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Trichoderma and Phosphite Elicited Distinctive Secondary Metabolite Signatures in Zucchini Squash Plants

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Abstract: Plant biostimulants are "green" solutions to improve crop production. *Trichoderma* spp. and phosphites, ordinarily used as biocontrol agents, can trigger phytostimulation, also promoting endogenous mechanisms involved in plant growth and development. The present study aimed at assessing the efficacy of a phosphite-based formulation (Phosphit-One) and *Trichoderma harzianum*-T22 on the morpho-physiological response and modulation of the metabolomics profile in zucchini squash plants (*Cucurbita pepo* L.) cultivated in controlled growth conditions (Fitotron®). The highest values of fresh biomass production (390.9 g plant^{-1}) and root dry weight (5.6 g plant^{-1}) were obtained for *Trichoderma*-treated plants. This last treatment resulted in an improved physiological performance (SPAD index, CO_2 assimilation rate, and F_v/F_m ratio) measured 30 days after transplanting. Both *Trichoderma* and phosphite treatments induced a broad metabolic reprogramming in leaves, evident also for the phosphite treatment that did not result in a growth promotion. The microbial and the non-microbial treatments showed distinctive signatures in secondary metabolism yet, common responses could be also highlighted. For instance, both *Trichoderma* and phosphite triggered ROS-mediated signaling processes, together with the accumulation of phenylpropanoids, glucosinolates, and phytoalexins. Furthermore, a significant alteration of phytohormones was observed, with terpenoid gibberellins and brassinosteroids showing the largest differences. The metabolomic signatures induced by *Trichoderma* and phosphite in zucchini squash provided molecular insights into the processes underlying elicitation of plant defense due to biostimulation. Interestingly, the modulation of plant secondary metabolism by both treatments did not impair plant growth.

Keywords: untargeted metabolomics; secondary metabolism; redox signaling; phytoalexins; phenylpropanoids; phytohormones; phytostimulants; open gas exchange chamber; *Cucurbita pepo* L.

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1. Introduction

In recent decades, the word "sustainability" has made an extraordinary rise to fame. The Neolithic agricultural revolution has paved the way for modern society by establishing systematic processes to harness natural resources based on plant products [1]. The environment represents the entire ecosystem on which a society depends for various services such as water, food, and energy [2]. Sustainability defines the ability of a system or process to endure over time [3]. However, when a system becomes inefficient in operating within its environment, it consumes more available resources and produces more entropy or waste, thus making it unsustainable [4]. For these reasons, modern agriculture must move towards sustainability while feeding an ever-increasing population [5] and preserving non-renewable natural resources (e.g., water and soil) [6,7]. In view of environmental

sustainability, agriculture is expected to rely heavily on innovative ecological solutions for crop nutrition and protection, such as plant biofertilizers, biocontrol agents, and biostimulants [8,9]. Biostimulants are among the most promising sustainable tools to improve yield and quality of horticultural products under sub-optimal growth conditions. Biostimulants also differ from synthetic fertilizers and pesticides because of their efficacy when applied at low doses [8,10,11]. The diffusion of biostimulants in agriculture is motivated by yield improvement and greater resistance to increasingly diffused biotic and abiotic stress factors [12,13]. The positive effects on crops seem to be related to the signaling mechanisms (stimulus-response) induced by the biostimulant-plant interaction, which triggers a defense response that can mitigate the effects of unfavorable environmental conditions [14].

Plant biostimulants include natural bioactive substances such as humic and fulvic acids, seaweed, plant extracts, and protein hydrolysates; inorganic compounds (e.g., chlorides, phosphites, silicates, and carbonates); and growth-promoting microorganisms such as beneficial bacteria and fungi [8]. Among the latter, *Trichoderma* spp., which are biocontrol agents with proven biopesticide activity, also exhibit strong biostimulant activity [15–18]. This can be attributed to the secretion of low-weight molecules (auxin-like compounds, peptides, and volatile organic compounds), which can re-modulate gene expression by inducing specific metabolic processes in both the epigeal and hypogeal systems, increasing photosynthetic efficiency, carbohydrate metabolism, and macro and microelements uptake [9,18–20]. Among different *Trichoderma* spp. marketed formulates, *T. harzianum*-T22 strain showed an excellent rhizosphere colonization activity independently of soil characteristics [21,22]. For instance, Carillo et al. [9] confirmed that the inoculation of *T. harzianum*-T22 is to be considered a valid eco-sustainable tool to increase the production of tomato in the Mediterranean environment.

Apart from microbial biostimulants, growers can also rely on commercial formulations based on inorganic salts such as chlorides, carbonates, silicates, and phosphites [8]. Due to its established elicitory and fungicidal activity, phosphite represents a sustainable alternative to promote crop defense and growth, considering also that it is not metabolized by eukaryotes [23]. Phosphite is the basis of different fungicides successfully controlling pathogens that usually cause significant production loss [24,25]. Specifically, different studies pointed out its effectiveness either on key fungal pathogens belonging to the Oomycetes class (*Phytophthora* spp., *Pythium* spp., *Peronospora* spp., and *Plasmopara*) or on the *Erwinia amylovora* bacterium, which is the causative organism of "fireblight" in Rosaceae [23,26,27]. Although the chemical structure of phosphite is analogous to phosphate, the absence of an oxygen atom prevents plants from directly utilizing it as a phosphorus source and thus, since it has no impact on plant primary metabolism, it is not classified as a fertilizer [24]. Even though phosphite is unmetabolized by plant tissues, it can elicit molecular, biochemical, and physiological responses acting also as a plant biostimulant [23]. For example, phosphite promotes root growth, improves nutrient uptake, and elicits mechanisms involved in stress response (biotic and abiotic stress), improving yield and quality of horticultural crops [28–30].

