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RESEARCH ARTICLE

Combination of humic biostimulants with a microbial inoculum improves lettuce productivity, nutrient uptake, and primary and secondary metabolism

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Abstract

Background and aims Biostimulants of natural origin represent a growing ecological strategy to increase crops productivity, especially when applied in combination with microbial bioeffectors. We studied the effect of biostimulants such as Potassium Humates (KH) from Leonardite and Compost Tea (CT) from green compost on both productivity and nutritional status of lettuce plants, as well as on the primary and secondary metabolism of treated plants, when amended either alone or in combination with a commercial microbial inoculum (M+), mainly based on arbuscular mycorrhizal fungi (Micosat TabPlus).

Results The biomass production as well as the uptake of both macro- and micronutrients by lettuce plants significantly increased when amended by the mixture of both humic materials (MIX) combined with the microbial inoculum. Similarly, the synergic MIX_M+ treatment significantly affected both the primary and secondary metabolism of lettuce more than their individual applications, by increasing, respectively, the biosynthesis of essential amino acids and carbohydrates, and that of antioxidant polyphenolic compounds, such as hydroxycinnamic acids, flavonols and coumarins.

Conclusions Our findings suggest that a calibrated mixture of humic bioactive molecules in combination with microbial consortia represents a potential tool to improve crop productivity and its nutritional and metabolic status.

Keywords Humic matter · Compost tea · Green compost · Arbuscular mycorrhizal fungi · Microbial bioeffectors · GC-MS · UHPLC-MS-IT-TOF

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Introduction

Over the past two decades, agricultural intensification was the principal strategy proposed to match the growing need for food production (Shennan et al.

2017). However, conventional agricultural practices, such as the extensive use of chemical products (i.e., fertilizers and pesticides), resulted in a negative impact on the environment and human health, by gradual increasing both water pollution and degradation of cultivated soils (Shahrajabian et al. 2021). It is therefore necessary to adopt innovative technologies to enhance the sustainability of agricultural production systems, by preserving soil fertility and minimizing the adverse environmental impact of current agricultural practices (Drobek et al. 2019). A promising and eco-friendly approach is represented by the application of plant biostimulants (PB) and microbial bioeffectors that are found to significantly increase plants productivity at low amendment rates and plants tolerance against a wide range of abiotic stress (du Jardin 2015). A major class of plant biostimulants are humic and fulvic acids, followed by protein hydrolysates, seaweed extracts, while microbial bioeffectors are beneficial fungi (i.e., arbuscular mycorrhizal fungi and *Trichoderma* spp.) and plant growth-promoting bacteria (Canellas et al. 2015). The promotion of plant biomass production by biostimulant products has been related to their impact on several biochemical or molecular pathways and physiological processes (Yakhin et al. 2017). Non-microbial and microbial plant biostimulants were found to positively influence nutrient use efficiency (NUE) by enhancing root system architecture and soil exploration, as well as increasing macro- and micronutrient solubilisation that can lead to increased plant productivity (Rouphael and Colla 2020). Recently, it has also been shown that bioactive molecules in various PB exhibit signalling activity in plants or induce signalling pathways in both primary and secondary metabolisms (Calvo et al. 2014). For instance, different type of plant biostimulants (E.g. humic and plant / seaweed extracts) have been demonstrated to contain auxin-like compounds, which could directly affect biological process such as plant development and growth regulation, or indirectly influence plants hormonal status (Colla et al. 2014; Kurepin et al. 2014; Scaglia et al. 2016).

Humic substances (HS) are composed of relatively small heterogeneous molecules resulting from the biotic transformation of plant and animal tissues and held together by multiple weak interactions in supramolecular associations (Piccolo 2002). HS are essential to maintain and improve soil physical, chemical and biological proprieties (Piccolo et al. 1997; Imbufe et al. 2005; Puglisi et al. 2008). Moreover, their direct influence on different bio-chemical mechanisms and physiological processes in plants has been repeatedly underlined (Nardi et al. 2002; Canellas and Olivares 2014). It was shown that the positive effect of both HS and humic-like-substances (HLS) on root elongation and lateral root emergence (Canellas et al. 2002, 2012; Savy et al. 2015, 2016), as well as on nutrient uptake (Nardi et al. 2000), is correlated to the growth and productivity of plants. Moreover, Jannin et al. (2012) revealed that the HS applications induce the overexpression of genes involved in the major metabolic plant functions (i.e. photosynthesis, nitrogen/sulphur, phytohormones, plant development), thus supporting the influence of humic materials on plants both primary and secondary metabolism (Nardi et al. 2007; Schiavon et al. 2010; Ertani et al. 2011; Pizzeghello et al. 2020). The mechanism implied in such biostimulation has been related to the incorporation into the humic suprastructure of hormone-like molecules (Muscolo et al. 2007), which are released to affect plant bioactivity when the humic supramolecular conformation is altered by the effect of small-size organic acids, as those commonly exuded by plants (Savy et al. 2017a, b; Piccolo et al. 2019). The main commercially available humic substances are leonardite Potassium Humates (KH), whose beneficial effect on both soil proprieties and plant productivity has been widely assessed (Piccolo et al. 1997; Kumar et al. 2013; Conselvan et al. 2017; Ertani et al. 2019). Potassium humate is a very concentrated form of humus in the naturally occurring lignite that is extensively used to increase soil fertility, since its application has been shown to improve both aggregates stability and water retention capacity of different soil types, thus increasing the resistance to runoff and erosion processes (Piccolo et al. 1997; Imbufe et al. 2005; Kumar et al. 2013). However, the use of HS from renewable resources, such as from composted biomasses or bio-refinery wastes, is spreading in organic farming worldwide due to their remarkable bioactivity (Monda et al. 2017, 2018; Spaccini et al. 2019). In this regard, compost tea (CT), the water-soluble fraction obtained through either aerated or non- aerated immersion of compost in water for few days, may have great potentials for a sustainable agricultural production (Eudoxie and Martin 2019). In fact, it has been shown that applications of CT has

multiple benefits as either fertilizer or biostimulant (Naidu et al. 2013; Pane et al. 2016; Zaccardelli et al. 2018), and as foliar spray against plants pathogens or antimicrobial product (Koné et al. 2010; Verrillo et al. 2021a, b). Moreover, Savarese et al. (2022) recently reported that the combined application of leonardite KH and CT from green compost on maize seedlings stimulated both root development and shoot growth due to a cage effect by which CT bioactive compounds are stored in the hydrophobic domains of KH suprastructures, and thereafter liberated to stimulate plant roots activity.

Microbial bioeffectors (BEs) have shown a powerful role in increasing both productivity and nutrients uptake of plants, as well as in mitigating stress conditions (Thonar et al. 2017; Giovannini et al. 2020; Moreira et al. 2020; Miceli et al. 2021). BE's growth-promoting effects on plants are related to several physiological processes, including hormonal regulation, balance of cell oxidative status, improved both photosynthetic response and water / nutrient use efficiency (Castiglione et al. 2021). Among the hormones regulating plant physiological processes, indole-3-acetic acid (IAA) is the major endogenous auxin in plants and is able to regulate multiple cell processes (Bhalerao et al. 2002). Scientific evidences have suggested that root colonization by AMF can increase the production of IAA, thus amplifying the physiological response of the plant to adverse conditions such as drought or salt stress (Abd-Allah et al. 2015; Zou et al. 2017). On the other hand, it has been shown that some strains of PGPR are capable of producing IAA or 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme involved in the biosynthesis of ethylene, thus representing a potential tool for the reduction of environmental stress effects on plants (Huang et al. 2015; Chandra et al. 2018). Additionally, soil inoculation by BEs has been recorded to enhance the levels of stress-related hormones, such as salicylic (SA) and jasmonic acid (JA), which play key roles in different signalling pathways involving abiotic plant responses (Kang et al. 2014; Bernardo et al. 2017). The effect of microbial bioeffectors in the induction of plants tolerance to drought or salinity conditions is also related to their influence on physiological processes such as apoplastic water flow, aquaporin genes modulation, leaf water potential and transpiration rate, which can improve plants water use efficiency (Bárzana et al. 2015; Ansari et al. 2019). Furthermore, beneficial microorganisms promote plants performance by affecting their nutritional status. In particular, AMF expand the roots-exploited soil volume by means of the extraradical mycelium, thus improving the nutrient absorption capacity of plants (Giovannini et al. 2020), while PGPR increase soil nutrients availability by fixing atmospheric N or solubilizing inorganic P, as well as producing ion-chelating compounds known as siderophores that promote plants uptake of micronutrients such as Fe (Backer et al. 2018).

Among BE, *Trichoderma* fungus was also shown to exhibit multiple beneficial effects, such as plant growth stimulation, promotion of nutrient uptake, suppression of plant pathogens and induction of plant defence mechanism (Woo et al. 2014; López-Bucio et al. 2015; Sanchez-Montesinos et al. 2020). The phytostimulation of Trichoderma has been attributed to several direct and indirect effects on plants, including the release of substances with auxin activity (i.e., IAA), small peptides and volatile organic compounds, which improve root system architecture and assimilation/solubilization of macro- (P) and micronutrients (Fe, Mn, and Zn) (Lorito and Woo 2015; Sanchez-Montesinos et al. 2020). Moreover, the beneficial effects to plants can be related to the capacity of many Trichoderma spp. to produce specific metabolites that are antimicrobial, thus contributing to phytopathogen control, and/or positively affecting the plant in aspects of growth promotion such as increased yield and other desirable characters like anti-oxidant properties (Vinale et al. 2009). Moreover, recent studies have pointed out the potential as plant biostimulants of microbial consortia, such as combined rhizobacteria and rhizofungi, which mimic the structured biological networks existing in native soils, through the empowerment of the natural microbiome (Kong et al. 2018; Woo and Pepe 2018; Bradáčová et al. 2019). Likewise, mixed application of HS and microbial bioeffectors has been recently explored for their possible synergistic effect on both plant development and soil biodiversity (Filho et al. 2020). Indeed, the combined application of humic extracts and PGPB, AMF or *Trichoderma* spp. was found to determine a boost effect on plants productivity, nutrients uptake and the overall metabolome (Ferreira et al. 2017; Vinci et al. 2018a, b; Canellas et al. 2019b; Torun and Toprak 2020; Cozzolino et al. 2021).

Despite the interest in developing functional products by combining different plant biostimulants, the effects of such mixed biostimulants on plants growth and physiology, as well as on their mechanisms of action are still poorly explored (Olivares et al. 2017). Lettuce (*Lactuca sativa* L.) is one of the most widely cultivated species of the horticultural groups of leafy vegetables, and the large availability of literature on biostimulant applications makes this crop a prime candidate for evaluation (Cristofano et al. 2021). Moreover, lettuce is considered a "healthy" food and its consumption is growing since the considerable content of phytochemicals has been shown to exert significant anti-oxidant and anti-inflammatory proprieties on human health (Pepe et al. 2015). Hence, the aim of the present work was to study the effect on both productivity and nutritional status of lettuce plants of different non-microbial (leonardite KH and CT from green compost) biostimulants and a commercial (Micosat TabPlus) microbial inoculum (M+), when applied to soil alone or in multiple combinations. Additionally, the changes in treated plants of primary and secondary metabolites were investigated by both GC-MS and UHPLC-MS-IT-TOF analysis, respectively, in order to evaluate the overall effect of the applied bio-treatments on the lettuce metabolome.

Materials and methods

Materials

Potassium Humates (KH) were provided by Hymato Products Ltd., Hungary, and were obtained by a KOH alkaline extraction from a leonardite ore and supplied in form of dried granules of about 0.5–1 mm size. Green compost was produced in the composting facility of the experimental farm of the University of Napoli Federico II at Castel-Volturno (CE), by 45-days composting process of 4×6 m static pile under forced air insufflation, followed by a two-month curing period. The composting pile consisted in residues of coffee production (coffee husks) (70% w/w) added with horticultural fresh residues (30% w/w). The compost was left to mature for 30 more days and then randomly sampled to collect 1 Kg. The compost sample was air dried, sieved at 2 mm and stored a 4 °C for further extraction processes. Compost Tea (CT) was extracted from the green compost as reported earlier (Savarese et al. 2022). Briefly, 200 g of compost was weighed into a gauze bag and suspended in 1 L of distilled water in a plastic becker (w/v 1/5). The compost containing gauze bag was subjected to air insufflation at regular intervals (15 min every 3 h) with an automatic aeration pump device. After seven days of aeration, the extraction was stopped, and compost tea solution was freeze-dried. A detailed chemical and molecular characterization of both KH and CT has been previously reported (Savarese et al. 2022).

The microbial inoculum employed here was the MICOSAT TABPLUS commercial product formulated by CCS Aosta s.r.l., and contained *Glomus coronatum* GU 53, *G. caledonium* GM 24, *G. mosseae* GP 11, *Gnaphalium viscosum* GC 41, *Rhizophagus irregularis* RI 31 1 (10%), plus *Trichoderma harzianum* TH01, *Trichoderma viride* TV 03, *Bacillus subtilis* BA 41, *Streptomyces* spp. SB 19, and *Pichia pastoris* PP59 (7.5% 10.2×10^7 C.F.U. /g).

The soil used in the pot experiment was collected from the surface layer (0–20 cm) of a farmland Vertic Xerofluvent clay-loam soil located at the Castel Volturno (CE) University of Naples experimental station. The soil showed a clay loam textural composition (44.6%, 28% and 27.4% sand, silt and clay, respectively), an alkaline pH (8.6) and a content of 1.11 g kg^{-1} of total nitrogen, 10.5 g kg^{-1} of organic carbon, and 11 mg kg^{-1} of $NaHCO_3$-extractable phosphorus.

Pot experiment

The pot experiment was performed from March to May 2020, under greenhouse conditions (15–25 °C, daily temperature range). Lettuce plants (*L. sativa* L. cv. capita "Meraviglia d'inverno") were grown on a mixture of soil/sand substrate (2:1 w/w, 1 kg pot^{-1}) sieved at 5 mm, and thoroughly homogenized. The basal nutrients supplied in powder form to the substrate of each individual pot and evenly mixed, consisted of 100 mg N kg^{-1} as ammonium nitrate NH_4NO_3, and of 75 mg P kg^{-1} and 160 mg K kg^{-1} added as dipotassium hydrogen phosphate (K_2HPO_4) (for K, we also added potassium sulphate, K_2SO_4, to get the right amount). The KH and CT were tested individually and in combination (1:1) (MIX) at the rate of 169 mg Kg^{-1}, applied

once as water suspension at transplant, concomitant to the soil inoculation with 250 mg of the commercial microbial product (M+) per pot. The same rate of microbial inoculum was added again after one month of plant growth, corresponding to final total concentration of 0.5 g Kg^{-1}.

The synergistic effect of humic materials and microbial inoculum was tested through the following experimental design: CTRL: Control (H_2O); CTRL_M+: Control plus microbial inoculum; KH: Potassium Humates; KH_M+: Potassium Humates plus microbial inoculum; CT: Compost Tea from green compost; CT_M+: Compost Tea from green compost plus microbial inoculum; MIX: KH plus CT; MIX_M+: KH plus CT plus microbial inoculum. All treatments were replicated five times, for a total of 40 pots. After two months of plant growth, the leaves were harvested by cutting the plant base at 1 cm above the soil surface with a sharp knife. The leaves were first rinsed with tap water and then with deionized water. Biomass was determined (fresh and dry weight) to provide the plants yield for each treatment. The leaves were divided in two equal groups. One group (dried) was stored for mineral analysis, while the second (fresh leaves) was immediately frozen in liquid nitrogen and stored at −80 °C for metabolic analysis.

Mineral content analysis

Dried lettuce leaves were ground using a PM 20-ball mill (Retsch) before undergoing chemical analyses. Total N and S concentration in plant tissues was determined using 3 mg of each sample by an UNICUBE elemental analyser (Elementar Analysensysteme GmbH, Germany). Total concentration of macro- (Ca, Mg, K) and micro- (Cu, Zn, Mn, Fe) elements in the plant tissues was ascertained by digesting 0.5 g of each sample with 6 mL of HNO_3 (65%) and 2 mL of H_2O_2 (35%) in a Milestone 900 microwave oven at 600 W for 24 min. Solutions were diluted to 25 mL with Milli Q water and analysed by an atomic absorption spectrometer (AAnalyst 700, Perkin-Elmer). The P content was measured calorimetrically in the same digested samples by the molybdenum blue assay method (Murphy and Riley 1962). All analyses were carried out induplicate from five biological replicates.

Extraction of primary metabolites and GC-MS determination

Lettuce leaves stored at −80 °C were freeze-dried and homogenized with a mortar. Then, 10 mg of homogenized plant samples were weighed into 2 mL Eppendorf tubes. The metabolites extraction was performed by adding on dried samples 1 mL of water/methanol/chloroform mixture (1:3:1 ratio) pre-cooled at −20 °C. Ribitol and Dodecanoic acid at the concentration of 13 mg L^{-1} were used as internal standards. Plant samples were vortexed for 2 minutes and incubated for 2 hours at −20 °C to increase extraction yields. After extraction, samples were incubated for 15 minute at 70 °C in order to inhibit the possible activity of possible enzymes, vortexed and centrifuged for 10 min at 12000 rpm and at 4 °C. Then, 900 µL of supernatant were recovered, transferred into 2 mL Eppendorf tubes, and mixed with 400 µL of Milli Q water to allow separation of polar and apolar phases. The chloroform phase was used to determine lipids, while the methanol/water upper phase was employed for polar compounds analyses. All extracts were stirred for 30 s and centrifuged for 10 min at 4 °C at 12000 rpm. Finally, 200 µL of each phase was transferred into 1.5 mL glass tubes for GC–MS analyses, dried under nitrogen and stored at −80 °C. All analyses were carried out in duplicate from five biological replicates.

