Inoculation of *Rhizoglomus irregulare* or *Trichoderma atroviride* differentially modulates metabolite profiling of wheat root exudates

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ABSTRACT

Root exudation patterns are linked to, among other things, plant growth, plant-microbe interaction and the priming effect. In this work, two complementary metabolomic approaches (both liquid and gas chromatography coupled to mass spectrometry) were applied to investigate the modulation of root exudation imposed by two beneficial fungi (substrate treatment of *Trichoderma atroviride* AT10, substrate application of *Rhizoglomus irregulare* BEG72 and seed treatment with *T. atroviride* AT10) on wheat (*Triticum aestivum* L.). The inoculation with *R. irregulare* elicited significant increases (by 18%, 39% and 20%) in the shoot, root dry biomass and root-to-shoot ratio compared to untreated plants, whereas inoculation with *T. atroviride*, as a substrate drench or as a seed coating, exhibited intermediate values for these parameters. The metabolomic approach demonstrated a broad chemical diversity, with more than 2900 compounds annotated in the root exudates. Overall, the Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) supervised modelling highlighted a distinctive modulation of the metabolic profile in the root exudates as a function of both fungal inoculation and means of application. Most of the differences could be ascribed to lipids (sterols and membrane lipids), phenolic compounds and terpenoids, siderophores and chelating acids, derivatives of amino acids and phytohormones, and as such, the interaction between the wheat roots and beneficial fungi resulted in a complex response in terms of root exudates, likely involving a cascade of processes. Nonetheless, the changes imposed by plant-microbe interactions can contribute to the support of the biostimulant effects of both *T. atroviride* and *R. irregulare*.

1. Introduction

The increasing concern over global food security coupled with the projections for global population increase to more than 9.5 billion by 2050, together with climate change projections, pose major challenges for the agricultural industry and scientists in their efforts to maximize crop productivity (Searchinger, 2013). Wheat is one of the world's leading grain crops, contributing about 22% of the global food requirement and ranking second in production volume (750 million tons) after maize (1060 million tons); it is cultivated on 220 million hectares worldwide (FAOSTAT, 2016). However, further to the availability of arable land and adequate precipitation, intensive wheat production relies heavily on the application of nitrogen and phosphorus-based fertilizers (Den Herder et al., 2010). Improving resource efficiency

through sustainable agricultural practice is an urgent need for both the production of food for the increasing human population and to minimize the impact of agriculture on the environment and on human health and longevity (Searchinger, 2013).

The use of microbial (i.e., arbuscular mycorrhizal fungi [AMF], *Trichoderma* spp. and plant growth promoting rhizobacteria) and non-microbial based biostimulants (i.e., humic and fulvic acids, protein hydrolysate, seaweed extracts and silicon) could be considered to be one of the most promising approaches to securing yield stability under low-input conditions (i.e., N and/or P deficiency) (Colla and Rouphael, 2015; Colla et al., 2015a, 2017; Conversa et al., 2007, 2013; du Jardin, 2015). According to Rouphael et al. (2015) and López-Bucio et al. (2015) the endophytic fungi AMF and *Trichoderma* represent two important categories of microbial-based biostimulants. The

phytostimulation effect of endophytic fungi under both optimal and suboptimal conditions can be attributed to several direct and indirect mechanisms including: i) improved uptake and translocation of macro and micronutrients, ii) more vigorous root system apparatus (higher root biomass, surface area and number of lateral roots), iii) improved water relations and photosynthetic capacity, iv) stronger antoxidative defence system, v) regulation of plant hormones and vi) production of enzymes and/or excretion of metabolites in the rhizosphere (Calvo et al., 2014; Colla et al., 2015b; Fiorentino et al., 2018; Fiorilli et al., 2018; López-Bucio et al., 2015). However, the quantity and quality of the root exudates is particularly important for improving the solubilization and assimilation of recalcitrant or poorly available macro and micronutrients (P, Fe, Mn and Zn) thus boosting crop performance and yield (Badri and Vivanco, 2009; Rouphael et al., 2015; Van Dam and Bouwmeester, 2016).

Plants invest a proportion of their photosynthetically fixed carbon into maintaining and shaping the microbial community in the rhizosphere. The quantitative and qualitative variation in low (amino acids, sugars, organic acids and phenolics) and high molecular weight organic compounds (mucilage and proteins) depends on several interacting variables such as microbial biostimulant strains as well as inoculation/application methods (Colla et al., 2015b,c; Fiorentino et al., 2018; López-Bucio et al., 2015; Rouphael et al., 2010, 2015). In extensive herbaceous species, microbial-based biostimulants are applied onto the plant/soil surfaces or used as a seed coating (Colla et al., 2015b). Soil or plant surface application of inocula requires a high quantity of the product to compensate for the dilution effect, whereas seed coating has proved an efficient and cost-effective way to introduce endophytic fungi to the rhizosphere (Tavares et al., 2013). However, the effectiveness of a seed coating with beneficial microorganisms is strongly dependent on the concentration of propagules on the surface of the seed. This issue however could be easily addressed in the case of *Trichoderma* inoculants, where the number of propagules can reach up to 4×10^{10} CFU g^{-1}. Therefore, *Trichoderma* can be applied either with a solid carrier at planting placed directly into the soil close to the seeds or with a film coating on the seed surface.

Taking this background into consideration, it is important to unravel the physiological and molecular bases of the enhanced crop performance imparted by endophytic fungi, as a function of both inoculation and application method, in order to substantiate a rationale for their extensive application on agronomic crops. In this context, untargeted metabolomics have been suggested for elucidating the profile of root exudates and investigating plant-microbe interaction in the rhizosphere (Van Dam and Bouwmeester, 2016). These authors suggest that, when there is no prior knowledge of the comprehensive exudate profiles, a combination of metabolomic platforms (e.g., LC/MS and GC/MS) may be used. Such information can support a better understanding of the *chemical dialogue* involved in plant-microbe interactions and shed light onto both latent and overt interactions. Hence, the aim of this study was to assess shoot and root morphological changes and to comprehensively profile the root exudates elicited by endophytic fungi (*Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride* AT10) using different application methods (substrate application or seed treatment) on wheat crop.

