Microbial-based biostimulant enhances sweet pepper performance by metabolic reprogramming of phytohormone profile and secondary plant metabolism

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Abstract

Microbial-based biostimulants can improve crop productivity by modulating cell metabolic pathways including hormonal balance. However, little is known about the microbial-mediated molecular changes causing yield increase. The present study elucidates the metabolomic modulation occurring in pepper (Capsicum annuum L.) leaves at the vegetative and reproductive phenological stages in response to microbial-based biostimulants containing the arbuscular mycorrhizal fungi Rhizoglomus irregularis and Funneliformis mosseae as well as Trichoderma koningii. Application of endophytic fungi significantly increased total fruit yield by 23.7% compared to that of untreated plants. Multivariate statistics indicated that the biostimulant treatment substantially altered the shape of the metabolic profile of pepper. Compared to the untreated control, the plants treated with microbial biostimulants presented with modified gibberellin, auxin, and cytokinin production and distribution. The biostimulant treatment also induced secondary metabolism and caused carotenoids, saponins, and phenolic compounds to accumulate in the plants. Differential metabolomic signatures indicated diverse and concerted biochemical responses in the plants following the colonisation of their roots by beneficial microorganisms. The above findings demonstrated a clear link between microbial-mediated yield increase and a strong up-regulation of hormonal and secondary metabolic pathways associated with growth stimulation and crop defence to environmental stresses.

Summary statement

Root inoculation with arbuscular mycorrhizal fungi *Rhizoglomus irregularis* and *Funneliformis mosseae* as well as *Trichoderma koningii* increased fruit yield of greenhouse pepper through a general up-regulation of hormonal and secondary metabolic pathways associated with growth stimulation and crop defence to environmental stresses.

Introduction

Three major current global challenges are food security, environmental degradation, and climate change. The first may be augmented and the latter two diminished by improving nutrient (nitrogen, phosphorus) use efficiency in agricultural crop production and stabilising yield by practicing sustainable agricul-

ture (Searchinger, Waite, Hanson, Ranganathan, & Dumas, 2018). The application of plant biostimulants such as beneficial microbes (arbuscular mycorrhizal fungi [AMF], *Trichoderma* spp., plant growth-promoting rhizobacteria [PGPR]), and bioactive substances (humic and fulvic acids, macroalgae and microalgae, protein hydrolysates, and silicon) used either separately or in combination may help crops contend with the aforementioned challenges (Rouphael & Colla, 2020).

Plant biostimulants were recently defined in the Regulations of the European Parliament and Council (Regulation EU 2019/1009) as "... *EU fertilising product(s) able to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: 1) nutrient use efficiency, 2) tolerance to abiotic stress, 3) quality traits, or 4) availability of confined nutrients in the soil or rhizosphere* ". AMF comprise a very important category of biostimulants (Rouphael et al., 2015). They are members of the *Glomeromycotina* family and establish mutualistic relationships with 74% of all terrestrial plant species (Spatafora et al., 2016). AMF boost productivity and enhance tolerance to abiotic stress (high temperature, drought, and salinity) in crops. AMF inoculation enhances the growth and vigour of the root apparatus in terms of biomass, length, density, and branching. It improves macronutrient (N, P, and Fe) and micronutrient (Mn and Zn) uptake and assimilation. It ameliorates water relations and photosynthetic activity, upregulates secondary metabolism, and releases low- and high-molecular-weight organic compounds such as amino acids, phenolics, organic acids, and proteins into the rhizosphere. It also modulates phytohormone signalling (Bernardo et al., 2018; Lucini et al., 2019; Rouphael and Colla, 2018; Rouphael et al., 2015; Yakhin, Lubyanov, Yakhin, & Brown, 2017). The indirect and direct mechanisms of AMF influence shoot and root function and augment crop agronomic performance. Other plant beneficial endophytic fungi include *Trichoderma* spp. Several of them are registered as microbial biological control agents (López-Bucio, Pelagio-Flores, & Herrera-Estrella, 2015). However, several studies reported that certain *Trichoderma* spp. including *T. atroviride* , *T. koningii* , *T. harzianum,* and *T. virens* are also plant biostimulants that boost crop performance and nutrient use efficiency and/or endue plants with abiotic stress tolerance (Colla, Rouphael, Di Mattia, El-Nakhel, & Cardarelli, 2015; Saia et al., 2019). The direct and indirect mechanisms of the biostimulant action of *Trichoderma* strains include i) improvement of lateral root development, ii) induction of plant mitogen-activated protein 6, and iii) production and rhizosphere excretion of auxins and secondary metabolites such as volatile and non-volatile substances that stimulate various plant responses and enhance crop nutrient uptake, resilience, and productivity (Lopez-Bucio et al., 2015).

The beneficial effects of combinations of AMF and *Trichoderma* on vegetable crops were previously demonstrated under both optimal and suboptimal conditions (Colla et al., 2015; Saia et al., 2019). However, the physiological and molecular mechanisms underlining biostimulant action have not been fully elucidated. One strategy to clarify biostimulant efficacy is to analyse metabolic profiling. In turn, this process serves as a basis for subsequent transcriptomic analyses. Metabolomic phytochemical characterisation could identify numerous physiological processes and metabolic pathways modulated by biostimulants (Yakhin et al., 2017).

It has been hypothesised that AMF and *Trichoderma* can induce an enhance fruit yield by modulating the hormonal balance and secondary metabolic pathways.

