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Research article

Metabolomic responses triggered by arbuscular mycorrhiza enhance tolerance to water stress in wheat cultivars

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ABSTRACT

Under global climate change forecasts, the pressure of environmental stressors (and in particular drought) on crop productivity is expected to rise and challenge further global food security. The application of beneficial microorganisms may represent an environment friendly tool to secure improved crop performance and yield stability. Accordingly, this current study aimed at elucidating the metabolomic responses triggered by mycorrhizal (*Funneliformis mosseae*) inoculation of durum (*Triticum durum* Desf.; cv. 'Mongibello') and bread wheat cultivars (*Triticum aestivum* L.; cv. 'Chinese Spring') under full irrigation and water deficit regimes. Metabolomics indicated a similar regulation of secondary metabolism in both bread and durum wheat cultivars following water limiting conditions. Nonetheless, a mycorrhizal fungi (AMF) x cultivar interaction could be observed, with the bread wheat cultivar being more affected by arbuscular colonization under water limiting conditions. Discriminant compounds could be mostly related to sugars and lipids, both being positively modulated by AMF colonization under water stress. Moreover, a regulation of metabolites related to oxidative stress and a tuning of crosstalk between phytohormones were also evidenced. Among the latter, the stimulation of the brassinosteroids biosynthetic pathway was particularly evident in inoculated wheat roots, supporting the hypothesis of their involvement in enhancing plant response to water stress and modulation of oxidative stress conditions. This study proposes new insights on the modulation of the tripartite interaction plant-AMF-environmental stress.

1. Introduction

Plant symbiotic association with arbuscular mycorrhizal fungi (AMF) represents the world's most widespread plant-microbe interaction, occurring in more of 80% of terrestrial plant species (Smith et al., 2010; Bonfante and Desirò, 2015; Berruti et al., 2016; Martin-Robles et al., 2018). Plant inoculum with beneficial symbiotic AMF could address the demand for sustainable food production strategies. In fact, AMF together with suitable agronomic practices can protect crops from adverse environmental constraints (Tenenboim and Brotman, 2016). The beneficial effects of AMF associated with crop roots are well known and include: i) improved uptake and translocation of macro- and micronutrients (Berruti et al., 2014; Rouphael et al., 2017; Bitterlich et al., 2018), ii) more vigorous root system apparatus (Berruti et al., 2014; Johri et al., 2015), iii) improved water relations and photosynthetic capacity (Berruti et al., 2014), iv) tolerance against multiple biotic/abiotic stressors (Colla et al., 2008; Berruti et al., 2014).

Drought is considered a major threat in many regions of the world including the Mediterranean area. Drought has been reported to disturb morphological, biochemical, physiological and metabolic processes leading to stunted growth and yield reduction (Daryanto et al., 2016). Similar to salinity stress, drought affects plant growth through changes in osmotic potential (Ruiz-Lozano, 2003). Symbiosis with AMF can ameliorate plant performance under diverse osmotic stresses (Augé, 2001). Santander et al. (2017) summarized the beneficial effects of AMF in improving osmotic responses to different stresses, through a better plant hydration and alteration of plant physiological processes. These authors also reported increasing mineral nutrient absorption,

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enhancing photosynthetic activity, accumulation of osmoprotectants, production of antioxidant enzymes, and modification of the rhizosphere environment" (Santander et al., 2017). Moreover, AMF hyphae can extend the explorable surface area (up to 50 times) and thus increase the uptake of water and several macro- and micronutrients (Smith and Smith, 2011; Berruti et al., 2014). The impact of AMF has been also demonstrated in different plant compartments subjected to drought stress. Specifically, Augè et al. (2014), through a meta-analysis, underlined that AMF symbioses often modify stomatal behavior and therefore play pivotal roles in plant productivity. In recent years, several studies reported the role of root-microbial association in alleviating drought effects on plants (Coleman-Derr and Tringe, 2014; Chitarra et al., 2016). The positive association of AM fungal species led to an improved tolerance to drought in tomato. Chitarra et al. (2016) reported that inoculation of tomato with *Funneliformis mosseae* (formerly *Glomus mosseae)* improved intrinsic water use efficiency and the levels of abscisic acid (ABA) were higher than in control (no AMF) under drought. Similar results were also reported for lettuce (Ruiz-Lozano et al., 2015). Beneficial symbionts are known to modulate plant hormonal pathways and existing host cross-talk stress response pathways (Glick et al., 2007; Coleman-Derr and Tringe, 2014).

Wheat is one of the world's leading grain crops contributing roughly to 22% of the global food requirements, ranking second in production volume (750 million tons) after maize (FAOSTAT, 2007). In addition, wheat production is estimated to rise in response to growing demand for food due to the global population growth [United Nations, Department of Economic and Social Affairs, FAOSTAT (2017). Therefore, research efforts to develop new strategies able to overcome water deficiency are needed. Among these strategies, the selection of new drought-tolerant varieties, innovative agronomic practices and soil management procedures are being investigated. Wheat follows different strategies for reacting to drought based on its genetic background (Marti and Slafer, 2014; Zhou et al., 2015). In our previous work, shotgun proteomics revealed a genotype-dependent modulation of the sulphur metabolism (Bernardo et al., 2017) even though the combination of fungal species and plant genotype commands a pivotal role (Graham and Abbott, 2000).

Although the benefits of AMF association for plants under drought conditions are evident, the biochemical mechanisms underlying improved wheat tolerance to stress are not yet fully disclosed. In this regard, the recent advances in metabolomics and data interpretation are offering the possibility to achieve a rather comprehensive picture of metabolites profile, thus opening new opportunities (Meier et al., 2017; Tsugawa, 2018). Indeed, metabolomics represents a close link between genotype and phenotype (Lamichhane et al., 2018) and has been proposed also for the comprehension of plant-environment interactions (Feussner and Polle, 2015).

To this aim, an MS-based metabolomic approach was applied to investigate mycorrhizal interaction with two different wheat genotypes, *i.e.* a durum and a bread wheat cultivar, under drought. Elucidating the plant-microorganism interaction and gaining knowledge on the metabolic mechanism(s) of action in AMF-plants can represent the basis for the further development of metabolic tools to help crops counteract drought.

2. Materials and methods

2.1. Plant material, experimental conditions, design and arbuscular mycorrhizal inoculation

The experiment was conducted in a growth chamber (Sanyo-Gallenkamp Model SGC970, Loughborough, UK) at Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics (CREA-GB, Fiorenzuola d'Arda, Italy).