2. Results

2.1. AM root colonization, Trichoderma concentration, growth responses and SPAD index

The percentage AM root colonization at the end of the experiment was significantly affected by the microbial inoculation treatment (Table 1). No AM fungi colonization was recorded in the roots of the control wheat plants or in the substrate or seed treatments with *T. atroviride*. However, in the plants supplied with *R. irregulare*, the percentage of AM was around 32% (Table 1). Moreover, the total number

of *Trichoderma* colonies recovered from the growing media and roots in the four treatments ranged between 1.1×10^{1} and 3.7×10^{4} CFU g^{-1}, with the highest values recorded in both the cases with substrate application and seed treatment with *T. atroviride* (Table 1).

Concerning the effects of microbial inoculation on growth responses, our results showed that inoculation with *R. irregulare* elicited significant increases (+18%, 39% and 20%) in the shoot, root dry biomass and root-to-shoot ratio compared to the untreated plants, whereas inoculation with *T. atroviride* via a substrate drench or as a seed treatment yielded intermediate values (Table 1). Similar to the effects on biomass production, the Soil Plant Analysis Development (SPAD) index in the microbial inoculation treatments was significantly higher (by 8% on average) than that obtained in the untreated wheat plants, irrespective of the microbial-biostimulant treatment (Table 1).

2.2. Profiling of root exudates by UHPLC/QTOF-MS

The wheat root exudates were analysed by UHPLC/QTOF-MS in order to understand how the metabolic profiling was influenced in each case of microbial inoculation treatment (untreated control, substrate application with *R. irregulare*, substrate application with *T. atroviride* and seed treatment with *T. atroviride*). The chemical diversity of the root exudates was wide, with more than 2900 compounds retained and used for statistics; the entire list of compounds is provided as Supplementary Material, together with individual composite MS spectra (mass and abundance combinations). Hierarchical clustering was formerly carried out, using the unsupervised multivariate approach, to elicit information on the relatedness of the treatments (Fig. 1). The clusters produced from the fold-change-based heatmap enabled the identification of four clusters, each of which included all replications within a treatment. Seed treatment of *T. atroviride* resulted to be the most distinctive result, followed by substrate application of *R. irregulare* and substrate application of *T. atroviride*.

OLPS-DA supervised modelling was applied, with the score plot showing a clear separation among the conditions under study (Fig. 2). The OPLS-DA regression parameters strongly supported the discrimination potential in the exudate profiles, with the goodness-of-fit R^2Y (correlation) and goodness-of-prediction Q^2Y (prediction ability) taking values of 0.99 and 0.82, respectively. Moreover, the CV-ANOVA coefficient (p < 0.001) was found to be satisfactory, the absence of outliers was confirmed by Hotelling's T2 test and overfitting could be excluded by permutation test. These outcomes supported the function of fungi associations in modulating wheat root exudates. Fig. 2 shows that the OPLS-DA score plot of the exudates derived from wheat root associated with substrate application with *R. irregulare* were completely separated from the exudates derived from substrate application with *T. atroviride* or seed treatment with *T. atroviride*.

Thereafter, Variable Importance in Projection (VIP) analysis was used to identify those compounds having the highest discrimination potential (VIP score > 1.3). This marker selection, combined with fold-change analysis, is provided in Table 2. This analysis highlighted nine compounds accumulated only in wheat root exudates associated with substrate application of *T. atroviride* [N-isopropylammelide; enterobactin (siderophore); juvenile hormone III and soyasapogenol B-3-*O*-beta-glucuronide (terpenoids); a glucosyloxyanthraquinone (quinone); salicyl-6-hydroxy-2-cyclohexene-on-oyl (aromatic compound); 5-[[4-methoxy-3-(phenylmethoxy)phenyl]methyl]-2,4-pyrimidinediamine; 4-sulfomuconolactone; N-alpha-methyl-L-histidine (amino acids)].

Three compounds were found to have accumulated in the exudates associated with substrate application with *R. irregulare* [(22R,23R)-28-homocastasterone (brassinosteroid); o-phenanthroline; (8Z,11Z,14Z)-3-oxo-icosatrienoyl-CoA (fatty acid)] while in both of the *Trichoderma* treatments contrasting regulations were highlighted. Despite the fact that the specific modulation of the root exudates was a function of the

Table 1
Effects of microbial inoculation with *Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride* on mycorrhiza root colonization and *Trichoderma* population in roots shoot dry weight and root dry weight, root to shoot ratio, leaf SPAD index of wheat plants at 60 days after sowing.

Treatment	Mycorrhiza root colonization (%)	Trichoderma spp. (CFU g^{-1})	Plant dry biomass (g plant^{-1})		Root to shoot	Leaf SPAD index
			Shoots	Roots		
Untreated control	0.0 b	1.1×10^{-1} b	17.2 c	1.8 c	0.10 c	41.5 b
Substrate application of *R. irregulare*	31.7 a	1.4×10^{-1} b	20.3 a	2.5 a	0.12 a	45.7 a
Substrate application with *T. atroviride*	0.0 b	3.7×10^{-4} a	19.0 b	2.2 b	0.11 b	44.8 a
Seed treatment with *T. atroviride*	0.0 b	5.0×10^{-3} a	18.6bc	2.1 b	0.11 b	43.9 a
Significance	***	**	**	*	*	*

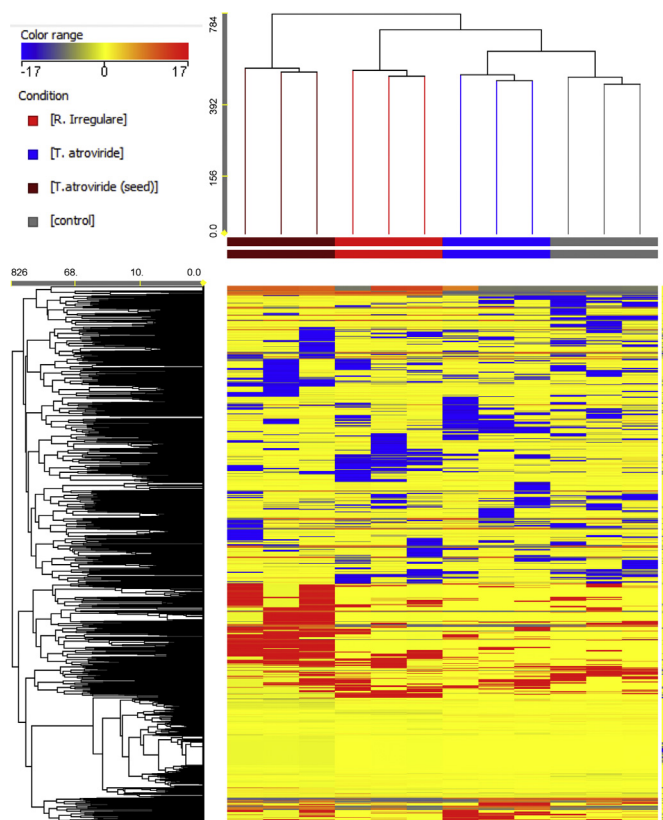*, **, ***, significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

