

Article

Multi-Omics Insights into Arbuscular Mycorrhizal Fungus-Mediated Enhancement of Sunflower Resistance to Salinity, Drought and *Orobanche* Stresses

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Abstract

Sunflower (*Helianthus annuus* L.) production is increasingly challenged by multiple environmental stresses, including salinity, drought, and parasitic plants such as *Orobanche cumana*. Arbuscular mycorrhizal fungi (AMF) are widely recognized as beneficial microorganisms that enhance plant stress tolerance. However, the molecular mechanisms underlying AMF-mediated adaptation to multiple stresses remain largely unclear. In this study, we investigated the effects of AMF inoculation on sunflower resistance to salinity, drought, and *O. cumana* parasitism through physiological, transcriptomic, metabolomic, and integrated multi-omics analyses. AMF inoculation promoted lateral root development in different sunflower varieties and induced physiological priming responses, characterized by enhanced antioxidant activity and improved regulation of reactive oxygen species homeostasis and osmotic balance under stress conditions. Transcriptomic analysis revealed a conserved AMF-responsive gene module across different stress environments, which was mainly associated with phenylpropanoid metabolism, cell wall organization, redox regulation, and lipid-related pathways. Metabolomic profiling further demonstrated that AMF extensively reshaped root metabolic networks, particularly affecting organic acids, amino acid derivatives, phenolic compounds, flavonoids, alkaloids, and lipid-associated metabolites. Integrated transcriptomic–metabolomic analysis identified key gene–metabolite associations involved in carbon metabolism, nutrient transport, hormone signaling, and membrane remodeling, providing potential molecular targets underlying AMF-mediated stress adaptation. Overall, this study reveals how AMF enhances sunflower resilience against biotic and abiotic stresses through coordinated physiological, transcriptional, and metabolic regulation. These findings highlight the potential application of AMF as a sustainable biological strategy for improving crop stress resistance and quality-related metabolic traits.

Keywords: *Helianthus annuus*; arbuscular mycorrhizal fungi; *Orobanche cumana*; abiotic stress; transcriptomics; metabolomics; stress adaptation



Academic Editor: Rosa Porcel

Received: 1 September 2026

Revised: 6 October 2026

Accepted: 14 October 2026

Published: 16 October 2026

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1. Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in the world. It provides high-quality edible oil and is also widely used in food, feed, and industry [1,2]. Improving sunflower yield and seed quality is therefore an important goal

of modern agriculture. However, sunflower production is often limited by several environmental stresses. Among them, drought and soil salinity are two major abiotic stresses that reduce water uptake, nutrient absorption, photosynthesis, and plant growth [3,4]. In addition, *Orobancha cumana* Wallr., an obligate parasitic weed, is one of the most serious biotic threats to sunflower production [4–6]. It attaches to sunflower roots and continuously takes water, nutrients, and photosynthetic products from the host, causing severe growth inhibition and yield losses [5,7,8]. In many sunflower-growing regions, these stresses may occur under the same production conditions, making crop management more difficult. Therefore, developing environmentally friendly methods to improve sunflower resistance to both abiotic and biotic stresses has become an important research topic [3,9].

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with the roots of most land plants. They improve the uptake of water and mineral nutrients and help plants grow better under unfavorable conditions. Many studies have shown that AMF also increase plant resistance to diseases, insects, and environmental stresses [10]. These positive effects are partly related to better plant nutrition and partly to antimicrobial compounds produced by AMF that can inhibit some pathogens [11]. More importantly, AMF modulate defense responses without disrupting the symbiotic relationship [12]. They keep plants in a primed state, allowing them to respond more quickly and more strongly when stress occurs [13]. AMF also promote the accumulation of phenolic compounds, strengthen cell walls, and activate antioxidant systems, collectively enhancing plant defense [14,15]. These advantages make AMF an effective and sustainable tool for improving crop production while reducing chemical inputs [16,17].