Derivatization for GC–MS analyses was conducted by suspending dried samples in 50 µL of a solution of methoxyamine hydrochloride solubilized in pyridine (20 mg mL^{-1}), that was gently shaken for 90 min at 30 °C. After methoximation, samples were silylated for 30 min at 37 °C by using 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). Then, 2 µL of each fully derivatized sample were analyzed through an Agilent 7683B Injector coupled to an Agilent HP6890 Series gas chromatograph system and a quadrupole 5973 Agilent Mass spectometer. The GC was carried out by RTX-5MS WCOT capillary column (Restek, 30 m×0.25 mm; film thickness, 0.25 mm) that was coupled, through a heated transfer line (250 °C), to a mass spectrometer. The gas chromatographic elution was carried out by applying a 1 min of isothermal phase at 70 °C, followed by a temperature increase from 80 to 300 °C (rate of 15 °C min^{-1}) and by a 10 minutes long

isothermal phase at 300 °C. Helium was the carrier gas at 1 mL min^{-1}, while the injector temperature was set at 250 °C and the split flow applied for the split-injection mode at 25 mL min^{-1}. Mass spectra were obtained in the EI mode (70 eV), scanning in the range included within 50 and 650 m/z, with a cycle time of 0.2 scan s^{-1}. The identification of mass spectra of polar and apolar compounds was carried out by analyzing standard compounds as well as by evaluating the mass spectra reported in the library NIST 11.

Extraction of phenolic metabolites and determination by UHPLC-MS-IT-TOF

About 30 mg of freeze-dried lettuce leaves were weighed into 1.5 mL Eppendorf tube and mixed with 400 µL of methanol/water mixture (8:2) pre-cooled at −20 °C. Umbelliferon at the concentration of 10 mg L^{-1} was used as internal standard. Plant samples were vortexed for 2 minutes and incubated overnight (16 h) at −20 °C to increase extraction yields. The mixture was centrifuged for 15 min at 12000 rpm at 4 °C, and 300 µL of supernatant were transferred into a new 1.5 mL Eppendorf tube. The residue was similarly extracted once again with 400 µL of MeOH. Supernatants of both extractions were combined, vortexed for 2 minutes and centrifuged for 15 min at 12000 rpm at 4 °C. Then, 100 µL of supernatant were transferred into a new 1.5 mL Eppendorf tube and added with 25 µL of 0.1% Formic acid. The mixture was vortexed for 2 minutes and centrifuged for 15 min at 12000 rpm at 4 °C. Finally, a volume of 100 µL was collected and transferred into 200 µL glass vials for UHPLC-MS-IT-TOF analyses. All analyses were carried out in duplicate from five biological replicates.

Secondary metabolites were separated by using a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, a DGU-20A5r degasser, a binary solvent system LC-20 AD, a SIL-20Axr autosampler and a column heater system CTO-20A. Chromatographic separation was obtained by a Kinetex EVO C18 column (150×2.1 mm, 2.6 µm particle size) coupled to a guard column with the same stationary phase, both from Phenomenex. The chromatographic conditions were the following: 2.0 µL injected sample, column maintained at 35.0 °C, flow rate of mobile phase at 0.3 mL min^{-1}. The mobile phase was a binary system of 0.1% formic acid aqueous solution (A) and acetonitrile containing 0.1% formic acid (B). The gradient elution was as it follows: 0–0.5 min, 1% B; 0.5–4.0 min, 13% B; 4.0–10.0 min, 45% B held for 2.0 min; 12.0–14.0 min, 60% B and then held for 1.5 min. The system was ri-equilibrated by reaching back to 1% B in 16.5 min and held it for 4.5 min before the next injection step.

The UHPLC system was coupled online to a hybrid IT-TOF mass spectrometer from Shimadzu Corp. (Tokyo, Japan), equipped with an electrospray ionization (ESI) source operating in negative mode under the following conditions: N_2 nebulizing gas flow of 1.5 L min^{-1}; interface voltage at 3.5 kV; curved desorption line (CDL) interface temperature of 200 °C; block heater temperature, 200 °C; detector voltage 1.57 kV; drying gas pressure of 110 kPa. Full scan MS data were acquired in the range of 80–1000 m/z (octopole ion accumulation time of 20 ms; IT, (repeat = 3). MS/MS experiments were conducted in a data dependent acquisition using a mass range of 50–900 m/z; precursor ions were acquired in the range 150–900 m/z; peak width, 1 Da; ion accumulation time, 40 ms; Collision Induced Dissociation (CID) energy, 50%, collision gas 50%, repeat = 1; execution trigger (BPC) intensity, at 95% stop level. LC–MS data elaboration was performed by LCMSsolution software (Version 3, Shimadzu), Formula Predictor software (Version 1.2, Shimadzu) and MetID solution software (Version 1.2, Shimadzu).

Statistical analysis

A normality test (Kolmogorov–Smirnov) was performed on the dataset derived from mineral analysis. Significant differences between the means were determined by one and two-ways analysis of variance, while application of LSD test to differentiate among results was given at the $p < 0.05$ probability level using the XLStat software v.9.0 (Addinsoft).

The semi-quantitative evaluation of both GC- and LC-chromatograms was obtained by normalizing the area of each peak to the area of the internal standard and further referring it to the sample fresh weight (mg). The Principal Component Analysis (PCA) was used here to reduce the dimensionality of the dataset and concomitantly preserve the useful information expressed in terms of variable variance. The XLStat software, version 9.0 (Addinsoft) was used to process

the PCA of the total dataset composed of 38 and 23 variables obtained by GC-MS analysis of polar and apolar phases, respectively, and 23 variables derived from UHPLC-MS-IT-TOF analysis of phenolic metabolites. Data were checked for normality and homogeneity of variance and transformed where necessary. Significant differences in metabolites amount among treatments were tested by one-way ANOVA, followed by LSD test (significant for p-values <0.05 at a significance α of 0.05). The Heatmapping was elaborated by the Heatmapper software (Babicki et al. 2016). Each Heatmap score represented the average value of nine replicates.

Results

Plant growth and nutrient content

All treatments significantly increased plant biomass as compared to control (CTRL) (Fig. 1). Without the microbial inoculum, the largest effect on plants yield was obtained for the MIX treatment, that is the combination of KH and CT, with a 33% shoot dry weight larger than control (Fig. 1b), whereas the application of the same materials alone showed an increase of only 15% (Fig. 1b). The same trend was visible in the shoot fresh weight (Fig. 1a). The inoculation with the microbial product (M+) significantly affected lettuce productivity that raised the shoot biomass for all treatments (Fig. 1). In particular, the combination of KH and CT with the microbial inoculum (MIX_M+) showed the largest shoot fresh and dry weight that increased by 41 and 52%, respectively, in comparison to control (Fig. 1).

The mineral content of lettuce plants resulted also affected by both the individual application of the microbial inoculum and biostimulants, and their combination (Tables 1 and 2). However, the effect on macronutrients uptake varied with the treatment (Fig. 2). In absence of microbial inoculum, the individual application of CT significantly increased leaf concentration of P, K, Ca, and Mg (Fig. 2c, e, i, m), whereas the combined MIX treatment provided a significant improvement of N and S leaf concentration (Fig. 2a, g). Moreover, plants treated with MIX showed a greater macronutrients leaf content (mg plant^{-1} DW) than both control and plants under single KH and CT applications (Fig. 2b, d, f, h, l, n). The inoculation with the microbial product (M+) significantly affected the macronutrients status of lettuce plants (Table 1), by increasing their uptake when applied either alone (except for CT), or in combination with the biostimulants (Fig. 2).

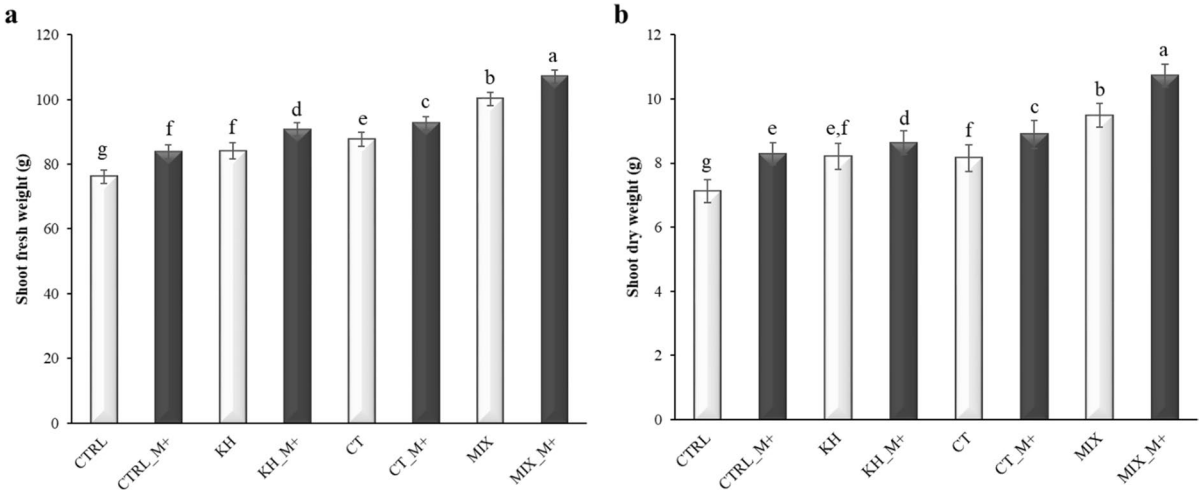


Fig. 1 Shoot fresh (**a**) and dry (**b**) weight of lettuce plants treated with different biostimulants. CTRL: Control; KH: Potassium Humates from leonardite; CT: Compost Tea from green compost; MIX: KH plus CT; MIX_M+: both biostimulants plus microbial inoculum (Micosat TabPlus). Dark grey indicates addition of microbial inoculation. Bars indicate standard deviation of means ($n=5$). Different letters above the bars indicate significantly different means according to LSD test ($p<0.05$)

Table 1 Two-way ANOVA results on the influence of biostimulants application (B), microbial inoculation (M+) and interaction between biostimulants and microbial inoculum (B*M+) on both leaf macronutrients concentration (mg g^{-1}) and content (mg $plant^{-1}$ Dry Weight) of lettuce plants

		Leaf concentration (mg g^{-1})			Leaf content (mg $plant^{-1}$ DW)		
Element	Treatment	df[a]	F[b]	p[c]	df[a]	F[b]	p[c]
N	B	3	57.690	**< 0.0001**	3	381.638	**< 0.0001**
	M+	1	5.105	**0.031**	1	137.908	**< 0.0001**
	B*M+	3	5.042	**0.006**	3	20.164	**< 0.0001**
	Error	32			32		
P	B	3	138.844	**< 0.0001**	3	1114.484	**< 0.0001**
	M+	1	58.672	**< 0.0001**	1	145.501	**< 0.0001**
	B*M+	3	141.013	**< 0.0001**	3	297.230	**< 0.0001**
	Error	32			32		
K	B	3	77.655	**< 0.0001**	3	1895.467	**< 0.0001**
	M+	1	8.830	**0.006**	1	2042.292	**< 0.0001**
	B*M+	3	53.015	**< 0.0001**	3	369.684	**< 0.0001**
	Error	32			32		
S	B	3	37.466	**< 0.0001**	3	279.772	**< 0.0001**
	M+	1	4.422	**0.043**	1	42.462	**< 0.0001**
	B*M+	3	1.058	0.380	3	10.518	**< 0.0001**
	Error	32			32		
Ca	B	3	916.008	**< 0.0001**	3	7489.782	**< 0.0001**
	M+	1	532.634	**< 0.0001**	1	5673.479	**< 0.0001**
	B*M+	3	898.377	**< 0.0001**	3	2648.269	**< 0.0001**
	Error	32			32		
Mg	B	3	94.341	**< 0.0001**	3	2291.876	**< 0.0001**
	M+	1	0.654	0.452	1	971.719	**< 0.0001**
	B*M+	3	112.707	**< 0.0001**	3	285.756	**< 0.0001**
	Error	32			32		

[a]Degrees of freedom; [b] Fisher test; [c] $p < 0.05$ probability level. The significant p values are in bold

In particular, the MIX_M+ treatment showed the largest effect on both macronutrients leaf concentration (mg g^{-1}) and content (mg $plant^{-1}$ DW) (Fig. 2). Plants treated with MIX_M+ revealed an increase in N, P, K, S, Ca and Mg leaf content (mg $plant^{-1}$ DW) of 55.2, 74.8, 33.9, 70.2, 140.8, and 73.6%, respectively, more than control plants (Fig. 2b, d, f, h, l, n).

The individual application of biostimulants slightly affected the micronutrients content of treated leaves, as compared to control (Fig. 3), whereas microbial inoculation (M+) greatly improved their uptake by lettuce plants (Table 2 and Fig. 3). In particular, the addition of KH together with the microbial product (KH_M+) significantly increased both leaf concentration (mg Kg^{-1}) and content (mg $plant^{-1}$ DW) of Cu and Fe (Fig. 3a, b, g, h). On the other hand, the concentration of Zn and Mn remarkably raised in plants under the MIX_M+ treatment (Fig. 3c, d, e, f). In fact, plants treated with MIX_M+ showed the greatest leaf content (mg $plant^{-1}$ DW) of Zn, Mn, and Fe, which resulted 50, 98 and 85%, respectively, larger than for control (Fig. 3d, f, h).

Primary metabolism

Differences in primary metabolism of lettuce plants grown under biostimulants alone or in combination with the microbial inoculum, were assessed by GC-MS. The main identified compounds in the polar fraction of leaves extracts were saccharides (mono- and di- saccharides), amino and organic acids (Table S3 and Fig. S1). To assess the effects of treatments on the leaves metabolome, GC-MS data of polar plants extracts were elaborated by the Principal Components Analysis (PCA). The PCA of plants inoculated with the microbial product (M+) in

Table 2 Two-way ANOVA results on the influence of biostimulants application (B), microbial inoculation (M+) and interaction between biostimulants and microbial inoculum (B*M+) on both leaf micronutrients concentration (mg g^{-1}) and content (mg $plant^{-1}$ Dry Weight) of lettuce plants

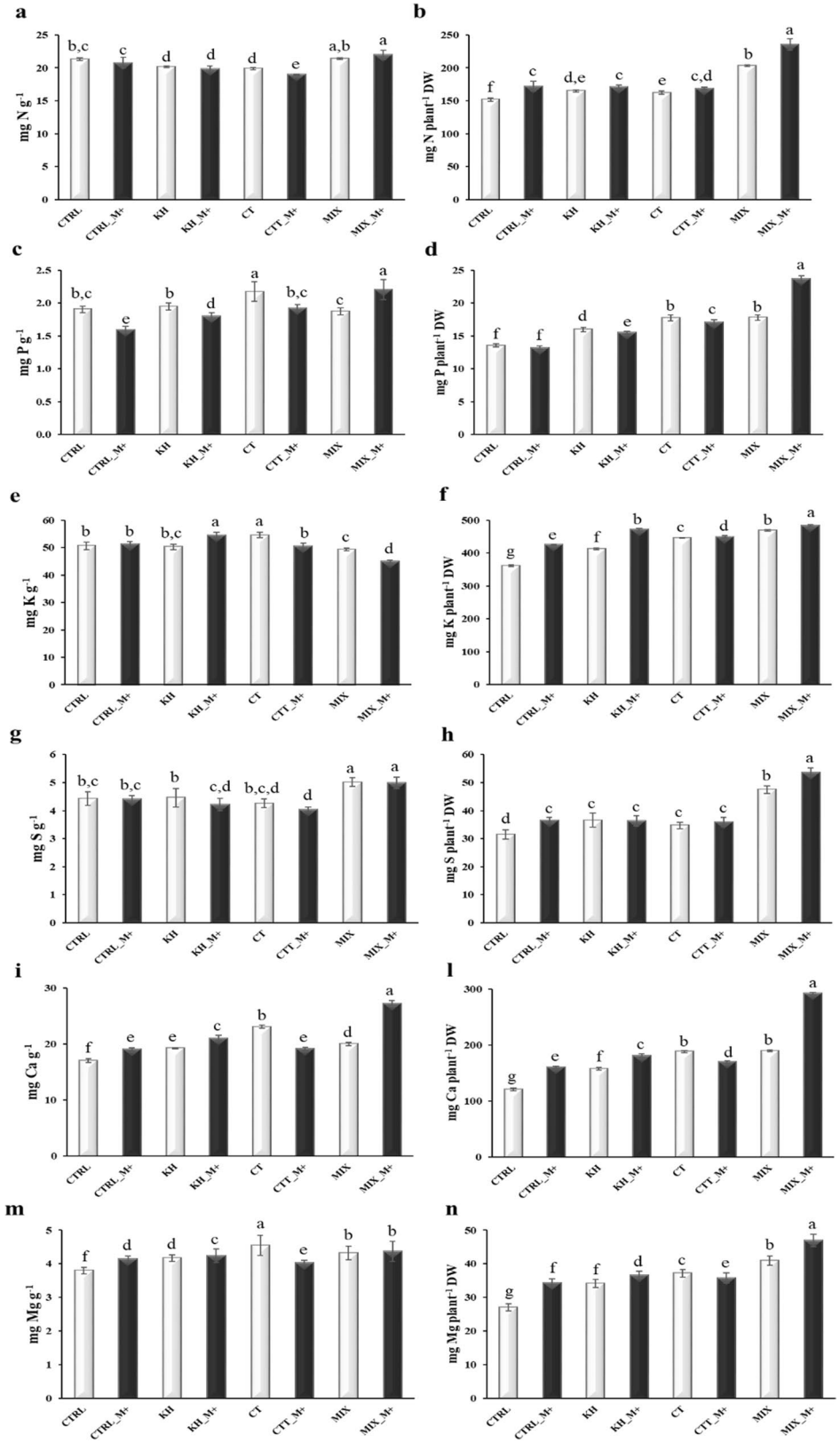
		Leaf concentration (mg Kg^{-1})			Leaf content (mg $plant^{-1}$ DW)		
Element	Treatment	df[a]	F[b]	p[c]	df[a]	F[b]	p[c]
Cu	B	3	229.870	**< 0.0001**	3	51.013	**< 0.0001**
	M+	1	44.925	**< 0.0001**	1	287.139	**< 0.0001**
	B*M+	3	88.736	**< 0.0001**	3	55.373	**< 0.0001**
	Error	32			32		
Zn	B	3	259.732	**< 0.0001**	3	706.522	**< 0.0001**
	M+	1	16.050	**0.0003**	1	2212.979	**< 0.0001**
	B*M+	3	310.818	**< 0.0001**	3	1014.692	**< 0.0001**
	Error	32			32		
Mn	B	3	903.346	**< 0.0001**	3	3851.439	**< 0.0001**
	M+	1	1445.613	**< 0.0001**	1	4348.142	**< 0.0001**
	B*M+	3	216.628	**< 0.0001**	3	229.508	**< 0.0001**
	Error	32			32		
Fe	B	3	5778.256	**< 0.0001**	3	6768.731	**< 0.0001**
	M+	1	20,099.934	**< 0.0001**	1	27,180.559	**< 0.0001**
	B*M+	3	1369.361	**< 0.0001**	3	10.307	**< 0.0001**
	Error	32			32		

[a]Degrees of freedom; [b] Fisher test; [c] $p < 0.05$ probability level. The significant p values are in bold

combination with different biostimulants explained 72.59% of the total variance, of which PC1 and PC2 accounted for 52.94 and 19.65%, respectively (Fig. 4a). The treatments were well separated and distributed in the loading plot. In particular, PC1 neatly separated CT and CT_M+ treatments from other ones, based on the low amount of almost all identified metabolites, except for cellobiose and glycerol (Table 3 and Fig. 4a). Moreover, the control and KH treatments, alone or in combination with the microbial inoculum (M+), were clearly separated along PC2 from CT and MIX application, due to a large amount in leaves extracts of myo-inositol and organic acids, such as malic, citric and fumaric acid (Table 3 and Fig. 4a). Finally, the distribution of MIX and MIX_M+ treatments in the lower right quadrant of PC1 was significantly correlated to a large amino acids content, particularly alanine, GABA and glutamic acid, and main carbohydrates, such as fructose, galactose and glucose (Table 3 and Fig. 4a).