Fig. 1. Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward) carried out from root exudate chemical profile following microbial inoculation with *Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride* AT10.

combination of the microbial association and the mode of application, it can be noted that metabolites could be grouped into a relatively small number of classes (Table 2). Phenolics and terpenoids were among the differential secondary metabolites most frequently identified, together with amino acid derivatives, phytohormones, sterols and membrane lipids. Notably, two carbohydrates, phosphate and a number of compounds known as siderophores or chelating agents (mainly muconic acid derivatives and acids related to the Krebs cycle), were also selected as discriminants.

2.3. Profiling of root exudates by GC/MS

Gas chromatography coupled with mass spectrometry and the post-acquisition filters described below retained overall more than 100 metabolites in the root exudates. Statistical analysis was then performed as described for the UHPLC/QTOF-MS. This further analysis confirmed that different microbial associations and different application methods resulted in a distinctive modulation of the wheat root

exudation patterns (Fig. 3). The correlation and prediction ability, R^2Y and Q^2Y, were 0.94 and 0.54, respectively, the validation parameters were adequate (CV-ANOVA, $p < 0.001$) and overfitting could be excluded. The following VIP score analysis allowed selection of the discriminant compounds (Table 3).

Although this analysis is expected to target mainly compounds from the primary metabolism, it is interesting to note that a few compounds discriminated the treatments, all of which were secondary metabolites. Among others, phenolics (catechol and 4-vinyl phenol), glycolic acid (i.e., hydroxyacetic acid), carbamic acid ethyl ester, as well as the aliphatic compounds heptacosane and hexadecane, were found to be modulated by fungal associations.

2.4. ChemRICH set enrichment statistical analysis

To date, there has been a lack of knowledge concerning the interpretation of metabolic changes and deeper analyses are needed to simplify the large list of metabolites found as a function of the treatment (Barupal and Fiehn, 2017). In this study, due to the difficulty of linking the findings obtained using the metabolomic approach and physiological explanation, a chemical enrichment analysis was carried out to better understand the complexity of the metabolite signatures.

Regarding *R. irregulare*, a ChemRICH set enrichment statistics plot identified 6 chemical clusters (see Supplementary Material), with phenols and isoflavones being the compounds that increased most in inoculated plants. Conversely, coumaric acids and lysophospholipids were the most repressed compounds in the treated plants. The *T. atroviride* treated plants presented an accumulation of anthraquinones and isoflavones, while the treated seeds showed an increase mainly of phenols and isoflavones. Coumaric acids were repressed in all of the treatments, although in different proportions.

3. Discussion

Over the past two decades, the application of microbial-biostimulants, and in particular endophytic fungi (mycorrhiza and *Trichoderma*), in agricultural cropping systems has been increasing, driven by the interest of farmers, breeding companies and researchers in boosting the crop productivity of important extensive crops such as wheat in a sustainable way (Colla et al., 2015b; Lorito and Woo, 2015). A biostimulant effect was observed in the current experiment with wheat, demonstrating that inoculation with endophytic fungi significantly improves the biomass production of wheat compared to untreated plants. In particular, substrate application of *R. irregulare* had the most positive effect on shoot and root dry biomass (Table 1). A presumed mechanism for the biostimulant activity of endophytic fungi in wheat performance is stimulation/modulation of the below-ground root system (i.e., root biomass), which may improve nutrient uptake and translocation, leading to higher biomass production (Colla et al., 2015b,c; Fiorentino et al., 2018; López-Bucio et al., 2015; Rouphael et al., 2015). Another putative mode of action responsible for the beneficial effect of microbial biostimulants could be related to the
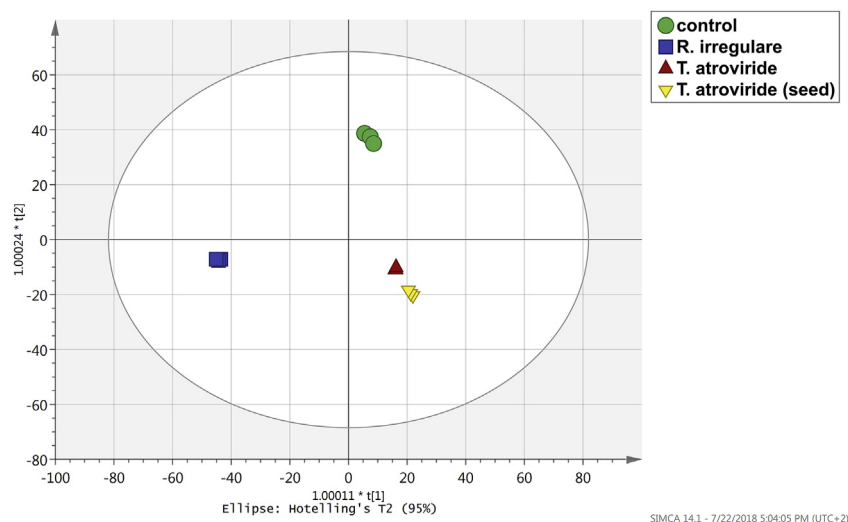
Fig. 2. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot on root exudate profiles gained through UHPLC-ESI/QTOF-MS analysis following microbial inoculation with *Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride* AT10.

better functioning of the photosynthetic apparatus and pigment biosynthesis. Such indirect effects agree with the SPAD values, that may trigger the translocation of photosynthates and thus enhance wheat performance (Colla et al., 2015b and references cited therein; Rouphael et al., 2015).