The beneficial effects of AMF are especially important under drought and salinity stress. During drought, AMF improve root development and form extensive hyphal networks in soil, which increase water and nutrient uptake [18]. AMF also promote the accumulation of osmotic regulators such as proline and improve antioxidant systems to reduce oxidative damage caused by water shortage [19,20]. Under salinity stress, AMF help plants maintain ion balance, improve photosynthetic performance, enhance antioxidant enzyme activities, and increase the uptake of essential nutrients such as phosphorus and zinc [21–24]. AMF also promote the accumulation of specific phenolic compounds such as chlorogenic acid, caffeic acid, and ferulic acid, strengthen cell walls, and activate antioxidant systems, which together improve plant defense [14,15]. These functions suggest that AMF may also contribute to resistance against parasitic weeds. *O. cumana* is one of the most serious biotic constraints in sunflower production because it directly connects with host roots and absorbs water, nutrients, and assimilates. Although AMF have been suggested to influence parasite infection through changes in root development and plant defense, the molecular mechanisms involved remain largely unknown. Therefore, further studies are needed to understand how AMF regulate sunflower resistance under different types of stress.

To elucidate the molecular mechanisms underlying AMF-mediated stress resistance, physiological analyses alone are insufficient. Transcriptomics can identify stress-responsive genes and biological pathways, while metabolomics directly reflects changes in metabolites associated with plant responses [25,26]. These two approaches provide complementary information and help connect changes in gene expression with metabolic regulation [27]. Integrated transcriptome and metabolome analyses have become powerful tools for studying plant adaptation to environmental stresses and for identifying important processes such as antioxidant defense, secondary metabolism, cell wall remodeling, and lipid metabolism [28]. However, most previous AMF studies have focused on individual stresses or single biological levels, and the common and stress-specific regulatory mechanisms activated by AMF remain unclear.

Although AMF have been widely studied for improving plant growth and stress tolerance, the mechanisms by which they coordinate responses to multiple abiotic and biotic stresses remain poorly understood [29,30]. In particular, integrated analyses of AMF-mediated resistance against drought, salinity stress, and *O. cumana* infection in sunflower are limited. It remains unclear whether AMF activate shared defense pathways or stress-specific regulatory networks under different challenges. In this study, we investigated AMF-mediated responses in sunflower under drought, salinity, and *O. cumana* stresses by combining physiological measurements with transcriptomic and metabolomic analyses. We aimed to (i) characterize changes in root traits and physiological responses induced by AMF, (ii) identify key genes and metabolic pathways involved in stress adaptation, and (iii) construct integrated gene–metabolite networks to reveal common and specific mechanisms underlying AMF-enhanced resistance. Our results provide new insights into the role of AMF in improving sunflower stress tolerance and support the application of AMF as a sustainable strategy for crop improvement and biofortification.

2. Materials and Methods

2.1. Plant Materials and AMF Inoculation

Four sunflower (*Helianthus annuus* L.) cultivars with different levels of resistance to *Orobancha cumana* were used in this study. The resistant cultivar Tonghui 15 and three susceptible cultivars, Shuangxing 60, SH363, and SH361, were provided by the Institute of Plant Protection, Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences. The susceptibility levels of these cultivars increased in the order of Tonghui 15, Shuangxing 60, SH363, and SH361. Seeds of *Orobancha cumana* Wallr. were obtained from related research institutions in Inner Mongolia.

The arbuscular mycorrhizal fungus (AMF) used in this study was *Funneliformis mosseae* (BGC NM02A, accession number 1511C0001BGCAM0045), with a spore density of 297 spores per 10 g inoculum. The AMF inoculum was propagated using white clover (*Trifolium repens* L.) as the host plant for approximately three months. After propagation, the rhizosphere substrate containing AMF spores, hyphae, and colonized root fragments was collected and mixed with sterilized sand at a volume ratio of 1:1. The resulting mixture was used as the AMF inoculum. AMF colonization was evaluated by root staining, and detailed colonization data are provided in Supplementary Table S1. The soil used in all experiments was sterilized by autoclaving at 121 °C for 30 min on two consecutive days before planting to eliminate indigenous soil microorganisms.