The Heatmap derived from metabolomics data confirmed that the relative amount of identified metabolites varied with treatments, thus determining a different placement of plant samples in the score-plot (Fig. 4b). The application of leonardite KH increased the accumulation of organic acids (Fig. S5), mostly malic and citric acid (Table 3), whereas CT treatments mainly enhanced the biosynthesis of cellobiose and glycerol (Table 3). Moreover, plants under the MIX treatment showed a greater amount of amino acids, such as aspartic and glutamic acid (Table 3), and carbohydrates like fructose and glucose, than for control (Table 3 and Fig. 4b). It is noteworthy that the inoculation with the microbial product (M+) significantly affected the primary metabolism of polar compounds, especially when applied in combination with the MIX treatment (Fig. 4b). Plants treated with MIX_M+ exhibited a larger amount of almost all amino acids and saccharides (Fig. S4 and S6) than lettuce plants subjected to all other biostimulants (Fig. 4b).

GC-MS analysis was also applied to identify the metabolites extracted in the apolar fraction of lettuce leaves under different treatments. The main identified compounds were long-chain fatty acids, alcohols, sterols, and terpenes (Table S4 and Fig. S2). When GC-MS data of apolar metabolic extracts were evaluated by PCA, it was found that the two first principal components captured 64.52% of total variance, and showed a certain separation among treatments (Fig. 5a). The PC1 (43.75% of total variance) distinguished among CT, KH_M+, MIX and MIX_M+


a
mg N g-1
b,c c d d d e a,b a
b
mg N plant-1 DW
f c d,e c e c,d b a
c
mg P g-1
b,c e b d a b,c c a
d
mg P plant-1 DW
f f d e b c b a
e
mg K g-1
b b b,c a a b c d
f
mg K plant-1 DW
g e f b c d b a
g
mg S g-1
b,c b,c b c,d b,c,d d a a
h
mg S plant-1 DW
d c c c c c b a
i
mg Ca g-1
f e e c b e d a
l
mg Ca plant-1 DW
g e f c b d b a
m
mg Mg g-1
f d d c a e b b
n
mg Mg plant-1 DW
g f f d c e b a
CTRL
CTRL_M+
KH
KH_M+
CT
CTT_M+
MIX
MIX_M+

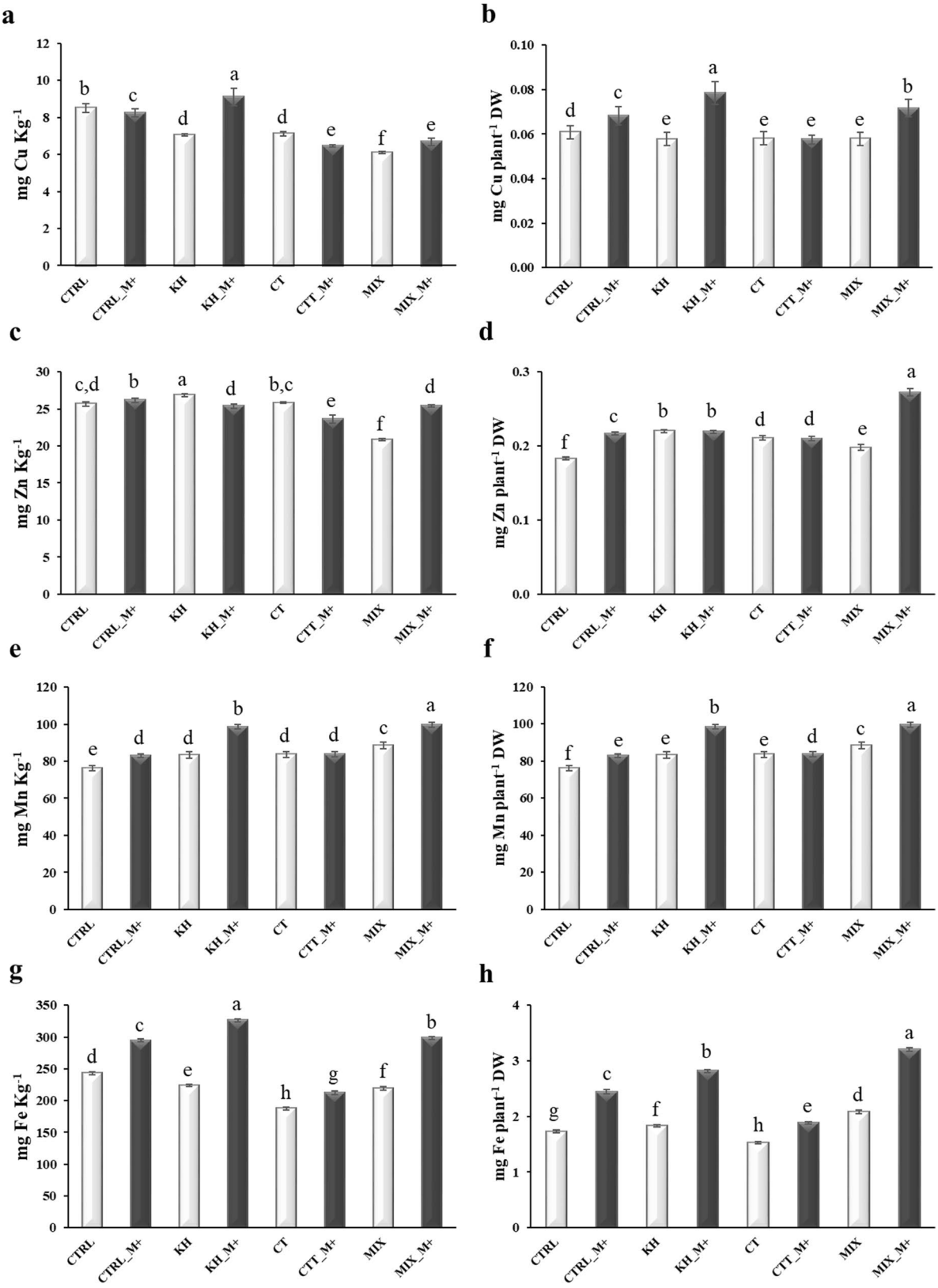
◂**Fig. 2** Effect of the biostimulants on macronutrients composition of lettuce leaves. Leaf nitrogen concentration and content (**a**, **b**), leaf phosphorus concentration and content (**c**, **d**), leaf potassium concentration and content (**e**, **f**), leaf sulphur concentration and content (**g**, **h**), leaf calcium concentration and content (**i**, **l**), leaf magnesium concentration and content (**m**, **n**). CTRL: Control; KH: Potassium Humates from leonardite; CT: Compost Tea from green compost; MIX: KH plus CT; MIX_M+: both biostimulants plus microbial inoculum (Micosat TabPlus). Dark grey indicates addition of microbial inoculation. Bars indicate standard deviation of means ($n = 10$). Different letters above the bars indicate significantly different means according to LSD test ($p < 0.05$)

treatments, due to more abundant metabolites such as myristic acid, docosanol, stearic acid and sterols (Table 3 and Fig. 5a). The placement of KH, CTRL and CTRL_M+ along the PC1 negative values was due to a lesser amount of all identified metabolites in the corresponding leaves extracts, except for amyrin, germanicol and lupenol (Table 3 and Fig. 5a). Moreover, the PC2 (20.78% of total variance) neatly separated plants under CT and CT_M+ from the other treatments, based on a greater amount of long-chain fatty acids, mainly linoleic and linolenic acid (Table 3 and Fig. 5a). These results were confirmed by applying the Heatmap elaboration on the metabolomics data (Fig. 5b). Untreated plants (CTRL) and plants under microbial inoculation alone (CTRL_M+) and leonardite KH application showed a similar lipidic profile (Fig. 5b), whereas the combined application of KH and M+ significantly enhanced the biosynthesis of fatty acids (Fig. S7), mainly lauric and myristic acid (Table 3). On the other hand, the application of CT, either alone or in combination with the microbial inoculum (CT_M+), remarkably raised the concentration of long-chain fatty acids (Fig. 5b and S7), principally linoleic and α-linolenic acid (Table 3). Interestingly, the lipidic profile of leaves treated with MIX was not so different from that of CT when applied alone (Fig. 5b). Conversely, the addition of the microbial inoculum to the mixed humic material (MIX_M+) significantly affected the concentration of all identified metabolites (Fig. 5b), mainly long-chain fatty alcohols and sterols (Table 3 and Fig. S8).

Polyphenols metabolism

The secondary metabolism of polyphenols in lettuce plants grown under different treatments were assessed by UHPLC-MS-IT-TOF. Identification of polyphenols was based on standards and comparison of fragmentation patterns with data present in literature. Molecular formulae were calculated by dedicated Formula Predictor softwares, setting a low tolerance so that most of the identified compounds were in the first position in the list of possible candidates. Results are shown in Table S5 in the progressive order of peak elution. The main identified compounds in the metabolic extracts were hydroxycinnamic and hydroxybenzoic acids, followed by flavones, flavonols and coumarins (Table S5). The most abundant compounds among hydroxycinnamic acids, peaks 11 and 15 (Fig. S3) showed two intense fragment ions at m/z 191 (quinic acid ion) and m/z 163 (p-coumaric acid ion), which were identified as chlorogenic and p-coumaroylquinic acid, respectively (Table S5). Moreover, isomeric form of dicaffeoyltartaric acid (chicoric acid) was found at m/z 473.0714 (Fig. S3), which yielded MS/MS fragment ions at m/z 311 and 293, indicating the successive loss of the caffeoyl moiety and caffeic acid, respectively, from the precursor ion (Table S5). On the other hand, glycosylated quercetin (flavonol) and luteolin (flavone) were the most representative compounds among the detected flavonoids. In particular, peak 22 and 23 were identified as luteolin-7-glucuronide and quercetin-3-glucuronide (Fig. S3), based on the corresponding fragment ions shown at m/z 285 and 301, respectively (Table S5). The semi-quantitative analysis of leaves polyphenols was performed by normalizing the extracted ion area of the most abundant identified compounds to the area of the internal standard (Fig. S3).

These metabolomics data were then processed by Principal Component Analysis (PCA), to detect the effect of different treatments on the polyphenols profile of leaves. The two first principal components explained 74.82% of the total variance, with PC1 and PC2 accounting for 59.59 and 15.23%, respectively (Fig. 6a). The PC1 neatly distinguished, from control samples (CTRL and CTRL_M+), the plants treated with the microbial inoculum (M+) in combination with KH, CT, or their mixture (MIX), and plants under the individual applications of both KH and CT (Fig. 6a). This separation was due to a greater amount in MIX of all identified compounds, and to a larger abundance of metabolites, such as chicoric acid, chlorogenic acid and luteolin-7-glucoside, in plants under KH_M+, CT_M+ and MIX_M+ treatments, respectively (Table 3). Moreover, lesser amount of


a
mg Cu Kg-1
b c d a d e f e
b
mg Cu plant-1 DW
d c e a e e e b
c
mg Zn Kg-1
c,d b a d b,c e f d
d
mg Zn plant-1 DW
f c b b d d e a
e
mg Mn Kg-1
e d d b d d c a
f
mg Mn plant-1 DW
f e e b e d c a
g
mg Fe Kg-1
d c e a h g f b
h
mg Fe plant-1 DW
g c f b h e d a
CTRL
CTRL_M+
KH
KH_M+
CT
CT_M+
MIX
MIX_M+

◂**Fig. 3** Effect of the biostimulants on micronutrients composition of lettuce leaves. Leaf copper concentration and content (**a**, **b**), leaf zinc concentration and content (**c**, **d**), leaf manganese concentration and content (**e**, **f**), leaf iron concentration and content (**g**, **h**). CTRL: Control; KH: Potassium Humates from leonardite; CT: Compost Tea from green compost; MIX: KH plus CT; MIX_M+: both biostimulants plus microbial inoculum (Micosat TabPlus). Dark grey indicates addition of microbial inoculation. Bars indicate standard deviation of means ($n = 10$). Different letters above the bars indicate significantly different means according to LSD test ($p < 0.05$)

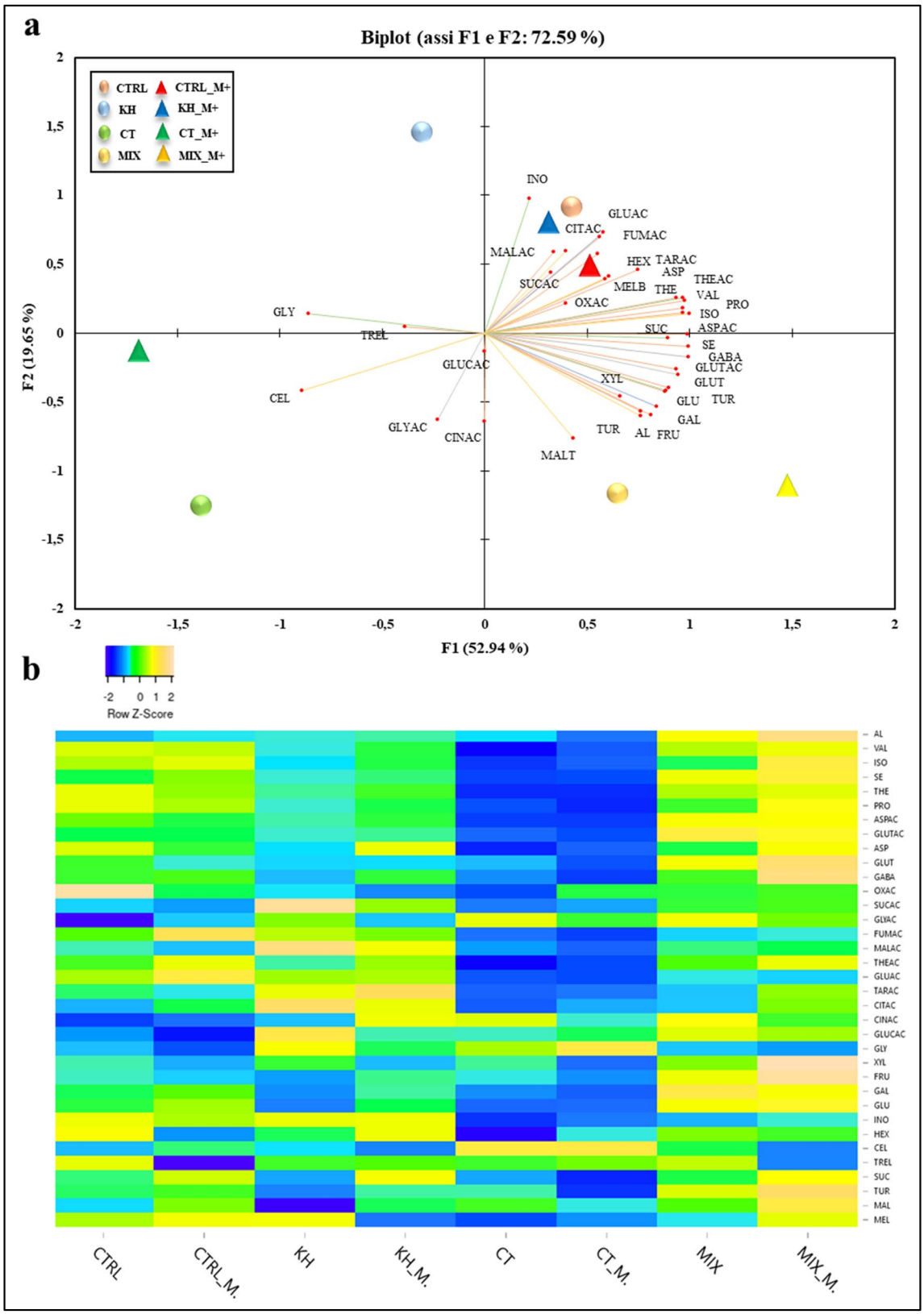
hydroxycinnamic acids, such feruloylquinic acid and caffeoyltartaric-p-coumaroyl acid (Table 3) determined the separation along the PC2 of CT and MIX_M+ treatments (Fig. 6a). The Heatmap deriving from these metabolomics data confirmed that the net dissimilarity in the relative amount of these compounds was a function of treatments (Fig. 6b). In particular, plants treated only with the microbial inoculum (CTRL_M+) or under the individual application of either KH or CT showed a metabolic profile similar to that of untreated plants (Fig. 6b), which was characterized by a reduced biosynthesis of polyphenols compounds (Fig. S9 and S10). Conversely, the MIX treatment significantly affected the leaves metabolome (Fig. 6b), by increasing the amount of all identified metabolites (Fig. S9 and S10). Moreover, the combined application of MIX with the microbial inoculum (MIX_M+), determined a significant increase of the accumulation of flavonoids in the corresponding leaves extracts (Fig. 6b and S10), mainly luteolin-7-glucoside and quercetin-3-O-glucoside (Table 3).