The durum wheat (*Triticum durum* Desf.) cv. 'Mongibello' and the bread wheat (*Triticum aestivum* L.) cv. 'Chinese Spring' were grown in square pots (15 × 15 cm) containing a mixture of sterile sand and pasteurized field soil in 1:1 vol ratio (Brito et al., 2009), with three seedlings per pot. The sand used as substrate was a quarz sand with particle size between 0.4 and 0.8 mm; such sand was an inert material with a neutral pH. The soil mixture resulted in a pH of 8.2 (pH was measured in a 1:2.5 soil-water suspension); 2.23% (w/w) organic matter (method proposed by Springer and Klee, 1954); C:N of 11.8; 0.11% (w/w) of total N (Kjeldahl method); 12.7% (w/w) carbonates (volumetric calcimeter method); 28.1, 15.2 and 12.9 g kg^{-1} of total, inorganic and organic carbon (Walkley-Black method), respectively; 35.4 mg kg^{-1} P_2O_5 (Olsen method) and a cation exchange capacity of 7.2 cmol (+) kg^{-1} (barium chloride method). The analytical methods used for determining the soil chemical characteristics were those described by Karla (1998). Day/night temperatures of 20/16 ± 2 °C were established with a 12 h photoperiod and a relative air humidity of 65–75%. Average photosynthetic photon flux at canopy level was of 450 µmol m^{-2} s^{-1}.

The growth chamber experiment was conducted with two mycorrhizal treatments (with AMF [+] or without AMF [-]) under water stress. Treatments were arranged in a completely randomized design with five replicates per treatment, amounting to a total of 20 pots.

Prior to seeding, half of the pots received a commercial mycorrhizal inoculum carrying *Funneliformis* (formerly known as *Glomus*) *mosseae* (MycAgro Lab., Technopole Agro-Environnement, Bretenière, France) by mixing 10 propagules per gram of soil. The remaining pots were not inoculated and used as control. After two weeks, a moderate drought stress was applied. The volumetric water holding capacity was 6.2%. The relative soil water content was allowed to drop to 45% on average within 20 days and was kept constant thereafter. The relative soil water content was controlled gravimetrically by weighing the square pots at two-to-three days intervals. The water stress treatment lasted for 29 days.

2.2. Sampling, biomass production, C/N analysis and AMF colonization

At the end of the experiment (43 days after sowing) durum and bread wheat plants were separated into leaves, stems and roots. The number of leaves and stems were recorded, and all aboveground tissues were dried at 65 °C for 72 h until they reached a constant weight to determine dry biomasses and water content. Shoot dry weight was calculated as the sum of the aerial vegetative parts (leaves + stems), and the root-to-shoot ratio was computed. The water use efficiency (WUE) was calculated as the ratio of total dry biomass to total water uptake during the growing cycle (Rizza et al., 2012).

The wheat roots were gently washed with fresh water until the roots were free from any soil particles. The root samples from both wheat cultivars were collected combining three plants per experimental pot. Root tissue samples were weighed, frozen in liquid nitrogen, ground into fine powder using pestle and mortar, and stored at −80 °C for subsequent analysis. The presence of *F. mosseae* DNA was evaluated with quantitative real time-PCR analysis, using the *F. mosseae* BEG12 primers according to Alkan et al. (2006). Thereafter, 0.5 g were analyzed for C and N content through Dumas combustion using an elemental analyzer (Elementar vario MAX CN, Langenselbold, Germany).

2.3. Leaf chlorophyll and flavonoids analysis

Leaf chlorophyll content (Chl, unitless index), epidermal flavonoids (Flav, unitless index) and a leaf Nitrogen Balance Index (NBI, unitless index), the ratio between Chl and Flav were evaluated on the youngest fully expanded leaves using the DUALEX 4 Scientific (FORCE-A, Orsay, France) optical leaf-clip instrument.

2.4. Metabolomic analysis

The ground root samples from both wheat cultivars were collected

combining three plants per experimental pot. Root tissue samples were weighed, frozen in liquid nitrogen, into fine powder using pestle and mortar, and stored at −80 °C for subsequent metabolomic analysis. Thereafter, 0.5 g of root were extracted in 10 mL of 0.1% HCOOH in 80% methanol using an Ultra-Turrax (Ika T-25, Staufen, Germany). Extracts were filtered through a 0.22 µm cellulose membrane syringe filter and transferred to an amber vial for analysis. Untargeted metabolite screening was then carried out using UHPLC liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry, with an electrospray ionization source (UHPLC-ESI/QTOF-MS). A 1290 UHPLC liquid chromatograph and a G6550 mass spectrometer (Agilent Technologies Santa Clara, CA, USA) equipped with a JetStream ionization system were used.

UHPLC-ESI/QTOF-MS untargeted metabolomics were optimized in previous experiments (Pretali et al., 2016). Briefly, UHPLC reverse phase chromatographic separation was carried out using a binary mobile phase consisting of water (A) and methanol (B), using an Agilent Zorbax Eclipse-plus column (75 × 2.1 mm i.d., 1.8 µm). The linear gradient started with 5% B and was increased to 90% B within 35 min, with a mobile phase flow of 220 µL min^{-1} at 35 °C. The mass spectrometer operated in positive polarity and scan mode (range of 100–1200 m/z) whereas electrospray conditions were as follows: capillary voltage was 4 kV, nebulizer pressure was 60 psig, sheath gas was nitrogen (10 L min^{-1}, 350 °C), and drying gas was nitrogen (10 L min^{-1}, 280 °C).

Raw data were processed with the software Profinder B.05 (from Agilent Technologies) for features deconvolution and annotations. Alignment was done for both mass and retention time, then features were annotated using the database PlantCyc 11 (Plant Metabolic Network, http://www.plantcyc.org; released November 2016). Compounds identification was achieved using the whole isotopic pattern, *i.e.* the combination of accurate monoisotopic mass, isotope ratio and isotope accurate spacing. According to the approach chosen, identification was carried out as Level 2 (putatively annotated compounds), as set out in COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi.html). A filter-by-frequency post-processing was applied to retain only those features that were present in 100% of replications within at least one treatment. Finally, compounds were filtered by abundance (area threshold: 10000 counts).

2.5. Statistical analysis of experimental data

Physiological and growth data were statistically analyzed by two-way analysis of variance using the R software package (R Core Team, 2014). To separate treatment means within each measured parameter, Least Significant Difference test was performed at a significance level of $p \leq 0.05$. Principal component analysis (PCA) was performed with the function prcomp of R (R Core Team, 2014).

Metabolomic data were elaborated using Agilent Mass Profiler Professional B.12.06 (from Agilent Technologies) as previously described (Lucini et al., 2017). Compounds abundance was Log2 transformed, normalized at the 75th percentile and baselined to the median of control. Unsupervised hierarchical cluster analysis was then carried out setting similarity measure as 'Euclidean' and 'Wards' as linkage rule (Rouphael et al., 2016). Fold-change analysis was also carried out, using a cut-off value of 1.3.

Thereafter, the dataset was exported into SIMCA 13 (Umetrics, Malmo, Sweden), Pareto-scaled and elaborated for Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modelling. Herein, predictive and orthogonal (*i.e.*, related to technical and biological variation) components of variability were separated during the supervised modelling, and then CV-ANOVA ($p < 0.01$) was done for validation and permutation testing done to exclude model overfitting. Outliers were checked using the Hotelling's T2 distance from the origin in the OPLS-DA model, adopting 95% and 99% confidence limits for suspect and strong outliers respectively. Model goodness-of-fit R^2Y and goodness-of-prediction Q^2Y were also calculated, adopting an acceptability threshold of > 0.5 for Q^2Y prediction ability, according to software recommendation and as set out in the literature (Rombouts et al., 2017). Variable importance in projection (VIP analysis) was finally used to rank metabolites according to their discrimination potential (VIP score ≥ 2).