To promote the dissemination of beneficial biostimulants and a more sustainable agriculture, comprehensive investigations of their effects on plant primary and secondary metabolism are mandatory. Moreover, it is important to account for possible detrimental effects on plant growth, since the elicitation of secondary metabolism could represent a metabolic cost for crops. To this end, together with morphological assays, untargeted metabolomics can represent a valuable tool for unveiling the eliciting effects of biostimulants on plant growth and development [25]. The capacity of metabolomics to unravel the molecular response of crops to environmental factors, is due to its ability to provide comprehensive information about the chemical phenotype of a crop subjected to specific factors [31]. Under this perspective, metabolomics can also provide insights into the intricated biochemical processes involved in plant response to elicitors.

Phosphite and *Trichoderma* spp. are ordinarily used in vegetable cropping systems to promote root and shoot growth, increase yield, and stimulate endogenous plant defenses

against biotic stresses. However, there is a lack of comparison in the scientific literature of these two types of biostimulants in terms of crop agronomic performance and especially metabolic reprogramming. A clear understanding of the agronomic and metabolomic effects induced by the two type of biostimulants is critical to understanding the best strategy for sustainably managing plant cropping systems.

Zucchini squash (*Cucurbita pepo* L.) is gaining in popularity in Europe and has become one of the most important grown and consumed horticultural crops, representing an economic resource for growers and the horticultural chain, ranking fourth among retail vegetables. Among Mediterranean coastal regions, Italy produces more than 200 tons of zucchini squash in protected crops and has an annual per capita consumption of about 9 kg [32,33].

The aim of our study was to comparatively assess the effects of an endophytic fungus (*Trichoderma harzianum*-T22) and a potassium phosphite elicitor (Phosphit-One, Italpollina, Rivoli Veronese, Italy) on epigeal and hypogeal biomass changes, physiological response, and metabolic reprogramming of zucchini squash (*Cucurbita pepo* L.) during the vegetative growth phase in a fully controlled Fitotron® chamber.

2. Materials and Methods

2.1. Plant Material, Experimental Design, Growth Conditions, and Sampling

The research was carried out in 2019 in a 28 m^2 open gas exchange chamber (Fitotron®; 7.0 m × 2.1 m × 4.0 m; W × H × D; ProcessC5, Spagnol Srl, Treviso, Italy) at the experimental farm of the University of Naples Federico II "Torre Lama", located in Bellizzi (Salerno, Italy). The Fitotron® was equipped with High Pressure Sodium lamps at 420 µmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD) with a light/dark regime of 12/12 h. Temperature (°C), relative humidity (RH), and ambient CO_2 concentration were set at 24/18 °C (day/night), 65–70%, and 380–400 ± 20 ppm, respectively. Environmental parameters were controlled using automated fog, heating, ventilation, and air conditioning systems.

At the two true-leaf stage, seedlings of zucchini squash (*Cucurbita pepo* L.) cv. 'San Pasquale' (Pagano Domenico & Figli, Scafati, Salerno, Italy) were transplanted into 16 cm plastic pots filled with 3L of Brill 3 substrate (Gebr. Brill Substrate GmbH & Co. KG, Georgsdorf, Germany). Transplanting was carried out on 25 June 2019. Pots were disposed at a plant density of 2.8 plants m^{-2}. The nutrient solution (NS) was provided through a drip irrigation system consisting of a main 16 mm polyethylene pipeline equipped with 4 L h^{-1} drippers. The NS was a modified Hoagland solution with the following composition: 9.6 mmol N L^{-1}, 1.5 mmol L^{-1} P, 4.5 mmol L^{-1} K, 6.5 mmol L^{-1} Ca, 2 mmol L^{-1} Mg, 20 µmol L^{-1} Fe, 9 µmol L^{-1} Mn, 0.3 µmol L^{-1} Cu, 1.6 µmol L^{-1} Zn, 20 µmol L^{-1} B, and 0.3 µmol L^{-1} Mo. The pH and EC of the NS were 6.0 ± 0.2 and 2 ± 0.1 dS m^{-1}, respectively. The experimental treatments consisted of the microbial application of *Trichoderma harzianum strain* T22 (hereafter *Trichoderma*; Koppert, Bussolengo, Italy) and the potassium phosphite-based elicitor Phosphit-One (hereafter Phosphit; Italpollina, Rivoli Veronese, Italy). At transplant, either 5 mL aqueous solution of *Trichoderma* at a dose of 10 g L^{-1} (i.e., 1×10^{10} spores g^{-1}) was manually supplied by a graduated cylinder, or a 5 mL aqueous solution of Phosphit (2.5 mL L^{-1}) was supplied at transplant (1) and 7, 14, 21 and 28 days after transplanting (DAT). The application rate of the two commercial *Trichoderma* and phosphite formulations used were based on the manufacturers' label recommendations. The experimental design was a randomized complete block design (RCBD) with three replicates for each treatment (*Trichoderma*, phosphite, and control) for a total of nine experimental units (plots). Each plot comprised 6 plants.

At the end of the experiment (24 July; 30 DAT), the plants were harvested and separated into leaves/stem and roots. For each plant, leaves and stem were weighed for fresh biomass determination (g $plant^{-1}$). A sub-sample of leaves tissue was immediately frozen in liquid nitrogen and stored at −80 °C until further use. All harvested tissues were oven-dried at 70 °C until constant weight (72 h) for dry biomass production and root dry weight determination (g $plant^{-1}$). Part of the dried leaves were ground with an MF10.1 cutting-

grinding head mill (IKA®, Staufen im Breisgau, Baden-Württemberg, Germany) and sieved through a MF0.5 sieve (0.5 mm hole size; IKA®, Staufen im Breisgau, Baden-Württemberg, Germany) for total nitrogen content and mineral composition determination.

2.2. Total Nitrogen and Mineral Content Analysis

Total nitrogen content was assessed according to Kjeldahl method described by Bremner [34]. Separation and quantification of zucchini squash leaves' mineral profile were performed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector according to the protocol described by Rouphael et al. [11]. Guard and analytical columns were purchased from Thermo Fisher Scientific™ Dionex™ (Sunnyvale, CA, USA). Cations (K^+, Ca^{2+}, and Mg^{2+}) were separated using an IonPac CG12A guard column and an IonPac CS12A analytical column while anions (PO_4^{3-} and SO_4^{2-}) were separated using an IonPac AG11-HC guard column and an IonPac AS11-HC analytical column. Three replicates were performed for each treatment.