2.2. Experimental Design and Stress Treatments

Pot experiments were conducted in plastic pots (20 cm diameter × 25 cm height). Plant growth conditions were maintained as described in our previous study [31,32], with modifications to the stress treatments as detailed in Supplementary Table S2. In the preliminary screening experiment, four sunflower cultivars were grown with or without AMF inoculation, with six biological replicates per treatment (48 pots in total). Among the susceptible cultivars, SH361 showed the highest susceptibility to *O. cumana*. In addition, Mantel analysis revealed stronger associations between AMF and *O. cumana* treatments and physiological traits in SH361 than in the other susceptible cultivars (Figure S2). Therefore, SH361 was selected for the subsequent physiological, transcriptomic, and metabolomic analyses.

Eight treatments were established: control (CK), salinity stress (S), drought stress (D), *O. cumana* parasitism (OR), AMF inoculation (A), AMF combined with salinity stress (AS), AMF combined with drought stress (AD), and AMF combined with *O. cumana* parasitism (AO), with three biological replicates per treatment. AMF inoculum and *O. cumana* seeds were applied according to established protocols, while salinity and drought treatments

were imposed at the designated growth stage. All plants were maintained under the same growth conditions except for the specific treatments.

Samples were collected at the six-leaf stage. Plants were carefully removed and washed, and roots and shoots were separated. Fresh tissues were immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses. Detailed information on stress intensity, treatment duration, and *O. cumana* inoculation is provided in Supplementary Table S2.

2.3. Physiological and Biochemical Measurements

2.3.1. Determination of Photosynthetic Pigments

The contents of chlorophyll and carotenoids were measured according to the acetone extraction method following our previously established protocol [32]. Briefly, 0.2 g fresh leaf samples were homogenized and extracted with 10 mL of 80% acetone in darkness at room temperature until the tissues became completely white. The absorbance values were measured at 645, 663, 652, and 470 nm using a spectrophotometer (Hitachi F-4600, Hitachi High-Tech, Tokyo, Japan).

2.3.2. Determination of Oxidative Stress and Antioxidant Enzyme Activities

Hydrogen peroxide (H_2O_2) content and antioxidant enzyme activities were determined using fresh plant tissues extracted under ice-cold conditions. For H_2O_2 determination, 0.5 g of fresh tissue was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA), followed by centrifugation at $12,000\times g$ for 15 min at 4°C . The H_2O_2 content was determined using the potassium iodide (KI) method by measuring absorbance at 390 nm.

Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities were determined using the NBT photoreduction, guaiacol oxidation, and H_2O_2 decomposition methods, respectively. The SOD reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and enzyme extract. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction. POD activity was determined by monitoring the increase in absorbance at 470 nm, while CAT activity was determined by monitoring the decrease in absorbance at 240 nm. Detailed procedures for the determination of H_2O_2 content and antioxidant enzyme activities were performed according to our previous study [33].

2.3.3. Determination of Osmotic Regulators and Secondary Metabolites

Proline, total phenolic, and flavonoid contents were determined to evaluate osmotic adjustment and secondary metabolic responses, following previously established protocols used by our group [32]. Proline content was measured using the sulfosalicylic acid–acid ninhydrin method, with absorbance recorded at 520 nm after toluene extraction. Total phenolic content was determined using the Folin–Ciocalteu method with gallic acid as the standard, and absorbance was measured at 760 nm. Flavonoid content was determined using the aluminum chloride colorimetric method with rutin as the standard, and absorbance was measured at 510 nm.

2.4. Transcriptome Sequencing and Analysis

Total RNA was extracted from sunflower root samples using a standard RNA extraction method. The quality and concentration of RNA were evaluated before library construction. Messenger RNA (mRNA) was enriched using oligo(dT)-attached magnetic beads and used for cDNA library preparation. RNA sequencing was performed using the Illumina NovaSeq platform with paired-end 150 bp sequencing (PE150). The sequencing data showed Q20 and Q30 values above 85% and 80%, respectively.

The raw sequencing reads were processed by removing adaptor sequences and low-quality reads to obtain clean reads. The clean reads were mapped to the sunflower reference genome (HanXRQr2), and gene expression levels were calculated based on transcripts per million (TPM). Differentially expressed genes (DEGs) were identified using statistical analysis with the criteria of $|\log_2(\text{fold change})| > 1.5$ and adjusted p -value (padj) < 0.01 .