Discriminant compounds were analyzed in PlantCyc (Schläpfer et al., 2017) to highlight enriched metabolic pathways and physiologically related compounds. The dataset was matched against the PlantCyc database (151 compounds matched; Supplementary Table S1) and then for a stronger correlation against the *Triticum aestivum* database (93 compounds matched, Supplementary Table S2). The analysis for both datasets were performed as enrichment with a Fisher Exact statistic and without correction (Supplementary Tables S3 and S4, respectively).

Identified compounds were analyzed by means of KEGG Mapper – Search&Color Pathway on-line tool (http://www.genome.jp/kegg/tool/map_pathway2.html) (Kanehisa et al., 2016, 2017) against the Kegg Orthology database using Kegg Identifiers as objects (Supplementary Figs. S1 and S2).

3. Results

3.1. Morphological and physiological parameters

The presence of *F. mosseae* was confirmed by real-time PCR in inoculated roots, whereas AMF DNA was not detected in non-inoculated wheat roots. Furthermore, most of the morpho-physiological parameters measured in the current experiment were mainly affected by genotype and to a lesser extent by mycorrhizal inoculation. Therefore, a cultivar *x* genotype interaction was postulated. When averaged over AMF treatments, the highest water content and root N concentration were recorded in 'Chinese Spring' cultivar (Fig. 1). However, an opposite trend was observed for the aboveground dry biomass as well as for the leaf chlorophyll and flavonoids, with the highest values recorded in the 'Mongibello' durum wheat cultivar (data not shown). From an overall perspective, irrespective of the genotype, the aboveground fresh and dry weight as well as N concentration in roots increased by 20.9%, 8.4% and 9.7% respectively, in AMF-inoculated (being 5.15 g, 1.03 g and 3.4%, respectively) in comparison to non-inoculated wheat plants (being 4.26 g, 0.95 g and 3.1%, respectively).

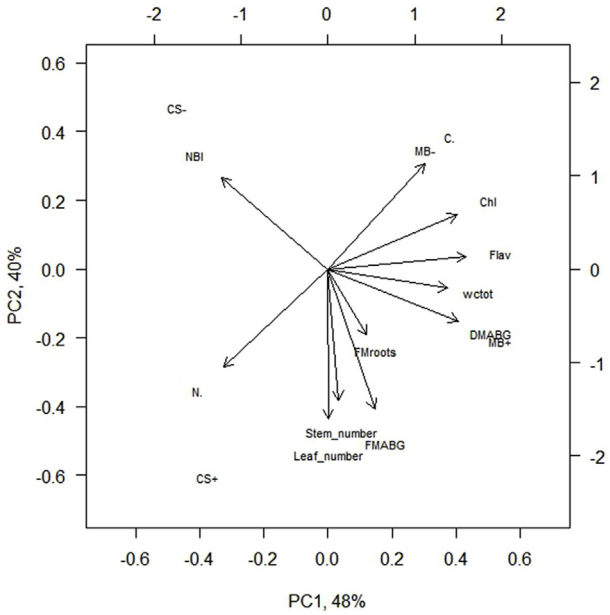

Fig. 1. C/N ratio of mycorrhized (+) or non-mycorrhized (−) Chinese Spring and Mongibello roots under drought.

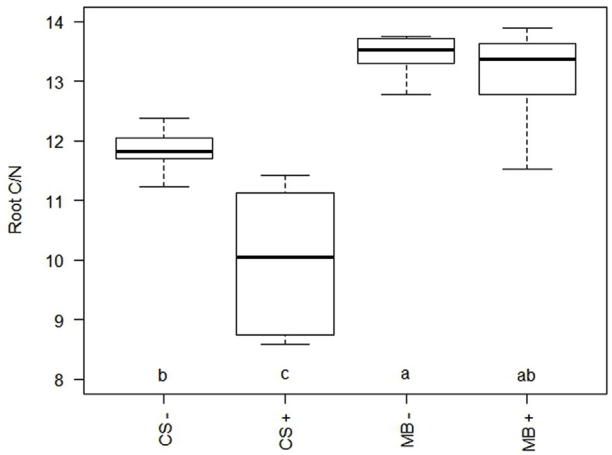

Fig. 2. Water use efficiency (g plant fresh mass per kg of water taken up during the experiment).

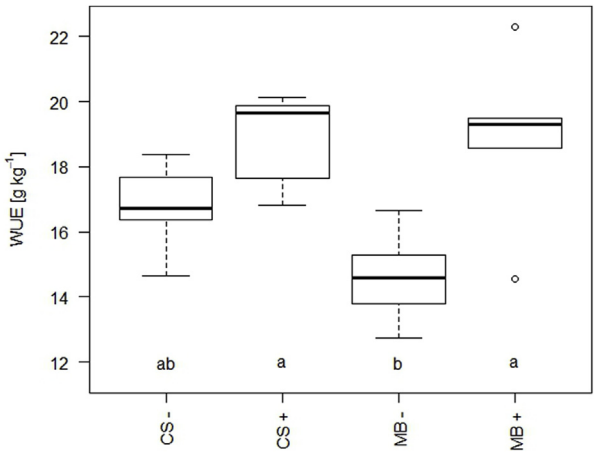

Fig. 3. Principal component loading plot and scores of principal component analysis (PCA) of morphological, physiological traits and mineral composition of two wheat cultivars, following two mycorrhizal treatments (with and without). CS- = Chinese Spring without mycorrhiza, CS+ = Chinese Spring with mycorrhiza, MB- = Mongibello without mycorrhiza, MB+ = Mongibello with mycorrhiza, C. = root carbon content, N. = root nitrogen content, Chl = leaf chlorophyll content, Flav = leaf epidermal flavonoid content, NBI = leaf nitrogen balance index, wctot = total water uptake during the whole experiment, FMroots = root fresh mass, FMABG = aboveground fresh mass, DMABG = aboveground dry mas.

The C/N ratio as well as the water use efficiency (WUE) were significantly affected by treatment and by genotype with interaction of these two variables (Figs. 1 and 2). The highest C/N ratio was observed in durum wheat 'Mongibello' with no significant differences between the AMF inoculation treatments, followed by non-inoculated plants of 'Chinese Spring' bread cultivar, whereas the lowest values were recorded for inoculated 'Chinese Spring' (Fig. 1). Finally, our results showed that inoculation with *F. mosseae* elicited significant increase in WUE in both wheat cultivars compared to non-inoculated 'Mongibello' plants, whereas non-inoculated 'Chinese Spring' treatment exhibited intermediate values (Fig. 2).