In the present study, then, an untargeted metabolomics approach was conducted on greenhouse pepper. The objectives were to illuminate metabolomic reprogramming by microbial biostimulants in leaf tissue at the vegetative and reproductive phenological stages, elucidate biostimulant regulation of key phytohormones, and correlate these molecular-level biostimulant-promoted changes to observed fruit yield and quality variations.

Materials and Methods

Growth conditions, plant material, crop management, and experimental design

The trial was conducted in a greenhouse located at Paraje Aguilas Bajas, Santa Maria del Aguila, Almeria, Spain (36deg47'39"N 2°46'32"W). The greenhouse was composed of polycarbonate walls and a roof made of trilaminated low-density polyethylene (LDPE) film (200 μm thickness) with ˜60% spectral transmittance in the photosynthetically active radiation (PAR) region. The greenhouse was unheated and passively ventilated

with lateral side panels and flap roof windows. It had an east-west orientation and a north-south crop row alignment. The air temperature and relative humidity (RH) inside the greenhouse were in the ranges of 12–32 °C and 50–70%, respectively. Transplants of the sweet pepper (*Capsicum annuum* L.) hybrid 'SV1204PB' (Seminis, Montornés del Vallés, Barcelona, Spain) at the 4–5 true-leaf stage were planted in "Enarenado" sandy soil commonly used in greenhouse production in Almería. The planting date was 19 July 2017 and the planting density was 2.0 m^{-2}. The soil composition was 13.5% (w/w) clay, 72.8% (w/w) sand, and 13.7% (w/w) silt. Its pH was 7.52, its organic matter content was 0.71%, and its total nitrogen, available phosphorus, and exchangeable potassium were 690 mg kg^{-1}, 51.4 mg kg^{-1}, and 321 mg kg^{-1}, respectively. Aerial drip irrigation was used. The in-line emitters were positioned at 0.30-m intervals and the emitter flow rate was 3.4 L h^{-1}. Preplant fertiliser was broadcast at 90 kg*ha^{-1} P, 120 kg*ha^{-1} K, and 15 kg*ha^{-1} Mg and incorporated into the soil. Additional fertiliser in the form of K$_2$SO$_4$ (80 kg*ha^{-1} K) was applied through the drip irrigation system. Nitrogen was applied via fertigation in the form of 27% NH$_4$NO$_3$ soluble fertiliser starting 10 d after transplanting until day 83. The total N supply was split into ten weekly dressings. Powdery mildew caused by *Leveillula taurica* was controlled by three foliar applications of penconazole (Topas 10EC; Syngenta, Madrid, Spain) at the label-recommended rate. Aphids and spider mites were controlled by one foliar application each of imidacloprid (Confidor 200 SL; Bayer Crop Science, Valencia, Spain) and fenpyroximate (Miro; Bayer Crop Science, Valencia, Spain), respectively. Bumblebees promoted flower pollination. Weeds were controlled by hand hoeing. The control and microbial-based biostimulant treatments were compared in a randomised block design with four replicates for a total of eight experimental plots. The microbial-based biostimulants were applied through a drip irrigation system. The first application was made at 15 d after transplanting (DAT) (3 August 2017) at the rates of 1x10^{-6} spores ha^{-1} *Rhizoglomus irregularis* BEG72 and 1x10^{-6} spores ha^{-1} *Funneliformis mosseae* BEG234 in the form of 2.0 kg ha^{-1} Team Horticola (Agrotecnologias Naturales, S.L., Tarragona, Spain) plus 1x10^{12} CFU ha^{-1} *Trichoderma koningii* TK7 in the form of 1.0 kg ha^{-1} Condor Shield (Agrotecnologias Naturales, S.L., Tarragona, Spain). The second treatment was applied 43 DAT (31 August 2017) at the rate of 5x10^{11} CFU ha^{-1} *Trichoderma koningii* TK7 as 0.5 kg Condor Shield (Agrotecnologias Naturales, S.L., Tarragona, Spain). Each experimental plot was 30 m^2 and contained 60 plants.

Yield measurements and arbuscular mycorrhizal fungi (AMF) root colonisation

Fully mature pepper fruits were harvested from 139 DAT (5 December 2017) to 272 DAT (17 April 2018). Mean fruit weight and number and marketable yield were determined for each experimental plot (replicate). Rotten fruit and those weighing < 100 g were considered unmarketable yield.

At the end of the trial, the roots of six pepper plants per experimental plot were rinsed and subsamples were used to evaluate AMF root colonisation. The root samples were cleared with 10% (w/v) KOH, stained with 0.05% (w/v) trypan blue in lactophenol, and microscopically (Stereo microscope Leica EZ4V, 32x - Leica Microsystems Srl, Buccinasco, Italy) examined for AMF colonisation. The percentage of colonised root segments was determined by the gridline intercept method (Giovannetti & Mosse, 1980).

Quantitative PCR determination of **Trichoderma** *concentration in rhizosphere soil*

At the end of the trial, rhizosphere soil was collected by shaking the roots collected from ten plants per plot. To determine the *Trichoderma* concentration in the rhizosphere, DNA was extracted from the soil samples with a DNeasy$^{(r)}$ Powersoil$^{(r)}$ kit (Cat. No. 12888-50; Qiagen, Hilden, Germany) according to Qiacube (Qiagen, Hilden, Germany) automation procedures. To amplify the transcripts, the genus-specific primer pair RM3/RM4 was used to detect *Trichoderma* spp. Every 4-μL DNA sample was amplified in a 20-μL reaction system in the presence of 10 μL QuaniNova^{TM} SYBR$^{®}$ Green Supermix (2x) and 0.14 μL of 25 μM primers on a Rotor-Gene Q (Qiagen, Hilden, Germany). The qPCR cycling conditions were as follows: initial incubation at 95 °C for 2 min, 45 cycles of 95 °C for 5 s each, and 60 °C for 12 s. Two technical replicates were performed per sample. After qPCR, the absolute values for the transcript quantities were calculated by interpolation of standard curves plotted using serial DNA dilutions, namely, undiluted and 1:1,000, 1:10,000, and 1:100,000 dilutions of standardised liquid culture at a total *Trichoderma* spp. concentration of 10^9 (CFU) mL^{-1}.

