-specific)] and functional categories (e.g., defense, growth and development, hormone metabolism, oxidative stress, protein metabolism, secondary metabolism, transcription, and transport). A close correlation (Pearson correlation coefficient, 0.93) between RNA-Seq and qPCR expression data was observed (**Figure S10**). In particular, expression profiles generated by qPCR and RNA-Seq agreed completely for eight genes and differed slightly for two genes (**Table S2**), possibly due to differences in the method sensitivity and discrimination capacity of multigene families (Perazzolli et al., 2016).

Endophytic Bacterial Strains Activate a Complex Transcriptional Response in Tomato Roots According to the Presence of Humic Acid

In tomato roots, 539 and 3,688 genes were upregulated and downregulated by HA in mock-inoculated plants, respectively (**Figure S11A and Table S5**). A significant enrichment of GO categories related to regulation of metabolic process and regulation of transcription was found for genes upregulated by HA (**Figure S11D**), such as transcription factors (e.g., 12 MYB, seven WRKY, five NAC domain-containing and two ethylene-

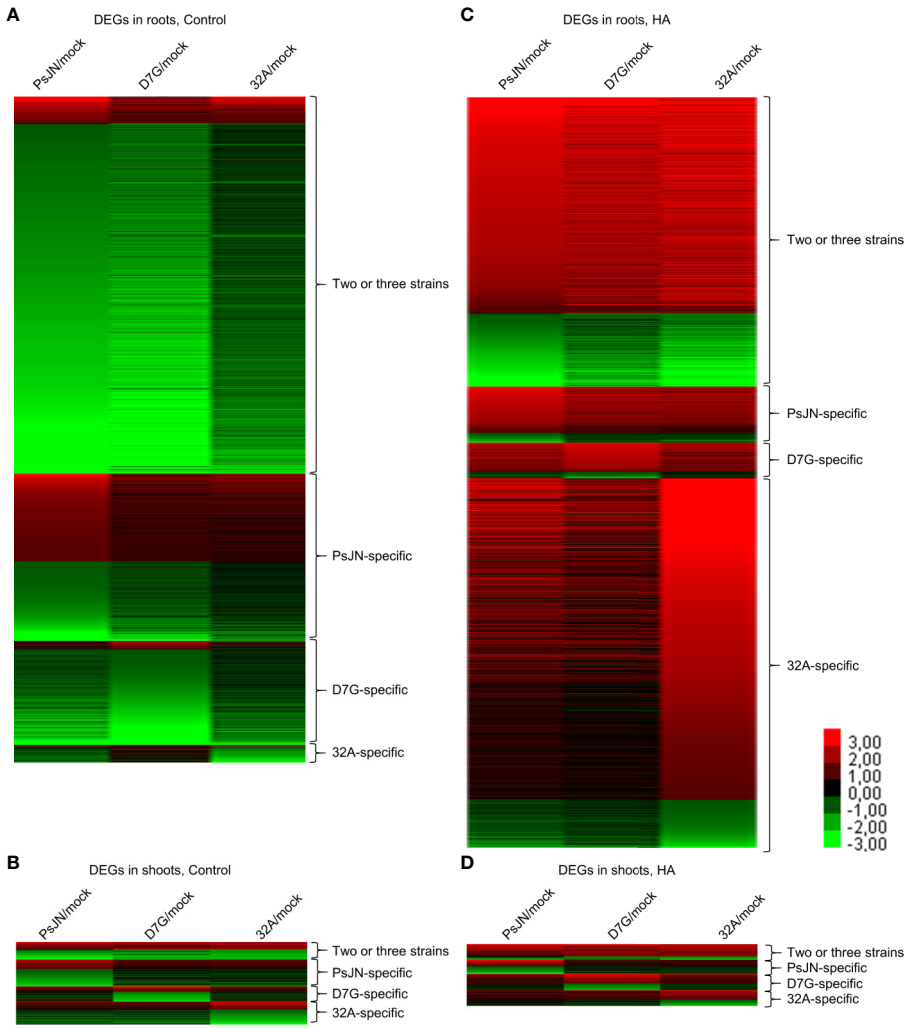

FIGURE 3 | Clustering of differentially expressed genes (DEGs) of tomato plants in response to endophytic bacterial strains and humic acid. Heat map diagram indicates the fold change values for DEGs identified in tomato roots **(A, C)** and shoots **(B, D)** 3 days after incubation with *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G), or *Enterobacter* sp. 32A (32A), calculated as compared to mock-inoculated plants (mock) in half-strength Hoagland with 0 mg L^{-1} (control; **A**, **C**) and 50 mg L^{-1} humic acid (HA; **B**, **D**). DEGs were classified as genes modulated by two or three strains or as genes modulated by only one bacterial strain (PsJN-, D7G-, or 32A-specific). The heat map diagram was visualized using Java Treeview according to color scale legend shown.

responsive transcription factors) and signal transduction-related genes (e.g., 14 kinases, eight calcium-binding proteins, and four receptor kinases; **Figure S11C** and **Table S5**). Moreover, genes downregulated by HA in tomato roots indicated global repression of cellular metabolic processes and energy-related processes (**Figure S11E**).

There were 119 and 926 genes upregulated by two or three strains in the control and HA condition, respectively (**Tables S6, S7**). Genes upregulated by two or three strains in tomato roots in the control condition were mainly involved in protein metabolism (e.g., one cysteine desulfurase, three F-box proteins and one tyrosine aminotransferase), transcription [e.g., two basic helix-loop-helix transcription factors, (bHLH), three zinc finger proteins and two WRKYs], transport (e.g., one heavy metal transport protein, one iron-regulated transporter, three potassium channels, one potassium transporter and two vacuolar iron transporters), signal transduction (e.g., four kinases, one receptor kinase and two serine/threonine-protein kinases), and defense [e.g., four defensin-like proteins, two nucleotide-binding site leucine-rich repeat proteins (NBS-LRRs) and three leucine rich repeat (LRR) receptorlike proteins; **Figure 4A** and **Table S6**]. As a possible common reaction to bacterial endophytes, a significant enrichment of GO categories related to defense response, response to stimulus and oxidative stress (e.g., five peroxidases and one glutaredoxin) was found for upregulated genes by two or three strains in the control condition (**Figure 4B and Table S6**). The presence of HA enhanced the transcriptional changes

activated in response to two or three strains, in terms of number of genes and FC values. Thus, genes related to protein metabolism, transcription [e.g., one WRKY, one ethylene (ET) response factor and two ET responsive transcription factors], transport, signal transduction (e.g., six receptor kinases, 17 protein kinases, three calcium transporting ATPases, one calcium-dependent protein kinase, one calcium/calmodulin-dependent serine/threonine-kinase, and 16 serine/threonine-protein kinases) and defense (e.g., four NBS-LRRs, four defensin-like proteins) were upregulated by two or three strains in the HA condition, together with genes implicated in the growth and development process (e.g., two cellulose synthases, six glycosyltransferases, one mannosyltransferase, two pectin lyases and three pectinesterases), in the hormone metabolism (e.g., four 1-aminocyclopropane-1-carboxylate synthases, three cytokinin riboside phosphoribohydrolases, one gibberellin oxidase, one gibberellin dioxygenase, one auxin efflux facilitator, and eleven small auxin responsive proteins), and response to oxidative stress (e.g., seven peroxidases, four glutaredoxins and one glutathione S-transferase; **Figure 4A and Table S7**).

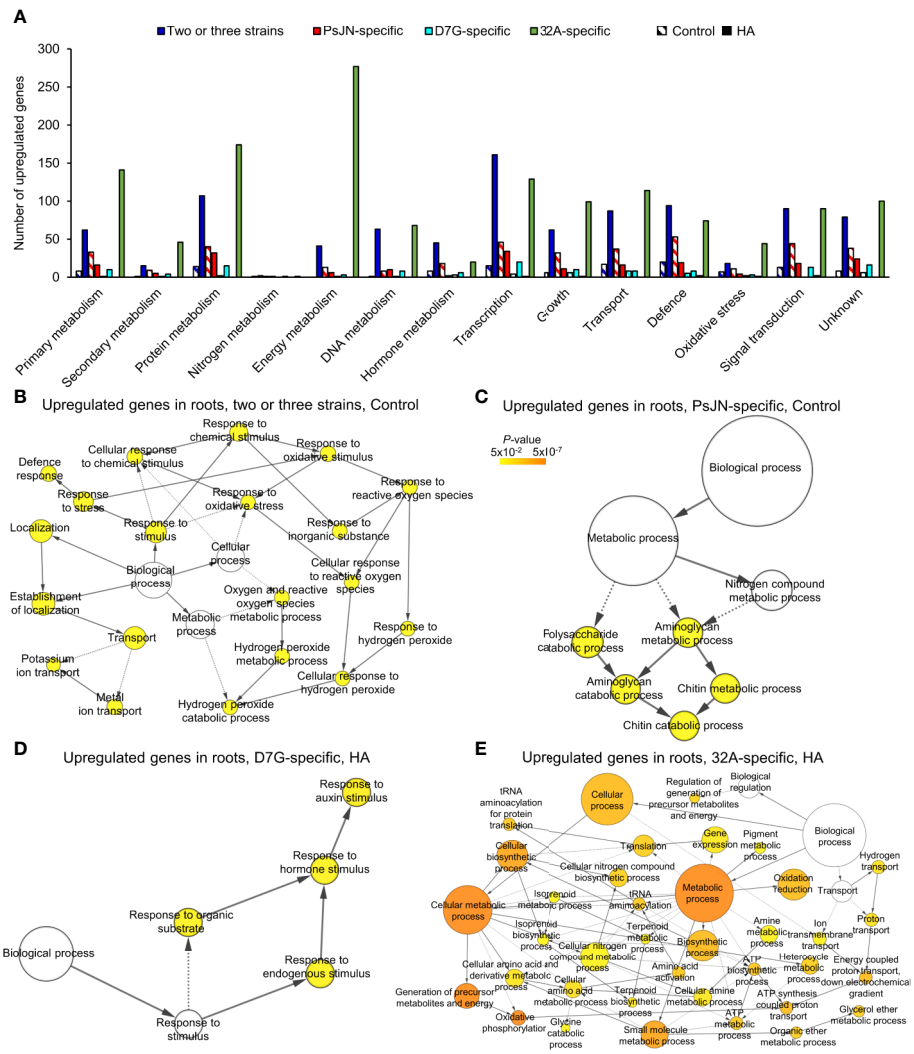

FIGURE 4 | Functional annotation of upregulated genes in tomato roots in response to endophytic bacterial strains. Functional classes **(A)** were assigned on the basis of the protein description of upregulated genes in tomato roots in response to two or three strains (blue) and specifically in response to *Paraburkholderia phytofirmans* PsJN (red), *Pantoea agglomerans* D7G (cyan), or *Enterobacter* sp. 32A (green) in half-strength Hoagland with 0 mg L^{-1} (control; stripped bars) and 50 mg L^{-1} humic acid (HA; solid bars). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO) terms of upregulated genes in tomato roots in response to two or three strains **(B)** or to PsJN **(C)** in the control condition and in response to D7G **(D)** or 32A **(E)** in the HA condition are reported. The color scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for upregulated genes in response to two or three strains and to PsJN in the HA condition, as well as in response to D7G or 32A in the control condition.

PsJN-specific genes revealed the upregulation of genes related to protein metabolism (e.g., one lysine-ketoglutarate reductase and one threonine aldolase), transcription [e.g., four basic helix loop helix transcription factors (bHLHs), two basic-leucine zipper family proteins (bZIP), six MYBs and three WRKY transcription factors and five zinc finger proteins], transport (e.g., two mannose transporter, one phosphate transporter and one potassium transporter), defense (e.g., two disease resistance proteins, four LRR receptor like proteins and six NBS-LRRs), signal transduction (e.g., nine kinases and eight receptor kinases), and hormone metabolism in the control condition (**Figure 4A**). As a consequence, the GO categories related to the chitin metabolic process and the aminoglycan and polysaccharide catabolic processes were enriched in the cluster of PsJN-specific genes in the control condition (**Figure 4C**). In addition, PsJN-specific genes upregulated in the HA condition were involved in protein metabolism (e.g., one cysteine desulfurase, one glutamate dehydrogenase, and 10 F-box proteins), transcription (e.g., two bHLHs, five zinc finger proteins, one MYB and two WRKYs), defense (e.g., one disease resistance proteins, three LRR receptor like proteins and one phenylalanine ammonia-lyase), and signal transduction (e.g., four serine/threonine-kinases, one histidine kinase, four protein kinases and one receptor kinase), as possible enhancement of tomato response in the HA condition (**Figure 4A**). D7G-specific genes upregulated in the control condition were involved in transport, defense, growth, and development (**Figure 4A**), while those upregulated in the HA condition were mainly involved in protein metabolism (e.g., one cysteine synthase and one glutamate dehydrogenase), transcription (e.g., one bHLH and two MYB transcription factors), and signal transduction (e.g., three protein kinases). In particular, D7G-specific genes involved in the response to hormone stimulus were upregulated in the HA condition (e.g., five small auxin responsive proteins and an ET receptor; **Figure 4D**) and control condition (e.g., 1-aminocyclopropane-1-carboxylate synthase, auxin-regulated IAA protein, cytokinin hydrolase). Likewise, 32A-specific genes upregulated in the HA condition were mainly involved in protein metabolism, energy metabolism (e.g., nine NADH dehydrogenases, one cytochrome c oxidase, and one ATPase) and transcription (e.g., three ankyrin repeat family proteins, seven bHLHs, eight zinc finger proteins; **Figure 4A**). In particular, the GO categories related to secondary metabolism and amino acid metabolism were enriched (**Figure 4E**) in the cluster of 32A-specific genes in the HA condition, together with genes related to oxidative stress response (e.g., 14 thioredoxins, three glutathione S-transferases, three superoxide dismutases, three glutaredoxins, and two peroxidases; **Figure 4A and Table S7**). Thus, the cellular processes involved in tomato root response to endophytic bacterial strains in the control and HA condition revealed the activation of a complex recognition machinery that involves signal transduction pathways and the consequent activation of transcription-, protein-, transport-, and defense-related pathways (**Figures 5**, **S12**). Different recognition processes were activated by PsJN, D7G, and 32A, and the presence of HA enhanced the upregulation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defense, and growth-related process in terms of number of DEGs and fold change values. Conversely, genes downregulated by endophytic bacterial strains in tomato roots suggest fine regulation of protein metabolism, DNA metabolism and secondary metabolism in the control and HA condition (**Figure S13**).

Endophytic Bacterial Strains Activate a Complex Transcriptional Response in Tomato Shoots According to the Presence of Humic Acid

HA incubation caused the upregulation and downregulation of 52 and 170 genes in tomato shoots of mock-inoculated plants, respectively (**Figure S11B and Table S8**). Tomato genes upregulated by HA were involved in primary metabolism (**Figure S11C**) and indicated the activation of the GO categories related to carbohydrate metabolism, alcohol metabolism and cell wall macromolecule metabolism (**Figure S11F**). Conversely, genes related to ROS metabolism were mainly downregulated by HA (**Figure S11G**).

Genes upregulated by two or three strains in tomato shoots in the control condition were mainly related to protein metabolism, defense, growth and development (**Figure 6A; Tables S9, S10**). A significant enrichment of the GO categories related to cell growth was found for upregulated genes by two or three strains in the control condition, such as cell wall-related processes (e.g., one glucan synthase and two xyloglucan endotransglucosylases; **Figure 6B**). In the HA condition, the enrichment of the GO categories related to aminoglycan metabolism and chitin metabolism was found for upregulated genes by two or three strains (**Figure 6C**). Genes associated to primary metabolism (e.g., two 2-oxoglutarate oxygenases and one lipase), protein metabolism (e.g., one calreticulin and one cysteine desulfurase), and transport (e.g., one calcium transporting ATPase) were upregulated by two or three strains in the HA condition (**Figure 6A**), in agreement with the shoot length promotion.

PsJN-specific genes modulated in tomato shoots were mainly related to protein metabolism, transcription and defense in the control condition (**Figure 6A**). Similarly, PsJN-specific genes upregulated in the HA condition were involved in transcription (e.g., one MYB and one zinc finger protein), defense, and transport. Tomato processes related to transcription, growth and development (e.g., one cyclin and one cell division cycle protein), transport, and defense were also upregulated by D7G in the control condition (**Figure 6A**), with the enrichment of the inositol and polyol GO processes (**Figure 6D**). In the HA condition, genes involved in transcription (e.g., one ET responsive transcription factor), growth and development (e.g., one expansin and two glycosyltransferases), and transport (e.g., one aluminium-activated malate transporter and two lipid transfer proteins) were upregulated by D7G. Moreover, 32A-specific genes upregulated in the control condition were mainly involved in the secondary metabolism and defense (**Figure 6A**) and the GO categories related to oxidative stress and phosphorylation were enriched (**Figure 6E**). 32A-specific genes related to transcription (e.g., one WRKY transcription factor),

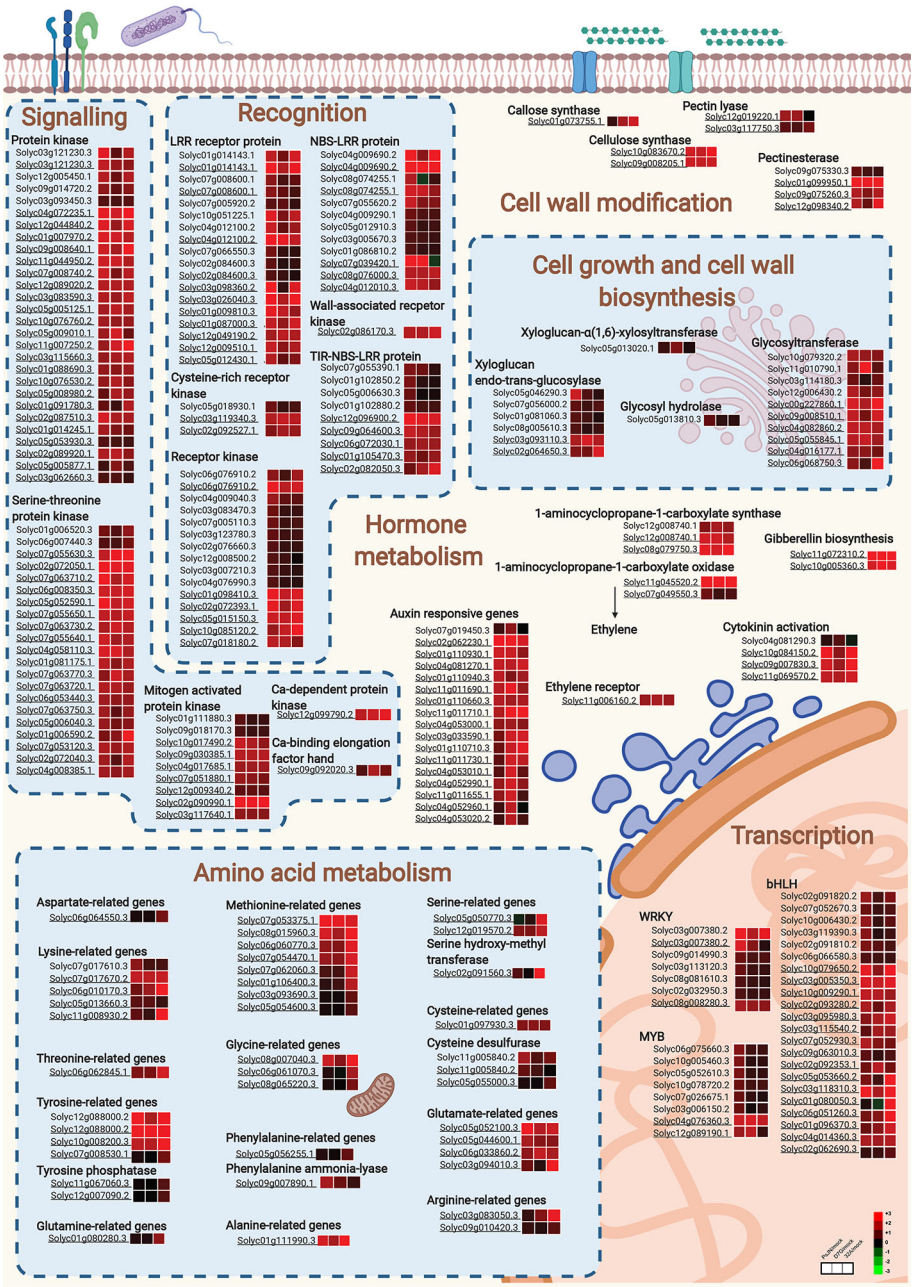

FIGURE 5 | Cellular processes activated by endophytic bacterial strains in tomato roots. Main cellular pathways of upregulated genes in tomato roots in response to *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G) or *Enterobacter* sp. 32A (32A) in half-strength Hoagland with 0 mg L^{-1} (control) and 50 mg L^{-1} humic acid (HA) were generated with Biorender. Not underlined and underlined gene codes indicate tomato genes modulated in the control and HA condition, respectively. For each gene, three squares represent the Log_2-transformed fold change values of PsJN-, D7G-, or 32A-inoculated plants calculated as compared to mock-inoculated plants respectively, according to the color scale reported. bHLH, basic helix-loop-helix; LRR, leucine-rich repeat; NBS-LRR, nucleotide-binding site leucine-rich repeat; TIR-NBS-LRR, non-toll-interleukin receptor nucleotide-binding site leucine-rich repeat; Ca, calcium.

growth and development (e.g., one expansin, one xyloglucan hydrolase and one xyloglucan endoglucanase inhibitor), signal transduction (e.g., two LRR kinases), and oxidative stress response (e.g., one glutathione S-transferase and one peroxidase) were upregulated in the HA condition (**Figure 6A** and **Table S10**). In summary, cellular processes activated in tomato shoots in response to endophytic bacterial strains included recognition-, signal transduction-, and transcription-related pathways, with an

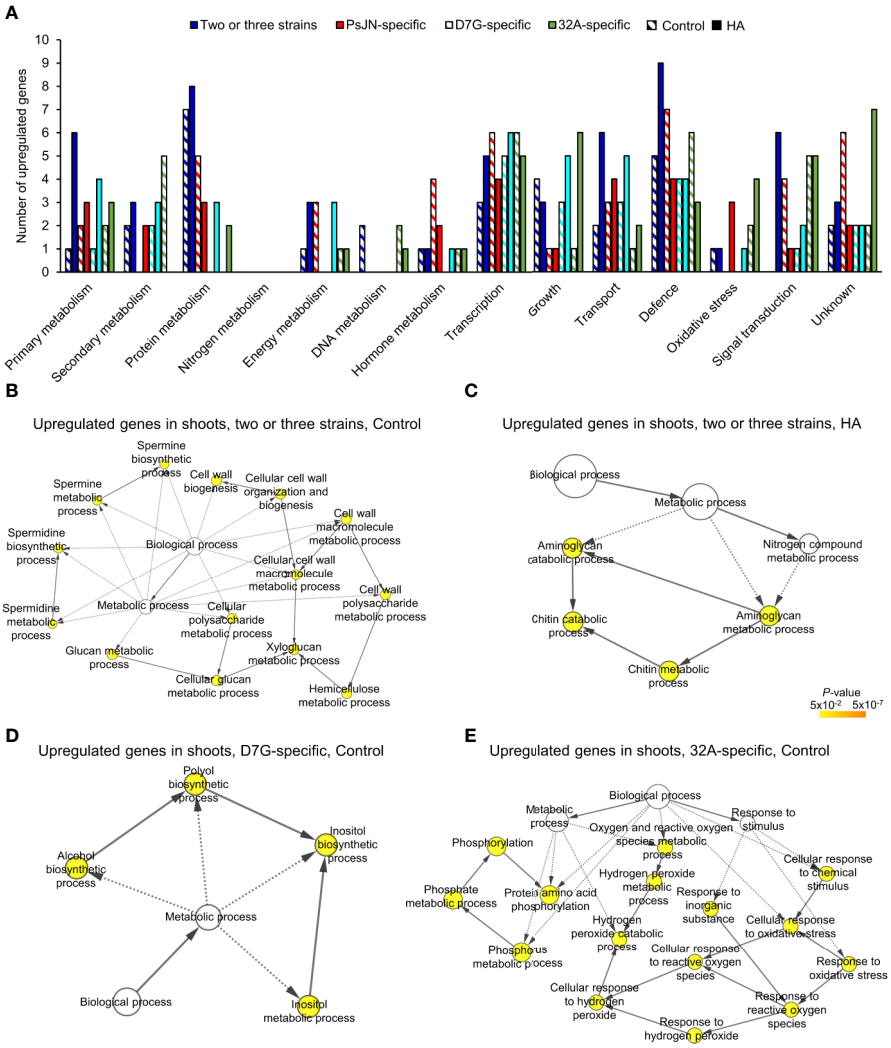

FIGURE 6 | Functional annotation of upregulated genes in tomato shoots in response to endophytic bacterial strains. Functional classes **(A)** were assigned on the basis of the protein description of upregulated genes in tomato shoots in response to two or three strains (blue) and specifically in response to *Paraburkholderia phytofirmans* PsJN (red), *Pantoea agglomerans* D7G (cyan), or *Enterobacter* sp. 32A (green) in half-strength Hoagland with 0 mg L^{-1} (control; stripped bars) and 50 mg L^{-1} humic acid (HA; solid bars). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO) terms of upregulated genes in tomato shoots in response to two or three strains in the control condition **(B)** and HA condition **(C)** and in response to D7G **(D)** or 32A **(E)** in the control condition are reported. The color scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for upregulated genes in response to PsJN in the control and HA condition, as well as in response to D7G or 32A in the HA condition.

enhancement of transport- and growth-related processes in the HA condition (**Figure 7**). On the other hand, downregulated genes in tomato shoots were related to stress response and DNA metabolism in the control condition, as well as lipid transport in the HA condition (**Figure S14**).

DISCUSSION

Some strains belonging to the bacterial genera *Enterobacter*, *Pantoea*, and *Paraburkholderia* had already been previously recognized as PGPB (Sessitsch et al., 2005; Campisano et al., 2014; Hardoim et al., 2015) and this study demonstrated that seed inoculation with PsJN, D7G, and 32A promotes tomato shoot growth. Inoculated tomato plants were efficiently colonized by the tested endophytic bacterial strains and HA did not increase the tissue colonization compared to the control condition. Moreover, the addition of HA (at the optimal concentration of 50 mg L^{-1}) enhanced the tomato growth induced by the endophytic bacterial strains, suggesting some possible complementation effects of HA to the tested PGPB. HA was known to improve nutrient uptake in tomato plants (Adani et al., 1998; Dursun et al., 2002), by

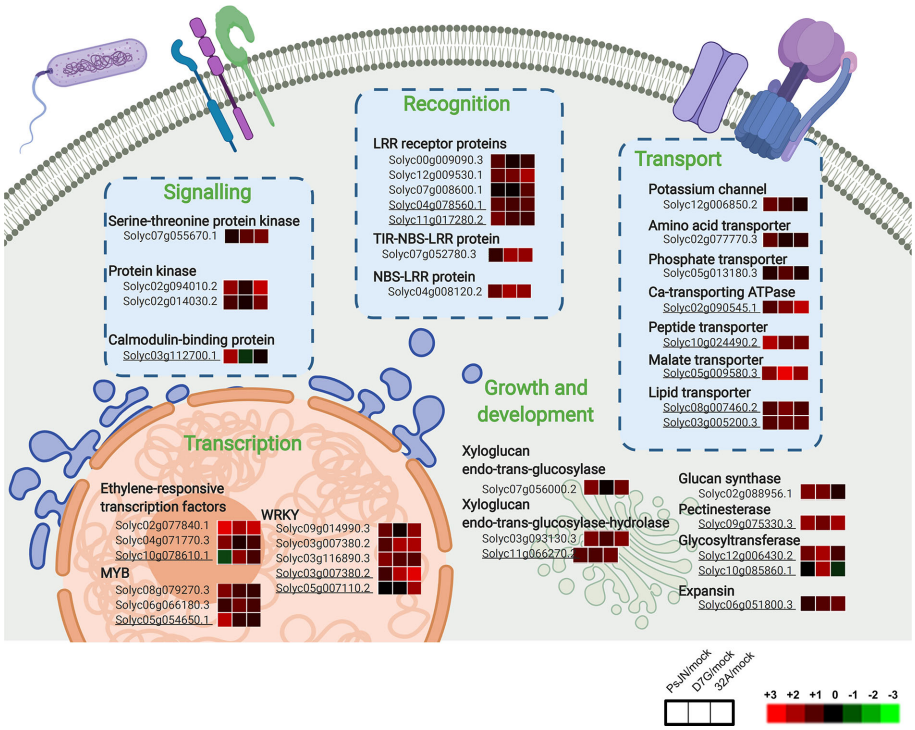

FIGURE 7 | Cellular processes activated by endophytic bacterial strains in tomato shoots. Main cellular pathways of upregulated genes in tomato shoots in response to *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G), or *Enterobacter* sp. 32A (32A) in half-strength Hoagland with 0 mg L^{-1} (control) and 50 mg L^{-1} humic acid (HA) were generated with Biorender. Not underlined and underlined gene codes indicate tomato genes modulated in the control and HA condition, respectively. For each gene, three squares represent the Log_2-transformed fold change values of PsJN-, D7G-, or 32A-inoculated plants calculated as compared to mock-inoculated plants respectively, according to the color scale reported. Ca, calcium; LRR, leucine-rich repeat receptor proteins; NBS-LRR, nucleotide-binding site leucine-rich repeat proteins; TIR-NBS-LRR, non-toll-interleukin receptor nucleotide-binding site leucine-rich repeat proteins; StkP, serine-threonine protein kinase.

increasing electrolyte leakage, cell permeability and nutrient accumulation (David et al., 1994) and activating primary and secondary metabolism (Aguiar et al., 2018; Canellas et al., 2019). HA incubation upregulated genes responsible for cellular regulations in mock-inoculated plants, such as transcription factors, receptors and kinases, and altered the transcriptional response of tomato plants to endophytic bacterial strains.

Tomato genes were modulated by endophytic bacterial strains mainly in roots (2,919 and 3,216 in the control and HA condition, respectively) compared to shoots (355 and 268 in the control and HA condition respectively), indicating major transcriptional regulations in belowground compared to aboveground tissues. The majority of DEGs was downregulated (79.4%) by endophytic bacterial strains in the control condition. Conversely, DEGs were mainly upregulated (80.0%) by endophytic bacterial strains in the HA condition, suggesting enhanced reactions of tomato plants to bacterial endophytes in the presence of HA. In particular, the majority of genes upregulated by the endophytic bacterial strains in roots in the HA condition was not modulated (64.2%) or downregulated (33.1%) in roots in the control condition, while only 2.7% was upregulated, but with lower extent, also in the control condition, indicating that specific genes are implicated in the tomato response to bacterial endophytes in the presence of HA. In particular, the presence of HA enhanced the activation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defense, and growth-related processes in response to PsJN, D7G, and 32A inoculation, as better discussed in the following paragraphs. Moreover, half of the DEGs (45.5%) was modulated by at least two endophytic bacterial strains and they represent possible common pathways modulated in response to bacterial endophytes.

Transcriptional Response of Tomato Roots and Shoots to Two or Three Endophytic Bacterial Strains and Humic Acid

Plant roots play a critical role in perception and recognition of the rhizosphere microorganisms (De Palma et al., 2019) and the presence of HA enhanced the activation of signal transduction and transcription processes in response to endophytic bacterial strains. These functional categories were activated by two or three strains and they included genes encoding receptor kinases, protein kinases and NBS-LRR proteins, indicating the activation

of a common recognition machinery to bacterial endophytes in tomato roots. In particular, serine/threonine kinases were upregulated by HA and two or three strains in roots, and protein kinases were also involved in HA-induced signaling in rice (Ramos et al., 2015) and *A. thaliana* (Trevisan et al., 2011). The elevation of intracellular calcium is also an indicator of plant response to beneficial microorganisms (Vadassery and Oelmüller, 2009) and a modulation of calcium- and calmodulin-related genes was found in response to HA alone and two or three strains in the HA condition.

Since beneficial effects of endophytic bacterial strains can derive from multiple mode of action (Glick, 2012; Ferreira et al., 2019), it is difficult to discriminate effects of microbial activities in providing nutrients to plants and/or direct stimulation of plant growth (e.g., modulation of the hormone levels). The increase of nutrient uptake was known as one of the mechanisms of plant growth promotion caused by PGPB (Glick, 2012) and HA (Zanin et al., 2019). In this study, tomato genes related to potassium and iron transport were upregulated by two or three strains in the control condition and genes related to magnesium, nitrogen, phosphate, sulphate, and zinc transport were upregulated in the HA condition, which makes them possible markers of tomato biostimulation. We found that ATPase-encoding genes were upregulated by two or three strains in the HA condition and by HA alone, and membrane pumps were previously found as activated by humic substances in tomato (Zandonadi et al., 2016) and maize (Quaggiotti et al., 2004), suggesting a positive effect of HA on tomato nutrient uptake.

Another mechanism of PGPB-dependent plant growth promotion is the modulation of the hormone levels (Glick, 2012). Tomato genes related to jasmonic acid (JA) response (e.g., WRKY transcription factors and defensins) were upregulated by two or three strains in the control and HA condition, while those related to ET synthesis (e.g., 1-aminocyclopropane-1-caroxylate synthases) and ET response (ET response factor and ET responsive transcription factors) were mainly upregulated in the HA condition. The interplay of auxin and ET signaling pathways was found also in the PsJN-dependent *A. thaliana* growth promotion (Poupin et al., 2016) and some auxin-responsive genes (e.g., auxin efflux facilitator and auxin responsive proteins) were upregulated by two or three strains in the HA condition and by HA alone. Likewise, the WAT1-related genes were upregulated by endophytic bacterial strains in both conditions and these genes are known to be involved in auxin transport and homeostasis, as well as in growth promotion and cell wall development (Irizarry and White, 2018), indicating a complex hormonal response to endophytic bacterial strains in the presence of HA. In particular, some genes implicated in gibberellin biosynthesis (e.g., copalyl diphosphate synthase, gibberellin oxidase, and gibberellin dioxygenase) and cytokinin metabolism (e.g., cytokinin riboside phosphoribohydrolases) were upregulated by HA alone and by two or three strains in the HA condition. PsJN, D7G, and 32A were able to produce auxin (Poupin et al., 2013; Campisano et al., 2014) and PsJN was able to induce gibberellin synthesis in *A. thaliana* (Poupin et al., 2013). Likewise, humic substances upregulated auxin responsive genes in *A. thaliana* (Trevisan et al., 2010) and showed cytokinin-like (Pizzeghello et al., 2012) and gibberellin-like (Nardi et al., 2000) activity in maize plants, suggesting additive effects of endophytic bacterial strains and HA in the stimulation of growth-related hormone metabolism in tomato.

As a possible consequence of hormonal changes, genes upregulated by at least two endophytic bacterial strains in the HA condition were involved in cell growth and cell wall biosynthesis, such as cellulose synthases, glycosyltransferases, mannosyltransferases, pectin lyases, pectinesterases, glucan synthase, and two xyloglucan endotransglucosylases as key markers of tomato biostimulation. Pectin and cellulose are implicated in cell wall expansion and the upregulation of genes encoding cell wall modification enzymes has been observed in growth promotion processes activated by *Pseudomonas fluorescens* in *A. thaliana* (Wang et al., 2005) and *Bacillus amyloliquefaciens* in cotton (Irizarry and White, 2018). Thus, the upregulation of genes encoding cell wall-loosening enzymes may be a common plant response to PGPB, in order to facilitate endophytic colonization and plant growth promotion (Irizarry and White, 2018). Markers of an attempted defense reaction and oxidative stress response were also upregulated by two or three strains in the control and HA condition. In particular, the upregulation of glutaredoxins, glutathione S-transferases, and peroxidases indicate the activation of the antioxidant machinery, as possible HA-dependent modulation of plant reaction to bacterial endophytes.

Transcriptional Response of Tomato Roots and Shoots Specifically Activated by *Paraburkholderia phytofirmans* PsJN, *Pantoea agglomerans* D7G, or *Enterobacter* sp. 32A

Different signaling pathways were activated by PsJN, D7G, or 32A, indicating a strain-specific response activated in tomato plants. Receptor kinases and transcription factors (e.g., bHLH, bZIP, MYB, and WRKY) were upregulated specifically by PsJN in roots in the control and HA condition. Similarly, PsJN induced the expression of receptor-like kinase genes in swtichgrass (Lara-Chavez et al., 2015) and bZIP, MYB, and WRKY transcription factors in *A. thaliana* (Timmermann et al., 2019) as possible key regulators of plant response to PsJN. Moreover, D7G- and 32A-specific genes upregulated in tomato roots and shoots included a distinctive signal transduction (e.g., protein kinases) and transcription (e.g., bHLH, MYB, WRKY, and zinc finger transcription factors) process responsible for plant reaction to endophytic bacterial strains.