3.2. Principal component analysis

A comprehensive overview of the morphological and physiological changes of the two wheat cultivars in response to AMF inoculation was obtained through the principal component analysis (PCA). The first two principal components explained 88% of the total variance (PC1, 48% and PC2 40%). The cultivar differences were mainly captured by PC1 while PC2 was more closely related to the differences between mycorrhized and non-mycorrhized plants (Fig. 3). Total water consumption during the experiment, aboveground dry biomass, leaf chlorophyll and flavonoid contents strongly loaded on PC1, while variation in leaf number, stem number and aboveground fresh mass dominated loading on PC2 (Fig. 3). The score plot of the PCA clearly separated the two genotypes along PC1 with 'Mongibello' characterized by higher chlorophyll, flavonoid and water consumption as well as aboveground dry biomass (Fig. 3). The AMF treatments were separated by PC2, with both non-inoculated 'Mongibello' and 'Chinese Spring' on the positive side of the PC2 that are characterized by slightly higher leaf chlorophyll contents, NBI and root C content. Inoculated plants were overall characterized by higher plant growth parameters. Finally, root N and C content loaded on both PC1 and PC2, associated with higher N content in mycorrhized plants and in 'Chinese Spring' (Fig. 3).

3.3. Metabolomic profiling of roots

The untargeted UHPLC-ESI/QTOF-MS analysis allowed annotating about 2900 putative metabolites (supplementary material). The unsupervised hierarchical clustering built on the fold-change based heat map, identified two main clusters, cultivar being the most relevant classification factor (Fig. 4). Two distinct sub-clusters were detected for the 'Chinese Spring' bread wheat cultivar, including samples from AMF inoculation and no inoculation, respectively. However, no distinct sub-clusters were produced for the durum cultivar 'Mongibello', where the two treatments were mixed.

To identify the metabolites better describing changes induced by AMF association under water stress, a subsequent supervised multivariate analysis (i.e., OPLS-DA) was carried out. Looking at OPLS-DA score scatter plots (Fig. 5), the modeling highlighted a clear separation of treatments according to AMF colonization; the contribution of the factor cultivars was still present but secondary (Fig. 5). OPLS-DA model fitting parameters were more than adequate, with a goodness-of-fit $R^2Y = 0.99$ and goodness-of-prediction Q^2Y 0.70. Both models provided 100% accuracy in class prediction (Fisher's probability: 1.1 E-05), cross validation CV-ANOVA ($p < 0.01$) was adequate and permutation testing excluded model overfitting. Outlier samples could be excluded by Hotelling's T2, using both 95% and 99% confidence limits (suspect and strong outliers, respectively).

Provided that multivariate modelling could adequately discriminate the treatments, VIP analysis was next used to reduce the number of discriminant compounds, adopting a score ≥ 2 as threshold (Table 1). Discriminant compounds were classified into biochemical classes to facilitate interpretations and then subjected to fold-change analysis to describe direction and intensity of regulation (Table 1). The more represented metabolic classes from VIP analysis were lipids, flavonoids, carbohydrates, S compounds, terpenoids, amino acids and hormones (with compounds related to brassinosteroid metabolism being the most represented). Interestingly, most of the compounds had the same regulation trend in both 'Chinese Spring' and 'Mongibello' cultivars. The metabolic enrichment was also analyzed with the PlantCyc database. Enrichment analysis allows us to identify whether a set of metabolites contains more modulated metabolites in a given metabolic pathway than one would expect to occur by chance.

Lipids represented the most intensely modulated class of compounds in response to mycorrhization under water stress, and in particular they were down accumulated in mycorrhizal roots. Several compounds were identified to fit in the cholesterol biosynthetic pathway (Supplementary Fig. 1: 4-beta-hydroxymethyl-4-alpha-methyl-5-alpha-cholest-7-en-3-beta-ol; 4-alpha-hydroxymethyl-5-alpha-cholesta-8,24-dien-3-beta-ol; 4-alpha-hydroxymethyl-5-alpha-cholesta-7,24-dien-3-beta-ol; 4-alpha-methyl-5-alpha-cholesta-8,14,24-trien-3-beta-ol; 3-dehydro-4-methylzymosterol; 4,4-dimethylzymosterol; 4-alpha,14-alpha-dimethyl-9-beta, 19-cyclo-5-alpha-cholest-24-en-3-