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Sample collection and untargeted metabolomics

Four leaves in the third position from the branch tip were harvested for untargeted metabolomics at 43 DAT (31 August 2017) and at 131 DAT (27 November 2017). The leaves were flash-frozen in liquid nitrogen and stored at -80 °C until subsequent metabolomic analysis.

An untargeted metabolomics approach was conducted in the UHPLC 1290 chromatographic system coupled to a hybrid quadrupole-time-of-flight (Q-TOF) G6550 mass spectrometer (UHPLC/Q-TOF) (Agilent Technologies, Santa Clara, CA, USA). A Waters Acquity UPLC® BEH C18 column (100×2.1 mm i.d., 1.7 μm) (Waters Corp., Milford, MA, USA) was used for reverse-phase chromatographic separation. The binary gradient consisted of water and acetonitrile and the Riken Plasma method was followed (Tsugawa et al., 2019). The injection volume was 2 μL and the mass spectrometer was run in positive polarity and SCAN mode (range: 100–1,700 m/z; extended dynamic range setting). Quality controls (QC) were prepared by pooling 10-μL samples. Five QCs were acquired in data-dependent mode (auto MS/MS) at 1 Hz, 10 precursors/cycle, collision energies of 10 V, 30 V, and 50 V), and in iterative mode with active exclusion to increase the number of compounds targeted for tandem MS fragmentation.

Alignment, blank filtration, and identification were performed in MSDIAL v. 4.0 (Riken, Tokyo, Japan) using the publicly available library MoNA (Mass Bank of North America) and an internal standard compound library as specified in the Supplementary Table 1. Compounds lacking experimental MS/MS spectra were annotated with MSFINDER (Riken, Tokyo, Japan) following the procedure described in Blazenovic et al. (2019). The alternatives were filtered by retention time prediction (Bonini et al., 2020). MSI (metabolomics standards initiative) levels for each identified compound are listed in Supplementary Table 1.

Statistics and data analysis

Data were statistically analysed with SPSS v. 21 (IBM Corp., Armonk, NY, USA). The microbial-based biostimulant effects were analysed by an unpaired Student's t-test. The compound intensity table exported from MSDIAL v. 4.0 (Riken, Tokyo, Japan) (Tsugawa et al., 2015) was uploaded into MS-FLO (Riken, Tokyo, Japan) (DeFelice et al., 2017) to reduce false positives and duplicates. The output was imported into R v. 3.6.0 for centring, scaling, PCA, and calculation of fold changes, Benjamini-Hochberg false discovery rates (FDR), and corrected p-values. Venn diagrams were plotted to identify metabolites common to two sampling points but not exclusive to a particular growth stage. Compounds with $P < 0.05$ were imported into ChemRICH (Barupal & Fiehn, 2017) for enrichment analysis based on their chemical similarity and MetaMapp (Barupal et al., 2012) for chemical network analysis. Cytoscape (Saito et al., 2012) displayed exported MetaMapp data and plotted the final images.

Results

Soil fungal concentration and crop yield

By the end of the trial, the percentage of mycorrhizal root colonisation was significantly ($P < 0.01$) higher under the microbial inoculation treatment (33.6%) than it was under the uninoculated control treatment (8.0%). The total number of *Trichoderma* colonies recovered from the rhizosphere under the microbial-based treatment was significantly ($P < 0.01$) higher than that recorded for the untreated control (2.2×10^5 *vs.* 1.2×10^3 CFU g^{-1}, respectively).

Relative to the uninoculated control, inoculation with AMF and *Trichoderma koningii* significantly increased fruit yield especially during the first part of the reproductive cycle, namely, early yield 139 DAT (P<0.05) and 174 DAT (P<0.01) (Table 1). The comparatively higher production rates measured at 139 DAT and 272 DAT for pepper plants inoculated with microbial-based biostimulant was due to an increase in mean fruit weight. In contrast, the relatively higher fruit yield determined for 174 DAT was attributed to increases in both fruit number per plant and mean fruit mass (Tables 1–3). The microbial-based biostimulant significantly improved cumulative fruit yield by 23.7% relative to uninoculated pepper plants (Table 1).

Modulation of metabolomic profile

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Here, ultra-high-performance liquid chromatography quadrupole-time-of-flight high-resolution mass spectrometry (UHPLC-QTOF) and annotation in publicly available databases and large metabolite groups were conducted to obtain wide metabolome coverage. We applied UHPLC-QTOF-based untargeted metabolomic profiling of crude extracts to assess relative differences in the vegetative stage (43 DAT) and reproductive stage (131 DAT) leaf metabolite profiles between inoculated and uninoculated plants. A principal component analysis (PCA) explained 79% of the overall variance. The PCA score plot (Figure 1) showed two main clusters accounting for the vegetative and reproductive stages, respectively. Within each cluster, good separation was achieved between the metabolomic profiles of leaves from inoculated plants and those of leaves harvested from the uninoculated control. This finding was anticipated as the metabolism dramatically shifts as the plant transitions from the vegetative to reproductive phases. This outcome was relevant as PCA provides unsupervised descriptions of relatedness/unrelatedness. We ran a t-test ANOVA (P < 0.01) to identify differentially accumulated metabolites at each plant growth stage. This analysis disclosed > 466 annotated metabolites (Supplementary Tables 2 and 3) that had significantly changed between the vegetative and reproductive stages. Of these, 327 were common to both the first and second sampling points (Figure 2). In contrast, 68 and 71 metabolites differentially accumulated during the vegetative and reproductive stages, respectively (Figure 2). The interactions between microbial inoculants and plants are complex. Nevertheless, metabolomics effectively included the metabolic responses and mechanisms involved in the plant-microbe interactions. As 70% of the metabolites were common to the vegetative and reproductive phenological stages (Figure 2), biostimulant-mediated shifts in the leaf metabolome were recorded for both of them. On the other hand, certain stage-specific responses could be identified as well.