Discussion

Effect of different biostimulants treatments on plants growth and nutrition

The promotion of both plants productivity and nutrient status by the individual application of KH, CT, and beneficial microorganisms (i.e. AMF, PGPB and Trichoderma spp.) has been widely demonstrated on different crops (Taha and Osman 2018; Priya et al. 2021; Saia et al. 2019, 2020). In our experiment, we observed that in absence of soil microbial inoculation, the MIX treatment showed a significanltly larger biomass production and nutrient uptake in lettuce than for control or individual KH and CT applications (Figs. 1, 2 and 3). These results could be ascribed to a synergistic effect of the two different humic extracts on plant growth. In particular, the most reported biological effect of HS and HLS is promotion of the plant root system development, due to stimulation of the pumping activity of the H+-ATPase through the root plasma membrane (PM), by the auxin-like molecules trapped into the supramolecular structure of HS and mobilized during roots exudation of organic acids (Piccolo 2002; Canellas et al. 2002, 2012; Muscolo et al. 2007). In addition, HS are known to improve plant nutrition, directly by upregulating the expression of transporter genes involved in nutrient primary uptake (Jannin et al. 2012; Jindo et al. 2016; Nardi et al. 2017), and indirectly by forming complexes with metallic cations, like Zn, Mn, Cu, Fe, which are no longer leached down and maintain their availability to plant absorption (Zanin et al. 2019).

Similarly to humic extracts by alkaline solutions, CT obtained from compost contains accessible nutrients, beneficial microorganisms (such as biostimulants, biofertilisers, and biopesticide microorganisms), and/or growth promoter compounds, like phytohormones, which stimulate root and vegetative growth of several crops, as well as their nutritional status (Naidu et al. 2013; Pane et al. 2016; Zaccardelli et al. 2018; Ros et al. 2020). Moreover, the occurrence of supramolecular interactions between hydrophobic and hydrophilic molecules in humic or humic-like matter were found essential for the environmental reactivity of dissolved organic molecules (Piccolo 2002) and their bioactivity on plants (Nardi et al. 2007; Canellas et al. 2010, 2012; Piccolo et al. 2019). In fact, Savarese et al. (2022) previously observed that the biostimulation of the MIX treatment on maize seedlings growth was positively correlated to a balance between the polar lignin derivatives in CT and the aromatic components in the mostly apolar KH. Their combination ensured a conformational flexibility of the mixed suprastructure that made effective the slow release of the trapped bioactive molecules, following the rearrangement of the humic supramolecular assembly by the action of root-exuded organic acids (Savarese et al. 2022). These findings are in accordance with the observed greater efficiency of the KH and CT mixed treatment in stimulating lettuce productivity and nutritional status (Figs. 1, 2 and 3), which may be related not only to


a
Biplot (assi F1 e F2: 72.59 %)
CTRL
CTRL_M+
KH
KH_M+
CT
CT_M+
MIX
MIX_M+
INO
GLUAC
CITAC
FUMAC
MALAC
HEX
TARAC
ASP
THEAC
SUCAC
MELB
THE
VAL
PRO
OXAC
ISO
GLY
SUC
ASPAC
TREL
SE
GABA
GLUCAC
GLUTAC
XYL
GLUT
CEL
GLU
TUR
GAL
GLYAC
CINAC
TUR
AL
FRU
MALT
F2 (19.65 %)
F1 (52.94 %)
b
-2 0 1 2
Row Z-Score
AL
VAL
ISO
SE
THE
PRO
ASPAC
GLUTAC
ASP
GLUT
GABA
OXAC
SUCAC
GLYAC
FUMAC
MALAC
THEAC
GLUAC
TARAC
CITAC
CINAC
GLUCAC
GLY
XYL
FRU
GAL
GLU
INO
HEX
CEL
TREL
SUC
TUR
MAL
MEL
CTRL
CTRL_M.
KH
KH_M.
CT
CT_M.
MIX
MIX_M.

◂**Fig. 4** PCA biplot (**a**) based on metabolites identified by GC-MS in the polar fraction of leaves extracts from lettuce plants treated with different biostimulants. The combination of the shapes and colors indicate different treatments. Light-Circle: single biostimulant; Dark-triangle: biostimulant plus microbial inoculum (M+). Red: Control (CTRL); Blue: Potassium Humates from leonardite (KH); Green: Compost Tea from green compost (CT); Yellow: KH plus CT (MIX). Heatmap (**b**) resulting from metabolomics data. Each row represents a metabolite feature and each column represents a treatment. The row Z-score or scaled expression value of each feature is plotted in orange-yellow-green-blue color scale. The orange color of the tile indicates high abundance and blue indicates low abundance. The PCA and Heatmap scores represent the average value of nine replicates. [1]AL: alanine; VAL: valine; ISO: isoleucine; SE: serine; THE: threonine; PRO: proline; ASPAC: aspartic acid; GLUTAC: glutamic acid; ASP: asparagine; GLUT: glutamine; GABA: 4-aminobutyrric acid; OXAC: oxalic acid; SUCAC: succinic acid; GLYAC: glyceric acid; FUMAC: fumaric acid; MALAC: malic acid; THEAC: threonic acid; GLUAC: glutaric acid; TARAC: tartaric acid; CITAC: citric acid; CINAC: cinnamic acid; GLUCAC: glucuronic acid; GLY: glycerol; XYL: xylose; FRU: fructose; GAL: galactose; GLU: glucose; INO: myo-inositol; HEX: hexose; CEL: cellobiose; TREL: trehalose; SUC: sucrose; TUR: turanose; MAL: maltose; MELB: melibiose

the individual bioactivity of the two different humic extracts on plant growth, but also to the synergistic interaction of their specific molecular components.

We observed that the MIX_M+ treatment combining both KH and CT with the microbial product (M+) significantly increased lettuce shoot dry weight by 52 and 30% more than control samples (CTRL and CTRL_M+), 31 and 13% more than application of biostimulants alone (KH, CT, and MIX), and 24 and 21% more than the KH_M+ and CT_M+ combinations, respectively (Fig. 1b). Additionally, the MIX_M+ treatment positively affected the uptake of both macro- and micro-nutrients by lettuce plants (Figs. 2 and 3), being the N, P, K, S, Ca and Mg leaf content (mg plant^{-1} DW) larger by 55, 75, 34, 70, 141, and 74% than non-treated plants, respectively (Fig. 2b, d, f, h, l, n), and the Mn and Fe content in lettuce leaves greater by about 98 and 85% than control, respectively, (Fig. 3d, f, h). It is well known that microbial bioeffectors act as plant growth promoters by improving their nutritional status (Castiglione et al. 2021). In particular, the extra-radical mycelium of AMF can facilitate the uptake and transfer of mineral nutrients from soil to their host plants (Giovannini et al. 2020), and PGPB can stimulate plant growth by either increasing nutrients availability or by producing bioactive hormone-like compounds (Hayat et al. 2010). Furthermore, *Trichoderma* spp. was found to boost growth and productivity of lettuce plants through multiple mechanisms of action, such as the solubilisation of soil micronutrients and the modulation of root growth by producing metabolites with hormone-like activities (Fiorentino et al. 2018)., Several works have recently highlighted the symbiotic effects of AMF and PGPB when applied in combination, due to an increased colonization of rhizospheric fungi on host plant roots, a concomitant production of metabolites that improves cell permeability, and an enhancement of root exudation that in turn stimulates further the growth of AMF hyphae (Saia et al. 2019; Moreira et al. 2020). It has been also reported that the combination of *Trichoderma* spp. and PGPB or AMF stimulated plant development through the production of growth-promoting substances or by increasing the availability of nutrients and their uptake (López-Bucio et al. 2015; Bonini et al. 2020). Scientific evidences have suggested that AMF promote the production of IAA in plant tissues (Castiglione et al. 2021), while both PGPB and *Thricoderma* sp. are capable of producing this phytohormone of the auxin class (Khalid et al. 2004; Sanchez-Montesinos et al. 2020), which plays a key role in the regulation of plant growth, including plant cell division and root formation, thus affecting nutrient uptake. Another possible mechanism by which PGPMs could help plants to absorb nutrients concerns their ability to synthesize organic molecules and secrete in the soil compounds such as organic acids, phytases and acid phosphatases, thus facilitating the solubilization and/or mineralization of inorganic P (Santos-Torres et al. 2021). An additional benefit of bio-inoculation is the ability of some PGP bacteria to produce ion chelating compounds known as siderophores that increase plants iron acquisition, which is an essential element in key biochemical processes like photosynthesis and respiration (Backer et al. 2018). Several studies also showed that both rhizospheric bacteria and *Thricoderma* sp. protect plants against soil-borne pathogens by producing antibiotics or antimicrobial metabolites, which contribute to phytopathogen control and positively affect the plant in aspects of growth promotion (Vinale et al. 2009; Giovannini et al. 2020). Likewise, the combined treatment with humic extracts and microbial inoculants was recently found to significantly increase both productivity and performance of

Table 3 Relative abundance (a/is = metabolite-to-internal standard total area ratio) of main discriminant variables from PCA of both GC-MS and UHPLC-MS-IT-TOF metabolomics data of lettuce plants treated with different biostimulants

Metabolite	Relative abundance (a/is)							
	CTRL	CTRL_M+	KH	KH_M+	CT	CT_M+	MIX	MIX_M+
Amino acids								
Alanine	0.144 ± 0.01 ^{d,e}	0.158 ± 0.01 ^{c,d}	0.161 ± 0.01 ^{c,d}	0.167 ± 0.01 ^{c}	0.155 ± 0.01 ^{c,d}	0.127 ± 0.01 ^{e}	0.234 ± 0.01 ^{b}	0.234 ± 0.01 ^{a}
Serine	0.173 ± 0.01 ^{d}	0.197 ± 0.01 ^{c}	0.157 ± 0.01 ^{e}	0.168 ± 0.01 ^{d}	0.108 ± 0.01 ^{f}	0.112 ± 0.01 ^{f}	0.220 ± 0.01 ^{b}	0.240 ± 0.01 ^{a}
Proline	1.109 ± 0.07 ^{b}	1.038 ± 0.08 ^{b}	0.745 ± 0.03 ^{d}	0.853 ± 0.05 ^{c}	0.451 ± 0.06 ^{e}	0.352 ± 0.06 ^{f}	0.918 ± 0.06 ^{c}	1.238 ± 0.09 ^{a}
GABA	0.05 ± 0.001 ^{b}	0.05 ± 0.001 ^{b}	0.04 ± 0.001 ^{c}	0.05 ± 0.001 ^{b}	0.03 ± 0.001 ^{c,d}	0.03 ± 0.001 ^{d}	0.05 ± 0.001 ^{b}	0.07 ± 0.001 ^{a}
Aspartic acid	0.342 ± 0.05 ^{b}	0.308 ± 0.05 ^{c}	0.285 ± 0.02 ^{c}	0.313 ± 0.05 ^{b,c}	0.187 ± 0.04 ^{d}	0.184 ± 0.05 ^{d}	0.397 ± 0.05 ^{a}	0.402 ± 0.04 ^{a}
Glutamic acid	0.631 ± 0.03 ^{b}	0.630 ± 0.01 ^{b}	0.598 ± 0.05 ^{b}	0.610 ± 0.06 ^{b}	0.478 ± 0.02 ^{c}	0.459 ± 0.06 ^{c}	0.832 ± 0.03 ^{a}	0.811 ± 0.06 ^{a}
Organic acids								
Malic acid	19.01 ± 0.6 ^{c,d,e}	18.57 ± 0.9 ^{d,e,f}	21.39 ± 0.6 ^{a}	20.47 ± 0.4 ^{b}	18.33 ± 0.7 ^{e,f}	17.96 ± 0.4 ^{f}	19.15 ± 0.8 ^{c,d}	19.28 ± 0.6 ^{c}
Citric acid	1.826 ± 0.2 ^{d}	2.096 ± 0.2 ^{c}	2.732 ± 0.3 ^{a}	2.427 ± 0.6 ^{b}	1.649 ± 0.5^{e}	1.816 ± 0.6 ^{d}	1.867 ± 0.3 ^{d}	2.274 ± 0.6 ^{b}
Fumaric acid	0.256 ± 0.05 ^{b}	0.307 ± 0.01 ^{a}	0.267 ± 0.01 ^{b}	0.259 ± 0.01 ^{b}	0.194 ± 0.03 ^{d}	0.182 ± 0.07 ^{d}	0.217 ± 0.02 ^{c}	0.225 ± 0.01 ^{c}
Tartaric acid	1.08 ± 0.2 ^{c,d}	1.012 ± 0.1 ^{d,e}	1.301 ± 0.4 ^{b}	1.465 ± 0.5 ^{a}	0.841 ± 0.2 ^{f}	0.864 ± 0.4 ^{e,f}	0.967 ± 0.2 ^{d,e,f}	1.215 ± 0.2 ^{b,c}
Threonic acid	0.161 ± 0.02 ^{b}	0.171 ± 0.01 ^{a}	0.152 ± 0.03 ^{c}	0.165 ± 0.02 ^{a,b}	0.126 ± 0.01 ^{e}	0.133 ± 0.03 ^{d}	0.162 ± 0.02 ^{b}	0.170 ± 0.01 ^{a}
Cinnamic acid	0.252 ± 0.02 ^{e}	0.1257 ± 0.01 ^{e}	0.266 ± 0.01 ^{d}	0.298 ± 0.01 ^{a,b}	0.294 ± 0.01 ^{b}	0.273 ± 0.03 ^{d}	0.303 ± 0.03 ^{a}	0.284 ± 0.02 ^{c}
Saccharides								
Glycerol	0.309 ± 0.03 ^{d,e}	0.284 ± 0.05 ^{e}	0.379 ± 0.02 ^{a,b}	0.333 ± 0.03 ^{c,d}	0.359 ± 0.04 ^{b,c}	0.394 ± 0.05 ^{a}	0.311 ± 0.03 ^{d,e}	0.302 ± 0.07 ^{e}
Fructose	28.26 ± 1.4 ^{c,d}	27.12 ± 1.5 ^{d,e}	26.01 ± 0.5 ^{e}	28.79 ± 0.6 ^{c}	27.89 ± 0.6 ^{c,d}	25.66 ± 0.7 ^{f}	33.36 ± 1.0 ^{b}	37.59 ± 0.7 ^{a}
Glucose	10.42 ± 0.5 ^{c}	11.04 ± 0.8 ^{b,c}	9.05 ± 0.5 ^{d}	10.29 ± 0.6 ^{c}	8.87 ± 0.8 ^{d}	8.91 ± 0.4 ^{d}	11.78 ± 0.2 ^{a,b}	11.97 ± 0.4 ^{a}
Galactose	28.12 ± 1.1 ^{b}	29.10 ± 0.8 ^{b}	26.21 ± 0.4 ^{c}	27.78 ± 1.0 ^{b}	26.21 ± 0.5 ^{c}	25.66 ± 0.6 ^{c}	31.48 ± 0.7 ^{a}	30.71 ± 0.8 ^{a}
Myo-inositol	15.63 ± 1.0 ^{a}	15.25 ± 0.8 ^{a,b}	15.48 ± 0.7 ^{a,b}	15.64 ± 0.8 ^{a}	12.99 ± 0.6 ^{c}	13.47 ± 0.7 ^{c}	13.82 ± 0.8 ^{c}	14.24 ± 0.5 ^{b,c}
Cellobiose	0.416 ± 0.03 ^{b,c}	0.445 ± 0.03 ^{b,c}	0.428 ± 0.03 ^{b,c}	0.399 ± 0.04 ^{b,c}	0.530 ± 0.03 ^{a}	0.531 ± 0.03 ^{a}	0.453 ± 0.05 ^{b}	0.398 ± 0.01 ^{c}
Trehalose	0.702 ± 0.04 ^{a}	0.567 ± 0.08 ^{c}	0.677 ± 0.07 ^{a,b}	0.683 ± 0.07 ^{a}	0.676 ± 0.03 ^{a,b}	0.686 ± 0.05 ^{a}	0.696 ± 0.03 ^{a}	0.618 ± 0.02 ^{b,c}
Lipids								
Myristic acid	3.61 ± 0.3 ^{c,d}	3.34 ± 0.2 ^{d,e}	3.62 ± 0.3 ^{c,d}	6.43 ± 0.3 ^{a}	4.21 ± 0.2 ^{b}	3.16 ± 0.1 ^{e}	3.86 ± 0.2 ^{b,c}	4.20 ± 0.2 ^{b}
Stearic acid	1.316 ± 0.2 ^{c,d}	1.273 ± 0.1 ^{d}	1.285 ± 0.2 ^{c,d}	1.441 ± 0.2 ^{b}	1.472 ± 0.1 ^{a,b}	1.236 ± 0.6 ^{d}	1.386 ± 0.3 ^{b,c}	1.552 ± 0.3 ^{a}
Linoleic acid	1.16 ± 0.1 ^{d,e}	1.091 ± 0.1 ^{e,f}	1.024 ± 0.1 ^{f}	1.26 ± 0.2 ^{c,d}	1.48 ± 0.2 ^{a,b}	1.538 ± 0.1 ^{a}	1.303 ± 0.2 ^{c}	1.434 ± 0.2 ^{b}
Linolenic acid	1.196 ± 0.2 ^{c,d}	0.949 ± 0.1 ^{e}	0.929 ± 0.3 ^{e}	0.969 ± 0.1 ^{e}	1.590 ± 0.2 ^{b}	1.835 ± 0.3 ^{a}	1.114 ± 0.4 ^{d}	1.259 ± 0.3 ^{c}
Hexadecenol	0.444 ± 0.05 ^{e}	0.47 ± 0.01 ^{e}	0.512 ± 0.01 ^{d}	0.456 ± 0.03 ^{e}	0.583 ± 0.01 ^{c}	0.451 ± 0.01 ^{e}	0.623 ± 0.03 ^{b}	0.722 ± 0.01 ^{a}
Docosanol	0.812 ± 0.01 ^{d}	0.959 ± 0.03 ^{c}	1.266 ± 0.2 ^{a}	1.095 ± 0.1 ^{b}	0.856 ± 0.06 ^{c,d}	0.621 ± 0.02 ^{e}	1.094 ± 0.1 ^{b}	1.321 ± 0.1 ^{a}
β-Sitosterol	1.34 ± 0.2 ^{c,d}	1.30 ± 0.2 ^{d}	1.29 ± 0.2 ^{d}	1.40 ± 0.2 ^{c}	1.55 ± 0.2 ^{b}	1.38 ± 0.1 ^{c,d}	1.63 ± 0.1 ^{b}	1.84 ± 0.1 ^{a}
β-amyrin	0.471 ± 0.02 ^{a}	0.374 ± 0.04 ^{c,d}	0.415 ± 0.07 ^{b,c}	0.433 ± 0.02 ^{a,b}	0.403 ± 0.02 ^{b,c}	0.385 ± 0.03 ^{c,d}	0.358 ± 0.02 ^{d}	0.384 ± 0.04 ^{c,d}
Phenolic compounds								
p-Coumaroylquinic acid	8.52 ± 0.3 ^{e}	10.13 ± 0.3 ^{d}	10.39 ± 0.7 ^{b,c}	11.47 ± 0.4 ^{b,c}	12.38 ± 0.7 ^{a,b}	12.99 ± 0.6 ^{a}	13.21 ± 0.3 ^{a}	11.89 ± 0.3 ^{c,d}
Feruloylquinic acid	0.341 ± 0.04 ^{c}	0.390 ± 0.05 ^{a,b}	0.391 ± 0.04 ^{a,b}	0.420 ± 0.06 ^{a}	0.210 ± 0.03 ^{d}	0.336 ± 0.02 ^{c}	0.370 ± 0.03 ^{b,c}	0.337 ± 0.07 ^{c}
Caffeoyltartaric p-coumaroyl acid	2.23 ± 0.2 ^{c}	2.25 ± 0.2 ^{c}	2.33 ± 0.3 ^{c}	2.68 ± 0.2 ^{b}	1.76 ± 0.1 ^{c}	2.68 ± 0.2 ^{b}	2.87 ± 0.3 ^{a}	1.86 ± 0.1 ^{d}
Chlorogenic acid	4.17 ± 0.9 ^{d}	5.87 ± 0.4 ^{b,c}	5.43 ± 0.6 ^{c}	5.75 ± 0.5 ^{b,c}	5.41 ± 0.5 ^{c}	6.01 ± 0.7 ^{b}	6.71 ± 0.4 ^{a}	5.81 ± 0.3 ^{b,c}
Coutaric acid	1.71 ± 0.1 ^{a,b}	1.75 ± 0.1 ^{a,b}	1.77 ± 0.2 ^{a}	1.69 ± 0.1 ^{a,b}	1.63 ± 0.2 ^{b}	1.78 ± 0.1 ^{a}	1.79 ± 0.1 ^{a}	1.71 ± 0.1 ^{a,b}
Chicoric acid	4.11 ± 0.3 ^{d}	5.28 ± 0.3 ^{c}	4.24 ± 0.4 ^{d}	6.24 ± 0.6 ^{a,b}	5.04 ± 0.9 ^{c}	6.68 ± 0.8 ^{a}	6.07 ± 0.8 ^{b}	6.07 ± 0.8 ^{b}
Luteolin-7-glucoside	1.35 ± 0.1 ^{c}	1.49 ± 0.1 ^{b}	1.50 ± 0.1 ^{b}	1.44 ± 0.1 ^{b,c}	1.77 ± 0.2 ^{a}	1.81 ± 0.1 ^{a}	1.84 ± 0.1 ^{a}	1.88 ± 0.1 ^{a}
Luteolin-7-glucuronide	3.37 ± 0.4 ^{e}	3.56 ± 0.2 ^{d,e}	3.11 ± 0.3 ^{f}	3.66 ± 0.3 ^{c,d}	3.08 ± 0.2 ^{f}	4.11 ± 0.2 ^{a,b}	4.22 ± 0.3 ^{a}	3.91 ± 0.3 ^{b,c}
Quercetin-3-O-glucoside	5.15 ± 0.7 ^{c}	6.11 ± 0.4 ^{b}	6.24 ± 0.6 ^{b}	6.62 ± 0.7 ^{b}	6.36 ± 0.3 ^{b}	7.86 ± 0.6 ^{a}	7.82 ± 0.7 ^{a}	7.72 ± 0.4 ^{a}