The strain-specific response of tomato involved the hormone metabolism. For example, the upregulation of salicylic acid (SA) biosynthesis (phenylalanine ammonia lyase) and SA responsive (e.g., pathogenesis-related genes) genes was found in PsJN-inoculated roots, suggesting SA accumulation in the HA condition, as previously shown in PsJN-inoculated switchgrass (Lara-Chavez et al., 2015). SA, JA, and ET were implicated in PsJN-induced resistance (Timmermann et al., 2019) and the interplay of ET with the auxin signaling pathways was

responsible for PsJN-dependent growth promotion in *A. thaliana* (Poupin et al., 2016). The auxin signaling- (indole-3-acetic acid inducible and dormancy associated/auxin-repressed) and transport-related (auxin efflux facilitator) genes were upregulated by PsJN in tomato roots in the control and HA condition, respectively. The hormone-related genes were upregulated also by D7G in the control and HA condition (e.g., auxin responsive genes, ET-related receptor and transcription factor, auxin and cytokinin metabolic genes) and this endophytic strain showed ACC-deaminase activity and auxin production activity *in vitro* (Campisano et al., 2014). As possible additive effect, the presence of HA can affect the auxin-related processes, as shown in *A. thaliana* (Canellas et al., 2010) and tomato (Canellas et al., 2011) plants, suggesting a complementation effect of endophytic bacterial strains and HA.

The protein metabolic pathways were activated by 32A in tomato roots in the HA condition, indicating the activation of nitrogen assimilation with upregulation of genes related to the metabolism of lysine, serine, glycine, cysteine, tyrosine, threonine, glutamine, alanine, arginine, and methionine. Likewise, the nitrogen and secondary metabolism was activated in *H. seropedicae* HRC54-inoculated tomato plants in the presence of HA (Olivares et al., 2015) and the increased concentration of amino acids and secondary metabolites was found in sugarcane plants inoculated with *H. seropedicae* HRC54 and *G. diazotrophicus* PAL 5 in the presence of HA (Aguiar et al., 2018; Canellas et al., 2019). In particular, 32A was able to fix atmospheric nitrogen *in vitro* (Campisano et al., 2014) and it caused the upregulation of a glutamine synthetase gene in tomato roots in the HA condition. Glutamine synthase encoding genes were also upregulated by endophytic diazotroph bacteria in sugarcane (Nogueira et al., 2005) and an increased amino acid content was found in sugarcane inoculated with the diazotroph *Pantoea* sp. 9C strain (Loiret et al., 2009), suggesting that the activation of the amino acid metabolism contributes to plant growth promotion. Amino acids are key precursors of secondary metabolites and genes related to secondary metabolism were induced by 32A in the HA condition. In accordance with these findings, a previous study had demonstrated that 32A affected the accumulation of secondary metabolites in grapevine plants as a possible mechanism for the successful host colonization (Lòpez-Fernàndez et al., 2015a). Likewise, the precise tuning of the plant defense by the endophytic bacterial strains could contribute to a successful host colonization. For example, the antioxidant machinery was activated in tomato roots mainly in the HA condition, indicating the activation of an attempted defense reaction against endophytic bacterial strains that is probably tuned by the endophytic bacterial strains to allow tissue colonization.

CONCLUSIONS

Growth promotion effects and transcriptional responses activated by bacterial endophytes in tomato plants were affected by the presence of HA, indicating a complementation effect of PGPB and the organic biostimulant under controlled conditions. In particular, HA enhanced the activation of pathways responsible for signal transduction, hormone metabolism, transcription, protein metabolism, transport, defense, and cell growth in response to the endophytic bacterial strains. Major transcriptional regulations occurred in tomato roots and involved global reactions activated by endophytic bacterial strains, including protein metabolism, transcription, transport, signal transduction, and defense processes. The optimized HA dosage and an in-depth knowledge of tomato reaction to bacterial colonization derived by this study represent key information for the further development of combined formulations of endophytic bacterial strains and HA as a tailored diet for tomato biostimulation. In addition, genes identified in this work may be the source of important markers of tomato biostimulation that can be used to monitor the plant response to bacterial endophytes and HA under field conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA622763.

AUTHOR CONTRIBUTIONS

NG carried out the functional experiments, performed DOPE-FISH experiments and microscopy analyses, and wrote the manuscript. SC performed DOPE-FISH experiments and microscopy analyses. MM analyzed the RNA-Seq data. CS helped in the functional experiments. AS and FW revised the manuscript. IP revised the manuscript and analyzed the data. GP conceived the study and revised the manuscript. MP conceived the study, supervised the experiments, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.582267/full#supplementary-material

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Novel Effects of Leonardite-Based Applications on Sugar Beet

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The present study aimed to explore the effects of foliar application of a leonardite-based product on sugar beet (*Beta vulgaris* L.) plants grown in the field. The approach concerned the evaluation of the community compositional structure of plant endophytic bacteria through a metabarcoding approach, the expression level of a gene panel related to hormonal metabolism and signaling, and the main sugar beet productivity traits. Results indicated that plants treated with leonardite (dosage of 2,000 ml ha^{-1}, dilution 1:125, 4 mg C l^{-1}) compared with untreated ones had a significant increase ($p < 0.05$) in (i) the abundance of *Oxalicibacterium* spp., recognized to be an endophyte bacterial genus with plant growth-promoting activity; (ii) the expression level of *LAX2* gene, coding for auxin transport proteins; and (iii) sugar yield. This study represents a step forward to advance our understanding of the changes induced by leonardite-based biostimulant in sugar beet.

Keywords: sugar beet, leonardite, 16S rRNA metabarcoding, gene expression, sugar yield

INTRODUCTION

Biostimulant products, applied to soil or plants, are recognized for improving plant health, quality, and yield (Nardi et al., 2018). They have been shown to influence plant metabolism through the enhancement of photosynthesis, water use efficiency, nutrient uptake, and assimilation (Calvo et al., 2014; Yakhin et al., 2017). Although the study of biostimulation mechanisms is still an ongoing task, available research highlighted a hormone-like activity and an enhancement of root and organ growth and development (Canellas et al., 2011). Moreover, biostimulants have an important role in promoting tolerance to abiotic stresses and plant recovery (Halpern et al., 2015; Van Oosten et al., 2017). Humic substances (HSs), such as leonardite, have prominent importance among biostimulant products. They are a dark brown natural organic compounds, ubiquitous in water, soil,

and sediments (Piccolo, 2002). Particularly, leonardite, originating from the atmospheric oxidation of lignite, is very rich in humic acids (David et al., 2014). Leonardite application has been shown to improve nutrient uptake, such as Fe, N, and K, and increase plant yield and quality (Ece et al., 2007; Fascella et al., 2015; Cieschi et al., 2017). Therefore, leonardite is generally used in agriculture as a soil conditioner, increasing the permeability of the stem cell membrane, nutrition rate, fruit quality, and crop yield (Ratanaprommanee et al., 2017). An improved production has been reported for leonardite-treated cherry, potato, corn, and ornamentals (Eyheraguibel et al., 2008; Sanli et al., 2013; Fascella et al., 2018; Demirer, 2019). Sugar beet (*Beta vulgaris* L.) plays a key role in the agricultural and economic scenario of 52 countries. In 2017, the world area harvested with sugar beet reached almost 5 Mh for a total production of 314 Mt (Food and Agriculture Organization (FAO), 2019), and the increasing trend is to move toward a sustainable cultivation. In this context, biostimulant products are classified as ecofriendly, minimizing the agricultural impact on the environment. Furthermore, these products not only protect microbes already present in the soil but also foster the growth of new rhizosphere bacteria communities and the related soil enzymatic activity (Du Jardin, 2015). Thus, the use of biostimulants is based on the knowledge of plant root and shoot bacterial communities.

The compositional structure of plant endophytic microbes is influenced by many factors. External environmental conditions, climate, biotic stresses, human practices, and the soil environment are the most important key factors altering the composition of plant endophytic communities (Reinhold-Hurek et al., 2015). The role of endophytic bacteria is crucial. Several studies revealed protective function from plant abiotic stresses, accelerating plant immune response following pathogen infection (Miliute et al., 2015). Furthermore, they can promote plant growth, development, and nutrient uptake (Liu et al., 2017). However, significant knowledge gaps remain, involving the cross-talk between plant and microbes and how the microbiome modulates gene expression in the plant (Liu et al., 2020).

Analysis of plant microbial communities requires suitable techniques and reproducible protocols. A rapidly emerging technique to explore complex bacterial populations is presented by the 16S rRNA gene metabarcoding. This approach, common between different sequencing platforms, involves the PCR amplification of the most taxonomically informative region of 16S rRNA gene followed by high-throughput sequencing. The 16S gene includes nine hypervariable regions (V1—V9) that are taxon-specific, flanked by conserved sequences. The selection of the most informative region is still a matter of scientific debate. V3 and V4 are the most commonly used regions for taxon identification (Yarza et al., 2014).

The present work aimed to explore the effects of leonardite treatment on sugar beet. For this purpose, we firstly compared the microbiome profiles of plants cultivated in hydroponics and field conditions. Then, we exploited the effect of foliar application on plants grown in the open field. Therefore, we investigated (i) the consequences of leonardite application on the composition of plant endophytic communities, (ii) the expression level of key genes related to hormonal and signaling metabolism, (iii) and its impact on yield traits using sugar beet (*B. vulgaris* L.) as a model crop.

MATERIALS AND METHODS

Plant Material

The sugar beet variety used for the experimental trials, both in the field and in hydroponics, was Smart-Briga (KWS, Einbeck, Germany), diploid and resistant to the herbicide Conviso, Cercospora leaf spot, Rhizomania, and nematodes.

Field Experiment

The field trials were carried out in four locations for 6 months, between March and August 2020. The geographical coordinates of the four locations involved are Pozzonovo, Padua, Italy (45°10'49.7"N, 11°47'48.0"E); Loreo, Rovigo, Italy (45°04'33.6"N, 12°10'36.2"E); Cavarzere, Venezia, Italy (45°06'37.7"N, 12°03'05.1"E); and San Martino di Venezze, Rovigo, Italy (45°06'12.9"N, 11°53'52.5"E). An experimental design constituted of four randomized blocks was applied. Each of the randomized blocks was divided into four subplots whose size was 2.7 × 10 m. A control plot was placed outside the randomized block, and plants were kept without treatments. Plants were subjected to foliar spray treatments with leonardite solution using a dosage of 2,000 ml ha^{-1} (dilution 1:125, 4 mg C l^{-1}). The novel leonardite formulation and non-commercial product used in this work was provided by Sipcam SpA (Italy). The leonardite formulation was analyzed by combustion (Elementar vario MACRO CNS, Elementar Analysensesystemse GmbH, Germany) for C, N, and S contents, ionomic analysis (inductively coupled plasma optical emission spectrometry, SPECTRO ARCOS II MV, SPECTRO, Germany) for elemental analysis, and NMR analysis (solid-state 13C MAS NMR spectra, fully proton-decoupled using a Bruker Avance II 400 MHz instrument, Bruker Corp., United States) for spectra and the distribution of the diverse forms of carbon. The results of this analysis were previously described by Barone et al. (2019). The first application was set for the stage BBCH 38 (leaves cover 80% of the ground), the second treatment was performed 40 days after the first, and the last treatment was applied 20 days after the second one. The untreated control plants were sprayed only with water. A 50-l backpack sprayer was used to uniformly distribute the leonardite solution. Four biological replicates consisting of three-leaf discs taken by plants randomly picked, inside the same subplot, were collected 48 h after treatment. Samples of approximately 50 mg of leaf tissue were placed in dry ice and taken to the laboratory for DNA extraction.

Hydroponic Experiment

Sugar beet seeds were sterilized by dipping in 76% ethanol for 5 min. The washing procedure with distilled water was repeated three times. To promote germination, seeds were kept inside a growing chamber in the dark on distilled water-moistened filter paper for 48 h at 25°C. Six days after germination, plants were transferred inside 500 ml glass pots with complete Hoagland

solution (Arnon and Hoagland, 1940). After 6 days, plants were divided into two different pots containing, respectively, 1 ml/l of leonardite (treated plants) and complete Hoagland solution (control plants). Leaf sampling was done 2 days after leonardite treatment. The experiment was repeated three times for validation aims.

DNA Extraction

DNA was extracted from 50 mg of fresh leaf material. Samples were homogenized inside the collection microtubes with 300 μl of Buffer RLT and 3 mm stainless steel beads. The homogenization step involved the use of Tissue Lyser (Qiagen, Hilden) for 5 min at 30 Hz. Homogenized samples were then transferred in a 96-well S-block plate containing also 200 μl of isopropanol and 20 μl of MagAttract magnetic beads (Qiagen). This plate was used for automatic DNA extraction using Biosprint 96 (Qiagen) together with five other plates respectively composed of 500 μl of Buffer RPW, 500 μl of 0.02% Tween, and two plates filled with 500 μl of 96% ethanol. DNA was eluted in 100 μl of nuclease-free water. Nucleic acid quantification was performed using Qubit (Thermo Fisher Scientific, Carlsbad, CA) with Qubit DNA High Sensitivity Assay Kit (Thermo Fisher Scientific).

RNA Extraction

mRNA was isolated using Dynabeads mRNA Direct Micro Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, starting from 50 mg of leaf material. mRNA was immediately analyzed with qPCRBIO SyGreen 1-step kit (Resnova-PCR Biosystem).

Metabarcoding of Bacterial 16S rRNA Gene by High-Throughput Sequencing

Library preparation was carried out using the 16S Ion Metagenomics Kit (Thermo Fisher Scientific). Briefly, the protocol consists of a first PCR amplification using two different primer sets (V2, V4, V8 and V3, V6, V7, V9) for the amplification of seven different hypervariable regions. The PCR program consisted of an initial denaturation of 95°C for 10 min, followed by 25 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 20 s, and a hold stage at 72°C for 7 min. Amplicons were quantified and pooled together to obtain a final concentration of 30 ng μl^{-1}. Subsequently, the protocol involved the use of the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific) and Ion Express Barcode Kit (Thermo Fisher Scientific) for bar code ligation. The library was amplified with six cycles of PCR at 58°C for 15 s and 70°C for 1 min, then 4°C for up to 1 h. The library was diluted to a concentration of 25 pM and used to prepare the template positive Ion sphere particles with Ion One Touch 2 instrument (Thermo Fisher Scientific). The enrichment process was done with the Ion ES instrument (Thermo Fisher Scientific) and the sequencing with Ion GeneStudio S5 using the Ion 520 chip kit (Thermo Fisher Scientific). The data were analyzed using the Ion Torrent Suite software, and the taxonomical assignment was performed by comparing operational taxonomic units (OTUs) against the Greengenes database (version 13.5) and the curated MircoSeq reference library v2013.1 on the Ion Reporter cloud (Thermo Fisher Scientific).

Real-Time PCR for Bacterial Detection

The obtained bacterial sequences were used to design Real-Time PCR primers with the software Primer Express V3.0 (Thermo Fisher Scientific). The primer sequences used in this work are reported in **Table 1**. Real-Time PCR was conducted using QuantStudio 5 (Life Technologies, United States) with the following mix: 5 μl of SYBR Green Real-Time PCR Master Mix, 0.1 μl of forward primer, 0.1 μl of reverse primer, 1.4 μl of nuclease-free water, and 1 μl of each sample. The PCR program was set as follows: 10 min of preincubation at 95°C and 50 cycles of 15 s at 95°C and 1 min at 60°C.

Real-Time Quantitative RT-PCR for Expressed Plant Genes

Eight sugar beet genes were used to test leonardite effects on plants. Primer design with Primer Express V3.0 (Thermo Fisher Scientific) was done starting from mRNA sequences downloaded from RefBeet_1.2[1]. **Table 2** shows the complete list of genes, their functional category, and gene product. Quantitative RT Real-Time PCR amplification and detection were conducted on a Quant Studio 12K Flex Real-Time PCR (Thermo Fisher Scientific) using qPCRBIO SyGreen 1-step kit (Resnova-PCR Biosystem). The 10 μl of reaction mixture contained 5 μl of SYBR Green, 0.5 μl retrotranscriptase, 0.4 μl of forward and reverse primers, 0.7 μl of nuclease-free water, and 1 μl of RNA. The threshold cycle (Ct) values obtained were normalized against the average transcript abundance of three housekeeping genes (*Tubulin*, Bv2_037220_rayf; *GAPDH*, Bv5_107870_ygnn; *Histone* H3, Bv6_127000_pera) using the formula: $2^{-\Delta Ct}$ in which ΔCt is obtained from the difference between the Ct of the target gene and the Ct of the control gene (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Yield Traits

The effect of leonardite on sugar beet yield traits such as root yield, sugar yield, and processing quality-related traits were evaluated between March and August 2020 in Pozzonovo, Padua, Italy (45°10'49.7"N, 11°47'48.0"E). The experimental design was divided into four randomized blocks, each one divided into four subplots whose size was 2.7 × 10 m. Outside the randomized block, a control plot was placed, and plants were kept without any treatments. The foliar spray treatments with leonardite solution were done using a dosage of 2,000 ml ha^{-1} (dilution 1:125, 4 mg $C\,l^{-1}$). Topped sugar beets from each subplot were collected after BBCH 49 (beet root has reached harvestable size) and analyzed to detect the mean of root yield, sugar yield, and processing quality-related traits as influenced by leonardite application. Roots from each collected plant were washed, and using a special sawing machine (AMA-KWS, AMA Werk GmbH, Alfeld, Germany), 1 kg of micronized tissues (brei) was obtained. About 70 g of representative homogenized brei samples were immediately

[1]http://bvseq.molgen.mpg.de

TABLE 1 | List of forward and reverse primer sets used for quantification of bacterial genera by Real-Time PCR on leonardite-treated and untreated samples.

Name	Forward primer 5′–3′	Reverse primer 5′–3′
Pseudomonas	GCGCGTAGGTGGCTTGATAA	GGATGCAGTTCCCAGGTTGA
Burkholderia	CCTCTGCCATACTCTAGCCC	ATGTGAAATCCCCGGGCTTA
Oxalicibacterium	GCGCAACCCTTGTCATTAGT	TGTCACCGGCAGTCTCATTA
Massilia	CAATGCCGCGTGAGTGAA	GAACCGTTTCTTCCCTGACAAA
Propionibacterium	GGGTTAAGTCCCGCAACGA	ACCATAACGTGCTGGCAACA
Methylobacterium	CTTCCGGTACCGTCATTATCG	GTGATGAAGGCCTTAGGGTTGT
Hymenobacter	AGGTGGCCCCGCAAGT	TCCATGGCAGTTCTGTAGTTGAG
Xanthomonas	AAGGTGGGGATGACGTCAAG	TGTGTAGCCCTGGTCGTAAG

frozen at −40°C. Sugar content and the main non-sugars were analyzed after cold digestion of the brei in lead acetate 0.75% (w/w) solution (Schneider, 1979) using an automated brei mixer (Venema Automation b.v., Groningen, Netherlands). To quantify the sugar content, a Thorn-Bendix 243 polarimeter (Bendix Corp., Nottingham, United Kingdom) was used, whereas K and Na concentrations were measured by a flame photometer (Model IL 754, Instrumentation Laboratory S.p.A., Milan, Italy). The α-amino N was quantified by colorimetric analysis (PM2K; Carl Zeiss GmbH, Oberkochen, Germany) following the procedure proposed by Kubadinow and Wieninger (1972) and Stevanato et al. (2010). The purity was calculated as the percentage of sugar from the roots extractable by the factory according to Wieninger and Kubadinow (1971) and Stevanato et al. (2010).

Data Analysis

Data analysis of community compositional structure of plant endophytic bacteria was conducted using Ion Torrent Suite software 5.16. This included the use of BaseCaller module to filter out low-quality sequences marked during the signal processing step followed by base calling, barcode assignment, and adaptor trimming at 3' end. The preprocessed fastq files were analyzed using Quantitative Insights into Microbial Ecology (QIIME) 1.9.1 pipeline. OTU clustering was done using a unique read abundance threshold of 10 and 97% sequence similarity against the curated Greengenes database v.13.8 and Curated MicroSEQ 16S Reference Library v2013.1. Microbial diversity was assessed using alpha and beta diversity using QIIME. The relative abundance of OTUs was calculated for both the family and genus level. Permutational multivariate analysis of variance (PERMANOVA), to test significance between groups, was performed using QIIME.

Data analysis of expression level of the gene panel and the main sugar beet productivity traits was conducted using Statistica v13.4 (Dell, Round Rock, TX, United States). Significant differences among the mean values were evaluated with Student *t*-test followed by *post hoc* analysis (Duncan's test). Significance was estimated at the $p < 0.05$ level. Data are expressed as mean ± standard error of the mean.

TABLE 2 | Details of genes used for quantitative RT Real-Time PCR showing their functional category and gene product.

Gene	Category	Gene product
AREB1	Hormone metabolism	Abscisic acid-insensitive 5-like protein
HAB1	Hormone metabolism	Serine/threonine phosphatases Mg dependent
AHG3	Hormone metabolism	Phosphatases 2C
AUX1	Hormone metabolism	Auxin transporter-like protein 1
ATTIR1	Hormone metabolism	Protein transport inhibitor response 1, auxin binding
LAX2	Hormone metabolism	Auxin transporter-like protein 2
PIN3	Hormone metabolism	Auxin efflux membrane carrier protein, component 3
CSD2	Hormone metabolism	Superoxide dismutase [Cu-Zn]

RESULTS

Bacterial 16S rRNA metabarcoding was performed on 14 untreated samples. We chose to sequence two groups of untreated plants, seven coming from the field (located in Pozzonovo, Padua, Italy) and seven grown in hydroponic solution, to study and compare the microbiome composition of sugar beet grown in two different environments without any treatment. Sequences have been deposited in the European Nucleotide Archive (ENA) browser under accession numbers PRJEB42500 and ERP126366.

A total number of 2,145,785 paired-end sequences were obtained, with an average length of 258 bp, and among them, 635,152 (29.6%) were rejected after the filtering process with the Torrent Suite software. Sequences were clustered into 139 OTUs at 97% identity cutoff. The remaining OTUs, divided into 34 different families and 37 genera, were subjected to the characterization of the endophytic bacterial communities. Alpha diversity, corresponding to the number of species or OTUs within samples (Willis, 2019), showed the highest number of sequences in samples grown in the field compared to hydroponics using the Chao indexes (**Figure 1**). A principal component analysis based on Euclidean distance was used to show how bacterial communities were distributed between field and hydroponics (**Figure 2**). Plants grown in hydroponic conditions (yellow dots) clustered separately from plants grown in the field (red dots) (PERMANOVA, $p < 0.05$).

The complete microbial profiles generated are shown in **Figure 3**. Bar-plot analysis showed that the majority of OTUs in the two groups were assigned to the genera *Pseudomonas*, followed by *Sphingomonas*, *Hymenobacter*, and *Methylobacterium*, as reported also by the percentage listed

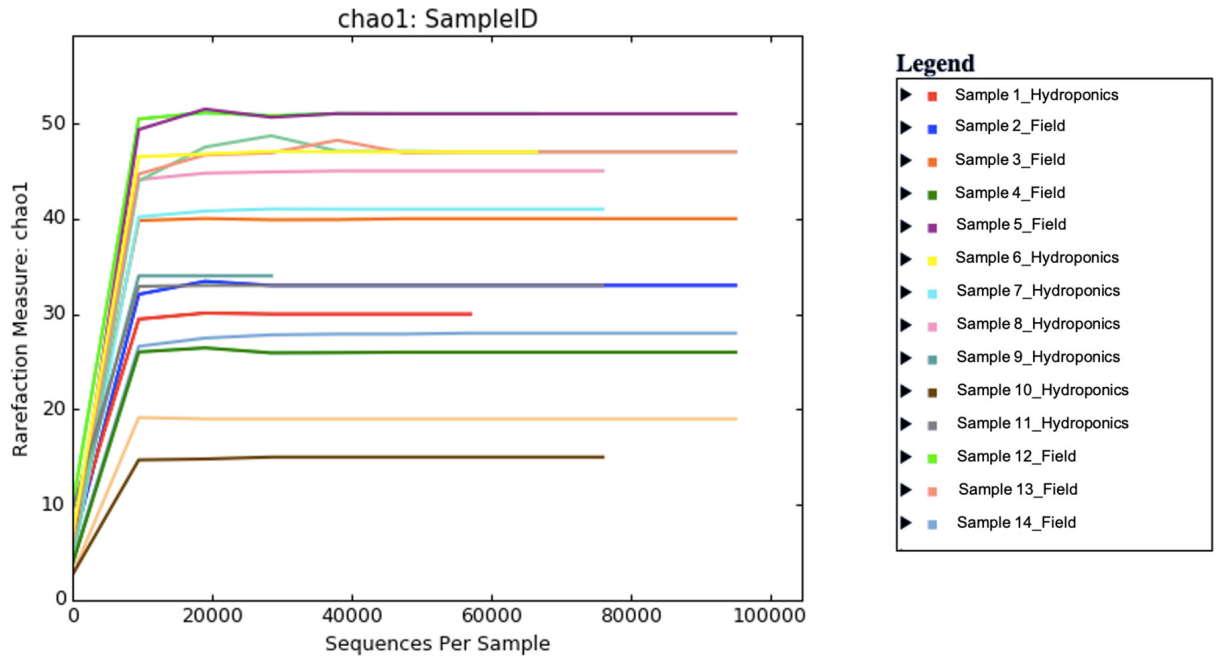

FIGURE 1 | Alpha diversity in seven field and hydroponics-grown plants calculated with the Chao diversity index.

in **Table 3**. The minority of the OTUs found belonged to *Propionibacterium*, *Burkholderia*, *Massilia*, *Oxalicibacter*, and *Xanthomonas* (**Table 3**). Moreover, the bar plot represented a remarkable variability in the field-grown plants at the genus level. This variability is directly related to a higher number of genera identified, 20 in the field-grown plants compared to the 14 genera identified in hydroponics-grown ones. Particularly, these additional genera included *Duganella*, *Stenotrophomonas*, *Ralstonia*, *Delftia*, *Microbacterium*, *Acidovorax*, *Aurantimonas*, *Spirosoma*, and *Rhizobium*. In **Figure 3**, "Others" represents bacterial genera that formed less than 1% of the total abundance.

Specific Real-Time PCR primer pairs were designed to detect eight genera, constituting the core microbiome of sugar beet, on leaf samples collected under field conditions (in four different locations) on 48 h leonardite-treated plants and untreated ones. All genera tested by Real-Time PCR were detected in both treated and untreated plants, without showing any significant variation, with exception of *Oxalicibacterium* spp. The average threshold cycle obtained for untreated samples was 24.20 with a standard error of 0.33, while samples treated with a dosage of 2,000 ml ha^{-1} (dilution 1:125) showed an average of 23.32 and a standard error of 0.29. Ct resulted from the mean of three biological replicates. Using a *p*-value threshold at 0.05, the treated samples have a significantly lower Ct value (indicating higher amounts of the template related to the presence of *Oxalicibacterium* spp.) compared to the untreated ones.

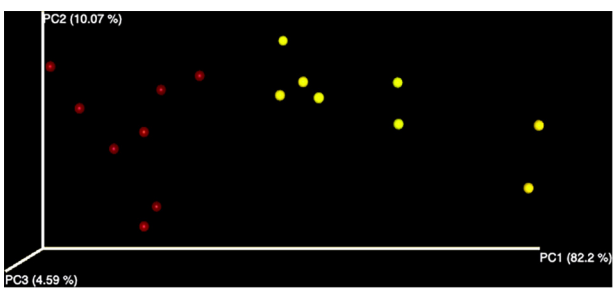

FIGURE 2 | Evaluation of beta diversity in field (red) and hydroponic (yellow) plants. The principal component analysis was performed using Quantitative Insights into Microbial Ecology (QIIME).

Quantitative RT Real-Time PCR was carried out to identify changes in gene expression profile between untreated and treated plants of the four locations. The selected genes had been detected in a previously published paper by Barone et al. (2019), where they were found responsive to leonardite treatment in hydroponic conditions. Among the complete dataset of 53 genes, we choose the ones involved in hormone metabolism. **Table 4** shows the percentage of variation in the gene expression level of treated samples with respect to the untreated ones. Samples were collected after 24 h from leonardite treatment using a dosage of 2,000 ml ha^{-1} (dilution 1:125). One of the analyzed genes, *LAX2*, showed a significantly different level of expression ($p < 0.05$) in treated vs. untreated samples. This gene encodes for an auxin transport protein. Particularly, 24 h after the leonardite application, an expression level of 38% over the control of the *LAX2* was observed.

Table 5 shows yield values and quality parameters as obtained from laboratory analyses on leonardite-treated and untreated

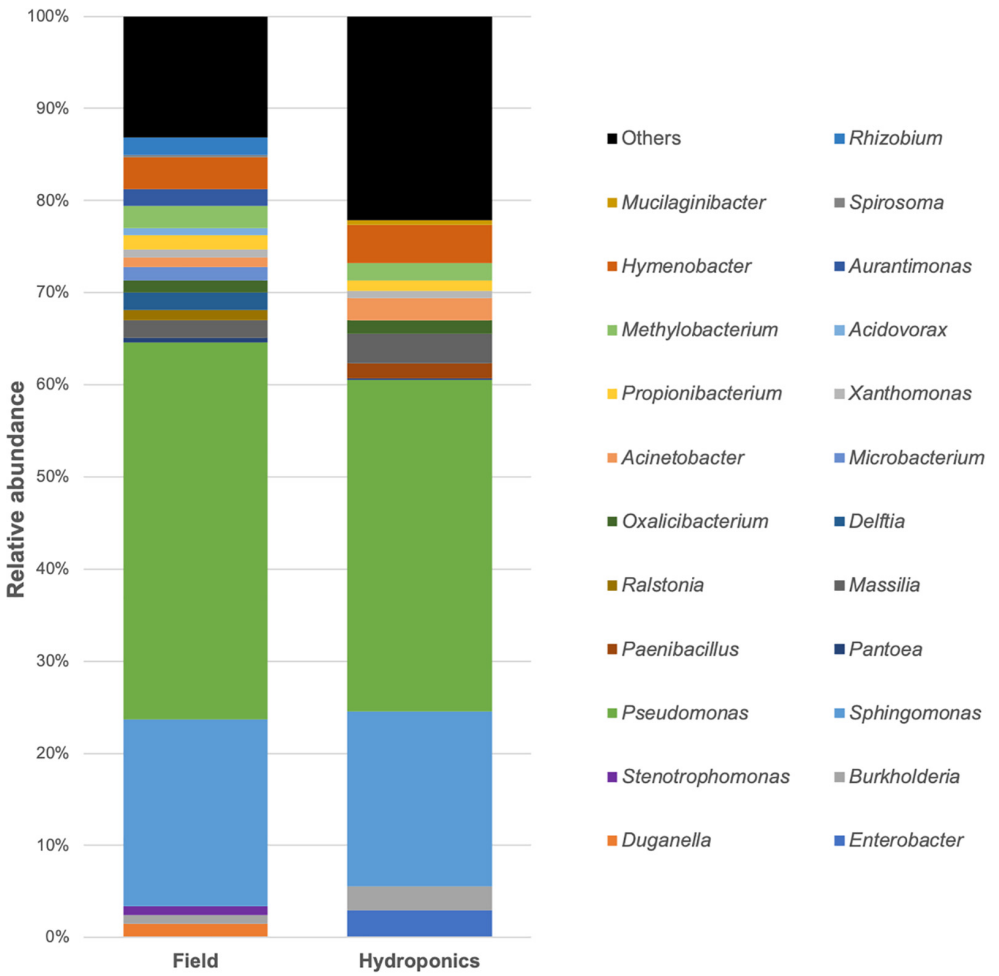

FIGURE 3 | Relative sequence abundance of bacterial genera associated with field and hydroponics-grown plants. The most represented operational taxonomic units (OTUs), with relative abundance higher than 1%, are reported. OTUs with less than 1% are assigned as "Others."

sugar beet coming from Pozzonovo, Padua, Italy. The sugar yield of plants treated with leonardite (12.30 ± 1.13 t ha^{-1}) was significantly higher ($p < 0.05$) compared to that of the untreated ones (11.40 ± 1.56 t ha^{-1}). No significant differences can be observed in quality parameters of juice such as Na, K, α-amino N content, and sugar purity.

TABLE 3 | Mean relative abundance (%) in each group at the genus level.

Genera	Field (%)	Hydroponics (%)
Pseudomonas	47.0	46.2
Sphingomonas	23.6	24.4
Hymenobacter	4.0	5.3
Methylobacterium	2.9	2.4
Massilia	2.2	4.1
Propionibacterium	1.8	1.4
Oxalicibacterium	1.4	1.9
Burkholderia	1.1	3.3
Xanthomonas	1.0	1.0

Bacteria with relative abundance higher than 1.0% are reported.

DISCUSSION

Maintaining a healthy environment, while increasing plant yield and quality, is one of the key aspects of sustainable agriculture. The application of chemical pesticides and fertilizers can undermine soil quality and invertebrate population (Liu et al., 2015). Therefore, the scientific community is studying the role and specific effects of organic plant biostimulants as a gradual and promising replacement of chemical products.

Among biostimulants, leonardite, due to the high percentage of humic acids, is considered a bioactive compound suitable to preserve soil integrity (Turgay et al., 2010). Organic molecules (phenolic and alcohol compounds) contained in leonardite can be used by microbes as a source of nitrogen and carbon (Conselvan et al., 2017; Zhang et al., 2020). Consequently, the microbiome change following leonardite applications may be

TABLE 4 | Percentage variation in the gene expression level of treated samples with respect to the untreated ones.

Genes	Percentage of variation	*p*-value
AREB1	31%	n.s.
HAB1	8%	n.s.
AHG3	16%	n.s.
AUX1	-4%	n.s.
ATTIR1	13%	n.s.
LAX2	38%	0.025
PIN3	-7%	n.s.
CSD2	37%	n.s.

Student t-test was applied to verify the statistical significance between groups ($p < 0.05$; n.s., not significant). Samples were collected after 24 h from leonardite treatment using a dosage of 2,000 ml ha^{-1} (dilution 1:125), in four different locations.

useful in elucidating the mechanism of action of this product (Yu et al., 2015). Therefore, the monitoring of bacterial species and their relative abundance is fundamental to understand the changes induced by biostimulant application.

In this study, the 16S rRNA metabarcoding analysis was performed on the pretreated microbiota of seven sugar beets grown in the field and seven grown in hydroponics. This comparison revealed nine shared bacterial genera between the two groups of plants. *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Propionibacterium*, *Burkholderia*, *Massilia*, *Oxalicibacterium*, *Hymenobacter*, and *Xanthomonas* constituted the core microbiome of seedlings grown in the two different environments. These, being found also in hydroponically grown seedlings, qualify as plant-borne and seed sterilization-resistant endophytes. As a result, these bacteria outline the seed microbiome of the sugar beet genotype used to compare the changes brought by leonardite treatments. These common bacteria are recognized to be seed endophytes with plant growth-promoting activity (Truyens et al., 2015), such as *Pseudomonas* and *Sphingomonas*, found also to be the most abundant genera. Other genera, including *Propionibacterium* and *Burkholderia*, are involved in seed germination and root and shoot growth (Johnston-Monje and Raizada, 2011; Rodríguez et al., 2020). Among total bacteria found through sequencing, many of them were unique of field-grown sugar beet, originating from soil and environment. These are *Duganella*, *Stenotrophomonas*, *Ralstonia*, *Delftia*, *Microbacterium*, *Acidovorax*, *Aurantimonas*, *Spirosoma*, and *Rhizobium*. They can be mostly divided into disease suppressive, such as *Duganella*, *Microbacterium*, *Rhizobium*, *Delftia*, and *Stenotrophomonas* that also have beneficial activity on plant growth and, on the other hand, *Acidovorax* and *Ralstonia* are recognized to be plant pathogens (Bergna et al., 2018; Woźniak et al., 2019).

The shared bacteria between the two groups were analyzed using quantitative Real-Time PCR on leonardite-treated and untreated sugar beet. Specific primers were designed to quantify their abundance. The results obtained showed that *Oxalicibacterium* spp. revealed a significant increase in abundance in plants treated with leonardite. *Oxalicibacterium* spp. belongs to the *Oxalobacteraceae* family, and among this family, we detected also the genus *Massilia*. *Massilia* is the richest genus of the *Oxalobacteraceae* family, isolated from roots and leaves, with plant growth-promoting activity and disease-suppressive abilities, while *Oxalicibacterium* is considered the most specialized oxalate degrader (Bonanomi et al., 2018; Raths et al., 2020). Oxalate is a secondary metabolite, widely reported in plants and soils, and a major component of root exudate with a key role in the recruitment of soil microbial species (Martin et al., 2012; Baldani et al., 2014). Typically, the root exudates contain acetate, succinate, lactate, fumarate, malate, citrate, isocitrate, aconitate, and oxalate. The release of these organic compounds increases microbial activity and nutrient exchange (Jones, 1998). Oxalotrophic bacteria metabolize oxalic acid, and the product of their metabolism leads to a strong local increase of soil pH (Martin et al., 2012). In *Arabidopsis thaliana* and *Phaseolus vulgaris* L., the degradation of oxalic acid has a protective function against pathogens, making the environment less favorable to fungi growth (Müller et al., 2016). Oxalate degrader microorganisms can increase the number of available phosphates influencing the phosphorus cycle and intensify the absorption of metals such as Fe and Al from soil (Morris and Allen, 1994). Other bacteria have been reported as oxalate degraders including *Burkholderia* spp., *Pseudomonas* spp., *Ralstonia*, and *Methylobacterium* spp. that we found as constituents of the core seed microbiome. Microbiome changes following leonardite treatment have already been studied in other plants, such as grapevine and potato (Cappelletti et al., 2016; Akimbekov et al., 2020). Also, Moreno et al. (2017) observed an increase of Gram-negative bacteria, such as *Proteobacteria*, as a consequence of the application of leonardite in barley.