2.3. SPAD Index, Carbon Dioxide Assimilation Rate, and Chlorophyll Fluorescence Determination

Soil Plant Analysis Development (SPAD) index, CO_2 assimilation rate (Pn), and Maximum quantum efficiency of Photosystem II (PSII; F_v/F_m ratio) measurements were carried out o four young fully expanded leaves (free of disease symptoms or visible defects) per replicate. At 16, 23, and 30 DAT, the leaf greenness (SPAD) was assessed using a portable chlorophyll meter SPAD-502 (Minolta Corp. Ltd., Osaka, Japan), and a single average SPAD value for each replicate was obtained by measuring ten randomly selected leaves.

On the same date, Pn-net photosynthesis was measured by an LCA-4 portable gas exchange analyzer (ADC BioScientific Ltd., Hoddesdon, UK), equipped with a 6.25 cm^2 broadleaf chamber. The flow rate was set to 400 mL s^{-1} while PPFD, RH, and CO_2 concentrations were set to growth chamber values. Maximum quantum efficiency of PSII of zucchini squash plants was assessed by a portable fluorometer F_v/F_m Meter (Opti Sciences Inc., Hudson, NH, USA) on dark-adapted (at least 10 min) leaves by special clips. According to Kitajima and Butler [35], the maximum quantum efficiency of PSII F_v/F_m, was calculated as $(F_m - F_o)/F_m$, where F_o was the ground fluorescence signal induced by a blu-LED internal light of 1–2 mol m^{-2} s^{-1} and F_m was the maximal fluorescence intensity in the dark-adapted state by a 1s saturating light pulse of 3000 mol m^{-2} s^{-1}.

2.4. Untargeted Metabolomics

For metabolomics analysis, leaves were processed as previously reported [36]. Briefly, freeze-dried samples (1.0 g) were extracted in 20 mL of 0.1% formic acid in 80% methanol aqueous solution using an Ultra-Turrax (Ika T-25, Staufen, Germany) and centrifuged (12,000× *g*). Untargeted metabolomics was carried out by ultra-high-pressure liquid chromatography coupled to a quadrupole-time-of-flight UHPLC-QTOF mass spectrometer from Agilent (Santa Clara, CA, USA), as previously described [36]. In brief, chromatography was done by a reverse-phase Agilent pentafluorophenylpropyl (PFP) column (2.0 × 100 mm, 3 µm) (Santa Clara, CA, USA) with a mobile phase of acetonitrile in water (6% to 94%) in a 33 min run with flow rate 200 µL min^{-1}. The mass spectrometer worked in SCAN mode (100–1000 m/z) and positive polarity. Raw spectral data were processed using the "find-by-formula" algorithm in Agilent Profinder B.07 (Santa Clara, CA, USA) followed by mass (5 ppm) and retention time (0.05 min) alignment [7]. Compounds were putatively annotated based on the PlantCyc 12.6 database (Plant Metabolic Network; Release: April 2018) by a combination of monoisotopic mass and isotopes ratio and spacing, according to Level 2 with reference to COSMOS Metabolomics Standards Initiative [37]. Compounds annotated in at least 75% of replicates within at least one treatment were retained for subsequent analysis.

2.5. Statistics and Chemometric Interpretation of Metabolites

The statistical analysis was performed using IBM SPSS Statistics (SPSS Inc., Chicago, IL, USA), version 20.0 package. The mean effect of plant biomass and partitioning, as well as mineral profile, SPAD index, Carbon dioxide assimilation rate and F_v/F_m ratio were subjected to One-way ANalysis Of VAriance (ANOVA). Statistical significance was determined at the $p < 0.05$ level using Tukey's HSD test. All data are presented as mean ± standard deviation, $n = 3$.

Chemometric interpretation of metabolite profiles was performed using Mass Profiler Professional B.12.06 from Agilent (Santa Clara, CA, USA) as described Corrado et al. [38]. Compound abundance was log2 transformed, normalized at the 75th percentile, and baselined against the median. The unsupervised hierarchical cluster analysis (HCA) was carried out based on fold-change values with the Wards agglomerative algorithm of the Euclidean distances. Multivariate reduction was carried out using a partial least squares discriminant analysis (PLS-DA) supervised method. Compounds having the highest discrimination score in first and second latent vector were finally exported from loading plots and then subjected to fold-change analysis (fold-change cut-off = 2). These compounds were then analyzed with the Omic Viewer Pathway Tool of PlantCyc (Stanford, CA, USA) to identify pathways affected by the treatments [39].

3. Results

3.1. Biometric Parameters

Biometric parameters recorded in response to phosphite and *Trichoderma* treatments are reported in Table 1. The application of *Trichoderma* resulted in increased fresh biomass production (14.9%) and root dry weight (115.4%) compared to the non-treated control as well as the highest dry biomass value (47.2 g plant^{-1}) compared to either the phosphite treatment or the control.

Table 1. Effects of phosphite and *Trichoderma* application on fresh biomass production, dry biomass production, and root dry weight of zucchini squash plants.

Treatment	Fresh Biomass Production	Dry Biomass Production	Root Dry Weight
	g plant^{-1}	g plant^{-1}	g plant^{-1}
Control	340.1 ± 10.17 b	43.3 ± 1.24 b	2.6 ± 0.36 b
Phosphite	364.5 ± 13.31 ab	43.7 ± 1.60 b	4.2 ± 0.96 ab
Trichoderma	390.9 ± 24.92 a	47.2 ± 1.75 a	5.6 ± 1.22 a
Significance	*	*	*

* significant at $p \leq 0.05$. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$.