The root exudate pattern plays an important role in establishing and maintaining plant-microbe interaction in the rhizosphere, since plant-secreted chemical compounds can act as signalling metabolites that mediate either positive or negative interactions, thus shaping root colonization (Bais et al., 2006; Van Dam and Bouwmeester, 2016). The recent advances in metabolomics and multivariate analysis contribute to a wider profiling of exudates, thus enabling a deeper understanding of the below-ground interactions (Haichar et al., 2014). In this regard, the combination of the liquid and gas chromatography platforms has been proposed in order to gain a holistic picture of the chemical profiles in exudates (Van Dam and Bouwmeester, 2016). Nonetheless, the changes in the root exudates following the plant-microbe interactions are reported to affect root branching and root architecture (Haichar et al., 2014). Soil structure at the rhizosphere level is also affected by rhizodeposition: plant-soil feedback is defined as a process whereby plants modify the biotic and abiotic factors of soils, subsequently modulating plant performance (Tsunoda and van Dam, 2017). The understanding of root exudation processes is therefore a pivotal and exciting area of research in the field of plant-soil-microbe interactions.

Although the full characterization and comprehension of the metabolite profile in the exudates is probably still not possible, a great diversity of compounds could be annotated through metabolomics. Our untargeted analyses revealed a distinctive and specific wheat root exudation pattern as a function of both fungal interaction and the means of application (Fig. 1). Considering that plants actively control exudation profiles (Tsunoda and van Dam, 2017), both microbial species/strains and the application method should be carefully considered when focusing on the effects of biostimulants (Figs. 2 and 3).

In turn, the differences in the fungal elicitation processes suggest that discriminant compounds might provide very useful insights into the biostimulant effect at the molecular level. It is likely that the differences in the exudation patterns actively contributed to the phenotype differences we observed (Table 1).

Lipids were the most represented chemical class in our findings (Table 2). An increase in ergosterol and cholesterol lipids, as well as impairment of other membrane lipids, was demonstrated under both fungal associations, underpinning a complex lipid exudation pattern (that also includes hydroxy-derivatives) by the roots (Strehmel et al.,

2014). The involvement of ergosterol could be expected when taking into account that this sterol is of fungal origin. In general, Rasmann and Turlings (2016a,b) reviewed the root exudation profiles of both lipids and terpenes, among others. Our results also found several differential terpenoids, including a phytoalexin (kauralexin A2) and the juvenile hormone III, both of which play a role in plant defence mechanisms.

Even though a number of differences in the extent and direction of the accumulations were observed with the treatments, phenolics in the root exudates were modulated by microbial interaction, in agreement with the previous literature on plant growth promoting rhizobacteria (Smith et al., 2015; Vacheron et al., 2013) and fungi (Zhang et al., 2012). A clear trend of accumulation could not be observed for phenolics, although several authors have observed that different flavonoids exhibit a specific accumulation in root exudates depending on the fungal genus and species (Scervino et al., 2005; Steinkellner et al., 2007). Phenolics in the rhizosphere are reported to have a wide range of activity, acting as chemo-attractants, nod gene inducers for rhizobia, inhibitors of nitrification, modulators of enzymes and cell receptors, as well as being involved in pathogen and allelopathic interactions (Cesco et al., 2012). On this basis, phenolics might affect the root structure and plant development, and during symbiosis, they can play a role in the selection of compatible rhizobia from plants (Hassan and Mathesius, 2012). Phenolics are also reported to be metal chelators, similar to the two metabolites that we classified as siderophores (enterobactin and *O*-citryl-ethanolamine), both of which accumulated in root exudates following fungal symbiosis. Both siderophores are secreted by rhizospheric microorganisms and could be used for metal uptake by the roots from the soil (González-Guerrero et al., 2016) in plants such as wheat that use strategy II for iron uptake under limiting conditions (Verbon et al., 2017). Furthermore, two sulpho derivatives of muconic acid (a hexa-dienoic acid) were identified, thus strengthening the involvement of chelating agents. Looking at discriminant compounds from GC/MS (Table 3), glycolic acid (having a potential functional role as an acidifier of the soil) and L-allo-threonine (the stereoisomer of the amino acid threonine) might also have a role as a chelator of poorly soluble mineral nutrients (Haichar et al., 2014).

These findings suggest that microbial biostimulants are able to act as elicitors of root exudates, increasing the production of several compounds related to the secondary metabolism. According to previous studies, plant roots exude several compounds which are not only associated with plant development, but are also able to mediate interactions with other organisms in the rhizosphere.

A reduction in sugar phosphates (i.e., a hexose and a ribulose

Table 2

Discriminant metabolites in wheat root exudates through UHPLC-ESI/QTOF-MS analysis. Following microbial inoculation with *Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride*. Compounds were selected through OPLS-DA supervised multivariate statistics followed by VIP analysis. and thereafter subjected to fold-change analysis (each treatment *vs* control).