To clarify and visualise the variations between metabolic profiles at the vegetative and reproductive stages, we performed a chemical enrichment analysis using ChemRICH (Figure 3; Tables 4 and 5) and plotted the output by MetaMapp Cytoscape (Figure 4) (Barupal & Fiehn, 2017). Most of the significantly upregulated and downregulated metabolites (fold-change values [?] 0.5 and [?] 1.5, respectively; P [?] 0.01) had a wide range of functions including growth stimulation, antifungal activity, pathogen resistance, energy sources, and secondary signalling cofactors.

Discussion

There is a growing interest in the use of beneficial microbial inoculants such as AMF, *Trichoderma* spp., and PGPR in horticulture as they have multiple beneficial effects on crops (Lopez-Bucio et al., 2015; Rouphael et al., 2015). In the present study, we inoculated pepper plants with the AMF species *Rhizoglomus irregularis* and *Funneliformis mosseae*. *Trichoderma koningii* enhanced mycorrhizal root colonisation whilst *Trichoderma* rhizosphere population accelerated and increased total crop yield by 24% relative to uninoculated plants (Table 1). The increase in pepper yield was attributed to gains in fruit weight and/or number. Colla et al. (2014) reported that compared with uninoculated field-grown zucchini plants, those supplied with live AMF *G. intraradices* and *T. atroviride* inocula presented with greater early and total yields. The beneficial fungi act as phytostimulants and improve foliar nutrient content. The phytostimulatory efficacy of beneficial fungi is explained by complex signal exchange and crosstalk between the host plants and the microorganisms affecting phytohormone balance and plant metabolism (Sbrana, Turrini, & Giovannetti, 2017). Metabolomics helps elucidate the metabolic pathways and processes involved in plant-microbe interactions. Growth stage has a hierarchically strong effect on the leaf metabolome. Nevertheless, microbial biostimulants significantly alter the metabolome such that it is readily distinguishable from the control (Figure 1).

The microbial treatments elicited several processes related to plant secondary metabolism. Microbial-based biostimulants promote the accumulation of carotenoids and other terpenes, saponins, phenolic compounds, and phospholipids (Figures 3 and 4).

Plant responses to microbial-based biostimulants involve modulations of the phytohormone network. Treatments with beneficial fungi alter auxins, cytokinins, and gibberellins. Modification of the hormone profile may have been associated with the observed yield increases. Several studies demonstrated that microbial biostimulants promote yield by changing the phytohormone balance, increasing nutrient availability and uptake, and enhancing abiotic stress tolerance (Rouphael et al., 2015; Saia et al., 2019, 2020). Certain putative

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mechanisms for the biostimulant activity of microbial-based inoculant (AMF + *Trichoderma*) in pepper have been proposed. Microbial-based inoculant augments root biomass, length, density, and branching which, in turn, increases macronutrient and micronutrient uptake and boosts crop productivity. It also regulates key phytohormones such as gibberellins, cytokinins, and auxins (Lopez-Bucio et al., 2015; Lucini et al., 2019; Rouphael et al., 2015).

Gibberellins are diterpenoid phytohormones that regulate plant development, flowering, and senescence (Shu, Zhou, Chen, Luo, & Yang, 2018). In response to microbial-based inoculant treatment, gibberellins A81, A36, A37, A12, and A20 increased by 1.3–16x relative to the control at both sampling points. Coordination between gibberellin biosynthesis and oxidation affects pollination and fruit set in tomato (Serrani, Sanjuan, Ruiz-Rivero, Fos, & Garcia-Martinez, 2007). Gibberellin A20 was recently linked to increased maize yield (Tucker et al., 2019).

Here, the microbial-based biostimulant treatment induced the auxins indole-3-acetamide and indole-3-pyruvic acid by 1.7-7.5x relative to the control. Auxins upregulate the genes encoding oxidases regulating gibberellin metabolism (Frigerio et al., 2006). Auxins and gibberellins overlap in terms of root growth and fruit set regulation (Bermejo et al., 2018). Microbial-based biostimulant also increased the accumulation of the cytokinin *trans* -zeatin by 2.2-5.1x in pepper leaves compared with the control. Cytokinins interact with auxins to fine-tune root and shoot development. *Trans* -zeatin modulates meristem activity and mediates plant responses to variable extrinsic factors such as abiotic stress (Werner & Schmulling, 2009). We postulate that coordinated auxin, gibberellin, and cytokinin recruitment may have contributed to the enhanced pepper fruit yield observed here. Earlier studies reported that beneficial microbes promote plant growth and productivity by altering phytohormone status (Bhattacharyya & Jha, 2012).