3.2. Total Nitrogen and Mineral Accumulation

Total nitrogen and mineral composition of zucchini leaves treated with *Trichoderma* and phosphite are presented in Table 2. The applied treatments did not significantly affect the total nitrogen content. Among the analyzed minerals, potassium was the most abundant, regardless of treatments, ranging from 23.9–24.3 g kg^{-1} dw, followed by calcium (14–16.1 g kg^{-1} dw), magnesium (3.7–4.5 g kg^{-1} dw), phosphorus (2.6–3.6 g kg^{-1} dw), and sulfur (1.9–2.5 g kg^{-1} dw) (Table 2). Potassium, calcium, and sulfur content did not vary significantly among all treatments, in contrast to phosphorus and magnesium. Particularly, the use of *Trichoderma* led to a 38.5 and 21.6% increase in phosphorus and magnesium content, respectively, compared to the control.

Table 2. Effects of phosphite and *Trichoderma* application on total nitrogen and minerals accumulation of zucchini squash plants.

Treatment	Total N	P	K	Ca	Mg	S
	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw
Control	23.9 ± 2.89	2.6 ± 0.24 b	24.1 ± 4.22	14.0 ± 4.32	3.7 ± 0.25 b	1.9 ± 0.38
Phosphite	21.4 ± 1.24	3.1 ± 0.11 ab	23.9 ± 2.20	15.3 ± 0.77	3.9 ± 0.22 ab	2.2 ± 0.42
Trichoderma	22.7 ± 2.75	3.6 ± 0.34 a	24.3 ± 2.50	16.1 ± 0.29	4.5 ± 0.31 a	2.5 ± 0.38
Significance	ns	*	ns	ns	*	ns

ns, * non-significant or significant at $p \leq 0.05$, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$. dw = dry weight.

3.3. Physiological and Biochemical Parameters

The main physiological parameters such as SPAD index, Pn, and F_v/F_m ratio, were significantly affected by the performed treatments in relation to the day after transplant (Table 3). The treatment with *Trichoderma* resulted in the highest SPAD index values both at 23 and 30 DAT compared with the untreated control. On the same dates, *Trichoderma*-treated plants showed higher Pn values of 38.7 and 53.8%, respectively, compared with the control. With respect to the F_v/F_m ratio, significant differences were recorded between treatments only at 30 DAT, with *Trichoderma* showing the highest value (0.78).

3.4. Metabolomics

The plant response to the application of *Trichoderma* and phosphite was then investigated using an untargeted metabolomics approach. This analysis resulted in the putative annotation of more than 2400 features, using the comprehensive database PlantCyc12.6 (www.plantcyc.org; Accessed on 3 December 2020). The Supplementary Table S1 provides the list of the annotated metabolites, with individual abundances and composite mass spectra (mass and abundance combinations). Thereafter, multivariate statistics were used to examine the simultaneous effect of the treatments on the multiple metabolomics variables. Unsupervised hierarchical cluster analysis allowed representing similarities/dissimilarities in metabolic profiles between the sample groups (i.e., control, *Trichoderma* and phosphite) grouping the samples according to the treatment. Despite being largely shared, the clustering clearly showed a diverse accumulation of compounds in plants after adding either *Trichoderma* or phosphite (Figure 1) suggesting that metabolic profile was influenced by the treatments in a distinctive manner.

This was further confirmed by the subsequent supervised PLS-DA, which separated the samples in the score space according to the treatments (Figure 2). The accuracy prediction of the model was 93%. To discriminate the compounds involved in the plant response and shed light into the effect(s) at a metabolism level, the compounds characterized by the highest score (score > 0.04) in the PLS-DA discrimination model underwent a fold-change analysis using a cut-off of 2 (Supplementary Table S2). To simplify the interpretation, these compounds were automatically classified by the Omic Dashboard tool of PlantCyc into the principal biosynthetic pathways, and the main classes of compounds were then used for interpretation.

Table 3. Effects of phosphite and *Trichoderma* application on SPAD index, Carbon dioxide assimilation rate (Pn; μmol CO^2 m^{-2} s^{-1}), and Fv/Fm ratio of zucchini squash plants.

Treatment	SPAD Index			Pn			F_v/F_m		
	16	23	30	16	23	30	16	23	30
	DAT			DAT			DAT		
Control	58.0 ± 1.22	51.1 ± 0.72 b	49.3 ± 0.84 b	9.3 ± 2.01	11.1 ± 1.23 b	8.0 ± 0.68 b	0.78 ± 0.02	0.75 ± 0.02	0.75 ± 0.01 b
Phosphite	55.7 ± 2.58	55.0 ± 0.87 a	50.3 ± 2.28 ab	10.2 ± 2.52	12.7 ± 1.15 ab	9.5 ± 0.78 b	0.78 ± 0.03	0.75 ± 0.02	0.75 ± 0.01 b
Trichoderma	60.0 ± 0.89	55.3 ± 0.78 a	54.0 ± 1.14 a	10.3 ± 1.03	15.4 ± 2.28 a	12.3 ± 1.45 a	0.80 ± 0.01	0.78 ± 0.02	0.78 ± 0.01 a
Significance	ns	**	*	ns	**	***	ns	ns	*

ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test ($p = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$. DAT = days after transplanting; SPAD = Soil Plant Analysis Development.