Table 3 (continued)

Metabolite	Relative abundance (a/is)							
	CTRL	CTRL_M+	KH	KH_M+	CT	CT_M+	MIX	MIX_M+
Esculetin-6-O-glucoside	0.630 ± 0.05 [d]	1.019 ± 0.08 [c]	0.963 ± 0.1 [c]	0.971 ± 0.07 [c]	0.958 ± 0.1 [c]	1.121 ± 0.1 [b]	1.247 ± 0.04 [a]	0.926 ± 0.1 [c]

CTRL Control, *KH* Potassium Humates from leonardite, *CT* Compost Tea from green compost, *MIX* KH plus CT, *M+* plus microbial inoculum (Micosat TabPlus). Values are the means of nine replicates ± standard deviation. Different letters indicate significant differences according to LSD test ($p < 0.05$)

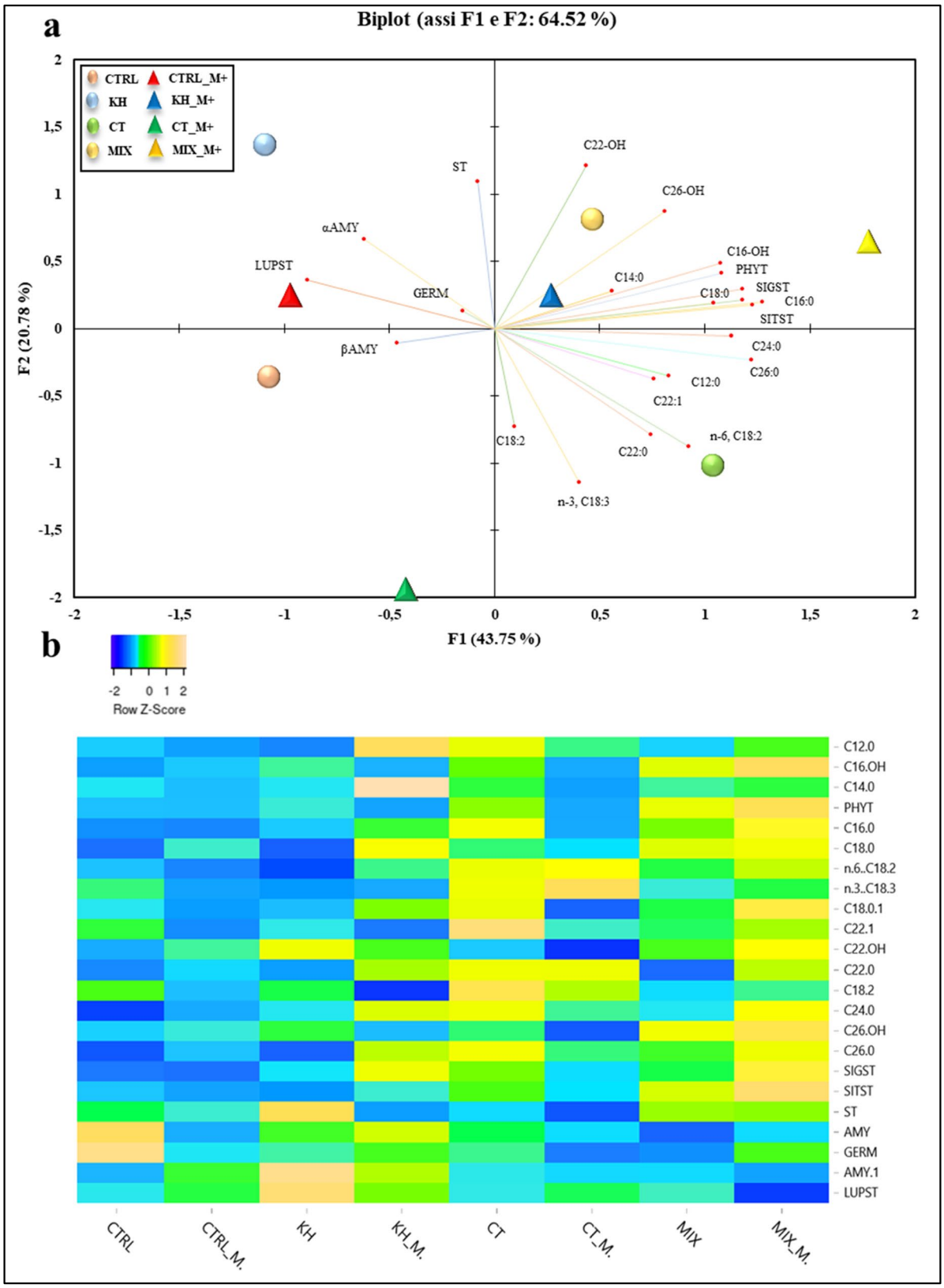
different crops (Canellas et al. 2013; Ekin 2019; Cozzolino et al. 2021).

The synergistic effect of humic materials and beneficial microorganisms is related to several mechanisms of action (Olivares et al. 2017). It is an acquired knowledge that HS influence root architecture and morphology, by inducing the formation of lateral roots and increasing root hair length and density (Canellas et al. 2002, 2012; Nardi et al. 2017). The natural openings due to the humic-induced new roots could promote the establishment of a plant-microorganism mutual symbiosis by favouring their entry into the host plant (Olivares et al. 2017). Moreover, HS application was reported to enhance the exudation of organic acids in maize (Canellas et al. 2008, 2019a), which may represent an advantage for the applied microbial inoculants over soil-dwelling microorganisms, since organic acids constitute a main nutrients source for soil microorganisms (Olivares et al. 2017; Nardi et al. 2021). The intensification of root exudation by HS can also lead to a change in the microbial community of the rhizosphere (Puglisi et al. 2008, 2009). In fact, Schoebitz et al. (2016) found that the treatment with both HA and a microbial consortium (bacteria and fungi) in blueberries (*Vaccinium corymbosum* L.), significantly increased shoot and root dry matter, while concomitantly altered the bacterial community of the rhizosphere, possibly due to changes in the root exudates pattern. Similarly, Cozzolino et al. (2021) recently showed that the combined application of humic acids, beneficial bacteria and two mycorrhizal fungi stimulated both maize growth and nutrient uptake (especially P), and positively influenced the native microbial community, favoring the development of arbuscular mycorrhizal populations. On the other hand, Siddiqui et al. (2008) and Naidu et al. (2013) also revealed that microbial-enriched compost teas significantly stimulated both root and shoot development of treated plants, as well as their nutritional status and photosynthetic activity. The authors highlighted that the available nutrients and beneficial microorganisms carried out by CT not only improved soil chemical and biological fertility, but also reduced the population of soil antagonists and induced a systematic resistance in the host plant, thereby enhancing root system expansion and vegetative growth (Siddiqui et al. 2008; Naidu et al. 2010, 2013).

These evidences well support the greater biological effectiveness of the microbial inoculum (M+) when it was applied in combination with leonardite KH or CT from green compost, and even more in synergy with their mixture (MIX_M+) (Figs. 1, 2 and 3). Furthermore, it should be noted that the biostimulation exerted by MIX_M+ on both lettuce productivity and nutrient uptake (Figs. 1, 2 and 3), may be accounted to the new supramolecular arrangement due to the interacting affinity between the polar bioactive molecules of CT from green compost and the apolar assemblies of leonardite KH (Savarese et al. 2022). It is reasonable to assume that the balanced molecular features of the MIX sample not only may provide a hydrophobic protection of the applied inoculant from soil antagonists, but also promote a tighter communication between preselected microorganisms and plants, through the adsorption on the host roots facilitated by the apolar humic components (Piccolo et al. 2019).

Effect of different biostimulants treatments on primary metabolism

The largest effect on plants growth and nutritional state observed for the MIX treatment with or without combination with microbial inoculum (MIX_M+), was also reflected by the primary metabolomics analysis of both polar and apolar fraction of lettuce leaves extracts (Figs. 4 and 5).


a
Biplot (assi F1 e F2: 64.52 %)
CTRL
CTRL_M+
KH
KH_M+
CT
CT_M+
MIX
MIX_M+
C22-OH
ST
C26-OH
αAMY
C16-OH
PHYT
LUPST
C14:0
GERM
C18:0
SIGST
C16:0
SITST
βAMY
C24:0
C26:0
C12:0
C22:1
C18:2
n-6, C18:2
C22:0
n-3, C18:3
F2 (20.78 %)
F1 (43.75 %)
b
-2 0 1 2
Row Z-Score
C12.0
C16.OH
C14.0
PHYT
C16.0
C18.0
n.6..C18.2
n.3..C18.3
C18.0.1
C22.1
C22.OH
C22.0
C18.2
C24.0
C26.OH
C26.0
SIGST
SITST
ST
AMY
GERM
AMY.1
LUPST
CTRL
CTRL_M.
KH
KH_M.
CT
CT_M.
MIX
MIX_M.

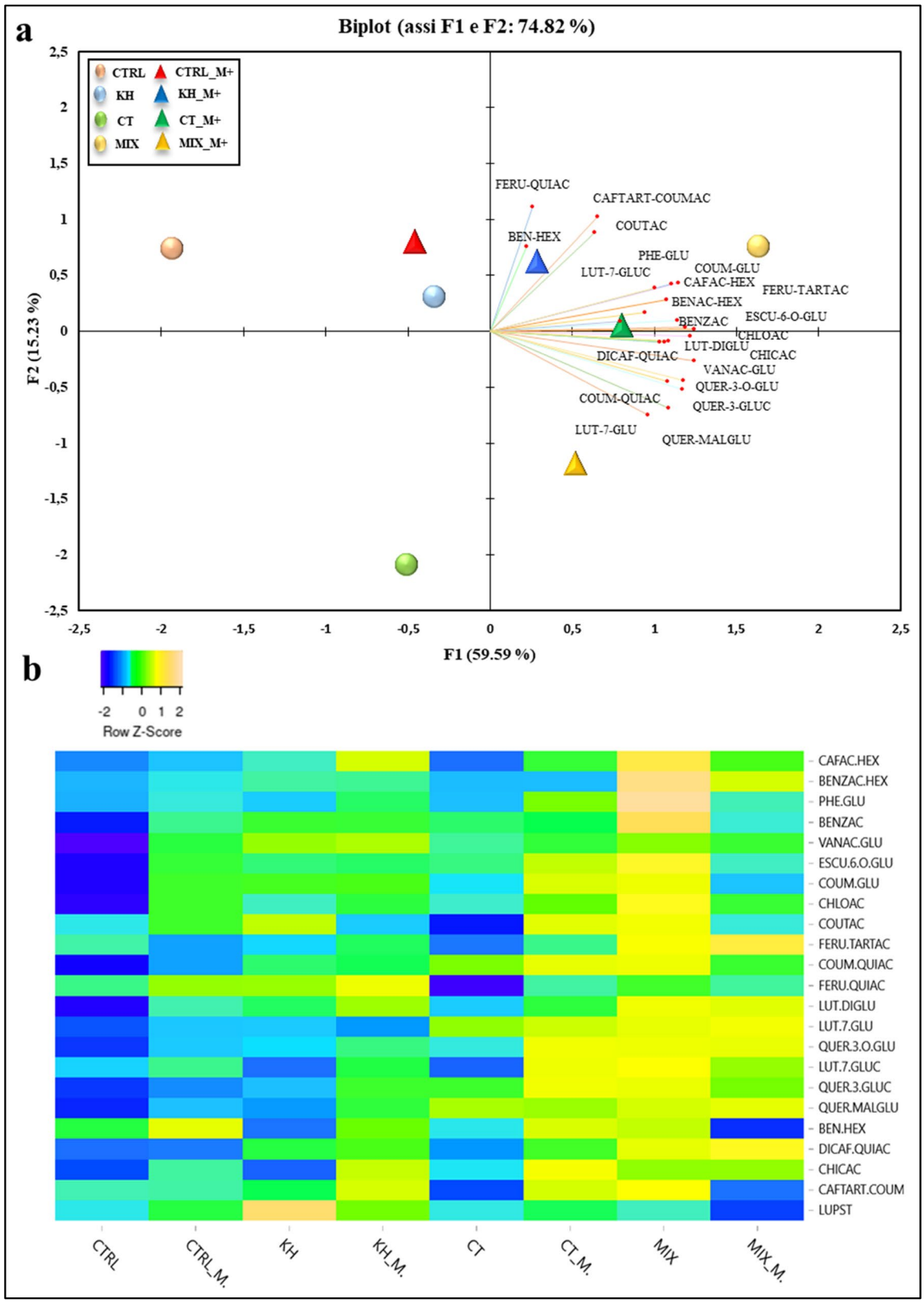
◂**Fig. 5** PCA biplot (**a**) based on metabolites identified by GC-MS in the apolar fraction of leaves extracts from lettuce plants treated with different biostimulants. The combination of the shapes and colors indicate different treatments. Light-Circle: single biostimulant; Dark-triangle: biostimulant plus microbial inoculum (M+). Red: Control (H_20); Blue: Potassium Humates from leonardite (KH); Green: Compost Tea from green compost (CT); Yellow: KH plus CT (MIX). Heatmap (**b**) resulting from metabolomics data. Each row represents a metabolite feature and each column represents a treatment. The row Z-score or scaled expression value of each feature is plotted in orange-yellow-green-blue color scale. The orange color of the tile indicates high abundance and blue indicates low abundance. The PCA and Heatmap scores represent the average value of nine replicates. [1]C12:0 lauric acid; C16-OH hexadecenol; C14:0 mirystic acid; PHYT phytol acetate; C16:0 palmitic acid; C18:0 stearic acid; n-6, C18:2 α-linoleic acid; n-3, C18:3 linolenic acid; C22:1 erucic acid; C22-OH docosanol; C22:0 docosanoic acid; C18:2 linoleic acid; C24:0 tetracosanoic acid; C26-OH hexacosanol; C26:0 hexacosanoic acid; SIGST sigmasterol; SITST sitosterol; ST steroid; αAMY α-amyrin; βAMY β-amyrin; GERM germanicol; LUPST lupenol acetate

An overall increase of amino acids biosynthesis, mainly alanine, glutamine, aspartic and glutamic acid, was detected in MIX_M+ treated plants (Table 3 and Fig. 4). Amino acids play an indispensable role in the metabolic pathways governing plant growth. The increase in alanine and aspartic acid content, which are involved in the carbon assimilation/fixation pathway, has already been reported after application of both single humic acids and combined microbial-humic treatments (Aguiar et al. 2018; Othibeng et al. 2021), as an indication of improved photosynthetic activity that promotes lettuce growth. In fact, the role of humic materials in the regulation at transcriptional and post-transcriptional levels of genes involved in the major metabolic plant functions, such as carbon/nitrogen metabolism and photosynthesis, has been widely demonstrated (Trevisan et al. 2011; Jannin et al. 2012).