Functional annotation of DEGs was performed using Gene Ontology (GO) enrichment analyses. To identify gene expression patterns associated with AMF-mediated stress responses, soft clustering analysis was performed using the Mfuzz (version 2.56.0) package in R (version 4.2.1). Gene clusters with similar expression trends were further analyzed to identify biological processes involved in stress adaptation.

2.5. Untargeted Metabolomics Analysis

Root samples from different treatments were collected for untargeted metabolomic analysis. Frozen samples were ground in liquid nitrogen, extracted with an appropriate solvent, and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) after centrifugation and filtration. Untargeted metabolomics analysis was performed by Beijing Tsingke Biotech Co., Ltd. (Beijing, China) using an ultra-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UPLC-MS/MS, Thermo Fisher Scientific, Waltham, MA, USA) platform.

Methanol (Merck, Darmstadt, Germany), acetonitrile (Shanghai Xingke, Shanghai, China), and formic acid (Aladdin, Shanghai, China) were used as analytical reagents. 2-Chlorophenylalanine (J&K Scientific, Beijing, China) was used as the internal standard. For the internal standard solution, 1 mg of the standard was dissolved in 1 mL of 70% methanol in water to prepare a 1000 $\mu\text{g/mL}$ stock solution. The stock solution was further diluted with 70% methanol to obtain an internal standard solution at a concentration of 250 $\mu\text{g/mL}$. For chromatographic separation, an ACQUITY UPLC HSS T3 column (1.8 μm , 2.1×100 mm; Waters) was used at 40 °C with a flow rate of 0.40 mL/min and an injection volume of 4 μL . The mobile phases were water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The gradient started at 95% A and 5% B, followed by a linear gradient to 25% A and 75% B within 2 min, and then to 1% A and 99% B within the next 2 min. This composition was maintained for 1.5 min. The mobile phase was then returned to 95% A and 5% B within 0.1 min and maintained for 1.4 min.

Mass spectrometric analysis was performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionization in both positive and negative ion modes. The acquisition alternated between full-scan MS and data-dependent MS/MS scans with dynamic exclusion. The full-scan mass range was m/z 84–1250 at a resolution of 35,000. The ion spray voltage was set to 3.5 kV in positive mode and 3.2 kV in negative mode. Sheath gas and auxiliary gas were set to 30 and 5 arbitrary units, respectively. The ion transfer tube and vaporizer temperatures were 320 °C and 300 °C, respectively. Collision energies of 30, 40, and 50 V were applied. The signal intensity threshold was set to 1×10^6 cps, and the acquisition was performed using a Top N setting of 10 with an exclusion duration of 3 s.

The raw mass spectrometry data were converted to mzML format using ProteoWizard and processed using XCMS for peak detection, alignment, and retention-time correction. Features missing in more than 50% of the samples were removed. For missing-value processing, features with blank values exceeding 50% were imputed using one-fifth of the minimum value, whereas other missing values were imputed using the K-nearest neighbors (KNN) method. Peak areas were further corrected using support vector regression (SVR). The corrected features were annotated by searching an in-house database, integrated public databases, prediction databases, and the metDNA-based metabolite identification

method. Metabolites with a comprehensive identification score ≥ 0.5 and a coefficient of variation (CV) < 0.5 in quality control (QC) samples were retained for subsequent analyses. Metabolites detected in both positive and negative ion modes were merged, and when the same metabolite was detected more than once, the metabolite with the highest identification level and score was retained. The resulting metabolite matrix was used for subsequent statistical and differential metabolite analyses.

Multivariate analyses, including OPLS-DA, were performed to assess metabolic differences among treatments. Differentially accumulated metabolites (DMs) were identified using variable importance in projection (VIP) values and statistical significance, with $|\log_2\text{FoldChange}| > 1.0$ and $p < 0.05$ as the screening criteria. Differential metabolites were further classified by chemical category, and pathway enrichment analysis was performed to identify metabolic pathways associated with AMF-mediated stress responses.

2.6. Integrated Transcriptome and Metabolome Analysis

To investigate the relationship between transcriptional regulation and metabolic changes, integrated transcriptome and metabolome analyses were performed. Differentially expressed genes and differentially accumulated metabolites identified from the same treatment comparisons were combined for correlation analysis.