Compound		OPLS-DA VIP analysis		Substrate application of *R. irregulare*		Substrate application of *T. atroviride*		Seed treatment with *T. atroviride*	
		Score	Std error	Log fold-change	Regulation	Log fold-change	Regulation	Log fold-change	Regulation
Phenolics	phaseoluteone	1.44	0.37	−15.87	down	−15.87	down	−15.87	down
	medicarpin-3-*O*-glucoside-6″-malonate	1.43	0.36	−5.69	down	−20.54	down	−4.94	down
	3,6,7,2′,4′-pentamethylquercetagetin	1.41	0.38	−0.25	down	14.14	up	15.01	up
	7-hydroxy-4′5′-dimethoxyisoflavone/ afrormosin/apigenin-7,4′-dimethyl ether	1.36	0.59	5.61	up	10.90	up	12.14	up
	esculin	1.38	0.69	0.37	up	0.50	up	1.93	up
	robustaquinone H	1.36	0.41	2.03	up	0.92	up	2.50	up
	coniferyl acetate	1.35	0.42	−1.98	down	−1.33	down	−0.65	down
Terpenoids	tetraprenyl-beta-curcumene	1.52	0.56	0.87	up	1.66	up	1.27	up
	soyasapogenol -3-*O*-beta-glucuronide	1.39	0.58	−5.36	down	−5.95	down	10.95	up
	juvenile hormone III	1.37	0.52	−2.55	down	−0.72	down	8.60	up
	3-hydroxylubimin	1.37	0.79	8.32	up	8.00	up	8.88	up
	kauralexin A2	1.34	0.35	−2.71	down	−9.06	down	−2.03	down
	phytyl monophosphate	1.34	0.44	−2.11	down	−0.70	down	−7.05	down
	Eudesmol/bisabolol	1.33	0.87	2.27	up	0.78	up	2.95	up
	(20S)-ginsenoside Rh2	1.53	0.50	−1.44	down	−1.85	down	−7.70	down
Hormones	dihydrozeatin-*O*-glucoside	1.50	0.54	15.45	up	0.00	down	17.60	up
	(22R,23R)-28-homocastasterone	1.40	0.51	0.75	up	−7.25	down	−0.06	down
	salicyl-6-hydroxy-2-cyclohexene-on-oyl	1.49	0.63	−0.97	down	−0.85	down	1.60	up
	indole-3-acetyl-beta-6-D-glucose	1.37	0.35	−0.86	down	−17.72	down	−17.72	down
Lipids	3-oxo-icosatrienoyl-CoA	1.36	0.49	0.79	up	−17.46	down	−16.69	down
	4-alpha-carboxy-ergosta-7,24(241)-dien-3beta-ol	1.57	0.58	0.39	up	0.20	up	1.85	up
	3-beta-hydroxy-4-beta-methyl-5alpha-cholest-7-ene-4alpha-carboxylate	1.55	0.50	0.20	up	0.27	up	1.61	up
	1-18:1-2-16:0-phosphatidylglycerol	1.49	0.38	−8.49	down	−1.49	down	−19.93	down
	sphingosine 1-phosphate	1.47	0.37	0.04	up	0.13	up	−6.69	down
	3-geranyl-4-hydroxybenzoate	1.46	0.50	−3.40	down	−1.85	down	−2.66	down
	1-16:0-2-lysophosphatidylcholine	1.37	0.64	−0.25	down	16.29	up	17.35	up
	4-alpha-formyl-4beta-methyl-5alpha-cholesta-8,24-dien-3beta-ol	1.35	0.31	17.05	up	15.76	up	16.41	up
	(13E)-11-alpha-hydroxy-9,15-dioxoprost-13-enoate/(5Z)-(15S)-11-alpha-hydroxy-9,15-dioxoprostanoate	1.41	0.39	−20.12	down	−2.10	down	−1.87	down
	alpha-tocotrienol	1.46	0.60	−12.47	down	6.32	up	7.73	up
	3-mercapto-1,2-propanediol	1.43	0.42	−16.50	down	−17.09	down	−16.31	down
Siderophores/ chelators	enterobactin	1.35	0.45	−1.72	down	−0.48	down	0.35	up
	3-sulfomuconate	1.41	0.66	0.05	up	−2.15	down	1.75	up
	4-sulfomuconolactone	1.37	0.57	−0.03	down	−16.07	down	1.61	up
	cis-aconitate	1.41	0.40	−17.07	down	−1.77	down	−17.05	down
	O-citryl-ethanolamine	1.34	0.60	5.11	up	−0.84	down	16.35	up
Amino acids	N-alpha-methyl-L-histidine	1.51	0.60	−0.25	down	−0.84	down	19.25	up
	N-alpha,N-alpha-dimethyl-L-histidine	1.35	0.43	−1.83	down	−1.20	down	−0.54	down
	N-acetyl-L-aspartate	1.40	0.47	−11.02	down	−16.43	down	−15.63	down
	S-ribosyl-L-homocysteine	1.38	0.37	−11.96	down	−7.50	down	−17.32	down
	N-hydroxytrihomomethionine	1.33	0.34	−18.41	down	−1.12	down	−1.49	down
Other nitrogen compounds	deoxypumiloside	1.46	0.56	10.88	up	−6.23	down	12.57	up
	7-methylxanthine	1.42	0.37	−1.61	down	−16.78	down	−16.78	down
	2-descarboxy-betanidin	1.38	0.23	−0.25	down	14.16	up	14.68	up
	miraxanthin V	1.34	0.58	−14.16	down	−9.12	down	−2.19	down
	(S)-reticuline/capsiconiate	1.33	0.60	0.08	up	6.27	up	7.11	up
	1,3,7-trimethyl-5-hydroxisourate	1.39	0.63	12.53	up	11.89	up	13.02	up
	cinnamoyltyramine	1.40	0.53	−14.17	down	−2.16	down	−8.29	down
Carbohydrates	hexose 1-phosphate	1.47	0.50	−0.05	down	−0.53	down	−10.34	down
	ribulose 5-phosphate	1.34	0.67	−11.40	down	−7.01	down	−16.25	down

Table 2 (*continued*)

| Compound		OPLS-DA VIP analysis		Substrate application of *R. irregulare*		Substrate application of *T. atroviride*		Seed treatment with *T. atroviride*	
		Score	Std error	Log fold-change	Regulation	Log fold-change	Regulation	Log fold-change	Regulation
Others	S-adenosyl-L-methionine	1.51	0.61	−0.09	down	4.87	up	8.13	up
	o-phenanthroline	1.38	0.70	0.38	up	−1.49	down	−7.45	down
	phloroglucinol	1.36	0.35	17.70	up	17.43	up	17.78	up
	a glucosyloxyanthraquinone	1.49	0.68	−0.25	down	−0.84	down	15.01	up
	pyrroloquinoline quinone	1.39	0.57	−0.25	down	9.69	up	15.97	up
	3.4-dihydroxymandelonitrile beta-D-glucoside	1.40	0.53	−9.22	down	−14.85	down	−3.94	down
	guaiacylglycerol-beta-guaiacyl ether	1.43	0.43	−15.93	down	−15.93	down	−15.93	down
	dihydroisopteroate	1.35	1.01	0.74	up	0.38	up	1.65	up
	5-[[4-methoxy-3-(phenylmethoxy)phenyl]methyl]-2,4-pyrimidinediamine	1.51	0.63	−5.82	down	−0.35	down	2.52	up
	1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one	1.41	0.80	−0.75	down	−0.93	down	−12.70	down
	N-hydroxy DAP	1.35	0.42	−1.80	down	−1.15	down	−0.56	down
	N-(3-acetamidopropyl)-4-aminobutanal	1.34	0.44	−12.64	down	−19.03	down	−8.04	down
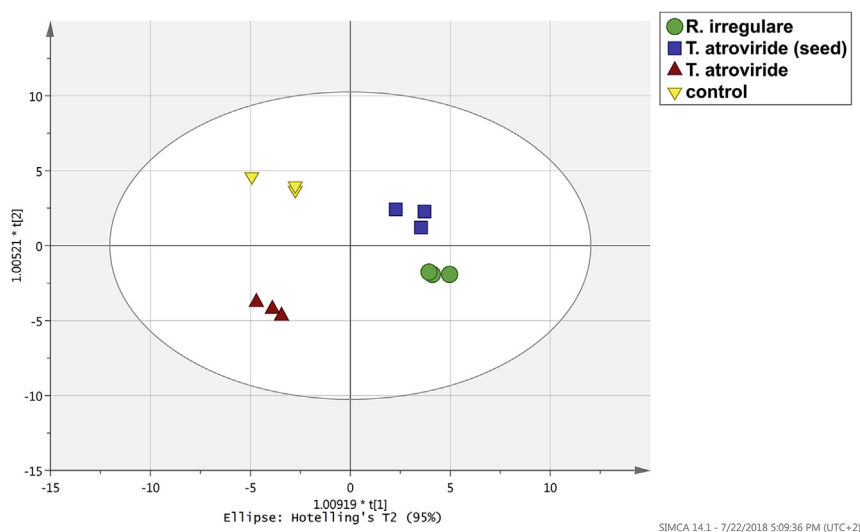
Fig. 3. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot on root exudate profiles gained through GC/MS analysis following microbial inoculation with *Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride* AT10.