The molecular analysis conducted in this work was done to evaluate hormonal gene responses, induced by leaf application of leonardite. The analyzed gene, belonging to hormonal metabolism, was selected among a larger set of 53 genes related to leonardite treatment on sugar beet and more

TABLE 5 | Mean of root yield, sugar yield, and processing quality-related traits in leonardite-treated and untreated sugar beet grown in Pozzonovo, Padua, Italy (45°10'49.7"N, 11°47'48.0"E).

Samples	Root yield (t ha^{-1})	Sugar yield (t ha^{-1})	Potassium (meq%°S)	Sodium (meq%°S)	α-amino N (meq%°S)	Sugar purity (%)
Untreated	75.70 ± 5.10	11.40 ± 1.56	24.38 ± 1.91	8.07 ± 0.90	6.69 ± 0.78	93.70 ± 8.19
Treated	80.70 ± 7.23	12.30* ± 1.13	23.54 ± 2.54	7.73 ± 0.65	7.04 ± 0.89	93.80 ± 10.17

These measurements were performed on four replicates with 60 plants each. ANOVA was used to evaluate differences between the two treatments with a 0.05 p-value threshold. Mean values followed by asterisk differ significantly from untreated samples ($p < 0.05$).

generally based on the already known activity of humic acids on plant growth and development (Canellas et al., 2015; Nardi et al., 2016; Barone et al., 2019; Hajizadeh et al., 2019). However, the aforementioned genes were tested only on plants grown in hydroponic conditions, showing significant variation compared to untreated samples after 24 h of treatment. Thus, a first evaluation of the data obtained revealed the complexity of leonardite effects on sugar beet grown in a dynamic and variable context such as the open field. Among eight evaluated genes, the *LAX2* gene, encoding for auxin transport protein, showed a significant change between treated and untreated plants, while the others showed high variability among replicates. The overexpression of the *LAX2* transporter at 24 h from the foliar application could be explained as a particular consequence of the ascertained auxin-like activity of humic substances contained in the product (Pizzeghello et al., 2001; Canellas et al., 2002). However, 72 h from leonardite treatments, the increasing trend in *LAX2* expression of treated samples is no longer observable (data are not shown). High variability, due to the open-field growth conditions, was observed for the other hormone-related genes and, although they showed a high percentage of variation, the statistical test resulted in no significant difference. However, these auxin-like substances are mainly transported through the phloem but are also exported and imported from cell to cell thanks to specific membrane transporters (Petrášek and Friml, 2009). The movement of auxins and the regulation of homeostasis of these substances within the plants are key processes in the modulation of growth and development such as tropism, embryogenesis, and organogenesis of roots, shoots, and vascular tissues.

Regarding the relationship between sugar beet yield traits and leonardite treatment, we did not find significant differences in the impurity content between control and treated plants unlike Rahimi et al. (2020) who observed a decrease in Na, K, and α-amino N following treatment with humic acid. However, we reported higher values of sugar yield on treated plants. This improvement in production is confirmed also in other treated crops with higher tuber yield in potato, higher root growth and yield in tomato, and a higher dry matter in canola (Akinremi et al., 2000; Pertuit et al., 2001; Sanli et al., 2013).

The present study provides important evidence for understanding the effects induced by leonardite-based biostimulant in sugar beet. Initially, the microbial populations of plants grown under hydroponic and field conditions were compared. After leonardite treatment, the most responsive genus was *Oxalicibacterium*, comprising endophytes with plant growth-promoting activity. Also, an upregulation of the *LAX2* gene, coding for auxin transport proteins, has been observed. This finding is in agreement with our previous work (Barone et al., 2019), which was entirely conducted on hydroponics-grown seedlings and the same gene was overexpressed after leonardite treatment. A significant increase in sugar yield was also observed in plants treated with leonardite compared with untreated ones. Thus, the present study represents a step forward to understand the changes induced by leonardite-based biostimulant in sugar beet.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession numbers can be found below: European Nucleotide Archive (ENA) Browser, https://www.ebi.ac.uk/ena/browser/home, PRJEB42500 and ERP126366.

AUTHOR CONTRIBUTIONS

AB, FM, AS, and PS: conceptualization. GCo, LS, and FM: supervision. MCDL, GB, LM, GM, SR, MC, CB, and GCa: methodology. CB and PS: writing original draft. AR: writing, review, and editing. All authors contributed to the article and approved the submitted version.

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Maize Growth and Grain Yield Responses to a Micronized Humic Product Across Soil Types and Annual Weather Patterns in Central Iowa, United States

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Despite growing interest in humic products as crop amendments, very few field evaluations have considered environmental factors of humic product efficacy. We determined the spatial and temporal variability in the efficacy of a micronized humic product on maize (*Zea mays* L.) growth and grain yield in two rainfed fields supporting a maize–soybean [*Glycine max* (L.) Merr.] rotation in 2012–2014, and 2016 in central Iowa, U.S. Crop management in both fields otherwise followed conventional farmer practices. In two dry growing seasons, mechanized combine measurements of grain yield increased significantly ($P < 0.10$) with humic product application on an eroded hilltop soil, amounting for two application rates to 930 and 1,600 kg ha^{-1} (11 and 19% of the control grain yield) in 2012, the droughtiest season, and 700 kg ha^{-1} (7% of the control) for the higher application rate in the somewhat droughty 2013 season. On a fertile side slope soil in the 2012 field, though, only a faint numeric response occurred in 2012, while on a toe slope soil the sole significant increase was in 2012, 870 kg ha^{-1} (14% increase above the control) for one application rate. With favorable rainfall in 2014 and 2016, significant grain yield increases with product application were small in the upland soil of 2014 and absent in 2016. Yield components analysis on 1-m row lengths of hand-collected samples attributed these yield boosts primarily to increased ear length, especially of the shorter ears. Combine grain yields, yield components, and total leaf area all demonstrated numerically slightly greater values for humic product treatments compared to the control in the vast majority of comparisons across years and soil types, with better distinction in the upland transects. Statistical significance, though, was reached only in the droughtier settings. The humic product had no consistent effects on nutrient concentrations of the grain, stover, or young leaves. Grain quality parameters showed a slight shift from protein to carbohydrates in the droughtier settings. Fifteen soil properties showed no response to the humic product. This humic

product demonstrated the capability to improve maize growth in rainfed conditions in a high-yielding region, and its efficacy varied predictably with environmental conditions. This finding provides one potential explanation for inconsistent reports elsewhere of crop responses to humic products.

Keywords: humic product, grain yield, landscape, maize, soil type, variability

INTRODUCTION

Humic products have received increasing attention as a potential field amendment for increasing crop growth and economic yield. Their efficacy in promoting plant growth has been demonstrated most commonly under controlled conditions (Chen and Aviad, 1990; Rose et al., 2014). A modest but increasing number of field studies has also demonstrated positive crop responses for horticultural (Canellas et al., 2015) and other agronomic and pasture crops (Verlinden et al., 2009; Calvo et al., 2014; Olk et al., 2018). These field studies have mostly involved only one or two site-year combinations. A smaller number of available studies reported no benefit of humic product application to crop growth in field settings (Hartz and Bottoms, 2010; Suddarth et al., 2019). The question then arises whether published studies represent only those intermittent cases where a positive response occurred, while an unknown number of unpublished trials failed to demonstrate any benefit. Information is lacking on the regularity of positive crop responses to humic products, especially under the range of environmental conditions that crops routinely encounter with field production.

Copious literature has demonstrated that agricultural amendments, including nitrogen (Cassman et al., 1996; Jaynes et al., 2004), other mineral fertilizers (Wollenhaupt et al., 1994; Havlin et al., 2013) and pesticides (Spark and Swift, 2002; Farenhorst, 2006) impact crop growth to varying degrees depending on local environmental and management factors. These can include crop type, soil type, compaction and other management-induced effects on soil properties, annual weather patterns, economic yield level, and tillage intensity. The efficacy of humic products might therefore also vary depending on these same factors, yet there have been no formal reports on such relationships.

In this study, we examined the field efficacy of a micronized humic product, Enersol[1], created through extremely fine grinding of leonardite ore. Product efficacy was evaluated during four growing seasons in two production fields owned and managed by the same farm operator but in opposite phases of a maize [*Zea mays* (L.)−soybean *Glycine max* (L.) Merr.] annual crop rotation in central Iowa, United States. Both fields featured multiple soil types lying along elevational changes in spatial patterns that allowed experimental treatments to equally traverse all soil types. Annual precipitation varied among the 4 years from severe drought to highly favorable. We hypothesized that crop responses to the humic product would vary over space and time, as affected by soil type and annual weather patterns. In-season plant measurements were leaf area, by which the area of each leaf is presumed to reflect the favorability of growing conditions at the time that leaf developed (Eik and Hanway, 1965), and nutrient concentrations of young leaves, which are presumed to represent in-season availability of soil nutrients (Whitney et al., 1985). At crop physiological maturity, we measured yield components through hand-collected samples, followed by grain yield determination through mechanized combine.

[1]Reference to any specific commercial product is for the information of the public and does not constitute endorsement or recommendation by the United States government or other sponsors of this report.

MATERIALS AND METHODS

Field Research Sites, Conditions and Operations

Two on-farm sites for field research were located in central Iowa near Ames, Story County (42° 02′ N, 93° 37′ W)–one slightly west of Ames and another near Kelley, IA, United States, that were separated by a distance of 5.5 km (**Figure 1**). Both fields are located within the same watershed and thus have similar geology, soils, and climate, together with similar historic land use and farming practices, all of which were described by Eidem et al. (1999) and Hatfield et al. (1999).

Both fields were in a maize−soybean crop rotation in alternating years, and all analyses were conducted in the maize phase. Both sites are mapped within the Clarion (fine-loamy, mixed, superactive, mesic Typic Hapludoll)−Nicollet (fine-loamy, mixed, superactive, mesic Aquic Hapludoll)−Webster (fine-loamy, mixed, superactive, mesic Typic Endoaquoll) soil association, which is further described by Hatfield et al. (1999). Field-long treatment strips were specifically located at the primary site near Ames to include this continuum of the hilltop Clarion loam (2 to 5% slopes), sideslope Nicollet loam, and lowland Webster silty clay loam. At the site near Kelley, the field-long treatment strips included both the hilltop Clarion loam (5 to 9% slopes, moderately eroded) and a lowland pattern of the Canisteo silty clay loam (fine-loamy, mixed, superactive, calcareous, mesic Typic Endoaquoll) and Harps loam (fine-loamy, mixed, superactive, mesic Typic Calciaquoll). The soil mapping units for this Kelley field did not include sideslope soils. Clarion soils are characterized as being upland and well-drained. Nicollet soils occur on sideslopes, are somewhat poorly drained and typically are the most productive soils in the Clarion−Nicollet−Webster soil association, owing to favorable fertility and soil-water relations. Webster, Canisteo and Harps soils occur in flat areas and are all poorly drained. The predominant textural classes in their uppermost 100 cm are loam for the Clarion, loam to clay loam for the Nicollet, silty clay loam and clay loam for the Webster, silty clay loam, clay loam,

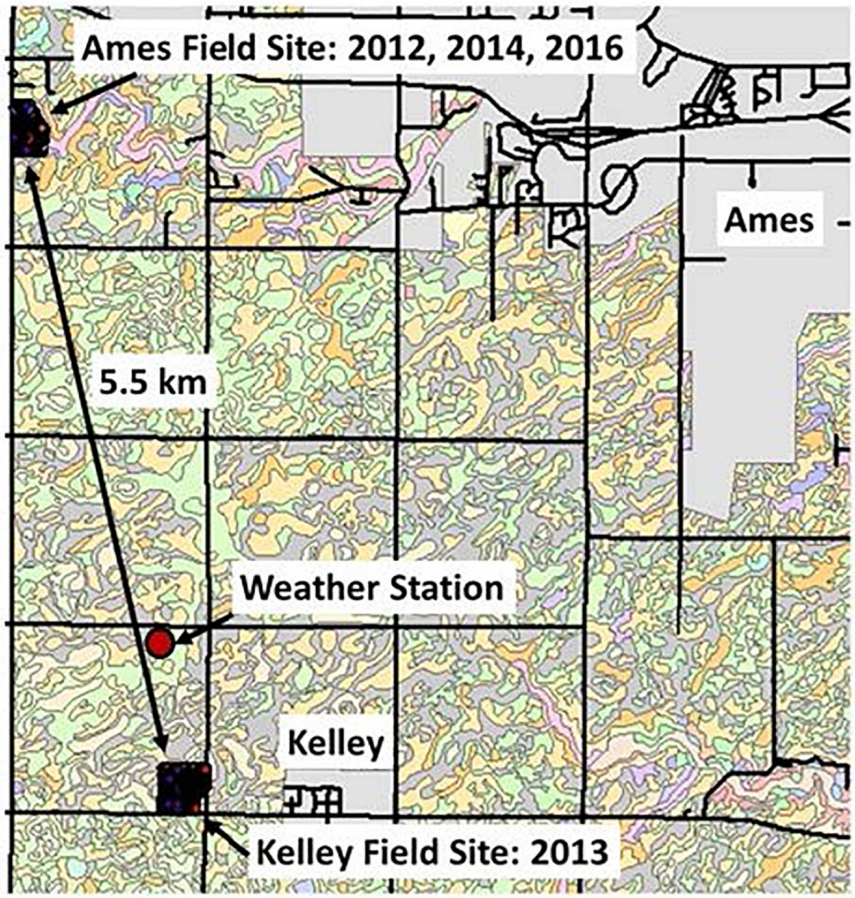

FIGURE 1 | The Ames and Kelley field sites, shown with nearby roads, communities, soil type boundaries, and the USDA-ARS weather recording station.

and loam for the Canisteo, and loam, clay loam, and sandy clay loam for the Harps (Soil Conservation Service, 1985), indicating increasingly finer soil textures downslope. Sampling transects for hand collection of plant and soil samples were established in two areas designated as either upland or lowland landscape in both fields, omitting the sideslope Nicollet soil in the Ames field. Henceforth each landscape will be presumed as interchangeable with its respective soil type.

This study included the maize crop years of 2012, 2014, and 2016 for the field site near Ames (**Figure 2**) and the 2013 maize crop year for the field site near Kelley (**Figure 3**). The Kelley field was not used in 2015 due to its change to continuous maize beginning in 2014. Both experimental designs were imbedded within production fields operated by a commercial farming family, who followed their normal farming operations for the duration of this study. Planting dates, seed varieties and planting populations for the three maize years at the Ames site were as follows: 26 April 2012, Pioneer 453AM variety, 104-day relative maturity (RM) at 84,000 seeds ha^{-1}; 23 April 2014, Pioneer 1151 AquaMax variety, 111-day RM at 85,000 seeds ha^{-1}; and 26 April 2016, DeKalb 54-40RIB variety, 104-day RM at 84,000 seeds ha^{-1}. Combine harvest dates for the Ames field were 01 October 2012, 13 November 2014, and 04 November 2016. At the site near Kelley, maize was planted on 18 May 2013, with DeKalb DKC62-97RIB variety and 112-day RM at 84,000 seeds ha^{-1}. Combine harvest of the Kelley field was 14 November 2013. For each of the four maize seasons, the field received both chisel plow tillage after soybean harvest the previous autumn and a secondary chisel plow tillage operation in the spring prior to planting. Tillage operations were not conducted during the growing season. Chisel plow tillage was performed again in the autumn after maize harvest and in the following spring prior to soybean planting. Maize row spacing was always at 0.76 m, and all field-long treatment strip-plots were eight rows wide.

At the Ames field there were four replications of four humic product treatments, whose application rates and timings were recommended by the Enersol manufacturer. This humic product is created through media milling of a naturally occurring leonardite ore from Gascoyne, North Dakota (United States). The native pH of the ore is 3.5–5.0; thus, the milling generates an acidic, aqueous suspension concentrate. It contains about 28% leonardite solid particles, which include at least 180 g kg^{-1} humic acid, at least 15 g kg^{-1} fulvic acid, 4 g kg^{-1} S, and 4 g kg^{-1} Ca. Application timings are reported here following the

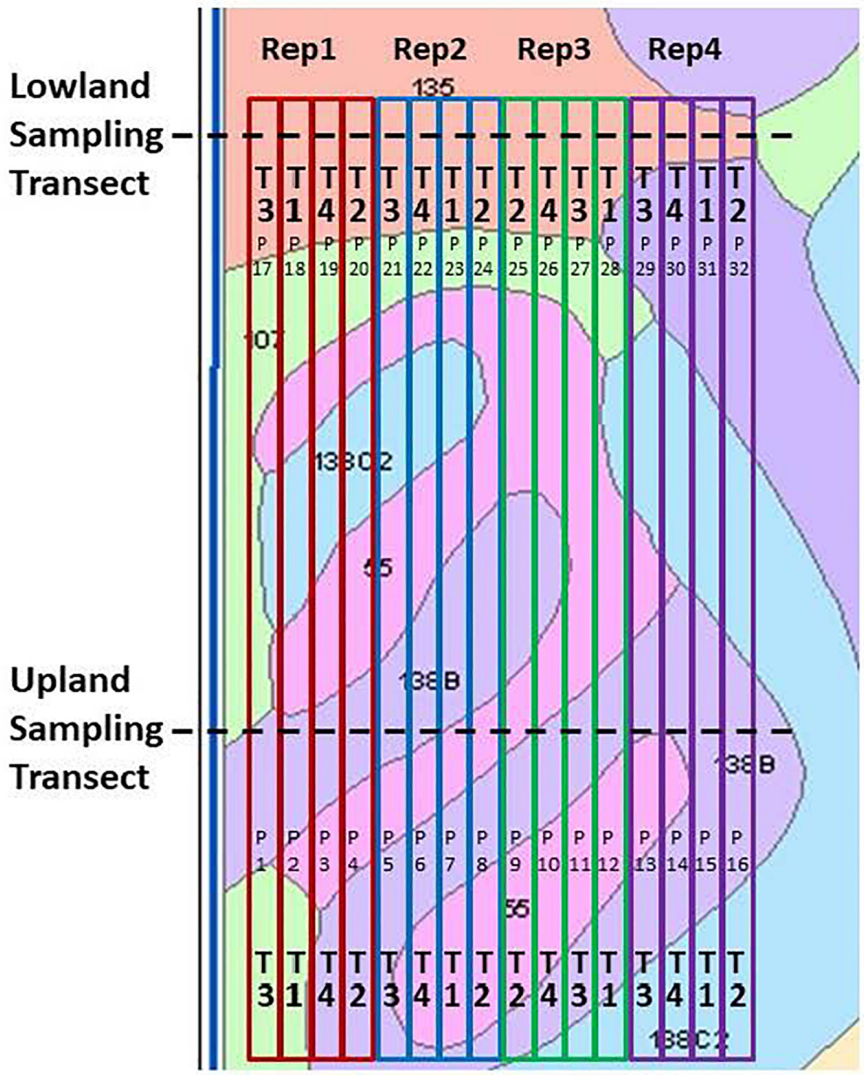

FIGURE 2 | Field design of the Ames on-farm field research site location, shown with soil mapping units and boundaries of the field-long treatment strips. Key to treatments ("T"): T1, Control lacking Enersol humic product application; T2, lower rate of Enersol humic product application; T3, higher rate of humic product application and T4, a separate alkali-extracted humic product. Exact application rates changed among years, as explained in the text. Plot numbers are shown as "P." Key to soil mapping units: 55, Nicollet loam, 1 to 3% slopes; 107, Webster silty clay loam, 0 to 2% slopes; 135, Coland clay loam, 0 to 2% slopes, 138B, Clarion loam, 2 to 5% slopes; and 138C2, Clarion loam, 5 to 9% slopes, moderately eroded.

leaf staging method that excludes the cotyledon leaf (Abendroth et al., 2011). The treatments in 2012 and 2014 were 2.5 L ha^{-1} Enersol humic product at the fourth maize leaf stage of vegetative growth (V4), 2 L ha^{-1} Enersol humic product at maize pre-emergence plus 1 L ha^{-1} Enersol humic product at V4, and 3 L ha^{-1} of a separate alkali-extracted humic product at V4, plus an unamended control (**Figure 2**). Based on crop responses to Enersol at other sites, in 2016 the application rates of the product were adjusted to 2.3 L ha^{-1} Enersol humic product at V4, 4.7 L ha^{-1} Enersol humic product at V4, and 4.7 L ha^{-1} of a separate alkali-extracted humic product at V4 (**Figure 2**). The alkali-extracted product treatment was more exploratory than were the other treatments, as the source of its extracted product varied among years. This treatment gave roughly analogous results as did the two Enersol treatments in the 3 years of the Ames field. Its presence in the field design affected the randomization of the other treatments within field replications, therefore it was included in statistical analyses of the whole field, including determination of main plot and soil type/landscape effects. Due to its variable sources, however, its results are not presented individually in this report.

At the Kelley field for 2013 (**Figure 3**), there were four replications of three treatments of the Ames field in 2012 and 2014, namely an untreated control, 2.5 L ha^{-1} humic product at V4, and 2 L ha^{-1} humic product at maize pre-emergence plus 1 L ha^{-1} humic product at V4.

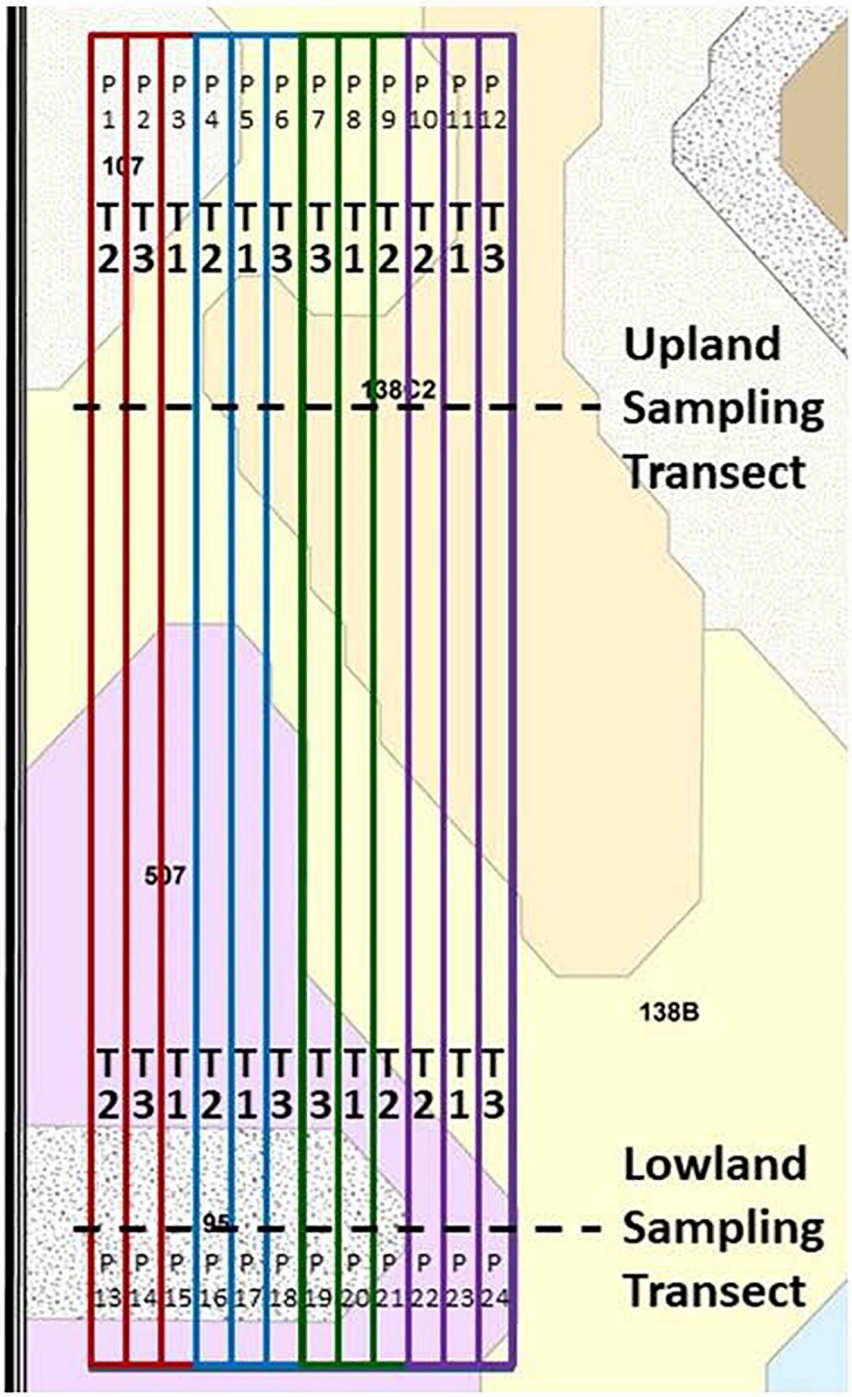

FIGURE 3 | Field design of the Kelley on-farm field research site location, shown with soil mapping units and boundaries of the field-long treatment strips. The treatments are explained in the caption of **Figure 2** and the main text. Plot numbers are shown as "P." Key to soil mapping units: 95, Harps loam, 1 to 3% slopes; 138B, Clarion loam, 2 to 5% slopes; 138C2, Clarion loam, 5 to 9% slopes, moderately eroded; and 507, Canisteo silty clay loam, 0 to 2% slopes.

The locations of the field-long strip plots and sampling transects in both fields were marked by global positioning system (GPS) and geographic information system (GIS) technologies in each year. Growing conditions were comparable among the field replicates within each transect except for the fourth field replicate in the upland transect of the Ames field, which was located on a less productive, eroded soil on a mild downward slope. In both fields, narrow walking paths were cut along the edges of the

hand-sampling transects. All hand samples were collected at least 5 m distance from the cleared transect paths, within areas having uniform crop growth.

Fertilization Rates and Timing

In the autumns prior to the spring plantings of the maize crops, N-phosphorus (P)-potassium (K) fertilizers were applied following soybean harvest at the respective rates of 112–90–134 kg ha^{-1}. An additional 67 kg N ha^{-1} was added in the spring just prior to maize planting in conjunction with pre-plant herbicide application, totaling 179 kg N ha^{-1} applied to the fields for maize production in the years 2012 through 2014. This fertilization procedure changed in 2016, when approximately 4.9 Mg ha^{-1} of chicken (*Gallus gallus* domesticus) manure was applied to the Ames field on 4 April 2016, which was determined by later nutrient analyses to represent an effective N–P–K application rate of 28–67–56 kg ha^{-1}. On 10 April 2016, an additional 134 kg N ha^{-1} of anhydrous ammonia (NH_4^+) was injected into the field, thus totaling 162 kg N ha^{-1} applied to the field for the 2016 maize growing season. No additional P and K was applied in 2016 beyond that contained in the chicken manure.

Plant and Soil Sampling

The Ames field was not sampled for soil prior to the humic product application in 2012, due to fertilizer applications that preceded field layout. Initial soil samples were instead taken from each treatment strip on 11 September 2012 in conjunction with hand-sampling of maize yield components.

Maize grain yield was measured by mechanical combine equipped with yield-monitoring GPS and GIS technologies. The electronic yield data were analyzed to generate yield maps with overlays of the plots, sampling transects, soil types/landscapes, and areas of poor growth and crop damage that had been manually marked with GPS equipment during the growing season. All areas with damaged crop growth were excluded from further data processing and statistical analyses. Ten consecutive geo-referenced yield data points that were clearly located within each of the three soil mapping units (also representing the differing landscape positions) were identified and used to estimate the combine yield data, including adjustment to the standard equivalent of 15.5% market moisture.

Maize stover and ear samples were hand-harvested at physiological maturity each year for all treatment strips near both landscape sampling transects. In each treatment strip within each landscape transect (analogous to a plot), a one-row length of 1 m was harvested in areas of uniform growth by cutting seven evenly spaced plants at ground level. Four soil cores were taken to the 15 cm depth in the untrafficked interrows adjacent to the 1 m-hand-harvested row with a 3.18-cm diameter probe, composited within each plot, and stored at 4°C until later analyses for soil properties. Maize stover samples were oven-dried at 59°C under forced air, then immediately measured for oven-dry weights and mechanically shredded. Composite subsamples were taken of the shredded stover for later grinding through a Wiley mill (1 mm mesh screen) and then a Cyclone mill (Udy Corporation, Fort Collins, CO, United States) to a powder consistency. Maize ears were placed in plastic mesh bags and hung for drying before storage for subsequent measurements. All maize ear grains were later hand-shelled and passed through a mechanical seed counter for determination of 100-kernel weight. Total kernel weights of the hand samples were recorded and kernel moisture was recorded by a moisture meter. Maize grain moisture content was also determined by a standard oven-drying method (ASAE, 1988). Grain weight per 1-m row was then calculated and extrapolated to a hectare basis. For the 2012 to 2014 seasons, subsamples of harvested grains were initially air-dried to no more than 100 g kg^{-1} moisture content and then stored in airtight plastic bags until later analysis for protein, oil and starch contents using near-infrared spectroscopic (NIRS) procedures (Iowa Grain and Quality Initiative, 2004). Ear lengths from air-dried cobs were measured, and the cobs were then oven-dried for 3 days at 120°C and immediately measured for dry weight. The dried cob weights were then added to those of the 1-m stover samples to report total aboveground stover weight. Harvest index was defined as the ratio of grain weight to total aboveground stover weight.

In-season leaf samples are used to determine in-season plant nutrient status (Whitney et al., 1985) and were collected in 2012, 2013, and 2014 near the sampling transects at three key periods of nutrient uptake: V10-11, V14-15, and the maize kernel blister stage of reproductive growth, or "R2" (Abendroth et al., 2011). For the first two sampling times, the second uppermost leaf that had a visible collar was taken from 16 plants for each plot by soil type/landscape combination from areas of representative and uniform growth. The second leaf was chosen instead of the uppermost visible collar leaf to avoid instances in which the collar of the uppermost leaf had just become visible within the previous night such that N and other essential nutrients had not yet been transported to the leaf to its fullest potential. At the R2 sampling, the ear leaf was sampled.

Plant and soil samples were analyzed for pre-determined sets of properties as offered by a commercial laboratory. Plant tissue and grain total N analyses were performed through micro-Kjeldahl digestion and colorimetric determination of the extracted total N content. Plant tissue and grain analyses for all other nutrients (P, K, Mg, Ca, S, Zn, Mn, Cu, Fe, and B) were performed using wet digestion in nitric acid with 30% hydrogen peroxide and determination by inductively coupled plasma−mass spectrometry. Sodium and Al were also measured, but their results are not reported here due to their erratic and at times absent concentrations.

Methods for measuring soil extractable nutrients, pH, buffer pH, organic matter, and cation exchange capacity followed Denning et al. (1998). Soil pH was determined in a 1:1 (w:v) slurry in water, and buffer pH followed the Sikora Buffer method. Soil organic matter content was determined through loss on ignition. Available soil P was determined colorimetrically from a Bray 1 extraction (Bray and Kurtz, 1945). Extractants for other available soil nutrients included cadmium reduction (nitrate-N), 1 M ammonium acetate (K, Ca, Mg), monocalcium phosphate (S), diethylenetriamene pentaacetate (DTPA, Fe, Zn, Mn, Cu) and hot water (B).

In both transects and all four seasons, maize leaves were destructively measured for total leaf area on three consecutive plants that were evenly spaced and in areas of uniform growth near the sampling transects. Triplicate groups of three plants were marked at the V6 crop stage for three in-field samplings. The first leaf area measurement was at the V5 or V6 growth stage, when flagging tape was used to mark the internode between the V6 and V7 leaves of the other two plant sets. One of these sets was later used for the second measurement of leaf area at the V11 or V12 growth stage. On the remaining plant set, flagging tape was used to mark the internode between the V11 and V12 leaves for the final leaf area measurement soon after full tassel. For each leaf, its length and maximum width were measured to calculate leaf area by the method of Montgomery (1911) using the equation:

$$\text{Leaf length (cm)} \times \text{maximum leaf width (cm)} \times 0.75 = \text{leaf area (cm}^2\text{)} \quad (1)$$

Total plant leaf area was the sum of the areas from all leaves on each plant.

Weather Recording Data Sites

Daily maximum and minimum temperatures (°C) and total rainfall (mm) were recorded at the 2-m height from a USDA-ARS weather station that was 4 km from the Ames site and 1.5 km from the Kelley site. These data were recorded for the period of 1 January 2012 through 31 December 2016. Each mean daily temperature was calculated as the mean of the daily maximum and daily minimum temperatures. Monthly mean high and low temperatures were calculated as the means of all daily values for their respective measures. The recent 30-year averages (1981–2010) for these same parameters were obtained from the U.S. Climate Data website for the Ames weather station, located at a distance of 6.5 km from the USDA weather station[2].

Statistical Analyses

Treatments were randomized by individual treatment strip within each replication, but not re-randomized by each soil type/landscape. Therefore, the experimental designs are treatments nested within treatment strips, and the program for SAS Proc Mixed (mixed models) program (SAS Institute, 2012) was accordingly adjusted to the proper degrees of freedom for this design. We used this program instead of the generalized linear models program for three reasons: (1) Proc Mix does not assume that observations are completely independent of each other, hence it tests for covariance and adjusts the levels of probability accordingly; (2) Proc Mix applies the same approach to errors; and (3) Proc Mix can allow the replication effect to be treated as a random variable instead of a fixed variable, which better represents field conditions. Repeated measures analyses, with time as an additional factor and with similarly adjusted degrees of freedom, were conducted for all data combined over multiple years from the Ames field, and for young leaf nutrient data, which were collected three times within each season at the same location. When time or soil type/landscape position were proven to be statistically significant factors, then additional analyses were conducted by individual time or soil type/landscape position to further examine treatment differences. Significance for all treatment and interaction terms was defined as $P < 0.10$. The Proc Corr procedure (SAS Institute, 2012) was used to correlate responses of several crop growth parameters to humic product application in the upland transect to an inverse index of drought stress. Lacking direct measurements of plant drought stress, we approximated drought stress as the ratio for each year of total rainfall from April to September in that year to the 30-year mean for total rainfall in those same months. We chose the Pearson correlation for this analysis. We did not attempt these correlations for the lowland transect, presuming a lack of correlation because this transect was less responsive to annual precipitation patterns than was the upland transect.

Several crop parameters showed consistent responses to the humic product that were not quite significant at $P < 0.10$ but would have been significant at less stringent thresholds. Due to their consistency, we also discuss these numeric trends, including the separation of results by soil type/landscape even in those cases having insignificant treatment—landscape interactions. We believe that to enable further refinement and development of site-specific, or precision, farming methods, all consistent information should be evaluated to improve production and environmental efficiencies of farming operations.

RESULTS

Weather Patterns

Over the 4 years of this study, the weather patterns ranged from severe drought in 2012 to quite favorable in 2014, as indicated by deviations of monthly mean maximum and minimum temperatures and total monthly precipitation from the 30-year (1981–2010) averages (**Table 1**). The months of May through August comprise the bulk of the growing season for row crops in the temperate climate of central Iowa. In 2012, these 4 months coincided with the greatest period of precipitation deficits for all 4 years. Simultaneously, temperatures were notably higher than normal in March, May and July. Precipitation for this 4-month span was only 44% of normal, which, given the increased temperatures, caused readily apparent symptoms of severe moisture stress for the 2012 maize crop. On an annual basis, total 2012 precipitation (572 mm) was 37% less than the 30-year average (910 mm).

In 2013, the spring was cooler than average, and May precipitation was 90% greater than average. With a planting date of 18 May, portions of the lowest-lying areas of the Kelley field were submerged early in the growing season, leading to their crop damage or loss. They were then excluded from sampling and calculation of grain yields. For the months of June through August, in contrast, precipitation was only 55% of normal, with near normal temperatures. Total annual precipitation in 2013 was 11% less than the 30-year average, and temperatures were slightly cooler than average.

In 2014 monthly precipitation varied within a narrow range and deviated little from normal means with just one

[2]https://www.usclimatedata.com/climate/ames/iowa/united-states/usia0026/2017/1

TABLE 1 | Deviations from the 30-year average (1981–2010; Ames, IA, United States) for monthly maximum (T_{max}) and minimum (T_{min}) temperature and precipitation (Pre) for the 4 years of the study and its two field sites near Ames and Kelley, Story County, IA, United States.