Modulation of plant signalling compounds in response to the microbial-based biostimulant treatment also involved membrane lipids. Phospholipids are plasma membrane components that play important roles in cell signalling, membrane trafficking, and apoptosis (Xue, Chen, & Mei, 2009). The microbial-based biostimulant treatment changed the phospholipids profile. It altered twenty foliar metabolites at the first sampling (vegetative stage) and 31 foliar metabolites in the second sampling (reproductive stage). Accumulation of phosphatidylethanolamines (PE(P-16:0/20:5)), phosphatidic acid (PA(15:0/22:6), PA(O-18:020:3)), phosphatidylinositol (PIM4(18:1/14:0)), and phosphatidylserine (PS(P-16:013:0)) increased by 1.5–30x in the biostimulant-treated plants compared to the control. Relative to the untreated leaves, lysophospholipids (PA(P-16:0e18:2)) increased by 6.5x in biostimulant-treated leaves from the second sampling (reproductive stage). Lysophospholipids release calcium from the endoplasmic reticulum, promote cell division, and inhibit apoptosis (Hou, Ufer, & Bartels, 2016; Ye, 2008).

The microbial treatment also modulated the biosynthesis of carotenoids, saponins, phenolic compounds, and purines. Secondary metabolism is often altered in response to plant interactions with the ambient environment including agronomic practices and plant-microbe interactions (Yang et al., 2018). Here, metabolomics identified substantial alterations in secondary metabolism. Hence, plant responses to microbial biostimulants entails the coordinated modulation of several unrelated pathways.

Compared to the control, by the second sampling date, foliar vitamin A and α-carotene were 1.5× and 8.5× higher, respectively, following microbial biostimulant treatment. Carotenoids absorb light energy, participate in photosynthesis, protect plants against oxidative damage, and are precursors of visual pigment chromophores and volatile apocarotenoids that attract pollinators (Heath, Cipollini, & Stireman, 2013; Sun, et al., 2018). Moreover, they are involved in plant responses to abiotic stresses and plant-microbe interactions (Felemban, Braguy, Zurbriggen, & Al-Babili, 2019). Blumenols comprise a class of apocarotenoids or cyclohexanone derivatives of carotenoid cleavage. They also accumulated in the biostimulant-treated plants. Blumenols accumulate in the roots and shoots of mycorrhized plants and have been proposed as arbuscular mycorrhizal fungi colonisation markers (Wang et al., 2018). Relative to the control, blumenol B was 2× and 2.5× higher at the first and second sampling dates, respectively, after biostimulant application. Their functions in processes other than allelopathy are unknown. However, their levels are strongly correlated with the degree of mycorrhization (Fester et al., 2002). Here, foliar saponins were 1.5–10× higher in plants treated

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with biostimulant than the untreated control. Saponins are produced constitutively in plants and comprise part of plant defence. They have both antifungal and antifeedant activity. Though they are generally associated with pathogenesis, it was recently reported that saponins may participate in mutualistic relationships among plants, rhizobacteria, and mycorrhizae (Szakiel, Paczkowski, & Henry, 2011).

Compared with the control, plants subjected to the microbial treatments presented with higher levels of phenolic compounds. Phenolic metabolites are essential for lignin and pigment biosynthesis and participate in plant responses to pathogens and external stimuli (Bhattacharya, Sood, & Citovsky, 2010). Mycorrhizae elicit phenolic biosynthesis in other plant species (Baslam & Goicoechea, 2012; Jugran et al., 2015). They also trigger plant defence against abiotic and biotic stresses and improve nutrient availability and use efficiency (Bernardo et al., 2019; Sharma & Sharma, 2017). Here, irrespective of growth stage, skullcapflavone I, pelargonidin-3-o-glucoside, kaempferol, genistein, apiin, and myricatomentoside I accumulated to levels 3–87× higher in the biostimulant-treated plants than the control. Phenolics are associated with plant defence mechanisms. Flavones may protect plants from both biotic and abiotic stress (Martinez et al., 2016). Lignans have high antioxidant activity (Durazzo, Turfani, Azzini, Maiani, & Carcea, 2013; Hu, Yuan, & Kitts, 2007). Compared with the uninoculated, the gibbilimbol B level was 1.5× and 4.2× higher at the first and second sampling dates, respectively, in the inoculated plants. Gibbilimbol B was reported to have fungicidal activity against *Fusarium oxysporum* f. sp. *dianthi* . Coumarin upregulation is related to iron nutrition (Curie & Mari, 2017), allelochemistry (Niro et al., 2016), and abiotic stress tolerance (Saleh & Madany, 2015) in plants. Plant coumarins may influence the shape of the root microbiome (Voges, Bai, Schulze-Lefert, & Sattely, 2019).

Relative to the control, the levels of several purines were altered in the plants treated with the microbial biostimulant here. Several studies have focused on the effects of increased levels of adenosine and purines. These compounds are recycled by the so-called "salvage pathway" (Ashihara, Stasolla, Fujimura, & Crozier, 2018). At the first and second samplings, we observed sharp increases in the guanosine (2.7× and 8.7×, respectively) and N6-threonylcarbamoyladenosine (3× and 7.8×, respectively) levels after microbial biostimulant inoculation. Nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) are reducing equivalent exchange cofactors that participate in several redox reactions. At both the first and second samplings, NAD and FAD had increased by 1.5–4.4× in the biostimulant-treated plants relative to the control.