Additionally, the activity of aspartate aminotransferase (AspAT) was found to be intensified in maize seedlings treated with compost tea, due to the presence in this extract of hydrophilic bioactive compounds (Vaccaro et al. 2009). Aspartate is a metabolically reactive amino acid that also serves as nitrogen donor in numerous aminotransferase reactions and is the precursor of essential amino acids such as threonine, lysine, isoleucine, and methionine (Coruzzi et al. 2015). In particular, the considerable amount of threonine and valine in the untreated lettuces, as compared to those under the biostimulants treatments (Fig. S4), may suggest a state of plants stress, since these two amino acids are involved in the BCAAs pathway, which are abundantly synthesized in response to abiotic stresses (Joshi et al. 2010). On the other hand, the significant increase of glutamine and glutamic acid in leaves extracts of the MIX_M+ treatment (Table 3) is in line with the already reported role of humic matter in the upregulation of key enzymes involved in N assimilation, such as glutamine synthetase and glutamate synthase (Ertani et al. 2011, 2019; Vaccaro et al. 2015). These enzymes are essential for the initial assimilation of nitrogen in plants, and perform several functions as substrates in protein biosynthesis, being both carrier and donor of N for the biosynthesis of amino acids, nucleotidic bases, and a host of other N-containing compounds (Coruzzi et al. 2015).

Other observed variations in the amino acidic profile included an increase of serine, proline, and GABA levels in MIX_M+ treated plants (Table 3 and Fig. 4). Apart from its proteinogenic function, serine plays an essential role in signalling mechanisms, plant photorespiration and biosynthesis of several biomolecules required for cell proliferation (Ros et al. 2014). Similarly, GABA acts as a signal to stimulate plant tissues to either accumulate or reduce energy and control C/N balance in plants (Michaeli and Fromm 2015), whereas proline is commonly recognized as an important osmolite that regulates plants response to a variety of abiotic stresses (Kavi Kishor and Sreenivasulu 2014). Othibeng et al. (2021) and Vinci et al. (2018a, b) have already reported an increase of serine and GABA levels in plants treated with either humic acids or a combination of beneficial microrganisms and compost. Moreover, Aguiar et al. (2018) recently related the increase in proline accumulation after the combined application of humic acids and PGPB, to the promotion of glutamate/glutamine synthesis in treated plants.

In respect to the biosynthesis of organic acids, MIX and MIX_M+ treated plants showed no significant differences from control samples (Fig. S5), except for a considerable amount of cinnamic acid in leaves of the MIX treatment (Table 3). This phenolic acid is synthetized in plants from phenylalanine through the action of the PAL/TAL (phenylalanine/tyrosine ammonia-lyase) enzyme, whose activity is recognized to increase after treatments with humic


a
Biplot (assi F1 e F2: 74.82 %)
CTRL
CTRL_M+
KH
KH_M+
CT
CT_M+
MIX
MIX_M+
FERU-QUIAC
CAFTART-COUMAC
COUTAC
BEN-HEX
PHE-GLU
LUT-7-GLUC
COUM-GLU
CAFAC-HEX
FERU-TARTAC
BENAC-HEX
BENZAC
ESCU-6-O-GLU
CHLOAC
LUT-DIGLU
CHICAC
DICAF-QUIAC
VANAC-GLU
QUER-3-O-GLU
COUM-QUIAC
QUER-3-GLUC
LUT-7-GLU
QUER-MALGLU
F2 (15.23 %)
F1 (59.59 %)
b
Row Z-Score
CAFAC.HEX
BENZAC.HEX
PHE.GLU
BENZAC
VANAC.GLU
ESCU.6.O.GLU
COUM.GLU
CHLOAC
COUTAC
FERU.TARTAC
COUM.QUIAC
FERU.QUIAC
LUT.DIGLU
LUT.7.GLU
QUER.3.O.GLU
LUT.7.GLUC
QUER.3.GLUC
QUER.MALGLU
BEN.HEX
DICAF.QUIAC
CHICAC
CAFTART.COUM
LUPST
CTRL
CTRL_M.
KH
KH_M.
CT
CT_M.
MIX
MIX_M.

◂**Fig. 6** PCA biplot (**a**) based on metabolites identified by LCMS-IT-TOF in the leaves extracts from lettuce plants treated with different biostimulants. The combination of the shapes and colors indicate different treatments. Light-Circle: single biostimulant; Dark-triangle: biostimulant plus microbial inoculum (M+). Red: Control (H_20); Blue: Potassium Humates from leonardite (KH); Green: Compost Tea from green compost (CT); Yellow: KH plus CT (MIX). Heatmap (**b**) resulting from metabolomics data. Each row represents a metabolite feature and each column represents a treatment. The row Z-score or scaled expression value of each feature is plotted in orange-yellow-green-blue color scale. The orange color of the tile indicates high abundance and blue indicates low abundance. The PCA and Heatmap scores represent the average value of nine replicates. [1]CAFAC-HEX: Dihydrocaffeic acid hexose; BENAC-HEX: Dihydroxybenzoic acid hexose; PHE-GLU: 4-hydroxyphenylacetyl glucoside; BENZAC: Hydroxybenzoic acid derivative; VANAC-GLU: Vanillic acid glucoside; ESCU-6-O-GLU: Esculetin 6-O-glucoside; COUM-GLU: p-coumaroyl glucoside; CHLOAC: Chlorogenic acid; COUTAC: Coutaric acid; FERU-TARTAC: Feruloyl tartaric acid; COUM-QUIAC: p-coumaroylquinic acid; FERU-QUIAC: Feruloylquinic acid; LUT-DIGLU: Luteolin diglucoside; LUT-7-GLU: Luteolin 7-glucoside; QUER-3-O-GLU: Quercetin 3-O-glucoside; LUT-7-GLUC: Luteolin 7-glucuronide; QUERC-3-GLUC: Quercetin 3-glucuronide; QUERC-MALGLU: Quercetin malonylglucoside; BEN-HEX: Hydroxybenzoyl dihydroxybenzoyl hexose; DICAF-QUIAC: Dicaffeoylquinic acid; CHICAC: Chicoric acid; CAFTART-COUMAC: Caffeoyltartaric-p-coumaroyl acid

substances (Schiavon et al. 2010). Although the presence of phenylalanine in leaves extracts was not detected (Table S3), the remarkable production of cinnamic acid in MIX treated plants suggests an increase in the polyphenol's metabolism induced by the combined humic biostimulants. On the other hand, treatment with KH and microbial inoculum (M+), either alone or in combined application (KH_M+), significantly increased the accumulation of malic, citric and fumaric acid, as compared to untreated plants (Table 3 and Fig. 4). The TCA-related compounds are essential for several plants physiological processes, such as photosynthesis, photorespiration, nitrogen metabolism, reductant transport and the maintenance of photosynthetic redox balance (Araújo et al. 2012). Humic substances are known to upregulate the enzymes involved in the tricarboxylic acids (TCA) cycle (Nardi et al. 2007). Similarly, it has been recently shown the role of microbial inoculants in the promotion of organic acids accumulation, as observed in the concomitant improvement of tomatoes quality (Bona et al. 2017, 2018). Furthermore, Canellas et al. (2019a, b) lately reported an increase in the biosynthesis of TCA-related compounds due to the combined application of humic acids and PGPB that is in line with the increase of organic acids accumulation observed here for the KH_M+ treatment (Fig. S5).

An overall increase of saccharides in lettuce leaves, mainly fructose, glucose, and galactose (Table 3 and Fig. 4), was also detected for the MIX_M+ treatment (Fig. S6). Soluble sugars, such as fructose and glucose, play a central role in plants structure and metabolism. According to Rosa et al. (2009) and Zeeman (2015), soluble sugars are involved in several metabolic events and act as molecular signals in the regulation of genes involved in photosynthesis, disaccharides metabolism and osmolyte synthesis. Although it is well recognized the increase of sugars accumulation in leaves induced by humic substances or microbial inoculants (Merlo et al. 1991; Nwodo et al. 2012; van Tol de Castro et al. 2021), their combined application produce diverse results. Canellas et al. (2013) showed that free carbohydrate content in leaf extracts was 60% less than control in maize plants treated with HS and PGPB, whereas Vinci et al. (2018a, b) found in the same specie an accumulation of glucose and fructose following the combined application of compost with beneficial bacteria or *Trichoderma* spp. In the latter work, the overproduction of soluble sugars was attributed to an efficient microorganisms-plant-compost synergism that led in treated plants to an increase in photosynthetic activity and shoots growth. This seems to be in line with our positive results for lettuce plants with under the MIX_M+ treatment, which improved the overall plants productivity, nutritional status and metabolism more than other treatments. Moreover, the role of CT in the MIX_M+ treatment should be considered, since compost teas application was shown to stimulate carbohydrates biosynthesis, due to improvement of the nutritional status of treated plants (Liguori et al. 2015; Ali 2015; Abou-el-hassan and El-batran 2020), thus also explaining the increase of cellobiose in both CT and CT_M+ treated leaves (Table 3 and Fig. 4). Interestingly, high levels of myo-inositol were found in control samples (Table 3 and Fig. 4). Myo-inositol derivatives are recognized as signal compounds in plants, as well as key metabolites under unfavourable conditions (Valluru and Van den Ende 2011), thus suggesting an onset of stress in untreated plants (Fig. S6).

The lipid metabolism was significantly affected by both non-microbial and microbial treatments (Fig. 5). The application of leonardite KH or CT from green compost in separate combination with the microbial inoculum (M+) greatly increased the accumulation of fatty acids (Fig. S7), whereas the MIX_M+ treatment remarkably improved the biosynthesis of fatty alcohols and sterols (Fig. S8). Lipids have essential functions in plants as the main structural components of cell membrane, substantial chemical reserve of free energy and cell signal messengers (Suh et al. 2015). In our study, plants under KH_M+ treatment synthetized a considerable amount of lauric and myristic acid, while CT_M+ showed an accumulation of both linoleic and α-linolenic acid in treated leaves (Table 3 and Fig. S7). These long-chain fatty acids are important constituent of storage lipids in plants, and the combined application of humic acids and PGPB has been reported to increase their biosynthesis in both maize and sugarcane seedlings (Canellas et al. 2019b). Similarly, Aguiar et al. (2018) previously revealed that plants accumulation of long-chain fatty alcohols are also enhanced by mixed humic-microbial biostimulants, which is in line with the considerable amount of hexadecenol, docosanol and hexacosanol found here in MIX_M+ treated plants (Table 3 and Fig. S8). The same authors found a large concentration of β-sitosterol in sugarcane leaves treated with HA and PGPB. This lipid compound is the main plants sterol involved into cellulose elongation chain and was also revealed to be significantly increased in lettuce plants under the MIX_M+ application (Table 3). Finally, leaves of untreated plants showed an accumulation of β-amyrin and germanicol (Table 3 and Fig. 5). Since terpenoids biosynthesis has recently been related to plants response to salt stress (Basyuni et al. 2009), this may suggest an initial stress condition of control samples that did not occur under treatments with humic biostimulants and microbial inoculum.

Therefore, our results suggest an overall capacity of mixed leonardite KH and CT from green compost, applied in combination with a microbial inoculum (MIX_M+), to positively affect the primary metabolism of lettuce plants (Figs. 4 and 5), in accordance with the observed greater nutritional status of MIX_M+ treated leaves (Figs. 1, 2 and 3). The biostimulant activity of the MIX_M+ treatment could be ascribed to a synergistic interaction between the two different humic extracts and the microbial bioeffectors, which may improve the biological effectiveness of each individual components of the mixture. This is in line with previous finding that showed that rhizosphere treatment with humic materials could favour both the survival of beneficial microorganisms in the soil environment and their colonization ability (Olivares et al. 2017; Canellas et al. 2019b). Similarly, other studies have reported the potential role of microbial-enriched compost tea in improving soil chemical and biological proprieties, thereby creating better conditions for the bioactivity of preselected microbes and plant root development (Naidu et al. 2010, 2013). In particular, the calibrated mixture of the readily available polar compounds of CT and the apolar domains of KH in our experiments, may have increased increased the performance of the inoculated microbial bioeffectors, by providing both an hydrophobic protection against soil antagonists and a carbon source for their multiplication.

Effect of different biostimulants treatments on secondary metabolism

The advantage in using UHPLC to separate polyphenolic compounds in plant extracts have recently been highlighted (Abu-Reidah et al. 2013). Our results showed that UHPLC-MS-IT-TOF analysis of leaves extracts allowed the identification of hydroxycinnamic acids and flavonols as the main phenolic compounds in lettuce plants (Table S5 and Fig. S3), right in line with previous studies conducted on the same species and with similar techniques (Pepe et al. 2015). In particular, the application of the MIX treatment provided the largest effect on the secondary metabolism of treated plants (Fig. 6). The accumulation of cinnamic acid derivatives, mainly chlorogenic and coutaric acid, as well as of flavonoids, such as the glucoside conjugates of both luteolin and quercetin, increased in MIX treated leaves significantly more than in all other treatments (Table 3). As recalled above, humic substances were found to upregulate the PAL/TAL activity, the enzyme that catalyses the first step in the biosynthesis of plant polyphenols (Schiavon et al. 2010). Conselvan et al. (2017) recently indicated that PAL activity can be also stimulated by the application of leonardite humic acids on lettuce seedlings, with a consequent accumulation of hydroxycinnamic acids, such as p-coumaric

and chlorogenic acid. Moreover, Cruz et al. (2014) reported an increase in total phenolic compounds of lettuce plants treated with espresso coffee residues. These findings are in line with the overproduction of polyphenols compounds observed by treating lettuce plants with a mixture of KH and CT from coffee wastes compost (Fig. 6).

These polyphenols are commonly recognized as antioxidant molecules in plants, playing a major role in ensuring plant growth under abiotic stresses (Shinozaki et al. 2015). Under unfavourable growth conditions, such as drought or salinity stress, the generation of Reactive Oxygen Species (ROS) is one of the primary responses in plants, causing significant cell damage (Djoukeng et al. 2008). Massive ROS production is potentially harmful, if not controlled by antioxidant mechanism, since it can induce bleaching of photosynthetic pigments, degradation and alteration of protein structure and function, lipid peroxidation, and damage to organic molecules, including DNA and RNA (Apel and Hirt 2004). Overproduction of antioxidants compounds such as ascorbic acid, glutathione and polyphenols is the major response of plants to the oxidative stress (Arbona et al. 2013). Among these molecules, hydroxycinnamic acids are precursors of lignin, which constitute an important stress defence mechanism, especially at the root level where they can modulate cell wall composition and stiffness (Peterson et al. 2010). Similarly, flavonoids, such as luteolin 7-O and quercetin 3-O glycosides, are potent free radical scavengers/antioxidants that effectively prevent ROS generation (Brunetti et al. 2013).

Another important mechanism of plants response to oxidative stress is the activation of enzymatic antioxidant systems. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are activated in plants to reduce the concentration of hydrogen peroxide and superoxide (Canellas et al. 2020). In this regard, Cordeiro et al. (2011) showed that the treatment with HS stimulated catalase activity in plants, whereas García et al. (2016) have shown the role of humic extracts in the upregulation of peroxidase that is capable to reduce ROS concentration in cell, thereby restoring the cytosolic redox homeostasis. In the latter study, the antioxidant defence induced in plants by HS was shown to be related to their biostimulant activity, as HS led to a state of eustress whose final effect is somehow beneficial to plants. In the search of an activity-structure relationship for humic matter (Savy et al. 2020; Nardi et al. 2021), it has been shown that bioactive polar humic molecules can interact with cell membrane in root surfaces and stimulate plant growth, while the conformational stability of the humic suprastructures defines the type and intensity of bioactivity in plants (García et al. 2019). Moreover, Monda et al. (2021) recently reported that molecules present in the supramolecular structure of HS such as lignin-derived fragments, aromatic structures and lipids, are potentially involved in the modulation of ROS level in plant by priming their defence systems, and resulting in increased root exploration and antioxidant production. In line with this, we earlier observed that the bioactivity of the MIX treatment on maize seedlings growth was positively correlated to the balanced molecular composition of this mixture, characterized by both lignin derivatives from CT and aromatic components from KH (Savarese et al. 2022).

These outcomes could also explain the overall improved secondary metabolism of MIX treated plants detected in this experiment (Fig. 6), which may arise from the mixture of humic extracts containing different type of bioactive molecules, whose stable supramolecular assembly guarantees a protection from biotic degradation but also their availability to plants following an alteration of the humic conformational arrangement upon the action of root-exuded organic acids (Savarese et al. 2022).

Furthermore, consumers' interest in lettuce has increased dramatically in recent years due to the considerable content of phytochemicals, such as polyphenols, having a positive role on human health. Phenolic compounds alone and vegetables containing polyphenols have shown beneficial effects against several human diseases (Kim et al. 2016). Cheng et al. (2014) showed the anti-diabetic effect of lettuce rich in phenolic compounds, especially chlorogenic acid, which determined less glucose content in blood. Lee et al. (2009) indicated the decrease of total plasma cholesterol in mice fed with red-pigmented lettuce, highlighting the potential role of this vegetable against the risk of cardiovascular disease. Pepe et al. (2015) outlined that polyphenols such as hydroxycinnamic acids derivatives, flavanols and coumarins in green lettuce have a potential anti-oxidant and anti-inflammatory effect on J774A.1 macrophage stimulated by *Escherichia coli* lipopolysaccharide. However, the

composition and abundance of these beneficial compounds in lettuce is highly variable and may be influenced by several factors such as cultivation practices, genetic makeup and harvesting stage (Adesso et al. 2016; Assefa et al. 2019). In fact, Ismail et al. (2019) have shown that transformation of *rol* genes significantly alters the metabolome of *L. sativa* by improving the biosynthesis of bioactive compounds, whereas Yang et al. (2017) reported an increase in total polyphenols accumulation, mainly luteolin and quercetin glycosides, by treating lettuce plants with exogenous glycine. Here, we similarly found that the application of different mixed humic extracts (MIX) stimulated the production of polyphenols in lettuce leaves (Fig. 6), which suggests that the combination of different biostimulants may be accounted to modulate plants secondary metabolism for the development of novel functional foods.