Month	2012			2013			2014			2016		
	T_{max}	T_{min}	Pre	T_{max}	T_{min}	Pre	T_{max}	T_{min}	Pre	T_{max}	T_{min}	Pre
	°C		mm	°C		mm	°C		mm	°C		mm
January	4.0	3.3	−5	1.1	0.3	8	−2.1	−4.8	−13	−1.7	0.8	2
February	1.8	2.6	16	−0.8	0.4	10	−6.2	−7.5	23	0.4	2.9	3
March	8.8	6.8	−1	−6.3	−3.3	−5	−4.3	−3.3	−32	3.0	3.1	17
April	1.0	1.8	8	−4.2	−2.3	42	−2.2	−1.2	46	−0.7	0.8	−7
May	2.7	1.9	−55	−2.5	−0.2	110	−0.1	0.1	−47	−0.6	−0.3	−52
June	0.9	0.5	−60	−1.1	0.7	−45	−0.9	0.4	105	2.4	1.7	−97
July	3.7	1.4	−87	0.3	−0.5	−93	−3.2	−3.0	−56	−1.1	−0.3	0
August	1.0	−2.5	−72	1.3	0.2	−90	−1.2	0.7	52	−0.5	0.4	61
September	0.5	−3.2	−39	1.7	1.2	−42	−1.9	−0.9	0	1.3	2.5	99
October	−2.1	−2.0	−14	−1.4	−0.2	26	−0.7	−0.5	17	1.9	2.0	−49
November	2.4	0.4	−29	−1.8	−2.6	−15	−4.6	−4.7	−29	5.5	2.8	−17
December	1.9	1.2	2	−3.8	−3.8	−9	1.4	4.2	−3	−0.2	1.0	22
Annual	2.2	1.0	−338	−1.4	−0.8	−104	−2.2	−1.7	64	0.8	1.4	−17

exception; June experienced 83% greater precipitation than average. Therefore, growing conditions in 2014 were very favorable for crop production, except for extended conditions of overly wet soils in a portion of the lowland transect.

In 2016 a dry period in May and June provided 60% less precipitation than normal for that period. Yet for the entire year, precipitation was within 2% of normal and temperatures were close to normal. For subsequent interpretations of results, we consider 2012 as having severe drought, 2013 as wet early followed by moderate drought, 2014 as favorable throughout the growing season except for seasonal wetness in a portion of the lowland transect, and 2016 as moderate drought early followed by favorable throughout the remainder of the growing season.

Combine Grain Yield

Ames Field (2012, 2014, 2016)

The only possible statistical analysis across multiple years was for 2012 and 2014, because the humic product rates and timing of application for the Ames site changed in 2016 from the earlier years, and a one-time application of chicken manure occurred shortly before the 2016 planting. In the combined analysis of 2012 and 2014, soil type/landscape, humic product treatment, and year effects and the soil type/landscape by year interaction were all highly significant ($P < 0.0001$). Non-significant interactions were found for treatment by soil type/landscape ($P = 0.42$), treatment by year ($P = 0.75$), and treatment by soil type/landscape by year ($P = 0.99$). Therefore, data will initially be presented by individual year and across the three soil types/landscapes.

Maize grain yields as measured by combine, and their statistical analyses for the Ames field site, are shown in **Table 2**, separately for 2012, 2014 and 2016. The field-averaged grain yield in 2012 was 9.4 Mg ha^{-1}, 21% greater than the national average of 7.8 Mg ha^{-1} (USDA-National Agricultural Statistics Service, 2018). The severe drought in 2012 coincided with the most significant benefit of the humic product to maize grain yield. Across the three soil types/landscape positions in 2012, the 2.5 L ha^{-1} V4 treatment increased grain yield by 690 kg ha^{-1} (8%) compared to the control (8.86 Mg ha^{-1}), and the 3 L ha^{-1} split application treatment increased yield by 780 kg ha^{-1} (9%). The main treatment effect was not significant ($P = 0.24$), but the soil type/landscape position effect was highly significant ($P < 0.0001$).

Further statistical analysis by individual soil type/landscape showed significant main treatment effects on yield for the upland Clarion loam ($P = 0.02$). Yield increases above the control (8.41 Mg ha^{-1}) there were 930 kg ha^{-1} (11%) for the 2.5 L ha^{-1} V4 treatment and 1.60 Mg ha^{-1} (19%) for the 3 L ha^{-1} split application treatment, and the corresponding levels of significance were 0.10 and 0.01, respectively. On the productive side slope Nicollet soil, there were slight numeric (2 and 3%) but insignificant increases by the humic product treatments above the control (overall soil mean 12.1 Mg ha^{-1}). On the lowland Webster soil, the main treatment was nearly significant ($P = 0.11$). Comparing individual treatments to the control (6.31 Mg ha^{-1}), the 2.5 L ha^{-1} V4 treatment had 870 kg ha^{-1} (14%) greater grain yield ($P = 0.07$), while the 3 L ha^{-1} split application treatment had only 390 kg ha^{-1} (6%) greater grain yield ($P = 0.37$). In all possible comparisons in 2012, grain yield did not differ significantly between the two humic treatments.

As opposed to the severe drought in 2012, growing conditions in 2014 were very favorable for crop production. Hence maize grain yield in 2014 was uniformly high across the Ames field, increasing above the corresponding 2012 yields by 33% for each humic treatment and by 42% for the control. Field-averaged grain yield was 12.7 Mg ha^{-1}, 18% above the national average (USDA-National Agricultural Statistics Service, 2018). Effects were non-significant for the main treatment, soil type/landscape position and their interaction (**Table 2**). Across the three soil type/landscape positions, the 2.5 L ha^{-1} V4 treatment increased

TABLE 2 | Humic product maize grain yield responses to humic product application for the Ames on-farm field trial extracted as georeferenced data from combine yield maps and shown by year (2012, 2014 and 2016) and three soil type/landscape positions. Two rates of humic product application (H1 and H2) were compared to an unamended control (C).

2012

	Maize grain yield (Mg ha^{-1})				Field-scale statistics	
Humic treatment	**Upland soil[a]**	**Sideslope soil[b]**	**Lowland soil[c]**	**Mean**		**LSD Pr > F[d]**
C	8.41	11.86	6.31	8.86	Humic treatment	0.24
H1[e]	9.34	12.12	7.18	9.55	Soil type/landscape	<0.0001
H2[f]	10.01	12.21	6.70	9.64	Treatment × soil type	0.86
Mean	9.25	12.06	6.73	9.35		
	Humic treatment comparisons (LSD Pr > F)					
	Upland soil	**Sideslope soil**	**Lowland soil**	**Field-scale**		
Main treatment	0.02	0.42	0.11	0.24		
C vs. H1	0.10	0.55	0.07	0.33		
C vs. H2	0.01	0.42	0.37	0.27		
H1 vs. H2	0.22	0.83	0.28	0.89		

2014

	Maize grain yield (Mg ha^{-1})					
Humic treatment	**Upland soil**	**Sideslope soil**	**Lowland soil**	**Mean**		**LSD Pr > F**
C	12.08	12.77	12.78	12.54	Humic treatment	0.47
H1	12.65	12.42	12.97	12.68	Soil type/landscape	0.74
H2	13.10	12.60	12.92	12.87	Treatment × soil type	0.71
Mean	12.61	12.60	12.89	12.70		
	Humic treatment comparisons (LSD Pr > F)					
	Upland soil	**Sideslope soil**	**Lowland soil**	**Field-scale**		
Main treatment	0.07	0.45	0.49	0.47		
C vs. H1	0.24	0.39	0.50	0.71		
C vs. H2	0.05	0.66	0.61	0.38		
H1 vs. H2	0.35	0.67	0.87	0.61		

2016

	Maize grain yield (Mg ha^{-1})					
Humic treatment	**Upland soil**	**Sideslope soil**	**Lowland soil**	**Mean**		**LSD Pr > F**
C	13.92	14.07	14.58	14.19	Humic treatment	0.37
H1[g]	14.04	14.34	14.76	14.38	Soil type/landscape	0.02
H2[h]	13.98	14.77	14.82	14.52	Treatment × soil type	0.95
Mean	13.98	14.39	14.72	14.36		
	Humic treatment comparisons (LSD Pr > F)					
	Upland soil	**Sideslope soil**	**Lowland soil**	**Field-scale**		
Main treatment	0.64	0.42	0.58	0.37		
C vs. H1	0.58	0.70	0.67	0.57		
C vs. H2	0.79	0.31	0.59	0.32		

(Continued)

TABLE 2 | Continued

	Humic treatment comparisons (LSD Pr > F)			
	Upland soil	**Sideslope soil**	**Lowland soil**	**Field-scale**
H1 vs. H2	0.77	0.52	0.90	0.66

[a] *Upland soil is the Clarion loam.*
[b] *Sideslope soil is the Nicollet loam.*
[c] *Lowland soil is the Webster silty clay loam.*
[d] *Probability of statistical significance as determined by the Least Significance Difference method.*
[e] *Enersol humic product broadcast applied in 2012 and 2014 at 2.5 L ha^{-1} at the fourth maize leaf stage.*
[f] *Enersol humic product broadcast split-applied in 2012 and 2014 at 2.0 L ha^{-1} at corn post-planting pre-emergence and 1 L ha^{-1} at the fourth maize leaf stage.*
[g] *Enersol humic product broadcast applied in 2016 at 2.3 L ha^{-1} at the fourth maize leaf stage.*
[h] *Enersol humic product broadcast applied in 2016 at 4.7 L ha^{-1} at the fourth maize leaf stage.*

grain yield by only 1% compared to the control, and the 3 L ha^{-1} split application treatment increased yield by 3%, hence providing more muted responses than in 2012. Statistical analyses by individual soil type/landscape position found a significant ($P = 0.07$) treatment effect only for the upland Clarion soil: the 3 L ha^{-1} split application treatment had significantly greater ($P = 0.05$) grain yield than did the control (12.1 Mg ha^{-1}) by 8%. The 2.5 L ha^{-1} V4 treatment had 5% greater grain yield than did the control for the upland Clarion soil, but the increase was not significant ($P = 0.24$). For the side slope Nicollet soil, both humic treatments had slight yield decreases compared to the control, although neither was significant (overall soil mean 12.6 Mg ha^{-1}). For the lowland Webster soil, slight yield gains with both humic product treatments were non-significant compared to the control (overall soil mean 12.9 Mg ha^{-1}).

Following manure application in early 2016, maize grain yields in 2016 increased by 13% above the 2014 grain yields for each humic product treatment and the control. Humic treatment effects in 2016 remained muted. Field-averaged grain yield was 14.4 Mg ha^{-1}, 31% above the national average (USDA-National Agricultural Statistics Service, 2018). Main treatment and its interaction with soil type/landscape were non-significant, but soil type/landscape had a significant ($P = 0.02$) effect, due to lower grain yields in the upland Clarion soil. Across the three soil type/landscape positions, the 2.3 L ha^{-1} treatment increased grain yield by only 1% above the control and the 4.7 L ha^{-1} treatment increased yield by 2%. Statistical analyses by individual soil type/landscape position found no significant treatment effects, although the grain yield increased numerically above the control for each humic treatment in each soil type/landscape position.

Kelley Field (2013)

In the single year of humic product treatments in the Kelley field (2013), the field-averaged grain yield was 11.1 Mg ha^{-1}, 10% above the national average (USDA-National Agricultural Statistics Service, 2018) and 19% greater than for the 2012 Ames field but 13% less than for the 2014 Ames field. Similar to 2016, the main treatment and its interaction with soil type/landscape position had non-significant effects on grain yield while soil type/landscape again had a significant ($P < 0.0001$) effect due to lower grain yields in the upland Clarion soil (**Table 3**). Field observations attribute this decrease to the droughty conditions that prevailed after early growth stages. Across both soil type/landscape positions, the 2.5 L ha^{-1} V4 treatment decreased grain yield by 3% compared to the control

TABLE 3 | Maize grain yield responses to humic product application for the 2013 Kelley on-farm field trial, extracted as georeferenced data from a combine yield map and shown by three soil type/landscape positions.

	Maize grain yield (Mg ha^{-1})		
Treatment	**Upland, Clarion soil**	**Lowland Canisteo/Harps soils**	**Mean**
Control	9.53	12.56	11.04
Humic 1[a]	9.69	11.81	10.75
Humic 2[b]	10.23	12.56	11.40
Mean	9.82	12.31	11.06

Statistical analyses

	Whole field		
	LSD Pr > F	**Comparisons**	**LSD Pr > F[c]**
Treatment	0.39	Control vs. Humic 1	0.53
Soil type/landscape	<0.0001	Control vs. Humic 2	0.45
Treatment × soil type	0.59	Humic 1 vs. Humic 2	0.18
	Upland Clarion soil		
	LSD Pr > F	**Comparisons**	**LSD Pr > F**
Treatment	0.17	Control vs. Humic 1	0.65
		Control vs. Humic 2	0.08
		Humic 1 vs. Humic 2	0.16
	Lowland Canisteo/Harps soils		
	LSD Pr > F	**Comparisons**	**LSD Pr > F**
Treatment	0.28	Control vs. Humic 1	0.18
		Control vs. Humic 2	0.99
		Humic 1 vs. Humic 2	0.17

Two rates of humic product application (Humic 1 and Humic 2) were compared to an unamended control (C).
[a] *Enersol humic product broadcast applied at 2.5 L ha^{-1} at the fourth maize leaf stage.*
[b] *Enersol humic product broadcast split-applied at 2.0 L ha^{-1} post-planting before maize emergence and 1 L ha^{-1} at the fourth maize leaf stage.*
[c] *Probability of statistical significance as determined by the Least Significance Difference method.*

while the 3 L ha^{-1} split application treatment increased yield by 3%. This paradox was resolved through statistical analyses by individual soil type/landscape position, which found numerically positive grain yield responses to both humic treatments in the upland Clarion loam, including a significant (P = 0.08) increase by 700 kg ha^{-1} (7%) with the 3 L ha^{-1} split application treatment compared to the control (9.5 Mg ha^{-1}). But in the lowland Canisteo silty clay loam/Harps loam complex, which encountered early season flooding, grain yield either decreased by 6% (P = 0.18) with the 2.5 L ha^{-1} V4 treatment compared to the control (12.6 Mg ha^{-1}), or it was unresponsive to the 3 L ha^{-1} split application treatment.

Summarizing across both fields and the 4 years, combine-measured grain yield numerically increased with application of a humic product compared to the control in 18 of 22 comparisons for either humic product treatment in a specific soil type/landscape position and in a single year. These increases were commonly modest, and their statistical significances were affected by year or soil type/landscape position.

Yield Components

Ames Field (2012, 2014, 2016)

Field-scale grain weights were calculated from the 1-m yield component samples for the upland Clarion and lowland Webster soils. These estimates consistently exceeded the combine-generated grain yields, as yield component samples were collected in areas of healthy crop growth, avoiding missing or damaged plants.

In the droughty 2012 season, cob length responded significantly to both main treatment (P = 0.08) and soil type/landscape (P = 0.07) for the whole field, and 100-kernel weight responded significantly to soil type/landscape (P = 0.02), while main treatment, soil type/landscape, and their interaction did not significantly affect whole field grain weights, stover weights or harvest index (**Table 4**). Statistical analyses by individual soil type/landscape position found the only significant responses to humic product application were positive for both grain weight (14.0 Mg ha^{-1} vs. 12.1 Mg ha^{-1} for the control) and cob length (17.1 cm vs. 16.0 cm for the control) for the 3 L ha^{-1} split application treatment in the upland soil. Summarizing all five yield components and both humic product application rates in 2012, the levels of significance for crop response to either humic product application were numerically stronger (smaller P-values) in the upland soil than in the lowland soil in seven of 10 cases.

In the favorable 2014 season, main treatment had no significant effects on any of the five yield component parameters for the whole field, but soil type/landscape significantly affected grain weight, stover weight and 100-kernel weight because of smaller values in the upland soil. Statistical analyses by individual soil type/landscape position found no significant responses to the humic product for any of the yield components, although for the upland landscape positive responses by cob length to the 2.5 L ha^{-1} V4 treatment and harvest index to the 3 L ha^{-1} split application treatment (both P = 0.12) neared the 0.10 threshold. Across all five yield components, the upland soil had numerically stronger levels of significance with humic product application than did the lowland soil in only five of 10 cases.

In the 2016 season, the humic main treatment did not significantly affect any of the five yield components for the whole field, while soil type/landscape significantly affected grain weight, cob length, and stover weight, due to mostly lower values in the upland soil. The soil type/landscape by main treatment interaction significantly affected grain weight and nearly significantly affected stover weight (P = 0.10). Statistical analyses by individual soil type/landscape position found the only significant increase with humic product application was for stover weight for the 4.7 L ha^{-1} treatment in the upland transect (10.2 Mg ha^{-1} vs. 9.3 Mg ha^{-1}). Yet this application rate also approached the P = 0.10 threshold of significance for grain weight, cob length, and harvest index in the upland soil and harvest index in the lowland soil, while the 2.3 L ha^{-1} treatment approached significance in the upland landscape for stover weight and harvest index. Across all five yield components, levels of significance with humic product application were numerically stronger (smaller P-values) in the upland soil than in the lowland soil in nine of 10 cases, with identical values in the 10th case. Most numeric differences were large.

Kelley Field (2013)

In the single year of humic product treatments in the Kelley field (2013), the main treatment and soil type/landscape both significantly affected grain weight, cob length, and harvest index, while soil type/landscape also significantly affected stover weight and 100-kernel weight (**Table 5**). These trends reflect yet more positive crop responses to humic product application in the lowland transect than in the upland transect. The main treatment by soil type/landscape interaction was significant only for stover weight. Statistical analyses by soil type/landscape position found significant increases for both humic application rates in the lowland transect for grain weight, cob length, and stover weight and also for 100-kernel weight for the 2.5 L ha^{-1} application treatment. In the upland transect, the sole response nearing significance was by harvest index to the 3 L ha^{-1} split application treatment (P = 0.10). Across all five yield components, the levels of significance were numerically stronger in the upland soil than in the lowland soil in only two of 10 cases. These trends are inconsistent with the combine grain yields, where the upland soil responded positively to the humic product and the lowland soil responded generally negatively. The lowland yield component samples were collected at slightly higher elevations than were the lowland combine yield data, probably lessening the growth limitations caused by early season wet conditions. Also, the elevational difference between upland and lowland was smaller in this field than in the Ames field.

Although yield component responses to humic product application were frequently non-significant, the responses were mostly numerically positive. For each yield component, of the 16 comparisons between humic product application and the control for all 4 years, both humic product rates, and both transects, numerically positive responses to the product occurred for grain weight and cob length in 14 cases each, for stover weight in 13

TABLE 4 | Maize yield component responses to humic product application for the Ames on-farm field trial (2012, 2014, and 2016).

2012

Grain weight (Mg ha^{-1})

Treatment	Upland[a]	Lowland[b]	Mean	Statistics	LSD Pr > F[c]	Tests	LSD Pr > F
C	12.11	13.00	12.56	Treatment	0.48	C vs. H1	0.80
H1[d]	12.39	13.47	12.93	Landscape	0.40	C vs. H2	0.34
H2[e]	13.99	13.26	13.62	Interaction	0.67	H1 vs. H2	0.47
Mean	12.83	13.24					

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.89	C vs. H1	0.68
C vs. H2	0.04	C vs. H2	0.85
H1 vs. H2	0.05	H1 vs. H2	0.82

Cob length (cm)

Treatment	Upland	Lowland	Mean	Statistics	LSD Pr > F	Tests	LSD Pr > F
C	16.01	16.74	16.38	Treatment	0.08	C vs. H1	0.15
H1	16.78	17.46	17.12	Landscape	0.07	C vs. H2	0.12
H2	17.11	17.23	17.17	Interaction	0.81	H1 vs. H2	0.92
Mean	16.63	17.14					

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.15	C vs. H1	0.26
C vs. H2	0.05	C vs. H2	0.44
H1 vs. H2	0.52	H1 vs. H2	0.71

Stover weight (Mg ha^{-1})

Treatment	Upland	Lowland	Mean	Statistics	LSD Pr > F	Tests	LSD Pr > F
C	9.57	10.51	10.04	Treatment	0.38	C vs. H1	0.57
H1	10.18	10.78	10.48	Landscape	0.20	C vs. H2	0.35
H2	10.64	10.75	10.70	Interaction	0.87	H1 vs. H2	0.72
Mean	10.13	10.68					

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.62	C vs. H1	0.44
C vs. H2	0.31	C vs. H2	0.47
H1 vs. H2	0.58	H1 vs. H2	0.96

Harvest index

Treatment	Upland	Lowland	Mean	Statistics	LSD Pr > F	Tests	LSD Pr > F
C	1.07	1.05	1.06	Treatment	0.89	C vs. H1	0.81
H1	1.04	1.07	1.05	Landscape	0.79	C vs. H2	0.64
H2	1.12	1.05	1.09	Interaction	0.77	H1 vs. H2	0.47
Mean	1.08	1.06					

(Continued)

TABLE 4 | Continued

	By upland				By lowland	
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**
	C vs. H1	0.38			C vs. H1	0.85
	C vs. H2	0.28			C vs. H2	0.98
	H1 vs. H2	0.07			H1 vs. H2	0.87
			100-Kernel Wt (g)			
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	27.78	26.20	26.99	Treatment	0.42	C vs. H1	0.56
H1	26.86	26.24	26.55	Landscape	0.02	C vs. H2	0.42
H2	27.52	25.22	26.37	Interaction	0.63	H1 vs. H2	0.81
Mean	27.39	25.89					
	By upland				**By lowland**	
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**
	C vs. H1	0.36			C vs. H1	0.95
	C vs. H2	0.79			C vs. H2	0.17
	H1 vs. H2	0.51			H1 vs. H2	0.16
			2014			
			Grain weight (Mg ha^{-1})			
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	16.20	17.24	16.72	Treatment	0.35	C vs. H1	0.34
H1	16.72	17.98	17.35	Landscape	0.03	C vs. H2	0.49
H2	16.79	17.57	17.18	Interaction	0.86	H1 vs. H2	0.79
Mean	16.57	17.60					
	By upland				**By lowland**	
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**
	C vs. H1	0.48			C vs. H1	0.42
	C vs. H2	0.43			C vs. H2	0.72
	H1 vs. H2	0.93			H1 vs. H2	0.65
			Cob length (cm)			
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	16.01	16.43	16.22	Treatment	0.29	C vs. H1	0.26
H1	16.46	16.48	16.47	Landscape	0.64	C vs. H2	0.46
H2	16.40	16.36	16.38	Interaction	0.57	H1 vs. H2	0.68
Mean	16.29	16.43					
	By upland				**By lowland**	
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**
	C vs. H1	0.12			C vs. H1	0.89
	C vs. H2	0.18			C vs. H2	0.84
	H1 vs. H2	0.80			H1 vs. H2	0.74
			Stover weight (Mg ha^{-1})			
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	11.33	11.91	11.62	Treatment	0.55	C vs. H1	0.42

(Continued)

TABLE 4 | Continued

Stover weight (Mg ha^{-1})							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
H1	11.67	12.45	12.06	Landscape	0.03	C vs. H2	0.83
H2	11.19	12.28	11.74	Interaction	0.94	H1 vs. H2	0.55
Mean	11.40	12.21					

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.56	C vs. H1	0.50
C vs. H2	0.81	C vs. H2	0.64
H1 vs. H2	0.41	H1 vs. H2	0.83

Harvest index							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	1.22	1.24	1.23	Treatment	0.74	C vs. H1	0.96
H1	1.22	1.23	1.22	Landscape	0.58	C vs. H2	0.53
H2	1.28	1.22	1.25	Interaction	0.62	H1 vs. H2	0.49
Mean	1.24	1.23					

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.91	C vs. H1	0.80
C vs. H2	0.12	C vs. H2	0.69
H1 vs. H2	0.14	H1 vs. H2	0.88

100-Kernel Wt (g)							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	24.56	25.17	24.86	Treatment	0.38	C vs. H1	0.33
H1	24.87	26.92	25.89	Landscape	0.08	C vs. H2	0.58
H2	25.21	25.68	25.44	Interaction	0.76	H1 vs. H2	0.67
Mean	24.88	25.92					

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.78	C vs. H1	0.22
C vs. H2	0.56	C vs. H2	0.71
H1 vs. H2	0.76	H1 vs. H2	0.38

2016

Grain weight (Mg ha^{-1})							
Treatment	Upland	Lowland	Mean	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	16.38	17.45	16.92	Treatment	0.58	C vs. H1	0.61
H1[f]	17.01	17.39	17.20	Landscape	0.02	C vs. H2	0.66
H2[g]	17.39	16.95	17.17	Interaction	0.08	H1 vs. H2	0.95
Mean	16.93	17.26					

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.34	C vs. H1	0.89

(Continued)

TABLE 4 | Continued

	By upland				By lowland		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H2	0.15			C vs. H2	0.41	
	H1 vs. H2	0.59			H1 vs. H2	0.49	
Cob length (cm)							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	17.4	18.3	17.8	Treatment	0.21	C vs. H1	0.21
H1	18.0	18.8	18.4	Landscape	<0.01	C vs. H2	0.36
H2	18.2	18.2	18.2	Interaction	0.19	H1 vs. H2	0.70
Mean	17.9	18.4					
	By upland				**By lowland**		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H1	0.23			C vs. H1	0.34	
	C vs. H2	0.11			C vs. H2	0.88	
	H1 vs. H2	0.63			H1 vs. H2	0.27	
Stover weight (Mg ha^{-1})							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	9.26	10.51	9.88	Treatment	0.64	C vs. H1	0.50
H1	9.98	10.29	10.13	Landscape	0.05	C vs. H2	0.88
H2	10.20	9.68	9.93	Interaction	0.10	H1 vs. H2	0.60
Mean	9.81	10.16					
	By upland				**By lowland**		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H1	0.12			C vs. H1	0.71	
	C vs. H2	0.05			C vs. H2	0.17	
	H1 vs. H2	0.60			H1 vs. H2	0.29	
Harvest Index							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	1.51	1.42	1.47	Treatment	0.96	C vs. H1	0.63
H1	1.46	1.45	1.45	Landscape	0.23	C vs. H2	0.70
H2	1.46	1.50	1.48	Interaction	0.19	H1 vs. H2	0.39
Mean	1.48	1.46					
	By upland				**By lowland**		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H1	0.12			C vs. H1	0.62	
	C vs. H2	0.10			C vs. H2	0.10	
	H1 vs. H2	0.94			H1 vs. H2	0.21	
100-Kernel Wt (g)							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	28.70	29.48	29.10	Treatment	0.73	C vs. H1	0.75
H1	29.17	29.40	29.28	Landscape	0.44	C vs. H2	0.77
H2	29.02	29.53	29.27	Interaction	0.86	H1 vs. H2	0.98
Mean	28.96	29.47					

(Continued)

TABLE 4 | Continued

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.61	C vs. H1	0.89
C vs. H2	0.73	C vs. H2	0.95
H1 vs. H2	0.87	H1 vs. H2	0.85

Two rates of humic product application (H1 and H2) were compared to an unamended control (C) for two landscapes/soil types.
[a]*Clarion loam.*
[b]*Webster silty clay loam.*
[c]*Probability of statistical significance as determined by the Least Significance Difference method.*
[d]*Enersol humic product broadcast applied in 2012 and 2014 at 2.5 L ha^{-1} at the maize fourth leaf stage.*
[e]*Enersol humic product broadcast split-applied in 2012 and 2014 at 2.0 L ha^{-1} at maize post-planting before emergence and 1 L ha^{-1} at the maize fourth leaf stage.*
[f]*Enersol humic product broadcast applied in 2016 at 2.3 L ha^{-1} at the maize fourth leaf stage.*
[g]*Enersol humic product broadcast applied in 2016 at 4.7 L ha^{-1} at the maize fourth leaf stage.*

cases, for 100-kernel weight in 12 cases, but for harvest index only in nine cases. Similar to the combine-based grain yields, these increases were mostly modest, and their statistical significances were affected by year and soil type/landscape position.

Comparing their proportional increases with humic product application, grain weight increases in 2012 and 2016 appear to result primarily from increased cob length, while grain weight increases in 2013 and 2014 appear to reflect both cob length and 100-kernel weight. For all comparisons of humic product vs. control, the increase in mean cob length resulted mostly from a shift in proportions from the shorter side to the longer side of cob lengths (15.0 – 19.5 cm) that were achieved by both the control and humic treatments (**Figure 4**). Only a modest proportion of the increase in mean cob length appears to result from cob lengths increasing to high values (19.5–20.5 cm) not reached by any control samples.

Grain Quality

Grain quality determination during the 2012–2014 seasons found that grain contents of protein, starch, and oil showed only few significant responses to humic product application. In the 2012 field season, the 3 L ha^{-1} split application of the humic product caused a significant ($P = 0.06$) decrease in grain protein content for the upland transect from 75.1 g kg^{-1} (control) to 70.2 g kg^{-1}, while accompanying decreases in the lowland transect for both humic product treatments (from 74.5 g kg^{-1} to 71.5 g kg^{-1}) were insignificant ($P = 0.67$). In the upland transect the 3 L ha^{-1} split application treatment also caused a significant ($P = 0.097$) increase in starch content from 612 g kg^{-1} to 617 g kg^{-1}. Starch concentration increased non-significantly for this treatment in the lowland transect and for the single application in both transects (data not shown). In 2013, both humic product treatments caused significant decreases in protein content for the upland transect (both $P < 0.05$), although both also caused non-significant protein increases in the lowland transect. Numeric increases in starch content with humic product application in the upland transect were insignificant (data not shown), while starch content decreases in the lowland transect were significant ($P = 0.096$) for the 3 L ha^{-1} split application. No significant responses to the humic product occurred in 2014 for protein, starch or oil contents (data not shown). In summary, a slight shift from protein toward starch accumulation likely occurred in the upland transect during the droughtier two of the three seasons, and the shifts were more pronounced in the droughty 2012 season. Elsewhere, humic product application in field conditions was also associated with increased carbohydrate accumulation by potato (*Solanum tuberosum* L.) (Selim et al., 2009) and sweet potato (*Ipomoea batatas* L.) (El-Sayed et al., 2011).

Plant Nutrients

Nutrient concentrations for young leaves sampled at the R2 reproductive growth stage were within acceptable ranges for normal maize growth (data not shown, Bryson and Mills, 2014) in at least two of the three 2012–2014 seasons for all nutrients except mild S and Zn deficiencies, which occurred in all three seasons and in both landscapes. Young leaf nutrients were not measured in 2016 due to the lack of consistent responses to humic treatments in the previous 3 years.

Across all 3 years, nutrient concentrations for all three leaf samplings and grain and stover at physiological maturity showed few significant responses to the humic product, none of which was broadly consistent across treatments, soil types/landscapes, and years. For example, simultaneous with the large grain yield responses to the humic product in 2012, the only significant response of grain nutrient concentrations to either humic application rate was decreased N in the upland transect (**Supplementary Table 1**). For stover nutrient concentrations, in the upland transect only B increased significantly, and in the lowland transect only P and K increased (**Supplementary Table 2**). Specific responses of young leaf nutrients for the 2012 season are discussed in the **Supplementary Material**. In summary, none of the trends found for one crop part in 2012 was reproduced for the other two crop parts.

This specific array of significant responses in 2012 was not reproduced in 2013, 2014, or 2016, which instead provided unrelated patterns of similarly scattered responses. Their details are discussed in the **Supplementary Material**.

In the two drier years of 2012 and 2013, B was a relatively responsive nutrient to both humic product treatments, mostly in the upland landscape. Further details are discussed in the **Supplementary Material**. We do not view enhanced B uptake as a mechanistic explanation for positive crop responses to humic products.

TABLE 5 | Maize yield component responses to humic product application for the Kelley on-farm field trial (2013).

Grain weight (Mg ha^{-1})

Treatment	Upland[a]	Lowland[b]	Mean	Statistics:	LSD Pr > F[c]	Tests	LSD Pr > F
C	15.00	15.04	15.02	Treatment	0.04	C vs. H1	0.02
H1[d]	16.20	19.00	17.60	Landscape	0.04	C vs. H2	0.03
H2[e]	16.13	18.58	17.35	Interaction	0.28	H1 vs. H2	0.79
Mean	15.78	17.54	16.66				

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.25	C vs. H1	0.01
C vs. H2	0.28	C vs. H2	0.02
H1 vs. H2	0.94	H1 vs. H2	0.73

Cob length (cm)

Treatment	Upland	Lowland	Mean	Statistics:	LSD Pr > F	Tests	LSD Pr > F
C	16.98	17.19	17.08	Treatment	0.08	C vs. H1	0.04
H1	17.60	19.27	18.43	Landscape	0.07	C vs. H2	0.01
H2	17.47	18.94	18.20	Interaction	0.81	H1 vs. H2	0.27
Mean	17.35	18.46	17.90				

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.35	C vs. H1	<0.01
C vs. H2	0.45	C vs. H2	<0.01
H1 vs. H2	0.85	H1 vs. H2	0.48

Stover weight (Mg ha^{-1})

Treatment	Upland	Lowland	Mean	Statistics:	LSD Pr > F	Tests	LSD Pr > F
C	10.10	10.60	10.35	Treatment	0.17	C vs. H1	0.09
H1	10.44	12.76	11.60	Landscape	0.07	C vs. H2	0.13
H2	10.17	12.74	11.46	Interaction	0.05	H1 vs. H2	0.72
Mean	10.24	12.04	11.14				

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.66	C vs. H1	0.02
C vs. H2	0.92	C vs. H2	0.02
H1 vs. H2	0.58	H1 vs. H2	0.96

Harvest index

Treatment	Upland	Lowland	Mean	Statistics:	LSD Pr > F	Tests	LSD Pr > F
C	1.26	1.20	1.23	Treatment	0.09	C vs. H1	0.06
H1	1.31	1.26	1.28	Landscape	<0.01	C vs. H2	0.05
H2	1.34	1.24	1.29	Interaction	0.69	H1 vs. H2	0.97
Mean	1.30	1.23	1.27				

By upland		By lowland	
Tests	LSD Pr > F	Tests	LSD Pr > F
C vs. H1	0.23	C vs. H1	0.13

(Continued)

TABLE 5 | Continued

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H2	0.10	C vs. H2	0.29
H1 vs. H2	0.57	H1 vs. H2	0.55

100-Kernel Wt (g)

Treatment	Upland	Lowland	Mean	Statistics:	LSD Pr > F	Tests	LSD Pr > F
C	27.35	28.74	28.04	Treatment	0.22	C vs. H1	0.13
H1	28.41	31.98	30.20	Landscape	0.04	C vs. H2	0.14
H2	29.06	31.13	30.10	Interaction	0.67	H1 vs. H2	0.94
Mean	28.27	30.62	29.44				

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.39	C vs. H1	0.09
C vs. H2	0.18	C vs. H2	0.19
H1 vs. H2	0.59	H1 vs. H2	0.62

Two rates of humic product application (H1 and H2) were compared to an unamended control (C) for two landscape positions.
[a]*Clarion loam.*
[b]*Canisteo silty clay loam/Harps loam.*
[c]*Probability of statistical significance as determined by the Least Significance Difference method.*
[d]*Enersol humic product broadcast applied at 2.5 L ha^{-1} at the fourth maize leaf stage.*
[e]*Enersol humic product broadcast split-applied at 2.0 L ha^{-1} post-planting before maize emergence and 1 L ha^{-1} at the fourth maize leaf stage.*

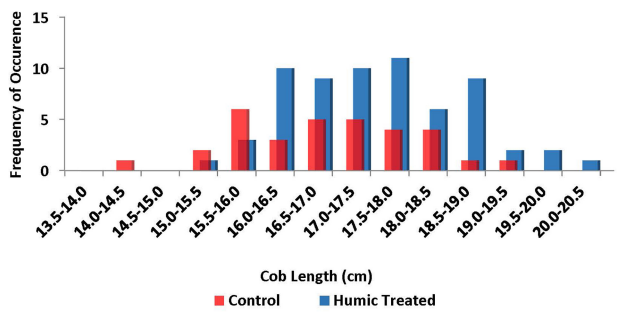

FIGURE 4 | Distribution of cob lengths at physiological maturity for plant samples hand-collected from all treatment strips in all 4 years. Both application rates of the humic product are grouped together into "Humic Treated." Total number of samples is 32 for the control and 64 for both humic product application rates combined.

In summary, plant nutrient concentrations responded irregularly and inconsistently to the humic product across years, soil types/landscapes, and plant parts. This randomness suggests enhanced availability and uptake of soil nutrients was not the causal mechanism for crop responses to the humic product that were observed in this study. Similarly, we note that the greater incidence of negative nutrient responses to the humic product in 2014, as opposed to greater incidences of positive nutrient responses in the drier years, did not inhibit crop growth in 2014.

Leaf Area

For the Ames field, total leaf area increased above the control with humic product application by small positive percentages in 10 of 12 cases for all soil type/landscape by treatment combinations in 2012, 2014, and 2016 (**Table 6**). The highest percent increase was 5.3% for the 3 L ha^{-1} split application treatment in the lowland transect of 2012. Across both landscapes, total leaf area did not respond significantly to either humic product application rate in any year, while soil type/landscape effect was significant only in 2014, with greater values in the lowland landscape. Within either soil type/landscape, total leaf area response to either application rate approached significance only in 2012 for both humic treatments in the lowland transect: $P = 0.11$ for the single application and $P = 0.02$ for the split application. Responses of individual leaf areas to the humic product are presented in the **Supplementary Material**.

For the Kelley field (2013), across both humic treatments total area of the 19 leaves was significantly greater ($P = 0.08$) in the lowland transect than in the upland transect (**Table 6**). Increases in total leaf area above the control were modest positive percentages for all four combinations of soil type/landscape by humic treatment. The highest percent increases were 5.8% and 5.4% for the 3 L ha^{-1} split application treatment in the lowland and upland transects, respectively. Across both transects, total leaf area responded significantly ($P = 0.09$) to the 3 L ha^{-1} split application treatment. Responses of individual leaf areas to the humic product are presented in the **Supplementary Material**.

Summarizing all four seasons, the largest proportional increases in total leaf area with humic product application

TABLE 6 | Total maize leaf area responses to humic product application for the Ames on-farm field trial (2012, 2014, and 2016) and for the Kelley on-farm field (2013).