Table 3
Discriminant metabolites in wheat root exudates through GC/MS analysis, following microbial inoculation with *Rhizoglomus irregulare* BEG72 or *Trichoderma atroviride*. Compounds were selected through OPLS-DA supervised multivariate statistics and thereafter subjected to fold-change analysis (each treatment *vs* control).

Compound	Substrate application of *R. irregulare*		Substrate application of *T. atroviride*		Seed treatment with *T. atroviride*	
	Log fold-change	Regulation	Log fold-change	Regulation	Log fold-change	Regulation
2-ethyltoluene	19.77	up	17.33	up	0.10	down
4-vinylphenol	2.34	up	3.85	up	3.44	up
Carbamic acid ethyl ester	−6.34	down	1.46	up	−1.78	down
Catechol	6.87	up	8.66	up	−10.31	down
Glycolic acid	1.95	up	1.95	up	1.21	up
Heptacosane	16.75	up	0.00	down	16.08	up
Hexadecene	1.03	up	−16.64	down	0.58	up
L-allo-threonine	1.14	up	3.47	up	3.47	up

derivative) was consistently observed across all treatments when compared to the control. Considering that these metabolites can be related to a nutritional imbalance (Tawaraya et al., 2014), their decrease might be related to the improved efficiency of nutrient use associated with fungal infection.

Verbon et al. (2017) reviewed the importance of iron uptake related to beneficial microorganisms also in the context of induced systemic resistance (ISR), albeit that the molecular mechanism remains unknown. The involvement of a salicyl-derivative (salicyl-6-hydroxy-2-cyclohexene-on-oyl), and the presence of hydroxy-derivatives of fatty acids (i.e., oxylipins), could be related to the actual recruitment of ISR in the response to fungal infection. It can be also speculated that the increase in S-adenosyl-L-methionine, involved in gibberellin inactivation and ethylene biosynthesis, further confirms the triggering of ISR

(Pretali et al., 2016; Verbon et al., 2017).

Looking at the phytohormone profiles (Table 2), the cytokine dihydrozeatin-*O*-glucoside and the conjugated auxin indole-3-acetyl-beta-6-D-glucose were identified among the discriminants. To some extent, the changes in these hormones might justify the stimulation of root development and the changes in the root-to-shoot ratio. Cytokines and ethylene can yield a negative feedback in fungal root penetration during the early stages, but their phytohormone homeostasis has positive effects in the later stages by regulating arbuscule development. The brassinosteroid 28-homocastasterone might also have been involved in the modulation of fungal colonization (Pozo et al., 2015).

More generally, the cluster plot based on chemical similarity, which summarizes the metabolites found in this study, demonstrates that the phenylpropanoid pathway has an important role in the response to fungal inoculation in all cases. It is known that flavonoids are commonly present in a high proportion in root exudates, and that phenylpropanoids are involved in plant response to biotic stimulus. In fact, it has been observed that the derivatives of flavonoids present antimicrobial properties in plants (Baetz and Martinoia, 2014), with recent literature highlighting the major roles that flavonoids play in legume and root nodule symbiosis (Gifford et al., 2018). In our experiments, phenolic compounds may be a mere consequence of plant-microorganism interaction rather than being involved in the establishment or maintenance of a mutual interaction. Further dedicated research is needed to better elucidate their role in root exudates.

Accumulation of coumaric acid was reduced in all of the inoculated plants, suggesting a modification of the carbon flux in the phenylpropanoid pathway, possibly due to the fungal inoculation. The alteration of this carbon flux might have led to the increase in phenolic compounds downstream. In fact, Gifford et al. (2018) postulated a host- and symbiont-specific accumulation of flavonoids.

Overall, a complex and distinctive shaping of the root exudate metabolic profile was achieved in wheat following fungal inoculation. Our results contribute to a better understanding of the complex metabolic changes related to the interactions between plants and beneficial microorganisms; however, such a complex biochemical interplay requires further investigation by considering other factors that might affect the mutual interaction. It is reported that the plant uses dedicated pattern recognition receptors (PRRs) to recognize its associated rhizomicrobiome (Venturi and Keel, 2016). Chemical signalling at the rhizosphere level presumably uses yet unknown root receptors to detect microorganisms, and root exudates might play a pivotal role in recruiting and maintaining root-associated microorganisms. It is also known that infection and development of symbiosis are independent processes and that the fungus physiology also changes during such processes (Scervino et al., 2005). However, this chemical signalling between plant and beneficial fungi is most probably continuous to establish a long-lasting colonization. From the fungus side, this dialogue is apparently not very specific, considering that there is no plant host specificity. This suggests that either the plant signals are conserved throughout the plant kingdom or that a broad range of signalling compounds are involved (Venturi and Keel, 2016). Furthermore, additional factors, such as plant genotypic traits (related to species and/or cultivar), plant developmental stage and nutrient availability, rather than the actual rhizomicrobiome, have an impact on the composition of root exudates (Huang et al., 2014; Pieterse et al., 2016; Rivero et al., 2018; Scervino et al., 2005; Venturi and Keel, 2016).