A nine-quadrant analysis was performed based on the fold changes in genes and metabolites. Genes and metabolites were divided into nine groups according to their expression patterns. The third quadrant and seventh quadrant, representing metabolites and genes with consistent decreasing or increasing trends, respectively, were selected as potential coordinated regulatory pairs.

To identify reliable gene–metabolite relationships, Spearman correlation analysis was performed among biological replicates. Gene–metabolite pairs with $|\text{Spearman correlation coefficient}| > 0.7$ were considered highly correlated and were used for further network construction. These integrated networks were used to identify candidate genes and metabolites potentially involved in AMF-mediated resistance responses.

2.7. RNA Isolation and qRT-PCR

Total RNA from the leaves and roots of sunflower plants was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China) following the manufacturer's instructions. The primers used for qRT-PCR are provided in Table S3. ACT was used as the reference gene. qPCR was performed using the SuperStar Universal SYBR Master Mix (CW3360) (CWBio, Beijing, China) in the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher). The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method with three biological replicates. The expression patterns of representative candidate genes were further examined by qRT-PCR, and the results were generally consistent with the transcriptome data (Figure S1), supporting the reliability of the RNA-seq analysis.

2.8. Statistical Analysis

All physiological experiments were performed with at least three biological replicates, and data were expressed as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using R software. Differences among treatments were evaluated using analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$. The radar chart was generated using normalized mean values to visualize overall response trends. Statistical analyses were performed on the original biological replicate data, and the results are presented in the corresponding figure panels and Supplementary Tables. Differentially expressed genes were identified using DESeq2. GO enrichment analyses were performed using clusterProfiler.

Metabolic pathway enrichment analysis was performed using the fast gene set enrichment analysis (fgsea) method. Metabolites were ranked according to their statistical ranking values obtained from the comparison between AMF-inoculated and non-inoculated samples, and the ranked metabolite list was used as the input for pathway enrichment analysis. KEGG metabolic pathway sets were used as the reference pathway database. For each pathway, the enrichment score (ES), normalized enrichment score (NES), nominal p value, and adjusted p value were calculated.

The fgsea analysis was used to evaluate coordinated changes in metabolites belonging to the same metabolic pathway and to provide pathway-level information complementary to the analysis of individual differential metabolites. Positive and negative NES values indicated enrichment toward the higher and lower ends of the ranked metabolite list, respectively. Because the pathway-level analysis was primarily used to explore coordinated metabolic trends, pathways with nominal p values < 0.1 and relatively strong NES values were considered indicative of pathway-level trends and were used for exploratory biological interpretation. Adjusted p values were calculated to account for multiple testing and were reported together with the nominal p values. Pathways that did not meet the multiple-testing significance threshold were not interpreted as statistically significant enrichment.

Correlation analysis and data visualization were performed using appropriate R packages. Heatmaps, enrichment analyses, and network visualization were generated based on normalized expression or metabolite abundance data.

3. Results

3.1. AMF-Associated Phenotypic and Physiological Responses of Sunflower Under Multiple Stresses

Root development is closely associated with plant nutrient acquisition and stress adaptation. In this study, differences in root phenotypes were visually observed between AMF-inoculated and non-inoculated plants across the four sunflower varieties (Figure 1). AMF-inoculated plants generally showed more developed root systems and more visible lateral root formation than non-inoculated plants. To provide quantitative support for these observations, root fresh weight was measured in the four sunflower varieties. AMF-inoculated plants showed higher root fresh weight than the corresponding non-inoculated plants in all four varieties ($p < 0.01$ for TH15 and SX60, and $p < 0.05$ for SH361 and SH363; Figure S3). The increase was particularly evident in TH15 and SX60, while moderate increases were observed in SH361 and SH363. These quantitative results support the observed differences in root development, whereas Figure 1 provides representative images of the associated root phenotypes.

To further investigate the physiological responses mediated by AMF under different stresses, the susceptible cultivar SH361 was selected for subsequent analysis based on its sensitivity to environmental variations. A radar chart was constructed to summarize the changes in antioxidant enzymes, osmotic adjustment substances, reactive oxygen species, and secondary metabolites in roots and leaves under salinity (S), drought (D), and *O. cumana* parasitism (OR) with or without AMF inoculation (Figure 2).