2012 (Ames field)							
Total leaf area (cm^2)							
Treatment	**Upland[a]**	**Lowland[b]**	**Mean**	**Statistics:**	**LSD Pr > F[c]**	**Tests**	**LSD Pr > F**
C	6827	6817	6821	Treatment	0.26	C vs. H1	0.51
H1[d]	6820	7043	6932	Landscape	0.18	C vs. H2	0.19
H2[e]	6912	7176	7044	Interaction	0.85	H1 vs. H2	0.48
Mean	6853	7012	6933				
	By upland				**By lowland**		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H1	0.75			C vs. H1	0.11	
	C vs. H2	0.50			C vs. H2	0.02	
	H1 vs. H2	0.69			H1 vs. H2	0.33	
2014 (Ames field)							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics:**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	6442	6846	6644	Treatment	0.21	C vs. H1	0.29
H1	6616	7045	6830	Landscape	<0.01	C vs. H2	0.26
H2	6593	7092	6842	Interaction	0.99	H1 vs. H2	0.94
Mean	6550	6994	6772				
	By upland				**By lowland**		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H1	0.48			C vs. H1	0.44	
	C vs. H2	0.54			C vs. H2	0.34	
	H1 vs. H2	0.92			H1 vs. H2	0.85	
2016 (Ames field)							
Treatment	**Upland**	**Lowland**	**Mean**	**Statistics:**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	6703	6938	6821	Treatment	0.86	C vs. H1	0.71
H1[f]	6827	6991	6909	Landscape	0.32	C vs. H2	0.94
H2[g]	6883	6720	6802	Interaction	0.59	H1 vs. H2	0.65
Mean	6804	6883	6844				
	By upland				**By lowland**		
	Tests	**LSD Pr > F**			**Tests**	**LSD Pr > F**	
	C vs. H1	0.61			C vs. H1	0.87	
	C vs. H2	0.47			C vs. H2	0.50	
	H1 vs. H2	0.82			H1 vs. H2	0.41	
2013 (Kelley field)							
Treatment	**Upland[a]**	**Lowland[h]**	**Mean**	**Statistics:**	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C	6306	6574	6440	Treatment	0.22	C vs. H1	0.34
H1[d]	6497	6772	6635	Landscape	0.08	C vs. H2	0.09
H2[e]	6648	6958	6804	Interaction	0.99	H1 vs. H2	0.40
Mean	6484	6768	6626				

(Continued)

TABLE 6 | Continued

By upland		By lowland	
Tests	**LSD Pr > F**	**Tests**	**LSD Pr > F**
C vs. H1	0.39	C vs. H1	0.45
C vs. H2	0.14	C vs. H2	0.17
H1 vs. H2	0.49	H1 vs. H2	0.48

Two rates of humic product application (H1 and H2) were compared to an unamended control (C).
[a]*Clarion loam.*
[b]*Webster silty clay loam.*
[c]*Probability of statistical significance as determined by the Least Significance Difference method.*
[d]*Enersol humic product broadcast applied in 2012 and 2014 at 2.5 L ha^{-1} at the maize fourth leaf stage.*
[e]*Enersol humic product broadcast split-applied in 2012 and 2014 at 2.0 L ha^{-1} at maize post-planting before pre-emergence and 1 L ha^{-1} at the maize fourth leaf stage.*
[f]*Enersol humic product broadcast applied in 2016 at 2.3 L ha^{-1} at the maize fourth leaf stage.*
[g]*Enersol humic product broadcast applied in 2016 at 4.7 L ha^{-1} at the maize fourth leaf stage.*
[h]*Canisteo silty clay loam/Harps loam.*

occurred in the droughtier 2012 and 2013 seasons. The corresponding levels of significance were generally numerically greater (smaller P levels) than those of 2014 and 2016 (**Table 6**).

Soil Properties

Across all 4 years, the lowland transect had greater concentrations of soil nutrients in the vast majority of comparisons with the upland transect (**Supplementary Tables 7–10**). Individual comparisons varied substantially among the years, though, suggesting random variation across time in either soil sampling and/or laboratory analyses. Manure application to the Ames field prior to the 2016 season (**Supplementary Table 10**) resulted in moderate to large numeric increases above the 2014 levels (**Supplementary Table 9**) for nearly all extractable nutrients other than Mn. Corresponding increases for SOM, CEC, and both pH parameters were muted or absent.

When partitioned by soil type/landscape within each year, soil properties in either humic product treatment differed significantly ($P < 0.10$) from the control in only 14 of 240 cases for all four growing seasons (**Supplementary Tables 7–10**). Of these differences, 13 were increases above the control. The 14 cases involved 10 soil properties, indicating inconsistent soil responses across soil types/landscapes and years. In short, soil properties showed no meaningful responses to humic product application.

DISCUSSION

Crop responses to agricultural inputs often vary across soil types/landscapes and time, and much research has sought to identify systematic causes of those variations in order to develop wiser management of the inputs. For example, variability in plant uptake of N and other nutrients was one rationale for development of site-specific or precision farming technologies (Pierce and Nowak, 1999). That a variable response exists across soil types/landscapes or time clearly does not exclude an agricultural input from being a viable tool for crop production. Similarly, inconsistent crop responses to humic product applications, as suggested by previous studies, do not justify a conclusion that humic products are unreliable. Instead, understanding the process-level causes of their variable effects becomes a worthy research objective. A first step toward that objective is to identify spatial and temporal patterns in their field efficacy.

The results of this study suggest that specific factors can be identified to explain variable crop responses to humic products. In our study, maize growth and grain yield responses to a humic product varied across time and space gradients within a high-yielding region. During the severe drought of 2012, grain yield responses to the humic product in the Ames field differed systemically among soil types/landscape positions and their associated soil types: the greatest gains in maize grain yield with the humic product occurred where the effects of drought should be most pronounced – the eroded upland soil with the coarsest texture and presumably lowest soil water availability compared to the sideslope and lowland areas. There was still significant benefit to maize grain yield with the lower rate of humic product application at the lowland landscape position. While we did not measure soil water relations during this study, we visually noted in the 2012 growing season that (i) the lowland landscape position had wetter soil conditions than did the upslope positions, and (ii) its onset of crop drought symptoms was delayed compared to the upland.

Enhancement of crop response to the humic product under droughtier conditions was also evident across growing seasons. The most pronounced responses in combine grain yield, hand-sampled grain weight, cob length, and total leaf area were in the droughtier years of 2012 and 2013, especially in the upland transects. In 2014 and 2016, by contrast, abundant rainfall and hence little environmental stress in this high-yield setting led to subdued maize growth responses. Numerically positive responses of several growth parameters to the humic product were commonly observed in all years and soil types/landscape positions, but they were more likely to reach statistical significance in the droughtier years and the upland landscape.

Partial alleviation of drought stress through application of humic materials, as suggested in our study, has already been demonstrated in controlled conditions, for example by studying maize seedlings (Bijanzadeh et al., 2019; Canellas et al., 2020), maize growth (Anjum et al., 2011) and creeping bentgrass (*Agrostis stolonifera*, L.) (Zhang and Ervin, 2004). In field studies,

crops being grown at suboptimal irrigation water rates responded significantly to humic product application for wheat (*Triticum* sp.) in Iran (Shahryari et al., 2012), barley (*Hordeum vulgare* L.) in Saudi Arabia (Almarshadi and Ismail, 2014), and pear (*Pyrus communis* L.) in Egypt (Ismail et al., 2007).

Our rainfed location did not allow such a design. Instead, we coarsely quantified the relationship of humic product use to drought stress alleviation by correlating (1) crop growth responses to humic product use in the upland transects in each of the 4 years to (2) the total precipitation amount during each growing season (April-September) expressed as a ratio to the 30-year (1981–2010) precipitation means for those months. Increasing drought stress would be represented as decreasing precipitation ratios. Including both humic product treatments in the correlation resulted in correlation coefficients of −0.609 (P = 0.109) for combine grain yield, −0.669 (P = 0.0697) for cob length, −0.390 (P = 0.339) for grain weight in the yield components, −0.338 (P = 0.413) for stover weight, and 0.403 (P = 0.323) for total leaf area (**Supplementary Table 11**). The negative correlation coefficients are consistent with drought stress (decreasing precipitation ratios) causing greater crop response to humic product use. The near significance of the combine grain yield correlation supports the association of humic product use with drought stress alleviation, as does the significance of the cob length correlation. The correlation of the yield component grain weight was improved to −0.947 (P = 0.0012) through deletion of the grain weight in the lowest yielding humic product treatment in the 4-year study, the 2.5 L ha^{-1} treatment in 2012. One possible explanation for this improved correlation is our observation that locating representative yield component samples was most difficult in low-yielding plots, as our yield component samplings were restricted to plants that were evenly spaced and had developed healthy, filled ears, even if this was not representative of growth throughout the low-yielding plots. Hence especially for low-yielding plots, we believe the combine grain yield trends are more reliable than are yield component trends. We did not attempt similar correlations for the lowland transects, where crop growth was less vulnerable to annual variations in drought stress.

We report these numeric responses by individual soil type/landscape even when the soil type/landscape effect was insignificant at $P < 0.10$. Selecting the threshold of significance in field work involves some discretion: milder thresholds than $P < 0.10$ are also justifiable (Carmer, 1976). At P levels less stringent than 0.10, more of our soil type/landscape effects and plant measurements would become significant. Hence their individual description can provide useful information, especially to researchers in other regions with less favorable growing conditions. For example, describing results of the upland landscapes each year emphasizes the large effect of annual precipitation, which will be useful for corn production in drought-prone regions. The consistency of small positive responses for most plant parameters is striking, and their magnitude differed numerically between soil types/landscapes and among years in a predictable manner. Reporting numerically consistent trends for individual year by soil type/landscape combinations enables the useful conclusion that this humic product has the capability to improve crop growth in field conditions, but significance at our P-value of 0.10 was reached only in droughtier conditions. Describing the incidences of these small responses by soil type/landscape is useful for understanding when and where a humic product is more likely to be effective, which is the main objective of site-specific management. It also guides our further research into mechanistic investigations, which we will report subsequently.

Annual changes in the maize cultivars cannot explain these variable crop responses to the humic product. The whole of each field was planted to only one cultivar in each year, yet in the drier 2012 and 2013 seasons the droughtier upland transect provided stronger crop responses than did the lowland transect.

That stress alleviation is a central reason for favorable crop responses to this humic product was further suggested by the finding that the grain yield response resulted primarily from a reduction in the number of shorter ears (**Figure 4**). In other words, the grain yield boost was achieved largely by enhancing growth of the weaker plants. This preferential support of smaller plants is also a form of environmental stress alleviation, in that the smaller plants would otherwise be disadvantaged in their competition against their larger neighbors for light, water, and nutrients, given the high population stands that characterize Corn Belt production.

This study was designed to establish the degree that environmental factors impact humic product efficacy in representative on-farm conditions. Its emphasis on field measurements was not suitable for identifying causal mechanism(s) of crop responses. Nevertheless, our results speak against the primary mechanism being nutrient-based. Positive responses of individual nutrient concentrations to humic product application were infrequent and inconsistent across nutrients, years, and soil types/landscapes, speaking against any single nutrient as the key mechanism. Young leaf nutrient concentrations at the R2 stage indicated that S and Zn were the only nutrient deficiencies that occurred in each of the 2012–2014 seasons. With humic product application, neither concentration of these limiting nutrients increased significantly in this young leaf sampling except for Zn in 2014, when the crop did not respond to humic product application. Sulfur and Zn concentrations in grain and stover increased sporadically and inconsistently with humic product application. Soil nutrients showed no consistent responses to humic product application. Manure application to the Ames field prior to the 2016 season caused large numeric increases for extractability of nearly all soil nutrients. Despite these more fertile soil conditions, crop yield component responses to the humic product were slightly clearer in 2016 than in 2014. Thus, we hypothesize that the fundamental mechanism for plant responses to a humic product is unrelated to soil nutrient availability. The unidentified actual mechanism might, however, stimulate plant nutrient uptake as a secondary benefit by increasing plant nutrient demand. These observations are consistent with the widely held view of humic products as biostimulants, which promote plant growth through stimulation of cellular-level plant processes, as discussed by Nardi et al. (2002), Mora et al. (2010), Zandonadi et al. (2013), Berbara and Garcia (2014), Calvo et al. (2014), and Olaetxea et al. (2018).

This study demonstrated some difficulties in field evaluations. First, production fields often have multiple soil types, each of which might support differing conclusions. For example, the droughtier upland soils provided more significant crop responses to the humic product than did downslope soils. Conversely, the most negative crop response was in an overly wet setting, the lowland soil of 2013. Future research will explore further such incidences of negative crop responses in seasonally wet soils that we have observed elsewhere. Second, high replicate variability is a challenge that must be considered when designing adequate field designs. For example, maize growth was limited in the eroded replicate 4 of the Ames upland transect compared to the other three upland replicates (data not shown). The resulting variability inhibited the establishment of statistical significance for some crop responses, despite appreciable numeric differences. For example, in the upland transect of 2016, combine grain yield showed increases of less than 1% with both humic product treatment, while grain weight of the yield component samples showed increases of 3.8% and 6.2%, which better aligned with field observations. Yield component samplings could more easily be fitted into areas of representative crop growth compared to combine grain yield.

A third and broader challenge posed by this research locale was the high-yielding nature of maize production in central Iowa. Field-average grain yields of 9.4 to 14.4 Mg ha^{-1} obtained here for each year (or 149 to 229 bushels $acre^{-1}$) surpassed the corresponding national average yields (USDA-National Agricultural Statistics Service, 2018) by 18 to 31% (mean 23%) for these years. The control treatments alone surpassed the national averages by 9 to 29% (mean 17%). This study reported significant crop responses to humic product application in cases despite these favorable conditions and high yield levels. Our mixed results confirm previous studies (Calvo et al., 2014) by suggesting that environmental stress mitigation is a large component of crop responses to humic products. Hence, field studies of humic products in settings less favorable than central Iowa might lead to yet more pronounced and frequent crop responses.

Our results demonstrate the capacity of the Enersol humic product to improve crop growth in field conditions, even in the high-yielding setting of the western Corn Belt. In addition, we also tested the hypothesis that environmental constraints predictably altered humic product efficacy. Our research measured multiple parameters both at in-season and harvest times, and they were repeated across time and space. The resulting large number of comparisons between an unamended control and humic product treatments allowed the nuanced observation that maize frequently showed positive but subtle growth responses to the humic product and that their magnitudes increased in droughtier settings. Given the vast potential array of environmental conditions, crop types and varied crop management practices, more such detailed studies are needed to fully assess field efficacies of humic products. As demand for increased crop production occurs over time with increasing global population, combined with diminishing availability of arable land, more pressure is exerted on marginally productive lands. Our research points to humic products as being a helpful tool in managing profitable crop production on those marginal lands, particularly where water is limiting to support crop production. This study also illustrates the rigor to which humic products should be evaluated. An adequate number of field replications is absolutely necessary to enable precise statistical analyses, and in-season plant analyses are necessary to depict the development of a grain yield response. Such information would more efficiently guide future research into the processes underlying crop responses to humic products.

CONCLUSION

Application of the Enersol humic product during four maize seasons in production fields of central Iowa led in cases to significant increases in maize grain yield, ear length, stover weight, and leaf areas. These beneficial crop responses were most evident in droughtier settings: the 2012 and 2013 growing seasons, and in the upland transect with its coarser textured soil. The yield increase resulted mostly from smaller proportions of short ears. In a high-yielding crop region having little environmental stress other than drought, our results support earlier research findings from controlled conditions and field studies that humic products can benefit crop growth through alleviation of environmental stresses. This relationship would help explain inconsistencies among results obtained by multiple studies. Our results suggest a systematic pattern to the field efficacy of humic products. Our results are also consistent with but do not prove the view of humic products as biostimulants that enhance crop physiological processes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will be uploaded to the In-house Database Management System of the USDA-ARS National Laboratory for Agriculture and the Environment, from where it will be made available for all reasonable requests.

AUTHOR CONTRIBUTIONS

DD and DO contributed to the design of the study. DD and DO managed the field experiments and together with CH and GR oversaw the sample analyses. DO and DD developed the interpretations. DO and DD drafted the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.672078/full#supplementary-material

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Bioactivity of Humic Acids Extracted From Shale Ore: Molecular Characterization and Structure-Activity Relationship With Tomato Plant Yield Under Nutritional Stress

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The increasing demands for biostimulants in the agricultural market over the last years have posed the problem of regulating this product category by requiring the industry to make available the information about efficacy and safety, including the explanation of mode of action and the definition of bioactive constituents. In the present study, we tested the biostimulant proprieties of a sedimentary shale ore-extracted humic acid (HA) on Micro Tom tomato plants under increasing nutritional stress and investigated the correlation with the chemical features of HA by means of ultra-high resolution FT-ICR MS, FT-ATR, and ^{13}C-NMR. Humic acid application proved effective in alleviating the nutritional stress by improving nutrient use efficiency, with results comparable to the control treatment supplied with higher NPK nutrition. Increased yield (up to +19%) and fruit quality (in the range +10–24%), higher ascorbic acid content and a better root growth were the main parameters affected by HA application. Molecular-level characterization identified the possible chemical drivers of bioactivity, and included flavonoids, quinones, and alkaloids among the most represented molecules, some of which exhibiting antioxidant, pro-oxidant, and antimicrobial activity. The redox effect was discussed as a determinant of the delicate homeostasis balance, capable of triggering plant defense response and eventually inducing a protective priming effect on the plants.

Keywords: humic acids, biostimulant activity, nutrient stress, FT-ICR MS of humic substances, reactive oxygen species, redox (bio)geochemistry, antioxidant and prooxidant, quinones and flavonoids

INTRODUCTION

Humic substances (HS) are the major component of natural organic matter (NOM), a complex mixture of organic compounds naturally occurring in soils, water, and sediments (Stevenson, 1994). The current use of the operationally based definition of HS fractions, although originally applied strictly to the soil, has been applied to a variety of different sources (Weber et al., 2018). Soil, peat, ores, sediments, leonardite, lignite, compost, and plant are just some sources out of a

possibly longer list, each one performing differently based on its own chemistry. During the last two centuries the knowledge about direct and indirect effects of HS on soil fertility and plant growth has evolved, but the complexity of their constituents and the diversity of each source did not allow the harmonization and standardization of the information accumulated. Conversely, supported by the lack of appropriate technologies and a vague operational definition, ambiguities, and uncertainties about HS origin and chemical structure arised (Kelleher and Simpson, 2006; Kleber and Lehmann, 2019; Olk et al., 2019; Hayes and Swift, 2020). As a consequence, the understanding of their mode of action has been delayed.

However, the biostimulant proprieties of HS have been gradually recognized by the agricultural community that contributed to pushing the market into a rising trend that is expected to increase the global returns at a rate between 9 and 13.4% by 2025 (Meticulous Market Research, 2019; Khillari, 2020). This fast expansion has led to the introduction of new government regulations requiring the elucidation of the mode of action to legitimize the biostimulant industry. A detailed characterization of the chemical composition becomes therefore critical in order to understand the structure-activity relationship and to finally supply farmers with effective products with claims based on science.

Nonetheless, the reduction of agrochemicals footprint and the adoption of an efficient nutrient management need to be implemented to promote a sustainable food production (Vitousek et al., 2009; Foley et al., 2011; Rouphael and Colla, 2018).

Much information has been accumulated regarding the mechanisms by which plants react to HS application (Nardi et al., 1991; Canellas et al., 2002, 2015; Zandonadi et al., 2010; Zanin et al., 2018; Pizzeghello et al., 2020; Olaetxea et al., 2021), as well as their interaction with the rhizospheric microbiome, ultimately leading to enhanced plant development (Puglisi et al., 2009, 2013; Maji et al., 2017; De Hita et al., 2020). However, when moving from short time lab-scale experiments to greenhouse or field experiments where final productivity is measured, impaired results are often reported. Azcona et al. (2011) found that pepper plants treated with HS from composted sludge did not show an improved nutrient uptake or differences in total fruit yield, despite an overall increased biomass produced. Similarly, Pilanal and Kaplan (2003) in a 2-year greenhouse experiment found that foliar application of HA did not affect nutrient uptake in mature strawberry leaves.

According to Rose et al. (2014) HS seem to be more effective when plants are grown under stress. A growing body of literature is accumulating about abiotic stress relief of HS, but little research is available on plants subjected to nutrient stress. Tavares et al. (2019) found that rice plants grown hydroponically and pre-treated with HA showed increased net influx of NO_3^- after a temporary nitrogen deprivation. The only paper found addressing the nutrient stress under field conditions reported an increased P uptake and yield along with the improvement of the antioxidant defense system in maize plants treated with leonardite HA under P deficiency (Kaya et al., 2020). The mitigation of stress has been linked to the ability of HS to prevent ROS induced oxidative damage by modulation of redox homeostasis (García et al., 2016a; Roomi et al., 2018). However, the role of HS chemical structure in biostimulation is not well understood and requires more investigations because an unequivocal relationship has not been identified, despite previous studies which demonstrated the importance of chemical composition and source in predicting the bioactivity of HS (Aguiar et al., 2013; Martinez-Balmori et al., 2014; Monda et al., 2018).

Relevant advances during the last decades, in elucidating the chemical nature of HS, have been achieved using several different techniques such as NMR, pyrolysis GC-MS, LC-MS, FT-IR, fluorescence spectroscopy, and HP-SEC. However, although assessing the general chemical nature of these materials by being marginally successful, none of these techniques has yielded molecular level information until the breakthrough introduction of ultra-high-resolution techniques. Fourier transform ion cyclotron resonance mass spectrometry in a high magnetic field (FT-ICR MS) has become one of the most important analytical tools for detailed characterization of complex mixtures due to its ultra-high mass resolving effectiveness. To date, extended literature has been produced on the application of FT-ICR MS to NOM and its fractions, mostly in relation to dissolved organic matter (DOM) (Brown and Rice, 2000; Kujawinski, 2002; Stenson et al., 2002; Sleighter and Hatcher, 2007; Remucal et al., 2012; Lv et al., 2016). But, when it comes to the terrestrial soil organic matter (SOM) and its fractions, only a few publications arise (Kramer et al., 2004; Ohno et al., 2010; Piccolo et al., 2010; Ohno and Ohno, 2013; Zherebker et al., 2019), whereas investigations on other sources such as ores or compost are rare. A recent innovative approach has been incorporated in the pipeline of the studies on biologically active metabolites, where molecular formulas obtained by FT-ICR MS were sourced from public online databases with valuable results that helped gathering insights into the chemistry of HS (Fedoros et al., 2018; Orlov et al., 2019; Zhernov et al., 2020).

The objective of the present study was to investigate in detail the chemical features of HA extracted from sedimentary ore with the aim of exploring the potential relationship of chemical function with biostimulant activity, and to evaluate the extent to which the priming effect of HA on tomato plants under nutritional stress was reflected on the yield gains.

MATERIALS AND METHODS

Ore Humic Acids Extraction and Elemental Composition

A sedimentary lignite ore (Idaho, USA), ground to pass a 1,000 μm sieve, was used as the source of HA (IDHA). Isolation of HA was obtained by alkaline extraction according to International Humic Substances Society (IHSS) procedure (Swift, 1996). Purification step through HCl/HF was performed to reduce the mineral ash content (Lamar et al., 2014).

The elemental composition of the purified HA extract was achieved by combustion analysis. Carbon and Nitrogen were determined by catalytic combustion with a Rapid CS Cube

combustion analyzer and a Rapid MAX N Exceed combustion analyzer both from Elementar Americas, Inc. (Elementar, Ronkonkoma, NY, USA).

ESI FT-ICR Mass Spectrometry

Extracted samples were analyzed with a custom-built 9.4 T FT-ICR mass spectrometer at the National High Magnetic Field Laboratory, equipped with a horizontal, 220 mm bore diameter operated at room temperature, and a modular ICR data station (Predator 32) facilitated instrument control, data acquisition, and data analysis (Blakney et al., 2011; Kaiser et al., 2014). A purified HA sample was first dissolved in NH_4OH (30%), followed by double dilution with MeOH:H_2O (1:1) to a concentration of 100 mg L^{-1} (Rostad and Leenheer, 2004). The mass spectrum was acquired in negative ionization mode with an introduction flow rate of 0.5 μL min^{-1}, ESI needle voltage of−3,000 V, 100 scan accumulation, and 400 ms event length. 100 individual transients of 5.8–6.1 s duration collected for crude extracts were averaged, apodized with a Hanning weight function, and zero-filled once prior to fast Fourier transformation. For all mass spectra, the achieved spectral resolving power approached the theoretical limit over the entire mass range, e.g., average resolving power, $m/\Delta m_{50\%}$, in which $\Delta m_{50\%}$ is mass spectral peak full width at half-maximum peak height was ∼1,000,000–1,300,000 for absorption mode at m/z 500 for all mass spectra and processed in absorption mode (Beu et al., 2004; Xian et al., 2010, 2012). Peaks with signal magnitude greater than six times the baseline root-mean-square (RMS) noise level were exported to a peak list. The spectrum was internally calibrated by using known methylene homologous series and molecular formula assignments of the resulting mass spectra considering $C_cH_hN_nO_oS_s$ chemical species (Savory et al., 2011; McKenna et al., 2019). Mass peaks with S/N>6 were processed for formula assignment by using the National High Magnetic Field Laboratory, Ion Cyclotron Resonance Facility PetroOrg© software (Corilo, 2018) by setting the following parameters: $^{12}C_{1-100}$ $^{1}H_{2-200}$, $^{6}O_{2-30}$, $^{14}N_{0-3}$, $^{32}S_{0-3}$ with a mass error threshold set at ≤0.5 ppm. Formulae having the least N and S were assigned first (Kujawinski et al., 2009). Generated formulae were filtered by O/C ratio (≤1) and H/C ratio (≤2) according to Koch et al. (2005). The degree of hydrogen and oxygen saturation and molecular heterogeneity were assessed within the assigned formulae and molecular reactivity analyzed based on H/C and O/C ratios by means of a Van Krevelen diagram (Van Krevelen, 1950) whose molecular compositional space was divided into the typical classes of discrete organic biomolecules found in organic matter according to the following rules: ($1.5 < H/C < 2$; $O/C \leq 0.3$) Lipid-like, ($1 < H/C < 2.2$; $0.1 < O/C < 0.67$; $N \geq 1$) Protein-like, ($0.7 < H/C < 1.5$; $0.1 < O/C < 0.67$) Lignin-like, (H/C >1.5; O/C >0.67) Carbohydrate-like, ($0.2 < H/C < 0.7$; $O/C \leq 0.67$) CAS Condensed aromatic structures, ($0.7 < H/C < 1.5$; $O/C \leq 0.1$) UHC Unsaturated hydrocarbons (Hockaday et al., 2009). Online databases such as ChEMBL and PubChem were used to tentatively estimate the potential isomeric structures of the most abundant group of molecules identified by FT-ICR MS data. The formulae most represented in each heteroatomic group were matched online and the most common structures selected when similar features were identified. It should be noted that structure identification might not be indicative of the actual isomer configuration.

^{13}C-CPMAS NMR Spectroscopy

A 300 MHz Bruker Avance spectrometer, equipped with a 4 mm wide-bore MAS probe, was used to run solid-state spectra of the HA sample. Powdered sample was packed into a 4 mm zirconium rotor, stoppered with a Kel-F cap and spun at a rate of 13,000 ±1 Hz. A ^{13}C-NMR spectrum was acquired through the Cross-Polarization Magic-Angle-Spinning (CPMAS) technique with the following parameters: 2 s of recycle delay, 1 ms of contact time, 30 ms of acquisition time, and 4,000 scans. The spectrum was processed by using both Bruker Topspin Software (v.2.1, Bruker Biospin, Rheinstetten, Germany) and MestReC NMR Processing Software (v.4.8.6.0, Cambridgesoft, Cambridge, Massachusetts, USA). Integration of the chemical shift was performed as follows: (0–45 ppm) Alkyl-C, (45–60 ppm) Methoxyl-C, (60–95 ppm) O-Alkyl-C, (95–110 ppm) O2-Alkyl-C, (110–145 ppm) Aryl-C, (145–165 ppm) O-Aryl-C, (165–210 ppm) Carbonyl-C. Structural indices that provided additional biochemical characterization were calculated as follows: hydrophobicity index, HB = (0–45 + 110 – 145 + 145 – 165 ppm)/(60 – 110 + 165 – 210 ppm), alkylic ratio, Alk-R = (0 – 45 ppm)/(60 – 110 ppm), lignin ratio, LigR = (45 – 60 ppm)/(145 – 165 ppm), aromaticity index, AI = (110 – 165 ppm)/(0 – 110 + 165 – 210 ppm) (Spaccini and Piccolo, 2007).

Molecular Mixing Model

Mathematical algorithms of the molecular mixing model (MMM) were used to extract relevant quantitative information from NMR and MS data as described by Baldock et al. (2004) and modified by Hockaday et al. (2009). Briefly, the model uses NMR peak areas to estimate the relative proportion of six components that represent the major biomolecule classes found in natural organic matter to describe the molecular composition of the sample. The six classes correspond to: Carbohydrate, protein, lignin, aliphatic, carbonyl and char. The model is built upon the empirical data obtained for terrestrial and marine environments. The linear combination of the six components allows the model to calculate the best fit to the measured NMR area distribution. As a means of quantitative matching, the MS data obtained by classification of molecular formulae into biochemical categories were used to run the MMM by reverse approach and predict the signal distribution for a ^{13}C-CPMAS NMR. In this way, it was possible to compare in a meaningful way the two analytical techniques and assess the degree to which the molecular distribution relates to the elemental composition.

FT-IR ATR Spectroscopy

An infrared (IR) spectrum was recorded on a Perkin-Elmer Spectrum Two Infrared Spectrometer using an attenuated total reflection (ATR) device equipped with a diamond/ZnSe crystal. About 5 mg HA powder was weighed and put in contact with the crystal by applying a strength of about 150 N on the sample. The spectrum was acquired by using 32 scans with resolution of 4 cm^{-1} from the 4,000 to 400 cm^{-1} region. The sample was

analyzed 3 times and the average of these spectra was used for data interpretation.

Tomato Plant Pot Experiment and Analysis

Tomato seeds (*Solanum lycopersicum* L. cv. Micro-Tom) were surface sterilized in 3% NaClO for 10 min and water rinsed thoroughly before individually sowing in pots containing a mixture of coconut coir and sand (2:1). Plants were grown for three months in a climate-controlled growth chamber set at 28°C with a light/dark cycle of 14/10 h, light intensity set at 300 μmol m^{-2} s^{-1} and relative humidity of 65%. At fifteen days seedlings started receiving nutrition as Hoagland solution with quarter (25), half (50) or full (100) NPK dose and watered at 70% of water holding capacity. Humic acids were added at the pre-plant stage to a concentration of 80 mg C L^{-1}. Nutritional dose and HA concentration were selected based on a previous experiment so that a nutritional stress condition was triggered at low nutrient dose (data not shown). A total of six treatments with eight replicates per treatment were arranged in a randomized complete block design. During the experiment plant height was tracked, and chlorophyll content measured by a chlorophyll meter MC-100 (Apogee Instruments, Logan, UT, USA). Chlorophyll fluorescence was determined at noon by using a OS30p+ pulse modulated fluorometer (Opti-Sciences, Hudson, NH, USA) after leaves were subjected to a dark adaptation period of 20 min, followed by the measure of the ratio F_V/F_0, were F_V is the difference between the maximum and minimum fluorescence and F_0 is the minimum fluorescence detected after dark adaptation. Actinic light intensity was set to 3,500 μmol·m^{-2}·s^{-1} according to Vredenberg (2011). At the end of the experiments roots and shoots were separated and fresh and dry weights determined. Tomato yield was evaluated by measuring the number of fruits and the fresh weight. Quality and antioxidant parameters were also assessed. Total acidity expressed as g L^{-1} of citric acid was obtained by manual titration of tomato juice extract to a pH of 8.2 with 0.1 M NaOH. Ascorbic acid was determined according to Nielsen (2017) and total soluble solids (TSS) by means of a MA871 Refractometer (Milwaukee Instruments, Woburn, MA, USA). Lycopene content was determined according to the reduced volumes of organic solvents described by Fish et al. (2002).

Data Analysis

Experimental data were tested for normality distribution (Shapiro–Wilk test) and the means compared through analysis of variance (ANOVA). *Post-hoc* test was performed to test the statistical significance (Tukey's HSD test, $P < 0.05$). Principal component analysis (PCA) was used as an exploratory tool to assess the correlation of variables with HA application. XLStat software (Addinsoft) was used for all statistical analyses.

RESULTS

ESI FT-ICR MS

FT-ICR MS analysis yielded 9,331 molecular formulae which were assigned with an RMS of 0.17 ppm. The compounds not assigned to a molecular formula represent 10.8% of the total (No hit), highlighted in red in **Supplementary Figure 1**. The relative abundance weighted average of the molecular weights was 384 m/z with an average of 22 C atoms and 16 equivalent double bonds (DBE) (**Supplementary Table 1**). The subdivision into groups showed that CHO formulae were the most abundant (47%), followed by CHON (33.6%), CHOS (4.94%), and CHONS (3.72%) (**Table 1**). The first two groups showed a similar average molecular weight which was lower in CHOS and CHONS where a smaller number of carbon atoms was also observed, thus indicating the presence of smaller molecules in the less represented groups. The low average molecular weight supports the hypothesis of supramolecular aggregation of small molecules dynamically associated through hydrogen bonds, π-π stacking and van der Waals interactions as previously suggested (Piccolo, 2001; Sutton and Sposito, 2005) and reported in heavy oil asphaltenes (Gray et al., 2011; McKenna et al., 2013).

Up to 50% of the relative abundance (scaled to the 100% peak in each spectrum) corresponded to species assigned to the CHO class having 3–10 oxygen atoms and the CHON class having 4–7 oxygens and 1 nitrogen atom (**Table 2**). The presence of 15–18 equivalent double bonds suggests the aromatic properties of these molecules. The elemental composition of HA calculated from the FT-ICR data was consistent with the combustion results. S content, however, was partially underestimated.

The Van Krevelen diagram containing all the peaks did not allow an immediate visual evaluation as the high number of identified components were superimposed and dispersed along both coordinates (**Figure 1A**). However, the comparison of single heteroatomic groups revealed that most of the molecules are grouped in proximity of the *x*, *y* intercepts, extending up to values of 0.65 for O/C and 0.9 for H/C, except in the CHO group (**Figures 1B–D**).

By assigning the compositional space to areas defined by specific H/C and O/C ratios, it was possible to group the molecules into typical classes of discrete organic molecules such as lignin, lipids, proteins, carbohydrates, condensed aromatic structures (CAS), and unsaturated hydrocarbons (UHC).

To further simplify the information visualized in the chart, the data reduction of Van Krevelen points was performed by gathering molecules in classes of compounds with the same heteroatomic number (**Figure 2**). Most of the classes belonging to the group of CHO molecules fell within the lignin compounds, particularly those with a lower number of oxygens ranging from 3 to 10 oxygen atoms, while those with a greater number of O atoms, the most abundant ones, lay in the CAS area (**Figure 2A**).

The classes belonging to the CHON compounds largely concentrated in the CAS area possessing 1 N atom and oxygen atoms ranging from 3 to 8 (**Figure 2B**). On the other hand, heteroatomic compounds showing 1 or 2 S atoms were distributed more uniformly among compounds belonging to the CAS category, lignin derivatives and protein-derived structures (**Figure 2C**).

Finally, the compounds showing the largest heteroatomic distribution appeared in the region belonging to the CAS area with a small number of classes representative of more labile structures such as carbohydrates (**Figure 2D**).

TABLE 1 | Group distribution of Idaho HA FT-ICR MS spectrum after molecular formula assignment.

Group	*N* peaks	% R.A.	Avg m/z	W. Avg m/z	W. Avg C#	W. Avg DBE	W. Avg H/C	AI
CHO	4,447	47.0	409	369	21	16	0.81	0.58
CHON	3,229	33.6	404	362	20	16	0.66	0.67
CHOS	882	4.94	343	326	17	13	0.76	0.73
CHONS	773	3.72	385	344	17	15	0.99	0.65

R.A., Relative abundance; Avg, Average; W. Avg, Weighted average; C#, Carbon number; DBE, Double bond equivalent; AI, Aromatic index.

TABLE 2 | Parameters for the main class distribution contributing up to 50% of the relative abundance.

Class	*N* peaks	% R.A.	W. Avg m/z	W. Avg C#	W. Avg DBE	W. Avg H/C
O6	348	6.32	363	21	16	0.77
O7	339	6.31	382	22	16	0.74
O5	379	6.27	346	21	15	0.80
O4	392	5.24	336	22	16	0.87
O8	297	4.70	398	22	16	0.75
O9	286	3.64	426	23	17	0.75
N1 O5	200	3.49	352	21	16	0.67
N1 O6	189	3.47	370	21	16	0.64
O3	378	3.24	325	22	15	0.99
N1 O4	172	3.01	325	20	15	0.67
N1 O7	183	2.44	393	22	17	0.65
O10	240	2.43	450	23	18	0.74

R.A., Relative abundance; Avg, Average; W. Avg, Weighted average; C#, Carbon number; DBE, Double bond equivalent.