Our metabolomics study revealed that microbial biostimulant treatment had two major effects on pepper. First, the biostimulant modulated the phytohormone profile and phospholipid signalling in the plants. Next, it altered various secondary metabolic processes involving saponins, blumenols, carotenoids, and phenolic compounds. Phytohormones and biochemical messengers are associated with various metabolic processes (Ashihara et al., 2018) and might account for the observed biostimulant-mediated increases in crop productivity. The secondary metabolites modulated by biostimulant treatment have numerous positive influences on plant productivity such as the enhancement of nutrient uptake and assimilation and biotic and abiotic stress tolerance. The elicitation of secondary metabolism by plant beneficial microbes merits further investigation in terms of abiotic stress tolerance and induced systemic response (ISR) induction. Carotenoids and phenolics improve quality and promote health in many fruits including pepper. Thus, the microbial biostimulant treatments applied here could have nutritional implications as well.

Conclusions

Recent scientific investigations have focused on improving sustainable farming practices that stabilise yield under optimal and suboptimal conditions and comply with changing legislation regarding the application of low-input agrochemicals. Microbial-based biostimulants (AMF and/or *Trichoderma*) may sustainably enhance crop productivity. Our greenhouse experiment on pepper confirmed that inoculation with a combination of AMF and *Trichoderma* increased fruit yield by 23.7% relative to that of the untreated control. A metabolomics analysis revealed that the biostimulant treatment reprogrammed the plant metabolome. Hence, several biochemical processes underly the observed increase in fruit yield. Here, we disclosed that the biostimulant modulated the phytohormone profile and induced secondary metabolism. Specifically, the

microbial-based biostimulant upregulated compounds such as carotenoids, saponins, and phenolics that participate in plant nutrition, defence and stress response. Thus, the results of the present study confirm that biostimulant amendments favour stable increases in fruit yield and lead the way towards future investigations into their effects on plants under challenging conditions such as abiotic and biotic stress, environmental perturbations, and physicochemical imbalances.

Data Availability

Acknowledgements

Conflict of Interest

Veronica Cirino and Gorka Erice are former employees of Atens Agrotecnologias Naturales.

Author Contributions

P.B., G.C., Y.R., V.C., M.C. and L.L. designed the experiment. G.C., M.C., Y.R. and G.E. measured and made the interpretation of agronomical data. P.B. and B.L. acquired the metabolomics and qPCR data. P.B., B.M.M. and L.L. analysed the metabolomics data. All authors discussed the results and contributed to the final manuscript.

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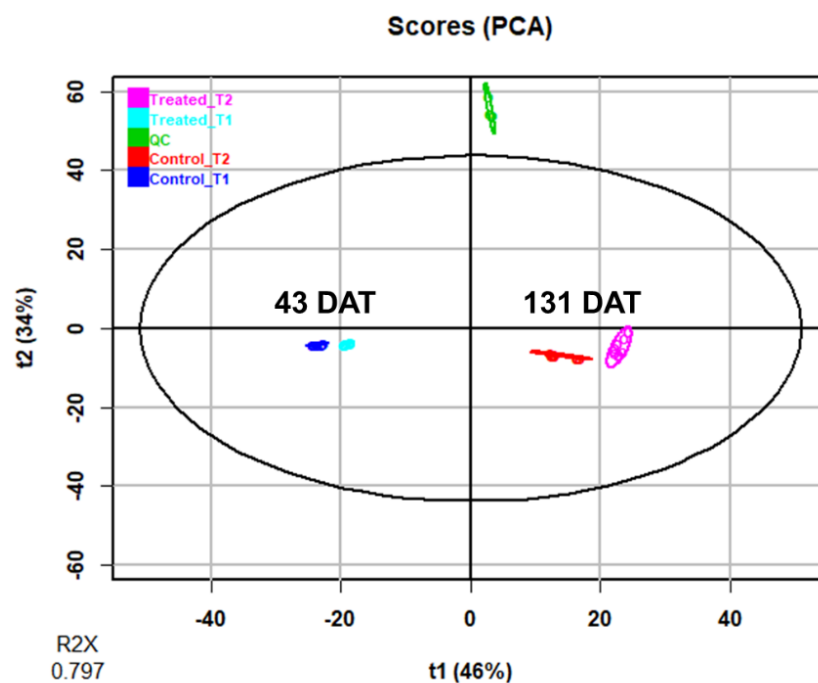
FIGURES:

Figure 1. Principal Component Analysis (PCA) of statistically different metabolites (P<0.05) in pepper plants following treatment with microbial biostimulants. Compounds were profiled by untargeted metabolomics and samples harvested at two dates: 43 days after transplanting (DAT; vegetative stage), and 131 DAT (reproductive stage).
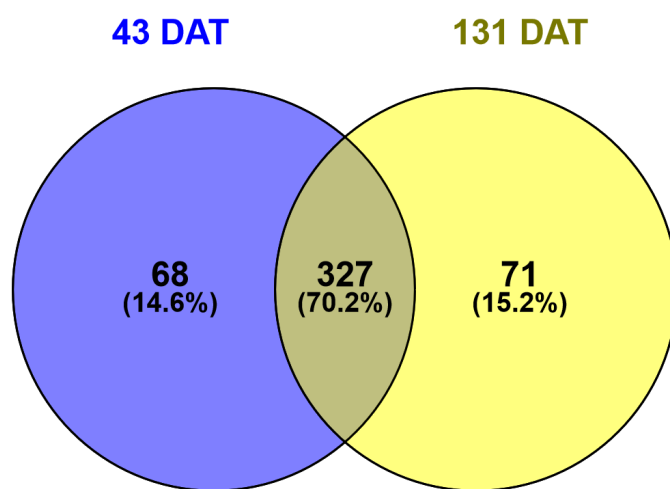
Figure 2. Venn diagram of statistically different metabolites (P<0.05) in pepper plants following treatment with microbial biostimulants. Compounds were profiled by untargeted metabolomics. Samples were harvested at two dates: 43 days after transplanting (DAT; vegetative stage), and 131 DAT (reproductive stage).