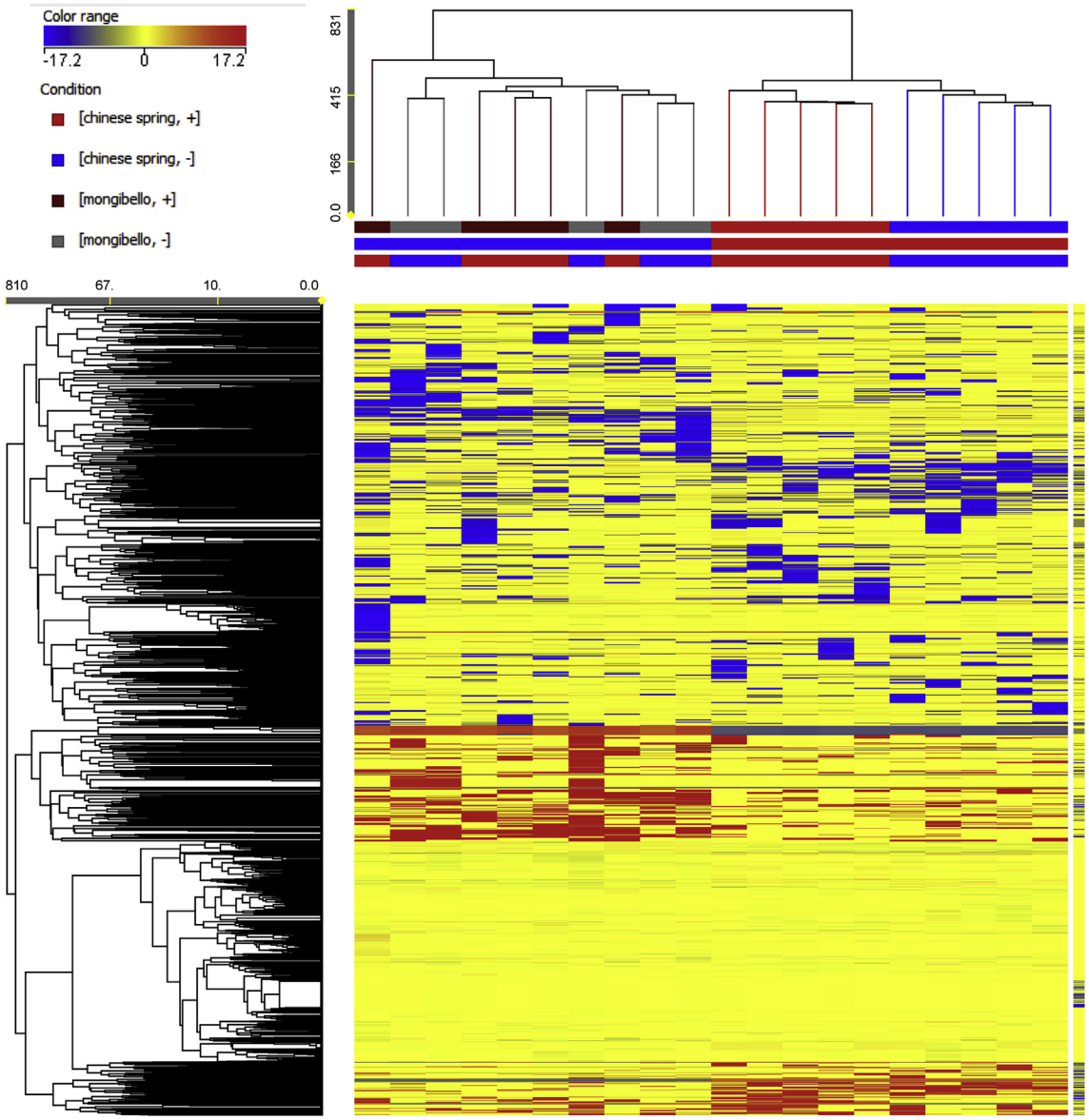

Fig. 4. Unsupervised hierarchical cluster analysis carried out from untargeted metabolomic profiles of wheat roots following inoculation with AMF. Dendrograms were produced on the basis of fold-change heat-maps (Euclidean similarity, Wards' linkage rule).

beta-ol) or cell membrane component (24-alkyl sterol 3; 24-alkyl sterol 2; 24-alkyl sterol 1) acting to regulate the membrane fluidity and flexibility. Altogether, these compounds were down represented in mycorrhizal roots from both wheat cultivars. Furthermore, solasodine 3-O-beta-D-glucopyranoside and decanoate were accumulated in both colonized wheat roots. Additionally, six (glycero) phospholipids were down represented in roots following AMF colonization (1-acyl-sn-glycero-3-phosphoethanolamine (n-C16:1); N-dimethylethanolamine phosphate; 2-acyl-sn-glycero-3-phosphoethanolamine (n-C16:1); 1-16:0-2-lysophosphatidylcholine; 1-palmitoylglycerone 3-phosphate; 1-hexadec-9-enoyl-sn-glycerol 3-phosphate).

Carbohydrates represented the second most modulated metabolic class; mannosylfructose-phosphate, 3-propylphosphoenolpyruvate, and glucose-1,6-bisphosphate were accumulated in both AMF-associated wheat roots. Our findings highlighted that the sugar alcohol pinitol and compounds related to pinitol biosynthesis, were down represented in the mycorrhizal roots of both wheat cultivars.

Only in few instances the two wheat cultivars differed in the modulation of metabolites, namely a heptose carbohydrate (D-glycero-D-manno-heptose 7-phosphate/D-sedoheptulose 7-phosphate), a flavonoid (apigenin-7,4′-dimethyl ether/7-hydroxy-4′5′-dimethoxyisoflavone/afrormosin), glycyrrhetaldehyde, and two sulphur-containing compounds (2-hydroxypropyl-CoM, indole-3-acetyl-methionine).

Various amino acids related compounds, linked to tyrosine or histidine metabolism (4-amino-2-methyl-5-phosphomethylpyrimidine; imidazole acetol-phosphate; O-phospho-L-tyrosine), were shown as discriminant compounds under AMF–root interaction. Among the metabolites with non-enzymatic antioxidative role, a modulation of alpha-tocopherol and 4-O-oxalyl-L-threonate (a catabolite of ascorbate) were identified by OPLS-DA. Similarly, accumulation of several flavonoids and flavonoid derivatives (luteolin 7-O-beta-D-glucuronide, cyanidin 5-O-beta-D-glucoside; isorhamnetin 3, 4′-bisulfate) was observed together with an increase of 3″-deamino-3″-oxonicotianamine, an intermediate for siderophores biosynthesis.

Several hormones were identified by VIP analysis as discriminant between AMF-colonized roots and control; these compounds are related to abscisic acid, brassinosteroids, gibberellins, auxins and jasmonic acid pathways (Table 1). An increase of GA and ABA catabolites such as β-D-glucopyranosyl abscisate, was also observed. However, brassinosteroid-related compounds (including cathasterone, deoxoteasterone,

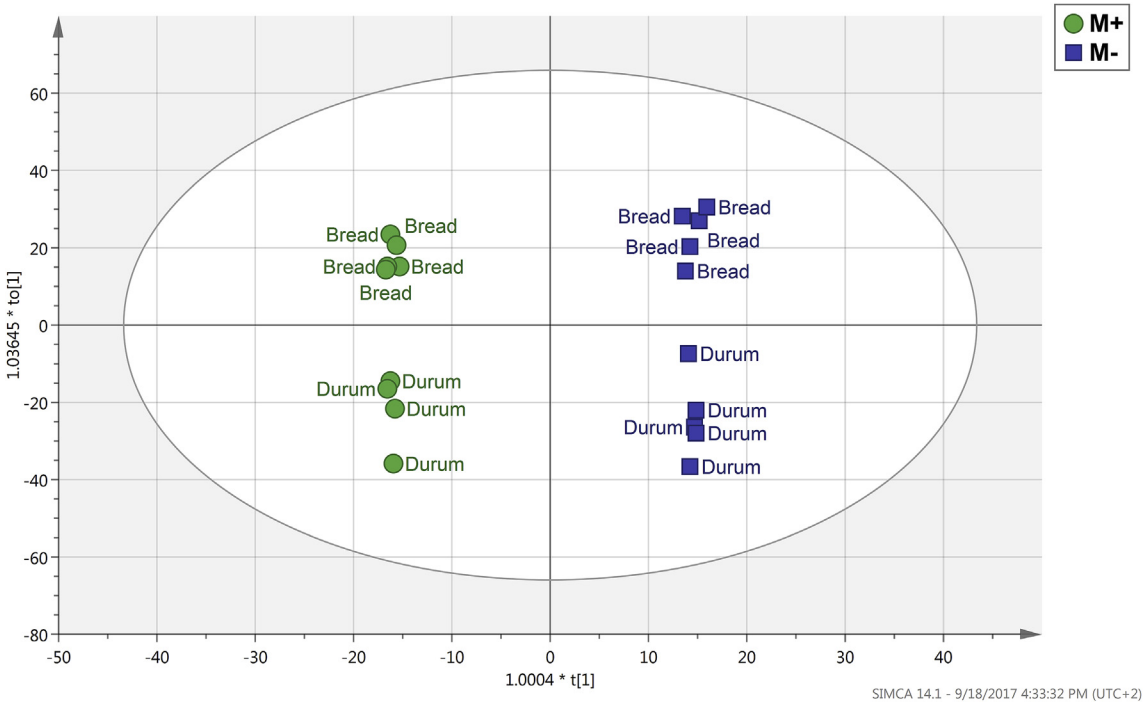

Fig. 5. Score plot of Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) supervised modeling carried out from untargeted metabolomic profiles of wheat roots following inoculation with AMF. The patterns observed in the score plot were used to identify discriminant compounds based on Variable of Importance in projection (VIP) analysis.

campesterol, campest-4-en-3-one and brassinolide derivatives) were the most frequently identified among hormones.