The molecular distribution for each class of compounds calculated by the H/C and O/C ratio are summarized in **Figure 3A**. The most represented compounds were those falling within the lignin and condensed aromatic structures, quantified at 29.5 and 28.6%, respectively, followed by a smaller proportion in unsaturated hydrocarbons, lipids, proteins, and carbohydrates.

The elemental composition of each biomolecule group is illustrated in **Figure 3B**. It is interesting to note the contribution of all heteroatomic classes to the CAS, lignin and UHC groups in which CHO structures add up to ∼39–55%, whereas the carbohydrate group mainly contained CHONS and CHOS structures but lacked CHO structures. As expected, CHON structures are largely associated with proteins. Lipids were almost exclusively composed of CHO molecules.

By matching FT-ICR molecular formulae by means of online chemical databases, we found that most of the lignin structures corresponding to FT-ICR assignments were represented by flavonoid and isoflavonoid phenolic compounds, some of them falling under bioactive plant and microbial metabolites (**Supplementary Table 1**). The major contribution came from both the CHO and CHON series, even though N-containing compounds belonged to more differentiated classes such as alkaloids, benzamides, and several nitrobenzene analogues in which the polar character of nitro groups confers a strong electron-withdrawing capacity and reactivity. In general, they can be classified as aromatic amines because of their relevant hydrogen-deficiency. Polyphenol-peptide reactions that produce condensed structures are likely to occur naturally. The CHON series occurred primarily in the area delimiting CAS and lignin structures (**Figure 2**). On the other hand, the CAS group contained mainly CHO and CHON formulae. Part of the nitrogen associated with HS is expected to be released when HA is separated from fulvic acid by acidic hydrolysis of peptide bonds. However, for amino acids directly bonded to phenolic rings, N may still exist as an acid-insoluble complex, as confirmed by the infrared spectra in the typical absorption of the peptide bond at 1,417 cm^{-1} (**Figure 6**), or as part of a heterocyclic ring as a stable –NH such as in indole (Stevenson, 1994). Condensed aromatic structures were the second most abundant group identified in the FT-ICR data and showed a substantial presence of quinone-derived structures along with alkaloids, flavonoids, and PAH, most of them identified as potentially bioactive compounds of plant, fungal, and bacterial origin (Senthamarai et al., 2003). Interestingly, some of them derive from a marine and freshwater environment and show antimicrobial and antioxidant activity (Namikoshi, 2006; Sturdy et al., 2010). Similarly, the UHC group also contained an abundance of quinone-derived structures where the presence of nitrogen gives pyridine aromatic analogues.

The most abundant structures identified in the lipid group were saturated fatty acids derived from fossilized plant waxes, hydroxy acids, and dicarboxylic acids. It is worth mentioning the large abundance in this list of polyunsaturated arachidonic acid whose inclination to react with molecular oxygen suggests its

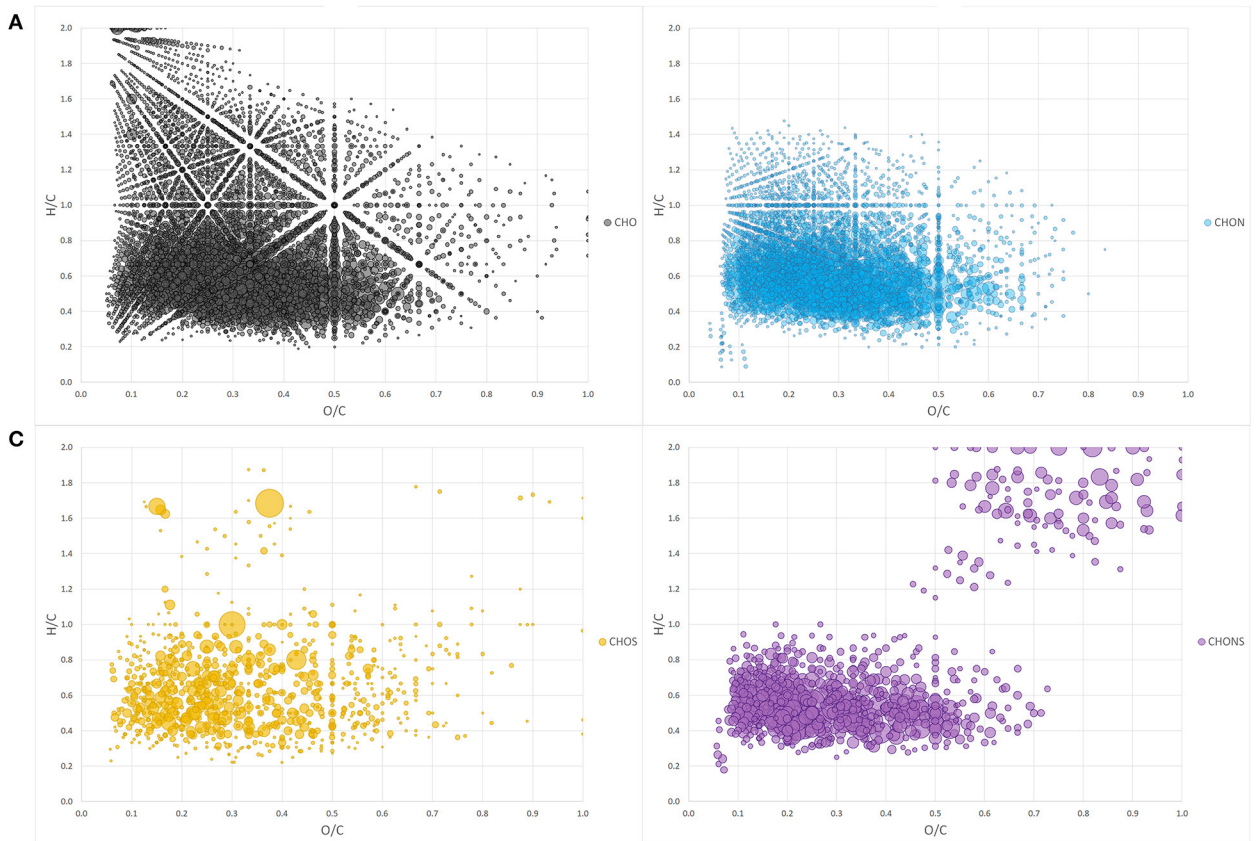

FIGURE 1 | Van Krevelen diagram of heteroatom class groups: CHO **(A)**, CHON **(B)**, CHOS **(C)**, CHONS **(D)**. Each bubble is a molecule assigned and the size of the bubbles is a measure of their relative abundance.

contribution to oxidative stress through the effect on H^+ channel activity (Henderson et al., 1997).

Protein class contributions were identified mainly in alkaloids, indoles, heterocyclic amines, and amino compounds and possibly bioactive quinoline derived structures, while the carbohydrate group was the less represented in FT-ICR and the most challenging to assign. However, even if small, this group was dominated by CHONS structures, probably sugar sulfates, sulfonates, thiocarbonates, or isothiocyanate derivatives such as glucosinolates, but the lack of specific structural analyses, makes it difficult to draw conclusions about the identity of these compounds.

^{13}C CPMAS NMR

The ^{13}C CPMAS NMR of the Idaho humic acid is shown in **Figure 4**. Two broad resonances appear to be predominant, the first in the range 10–45 ppm where the highest peak at 33 ppm was indicative of methyl groups belonging to alkylic structures such as lipidic compounds, and the second in the range of 110–145 ppm where the highest signal appearing at 127 ppm indicated the abundance of protonated aromatic rings.

The relative carbon distribution over the chemical shift is summarized in **Table 3**. The functional groups most represented were the Alkyl-C and Aryl-C, whose regions accounted for 23.6 and 31% of the total area, respectively, followed by the O-Alkyl-C (12.2%), the O-Aryl-C (9.8%), and Carbonyl-C (8.8%) as the most abundant groups. The lower resonances in the O-Alkyl regions, assigned to mono- and polysaccharidic structures mainly derived from plant cellulose, are not resolved in any predominant peak. However, their presence was indicated by the peak at 99 ppm assigned to the anomeric carbons. Methoxyl-C in the range 45–60 ppm, which accounted for 8% of the total area, confirmed the presence of lignin material as the peak at 55 ppm was associated with methoxyl groups substituted on the aromatic core as well as the side chain of lignin monomers. Phenolic compounds added up to lignin aromatic rings as O-substituted C, however this region was mostly overlapped by the Aryl-C region.

Carbonyl-C was visible as a shoulder in the range 165–210 ppm and the peak at 165 ppm was indicative of carboxylated functions in aliphatic chains as well as in protein derived compounds.

Structural indices calculated from the ^{13}C spectrum indicated the mainly aromatic character of the HA where the LigR suggested that preservation of lignin structures happened through the advanced oxidative transformation and stabilization degree of this material. In addition, the AlR and HB indices highlighted the contribution of aliphatic and olefinic structures to the hydrophobicity degree (**Table 3**).

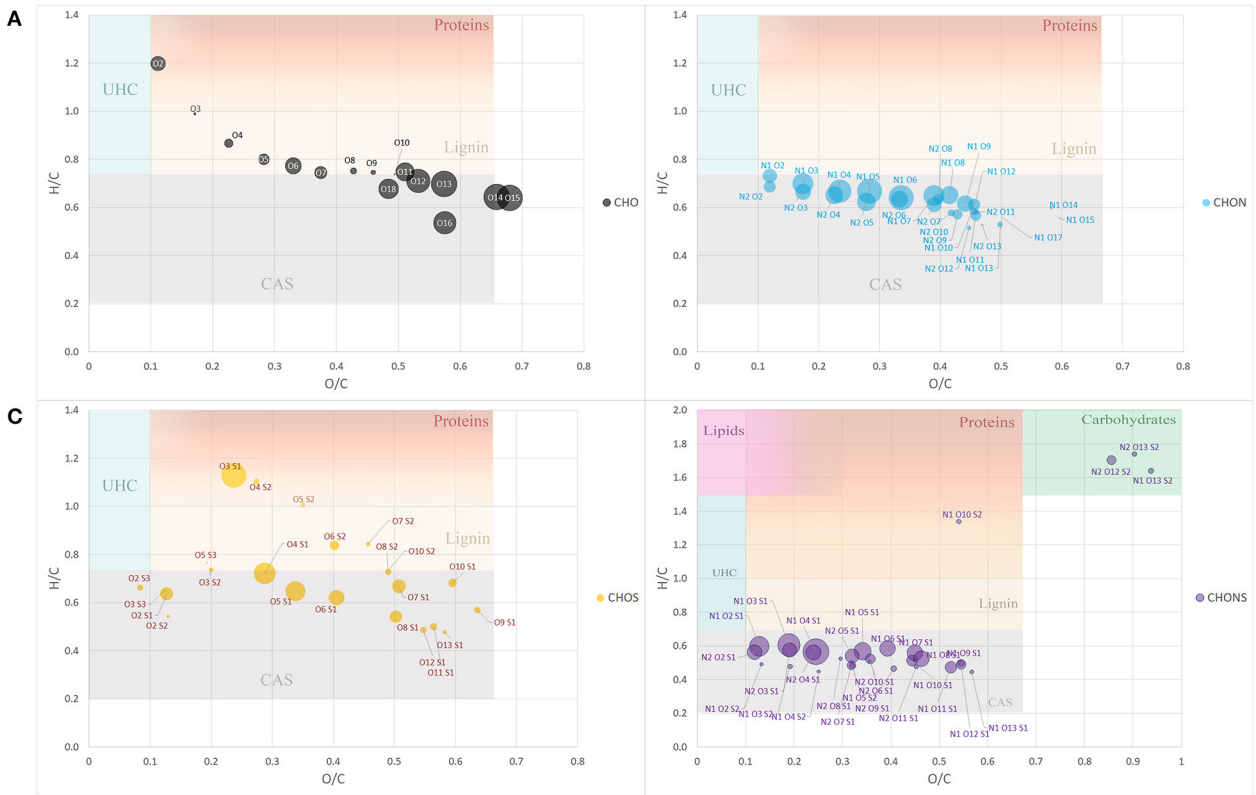

FIGURE 2 | Van Krevelen diagram of class group molecules arranged by the same heteroatoms number. CHO **(A)**, CHON **(B)**, CHOS **(C)**, and CHONS **(D)**. Size of the bubbles is a measure of their relative abundance. Area identified by H/C and O/C ratios and belonging to different type of organic compounds (proteins, lignins, catbohydrates, lipids, CAS, and UHC) have different background color. Fading colors indicate overlapping of different area.

Molecular Mixing Model: MS and NMR Data Comparison

Elemental Composition

The bulk elemental composition comparison suggested that the FT-ICR values were in good agreement with the elemental analysis in relation to the capacity of this instrument to delve deeper into carbon chemistry (**Table 4**). Discrepancies arose when heteroatoms were considered as the N and S content appeared to be underestimated in the MS data. However, the conservative approach used in the ESI MS analysis was intended to preserve the quality and robustness of the results while keeping the error as low as possible. This was consequently reflected in the molar ratios calculated by each technique and suggested a H– deficiency in the MS data, possibly due to the exclusion of hydrocarbons with only C– and H– from the analysis, which are generally not considered as constituents of organic matter. O/C and N/C ratios were overall in agreement across the analyses as well as the aromaticity index that resulted in a slightly higher value when the FT-ICR result was compared to the NMR mixed model value.

Molecular Distribution

Data reduction and aggregation along with the molecular mixing model approach allowed the comparison of NMR and FT-ICR data in terms of individual bio-molecule structures. To make the comparison matching accordingly, the NMR carbonyl value was added to the aliphatic value, while the MS UHC value was combined with the lipid group. Finally, the Char classification in the NMR is referred to as CAS in the MS analysis. There was a close agreement of NMR and MS biomolecule groups, except for the carbohydrates class, which was underrated in the FT-ICR results (**Figure 5**). This trend has been observed previously by other authors and seems to be ascribed to ionization efficiency of this biochemical class (Hockaday et al., 2009). The difference observed in the CAS value between the two techniques can be ascribed to a minor efficiency of cross-polarization technique to correctly represent carbons that are not closely associated with protons, like those of condensed aromatic rings. Consequently, aliphatic carbons could have been preferentially cross-polarized over condensed aromatics and that could have resulted in the underestimation (Kramer et al., 2004).

FT-ATR

The spectrum of Idaho HA (**Figure 6**) showed the typical adsorption bands of humic material. The two peaks appearing at 2,920 and 2,853 cm^{-1} were assigned to both the symmetrical and asymmetrical stretching vibrations of methyl and methylene functions of aliphatic structures, including fatty acids, waxes, higher alkanes and other naturally occurring polyesters. The broad shoulder ranging from 2,500 to 3,500 cm^{-1} represented

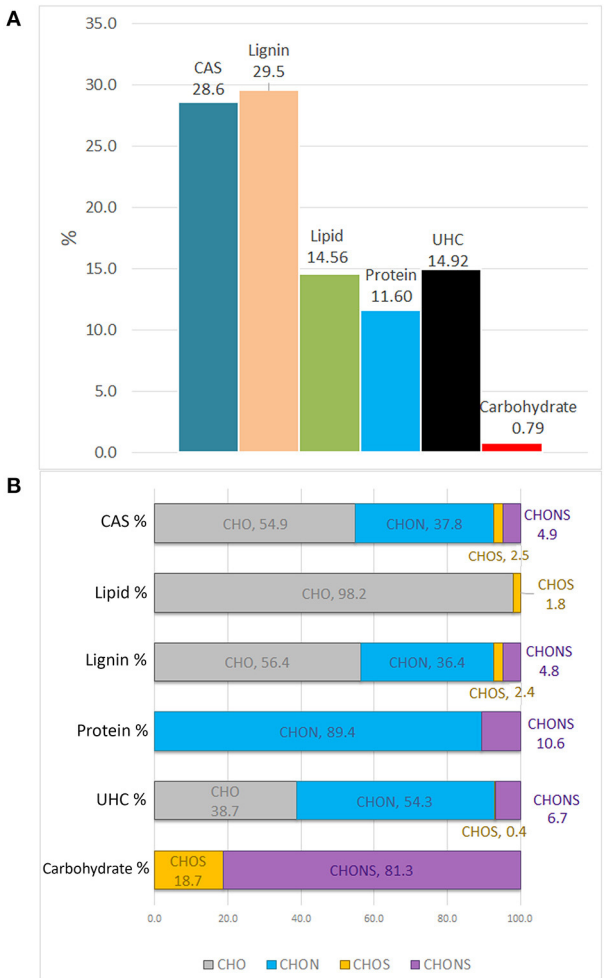

FIGURE 3 | Van Krevelen proportion of different organic compound classes expressed in percentage, as calculated by H/C and O/C ratios **(A)**. Contribution of heteroatoms groups to each compound class **(B)**.

hydroxyl groups belonging to alcohols, phenols, and carboxylic acids. The fingerprint region showed two typical adsorptions at 1,703 and 1,605 cm^{-1} where the stretching of C=O and the vibrations of carbonyl occur, indicating the presence of carboxylic acids, further confirmed by the weak peak at 1,417 cm^{-1}, which can be alternatively attributed to amide II bond stretch. The quite intense peak at 1,198 cm^{-1} and the shoulder peak appearing at 1,041 cm^{-1} were assigned respectively to phenolic –OH of lignin structures and the C–O stretching of polysaccharidic compounds, such as cellulose and hemicellulose derivatives. The two peaks in the lower fingerprint region at around 803 and 763 cm^{-1} were assigned to –CH vibrations of substituted benzene rings belonging to both aromatic and phenolic derivatives.

Plant Growth Bioassay

To test the biological activity of the HA extract, plant morphological traits were investigated. The final goal of the application of HA is the positive economic impact on production. Therefore, yield and fruit quality parameters were also evaluated.

Plants grown under HA treatment showed the best general performances, especially when NPK nutrient supply was reduced to half or a quarter in combination with HA application. The differences observed in shoot biomass showed that the largest value was produced by the 50 HA treatment, followed by 25 HA treatment (**Table 5**). Root biomass was generally positively affected by the application of HA. Root weight, in particular the dry weight, was significantly affected by the presence of HA. The best overall performance was displayed by the 50 HA treatment. Even though the HA supplied under full nutrition resulted in a detrimental effect for several parameters observed, root growth was still positively influenced by the addition of the extract. Chlorophyll content showed an increasing trend when HA was supplied at increasing nutritional doses, however, the differences were not statistically significant. A decrease in chlorophyll fluorescence is an indicator of an ongoing physiological stress. As expected, the control treatment under full nutrition showed the least stress condition, followed by 25 HA and 50 HA that showed the ability to better cope with the stress when compared to the relative controls. Conversely, the drastic decrease in the fluorescence signal measured for 100 HA treatment indicated a reduced photosynthetic efficiency (**Table 5**).

Tomato production and quality assessment results are summarized in **Table 5**. Yield obtained by HA-treated plants showed an increased number of tomatoes produced, up to +19 and +16% in 25 HA and 50 HA treatments, respectively. The trend was not continued when full nutrition was supplied, in which the application of HA decreased the numbers of tomatoes by 13% when compared to the relative control. However, tomato fresh weight was increased in all HA treatments, up to a +24% in the 25 HA treatment.

The fruit quality assessment involved the analysis of acidity, the total soluble solid content (i.e., Brix) and the antioxidant activity measured by lycopene and ascorbic acid production (**Table 5**). Total acidity increased as a result of the application of HA at half and full nutritional strength, while the soluble solid content increased in the 25 HA and 100 HA treatments, +12 and +24%, respectively, as compared to non-treated plants. Lycopene content increased significantly only in the 50 HA and 100 HA treatments. Except for the full nutritional level (100 HA) where there was a significant decrease, the application of HA increased the ascorbic acid concentration up to 10% in the 50 HA treatment as compared to respective control.

The data reduction through principal component analysis allowed the determination of the variables most influenced by the HA application. Humic treatments were clearly separated from the controls along the first principal component that explained 58.8% of variability (**Figure 7**). All the variables were spread mostly along the second principal component and showed a positive correlation with 25 HA and 50 HA treatments. Root dry weight was the strongest correlating parameter, while chlorophyll, root fresh weight and ascorbic acid appeared less important for the purpose of biostimulation.

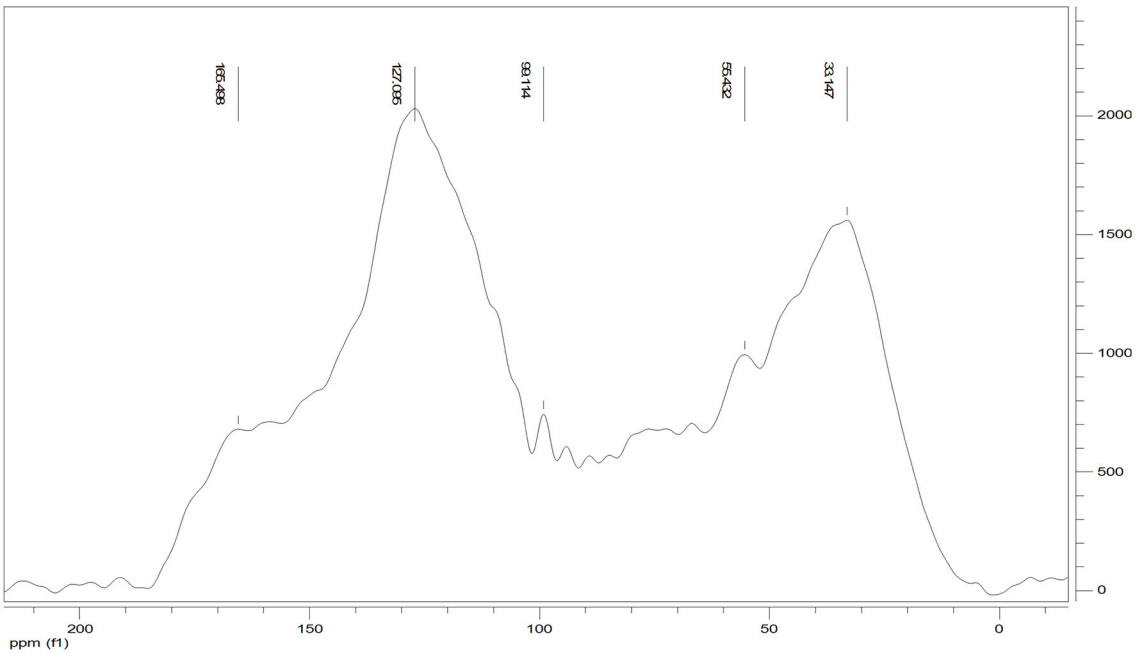

FIGURE 4 | ^{13}C CPMAS NMR spectrum of Idaho HA.

TABLE 3 | Relative contribution (%) of main C structures over chemical shift regions (ppm) calculated from ^{13}C CPMAS NMR of Idaho HA sample and structural indices derived from spectral areas.

Sample	Carbonyl-C 210–165	O-Aryl-C 165–145	Aryl-C 145–110	O2-Alkyl-C 110–95	O-Alkyl-C 95–60	Methoxyl-C 60–45	Alkyl-C 45–10	HB	AlR	LigR	AI
IDHA	8.8	9.8	31.0	6.7	12.2	8.0	23.6	1.8	1.25	0.81	0.5

HB, Hydrophobicity index; AlR, Alkylic ratio; LigR, Lignin ratio; AI, Aromaticity index.

TABLE 4 | Elemental composition of Idaho HA as determined by elemental analysis (EA), FT-ICR ESI MS, ^{13}C CPMAS NMR (through molecular mixing model MMM).

	C%	H%	O%	N%	S%	H/C	O/C	N/C	S/C	AI
EA	50.1	–	–	2.03	1.59	–	–	0.04	0.032	–
FT-ICR	50.2	32.9	15.2	1.38	0.29	0.65	0.30	0.03	0.006	0.66
^{13}C NMR MMM	–	–	–	–	–	1.18	0.34	0.08	–	0.5

DISCUSSION

Optimization of nutrient use efficiency represents an important strategy to reduce the environmental cost generated by harmful contamination of groundwater and atmosphere that mineral fertilizers produce when used in excess to maximize crop production (Conley et al., 2009). The use of HS as biostimulant represents a cost-effective and environmental-friendly tool to improve nutrient uptake by promoting sustainable agricultural practices. Indeed, HS affect nutrient complexation and act as natural chelates (Garcia-Mina et al., 2004; Tomasi et al., 2014) but they also induce plant metabolism changes. HS stimulate active proton extrusion from the root plasma membrane by the activity of H^+ –ATPase resulting in the generation of a transmembrane potential involved in the cell elongation and active uptake of nutrients (Varanini et al., 1993; Canellas et al., 2002; Zandonadi et al., 2007; Jannin et al., 2012). More recently it has been observed that HS performances increase when a stress condition is present. Jindo et al. (2016) demonstrated that application of HS in the presence of low phosphorus availability induces high-affinity P_i transporters in plant roots thereby enhancing P uptake. Tavares et al. (2019) found that HA stimulated NO_3^- uptake after 96 h of N deprivation. Additionally, the chemical nature of HS that differs for each source plays a key-role and often leads to practical ineffective results. In this SAR study the biostimulant activity of an ore-extracted and

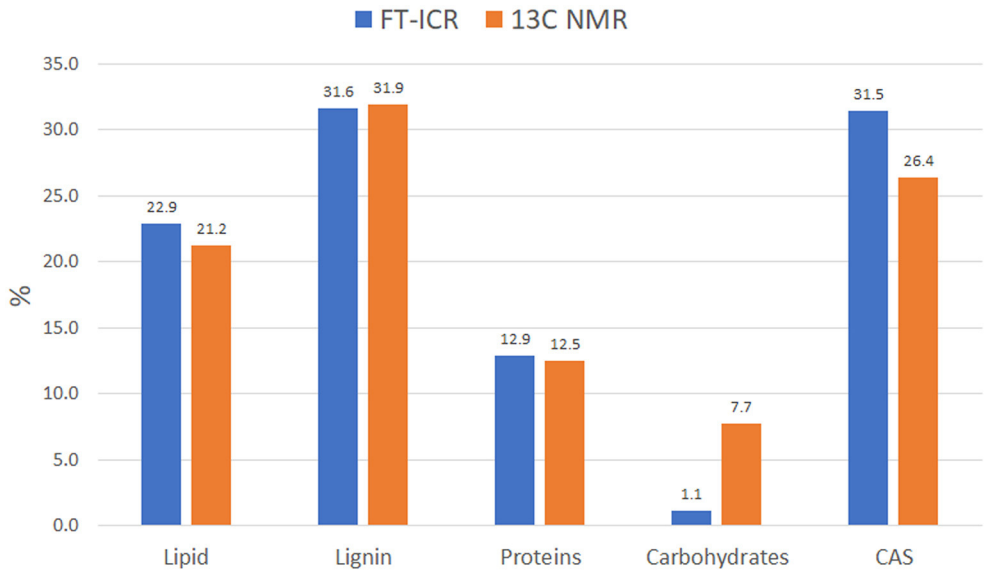

FIGURE 5 | Idaho HA molecular distribution into the main biomolecule structures as observed by FT-ICR and ^{13}C NMR. Data computed through MMM for both analyses.

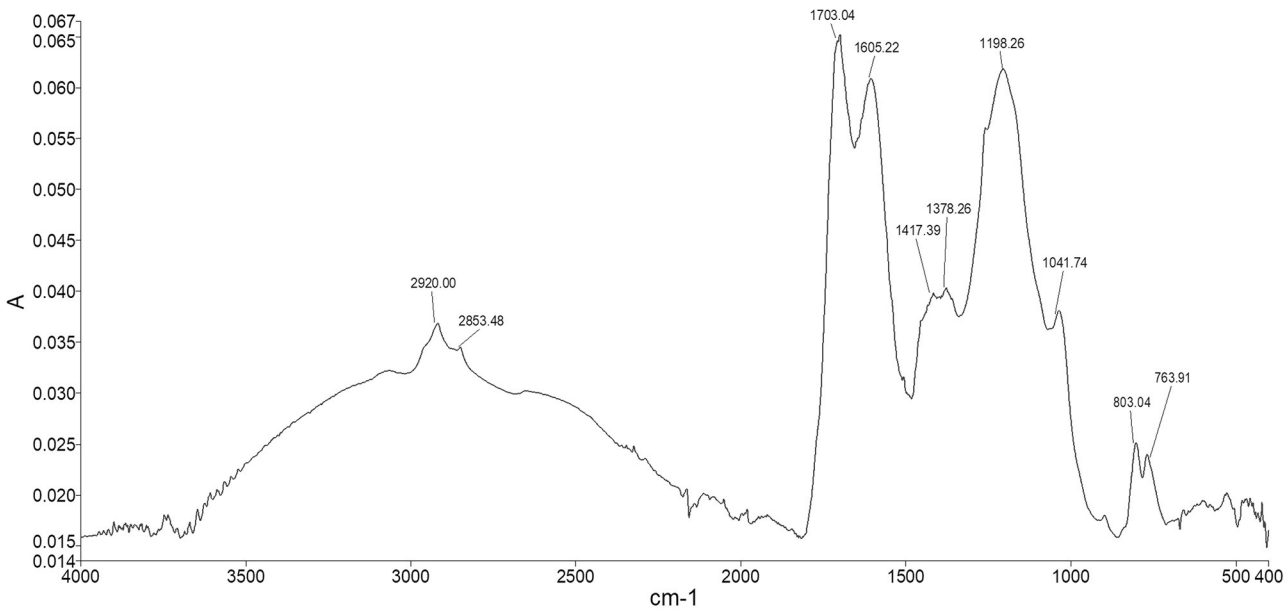

FIGURE 6 | Idaho HA FT-ATR spectrum.

purified HA was evaluated in a tomato pot experiment and the HA chemical proprieties were characterized by means of high-resolution MS, ^{13}C NMR, and FT-IR. The chemical nature of HA was further analyzed for the recognition of specific biochemical structures potentially involved in tomato plant morphological and productivity responses, including an increase in defense mechanism parameters.

The application of HA confirmed its ability to stimulate tomato plant growth. HA treated plants yielded more tomatoes than control plants when combined with lower nutritional dose. Comparison of the control under 50% of nutritional dose with the HA treatment at 25% of nutrition supplied showed a similar production rate and similar photosynthetic activity, indicating the ability of HA to alleviate the stress condition and to partially reduce the amount of fertilizer required to obtain comparable results.

Tomato fruit quality improvements were also reported in all HA treatments. Although the application of HA was less effective under full nutrition in terms of some morphological parameters in this treatment, HA may have caused the plants to shift to an

TABLE 5 | Plant morphological mean data, tomatoes yield, and quality parameters.

Treatment	H cm	CHL μmol m^2	CHL FL Fv/F0	SHOOT FW g	SHOOT DW g	ROOT FW g	ROOT DW g	TOM N	TOM FW g	TA g/L citric a.	TSS Brix	AsA mg/g FW	Lycopene mg/100 g FW
25	6.85	401.9	2.95	7.76	1.73	4.45	0.51	13	3.25	4.0	5.5	0.46	11.5*
50	7.3	403.8	2.94	8.04	1.69	4.21	0.78	16	5.06	4.0	6.2	0.48	10.0
100	7.36	423.0	3.02*	9.10*	1.88	5.14	0.66	32	5.65	4.1	5.4	0.52*	11.8
25 HA	7.39	425.3	3.00*	9.93*	2.07*	3.94	0.79*	16	4.29*	3.9	6.2*	0.49*	10.6
50 HA	7.84	428.1	2.96	10.93*	2.12*	5.09*	0.91*	19	5.22	5.5	6.0	0.53*	12.3*
100 HA	7.02	399.3	2.73	7.83	1.68	4.57	0.81*	28	6.29	5.2	7.1*	0.47	12.3*

H, height; CHL, Chlorophyll; CHL FL, Chlorophyll fluorescence; FW/DW, Fresh/Dry weight; TOM N, Tomatoes number; TA, Total acidity; TSS, Total soluble solids; AsA, Ascorbic acid. Significant difference at P < 0.05 (Tukey's test) are indicated by asterisk.

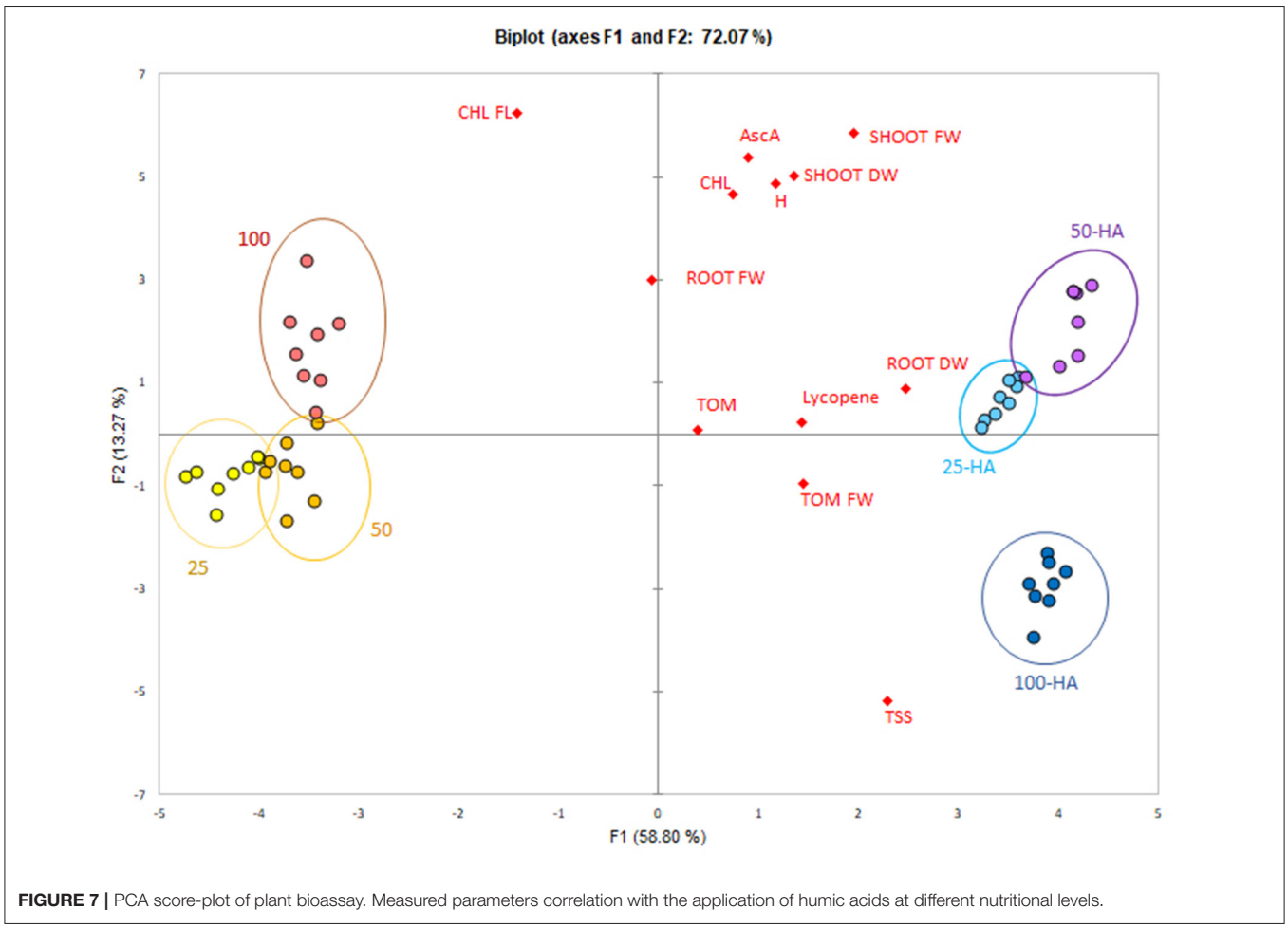

FIGURE 7 | PCA score-plot of plant bioassay. Measured parameters correlation with the application of humic acids at different nutritional levels.

energy conservation strategy that entailed less vegetative growth but promoted mainly fruit development. In fact, even if tomatoes produced in HA100 were slightly less in number than control, their fresh weights were larger by 10%. This conclusion was also supported by the content of total soluble solids, lycopene, and total acidity that in 100 HA tomatoes outperformed the respective control.

The application of HA overall increased the content of measured antioxidants as a result of plant defense system activation. Ascorbic acid, which as the primary plant antioxidant contributes to the reduction of the oxidative damage, may have allowed a stronger response of HA treated plants to the nutrient stress condition. When compared to relative controls, the HA treatments showed a significant lycopene increase only at higher nutritional input. The increased stress due to deficient nutrition is supposed to increase the antioxidant content at lower NPK supply, but conversely to what expected, lycopene increased as nutritional dose increases, in both controls and HA

treatments. Our results are in accordance with Koleška et al. (2017). The higher K and P fertilization could be potentially responsible for the described trend because they have been reported to positively influence lycopene content in tomato as their supply is increased (Zdravković et al., 2004; Ramírez et al., 2009).