Conclusions

This work showed that the interaction between different humic materials and a commercial microbial consortium significantly enhanced the plants uptake of both macro- and micronutrients in respect to their individual application, with a consequent increase of lettuce biomass production. Moreover, the MIX treatment in combination with the microbial inoculum stimulated the accumulation in lettuce leaves of essential primary metabolites, mainly amino acids and saccharides. We also evaluated the changes in the polyphenolic secondary metabolism of treated lettuce plants and found that the mixture of KH and CT materials significantly increased leaf content of potent antioxidant compounds, as compared to other treatments. This stimulatory effect of the mixed humic extracts could be related to an optimum balanced molecular composition of bioactive molecules and protective hydrophobic domains, capable of modulating the humic molecular bioactivity (Savarese et al. 2022). Therefore, this study indicates that a proper mixture of humic biostimulants containing different type of humic bioactive molecules and their combination with microbial bioeffectors has great potentials to improve both the productivity and nutritional status of plants, as well as to modulate their metabolome for the development of novel crops functionalities.

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References

Abd-Allah EF, Hashem A, Alqarawi AA, Bahkali AH, Alwhibi MS (2015) Enhancing growth performance and systemic acquired resistance of medicinal plant Sesbania sesban (L.) Merr using arbuscular mycorrhizal fungi under salt stress. Saudi J Biol Sci 22:274–283

Abou-el-hassan S, El-batran HS (2020) Integration of some bio compounds with compost tea to produce sweet corn without mineral Fertilizers. Middle East J Agric Res 645–652. https://doi.org/10.36632/mejar/2020.9.3.51

Abu-Reidah IM, Contreras MM, Arráez-Román D et al (2013) Reversed-phase ultra-high-performance liquid chromatography coupled to electrospray ionization-quadrupole-time-of-flight mass spectrometry as a powerful tool for metabolic profiling of vegetables: *Lactuca sativa* as an example of its application. J Chromatogr A 1313:212–227. https://doi.org/10.1016/j.chroma.2013.07.020

Adesso S, Pepe G, Sommella E et al (2016) Anti-inflammatory and antioxidant activity of polyphenolic extracts from *Lactuca sativa* (var. Maravilla de Verano) under different farming methods. J Sci Food Agric 96:4194–4206. https://doi.org/10.1002/jsfa.7622

Aguiar NO, Olivares FL, Novotny EH, Canellas LP (2018) Changes in metabolic profiling of sugarcane leaves induced by endophytic diazotrophic bacteria and humic acids. PeerJ 6:e5445. https://doi.org/10.7717/peerj.5445

Ali OAM (2015) Role of humic substances and compost tea in improvement of endogenous hormones content, flowering and yield and its components of Faba bean (Vicia faba L.). Ann Agric Sci Moshtohor 53:373–384. https://doi.org/10.21608/assjm.2015.109889

Ansari FA, Ahmad I, Pichtel J (2019) Growth stimulation and alleviation of salinity stress to wheat by the biofilm

forming Bacillus pumilus strain FAB10. Appl Soil Ecol 143:45–54

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399. https://doi.org/10.1146/annurev.arplant.55.031903.141701

Araújo WL, Nunes-Nesi A, Nikoloski Z et al (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant Cell Environ 35:1–21. https://doi.org/10.1111/j.1365-3040.2011.02332.x

Arbona V, Manzi M, de Ollas C, Gómez-Cadenas A (2013) Metabolomics as a tool to investigate abiotic stress tolerance in plants. Int J Mol Sci 14:4885–4911. https://doi.org/10.3390/ijms14034885

Assefa AD, Choi S, Lee JE et al (2019) Identification and quantification of selected metabolites in differently pigmented leaves of lettuce (*Lactuca sativa* L.) cultivars harvested at mature and bolting stages. BMC Chem 13:1–15. https://doi.org/10.1186/s13065-019-0570-2

Babicki S, Arndt D, Marcu A et al (2016) Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res 44:W147–W153. https://doi.org/10.1093/nar/gkw419

Backer R, Rokem JS, Ilangumaran G et al (2018) Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front Plant Sci 871:1–17. https://doi.org/10.3389/fpls.2018.01473

Bárzana G, Aroca R, Ruiz-Lozano JM (2015) Localized and non-localized effects of arbuscular mycorrhizal symbiosis on accumulation of osmolytes and aquaporins and on antioxidant systems in maize plants subjected to total or partial root drying. Plant Cell Environ 38:1613–1627

Basyuni M, Baba S, Inafuku M et al (2009) Expression of terpenoid synthase mRNA and terpenoid content in salt stressed mangrove. J Plant Physiol 166:1786–1800. https://doi.org/10.1016/j.jplph.2009.05.008

Bernardo L, Morcia C, Carletti P, Ghizzoni R, Badeck FW, Rizza F, Lucini L, Terzi V (2017) Proteomic insight into the mitigation of wheat root drought stress by arbuscular mycorrhizae. J Proteome 169:21–32

Bhalerao RP, Eklof J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in Ara- bidopsis seedlings. Plant J 29:325–332

Bona E, Cantamessa S, Massa N et al (2017) Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads improve yield, quality and nutritional value of tomato: a field study. Mycorrhiza 27:1–11. https://doi.org/10.1007/s00572-016-0727-y

Bona E, Todeschini V, Cantamessa S et al (2018) Combined bacterial and mycorrhizal inocula improve tomato quality at reduced fertilization. Sci Hortic (Amsterdam) 234:160–165. https://doi.org/10.1016/j.scienta.2018.02.026

Bonini P, Rouphael Y, Miras-Moreno B et al (2020) A microbial-based biostimulant enhances sweet pepper performance by metabolic reprogramming of Phytohormone profile and secondary metabolism. Front Plant Sci 11:1–13. https://doi.org/10.3389/fpls.2020.567388

Bradáčová K, Florea AS, Bar-Tal A et al (2019) Microbial consortia versus single-strain inoculants: an advantage in PGPM-assisted tomato production? Agronomy 9. https://doi.org/10.3390/agronomy9020105

Brunetti C, Di Ferdinando M, Fini A et al (2013) Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. Int J Mol Sci 14:3540–3555. https://doi.org/10.3390/ijms14023540

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant Soil 383:3–41. https://doi.org/10.1007/s11104-014-2131-8

Canellas LP, Olivares FL (2014) Physiological responses to humic substances as plant growth promoter. Chem Biol Technol Agric 1:1–11. https://doi.org/10.1186/2196-5641-1-3

Canellas LP, Olivares FL, Okorokova-Façanha AL, Façanha AR (2002) Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H+-ATPase activity in maize roots. Plant Physiol 130:1951–1957. https://doi.org/10.1104/pp.007088

Canellas LP, Teixeira LRL, Dobbss LB et al (2008) Humic acids crossinteractions with root and organic acids. Ann Appl Biol 153:157–166. https://doi.org/10.1111/j.1744-7348.2008.00249.x

Canellas LP, Piccolo A, Dobbss LB et al (2010) Chemosphere chemical composition and bioactivity properties of size-fractions separated from a vermicompost humic acid. Chemosphere 78:457–466. https://doi.org/10.1016/j.chemosphere.2009.10.018

Canellas LP, Dobbss LB, Oliveira AL et al (2012) Chemical properties of humic matter as related to induction of plant lateral roots. Eur J Soil Sci 63:315–324. https://doi.org/10.1111/j.1365-2389.2012.01439.x

Canellas LP, Balmori DM, Médici LO et al (2013) A combination of humic substances and Herbaspirillum seropedicae inoculation enhances the growth of maize (Zea mays L.). Plant Soil 366:119–132. https://doi.org/10.1007/s11104-012-1382-5

Canellas LP, Olivares FL, Aguiar NO et al (2015) Humic and fulvic acids as biostimulants in horticulture. Sci Hortic (Amsterdam) 196:15–27. https://doi.org/10.1016/j.scienta.2015.09.013

Canellas LP, Olivares FL, Canellas NOA et al (2019a) Humic acids increase the maize seedlings exudation yield. Chem Biol Technol Agric 6:1–14. https://doi.org/10.1186/s40538-018-0139-7

Canellas NOA, Olivares FL, Canellas LP (2019b) Metabolite fingerprints of maize and sugarcane seedlings: searching for markers after inoculation with plant growth-promoting bacteria in humic acids. Chem Biol Technol Agric 6:1–10. https://doi.org/10.1186/s40538-019-0153-4

Canellas LP, Canellas NOA, Luiz Eduardo LES et al (2020) Plant chemical priming by humic acids. Chem Biol Technol Agric 7:1–17. https://doi.org/10.1186/s40538-020-00178-4

Castiglione AM, Mannino G, Contartese V et al (2021) Microbial biostimulants as response to modern agriculture needs: composition, role and application of these innovative products. Plants 10:1533. https://doi.org/10.3390/plants10081533

Chandra D, Srivastava R, Glick BR, Sharma AK (2018) Drought-tolerant Pseudomonas spp. improve the growth Perfor- mance of finger millet (*Eleusine coracana* (L.) Gaertn) under non-stressed and drought-stressed conditions. Pedosphere 28:227–240

Cheng DM, Pogrebnyak N, Kuhn P et al (2014) Development and phytochemical characterization of high polyphenol red lettuce with anti-diabetic properties. PLoS One 9:1–10. https://doi.org/10.1371/journal.pone.0091571

Colla G, Rouphael Y, Canaguier R et al (2014) Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. Front Plant Sci 5:1–6. https://doi.org/10.3389/fpls.2014.00448

Conselvan GB, Pizzeghello D, Francioso O et al (2017) Biostimulant activity of humic substances extracted from leonardites. Plant Soil 420:119–134. https://doi.org/10.1007/s11104-017-3373-z

Cordeiro FC, Santa-Catarina C, Silveira V, De Souza SR (2011) Humic acid effect on catalase activity and the generation of reactive oxygen species in corn (*Zea mays*). Biosci Biotechnol Biochem 75:70–74. https://doi.org/10.1271/bbb.100553

Coruzzi G, Last R, Dudareva N et al (2015) Amino Acids. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and molecular biology of plants, American Society of Plant Biologists, 2nd edn. Wiley-Blackwell, Hoboken, pp 289–336

Cozzolino V, Monda H, Savy D et al (2021) Cooperation among phosphate-solubilizing bacteria, humic acids and arbuscular mycorrhizal fungi induces soil microbiome shifts and enhances plant nutrient uptake. Chem Biol Technol Agric 8:1–19. https://doi.org/10.1186/s40538-021-00230-x

Cristofano F, El-Nakhel C, Rouphael Y (2021) Biostimulant substances for sustainable agriculture: origin, operating mechanisms and effects on cucurbits, leafy greens, and nightshade vegetables species. Biomolecules:11

Cruz R, Gomes T, Ferreira A et al (2014) Antioxidant activity and bioactive compounds of lettuce improved by espresso coffee residues. Food Chem 145:95–101. https://doi.org/10.1016/j.foodchem.2013.08.038

Djoukeng JD, Arbona V, Argamasilla R, Gomez-Cadenas A (2008) Flavonoid profiling in leaves of citrus genotypes under different environmental situations. J Agric Food Chem 56:11087–11097. https://doi.org/10.1021/jf802382y

Drobek M, Frąc M, Cybulska J (2019) Plant biostimulants: Importance of the quality and yield of horticultural crops and the improvement of plant tolerance to abiotic stress—A review. Agronomy 9(6):335

du Jardin P (2015) Plant biostimulants: definition, concept, main categories and regulation. Sci Hortic (Amsterdam) 196:3–14. https://doi.org/10.1016/j.scienta.2015.09.021

Ekin Z (2019) Integrated use of humic acid and plant growth promoting rhizobacteria to ensure higher potato productivity in sustainable agriculture. Sustain 11. https://doi.org/10.3390/su10023417

Ertani A, Francioso O, Tugnoli V et al (2011) Effect of commercial lignosulfonate-humate on Zea mays L. metabolism. J Agric Food Chem 59:11940–11948. https://doi.org/10.1021/jf202473e

Ertani A, Nardi S, Francioso O et al (2019) Metabolite targeted analysis and physiological traits of zea mays L. in response to application of a leonardite-humate and lignosulfonate-based products for their evaluation as potential biostimulants. Agronomy 9:1–18. https://doi.org/10.3390/agronomy9080445

Eudoxie G, Martin M (2019) Compost tea quality and fertility. Org Fertil - Hist Prod Appl. https://doi.org/10.5772/intechopen.86877

Ferreira S, Olivares FL, Canellas LP (2017) The biostimulant manufactured using diazotrophic endophytic bacteria and humates is effective to increase sugarcane yield. Chem Biol Technol Agric 1–6. https://doi.org/10.1186/s40538-017-0106-8

Filho JFDCL, Thomason WE, Evanylo GK et al (2020) The synergistic effects of humic substances and biofertilizers on plant development and microbial activity: a review. Int J Plant Soil Sci 32:56–75. https://doi.org/10.9734/ijpss/2020/v32i730306

Fiorentino N, Ventorino V, Woo SL et al (2018) Trichoderma -based biostimulants modulate rhizosphere microbial populations and improve N uptake efficiency, yield, and nutritional quality of leafy vegetables. 9:1–15. https://doi.org/10.3389/fpls.2018.00743

García AC, Santos LA, de Souza LGA et al (2016) Vermicompost humic acids modulate the accumulation and metabolism of ROS in rice plants. J Plant Physiol 192:56–63. https://doi.org/10.1016/j.jplph.2016.01.008

García AC, van Tol de Castro TA, Santos LA et al (2019) Structure-property-function relationship of humic substances in modulating the root growth of plants: a review. J Environ Qual 48:1622–1632

Giovannini L, Palla M, Agnolucci M et al (2020) Arbuscular mycorrhizal fungi and associated microbiota as plant biostimulants: research strategies for the selection of the best performing inocula. Agronomy 10. https://doi.org/10.3390/agronomy10010108

Hayat R, Ali S, Amara U et al (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598. https://doi.org/10.1007/s13213-010-0117-1

Huang XF, Zhou D, Guo J, Manter DK, Reardon KF, Vivanco JM (2015) Bacillus spp.: from rainforest soil promote plant growth under limited nitrogen conditions. J Appl Microbiol 118:672–684

Imbufe AU, Patti AF, Burrow D et al (2005) Effects of potassium humate on aggregate stability of two soils from Victoria, Australia. Geoderma 125:321–330. https://doi.org/10.1016/j.geoderma.2004.09.006

Ismail H, Gillespie AL, Calderwood D et al (2019) The health promoting bioactivities of *Lactuca sativa* can be enhanced by genetic modulation of plant secondary metabolites. Metabolites 9:1–18. https://doi.org/10.3390/metabo9050097

Jannin L, Arkoun M, Ourry A et al (2012) Microarray analysis of humic acid effects on Brassica napus growth: involvement of N, C and S metabolisms. Plant Soil 359:297–319. https://doi.org/10.1007/s11104-012-1191-x

Jindo K, Soares TS, Peres LEP et al (2016) Phosphorus speciation and high-affinity transporters are influenced by

humic substances. J Plant Nutr Soil Sci 179:206–214. https://doi.org/10.1002/jpln.201500228

Joshi V, Joung JG, Fei Z, Jander G (2010) Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress. Amino Acids 39:933–947. https://doi.org/10.1007/s00726-010-0505-7

Kang SM, Khan AL, Waqas M, You YH, Kim JH, Kim JG, Hamayun M, Lee IJ (2014) Plant growth-promoting rhizobacteria reduce adverse effects of salinity and osmotic stress by regulating phytohormones and antioxidants in Cucumis sativus. J Plant Interact 9:673–682

Kavi Kishor PB, Sreenivasulu N (2014) Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? Plant Cell Environ 37:300–311. https://doi.org/10.1111/pce.12157

Khalid A, Arshad M, Zahir ZA (2004) Screening plant growth-promoting rhizobacteria for improving groth and yield of wheat. J Appl Microbiol 96:473–480

Kim MJ, Moon Y, Tou JC et al (2016) Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). J Food Compos Anal 49:19–34. https://doi.org/10.1016/j.jfca.2016.03.004

Koné SB, Dionne A, Tweddell RJ et al (2010) Suppressive effect of non-aerated compost teas on foliar fungal pathogens of tomato. Biol Control 52:167–173. https://doi.org/10.1016/j.biocontrol.2009.10.018

Kong W, Meldgin DR, Collins JJ, Lu T (2018) Designing microbial consortia with defined social interactions. Nat Chem Biol 14:821–829. https://doi.org/10.1038/s41589-018-0091-7

Kumar D, Singh AP, Raha P et al (2013) Potassium Humate: a potential soil conditioner and plant growth promoter. Int J Agric Environ Biotechnol 6:441. https://doi.org/10.5958/j.2230-732x.6.3.015

Kurepin LV, Zaman M, Pharis RP (2014) Phytohormonal basis for the plant growth promoting action of naturally occurring biostimulators. J Sci Food Agric 94:1715–1722. https://doi.org/10.1002/jsfa.6545

Lee JH, Felipe P, Yang YH et al (2009) Effects of dietary supplementation with red-pigmented leafy lettuce (*Lactuca sativa*) on lipid profiles and antioxidant status in C57BL/6J mice fed a high-fat high-cholesterol diet. Br J Nutr 101:1246–1254. https://doi.org/10.1017/S0007114508073650

Liguori L, Pane C, Albanese D et al (2015) Compost and compost tea Management of Mini Watermelon Cultivations Affects the chemical, physical and sensory assessment of the fruits. Agric Sci 06:117–125. https://doi.org/10.4236/as.2015.61009