Nonetheless, the use of quartziferous sand as a growth substrate was effective in supporting fungal colonization in the long term (60 days), representing a simplified model in which exuded compounds are more easily available. The application of untargeted metabolomics followed by orthogonal signal corrected discriminant analysis (OPLS-DA) highlighted a wide diversity of chemical compounds in root exudates and represents a powerful tool that may be used to better understand the exudation processes.

Finally, regardless the specific responses highlighted by

metabolomics, it is important to consider that the plant-symbiont interactions studied also involve a tight association between fungi and a vast community of sporosphere bacteria (Battini et al., 2016). Although microbial communities were not studied in the present work, their direct or indirect contribution to exudation patterns cannot be excluded. Indeed, such microbial communities are reported to possess diverse physiological characteristics and to affect the performance of AMF isolates (Agnolucci et al., 2015). Additionally, some of the compounds identified (e.g., siderophores) might also be derived from bacteria (Andrade et al., 1997; Battini et al., 2016).

4. Conclusions

Two complementary metabolomic approaches were employed to investigate the changes in root exudation patterns imposed by substrate treatment of *T. atroviride*, substrate application of *R. irregulare* and seed treatment with *T. atroviride*.

Overall, the root exudate metabolic profile was diverse and was distinctively modulated in wheat following fungal inoculation. Despite the fact that the processes underlying the changes related to these below-ground chemical cues are yet not fully elucidated, our results clearly demonstrate that both different microbial inoculations and different means of application can modulate the exudate profile. To the best of our knowledge, no information has previously been available in respect of this latter point.

Research is still necessary to depict the changes in exudation patterns during the colonization process (i.e., versus time); nevertheless, our findings contribute to the elucidation of such complex processes, and they support several biostimulant effects, mainly in terms of nutrient uptake efficiency and the regulation of plant growth. Therefore, we can postulate that the modulation of root exudate composition is among the processes underlying the promotion of plant growth induced by microbial biostimulants. From a practical point of view, our results might be of interest in the context of developing/implementing novel strategies aimed at addressing crop tolerance to abiotic/biotic stresses rather than improving the efficiency of nutrient use. In fact, given the variety of plant processes related to either biostimulants or root exudation, sustainable strategies for crop production could be supported by our findings. Potentially, several other interesting aspects related to the priming effect and the plant immune response could be explored, but these fall outside of the scope of this work and deserve dedicated future experiments.

5. Experimental

5.1. Greenhouse conditions, plant material and experimental design

The trial was conducted during the 2016 growing season in a polyethylene greenhouse located at the Experimental Farm of Tuscia University, central Italy (latitude 42°25′N, longitude 12°08′E, altitude 310 m). Inside the greenhouse, ventilation was provided automatically when the air temperature exceeded 26 °C, and the only light was natural solar radiation. The mean air temperature and relative humidity inside the greenhouse were 20 °C and 59%, respectively. The crop selected for the current greenhouse experiment was wheat (*Triticum aestivum* L.) cv. 'Chinese Spring'. Seeds of wheat were surface sterilized with a solution containing 80% ethanol. After sterilization (10 min), the seeds were washed twice with sterile distilled water. On May 4, wheat seeds were sown in plastic pots (diameter of 18 cm) filled with 4.0 L of quarziferous sand at a rate of four seeds per pot. The experiment included the following four treatments: 1) 80 spores of *Rhizoglomus irregulare* BEG72 (Aegis produced by Atens, Agrotecnologías Naturales, S.L., Tarragona, Spain) per pot mixed on the substrate surface (at a depth of 3 cm) just before sowing; 2) 5×10^7 CFU of *Trichoderma atroviride* AT10 (= 0.05 g of a biological seed inoculant 'Condor' produced by Atens, Agrotecnologías Naturales, S.L., Tarragona, Spain) per

pot mixed on the substrate surface (at a depth of 3 cm) just before sowing; 3) seed treatment with 4×10^{10} CFU of *Trichoderma atroviride* AT10 (= 20 g of a biological seed inoculant 'Covenant Trichoderma' produced by Atens, Agrotecnologías Naturales, S.L., Tarragona, Spain) per 1 kg of wheat seeds; 4) untreated control. Seed treatment was performed with a seed-treatment machine capable of automatically spraying the seed surface with a water suspension containing the biological seed inoculant at a rate of 10 mL per kg of seed. Treatments were arranged in a randomized block design with three replicates. Each experimental unit consisted of six pots. There were 12 independent closed systems (4 treatments × 3 replicates) composed of 6 pots each. Pots were placed in a trough with a 1% slope. In each trough (experimental unit) an independent tank was provided to supply the plants with nutrient solution through a drip irrigation system. After application, each solution was returned to its tank for later recirculation (closed system). In addition to the required pots (18 pots per treatment), ten extra pots per treatment were also prepared. One week after the initial emergence of the seedlings, pots having less than 4 plants per pot were discarded.

Plants were fertigated starting one week after sowing. The basic nutrient solution used was a modified Hoagland and Arnon formulation having the following mineral composition: 7.0 mM N–NO_3^-, 1.5 mM S, 0.2 mM P, 2.7 mM K, 5.5 mM Ca, 1.5 mM Mg, 20 μM Fe, 9 μM Mn, 0.3 μM Cu, 1.6 μM Zn, 20 μM B and 0.3 μM Mo. The nutrient solution was prepared using de-mineralized water and its electrical conductivity (EC) and pH were 1.8 dS m^{-1} and 6.0, respectively.

5.2. Biomass production, SPAD index, arbuscular mycorrhiza root colonization and quantification of Trichoderma

At the end of the experiment (60 days after sowing, June 29) the Soil Plant Analysis Development (SPAD) index was measured on fully expanded functional leaves (the third from the apex) by means of a portable chlorophyll meter SPAD-502 (SPAD-502, Minolta corporation, Ltd., Osaka, Japan). Eight healthy leaves were randomly measured and averaged to a single SPAD value for each experimental plot. On the same date, shoots and roots were harvested. Roots were rinsed from vermiculite, and subsamples were saved for assessment of arbuscular mycorrhiza-fungi root colonization. All plant tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination.

In order to determine the root colonization by arbuscular mycorrhizal fungi (AMF), root samples were cleared with 10% KOH, stained with 0.05% trypan blue in lactophenol, as described by Phillips and Hayman (1970), and microscopically examined for AMF colonization by determining the percentage of root segments containing arbuscules + vesicles using a gridline intercept method (Giovannetti and Mosse, 1980).