AMF colonization rates were quantified and are presented in Supplementary Table S1. The colonization rate was significantly higher in AMF-inoculated plants compared to non-inoculated controls, confirming successful establishment of the symbiosis.

Different stresses induced distinct physiological response patterns in SH361, with clear organ-specific characteristics. Under salinity stress, root tissues exhibited increased activities of SOD and POD, suggesting that roots were actively involved in the perception and mitigation of salt-induced oxidative stress. Under drought conditions, leaf tissues showed enhanced accumulation of proline, accompanied by increased antioxidant activity,

indicating the involvement of osmotic regulation and ROS scavenging mechanisms. In contrast, *O. cumana* parasitism induced a stronger secondary metabolic response, with elevated POD activity and total phenolic accumulation in leaves, reflecting the activation of defense-related metabolic pathways against biotic stress.



Figure 1. Effects of arbuscular mycorrhizal fungi (AMF) on root phenotypes of different sunflower varieties. Panels (A–H) show the root phenotypes of four different resistant sunflower varieties under control (uninoculated) and AMF inoculation, respectively. ((A,C,E,G) are controls; (B,D,F,H) are AMF treatments).

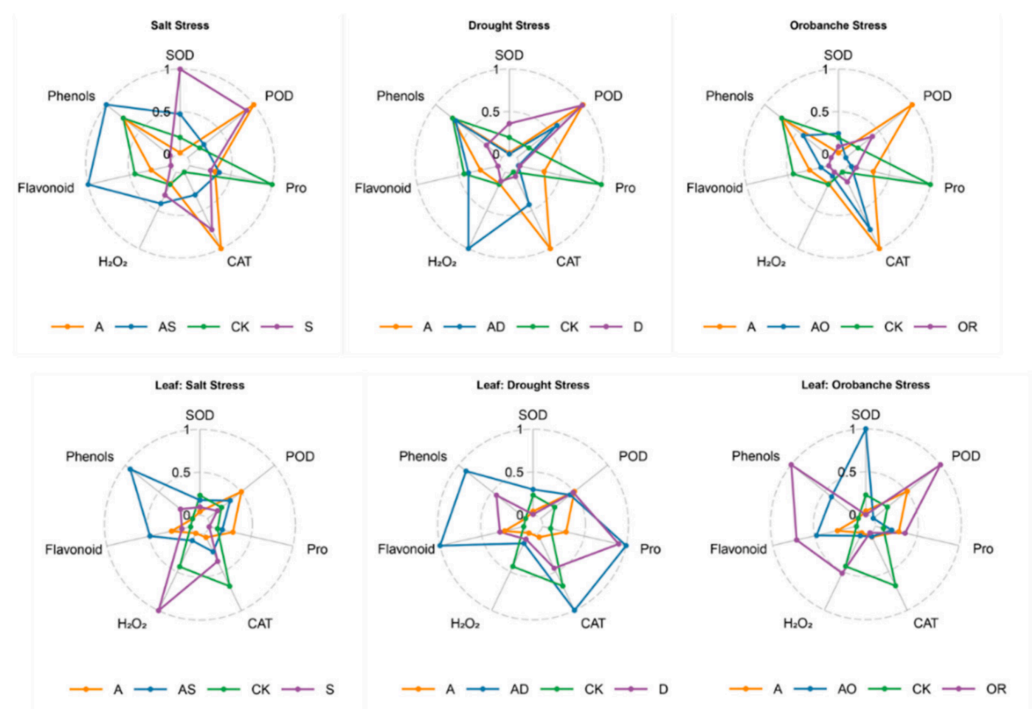


Figure 2. Radar chart analysis of physiological characteristics of roots and leaves of sunflower SH361 under multiple stresses and AMF regulation. Polygon vertices represent the levels of antioxidant enzymes (SOD, POD, CAT), osmotic regulators (Pro), and secondary metabolites (Flavone, total phenol Phen) and reactive oxygen species (H_2O_2) in roots and leaves, respectively. The data were normalized. Control (CK), salinity stress (S), drought stress (D), *O. cumana* parasitism (OR), AMF inoculation (A), AMF combined with salinity stress (AS), AMF combined with drought stress (AD), and AMF combined with *O. cumana* parasitism (AO).