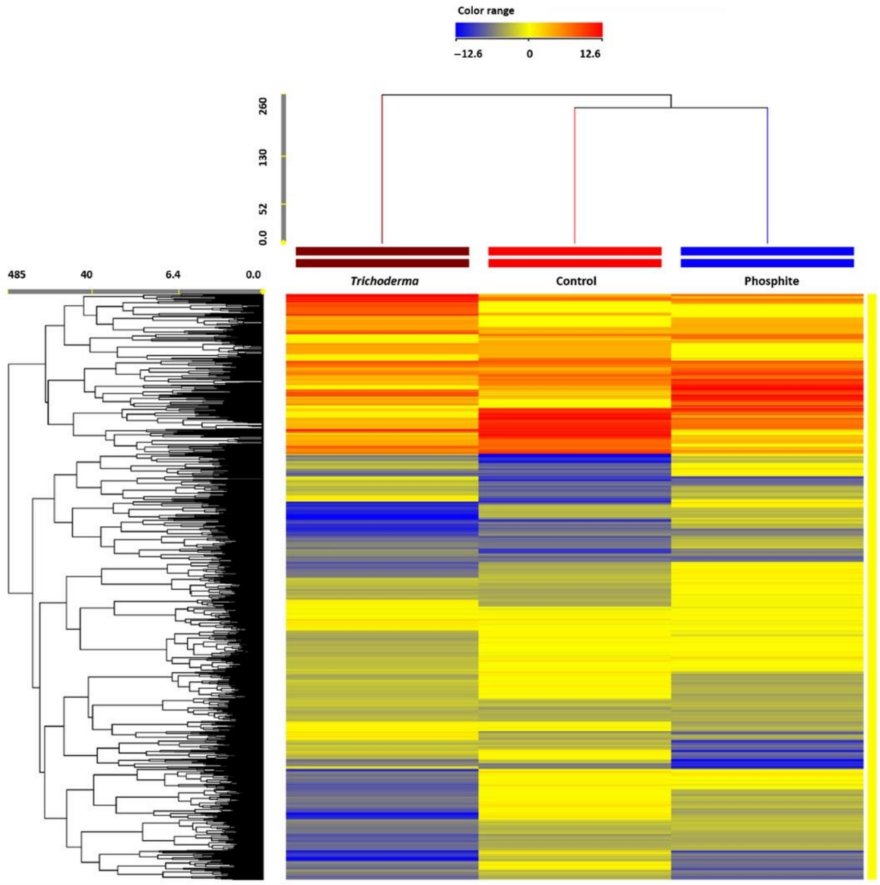

Figure 1. Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward) carried out from chemical profiles of plants treated with *Trichoderma* and phosphite. Metabolites were obtained by UHPLC-ESI/QTOF-MS untargeted analysis, and their normalized intensities were used to build up the heatmaps.

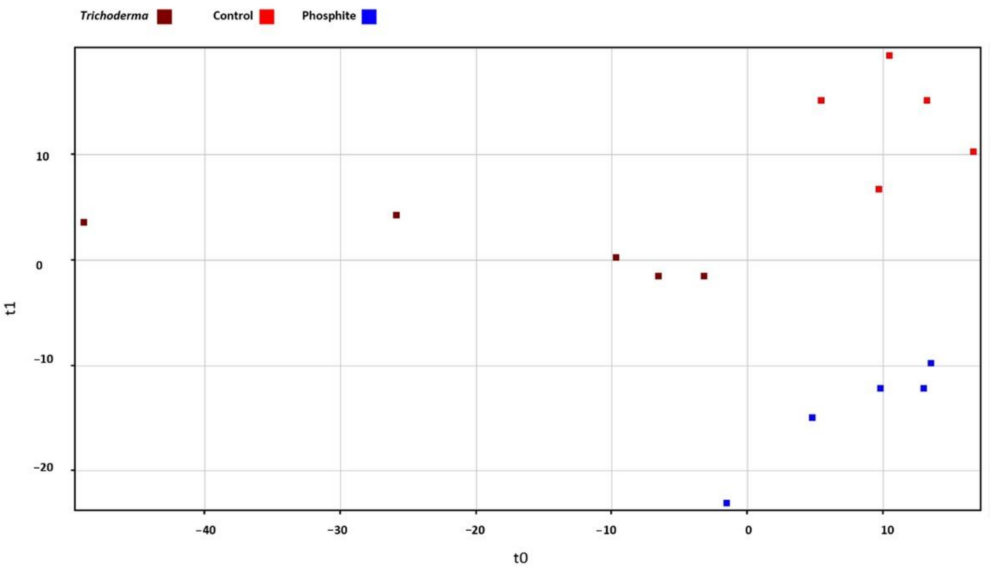

Figure 2. Partial least squares discriminant analysis (PLS-DA) on plants according to their metabolic response to *Trichoderma* and phosphite. Individual replications are given in the class prediction model score plot.

The large number of discriminant compounds in separation are evocative of the intricate network of processes involved in plant response after treatment The distinctive nature of the tested product associated to different plant responses. Nonetheless, regardless of the quantitative variation, most of the pathways were affected by both the *Trichoderma*- and phosphite-treated plants (Figure 3).

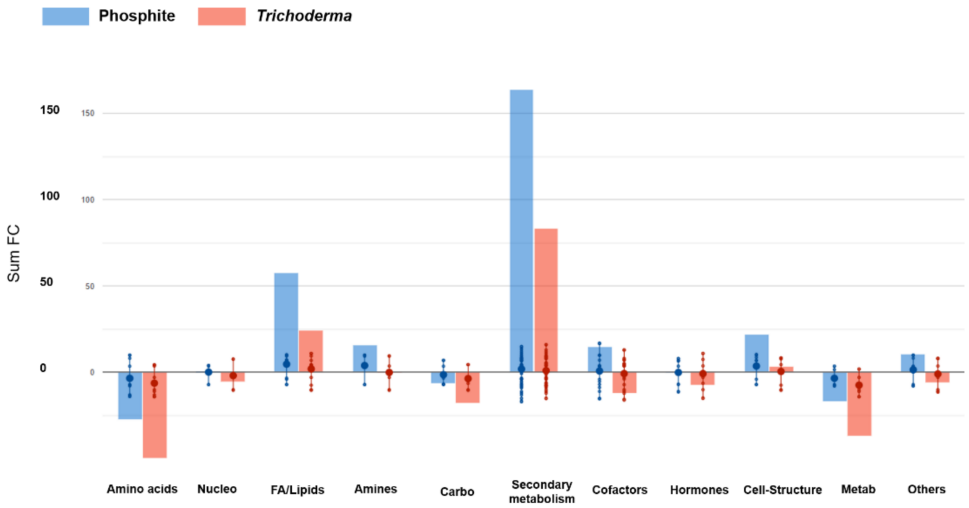

Figure 3. Biosynthetic pathways affected in plants treated with *Trichoderma* and phosphite. Differential metabolites and their fold-change (FC) values were elaborated using the Omic Viewer Dashboard of the PlantCyc pathway Tool software (www.pmn.plantcyc.com; Accessed on 3 December 2020). In each class, the large dot represents the average (mean) logFC of the metabolites. Small dots represent the individual logFC for each metabolite. The abbreviated subcategory names on the x-axis correspond to the biosynthesis of: Nucleo: nucleosides and nucleotides; FA/Lipids: fatty acids and lipids; Amines: amines and polyamines; Carbo: carbohydrates; Cofactors: cofactors, prosthetic groups, electron carriers, and vitamin; Metab: metabolism regulators.