López-Bucio J, Pelagio-Flores R, Herrera-Estrella A (2015) Trichoderma as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. Sci Hortic (Amsterdam) 196:109–123. https://doi.org/10.1016/j.scienta.2015.08.043

Lorito M, Woo SL (2015) Trichoderma: a multi-purpose tool for integrated pest management. In: Lugtenberg B (ed) Principles of plant-microbe interactions. Springer International Publishing, Cham, pp 345–353. https://doi.org/10.1007/978-3-319-08575-3_36

Merlo L, Ghisi R, Passera C, Rascio N (1991) Effects of humic substances on carbohydrate metabolism of maize leaves. Can J Plant Sci 71:419–425. https://doi.org/10.4141/cjps91-058

Miceli A, Moncada A, Vetrano F (2021) Use of microbial biostimulants to increase the salinity tolerance of vegetable transplants. Agronomy 11. https://doi.org/10.3390/agronomy11061143

Michaeli S, Fromm H (2015) Closing the loop on the GABA shunt in plants: are GABA metabolism and signaling entwined? Front Plant Sci 6:1–7. https://doi.org/10.3389/fpls.2015.00419

Monda H, Cozzolino V, Vinci G et al (2017) Molecular characteristics of water-extractable organic matter from different composted biomasses and their effects on seed germination and early growth ofmaize. Sci Total Environ 590–591:40–49. https://doi.org/10.1016/j.scitotenv.2017.03.026

Monda H, Cozzolino V, Vinci G et al (2018) Molecular composition of the Humeome extracted from different green composts and their biostimulation on early growth of maize. Plant Soil 429:407–424. https://doi.org/10.1007/s11104-018-3642-5

Monda H, McKenna AM, Fountain R, Lamar RT (2021) Bioactivity of humic acids extracted from shale ore: molecular characterization and structure-activity relationship with tomato plant yield under nutritional stress. Front Plant Sci 12:1–17. https://doi.org/10.3389/fpls.2021.660224

Moreira H, Pereira SIA, Vega A et al (2020) Synergistic effects of arbuscular mycorrhizal fungi and plant growth-promoting bacteria benefit maize growth under increasing soil salinity. J Environ Manag 257:109982. https://doi.org/10.1016/j.jenvman.2019.109982

Murphy J, Riley JP (1962) A modified single solution method for the deter- mination of phosphate in natural waters. Anal Chim Acta 27:31–36

Muscolo A, Sidari M, Francioso O et al (2007) The auxin-like activity of humic substances is related to membrane interactions in carrot cell cultures. J Chem Ecol 33:115–129. https://doi.org/10.1007/s10886-006-9206-9

Naidu Y, Meon S, Kadir J, Siddiqui Y (2010) Microbial starter for the enhancement of biological activity of compost tea. Int J Agric Biol 12:51–56

Naidu Y, Meon S, Siddiqui Y (2013) Foliar application of microbial-enriched compost tea enhances growth, yield and quality of muskmelon (Cucumis melo L.) cultivated under fertigation system. Sci Hortic (Amsterdam) 159:33–40. https://doi.org/10.1016/j.scienta.2013.04.024

Nardi S, Pizzeghello D, Gessa C et al (2000) A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. Soil Biol Biochem 32:415–419. https://doi.org/10.1016/S0038-0717(99)00168-6

Nardi S, Pizzeghello D, Muscolo A, Vianello A (2002) Physiological effects of humic substances on higher plants. Soil Biol Biochem 34:1527–1536. https://doi.org/10.1016/S0038-0717(02)00174-8

Nardi S, Muscolo A, Vaccaro S et al (2007) Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and Krebs cycle in maize

seedlings. Soil Biol Biochem 39:3138–3146. https://doi.org/10.1016/j.soilbio.2007.07.006

Nardi S, Ertani A, Francioso O (2017) Soil–root cross-talking: the role of humic substances. J Plant Nutr Soil Sci 180:5–13. https://doi.org/10.1002/jpln.201600348

Nardi S, Schiavon M, Francioso O (2021) Chemical structure and biological activity of humic substances define their role as plant growth promoters. Molecules 26. https://doi.org/10.3390/molecules26082256

Nwodo UU, Green E, Okoh AI (2012) Bacterial exopolysaccharides: functionality and prospects. Int J Mol Sci 13:14002–14015. https://doi.org/10.3390/ijms131114002

Olivares FL, Busato JG, Paula AM De, et al (2017) Plant growth promoting bacteria and humic substances : crop promotion and mechanisms of action. Chem Biol Technol Agric 1–13. https://doi.org/10.1186/s40538-017-0112-x

Othibeng K, Nephali L, Ramabulana A-T, Steenkamp P, Petras D, Kang KB, Opperman H, Huyser J, Tugizimana F (2021) A metabolic choreography of maize plants treated with a humic substance-based biostimulant under Normal and starved conditions. Metabolites 11:403. https://doi.org/10.3390/metabo11060403

Pane C, Palese AM, Spaccini R et al (2016) Enhancing sustainability of a processing tomato cultivation system by using bioactive compost teas. Sci Hortic (Amsterdam) 202:117–124. https://doi.org/10.1016/j.scienta.2016.02.034

Pepe G, Sommella E, Manfra M et al (2015) Evaluation of anti-inflammatory activity and fast UHPLC-DAD-IT-TOF profiling of polyphenolic compounds extracted from green lettuce (*Lactuca sativa* L.; Var. Maravilla de Verano). Food Chem 167:153–161. https://doi.org/10.1016/j.foodchem.2014.06.105

Peterson M, Hans J, Matern U (2010) Biosynthesis of phenypropanoids and related compounds. In: Wink M., Ed., Biochemistry of Plant Secondary Metabolism (2nd ed.), Annual Plant *reviews*, Vol. 40, Wiley-Blackwell, 182–230

Piccolo A (2002) The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. Adv Agron 75:57–134. https://doi.org/10.1016/s0065-2113(02)75003-7

Piccolo A, Pietramellara G, Mbagwu JSC (1997) Use of humic substances as soil conditioners to increase aggregate stability. Geoderma 75:267–277. https://doi.org/10.1016/S0016-7061(96)00092-4

Piccolo A, Spaccini R, Savy D, Drosos M, Cozzolino V (2019) The soil Humeome: chemical structure, functions and technological perspectives. In: Vaz S Jr (ed) Sustainable Agrochemistry: a compendium of technologies. Springer Nature, Heidelberg, pp 183–222

Pizzeghello D, Schiavon M, Francioso O et al (2020) 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. Front Plant Sci 11:1–17. https://doi.org/10.3389/fpls.2020.01203

Priya S, Mk K, Roshan C, Deepak K (2021) Effect of soil and foliar applications of compost tea on growth characters , quality and nutrient content of soybean [Glycine max . (L.) Merrill] under organic cultivation. J Pharmacogn Phytochem 10:2566–2569

Puglisi E, Fragoulis G, Del Re AAM et al (2008) Carbon deposition in soil rhizosphere following amendments with compost and its soluble fractions, as evaluated by combined soil-plant rhizobox and reporter gene systems. Chemosphere 73:1292–1299. https://doi.org/10.1016/j.chemosphere.2008.07.008

Puglisi E, Fragoulis G, Ricciuti P et al (2009) Effects of a humic acid and its size-fractions on the bacterial community of soil rhizosphere under maize (*Zea mays* L.). Chemosphere 77:829–837. https://doi.org/10.1016/j.chemosphere.2009.07.077

Ros R, Muñoz-Bertomeu J, Krueger S (2014) Serine in plants: biosynthesis, metabolism, and functions. Trends Plant Sci 19:564–569. https://doi.org/10.1016/j.tplants.2014.06.003

Ros M, Hurtado-Navarro M, Giménez A et al (2020) Spraying agro-industrial compost tea on baby spinach crops: evaluation of yield, plant quality and soil health in field experiments. Agronomy 10. https://doi.org/10.3390/agronomy10030440

Rosa M, Prado C, Podazza G et al (2009) Soluble sugars-metabolism, sensing and abiotic stress a complex network in the life of plants. Plant Signal Behav 4:388–393. https://doi.org/10.4161/psb.4.5.8294

Rouphael Y, Colla G (2020) Editorial: Biostimulants in Agriculture. Front Plant Sci 11:1–7. https://doi.org/10.3389/fpls.2020.00040

Saia S, Colla G, Raimondi G et al (2019) An endophytic fungi-based biostimulant modulated lettuce yield, physiological and functional quality responses to both moderate and severe water limitation. Sci Hortic (Amsterdam) 256:108595. https://doi.org/10.1016/j.scienta.2019.108595

Saia S, Aissa E, Luziatelli F et al (2020) Growth-promoting bacteria and arbuscular mycorrhizal fungi differentially benefit tomato and corn depending upon the supplied form of phosphorus. Mycorrhiza 30:133–147. https://doi.org/10.1007/s00572-019-00927-w

Sánchez-Montesinos B, Diánez F, Moreno-Gavíra A, Gea FJ, Santos M (2020) Role of Trichoderma aggressivum f. europaeum as Plant-Growth Promoter in Horticulture. Agronomy 10(7):1004

Santos-Torres M, Romero-Perdomo F, Mendoza-Labrador J, Gutiérrez AY, Vargas C, Castro-Rincon E, Caro-Quintero A, Uribe-Velez D, Estrada-Bonilla GA (2021) Genomic and phenotypic analysis of rock phosphate-solubilizing rhizobacteria. Rhizosphere 17:100290

Savarese C, di Meo V, Cangemi S et al (2022) Bioactivity of two different humic materials and their combination on plants growth as a function of their molecular properties. Plant Soil 472:509–526. https://doi.org/10.1007/s11104-021-05267-3

Savy D, Cozzolino V, Vinci G et al (2015) Water-soluble lignins from different bioenergy crops stimulate the early development of maize (*Zea mays* L.). Molecules 20:19958–19970. https://doi.org/10.3390/molecules201119671

Savy D, Cozzolino V, Nebbioso A et al (2016) Humic-like bioactivity on emergence and early growth of maize

(*Zea mays* L.) of water-soluble lignins isolated from biomass for energy. Plant Soil 402:221–233. https://doi.org/10.1007/s11104-015-2780-2

Savy D, Canellas L, Vinci G et al (2017a) Humic-like water-soluble Lignins from Giant reed (*Arundo donax* L.) display hormone-like activity on plant growth. J Plant Growth Regul 36:995–1001. https://doi.org/10.1007/s00344-017-9696-4

Savy D, Mazzei P, Drosos M et al (2017b) Molecular characterization of extracts from biorefinery wastes and evaluation of their plant biostimulation. ACS Sustain Chem Eng 5:9023–9031. https://doi.org/10.1021/acssuschemeng.7b01928

Savy D, Brostaux Y, Cozzolino V et al (2020) Quantitative structure-activity relationship of humic-like biostimulants derived from agro-industrial byproducts and energy crops. Front Plant Sci 11:1–13. https://doi.org/10.3389/fpls.2020.00581

Scaglia B, Nunes RR, Rezende MOO et al (2016) Investigating organic molecules responsible of auxin-like activity of humic acid fraction extracted from vermicompost. Sci Total Environ 562:289–295. https://doi.org/10.1016/j.scitotenv.2016.03.212

Schiavon M, Pizzeghello D, Muscolo A et al (2010) High molecular size humic substances enhance phenylpropanoid metabolism in maize (*Zea mays* L.). J Chem Ecol 36:662–669. https://doi.org/10.1007/s10886-010-9790-6

Schoebitz M, López MD, Serrí H et al (2016) Combined application of microbial consortium and humic substances to improve the growth performance of blueberry seedlings. J Soil Sci Plant Nutr 16:1010–1023. https://doi.org/10.4067/S0718-95162016005000074

Shahrajabian MH, Chaski C, Polyzos N, Petropoulos SA (2021) Biostimulants application: a low input cropping management tool for sustainable farming of vegetables. Biomolecules 11. https://doi.org/10.3390/biom11050698

Shennan C, Krupnik TJ, Baird G et al (2017) Organic and conventional agriculture: a useful framing? Annu Rev Environ Resour 42:317–346. https://doi.org/10.1146/annurev-environ-110615-085750

Shinozaki S, Uemura M, Bailey-Serres J et al (2015) Responses to abiotic stress. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and molecular biology of plants, American Society of Plant Biologists, 2nd edn. Wiley-Blackwell, pp 1051–1099

Siddiqui Y, Meon S, Ismail R et al (2008) Bio-efficiency of compost extracts on the wet rot incidence, morphological and physiological growth of okra (*Abelmoschus esculentus* [(L.) Moench]). Sci Hortic (Amsterdam) 117:9–14. https://doi.org/10.1016/j.scienta.2008.03.008

Spaccini R, Cozzolino V, Di Meo V et al (2019) Bioactivity of humic substances and water extracts from compost made by ligno-cellulose wastes from biorefinery. Sci Total Environ 646:792–800. https://doi.org/10.1016/j.scitotenv.2018.07.334

Suh MC, Hahne G, Liu JR et al (2015) Plant lipid biology and biotechnology. Plant Cell Rep 34:517–518. https://doi.org/10.1007/s00299-015-1780-2

Taha SS, Osman AS (2018) Influence of potassium humate on biochemical and agronomic attributes of bean plants grown on saline soil. J Hortic Sci Biotechnol 93:545–554. https://doi.org/10.1080/14620316.2017.1416960

Thonar C, Lekfeldt JDS, Cozzolino V et al (2017) Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils. Chem Biol Technol Agric 4:1–16. https://doi.org/10.1186/s40538-017-0088-6

Torun H, Toprak B (2020) Arbuscular mycorrhizal Fungi and K-Humate combined as biostimulants: changes in antioxidant defense system and radical scavenging capacity in Elaeagnus angustifolia. J Soil Sci Plant Nutr 20:2379–2393. https://doi.org/10.1007/s42729-020-00304-z

Trevisan S, Botton A, Vaccaro S et al (2011) Humic substances affect Arabidopsis physiology by altering the expression of genes involved in primary metabolism, growth and development. Environ Exp Bot 74:45–55. https://doi.org/10.1016/j.envexpbot.2011.04.017

Vaccaro S, Musculo A, Pizzeghello D et al (2009) Effect of a compost and its water-soluble fractions on key enzymes of nitrogen metabolism in maize seedlings. J Agric Food Chem 57:11267–11276. https://doi.org/10.1021/jf901808s

Vaccaro S, Ertani A, Nebbioso A et al (2015) Humic substances stimulate maize nitrogen assimilation and amino acid metabolism at physiological and molecular level. Chem Biol Technol Agric 2:1–12. https://doi.org/10.1186/s40538-015-0033-5

Valluru R, Van den Ende W (2011) Myo-inositol and beyond - emerging networks under stress. Plant Sci 181:387–400. https://doi.org/10.1016/j.plantsci.2011.07.009

van Tol de Castro TA, Berbara RLL, Tavares OCH et al (2021) Humic acids induce a eustress state via photosynthesis and nitrogen metabolism leading to a root growth improvement in rice plants. Plant Physiol Biochem 162:171–184. https://doi.org/10.1016/j.plaphy.2021.02.043

Verrillo M, Cozzolino V, Spaccini R, Piccolo A (2021a) Humic substances from green compost increase bioactivity and antibacterial properties of essential oils in basil leaves. Chem Biol Technol Agric 8:1–14. https://doi.org/10.1186/s40538-021-00226-7

Verrillo M, Salzano M, Cozzolino V et al (2021b) Bioactivity and antimicrobial properties of chemically characterized compost teas from different green composts. Waste Manag 120:98–107. https://doi.org/10.1016/j.wasman.2020.11.013

Vinale F, Flematti G, Sivasithamparam K (2009) Harzianic acid, an antifungal and plant growth promoting metabolite from Trichoderma harzianum. J Nat Prod 72(11):2032–2035

Vinci G, Cozzolino V, Mazzei P et al (2018a) Effects of Bacillus amyloliquefaciens and different phosphorus sources on maize plants as revealed by NMR and GC-MS based metabolomics. Plant Soil 429:437–450. https://doi.org/10.1007/s11104-018-3701-y

Vinci G, Cozzolino V, Mazzei P et al (2018b) An alternative to mineral phosphorus fertilizers: the combined effects of Trichoderma harzianum and compost on Zea mays, as revealed by 1 H NMR and GC-MS metabolomics.

PLoS One 13:1–15. https://doi.org/10.1371/journal.pone.0209664

Woo SL, Ruocco M, Vinale F, et al (2014) Trichoderma -based products and their widespread use in agriculture. 71–126

Woo SL, Pepe O (2018) Microbial consortia: promising probiotics as plant biostimulants for sustainable agriculture. Front Plant Sci 9:7–12. https://doi.org/10.3389/fpls.2018.01801

Yakhin OI, Lubyanov AA, Yakhin IA, Brown PH (2017) Biostimulants in plant science: a global perspective. Front Plant Sci 7. https://doi.org/10.3389/fpls.2016.02049

Yang X, Cui X, Zhao L et al (2017) Exogenous glycine nitrogen enhances accumulation of glycosylated flavonoids and antioxidant activity in lettuce (*Lactuca sativa* L.). Front Plant Sci 8:1–16. https://doi.org/10.3389/fpls.2017.02098

Zaccardelli M, Pane C, Villecco D et al (2018) Compost tea spraying increases yield performance of pepper (*Capsicum annuum* L.) grown in greenhouse under organic farming system. Ital J Agron 13:229–234. https://doi.org/10.4081/ija.2018.991

Zanin L, Tomasi N, Cesco S et al (2019) Humic substances contribute to plant iron nutrition acting as chelators and biostimulants. Front Plant Sci 10:1–10. https://doi.org/10.3389/fpls.2019.00675

Zeeman S (2015) Carbohydrate metabolism. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and molecular biology of plants, American Society of Plant Biologists, 2nd edn. Wiley-Blackwell, Hoboken, pp 567–608

Zou YN, Wang P, Liu CY, Ni QD, Zhang DJ, Wu QS (2017) Mycorrhizal trifoliate orange has greater root adaptation of morphology and phytohormones in response to drought stress. Sci Rep 7:1–10