Molecular detection and quantification of *Trichoderma* from 100 g of root/substrate mixture per plot were carried out at the end of the experiment using Real Time PCR (polymerase chain reaction). The methodology used was described previously by Colla et al. (2015b).

5.3. Collection of root exudates

The collection of water-soluble exudates was carried out at the end of the trial (60 days after sowing) through a leaching procedure (Begum et al., 2016). In each closed system, elution of exudates was performed by continuous irrigation with sterilized, deionized water for 6 h starting from 9:00 am. After 6 h, 1 litre of drainage solution per plot containing root exudates was collected from each tank, filtered and frozen at −20 °C until metabolomic analysis.

5.4. Metabolomic analysis by UHPLC/QTOF-MS

Freeze dried root exudates (50 mL) were suspended in 5 mL of 0.1% HCOOH in 80% methanol, filtered through a 0.22 μm cellulose membrane and transferred to an amber vial for analysis. The screening of

plant metabolites was carried out using a UHPLC chromatographic system coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS). Specifically, a 1290 series liquid chromatography system, equipped with a binary pump, degasser and a JetStream Electrospray ionization system, was interfaced to a G6550 iFunnel mass spectrometer (all from Agilent technologies Santa Clara, CA, USA). The instrument was operated as previously reported (Kumar et al., 2015). Briefly, the QTOF was run in SCAN mode (positive polarity, 100–1200 m/z range) and extended dynamic range mode. Chromatographic separation was achieved in reverse phase, using water and methanol as the mobile phases, on an Agilent Zorbax Eclipse-plus column (75 × 2.1 mm i.d., 1.8 μm). The elution gradient started from 5% to 90% methanol within 35 min, injection volume was 3.5 μL and flow rate was 220 μL min^{-1} (Lucini et al., 2016). Deconvolution, mass and retention time alignment, as well as filtering (mass accuracy < 5 ppm, single ion ID disabled) were carried out using the software Profinder B.06 (from Agilent Technologies). Post-acquisition processing was performed according to Rouphael et al. (2016): compound annotation was based on accurate mass, isotope spacing and isotope ratio, against a custom database produced by combining compounds exported from PlantCyc 9.5 (Plant Metabolic Network, http://www.plantcyc.org; accessed April 2017), Phenol-Explorer 3.6 (http://www.phenol-explorer. eu; accessed April 2017), as well as additional compounds that might be present in root exudates. Finally, compounds were filtered by frequency: those compounds that were not present in 100% of replications within at least one treatment were discarded.

5.5. Metabolomic analysis by GC/MS

A complementary approach, based on gas chromatography mass spectrometry (GC/MS), based on previous literature (Lisec et al., 2006) with minor changes, was also applied. An aliquot (200 μL) of root exudate was transferred to microtubes containing 5 μL of myristic-d$_{27}$ acid (Sigma- Aldrich, St. Louis, MO, USA). The mixture was dried overnight in a speed vacuum concentrator. Then, 60 μL of 2% methoxyamine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) in pyridine was added to the dried extracts. Samples were incubated and mixed in a ThermoMixer (Eppendorf; Hamburg, Germany) at 400 rpm for 3 h 30′ at 37 °C. Thereafter, they were derivatized with 90 μL of *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Sigma-Aldrich) incubated and mixed in the ThermoMixer (at 400 rpm for one hour at 55 °C). Finally, the samples were transferred into vials to be analysed.

GC/MS analysis was performed using an Agilent 6890 gas chromatograph equipped with a 30 m DB-5MS capillary column and coupled to an Agilent 5977 quadrupole mass spectrometer. Derivatized extracts (1 μl) were injected in splitless mode (250 °C), using helium as the carrier gas (1 ml/min) and electron impact for ionization purposes. A GC oven temperature program was adopted, starting from 100 °C (maintained for 2 min) up to 325 °C at 10 °C min^{-1}. A fatty acid methyl ester mixture was used for retention time locking purposes (FAME mix, Agilent Technologies). Features were deconvoluted using the software 'Unknown Analysis' (Agilent Technologies) and identification was based on spectral comparison against the commercially available database known as the 'Fiehn Library' (Agilent Technologies, released May 2016).

5.6. Statistics

Analysis of variance (one way-ANOVA) of the experimental data (plant biomass and partitioning, root biomass, root-to-shoot ratio and SPAD index) was performed using the SPSS software package (IBM SPSS Statistics version 20.0.0). To separate treatment means within each measured parameter, Duncan's multiple range test was performed at $p \leq 0.05$.

Metabolomics data were initially interpreted in Agilent Mass

Profiler Professional B.12.06 (from Agilent Technologies). Post-acquisition processing included compound filtering by abundance (area > 10000 counts), normalization at the 75th percentile and baselining to the median of the control. Unsupervised hierarchical cluster analysis was then carried out on the basis of fold-change-based heatmaps, setting the similarity measure as 'Euclidean' and using 'Wards' as the linkage rule. Thereafter, the dataset was exported into SIMCA 13 (Umetrics, Malmo, Sweden), UV-scaled and elaborated for Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modelling. The use of class membership in the supervised OPLS-DA statistics was chosen to effectively separate, in the score plot hyperspace, the Y-predictive from the uncorrelated (i.e., ascribable to technical and biological variability) variation in X. The model was validated through cross-validation CV-ANOVA ($p < 0.01$) whereas overfitting was excluded by permutation testing (n = 100). Model parameters (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y) were also produced. Outliers were finally investigated using Hotelling's T2 and the distance from the origin in the OPLS-DA model (95% and 99% confidence limits for suspect and strong outliers, respectively).

Starting from OPLS-DA modelling, Variable Importance in Projection (VIP analysis) was used to identify those metabolites having the highest discrimination potential (VIP score > 1.3). These latter underwent a fold-change analysis to determine the direction and extent of accumulation across treatments.

Chemical Similarity Enrichment Analysis (ChemRICH) was performed, as described by Barupal and Fiehn (2017), based on chemical similarities. Tanimoto substructure chemical similarity coefficients were determined to cluster metabolites into non-overlapping chemical groups. Statistical significance p-values were obtained by self-contained Kolmogorov–Smirnov tests. The online web-app tool (http://chemrich.fiehnlab.ucdavis.edu) was used for this analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2018.10.033.

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