Figure 3. Chemical similarity enrichment analysis (ChemRICH) of statistically different annotated metabolites in microbial-based biostimulant treated leaves compared to untreated control at vegetative (43 days after transplanting; DAT) and reproductive stage (131 DAT).

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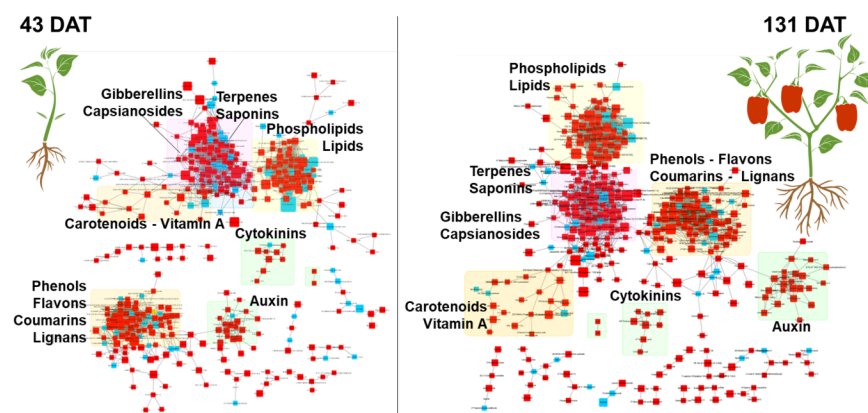
Figure 4. MetaMapp Metabolomic network maps of pepper leaves at vegetative (43 days after transplanting; DAT) and reproductive stage (131 DAT). Microbial-based biostimulant treated plants were compared to control ones. Red dots are compounds with an increase of fold change, while blue ones represent compounds with a decrease in fold changes. Chemical similarity and KEGG reaction were utilised to draw the clusters and nodes.

Table 1

Effect of microbial-based biostimulant application on fruit yield of greenhouse-grown peppers at different days after transplanting (DAT)

Treatment	Fruit yield (kg plant^{-1})	Fruit yield (kg plant^{-1})	Fruit yield (kg plant^{-1})	Fruit yield (kg plant^{-1})	Fruit
	139 DAT	174 DAT	243 DAT	264 DAT	272 D
Control	0.57	0.85	0.59	0.73	0.63
Biostimulant	0.73	1.41	0.48	0.74	0.81
Significance	*	**	ns	ns	*

Two-tailed unpaired Student t- test, ns= nonsignificant, *P<0.05, and **P<0.01.

Table 2

Effect of microbial-based biostimulant application on fruit number of greenhouse-grown peppers at different days after transplanting (DAT)

Treatment	Fruit number (n. plant^{-1})	Fruit number (n. plant^{-1})	Fruit number (n. plant^{-1})	Fruit number (n. plant^{-1})
	139 DAT	174 DAT	243 DAT	264 DAT
Control	2.35	3.65	2.90	2.20
Biostimulant	2.75	5.56	1.92	1.98
Significance	ns	**	ns	ns

Two-tailed unpaired Student t- test, ns= nonsignificant, *P<0.05, and **P<0.01.

Table 3.

Effect of microbial-based biostimulant application on fruit mean weight of greenhouse-grown peppers at different days after transplanting (DAT)

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Treatment	Fruit mean weight (g fruit^{-1})	Fruit mean weight (g fruit^{-1})	Fruit mean weight (g fruit^{-1})	Fruit mean we
	139 DAT	174 DAT	243 DAT	264 DAT
Control	244.0	232.4	204.4	335.1
Biostimulant	264.7	254.4	250.4	368.3
Significance	**	**	ns	*

Two-tailed unpaired Student t- test, ns= nonsignificant, *P<0.05, and **P<0.01.

Table 4.

Effect of microbial-based biostimulant application on compound chemical classes (CHEMRICH) of greenhouse-grown peppers at vegetative stage (43 DAT)