The analysis of the biosynthetic pathways further confirmed the ability of both *Trichoderma* and phosphite to elicit plant defenses. Although the untargeted metabolomic approach mainly focused on secondary metabolites, several compounds classified within the primary metabolism.

Lipids and, in particular fatty acids, were largely up-accumulated in phosphite-treated plants and, to a lesser extent, in *Trichoderma*-treated plants. In fact, fatty acids, mainly hydroxylated and unsaturated fatty acids and phospholipids were up-accumulated in treated plants compared to the control as well as plant cell structures (i.e., trans-5-*O*-caffeoyl-D-quinate, 18-hydroxystearoyl-CoA and 2-(2,8-dihydroxytridecyl)-6-oxopyran-4-olate) Riboflavin was up-accumulated and tocotrienol was down-accumulated in the presence of *Trichoderma*. Compounds involved in chlorophyll biosynthesis and degradation (phytyl diphosphate, chlorophyll, 3,8-divinyl protochlorophyllide and epoxypheophorbide) presented similar regulation for both treatments.

The most remarkable metabolomics' feature was the elicitation of secondary metabolism after treatments applications (Figure 4).

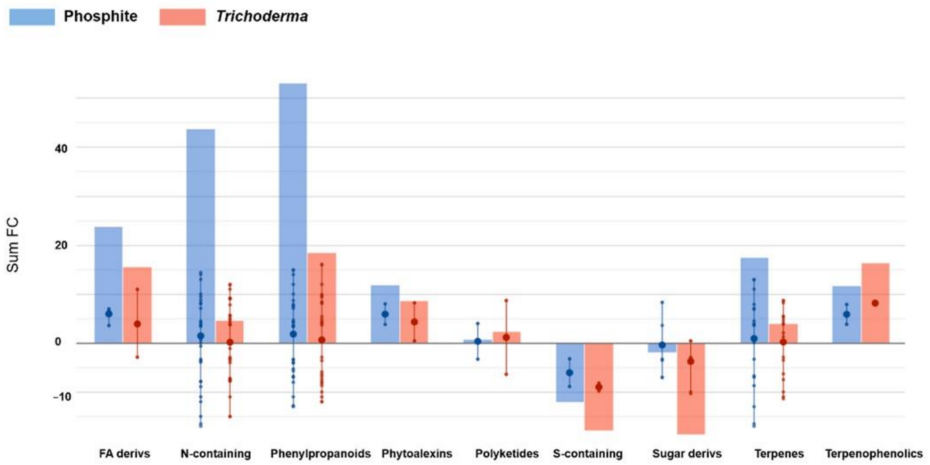

Figure 4. Biosynthetic pathways of secondary metabolites affected in plants treated with *Trichoderma* and phosphite. Differential metabolites and their fold-change (FC) values were elaborated using the Omic Viewer Dashboard of the PlantCyc pathway Tool software (www.pmn.plantcyc.com; Accessed on 3 December 2020). In each class, the large dot represents the average (mean) logFC of the metabolites. Small dots represent the individual logFC for each metabolite. The abbreviated subcategory names on the x-axis correspond to: FA derivs: fatty acid derivatives; N-containing: Nitrogen-containing secondary metabolism; S-containing: Sulfur-containing secondary metabolites; Sugar derivs: sugar derivatives.

The principal classes of secondary metabolites, those derived from the shikimate pathway, nitrogen-containing secondary metabolites and isoprenoids, were strongly promoted. Flavonoids, including anthocyanins and conjugate forms, were the most represented subclass of phenylpropanoids. Terpenes and alkaloids also increased in the presence of phosphite and, to a lesser extent, in the presence of *Trichoderma*. The phytoalexins 15-hydroxysolavetivone and (indole-3-yl) acetonitrile, an indole derivative, increase in treated plants. Secondary metabolites derived from tryptophan seemed to be particularly modulated as well as sulfur-containing secondary metabolites (i.e., cysteine conjugate forms, as well as both indole and aliphatic glucosinolates). Interestingly, indole-3-carbinol, a phytoalexin derived from glucosinolate degradation, was strongly accumulated while its precursor was highly down-accumulated in phosphite-treated. A similar trend was observed for *Trichoderma* although its LogFC accumulated was lower. In addition to secondary metabolites derived from Trp, several degradation products were found up-accumulated confirming the implication of this amino acid in plant response to both treatments.

Consistently, some auxins and related compounds were strongly modulated. Besides the above-mentioned phytoalexin indole-3-yl acetonitrile, the inactive auxin form (indol-3-yl)acetyl-L-valine were stimulated after phosphite addition while the IAA precursor 3-hydroxy-indole-3-butyryl-CoA increased in both *Trichoderma*- and phosphite-treated plants. The inactive forms 7-hydroxy-2-oxindole-3-acetate increase in *Trichoderma*-treated plants while 7-hydroxy-2-oxindole-3-acetate glucoside increase in phosphite-treated plants. Overall, variation in phytohormones could be observed in treated plants. As in the case of other metabolic features, a similarity of the hormone responses was observed in the two treatments. The metabolic pathway of brassinosteroids was modulated leading to the biosynthesis of the active form homobrassinolide in all treated plants. Cytokinin N6-(Δ2-isopentenyl)-adenosine 5′-diphosphate decreased in the presence of both *Trichoderma* and phosphite. Dihydroxyphaseic acid increased in both *Trichoderma*- and phosphite-treated plants while phaseic acid increased in the presence of phosphite and decreased in the presence of *Trichoderma*. The down-accumulation of salicin in *Trichoderma*-treated plants and the up-accumulation of 2,5-dihydroxybenzoate 2-*O*-β-D-glucoside in both *Trichoderma*- and phosphite-treated plants suggested a modulation of the salicylic acid (SA) biosynthetic

pathway. Similarly, the biosynthetic pathway of jasmonates was promoted by the increase of the jasmonate precursors 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-(E-octa-2-enoyl)-CoA and 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-hexanoyl-CoA.