Furthermore, S-containing compounds were identified to be accumulated in mycorrhizal wheat roots (viz. homotaurine; S-9-methylthiononylhydroximoyl-L-cysteine; carboxin; glutathione; methionine). Conversely, a compound related to methionine (N-acetyl- methionine) was down-represented in mycorrhizal root. Glucosinolates were among the metabolites having the greatest VIP scores, able to differentiate the non-mycorrhizal roots. In more detail, 1/4-methoxy-3-indolylmethyl-glucosinolate, 8-methylthiooctyl glucosinolate, 3-methylthiopropyl-desulfoglucosinolate, 2-propenyl-glucosinolate and phenylacetothiohydroximate were down-represented, while 3-butenylglucosinolate was accumulated. Finally, further metabolites classified into terpenoids and alkaloids were also negatively modulated by AMF-root association under water limiting conditions.

4. Discussion

A primary issue for the modern extensive agriculture will be to grow staple food crops (including wheat) under suboptimal conditions dictated by global climate change. The application of microbial-based biostimulants in agricultural cropping systems is gaining an increasing interest among growers, extension specialists, scientists as well as breeding companies to secure yield stability under stress conditions (Fiorentino et al., 2018; Rouphael and Colla, 2018).

In our experiments, a beneficial effect was observed for both wheat cultivars, demonstrating that inoculation with *F. mosseae* significantly improved the biomass production under limited water availability in wheat (Fig. 3). A putative mechanism behind the stimulation activity of arbuscular mycorrhizal fungi on wheat performance is the modulation of the root system architecture and a better uptake of water and low mobile macro- and micronutrients (Smith and Smith, 2011; Rouphael et al., 2015). Interestingly, our findings showed a positive trend for water use efficiency (WUE) of the host plant following AMF inoculation (Fig. 2), although differences were significant only in 'Mongibello'. A possible mode of action behind the higher WUE in inoculated wheat plants could be linked to the positive trend for roots-to-leaves N content, in line with Chl and NBI trends, as a balance between net photosynthetic to transpiration rates (Santander et al., 2017 and references therein).

The metabolomic analysis suggests a comparable metabolic regulation in both bread and durum wheat cultivars under water limiting conditions, for the majority of identified metabolites (Table 1). The unsupervised analysis suggested that the AMF colonization had an impact on root secondary metabolome, with the bread wheat cultivar resulting to be more affected by AMF colonization. The *F. mosseae* colonization had a strong impact in reprogramming the wheat roots metabolism of sugars, lipids and plant phytohormones, besides flavonoids, metabolites related to histidine biosynthesis and lignans that were known to accumulate in mycorrhizal roots (Schliemann et al., 2008; Rivero et al., 2015) (Fig. 5).

Sugars are known to be the primary C source for AMF, being provided by the host plants; however, in last decade, researchers proposed lipids as additional supply of C to fungi (Wang et al., 2017). With this regard, C16:0 was the main fatty acid transferred from plant hosts to AMF, according to a so called "bread-and-butter" hypothesis (Roth and Paszkowski, 2017; Wang et al., 2017). An over representation on sugars, amino acids, and phenolics was found in tomato roots colonized by *F. mosseae* compared to *Rhizophagus irregularis* (Rivero et al., 2015) and sucrose metabolism is known to be associated to drought tolerance in leaves of trifoliate orange (Wu et al., 2017).

In our study, phosphate sugars were accumulated in mycorrhizal roots from both cultivars under water stress, while glycerol-heptoses were accumulated only in 'Chinese Spring'. This latter pinitol-related lipopolysaccharide is reported to have a double role both for C exchange and osmoprotection/osmoregulation under water-limited conditions (Rapparini and Penuelas, 2014; Bárzana et al., 2015).

Among lipids, higher abundance of compounds involved in phytosterols, glycerophospholipids and cholesterols biosynthesis characterized the non-mycorrhized roots. As reported by Siebers et al. (2016), the AMF accumulation of fatty acids directly synthetized by fungi or following plant supply is nowadays under debate. Our analyses

Table 1
Discriminant compounds (VIP analysis, score > 2) gained from Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) supervised modeling carried out from untargeted metabolomic profiles of wheat roots following inoculation with AMF. Compounds are provided together with fold-change values and regulation for bread and durum wheat (Chinese Spring and Mongibello cultivars, respectively). Missing values denote fold-change values < 1.5.