Cluster name	Cluster size	p-values	FDR	Key compound	Inc
Carotenoids	10	2.2E-20	9.2E-20	Fucoxanthinol, Vit A, Alpha Carotene	7
Diterpenes	17	2.2E-20	9.2E-20	NCGC00385284-01_C32H54O13	17
Flavonoids	9	2.2E-20	9.2E-20	Skullcapflavone I 2'-(2''-E-cinnamoylglucoside)	7
Isoflavones	6	2.2E-20	9.2E-20	Genistein	6
Lignans	4	2.2E-20	9.2E-20	Myricatomentoside I	3
Phosphatidic Acids	9	2.2E-20	9.2E-20	PA(18:0/18:2)	8
Phosphatidylethanolamines	12	2.2E-20	9.2E-20	PE(P-16:0/20:5)	4
Phosphatidylinositols	5	2.2E-20	9.2E-20	PIM4(18:1/14:0)	5
Phosphatidylserines	12	2.2E-20	9.2E-20	PS(P-16:0/13:0)	7
Saponins	15	2.2E-20	9.2E-20	Borassoside A	8
Triterpenes	14	2.2E-20	9.2E-20	Cussoracoside F	10
Glucosides	5	1.1E-16	3.9E-16	Luteolin-4'-O-glucoside	5
Amino Acids	5	7.8E-16	2.2E-15	Arginine	2
Phenols	6	1.7E-15	4.5E-15	Gibbilimbol B	5
Glycosides	4	7.7E-14	1.7E-13	Melissoidesin D	2
Macrolides	10	2.6E-14	5.9E-14	Capsianoside	8
Piperidines	3	9.1E-13	1.9E-12	Andrachcinidine	3
Iridoids	3	1.5E-12	3E-12	NCGC00168877-02_C15H20O8	3
Catechols	3	5.4E-12	9.9E-12	(S)-[8]-Gingerol	3
Flavonols	3	1.6E-11	2.7E-11	Kaempferol	3
Auxins	12	6E-11	9.8E-11	Indole-3-acetamide	12
Glycerides	4	3.7E-09	5.4E-09	DG(19:1(9Z)/22:4(7Z,10Z,13Z,16Z)/0:0)[iso2]	3
Limonins	4	5.9E-09	8.4E-09	11beta-Acetoxydihydrocedrelone	2
Dipeptides	6	1.2E-08	1.7E-08	Ala-Phe	4
Sesquiterpenes	4	4.5E-08	6.1E-08	Leucascandrolide A	4
Coumarins	3	5.8E-08	7.6E-08	Coumarin	2
Monoterpenes	4	9.6E-08	1.2E-07	NCGC00384740-01_C21H34O9	4
Amino Acids, Aromatic	5	0.0000002	2.5E-07	Tryptophan	4
Glycolipids	3	3.1E-07	3.6E-07	Lyciumoside IV	2
Anthocyanins	3	3.7E-07	4.3E-07	Cyanidine-3-O-sambubioside	3
DiHODE	3	6.8E-07	7.6E-07	8(R)-Hydroperoxylinoleic acid	3
Gibberellins	4	0.0000019	0.0000021	Gibberellin A20	4
Oligopeptides	3	0.0000047	0.000005	Indole-3-acetyl-L-isoleucine	3
Saturated FA	3	0.000024	0.000025	Capric acid	2
Phosphatidylglycerols	4	0.00037	0.00037	PG(18:2/13:0)	3

Table 5.

Effect of microbial-based biostimulant application on compound chemical classes (CHEMRICH) of greenhouse-grown peppers at reproductive stage (131 DAT)

Cluster name	Cluster size	p-values	FDR	Key compound
Carotenoids	12	2.2E-20	1.3E-19	Fucoxanthinol, Vit A, Alpha Carotene
Diterpenes	15	2.2E-20	1.3E-19	Traversianal
Flavonoids	9	2.2E-20	1.3E-19	Skullcapflavone I 2'-(2"-E-cinnamoylglucoside)
Lignans	5	2.2E-20	1.3E-19	Myricatomentoside I
Macrolides	9	2.2E-20	1.3E-19	Capsianoside
Phosphatidic Acids	9	2.2E-20	1.3E-19	PA(O-18:020:3(8Z11Z14Z))
Phosphatidylserines	12	2.2E-20	1.3E-19	PS(P-16:013:0)
Triterpenes	15	2.2E-20	1.3E-19	Tricalysioside T
Phosphatidylglycerols	8	3.3E-16	1.7E-15	PG(P-18:017:2(9Z12Z))
Phosphatidylethanolamines	8	4.9E-15	2.1E-14	PE(P-16:020:5(5Z8Z11Z14Z17Z))
Saponins	9	1.2E-14	4.9E-14	Namonin E
Isoflavones	6	1.8E-14	6.8E-14	Genistein
Amino Acids	5	1.6E-12	5.4E-12	L-Valine
Phosphatidylinositols	5	3.1E-12	1E-11	PIM4(18:1(9Z)14:0)
Glucosides	6	4.3E-12	1.3E-11	Daedaleaside D
Glycolipids	3	5.3E-12	1.5E-11	Capsoside A
Phenols	6	7E-12	1.9E-11	Gibbilimbol B
Glycosides	5	3.2E-11	7.6E-11	Cyclopassifloside VII
Auxins	12	5.1E-11	1.2E-10	INDOLE-3-PYRUVIC ACID
Monoterpenes	5	1.2E-10	2.5E-10	beta-Thujaplicin
Amino Acids, Aromatic	4	2.3E-10	4.6E-10	34-Dihydroxy-L-phenylalanine
Purine Nucleosides	4	6.9E-10	1.2E-09	Adenosine
Sesquiterpenes	4	1.4E-09	2.4E-09	(+)-vulgraon B
Oligopeptides	5	2.7E-08	4.3E-08	Indole-3-acetyl-L-isoleucine
Limonins	4	2.9E-08	4.4E-08	Toonaciliatin D
Xanthophylls	3	5.6E-08	8.3E-08	Spirilloxanthin
Chlorophyllides	3	8.2E-08	1.2E-07	chlorophyllide a
Anthocyanins	3	8.4E-08	1.2E-07	Delphinidin-3-O-sambubioside
Saturated FA	3	1.8E-07	2.5E-07	Petroformyne 1
Iridoids	4	4.1E-07	5.1E-07	Eleganoside B
Flavonols	3	5E-07	6.1E-07	Kaempferol
Coumarins	3	1.3E-06	1.5E-06	Coumarin
Diglycerides	3	1.7E-06	0.000002	DG(15:1(9Z)22:6(4Z7Z10Z13Z16Z19Z)0:0)iso2
Saturated_Fatty Acids	3	2.5E-06	2.8E-06	Capric acid
Catechols	3	4.3E-06	4.8E-06	33'44'-Tetrahydroxy-55'-diisopropyl-22'-dimethylbiphe
Lysophospholipids	3	4.9E-06	5.2E-06	PC(O-17:00:0)
Cinnamates	6	8.2E-06	8.5E-06	Sinapine
Disaccharides	3	0.000011	0.000011	Melibiose
Gibberellins	3	0.000036	0.000036	Gibberellin A20