4. Discussion

Endophytic fungi such as *Trichoderma* spp. and inorganic phosphite-based compounds are used mainly to control phytopathogens in agriculture [40,41]. Recent studies have highlighted their promising biostimulant and/or elicitor activities, since they modulate plant primary and secondary metabolism under sub-optimal growth conditions [42]. Their dual role has attracted the interest of producers and growers, driving researchers towards a better understanding of the molecular mechanisms associated with their mode of action, to promote their use in an ever-increasing eco-sustainable agriculture.

Our results indicated that root inoculation with *Trichoderma* promoted vegetative growth, as reported for other crops [42–46]. *Trichoderma* increased fresh and dry biomass production by 14.9% and 9.0%, respectively, compared to the untreated control. The increased biometric parameters were likely due to the well-recognized direct and indirect phytostimulatory effects of signal molecules secreted by the fungal mycelium [18]. For instance, *Trichoderma* releases into the rhizosphere exudates with a hormone-like role (e.g., auxins and ethylene), volatile organic compounds, and low-molecular-weight peptides [7,42,47]. In our study, the significant increase in dry root weight after *Trichoderma* inoculation supports previous findings, and it is a major driver of the higher biometric parameters obtained. The hypogeal system improvement leads to a more efficient use of micro- and macronutrients (P), resulting in their higher bioavailability [48]. On the other hand, the application of phosphite on zucchini plants did not result in significant changes in the biometric parameters investigated, in contrast to the results recorded in different horticultural species [23]. The controlled environmental conditions guaranteed by the growth chamber could have restrained the effect of phosphite, which, as suggested by other authors, better performs in sub-optimal growth conditions [49,50]. Similarly, the differences in the analyzed leaf minerals were related to *Trichoderma* application, which significantly increased the P and Mg content compared to the control. These findings suggest that the enhanced root biomass elicited by *Trichoderma* led to increased uptake and accumulation of phosphorus and magnesium [7,48,51]. In addition to cell turgor regulation, magnesium is also involved in photosynthesis, as a central atom of chlorophyll pigments [52]. The highest SPAD values, which is an indicator of chlorophyll content, were recorded in plants inoculated with *Trichoderma* (30 DAT) and correlated to the highest leaf magnesium content, as also observed in lettuce [53]. In addition, several compounds related to chlorophyll cycle were impaired mainly in the presence of *Trichoderma*. Similarly to magnesium, phosphorus plays a crucial role in the photosynthetic process by regulating gene expression that protects photosystem II from photoinhibition [54]. *Trichoderma* root colonization induces gene and chloroplast components up-regulation in plants, leading to improved photosynthetic process [55]. Investigations carried out on tomato showed that inoculations with *Trichoderma afroharzianum*-T22 and *Trichoderma virens* 41 resulted in improved photosynthetic rates [55]. In our experimental system, the highest net CO_2 assimilation rate (Pn) at 23 and 30 DAT and the highest maximum quantum efficiency of open Photosystem II (F_v/F_m ratio) at 30 DAT were obtained in *Trichoderma*-treated plants, reflecting the improved assimilation and translocation of magnesium and phosphorus, thereby accounting for the higher vegetative growth.

Considering the different nature of elicitors (i.e., microbial and non-microbial), a differential response of zucchini plants to both *Trichoderma* and phosphite was expected. However, both elicitors triggered similar plant responses. The untargeted metabolomics confirmed the elicitation of plant defense by both *Trichoderma* and phosphite.

In our study, L-cysteine-S-conjugates involved in glutathione detoxification and in the biosynthesis of glucosinolates were found as discriminant compounds in both *Trichoderma*- and phosphite-treated plants. Both aliphatic and indole glucosinolates were involved in

plant response to elicitors. Although it is not expected to find glucosinolates as a main response in zucchini plants, these N- and S-containing secondary metabolites have been elicited in several crops under stress conditions as previously reported [56,57]. For instance, Bernardo et al. [58] revealed that S-containing compounds, including glutathione and glucosinolates, were accumulated in AMF-treated roots of wheat. On the other hand, Lucini et al. [59] found glucosinolates within the main classes of differential compounds in another Cucurbitaceae treated with a vegetal biopolymer-based biostimulant. Many of these compounds are involved in the detoxification of glutathione (GSH), which is involved in reactive oxygen species (ROS) mitigation damage and in the maintenance of the cellular redox balance [60]. In this line, the modulation of tryptophan-derived compounds appeared as a common response to both microbial and non-microbial treatment. In fact, Ishihara et al. [61] reported that the Trp pathway is involved in the defense responses of rice against pathogenic infection, via serotonin production. These authors observed an increase of anthranilate synthase activity, which regulates metabolic flux in the Trp pathway, and increasing values of Trp and derived compounds. Moreover, it has been previously observed an increase of Trp in maize treated with potassium phosphite [62]. Several authors reported the role of Trp in minimizing oxidative stress acting as alternative electron donors for the mitochondrial electron transport chain [63].