Compound		VIP score	Chinese Spring cultivar		Mongibello cultivar	
			Log FC	Regulation	Log FC	Regulation
Amino acids	2-amino-3-oxobutanoate	3.23	−0.94	down	−0.75	down
	4-amino-2-methyl-5-phosphomethylpyrimidine	2.81	1.28	up	1.01	up
	seleno-L-methionine	2.78	-	-	-	-
	N-acetyl-DL-methionine	2.72	−6.77	down	−0.47	down
	3-phospho-hydroxypyruvate	2.57	14.98	up	8.47	up
	cyclogutamate	2.53	−7.16	down	−4.25	down
	imidazole acetol-phosphate	2.28	16.41	up	0.44	up
	penicillamine	2.16	1.38	up	0.76	up
	L-methionine/S-ethyl-L-cysteine	2.13	4.18	up	0.76	up
	O-phospho-L-tyrosine	2.03	15.91	up	15.60	up
	3″-deamino-3″-oxonicotianamine	2.01	0.67	up	0.35	up
Alkaloids	cyclo-acetoacetyl-L-tryptophan	2.56	−0.41	down	−0.24	down
	1,3,7-trimethyl-5-hydroxyisourate	2.27	−11.34	down	−14.55	down
	staurosporine	2.17	12.12	up	0.79	up
	caffeine	2.08	−15.23	down	−4.50	down
Carbohydrates	mannosylfructose-phosphate	3.02	5.27	up	2.74	up
	3-propylphosphoenolpyruvate	2.65	1.48	up	14.54	up
	2-O-(6-phospho-alpha-D-mannosyl)-D-glycerate	2.61	−8.11	down	−4.58	down
	2′-O-methyl-rhamnosyl tetracyclic spinosyn pseudoaglycone	2.33	−0.46	down	−0.85	down
	N4-(beta-N-acetyl-D-glucosaminyl)-L-asparagine	2.17	−0.18	down	−0.68	down
	glucose-1,6-bisphosphate	2.12	0.21	up	1.65	up
	D-sedoheptulose 7-phosphate/D-glycero-D-manno-heptose 7-phosphate	2.06	0.49	up	−2.05	down
	methyl-D-glucoside/methyl-D-galactoside	2.01	−4.39	down	−4.43	down
Phenolics - flavonoids	luteolin 7-O-glucuronide	2.90	11.43	up	15.02	up
	myricetin 3-O-gentiobioside	2.71	−12.46	down	−0.25	down
	2-protocatechuoylphloroglucinolcarboxylate	2.63	−3.84	down	−4.15	down
	7-hydroxy-4′5′-dimethoxyisoflavone/apigenin-7,4′-dimethyl ether/afrormosin	2.51	−5.24	down	3.00	up
	cyanidin 3-O-beta;-(2-O-beta-D-glucuronosyl)-beta-D-glucoside	2.50	−1.01	down	−0.65	down
	quercetagetin 7-O-glucoside	2.39	−1.69	down	−0.38	down
	quercetin 3-O-rhamnoside/orientin/a kaempferol-glucoside	2.34	0.41	up	0.51	up
	cyanidin 5-O-beta-D-glucoside	2.34	0.41	up	0.51	up
	3′,4′,5′-O-trimethyltricetin	2.12	5.51	up	10.94	up
	protohypericin	3.00	−1.11	down	−0.35	down
	scutellarin	2.90	11.43	up	15.02	up
	bis-noryangonin	2.67	−0.87	down	−0.17	down
	(+)-secoisolariciresinol monoglucoside	2.13	0.18	up	0.42	up
	4-coumaroyl-3′,4′-dihydroxyphenyllactate	2.12	5.51	up	10.94	up
	N-caffeoylputrescine	2.05	−5.68	down	−0.65	down
	cinnamoyl-beta; -D-glucoside	1.99	−6.92	down	−11.81	down
	isorhamnetin 3, 4′-bisulfate	2.03	0.26	up	0.71	up
	(+)-sesaminol 2-O-beta-D-glucoside	2.02	−0.24	down	−4.78	down
Glucosinolates	1/4-methoxy-3-indolylmethyl-glucosinolate	2.99	-	-	-	-
	8-methylthiooctyl glucosinolate	2.58	−1.19	down	−3.24	down
	3-methylthiopropyl-desulfoglucosinolate	2.57	−6.03	down	−5.05	down
	2-propenyl-glucosinolate	2.23	−0.39	down	−0.37	down
	3-butenylglucosinolate	2.22	1.06	up	6.19	up
	phenylacetothiohydroximate	2.35	−8.81	down	−8.58	down
Hormones	24-epicathasterone/3-dehydro-6-deoxoteasterone/22,23-dihydroxycampesterol	2.46	−8.34	down	−8.27	down
	(22alpha)-hydroxy-campest-4-en-3-one	2.46	−4.42	down	−4.40	down
	(22R,23R)-28-homobrassinolide 22-sulfate	2.22	-	-	-	-
	6alpha-hydroxy-castasterone	2.18	7.62	up	0.53	up
	brassinolide-23-O-glucoside	2.15	0.21	up	0.83	up
	cathasterone	2.49	−4.45	down	−4.33	down
	episterone	2.37	−19.75	down	−3.21	down
	D-glucopyranosyl abscisate	2.12	1.63	up	4.70	up
	indole-3-acetyl-valine	2.05	−1.67	down	−0.74	down
	indole-3-acetyl-methionine	2.10	2.29	up	−4.38	down
	gibberellin A29-catabolite/gibberellin A34-catabolite	2.04	0.27	up	11.62	up
	tuberonic acid glucoside	2.03	0.67	up	0.48	up
	OPC8-CoA	2.14	−6.97	down	−0.90	down
Lipids	4beta-hydroxymethyl-4alpha-methyl-5alpha-cholest-7-en-3beta-ol	2.96	−0.89	down	−5.71	down
	4alpha-hydroxymethyl-5alpha-cholesta-8,24-dien-3beta-ol	2.73	−0.47	down	−4.40	down
	4alpha-hydroxymethyl-5alpha-cholesta-7,24-dien-3beta-ol	2.46	−4.42	down	−4.40	down
	solasodine 3-O-beta; -D-glucopyranoside	2.45	0.23	up	0.84	up

(*continued on next page*)

Table 1 (*continued*)

Compound		VIP score	Chinese Spring cultivar		Mongibello cultivar	
			Log FC	Regulation	Log FC	Regulation
	decanoate	2.38	0.89	up	10.36	up
	4alpha-methyl-5alpha-cholesta-8,14,24-trien-3beta-ol/3-dehydro-4-methylzymosterol/5-dehydro episterol	2.37	−19.75	down	−3.21	down
	sn-1-lyso-2-16:0-monogalactosyldiacylglycerol	2.29	−0.54	down	−0.58	down
	a 1-acyl-sn-glycero-3-phosphoethanolamine (n-C16:1)	2.36	−9.14	down	−12.60	down
	A poriferstа-dienol/4,4-dimethylzymosterol/4alpha,14alpha-dimethyl-9beta,19-cyclo-5alpha-cholest-24-en-3beta-ol	2.31	−5.51	down	−5.13	down
	Delta24-25-sitosterol/stigmasterol	2.31	−5.51	down	−5.13	down
	(22alpha)-hydroxy-sitosterol	2.86	−5.51	down	−5.71	down
	N-dimethylethanolamine phosphate	2.20	−0.37	down	−0.58	down
	a 2-acyl-sn-glycero-3-phosphoethanolamine (n-C16:1)	2.11	−9.03	down	−8.84	down
	a hexadecenoyl-CoA (n-C16:1CoA)	2.09	−0.32	down	−4.01	down
	1-16:0-2-lysophosphatidylcholine	2.09	−0.37	down	−5.14	down
	1-palmitoylglycerone 3-phosphate	2.03	−4.58	down	−10.90	down
	a 1-hexadec-9-enoyl-sn-glycerol 3-phosphate	2.01	−4.58	down	−4.63	down
Terpenoids	a limonene-1,2-diol	2.36	0.51	up	3.78	up
	7-deoxyloganate	2.31	−0.64	down	−1.17	down
	(20S)-ginsenoside Rh2	2.18	0.22	up	0.83	up
	hemigossypol-6-methyl ether	2.07	−8.05	down	−7.08	down
	glycyrrhetaldehyde	2.05	2.91	up	−11.62	down
Nucleosides	6-amino-6-deoxyfutalosine	2.97	−13.12	down	−4.54	down
	ribavirin-5′-monophosphate	2.93	−1.32	down	−1.15	down
	7-methylxanthosine	2.57	−0.61	down	−6.83	down
	2′-deoxyuridine 3′-monophosphate	2.31	0.68	up	0.69	up
	dUMP	2.31	0.68	up	0.69	up
	uridine	2.07	0.22	up	0.83	up
	pseudouridine	2.07	0.22	up	0.83	up
	guanosine	2.01	0.67	up	0.35	up
	8-oxo-deoxyguanosine	2.01	0.67	up	0.35	up
Pterins	[1-(2-amino-7-methyl-4-oxo-7,8-dihydro-3H-pteridin-6-yl)]ethyl-4-(beta-D-ribofuranosyl)aminobenzene 5′-phosphate	3.13	−0.87	down	−0.55	down
	7,8-dihydropterin-6-ylmethyl-4-(beta-D-ribofuranosyl)aminobenzene 5′-phosphate	2.18	−10.92	down	−7.85	down
Sulphur compounds	2,4-diamino-6-ethyl-5,3'-(2-trifluoromethyl-4-sulphonamidophenoxy)prop-1′-yloxypyrimidine	2.99	−2.51	down	−1.67	down
	diethyldithiocarbamate	2.77	−0.78	down	−0.36	down
	homotaurine	2.74	14.27	up	15.13	up
	a 2-hydroxypropyl-CoM	2.63	1.53	up	−0.73	down
	S-9-methylthiononylhydroximoyl-L-cysteine	2.36	2.53	up	0.71	up
	dimethylsulfoniopropanoate	2.23	−0.85	down	−0.13	down
	steroid O sulfate	2.20	−7.83	down	−0.69	down
	carboxin	2.12	2.09	up	7.02	up
	glutathione	2.09	0.67	up	0.37	up
Others	alpha-tocopherol	2.86	−5.51	down	−5.71	down
	4-O-oxalyl-L-threonate	2.29	0.75	up	0.88	up
	D-pinitol	2.01	−4.39	down	−4.43	down
	sequoyitol	2.01	−4.39	down	−4.43	down
	D-ononitol	2.01	−4.39	down	−4.43	down
	1-O-methyl-scyllo-inositol	2.01	−4.39	down	−4.43	down
	calcitetrol	1.99	0.23	up	0.43	up
	diprenylphlorisovalerophenone	2.08	7.63	up	4.14	up
	N-phenylsuccinimide	2.08	−17.67	down	−6.57	down