The FT-ICR MS analysis of the HA helped provide an understanding of the composition and distribution of major biomolecule classes present. This ore-extracted HA consisted of more than 10,000 small molecules with an average m/z of 384 and are representative of plant and microbial aromatic biomolecule derivatives. Together with a marked presence of aliphatic compounds, these components confer on this HA a distinctive aromatic and hydrophobic character. The ^{13}C CPMAS NMR supported this outcome showing that relative carbon distribution is concentrated mostly in the aliphatic and aromatic region where the presence of peaks at 56, 127, and 165 ppm have been previously associated with lateral root stimulation bioactivity (Aguiar et al., 2013). On the other hand, it highlighted the preservation of a modest carbohydrate component that was not found in the FT-ICR MS. The molecular mixing model was used to predict the molecular allocation of biomolecules by applying FT-ICR MS data to the model and comparing the results to the NMR data. The model showed a close match in the distribution of biomolecules, except for carbohydrates, confirming the preferential ionization, in the ICR cell, of some classes of compounds such as CAS and lipids. Nonetheless, the correspondence in all the other groups overall proved the validity of the model comparison.

Online database searches of the molecular formulae identified several bioactive molecules belonging to the lignin derived flavonoids class, quinone-derived structures, and other molecules belonging to CAS and lipids that are potentially involved in the oxidative stress modulation.

Flavonoids are a big family of phytochemicals involved in the plant defense mechanism while coping with a stress condition (Cetinkaya et al., 2017; Trejo-Téllez et al., 2019). They are synthesized through the phenylpropanoid pathway and exhibit ROS scavenging properties assisting plants in tolerating and escaping external biotic and abiotic stresses (Treml and Šmejkal, 2016). As their role as antioxidants is widely recognized (Pietta, 2000), their occurrence in this humic extract supports the biostimulant action that HA exert on plant fitness. However, they can also be involved in priming plant stress machinery as described by Canellas et al. (2020).

Several authors described the prooxidant and antioxidant properties of phenols, alkaloids, and quinones (Azam et al., 2003; Pietsch et al., 2011; Kurutas, 2015). Polyphenols are considered antioxidants, but they not only might undergo oxidative reactions, but when applied externally, their allelochemical biological role and negative impact on target organisms should be considered. These includes impacts such as ROS generation, inhibition of cell division and reduced photosynthetic rates, among others. Secondary metabolites and particularly phenols can often demonstrate prooxidant activity by releasing a hydrogen atom and producing a reactive semiquinone radical capable of reducing oxygen to $^{\cdot}O_2^-$ which can be further converted to other detrimental ROS including H_2O_2 and $^{\cdot}OH^-$ by scavenging of other phenols (Grace, 2005; Gniazdowska et al., 2015).

Quinones on the other hand can act as prooxidant and have been proposed as potentially responsible for triggering the ROS production in plant, by acting as electron shuttles due to their oxidizing/reducing capabilities (Lamar, 2010; Lv et al., 2018). However, the antioxidant function of isoprenoid quinones has been recently described by Kruk et al. (2016). They have, indeed, an important biological role as redox co-enzymes and vitamin constituents. Zhang et al. (2018) demonstrated that HA contained redox-active groups and exhibited redox potentials between −0.36 and −0.28 V suggesting their role as redox mediator in enhancing multiple microbial reductions, thereby affecting various biogeochemical processes. Nonetheless, Zykova et al. (2018) attributed the radical scavenging property of several HA to the presence of condensed aromatic structures such as semi-quinone type and phenoxyl type radicals.

The presence in HA of molecules that can act transiently as antioxidant or prooxidant depending on the environmental constraints, could explain, at least in part, the bioactivity effect through the modulation of ROS accumulation in plant. Despite the damage that ROS exposure might have in the oxidative process, ROS have an important role as signaling molecules, often leading to the conferment of tolerance to environmental stresses (Balasubramaniyam, 2015). Whether the exposure to stress promotes toxicity or acclimation strategy depends on the homeostasis balance between ROS production and ROS scavenging that eventually produces a shift in the regulatory role of ROS from cell signaling to the negative physiological effects (García et al., 2016a).

HS have been found to increase the ROS levels by acting as a mild stressor by triggering the plant defense system. García et al. (2012) described the increase of ROS in rice roots as a consequence of HA application. Similarly, Mehrasbi et al. (2018) found that HA affected ROS production in algae. However, the pre-teatment with HA has been found to mitigate the presence of major abiotic stresses induced by PEG (García et al., 2016b) as well as salinity, drought and heavy metals (Canellas et al., 2020), resulting in higher transcription level of genes involved in stress perception.

All the molecules found in this study could be involved in both the determination of a eustress, where the final effect is somehow beneficial for the plant, or in the establishment of a distress, leading to detrimental and irreversible tissue damage (Vargas-Hernandez et al., 2017).

In our study, plants under nutritional stress performed better when HA was supplied, while plants at full nutrition were not showing a clear advantage from HA application, which might have behaved as a stressor when no other stress was present. On the other hand, all HA treatments showed faster adaptation to the stress condition, particularly when nutrient deficiency occurred. The resulting increased nutrient accumulation and growth of tomato seedlings by application of humic under limited nutrient availability solution was reported by David et al. (1994) and supports our observations. Indeed, the increased root biomass observed was indicative of a better nutrient uptake efficiency and

could have resulted from ROS sensitive signaling response to nutrient deprivation that leads to cell-wall relaxation and root growth (Schachtman and Shin, 2007), a process strictly correlated to the activation of plasma membrane H^+ ATPase reported by several authors (Nardi et al., 2017; Tavares et al., 2017). This hypothesis is in accordance with Cordeiro et al. (2011) that found ROS level increased in the maize root apex upon HA application and a higher transcription of catalase antioxidant enzyme when nitrogen supply was low.

Because the final effect of HS is not solely the consequence of the presence of a single molecule but relies on the complex mix of constituents, how the final effect on plant is modulated is still difficult to predict, but it is likely to be associated with the emerging properties defined by the interaction with plant defense system and the biochemical environment. In fact, lignin, and CAS derived molecules can participate in electron transfer reactions either as donors or acceptors, depending on the presence or absence of specific functional groups. For example, electron transfer mediated by one phenolic hydroxyl group can lead to an oxidized radical, while the presence of two hydroxyl groups on catechol can reduce H_2O_2 to H_2O. However, if ROS are not present, the catechol can reduce molecular oxygen to H_2O_2, while the presence of ROS drives the process toward the scavenging reactions as long as homeostasis restoration is achieved (Hadacek et al., 2011). The role of HS electron accepting capacity has been investigated by Yang et al. (2016) who found that quinone moieties were responsible for the high reducing ability of low molecular weight HA such as the ones described in our study. Lv et al. (2018) demonstrated that polyphenol-like compounds with medium oxygen content were the major compounds acting as electron donors in HS. Furthermore, polyphenols such as flavonoids can be involved in nutrient uptake as they form stable complexes with Fe and Al present in insoluble Fe- and Al-phosphates thereby increasing the P solubility for plant uptake (Cesco et al., 2010). Nonetheless, they can also prevent microbial degradation of extracellular phosphatases and organic acids released by roots as a response to nutritional deficiencies (Neumann and Römheld, 2007).

Based on our results, here, we suggest that the balance of flavoinoids and quinones found in the humic extract could have positively modulated ROS signaling involved in plant nutrient uptake and therefore triggered the biostimulant effect observed. While the understanding of mode of action will require further investigation, plant pre-conditioning with HS might represent an important determinant in the adaptive plant defense response and an effective strategy to improve nutrients management and plant productivity.

CONCLUSION

The outcomes of this study highlighted the role of HA in enhancing nutrient efficiency uptake. The application of HA at low NPK supply improved tomato yield and plant ability to cope with nutritional stress. Chemical composition revealed the presence of both antioxidants and prooxidant molecules such as flavonoids and quinones and suggested their role as modulators of ROS level in plant by priming plant defense systems and resulting in increased root exploration and antioxidant production. Our results proved that use of HA ultimately leads to a fast and effective response to nutrient deficiency based on increases in plant morphology and productivity.

The implementation of *in silico* technologies represents a valid tool and a promising strategy where combinations of ultra-high mass resolution and complementary techniques will allow a more extensive understanding of molecular composition of HS from different source environments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s. FT-ICR MS data is publicly-available on the Open Science Framework through DOI: 10.17605/OSF.IO/TF7QB.

AUTHOR CONTRIBUTIONS

HM and RL conceptualized and designed the study. HM performed the experimental work, the plant experiment, the analytical data analysis, and statistical tests. AM supported the FT-ICRMS data interpretation. RF did the elemental analysis. HM wrote the manuscript. RL and AM supervised the article. All authors approved the submitted version.

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The authors declare that this study received funding from Bio Huma Netics, Inc. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article. It was involved in the decision to submit it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.660224/full#supplementary-material

Supplementary Figure 1 | Idaho HA FT-ICR spectrum. "No hit" highlighted in red **(A)**. Distribution of molecular weights vs. intensity for the different heteroatom groups **(B)**.

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Maize Growth Responses to a Humic Product in Iowa Production Fields: An Extensive Approach

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Field evaluations of commercial humic products have seldom involved replication across location or year. To evaluate the consistency of humic product efficacy in field conditions, we determined the effects of a humic product on maize (*Zea mays* L.) growth in high-yielding Midwestern (US) fields through the following two extensive approaches: (i) replicated strip plots in five site—year combinations from 2010 to 2013; and (ii) demonstration strips in 30–35 production fields annually from 2009 to 2011 that covered major areas of Iowa. Mechanized combine measurements of grain yield showed increases of 0.2–0.4 Mg ha^{-1} (1–4%) with humic product application for all five site—year combinations of the replicated strip plots. Six of 10 humic treatments within the fields responded positively ($P < 0.07$), and the positive responses of two more treatments approached significance at the benchmark of $P = 0.10$. In the demonstration strips, maize grain weight in hand-collected samples increased significantly ($P < 0.004$) with humic product application in each of the three growing seasons, and across all the three seasons by 6.5% ($P < 0.001$). Grain weight increased numerically for 76 of the 98 demonstration strips. Yield component analysis for both the replicated strip plots and the demonstration strips attributed the yield boosts largely to increased ear length, especially of the shorter ears. Humic product application caused significantly ($P < 0.10$) greater total leaf area in all eight field treatments at three site—year combinations. Humic product application did not consistently affect nutrient concentrations of the grain or stover or any measured soil property. These results represent among the widest geographic evaluations published on field efficacy of a humic product. They demonstrate the capability of a humic product to improve maize growth in high-yielding conditions.

Keywords: humic product, maize, grain yield, leaf area, on-farm survey

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INTRODUCTION

Humic products have received increasing attention as a management tool for increasing crop growth. Plant responses have been demonstrated most often under controlled conditions (Chen and Aviad, 1990; Rose et al., 2014). A growing number of published studies address the field efficacy of humic products for horticultural crops (Bryla and Vargas, 2013; Shahin et al., 2015; Suman et al., 2016; Popescu and Popescu, 2018), agronomic crops (Herrera et al., 2016; Chen et al., 2017;

Abbreviations: ANOVA, analysis of variance; LSD, least significant difference; V, leaf vegetative growth stage.

Lenssen et al., 2019; Izquierdo and Pintos, 2021; Pačuta et al., 2021), and in the alleviation of environmental stresses (Osman and Rady, 2012; Bezuglova et al., 2019; Nazli et al., 2020; Qin and Leskovar, 2020; Fallahi et al., 2021; Lindsey et al., 2021). Reviews of field studies were provided by Calvo et al. (2014), Canellas et al. (2015), and Olk et al. (2018). The field studies, however, largely involved only one or two site–year combinations. A notable exception reported significant increases in soybean yield components collected by hand in Uruguayan farm trials, which across 6 years amounted to 85 sites (Izquierdo and Pintos, 2021). Overall, there is a paucity of results on humic product efficacy for numerous field locations and across years. Hence the question of whether positive crop responses to humic products can be generally expected across wide settings in crop production remains unanswered.

To address the above question, this study was conducted in US Midwestern production fields in the maize [*Zea mays* (L.)] phase of a maize—soybean [*Glycine Max* (L.) Merr.] rotation, primarily in central Iowa. Little published evidence exists on humic product efficacy in this region. We measured the maize crop responses to a previous formulation of a liquid humic product, Yield Igniter®,[1] created through alkaline extraction of leonardite ore. Humic product efficacy was evaluated through two complementary approaches. First, we measured maize grain yield through mechanized-combine and yield-component samples for five site—year combinations at three production field trials in central Iowa. These studies had replicated field-long treatment strips which compared humic product applications to unamended controls. In three of these five, we measured leaf area, presuming that the area of each leaf reflects the favorability of growing conditions at the time when the leaf developed (Eik and Hanway, 1965). Second, for a much broader survey of on-farm fields, we determined maize biomass and grain weight in yield-component samples that were hand-collected at physiological maturity from demonstration strips of humic product application, paired with corresponding yield-component samples from adjacent, unamended maize rows. Such paired samples were collected from 30 to 35 production fields annually for three growing seasons (2009–2011), mostly in central Iowa but also including additional sites across Iowa and from Nebraska (NE) and South Dakota (SD). This supplemental approach is intended to determine the trends across a wider geographic region, but limited resource and logistical challenges during its implementation compelled some sacrifices in scientific rigor. The combination of both approaches is intended to provide a uniquely extensive yet replicated database for evaluating the magnitude and reproducibility of maize grain yield responses to this humic product under conventional on-farm production practices in a high-yielding region. This study does not address potential mechanistic explanations for such responses.

[1]Reference to any specific commercial product is only for the information of the public and does not constitute endorsement or recommendation by the US government.

MATERIALS AND METHODS

Study Sites

Weather Patterns

Field experiments with replicated field-long treatment strips were conducted in 2010, 2011, and 2013 at three sites near the communities of Conrad and Radcliffe in central Iowa. The region is characterized by warm, subhumid summers and cold winters. Maize production in Iowa is rainfed and has traditionally displayed drought stress symptoms in July and August. In this study, annual weather patterns are described locally by measurements collected at the National Oceanic and Atmospheric Administration-National Weather Service weather station site in Marshalltown, about 22 km south of Conrad and 52 km southeast of Radcliffe.

In 2010, the total annual precipitation was 176 mm above the 30-year annual average (1971–2000) (**Table 1**). Monthly totals during the growing season (April–September) were all above average. Monthly mean temperatures during these same months did not vary dramatically from the 30-year means except for the warm August. In short, growing season conditions were mostly favorable for crop production, aside from the customary summer drought. In 2011, a dry period extended from June through October. Total annual precipitation in 2011 was 179 mm below the average. Both sites experienced the same conditions as most of Iowa: favorable growing conditions in the early part of the growing season, followed by crop drought stress during the second half. In 2013, 566 mm of precipitation fell in April and May, nearly triple the long-term average (192 mm). The remainder of the 2013 growing season, June–October, reverted to drier than normal conditions with a total of 259 mm of precipitation, 277 mm below the average. The wet soil conditions of the early growing season thus abruptly turned to dry conditions beginning in June. The annual mean temperature for 2013 was only 0.9°C below the average.

The on-farm survey was conducted in 2009–2011, for which we describe state-averaged weather patterns in Iowa. Temperatures in 2009 were mostly cool (Iowa Department of Agriculture and Land Stewardship, 2009), especially in the midsummer months. State annual precipitation averaged 1,017 mm, 10% above the long-term average of 927 mm. This combination of little heat stress and moderate precipitation, particularly during the growing season, made 2009 a favorable year for crop production.

In 2010, the state annual precipitation was 1,146 mm statewide, 24% above the long-term average and the second wettest year in the 138-year record of the state at that time. Every month, except October, had greater than average precipitation and the year began with a heavy amount of snowpack that served to saturate the soil profiles in the early growing season. Temperatures in 2010 during the summer months were marginally warmer than the average, except for the month of August when the monthly mean temperature was 2.2°C greater than the 30-year mean. For 2011, temperatures were moderate to slightly cooler than normal for January through June. That trend was broken in July with the temperature above the normal, and episodic high temperatures over 38°C at some locations in the

state in August. Precipitation across the state varied widely but was generally dry, similar to the Marshalltown weather station. By November, 68% of Iowa was classified as being in a drought condition. Hence, conditions were mostly favorable for the first half of the growing season, followed by soil moisture deficits in the second half.

Soil Types

Central Iowa Trials With Replicated Treatment Strips

Soils in central Iowa were formed on recent glacial till of the Des Moines Lobe (Wisconsin glaciation period), with a cover of wind-blown loess, and are highly productive for crop production. Treatment strips in a study near Radcliffe, IA, traversed all three Mollisols of the Clarion (fine-loamy, mixed, mesic Typic Hapludoll)-Nicollet (fine-loamy, mixed, mesic Aquic Hapludoll)-Webster (fine-loamy, mixed, mesic Typic Haplaquoll) soil association (USDA Soil Conservation Service, 1985b). All three soils have deep and fertile surface soil horizons, with high soil organic matter and good water-holding capacity. For example, the 2010 soil sampling found a mean soil organic matter content (loss on ignition) of 37.9 g kg^{-1}, pH (1:1 water) of 6.42, and cation exchange capacity (sum of NH_4-extractable cations) of 17.4 $cmol_c$ kg^{-1}.

Two replicated studies were also conducted within 2 km of each other near Conrad, IA. Both fields were mapped within the Tama-Muscatine-Downs soil association (USDA Soil Conservation Service, 1977), which are Mollisols with deep surface horizons of high fertility, soil organic matter content, and water-holding capacity. The field on the Ag Logic Distributors research farm ("Conrad" field) consisted predominantly of the Tama soil (fine-silty, mixed, mesic Typic Argiudoll), with a small inclusion of Sawmill silt loam soil (fine-silty, mixed, mesic Cumulic Haplaquoll) in a natural drainage path. Treatment strips at the nearby on-farm "Whitten" field included the Tama, Muscatine (fine-silty, mixed, mesic Aquic Hapludoll), Garwin (fine-silty, mixed, mesic Typic Haplaquoll), and Sawmill soil types. A 2010 soil sampling in this field reported a mean soil organic matter content of 52.1 g kg^{-1}, pH of 6.54, and cation exchange capacity of 23.6 $cmol_c$ kg^{-1}.

On-Farm Survey

The exact locations of the on-farm demonstration strips as recorded by global positioning system (GPS) technology were lost during personnel changes. Hence, we describe in general terms their local landscapes and soil types (Prior, 1991). Most demonstration strips were in maize—soybean rotation fields in the Des Moines Lobe, Iowan Surface, and the Southern Iowa Drift Plain. These three landforms are characterized by Mollisols. A large majority of the soils within these landforms were formed under tallgrass prairie. While most surface soils in the Des Moines Lobe area were formed in glacial till, some soils of the Iowa Surface have overlying mantles of loess, and the Southern Iowa Drift Plain largely consists of loess surface soils over older glacial till deposits and are more eroded with deeper valleys than the other two landforms.

In 2011, six sites were also sampled in the Sand Hills region of north-central NE and south-central SD. Three were dryland, and three were irrigated due to low annual precipitation (508–570 mm yr^{-1}). The six fields were located within Rock County NE, and Tripp County, SD. Soil orders in Rock County range from relatively young soil orders of Entisols and Inceptisols, to a few Mollisols (USDA Soil Conservation Service, 1985a). The Els-Valentine-Tryon soil association dominates the county. These are somewhat excessively to well-drained soils of sandy texture having low fertility and water-holding capacity. Tripp County, SD, has more diverse soils ranging in texture from fine sands to loams and clayey soils that are mostly of the Entisol and Mollisol soil orders (USDA Soil Conservation Service, 1979). The Millboro–Lakoma soil association is predominant, which has well-drained silty clays of moderate to low fertility.

Field Designs and Management Practices

The Radcliffe field experiment in 2010 and 2011 and the Whitten field experiment in 2010 were each organized in randomized complete block designs. The plots were field-long treatment strips with maize rows at 76.2 cm spacing. Treatments in both fields compared different application timings of the previous formulation of the Yield Igniter® humic product. This product was created through alkaline extraction of leonardite ore and contained about 30 g kg^{-1} of humic acid and 1.2 g kg^{-1} of fulvic acid (California Department of Food and Agriculture test). The rate of humic product application was 3.5 L product ha^{-1}, following the recommendation of the manufacturer. The humic product was diluted with tap water to 94 L ha^{-1} and applied to the fields using standard agricultural sprayers, except for the in-furrow treatment at Conrad. In most cases, the nozzles were TeeJet XRC, and in some cases TeeJet drift guard (DG) nozzles were used, depending on the daily wind conditions, to maximize leaf interception and minimize wind drift. The pressure ranged from 207 to 310 kPa. At Radcliffe, the treatments compared a sole application at either preemergence, third leaf growth stage (V3), as defined by the leaf staging method that excludes the cotyledon leaf (Abendroth et al., 2011), or the sixth leaf stage (V6), compared to the unamended control. In the Whitten field, the treatments compared V3 and V6 applications against an unamended control. Both field experiments had four replications. In the 2011 Radcliffe field, one replicate was removed from the statistical analysis of the combine-measured grain yield because saturated soil conditions impaired the early season growth of maize in this replication. Each treatment strip contained 6 rows with 76.2-cm spacing in the Radcliffe field and 24 rows with 76.2-cm spacing in the 2010 Whitten field. Row length in both the fields was about 760 m. The 2011 Radcliffe plots were placed in the same locations as in 2010 by using the GPS and geographic information system technologies.

The Conrad field in 2013 contained two adjacent studies. Each was organized in a randomized split-plot design with four (north block) or five (south block) replicates. This design was intended to minimize data variability that could have arisen from soil drainage differences across this field. Main plot treatments in the north block compared three maize cultivars having relative maturity (RM) ratings (in days) of 100, 105, and 110, and subplots

compared an unamended control to in-furrow application of the humic product with planting at the recommended rate. An adjacent south block had the same design except that the 105-day variety was omitted and the humic product was broadcast applied at the V5 growth stage. Row lengths in each Conrad block were about 62 m, and each plot had four maize rows of 76.2 cm spacing.

Thus, the timing of the humic product applications at the replicated field sites varied from in-furrow application with planting to V6. All other crop management practices across the entire fields were decided by the land managers, including cultivar, planting date, population density, fertilizer application rates, pest management, and harvesting practices. They followed management practices that are conventional for US maize production, and all fields received conventional tillage.

In each year of the on-farm survey, the Yield Igniter® humic product was applied as demonstration strips in maize fields of collaborating farmers across much of central, southern, and northern Iowa, and also in 2011 at the six sites in SD and NE. The product was applied at post emergence through standard pesticide sprayers at early maize growth stages, not later than V6. The humic product was applied by the manufacturer in demonstration strips for all survey fields in 2009 and 2010 at their recommended rate of 3.5 L product ha^{-1}, diluted with tap water to field-relevant volumes, while in 2011, some farmers performed the demonstration strips in their own fields. Following product application, the demonstration strips were not visited again and were left to farmer supervision until sampling time. While a few cooperating farmers participated in multiple years, their demonstration strips were not located on the same rows within those fields in all the years. Therefore, each paired comparison in each year represents a novel site location. Conventional crop management practices were followed and were selected by the managing farmer, including maize cultivar.

At crop physiological maturity in 2009, 2010, and 2011, about 30–35 production fields were hand-sampled for yield components across distinct regions in Iowa or adjacent states. All their data are presented here except for two fields in 2009, due to uncertain plot labels, and two fields in 2011, due to uncertain sample labels. In most cases, each field had only one demonstration strip. For the few fields where multiple demonstration strips were established, either one strip was randomly selected for sampling or all strips within each field were sampled and their means were calculated to represent that field.

Plant and Soil Sampling

Maize Grain Yield Measurements by Combine and Weigh Wagon

For the central Iowa trials with replicated treatments strips, grain yield and moisture were recorded by mechanized combine. Yield monitor was used at the Whitten site for each field-long treatment strip, and we report the means of each treatment strip. At the Radcliffe and Conrad sites, weigh wagons were used to record grain mass and grain moisture (measured with a hand-held meter) along with yield monitor data that were hand-recorded for the field-long treatment strips. Weigh wagons were calibrated annually to the nearest 0.9 kg by their manufacturer, and then the weigh wagons were calibrated against the combine-yield monitors in each field prior to harvesting. Grain yield data from all sites were expressed as dry volume by adjusting them to the standard equivalent of 15.5% market moisture. For the on-farm survey, grain yield measurements by either combine-yield monitor or weigh wagon were not made available by any of the collaborating farmers. We chose not to confront their reluctance, as public and private sector advisors often discourage farmers from sharing their data.

Yield Components

For both years at the Radcliffe site and the on-farm surveys, plant samples were hand-harvested after maize kernels had achieved physiological maturity to determine yield components. They were collected in areas of uniform growth and similar soil type across all treatment strips and unamended controls for each field. Samples were collected from the Radcliffe field in the Nicollet soil for all treatment strips, and from the Whitten field in the Tama soil type.

Specifically, except the 2009 on-farm survey, a 1-m length section of one maize row was harvested by selecting an area of representative crop growth in each demonstration strip, then cutting seven evenly spaced healthy plants at ground level, and then separating the ears from the stover. This procedure was repeated nearby, within a limited number of maize rows outside the demonstration strip, to collect an unamended control sample while avoiding both edaphic differences and border effects. A more laborious method was used in the initial 2009 on-farm survey, by which a representative plant was sampled in each of eight consecutive rows at predetermined distances into each demonstration strip and, similarly, into the area of untreated plants immediately next to each demonstration strip. Soil samples were collected from the Radcliffe and Whitten fields in 2010 and from the on-farm survey in 2010 and 2011. Specifically, four soil cores were taken to the 15-cm depth with a 3.18-cm diameter probe in a row traversing the 1 m-hand-harvested section or (2009 survey only) in an untrafficked interrow at the final sampled plant, then composited within each treatment strip, and stored at 4°C until later analyses for nutrient contents and other soil properties.

All maize stover samples were oven-dried at 55°C in forced air dryer rooms, then immediately measured for oven-dry weights and mechanically shredded. Subsamples were taken from the shredded stover for later grinding through a Wiley mill (1 mm mesh screen) and then from a Cyclone mill (Udy Corporation, Fort Collins, CO) to a powder consistency. Maize ears were dried in 2009 in the same dryer rooms as were the stover, but in all subsequent years, they were placed in plastic mesh bags and hung for drying at ambient temperatures before being stored in airtight bins for subsequent measurements. Maize ear grains for the replicated field trials and the 2011 on-farm survey were later hand-shelled and passed through a mechanical seed counter for determining the 100-kernel weight. Total kernel weights of the hand samples were recorded, and kernel moisture was recorded by a moisture meter. Maize grain moisture content was also determined by a standard oven-drying method (ASAE, 1988). For all sites, the grain weight of each sample was then calculated and

TABLE 1 | Monthly precipitation amounts and mean temperatures in 2010, 2011, and 2013, and their deviations from 30-year means (1971–2000), for the replicated field trials in central Iowa.

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Annual
Monthly precipitation (mm)													
30-Yr Mean	24	27	61	84	108	142	116	122	90	67	55	31	927
2010	19	26	15	110	140	201	156	150	204	13	44	20	1103
Deviation	*–5*	*–1*	*–46*	*26*	*32*	*59*	*40*	*28*	*114*	*–54*	*–11*	*–11*	*176*
2011	20	10	29	104	130	117	83	31	69	36	58	61	748
Deviation	*–4*	*–17*	*-32*	*20*	*22*	*–25*	*–33*	*–91*	*–21*	*–31*	*3*	*30*	*–179*
2013	30	34	59	161	405	100	40	4	55	60	62	20	1029
Deviation	*6*	*7*	*–2*	*77*	*297*	*–42*	*–76*	*–118*	*–35*	*–7*	*7*	*–11*	*102*
Monthly mean temperature°C													
30-Yr Mean	–8.2	–4.7	2.1	9.1	15.6	21.0	23.1	21.6	17.0	10.3	2.1	–5.4	8.6
2010	–10.4	–9.8	2.8	12.4	15.5	21.9	2.5	23.7	16.0	11.3	3.0	–7.6	8.5
Deviation	*–2.2*	*–5.1*	*0.7*	*3.3*	*–0.2*	*0.9*	*0.5*	*2.2*	*–1.0*	*1.0*	*1.0*	*–2.2*	*–0.1*
2011	–10.0	–5.2	0.8	7.7	14.8	20.7	25.4	21.8	15.1	11.5	4.1	–1.6	8.8
Deviation	*–1.8*	*–0.5*	*–1.3*	*–1.4*	*–0.8*	*–0.3*	*2.3*	*0.2*	*–1.9*	*1.2*	*2.0*	*3.8*	*0.1*
2013	–6.3	–5.4	–2.8	6.1	15.0	20.7	22.1	22.1	19.2	10.2	0.4	-9.0	7.7
Deviation	*2.0*	*–0.7*	*–4.9*	*–3.0*	*–0.7*	*–0.3*	*–1.0*	*0.5*	*2.2*	*–0.1*	*–1.7*	*–3.6*	*–0.9*

extrapolated to a hectare basis to present the grain yield as if each field were wholly homogenous. Given the soil type variability that can occur within field-long treatment strips, such extrapolations primarily express the yield response to the humic product only at the sampling site. Grain weights at all sites were expressed as dry volume by adjusting to the standard equivalent of 15.5% market moisture.

The lengths of air-dried cobs were measured for all hand-samples, and the cobs were then oven-dried for 3 days at 120°C and immediately measured for dry weight. The dried cob weights were then added to those of the 1-m stover samples to report total aboveground stover weight.

From the replicated trials at the Radcliffe and Whitten fields, and from the 2010 on-farm survey, subsamples of harvested grains were initially air-dried to no more than 100 g kg^{-1} moisture content and then stored in airtight plastic bags until later analysis for protein, oil, and starch contents using near-infrared spectroscopic procedures (Iowa Grain Quality Initiative, 2004).

Plant and soil samples were analyzed for predetermined sets of properties as offered by a commercial analytical laboratory. Total N analyses were performed on plant stover and grain through micro-Kjeldahl digestion and colorimetric determination of the extracted N content. Plant stover and grain analyses for all other nutrients (P, K, Mg, Ca, S, Zn, Mn, Cu, Fe, and B) were performed using wet digestion in nitric acid with 30% hydrogen peroxide and determination by inductively coupled plasma-mass spectrometry. Plant Na and Al were also measured, but their results are not reported due to their erratic, and at times absent, concentrations and relatively low precision of analysis.

Methods for measuring soil extractable nutrients, pH, buffer pH, organic matter, and cation exchange capacity followed the Recommended Chemical Soil Test Procedures for the North Central Region, Publication No. 221 Revised (Denning et al., 1998). Soil pH was determined in a 1:1 (w:v) slurry in water, and buffer pH by the Sikora Buffer method. Soil organic matter content was determined through loss on ignition. Available soil P was determined colorimetrically from a Bray 1 extraction (Bray and Kurtz, 1945). Available soil cations were extracted with 1 M ammonium acetate and analyzed by inductively coupled plasma-mass spectrometry.

In the Radcliffe and Whitten field trials, all maize leaves were destructively measured for leaf area measurement on selected plants in areas of uniform growth. Triplicate sets of three plants were marked at the V5 or V6 crop stage for three in-field samplings. The first leaf area measurement was at the V5 or the V6 growth stage. At the same time, flagging tape was used to mark the internode between the V6 and V7 leaves of the other two plant sets. One of these sets was later used for the second measurement of the leaf area at the V11 or V12 growth stage. Flagging tape was also used then to mark the internode between the V11 and V12 leaves of the final plant set for the third leaf area measurement soon after full tassel (transition from vegetative to reproductive growth stage). For each leaf, its length and maximum width were measured to calculate leaf area by the method developed by Montgomery (1911) using the following equation:

$$\text{Length (cm)} \times \text{Maximum Leaf Width (cm)} \times 0.75 = \text{Leaf Area (cm}^2\text{)} \quad (1)$$

Total plant leaf area was the sum of the areas from all the leaves of each plot. The first two leaves of each plant were often lost already at the first leaf area measurement, due to senescence or physical damage.

Statistical Analyses

All experimental data from the central Iowa trials with replicated treatment strips were analyzed by ANOVA *via* the Proc Mixed

procedure of SAS Version 9.2 software (SAS Institute, 2010) with randomized complete block or split-plot design programs to examine main plot treatment, split-plot treatment, and interaction effects. Paired *t*-tests were conducted by the least significant difference method. At the Conrad site, the cultivar—humic product interaction terms for both the blocks were insignificant ($P > 0.10$) and are not shown (**Table 2**).

For the on-farm survey, we used SAS Version 9.2 (SAS Institute, 2010) to perform ANOVA for evaluating humic product application as the independent variable and the difference between humic-treated and control plant samples at each site to calculate each of the dependent variables: maize yield components and nutrient concentrations in grain, stover, and soil. Each site was treated as a single replication. The site factor was treated as a random effect in a two-factor ANOVA comparing the control and humic treatment group (Factor 1) and three specific years of 2009–2011 (Factor 2), these two factors being treated as fixed effects, and then examining the interaction between the group and the year.

Field crop responses to humic products can in cases be modest, but they can also change gradationally with local environmental conditions (Olk et al., 2021). For example, Olk et al. (2021) found that maize growth responses to a humic product were frequently weakly positive across three soil types in four growing seasons, but they were much more likely to reach statistical significance ($P < 0.10$) in droughty conditions. Adhering to a preselected level of significance is somewhat a subjective decision, and useful information can be lost regarding the patterns of gradational responses. Therefore, we report individual levels of significance (P) for key plant growth parameters to depict gradational responses more accurately. At the same time, we summarize large datasets of plant and soil parameters having secondary value by setting a benchmark level of significance at $P = 0.10$.

RESULTS

Replicated Field Trials in Central Iowa

Mechanized Grain Yield

In the 2010 Radcliffe field, all three timings of product application provided for grain yields (measured by weigh wagon) that were 0.29 to 0.38 Mg ha^{-1} (2 to 3%) greater than the grain yield of the unamended control (**Table 2**). The main plot treatment was highly significant ($P = 0.0095$). When comparing each treatment with the control by paired *t*-tests, all differences ranged from significant ($P < 0.05$) to highly significant ($P < 0.01$). In 2011, the three treatments similarly provided yield increases of 0.22–0.35 Mg ha^{-1} (2–3%). With only three field replications in 2011, the main plot treatment approached benchmark significance ($P = 0.125$). Paired *t*-tests for individual treatments found levels of significance varying from 0.033 to 0.126.

In the 2010 Whitten field, the two application timings increased the grain yield (measured by combine yield monitor) by 0.15 and 0.19 Mg ha^{-1} (1%) more than the control in this traditionally high-yielding field (**Table 2**). The main plot treatment was insignificant ($P = 0.283$). Paired *t*-tests were insignificant ($P = 0.227$ and 0.152).

In the 2013 Conrad field northern block, maize grain yield was significantly greater ($P = 0.038$) for the 105-RM and 110-RM varieties than for the 100-RM variety (**Table 2**). At the subplot level, humic product application increased the grain yield across all three maize varieties by 0.42 Mg ha^{-1} (4%), which was significant at $P = 0.064$. Paired *t*-tests found significant ($P < 0.05$) differences among varieties when comparing the 100-RM variety against each of the longer-duration varieties. In the Conrad field southern block, the 110-RM maize variety again had significantly ($P = 0.037$) greater grain yield than did the 100-RM variety (**Table 2**). Humic product application again provided for a numeric increase in the grain yield above the unamended control across both varieties, but only by an insignificant amount of 0.23 Mg ha^{-1} (2%, $P = 0.212$) (**Table 2**). Summarizing the replicated field trials, combine-measured grain yield increased numerically with humic product application in all five site—years, and its magnitude was generally larger in those site—years where the control had relatively lower grain yields. Thus, the yield response was larger in the lower-yielding Radcliffe and Conrad North fields but was of the smallest magnitude in the high-yielding Whitten field. These variable responses, in turn, affected the degree of statistical significance of the yield response for each site—year. In all cases, they were modest proportional increases.

Yield Components at the Radcliffe Field

In 2010, grain weights of the hand-collected samples, as extrapolated to a hectare basis, increased numerically with humic product application by 0.36 to 1.18 Mg ha^{-1} (2–7%), and the increases were largest with the earlier application (**Table 3**). But the main plot treatment was insignificant ($P = 0.70$), and paired *t*-tests between the control and each application time also showed no significant differences ($P > 0.10$). In 2011, for the same field, grain weights again increased numerically with humic product application, by 0.44 to 1.31 Mg ha^{-1} (3–8%), and the increases were, again, largest with the earlier application. In this year, the main plot treatment approached benchmark significance ($P = 0.156$), and paired *t*-tests showed a significant difference ($P = 0.04$) between the V3 application and the control.

In 2010, all application treatments had non-significant effects ($P > 0.10$) on cob length, as determined by paired *t*-tests with the control. In 2011, however, both the V3 and V6 applications of the humic product caused significant ($P = 0.074$ and $P = 0.026$, respectively) increases in the cob length. The preemergence application caused a slightly weaker yet still positive response that approached benchmark significance ($P = 0.13$), and the overall main plot treatment similarly approached benchmark significance ($P = 0.110$).

For 100-kernel weight in 2010, the main plot treatment was significant at $P = 0.108$, and preemergence application in 2010 caused a significant positive ($P = 0.022$) response, as determined by a paired *t*-test with the control. For the other treatments in 2010 and all treatments in 2011, the paired *t*-tests showed non-significant ($P > 0.10$) differences from the control. All differences from the control in 2011 were 1% or less.

TABLE 2 | Maize grain yield measured by combine for replicated field trials at Radcliffe, Whitten, and Conrad with the application of the humic product at preemergence, third leaf stage (V3), fifth leaf stage (V5), or the sixth leaf stage (V6).