Besides indole-glucosinolates, other Trp derived compounds were founds discriminants in the plant response. The biosynthesis of some phytoalexins were promoted in the presence of phosphite and, to a lesser extent, in the presence of *Trichoderma*. It is worth noting the presence of indole-3-carbinol, an indole glucosinolate degradation product, which acts as a signaling molecule by direct competition with auxins, adjusting the balance between plant growth and plant defense. Indeed, auxins were modulated by the treatments, leading to the encouragement of auxin precursors and inactive forms [64]. Nonetheless, the literature reveals the complex link between glucosinolate metabolism and auxin homeostasis [65], suggesting that indole glucosinolates might have a role as auxin precursor [66]. These results highlight the complex network between secondary metabolism and hormones which takes place as a response to external perturbations. In fact, this modulation of Trp derivatives might be linked to other phytohormones. For instance, the modulation of glucosinolates leading to changes in the flux of auxin biosynthesis could imply a disorder in cytokinin homeostasis as an indirect consequence [65]. In addition, it is well established that jasmonic acid (JA) and SA are involved in plant defense. In particular, jasmonates play a key role in glucosinolates regulation [67]. Similarly, abscisic acid and SA seem to affect glucosinolates content [68]. Thus, a clear reprograming of secondary metabolism took place following *Trichoderma* and phosphite treatment probably triggered by the hormonal imbalance. Moreover, phytohormones may crosstalk to the ROS signaling cascade. It has been reported that several genes involved in phytohormones biosynthesis are early activated to lead to changes al transcriptome level as a general response to elicitors [69]. In our study, terpene hormones were also involved in plant response. On one hand, gibberellins control cellular redox homeostasis being crucial for the ROS signaling pathways. They also play a critical role in stress response, through ROS signaling pathways thus controlling cellular redox homeostasis. For instance, the DELLA proteins, which are negative regulators of GA signaling, increase during stresses to enable quenching of excessive ROS [69]. On the other hand, brassinosteroids are linked to the response of plants to stress including an antagonistic role towards ABA. In fact, Lucini et al. [59] pointed out the role of brassinosteroids and their interaction with other hormones as a key in the melon response to a biostimulant able to enhance the plant defense. These authors suggested not only a correlation between impairment of brassinosteroids accumulation and other plant hormones profile, but also a correlation with altered photosynthesis, in agreement with our results. Similarly, auxins could be involved in the complex response to elicitors at the photosynthetic level, reinforcing the idea of a multilevel shaping of hormonal network [70].

ROS are involved in the early events that take place in response to a variety of elicitors [69]. Consistently, we observed differences in compounds involved in the response to

oxidative stress in both phosphite and *Trichoderma* treated plants, including the radical scavengers phenylpropanoids, glutathione intermediates and tocotrienols, epoxy and hydroxy derivatives of fatty acids, together with green leaf volatile compounds (i.e., hexanal) and detoxifying compounds as pentanal. In addition, the modulation of the citrulline and N-ω-hydroxy-L-arginine, both involved in NO production, might indicate the implication of this pathway in the general plant response to biostimulation. Despite several clues suggest their involvement, the role ROS worth future and dedicate investigation. Nonetheless, it can be considered that they represent a common response to biotic and abiotic stress, since they trigger signaling pathways cascades that regulate acclimatory and defense responses in plants [71] and that they act synergistically with NO [72]. Moreover, the modulation of lipid metabolism, which included phosphocholine and fatty acids, was also observed. Besides their role during plant-microbial communication, lipid signaling is associated to plant responses to adverse conditions [73].

Although no differences were observed regarding photosynthetic and morphological parameters following phosphite treatment, this product strongly elicited defense compounds in zucchini plants, also suggesting a plant protection effect. Our findings agree with studies describing the protective effect of potassium phosphite on potato plants against different pathogens [59]. Lobato et al. [60] observed and induction of the systemic defense response in potato tubers after applying potassium phosphite. These authors reported an increase in phytoalexin and chitinase contents, as well as an increase in peroxidase and polyphenol-oxidase activities after treating the leaves. Noteworthy, this elicitation of plant defense did not imply a detriment of the plant growth, in agreement with our results [60]. On the other hand, it has been suggested that phosphite might affect sugar metabolism, the shikimic acid pathway and could alter the hormonal level in agreement with our results [23].

In agreement with our results, changes in SA and JA have been previously described in plants inoculated with *Trichoderma*. Contreras-Cornejo et al. [74] indicate a tricky network of SA, JA and the indole derivative camalexin in plant, as a response elicited by *Trichoderma* in Arabidopsis that boosted plant immunity. These authors also pointed out that this microorganism triggers plant defense responses through ROS by promoting an accumulation of H_2O_2 in Arabidopsis roots-colonized. In fact, they found a correlation between defense gene expression, H_2O_2 induction, SA and JA accumulation, camalexin production, and reduced disease symptoms in Arabidopsis colonized by *Trichoderma*, suggesting a combined activation of these defense pathways. These authors suggest that biotic interactions capable of regulating multiple defense responses may increase the fitness of plants when challenged with pathogens [74].

Unexpectedly, a similar response was obtained by adding phosphite. However, the enhance of ROS leading to increase the plant defense has been postulated as one of roles of phosphites in plants. Moreover, several authors revealed that the elicitation of plant defense by potassium phosphite depend on the action of phytohormones such as SA, JA, auxins, and ethylene supporting our conclusion of intricate networks of response after elicitor addition [75]. In addition, these authors showed the differentia expression of miRNA targets genes related to pathogen resistance, transcription factors, and oxidative as a response to potassium phosphite in potato.

5. Conclusions

Biostimulants are a useful eco-friendly tool for growers oriented towards sustainable agriculture. Our results suggest that endophytic fungi inoculation leads to improved growth and physiological performance of zucchini squash plants. Specifically, plants inoculated with *Trichoderma harzianum*-T22 increased fresh and dry biomass production, root dry weight, phosphorus, and magnesium buildup, compared to untreated plants. Intriguingly, untargeted metabolomics confirms the potential of both microbial and non-microbial treatments as enhancers of plant defenses by eliciting secondary metabolism in zucchini. Interestingly, such elicitation of secondary metabolism did not involve a

detriment of plant growth, suggesting that this process represents a sustainable metabolic cost for plants.

Our results pointed out that the plant response to elicitors is based on a complex and broad biochemical modulation. However, the plant response showed similarity between the two treatments, suggesting that both *Trichoderma* and phosphite triggered early signaling related to oxidative stress. This signaling cascade appears to involve an intricate hormonal network following the similar trend but with a different intensity, which could explain the differences observed in morphological and physiological parameters.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11061205/s1, Table S1. Dataset from untargeted metabolomics of plants treated with *Trichoderma* and Phosphite. Compounds are presented with individual intensities and with composite mass spectra; Table S2. List of discriminant metabolites possessing the highest score (score > 0.04) in the PLS-DA model and subjected a fold-change analysis (FC > 2); these metabolites were uploaded into the PlantCyc pathway Tool software and used for interpretations.

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