suggest a reduction in carbohydrates possibly depending on their conversion in lipids that are afterwards available to AMF (Wang et al., 2017).

A beneficial impact of AMF on wheat roots of both cultivars was represented by the decrease of alkaloids and flavonoids, compounds known to have many roles in plants to react with other organisms or to mediate environmental changes, like a biomarker of plant stresses thanks to their antioxidative properties (Mierziak et al., 2014).

Drought is associated to osmotic and oxidative stress, because of an impaired balance of ions, resulting in oxidative damage and alteration of several cellular functions such as cell membrane stability and production of Reactive Oxygen Species (ROS) (Ruiz-Lozano, 2003; Ghosh and Xu, 2014; Bernardo et al., 2017; Abid et al., 2018). The excess in ROS leads to membrane damage, lipid peroxidation, protein denaturation and DNA modification (Abid et al., 2018). Compounds known to play an antioxidative role by directly reacting with ROS were accumulated in roots from both wheat cultivars, namely glutathione and 4-O-oxalyl-L-threonate, the latter indicating ascorbate degradation. In fact, AMF colonization has been demonstrated to counteract oxidative stress during abiotic stress, thus improving antioxidant defence and drought tolerance, with reduced accumulation of ROS in plants (Nath et al., 2016).

S is an important macro-element for plant growth, as component of cysteine and methionine, precursor of ethylene, and as part of glucosinolates (Gahan and Schmalenberger, 2014). Normally, organic S is synthetized by soil microorganisms and mycorrhizal plants are able to

obtain S from organic source (Allen and Shachar-Hill, 2009). Among S-containing compounds, glutathione and homotaurine were accumulated in both mycorrhizal wheat cultivars. Glutathione (GSH) plays important roles in the intrinsic responses of plants to abiotic stresses by acting in the maintenance of the cellular redox balance and mitigating damage caused by ROS (Zechmann, 2014). The involvement of S-related metabolites agrees with previous findings based on proteomic investigation in AMF-inoculated wheat roots under drought stress (Bernardo et al., 2017).

A complex and coordinate alteration of phytohormone profile was imposed by AMF colonization in roots of both wheat cultivars. The regulation of AMF colonization depends on environmental conditions, on the genotypes of both plant and AMF, and occurs under a strict coordination of different plant hormones such as ABA, auxins and GA. ABA regulates plant growth and responses to abiotic stresses, and promotes AMF colonization (Fernández et al., 2014; Nadeem et al., 2014). A low ABA concentration stimulates hyphae branching for water perception (Foo et al., 2013; Stec et al., 2016). Conjugated (inactive) auxins were among discriminant metabolites for non-mycorrhizal roots. The possible role of auxin in AMF formation and development is under debate since many years, and some researchers suggested that it partially regulated AMF root colonization (Foo et al., 2013 and references therein; Etamadi et al., 2014). Gibberellins are also involved in AMF development (Santander et al., 2017) and are reported to suppress arbuscule formation in the latter stages of colonization (Foo et al., 2013). In our metabolic profiling, an increase in GA catabolites supports the conclusion that active gibberellins decreased. Jasmonic acid (JA) is a stress-related phytohormone entailing effects on AMF colonization. The presence of its precursor (tuberonic acid glucoside) in mycorrhizal wheat roots might confirm its function as stress hormone in response to drought as well as its active role in the establishment of symbiotic association with AMF as previously postulated (Hause et al., 2002; Stumpe et al., 2005; Bernardo et al., 2017).

Besides others, brassinosteroids were the phytohormones most frequently identified by our analyses (Supplementary Tables 1 and 2; Supplementary Fig. 2). In more detail, intermediates of brassinosteroid biosynthesis were accumulated in inoculated plants. Brassinosteroids play a pivotal role in regulating plant growth and root development and elongation. Extensive knowledge about their role in mycorrhizal symbiosis is still missing, but a recent study suggested their contribution in AMF colonization (Wei and Li, 2016). Brassinosteroids act antagonistically with other phytohormones, such as ABA, auxins and gibberellins, as reviewed by Sharma et al. (2017). This is in line with our findings, where the increase in brassinosteroids was coupled with higher ABA and GA catabolites. Some studies reported the role of brassinosteroids also to alleviate detrimental effects of drought by enhancing antioxidants such as GSH (Vardhini and Anjum, 2015), which was actually found to increase in our study.

5. Conclusions

Recent research on plant-symbiotic associations ushers to sustainable practices for enhancing plant fitness and mitigating the effects of abiotic stress under the specter of climate change. The present study shed light onto the changes in wheat root metabolome following association with *F. mosseae* under drought, adding a piece to the complex puzzle of the tripartite interaction plant-AMF-environmental stress. Our present findings suggest that the AMF colonization triggers a wide and diverse modulation of root metabolic processes, beyond aspects of mere nutrient availability. An inoculation *x* cultivar interaction could be observed, with bread wheat benefiting more from AMF colonization under limited water availability. Despite differences in the extent of regulation, the metabolic reprogramming in both bread and durum wheat under water limiting conditions shared the majority of discriminant metabolites. Above all, the ability to cope with ROS-mediated oxidative stress and the regulation of hormone crosstalk were suggested to play a pivotal role in increasing wheat resilience to drought stress following AMF colonization.

Authorship contribution statement

L. Bernardo: formal analysis, data curation, writing original draft.
P. Carletti and F.W. Badeck: data curation.
F. Rizza, C. Morcia, R. Ghizzoni: formal analysis.
Y. Rouphael and G. Colla: data curation, writing (review & editing).
V Terzi: conceptualization, data curation.
L. Lucini: conceptualization, data curation, writing (review & editing).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.007.

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