2010 Radcliffe			
Humic treatment	**Maize grain yield (Mg ha^{-1})**	**Probability of statistical significance**	
		Humic main plot	0.010
Control	13.20		
Pre-emergence	13.49	Paired *t*-test vs. Control	0.012
V3	13.54	Paired *t*-test vs. Control	0.002
V6	13.58	Paired *t*-test vs. Control	0.005
2011 Radcliffe			
		Humic main plot	0.125
Control	12.54		
Pre-emergence	12.89	Paired *t*-test vs. Control	0.033
V3	12.76	Paired *t*-test vs. Control	0.126
V6	12.83	Paired *t*-test vs. Control	0.066
2010 Whitten			
		Humic main plot	0.283
Control	13.90		
V3	14.05	Paired *t*-test vs. Control	0.227
V6	14.09	Paired *t*-test vs. Control	0.152
2013 Conrad North block[a]			
		Varietal main plot	0.038
100 RM[b]	9.95		
105 RM	11.17	Paired *t*-test vs. 100 RM	0.017
110 RM	10.93	Paired *t*-test vs. 100 RM	0.043
Control	10.48		
Humic at Planting	10.90	Paired *t*-test vs. Control	0.064
2013 Conrad South Block[a]			
		Varietal main plot	0.037
100 RM	10.39		
110 RM	11.13	Paired *t*-test vs. 100 RM	0.037
Control	10.65		
Humic at V5	10.88	Paired *t*-test vs. Control	0.212

[a] *Cultivar—humic product interaction terms for both blocks were insignificant ($P > 0.10$) and are not shown.*
[b] *Relative maturity rating (estimated in day units).*

In 2010, all application treatments had non-significant effects ($P > 0.10$) on stover weight. In 2011, the V3 application increased stover weight by 12% ($P = 0.099$). The preemergence treatment increased stover weight by 7% but was not significant ($P = 0.30$). Across both the years, the only cases of humic product application that significantly ($P < 0.10$) affected the grain content of protein, oil, or starch were found in 2010 (data not shown). Specifically, V3 application increased ($P = 0.085$) the protein content, and V6 application increased ($P = 0.099$) the starch content. No numeric trends were apparent in the remaining results.

For grain or stover concentrations of N, P, K, Mg, Ca, S, Zn, Mn, Cu, Fe, and B in either year, the only nutrients that significantly responded ($P < 0.10$) to any humic product treatment were decreases in stover Mg ($P < 0.050$) with V3 application and stover Zn ($P < 0.097$) with V6 application in 2010, and an increase in grain Mg ($P = 0.041$) with V3 application (data not shown) in 2011. No numeric trends were apparent in the remaining results (data not shown).

Of the soil properties measured in the 2010 Radcliffe and Whitten fields (soil organic matter content, pH, buffer pH, cation exchange capacity, total N, extractable P, K, Mg, Ca, S, Fe, Zn, Mn, Cu, and B), the humic product effects were significant ($P < 0.10$) at Radcliffe for only increased extractable Cu ($P = 0.065$) and at Whitten for only increased extractable Mn (0.088) (data not shown). Paired *t*-tests for individual treatments found significant ($P < 0.10$) increases at the Radcliffe field for only Cu with preemergence ($P = 0.018$) and V3 applications ($P = 0.027$) and at the Whitten field for only K ($P = 0.083$), Mn ($P = 0.016$), and Cu ($P = 0.049$) with the V3 application. Only occasional numeric trends were apparent in the remaining results, in no meaningful pattern (data not shown).

TABLE 3 | Maize yield components at the Radcliffe site in 2010 and 2011.

Humic treatment		Probability (P) of statistical significance	
2010 Grain weight (Mg ha^{-1})			
		Humic Main plot	0.697
Control	16.50		
Pre-emergence	17.68	Paired *t*-test vs. Control	0.286
V3	17.31	Paired *t*-test vs. Control	0.460
V6	16.86	Paired *t*-test vs. Control	0.740
2011 Grain weight (Mg ha^{-1})			
		Humic Main plot	0.156
Control	16.25		
Pre-emergence	16.70	Paired *t*-test vs. Control	0.403
V3	17.57	Paired *t*-test vs. Control	0.037
V6	17.00	Paired *t*-test vs. Control	0.183
2010 Cob length (mm)			
		Humic Main plot	0.969
Control	158.6		
Pre-emergence	159.9	Paired *t*-test vs. Control	0.797
V3	157.6	Paired *t*-test vs. Control	0.833
V6	159.2	Paired *t*-test vs. Control	0.945
2011 Cob length (mm)			
		Humic Main plot	0.110
Control	160.1		
Pre-emergence	164.4	Paired *t*-test vs. Control	0.128
V3	165.3	Paired *t*-test vs. Control	0.074
V6	167.2	Paired *t*-test vs. Control	0.026
2010 One hundred-kernel weight (g 100 kernel^{-1})			
		Humic Main plot	0.108
Control	25.13		
Pre-emergence	27.15	Paired *t*-test vs. Control	0.022
V3	25.70	Paired *t*-test vs. Control	0.460
V6	25.90	Paired *t*-test vs. Control	0.323
2011 One hundred-kernel weight (g 100 kernel^{-1})			
		Humic Main plot	0.722
Control	31.66		
Pre-emergence	31.34	Paired *t*-test vs. Control	0.495
V3	31.77	Paired *t*-test vs. Control	0.804
V6	31.39	Paired *t*-test vs. Control	0.556
2010 Stover weight (Mg ha^{-1})			
		Humic Main plot	0.650
Control	11.80		
Pre-emergence	12.24	Paired *t*-test vs. Control	0.519
V3	11.49	Paired *t*-test vs. Control	0.651
V6	11.50	Paired *t*-test vs. Control	0.669
2011 Stover weight (Mg ha^{-1})			
		Humic Main plot	0.322
Control	12.76		
Pre-emergence	13.66	Paired *t*-test vs. Control	0.295
V3	14.28	Paired *t*-test vs. Control	0.099
V6	13.13	Paired *t*-test vs. Control	0.653

Leaf Area

At the Radcliffe site in both 2010 and 2011 and at the 2010 Whitten field, all humic product treatments provided significantly ($P < 0.10$) greater total leaf area than did the unamended control (**Table 4**). The increases reached as high as 12% for the preemergence application at the 2011 Radcliffe site. Main plot humic treatment effects were also significant for the 2010 Radcliffe ($P = 0.0138$), 2011 Radcliffe ($P = 0.0701$), and 2010 Whitten ($P = 0.0103$) sites.

Leaf area by individual leaves showed infrequent positive responses to the humic product by the earliest leaves; we attribute them to random variation among plots when selecting healthy plants at an early growth stage. Positive responses to humic product application became consistent no earlier than the 7th leaf for the preemergence and V3 applications and the 10th or 11th leaf for the V6 applications (**Figures 1A–C**). The increases became consistently significant ($P < 0.10$) for the Radcliffe preemergence application at about the 7th leaf (2011) or 10th leaf (2010) and remained significant for most leaves through the 17th or the 18th leaf (**Table 4**). The V3 application showed a weaker response but of comparable timing. Significant increases for the V6 application became consistent at all sites starting at about the 12th leaf and remained significant for most leaves until the 15th (Whitten) or 18th leaf (Radcliffe). Thus, the benefit to leaf area of the V6 application was somewhat delayed compared to those of earlier applications. Numeric trends suggested that leaf area growth might have been depressed briefly after the foliar applications compared to the control, specifically for both the V3 and V6 applications at the 2010 Radcliffe site, V6 application at the 2011 Radcliffe site, and V3 application at the 2010 Whitten site (**Figures 1A–C**). This decrease reached statistical significance ($P = 0.037$ and $P = 0.004$, respectively) for V6 applications in both years at Radcliffe.

On-Farm Survey

The vast majority of the sites were located on the Des Moines Lobe, with smaller numbers of farms on the Iowa Surface and very few on the Southern Iowa Drift Plain. Maize responses did not clearly differ among these three geomorphic surfaces; therefore, all Iowan sites are presented as one set. The 2011 NE and SD sites did differ clearly from the Iowa sites, so we present the 2011 results both as one complete set and also with the NE and SD sites separated from the Iowa sites.

In multi-year combined statistical analyses for the on-farm survey data, agronomic yield components showed a statistical significance. However, the year factor was significant for all measures. This was not surprising, given that weather patterns substantially affect crop growth and soil nutrient availability. In addition, for each year, many sample sites were not in the same fields as in the previous years. Therefore, we initially present these on-farm survey measures by individual year. Humic product—year interactions were insignificant ($P > 0.10$) for all plant and soil measurements, which are not shown.

In each year, grain weight per hectare, as extrapolated from the yield component samples, increased numerically with humic product application for the vast majority of farms. In 2009,

TABLE 4 | Total leaf area and level of statistical significance (P)[#] by individual leaf areas for three replicated field trials.

	2010 Radcliffe				2011 Radcliffe				2010 Whitten		
Treatment	**Control**	**Pre-emerge**	**V3**	**V6**	**Control**	**Pre-emerge**	**V3**	**V6**	**Control**	**V3**	**V6**
Total area (cm^2)	7040	7516	7341	7282	6625	7401	7271	7389	6694	7427	7362
P		*0.002*	*0.024*	*0.057*		*0.025*	*0.048*	*0.026*		*0.010*	*0.009*
Leaf	Level of statistical significance (P) by individual leaf										
V1	–	–	–	–	–	–	–	–	–	–	–
V2	–	–	–	–	–	0.301	**0.018**	0.517	–	–	–
V3	–	0.466	0.568	0.923	–	0.804	0.575	0.673	–	0.446	0.929
V4	–	0.412	**0.039**	**0.055**	–	0.828	0.604	0.629	–	0.736	0.192
V5	–	0.786	0.152	0.258	–	0.501	0.278	0.418	–	0.871	0.460
V6	–	0.758	**0.096**	**0.037**	–	0.850	0.655	0.700	–	0.882	0.304
V7	–	0.906	0.681	0.214	–	**0.004**	**0.001**	**0.004**	–	0.984	0.491
V8	–	0.345	0.373	0.970	–	**0.004**	**0.008**	0.190	–	0.540	0.612
V9	–	**0.072**	**0.084**	0.661	–	0.192	0.837	0.458	–	0.939	0.930
V10	–	**0.006**	**0.034**	0.367	–	0.228	0.479	0.728	–	0.947	0.715
V11	–	**0.005**	**0.019**	0.119	–	**0.033**	0.760	0.325	–	0.415	0.272
V12	–	**0.002**	**0.021**	**0.049**	–	**0.014**	0.228	**0.006**	–	0.406	**0.063**
V13	–	**0.004**	**0.055**	**0.025**	–	**0.044**	0.119	**0.012**	–	**0.012**	**0.007**
V14	–	**0.010**	0.165	**0.092**	–	0.139	0.144	**0.059**	–	**0.027**	0.174
V15	–	**0.034**	0.368	**0.006**	–	**0.054**	**0.029**	**0.017**	–	**0.014**	**0.039**
V16	–	0.119	0.372	**0.095**	–	**0.042**	0.111	**0.033**	–	0.260	0.458
V17	–	0.408	0.538	0.323	–	**0.057**	**0.091**	**0.036**	–	0.167	0.286
V18	–	**0.037**	0.295	**0.023**	–	0.136	0.125	**0.047**	–	0.102	**0.058**
V19	–	0.675	0.696	0.640	–	–	–	–	–	0.152	0.164
V20	–	0.828	0.656	0.427	–	–	–	–	–	–	–

Significant values (P < 0.100) for individual leaves are shown in bold font.
[#]*Statistical significance for total leaf areas and individual leaves determined by paired t-tests against the control.*

the grain weight increase occurred at 25 of 30 farms, or 83% (**Figure 2A**). Mean grain weight across all 30 farms increased with the product application by 5.7%, or 0.98 Mg ha^{-1} ($P < 0.0001$) (**Table 5**). In 2010, grain weight was numerically greater for 29 of 35 farms, or 83% (**Figure 2B**), and grain weight increased across the 35 farms with product application by 6.7%, or 1.05 Mg ha^{-1} ($P = 0.0002$). In 2011, 22 of 33 farms (67%) had numerically greater grain weight with humic product application (**Figure 2C**). The coarser textured, dryland production and irrigated sites in SD and NE were among the more responsive sites to product application in 2011, averaging 22% increase, or 2.9 Mg ha^{-1} ($P = 0.041$), while for the Iowa 2011 sites, the mean increase was 4.2%, or 0.68 Mg ha^{-1} ($P = 0.043$) (**Table 5**). Mean grain weight in the unamended controls of the SD and NE sites was only 81% of the mean for the Iowa controls. Across all the 3 years, grain weight increased with humic product application in 76 of 98 cases (78%). We do not propose a single explanation for the negligible or negative responses for 22 of the 98 cases, other than the observation that a few sites were excessively wet, and limited evidence suggests humic product efficacy is sharply impaired in excessively wet soils (Olk et al., 2021).

To combine the data across all the 3 years, grain weights from the 8 plants collected from the 2009 plots were adjusted to the 7-plant basis of the 2010 and 2011 seasons. The adjusted data from 2009 to 2011 were analyzed collectively for their distribution across 10 intervals of grain weights for the humic product-treated samplings and separately for the controls (**Figure 3**). The grain weights from both the unamended plots and also the humic-treated plots occurred mostly in the same ranges of grain weights; the humic product scarcely increased the grain weight beyond the maximum values achieved in the control plots. Instead, product application led to greater proportions of the medium- and high-grain weights and lesser proportions of the lower-grain weights. Mean mass across all 98 paired comparisons was 1.23 kg m^{-1} for the control and 1.31 kg m^{-1} for the treated plots, a highly significant ($P < 0.001$) increase of 0.08 kg m^{-1} (6.5%), or 1.05 Mg ha^{-1}. In short, humic product application significantly increased the grain weight, mostly by increasing what would have been lesser grain weights to more moderate weights.

Similar to grain weights, humic product application did not alter the range of cob lengths compared to that of the control for 95 of the same 98 farms across all the 3 years (**Figure 4**). Instead, humic product application again caused greater proportions of the medium-length and long cobs, with smaller proportions of the shorter cobs, compared to the control plots. With humic product application, cob length for all 95 farms increased by 3% from 16.5 to 17.0 cm, which was highly significant ($P = 0.0053$). Cob length also increased significantly for each of the 3 years from 2009 to 2011 ($P = 0.0005$, 0.0026, and 0.0033, respectively,

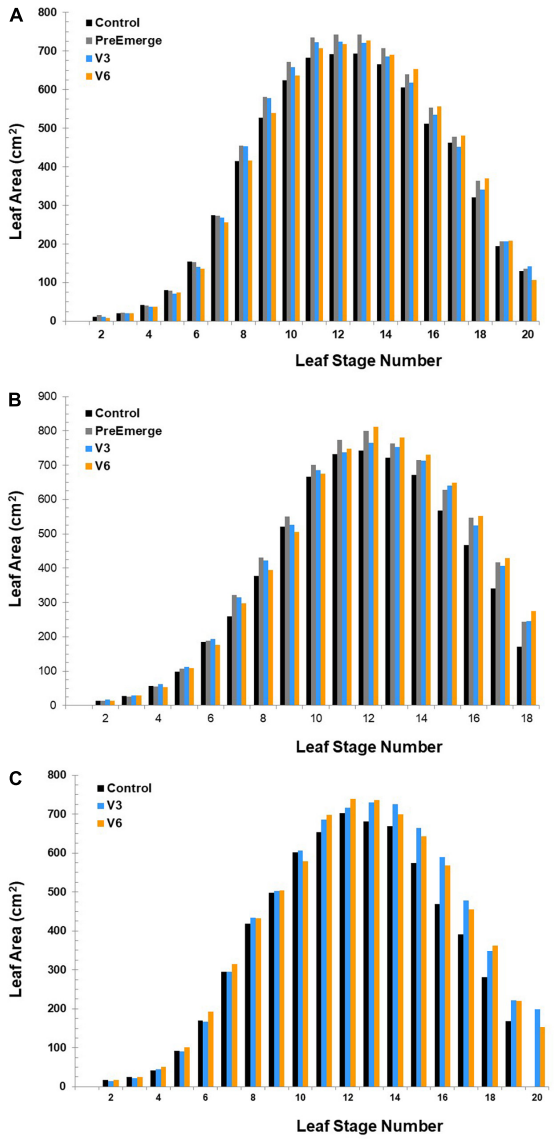

FIGURE 1 | Leaf area for the **(A)** 2010 Radcliffe field, **(B)** 2011 Radcliffe field, and **(C)** 2010 Whitten field by individual stage and time of humic product application. The V3 time of humic application is the third leaf vegetative growth stage, and V6 is the sixth leaf vegetative growth stage.

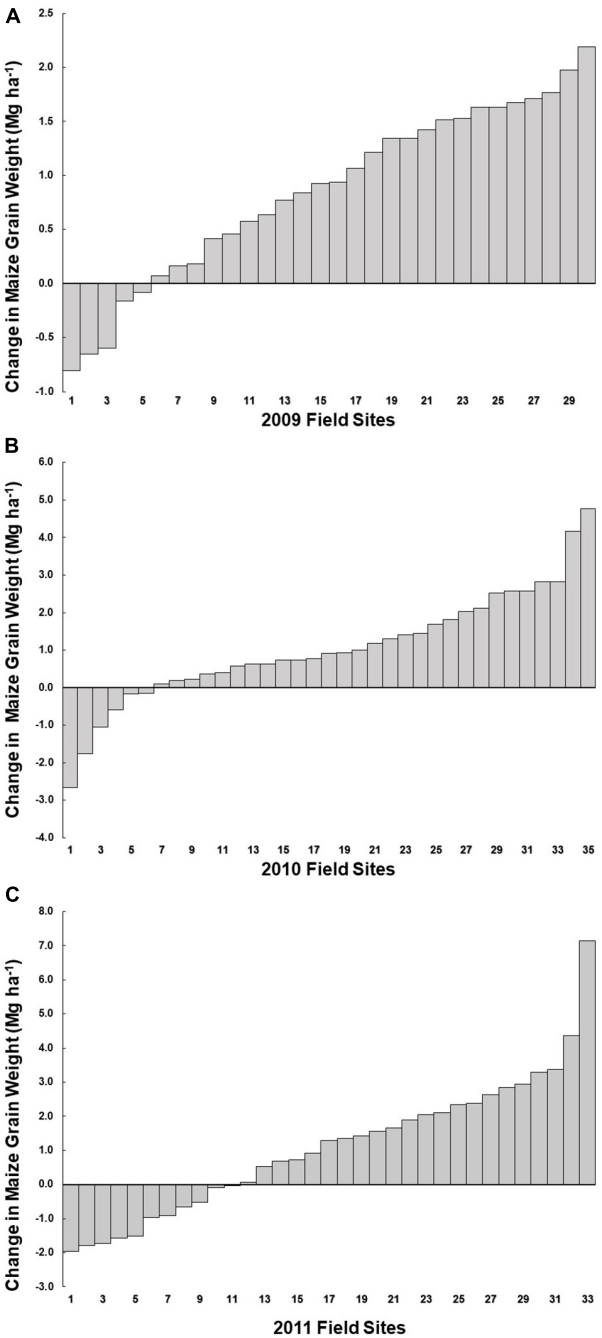

FIGURE 2 | Maize grain weight response to humic product application compared to an adjacent unamended control at on-farm survey sites in **(A)** 2009, **(B)** 2010, and **(C)** 2011. In the 2011 survey, Nebraska (NE) sites are numbered 10, 25, and 33, and South Dakota (SD) sites are numbered 14, 29, and 32.

Table 5). Like grain weight, cob length at the irrigated sites in NE and SD in 2011 responded especially well, with a 5% increase ($P = 0.031$). Using calculations presented by Nielsen (2018), our observed increase in the cob length of 0.5 cm across all 95 farms translates into an increase in the grain weight of about 0.5 Mg ha^{-1}, presuming a grain diameter of 0.4 cm, 14 rows of grain per cob, and complete kernel filling. Thus, the increased cob length with humic product application accounted for about half of the measured increase in the grain weight. The remaining yield increase might be partially attributable to a more complete grain filling of the cob, which we observed routinely.

Of the other yield components, the stover mass responded similarly as did the grain weight. Across all the 3 years ($n = 98$), it increased significantly ($P = 0.002$) with humic product application by 6.2% (data not shown). For each of the 3 years, its increases were in the sequence of 5.8% ($P = 0.002$), 7.3%

($P = 0.0009$), and 5.6% ($P = 0.016$) (**Table 5**). For the 2011 dryland and irrigated sites in NE and SD, the increase in stover mass was a vigorous 20% ($P = 0.053$). The 100-kernel weight was recorded only in 2011. Humic product application caused a 2% increase in the 100-kernel weight across all sites, which approached benchmark significance ($P = 0.17$). The NE and SD sites showed a numerically more vigorous response that more closely approached benchmark significance ($P = 0.12$). Three parameters of grain quality were measured only in 2010. Their responses to humic product application were generally insignificant for oil content ($P = 0.162$), starch content ($P = 0.54$), and protein content ($P = 0.90$) (data not shown). Field observations found that the number of developed ears never changed with the humic product application. Frequent checks in 2009 found no effect of the humic product on the number of kernel rows on each ear (data not shown).

Humic product application did not significantly ($P > 0.10$) affect the total concentrations of N, P, K, Mg, Ca, or Fe in either the grain or the stover (data not shown). Neither did it statistically affect the amounts of any of these same nutrients extracted from soil either in 2009 or 2010 or across both the years, nor soil organic matter content, pH, buffer pH, or cation exchange capacity (data not shown). Similarly, concentrations of S, Zn, Mn, Cu, and B as total plant nutrients or extractable soil nutrients showed no numeric trends with humic product application (data not shown).

DISCUSSION

A major knowledge gap constraining the widespread use of humic products concerns their reliability over time and space in benefiting crop growth. Humic products do not appear to promote crop growth in all situations, given the variable results reported to date (Olk et al., 2021). Thus, the need arises to determine whether there is a predictable pattern in when and where the humic products improve crop growth and provide economically viable returns. As the first step, this study provided a wider scope of field settings for measuring agronomic benefits to maize production in the US Midwest than has been presented previously.

First, our results show that the recommended rates of humic product application have the capacity to boost maize growth in field conditions, even in a high-yielding region like central Iowa. In all eight treatment—year combinations of the replicated field trials, where leaf area was measured, total leaf area increased significantly ($P < 0.10$) with humic product application (**Table 4**), indicating that the humic product created improved growing conditions for the crop (Eik and Hanway, 1965). Statistically significant ($P < 0.10$) responses by individual leaves occurred mostly in the second half of vegetative growth, indicating that these growth stages might occur at the time of maximum product effect on plant processes, at least for the application times used in this study.

The enhancement of crop growth leading to increased grain yield may well depend on multiple factors, especially on the severity of other yield constraints. Among the replicated field trials, combine-measured grain yield responded most to product application at the slightly lower yield levels of the Radcliffe and North Conrad fields. The most productive field, Whitten, in the favorable 2010 growing season, showed only a slight numeric yield response to the humic product. The South Conrad field also showed only a slight yield response. This field tended to be seasonally wet, and abnormally high precipitation amounts fell in the 2013 early season. Olk et al. (2021) postulated that humic product benefits to upland crop growth are diminished in seasonally wet soils. Overall, all sites gave numerically positive yield increases.

Among the results from the on-farm survey of hand-harvested plant samples, especially notable is the frequency of grain weight increases in all 3 years of the on-farm survey, reaching 78% of all cases. The frequency of numeric increases was high in each year, varying only between 67 and 83% of all cases in each of the 3 years. Thus, although the three growing seasons varied somewhat in their precipitation patterns and perceived drought stress, no consistent effect of the weather variability was observed. A wide range of responses across the farms was recorded within each year, but like the replicated field trials, the responses were numerically mostly positive. Grain weight responses to the humic product were not clearly different among the three Iowa landforms—Des Moines Lobe, Iowan Surface, and the Southern Iowa Drift Plain. Among the most responsive sites were the dryland and irrigated sites in the sandier, less fertile soils of NE and SD of 2011, where maize growth in the controls was clearly less than in the controls in the more fertile Iowan soils. Many of the individual grain weight responses in the on-farm survey would not be statistically significant in a study having limited replication. Similarly, the maize growth responses in the replicated field trials were often weak statistically. Yet with the high number of field replicates in this on-farm survey, these differences became highly significant ($P < 0.001$). Hence, inconsistent field evaluations of humic products might in cases be due to an inadequate number of field replications to discern a potentially modest benefit. The number of recommended replicates may well vary by study, depending on the crop type, soil type(s), and general yield level in the local region.

This study presents both the replicated field trials and the on-farm survey to highlight their common findings. An extensive on-farm survey carries inherent research limitations and is presented here as supplementary to the replicated field trials. Researchers did not perform or supervise the application of the humic product at the survey sites, although we collected all plant and soil samples. Mechanized grain yield estimates were not made available by the farmers; hence, the sampled area was much smaller than a field-long strip. Location of the yield component sampling within each field involved some judgment, and as previously noted, the obtained grain weights represent maize response only from the sampled area, and not from the entire field.

Yet the consistency in results gained from both the replicated field trials and the on-farm survey merit noting. The replicated field trials and the on-farm survey shared the findings of generally positive grain responses to the humic product. Similarly, Olk et al. (2021) reported mostly positive responses of maize-combine

TABLE 5 | Maize yield components for individual years of the on-farm survey.

Humic treatment	Grain weight (Mg ha^{-1})	Cob length (cm)	Stover weight (Mg ha^{-1})	100-kernel weight (g 100 kernel^{-1})
	2009			
Control	17.15	17.23	12.82	–
Humic treated	18.13	17.62	13.56	–
F-test *P*-value	*<0.001*	*0.001*	*0.003*	–
	2010			
Control	15.77	16.06	11.20	–
Humic treated	16.82	16.62	12.02	–
F-test *P*-value	*<0.001*	*0.003*	*0.001*	–
	2011—All sites			
Control	15.52	16.30	11.27	29.94
Humic treated	16.61	16.75	11.90	30.60
F-test *P*-value	*0.004*	*0.003*	*0.016*	*0.170*
	2011—Nebraska and South Dakota sites only			
Control	12.95	16.79	9.08	24.52
Humic treated	15.85	17.65	10.87	27.20
F-test *P*-value	*0.041*	*0.031*	*0.053*	*0.118*
	2011—Iowa sites only			
Control	16.09	16.19	11.76	31.15
Humic treated	16.77	16.55	12.13	31.36
F-test *P*-value	*0.043*	*0.032*	*0.129*	*0.639*

Number of observations was 98 for grain weight, 95 for cob length and stover weight, and 33 for 100-kernel weight (2011 only).

grain yields, yield components, and total leaf area to a different humic product across 4 years and two landscape positions within two central Iowan fields. Annual precipitation patterns varied more in that study than the present one, ranging from severe drought to nearly ideal conditions. Olk et al. (2021) reported grain yield responses that were statistically significant only in droughty conditions. Both the studies suggest that humic products can promote crop growth in field conditions, as represented by the leaf area data found here, but whether that promotion leads to significantly greater economic yield depends on additional localized factors.

Combine-measured grain yields in the replicated field trials averaged an increase of about 0.3 Mg ha^{-1} with humic product application, while hand-sampled yield components from both years at the Radcliffe site increased by about 0.8 Mg ha^{-1}, and the mean yield increase for hand-sampled plants of the on-farm survey reached 1.0 Mg ha^{-1}. Three apparent explanations for this discrepancy between combine and hand-sampling are that, first, the hand-sampling avoided areas within a field where maize growth was visibly stunted by local environmental conditions, including potholes and eroded soils. Second, hand-sampling targeted plants of healthy growth, thus avoiding plants whose growth was limited by disease, insects, wind damage, or irregular plant spacing. Third, maize grain loss with hand-sampling was essentially non-existent, while with mechanized harvesting, ears can be dropped. For all these potential explanations, hand-sampling served to avoid conditions that might diminish the observed plant capacity to respond to the humic product. In contrast, mechanized combining would have harvested such growth-limited plants, possibly lowering the overall crop responsiveness to the humic product. It is informative to present both types of grain yield responses, as they show the potential and also the actual crop responses to humic product application in field conditions.

Cob length was a responsive yield component to humic product application in the on-farm survey, and it was also responsive in one of the 2 years at Radcliffe. In the maize field study by Olk et al. (2021) discussed previously, cob length was the yield component that was mostly responsible for increases in grain yield with humic product application. Potential ear length is determined by at least V15 and maybe as early as V12 (Strachan, 2004), and it is strongly affected by environmental stresses. Favorable leaf area responses to the humic product in these growth stages (**Figures 1A–C**) indicate that with humic product application the plant perceived better growing conditions involving less stress than in the control, thus possibly promoting the development of longer ears.

In both the studies, humic product application led to greater responsiveness of the smaller cobs in cob length and grain weight than that of the larger cobs, creating more homogenous ear sizes. This trend is seen in the shift of those measures in class size distributions (**Figures 3**, **4**). As discussed by Olk et al. (2021), greater responsiveness of the smaller ears indicates that the primary benefit of the humic product may be to help smaller plants better compete with their larger neighbors for growth requirements. This hypothesis can be phrased as an example of stress alleviation, which would match the statements made by Calvo et al. (2014) that humic product benefits to plant growth often consist of alleviating environmental stresses. Also, the consistency of these findings across both maize studies, despite the use of different humic products, provides some evidence to the

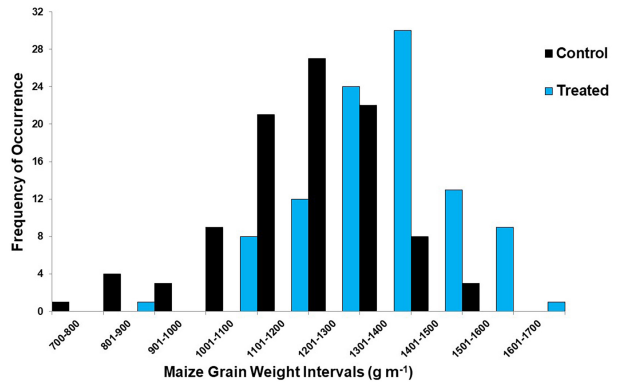

FIGURE 3 | Frequency of occurrence for 10 intervals of maize grain weight for the humic product vs. unamended control treatments at 98 locations in the on-farm survey, 2009–2011.

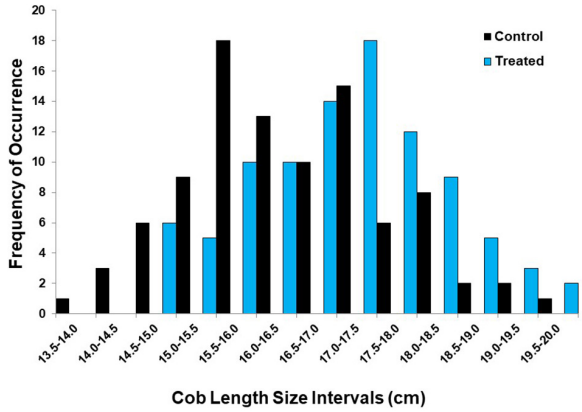

FIGURE 4 | Frequency of occurrence for 13 cob length intervals for the humic product vs. unamended control treatments at 98 locations in the on-farm survey, 2009–2011.

fact that the hand-sampling in this on-farm survey provided plausible results.

We presented limited data on the response of 100-kernel weight to the humic product. Its response was positive in the 2010 Radcliffe treatments and in the sole year when it was measured for the on-farm survey. These responses were mostly weak statistically. Such results are consistent with the results of Olk et al. (2021), who also found that the 100-kernel weight responses to humic product application were frequently positive but statistically often insignificant. Hence 100-kernel weight does not appear to be the primary driver for grain yield increase with humic product application in central Iowa, which has fertile soils and generally favorable climate for crop production. But it may well provide a secondary contribution. For regions where soils are less fertile and water deficits are more common, yield component responses to a humic product application could vary from those of this study, or they could be generally more pronounced, as we observed for the six NE and SD sites.

Further evidence that humic product use affected basic processes of plant growth was suggested by the beginning and end dates of the grain-filling period; that is, ear pollination and physiological maturity as represented by necrosis of kernel tips ("black layer"), respectively. In four 2009 maize production fields, pollination dates were scored visually as complete darkening of the ear silks through necrosis for humic product treated vs. control plots. In all fields, silk darkening (and hence pollination) occurred on average 3 days earlier for the treated plots than for the control. Yet in the three fields that were monitored at the end of the season, physiological maturity with product application was delayed by about 6 days. Thus, the grain-filling period was extended by about 10% (Abendroth et al., 2011) through both an earlier start and delayed finish. We speculate that extended grain-filling time was prompted by the previous development of larger ears, which would require more time for optimal grain-filling.

Our nutrient uptake data showed no consistent responses to humic product application for any nutrient concentration in either the grain or stover. Olk et al. (2021) also reported a similar lack of consistent nutrient response for maize growth in Central Iowa. Soil properties showed no consistent effect of the humic product on soil nutrient availability, although most plots received only 1 year of humic product use. Olk et al. (2021) reported similar results. These findings speak against a common industry belief that humic products enhance soil nutrient availability and instead point toward a plant-based mechanism for improved crop growth.

In summary, this study reported numerically positive responses of maize growth and grain yield in a high-yielding region. Yet, their statistical significances varied considerably, likely in part due to local conditions. Olk et al. (2021) reported the same findings. Modest agronomic responses can still be profitable economically if commodity prices are favorable, as the cost of many humic products is low. If this study were repeated on maize in a lower-yielding region or on another crop, a different array of results may well be found. More work is needed to determine the efficacy of humic products in promoting plant growth for the wide ranges of crop types, soil types, and environmental conditions that typify production agriculture.

CONCLUSION

Application of the Yield Igniter® humic product to maize production fields in the western US Maize Belt resulted in frequent positive responses by maize growth. Total leaf area increased significantly in all the eight field treatments where it was measured. Grain yield, as measured by combine for five site—year combinations, increased in all cases, and grain weight based on hand-sampled yield components for the on-farm survey increased in each of the 3 years. Increases were modest agronomically in this high-yielding region and varied

in statistical significance, but the low cost of the humic product meant that it could provide profitable returns, depending on grain prices. Other yield components responded generally in positive manners, but as with the combine grain yield, their statistical significance varied and were often of modest magnitude. Even in this high-yielding region, the humic product demonstrated the capability to improve crop growth. Results could differ in other field studies depending on multiple factors, including humic product, crop type, crop management practices, and environmental conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DD and DO contributed to the design of the studies and conducted or supervised all plant and soil samplings, oversaw the sample analyses, developed the interpretations, and drafted the manuscript. CC coordinated field management practices and harvesting of some of the replicated field trials in Central Iowa. All authors reviewed and approved the manuscript.

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RETRACTION
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Retraction: Use of Ore-Derived Humic Acids With Diverse Chemistries to Elucidate Structure-Activity Relationships (SAR) of Humic Acids in Plant Phenotypic Expression

*Frontiers Editorial Office**

A Retraction of the Original Research Article

Use of Ore-Derived Humic Acids With Diverse Chemistries to Elucidate Structure-Activity Relationships (SAR) of Humic Acids in Plant Phenotypic Expression
by Lamar, R. T., Monda, H., and Sleighter, R. (2021). Front. Plant Sci. 12:758424. doi: 10.3389/fpls.2021.758424

The journal retracts the 01 December 2021 article cited above.

Following publication, concerns were raised regarding the inclusion of proprietary information in the article. The data in question was generated whilst the corresponding author was working for another commercial company and was included in the article without proper authorization.

This retraction was approved by the Chief Editors of Frontiers in Plant Science and the Chief Executive Editor of Frontiers. All authors agree to this retraction.

OPEN ACCESS

Approved by:
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North Carolina State University,
United States

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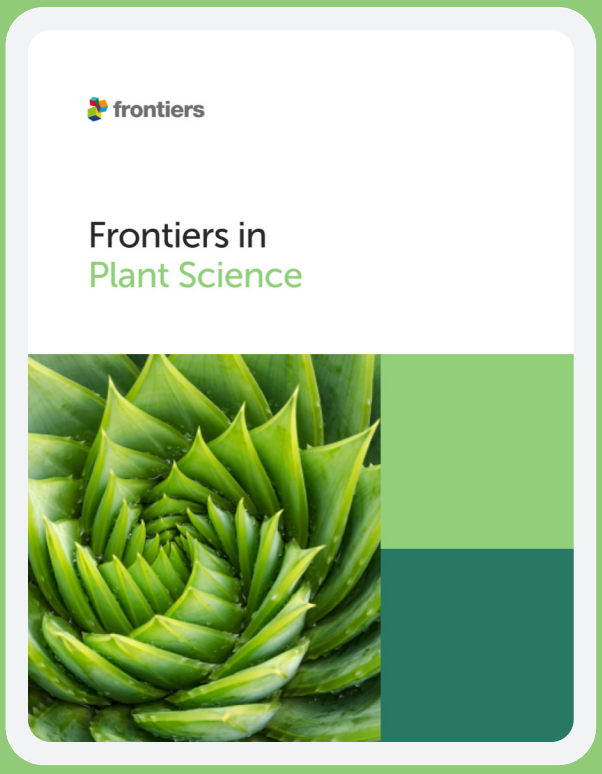

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