Interestingly, AMF inoculation alone increased the activities of CAT and POD even under non-stress conditions, suggesting that AMF colonization promoted a primed defense state in sunflower. When combined with stress treatments, AMF further enhanced stress-responsive physiological adjustments. Under salinity stress, AMF inoculation increased the accumulation of flavonoids and total phenolics in roots while reducing H_2O_2 accumulation in leaves. Under drought conditions, AMF treatment increased leaf proline accumulation and CAT activity, suggesting improved osmotic adjustment and antioxidant defense.

Overall, these results demonstrate that AMF improved sunflower stress adaptation through coordinated regulation of root development, antioxidant defense, osmotic adjustment, and secondary metabolism. The physiological responses induced by AMF were not limited to a single stress type but showed a broad protective effect against salinity, drought, and *O. cumana* parasitism, providing a physiological foundation for further investigation of AMF-mediated molecular regulation.

3.2. Transcriptomic Analysis Reveals AMF-Mediated Gene Regulatory Networks

To investigate how AMF regulates sunflower responses to different environmental stresses, transcriptome sequencing was performed using root samples of SH361 under control, salinity, drought, and *O. cumana* parasitism conditions with or without AMF inoculation. Sample correlation analysis revealed distinct expression patterns among treatment groups, while biological replicates exhibited high similarity, indicating the reliability of the transcriptomic dataset (Figure 3A).

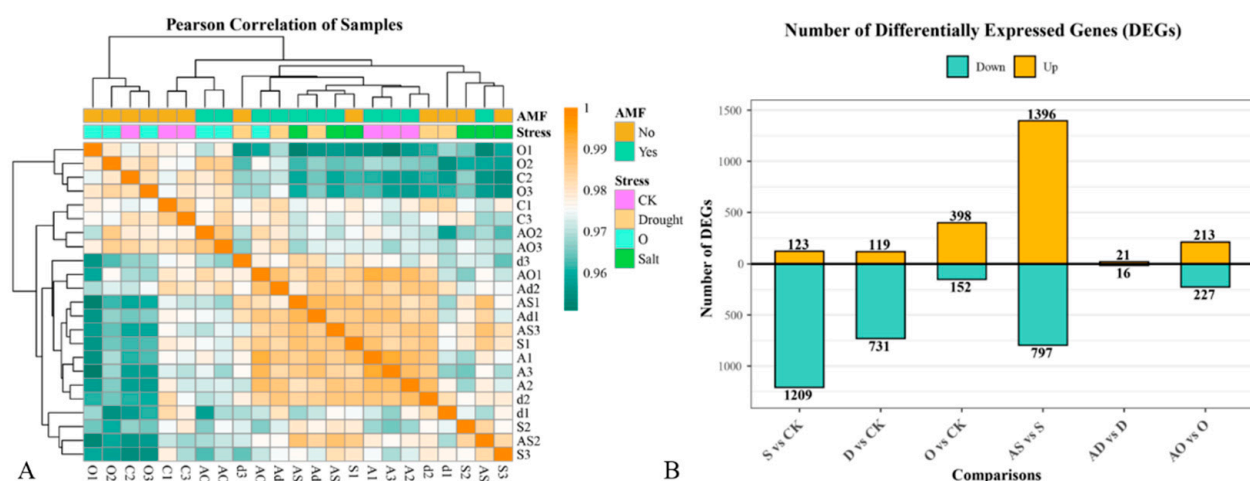


Figure 3. Correlation and differential gene expression profiles of sunflower root transcriptome samples. (A) Pearson correlation heat map between samples of each treatment group shows the clustering and data homeostasis of biological repetition of samples under different treatments. (B) Statistical map/volcano map of differentially expressed genes (DEGs) under different stress and AMF inoculation conditions. The control group included salinity stress (S vs. CK), drought stress (D vs. CK), *Orobancha parasitica* (O vs. CK), and response to AMF inoculation under stress background (AS vs. S, AD vs. D, AO vs. O). The up-regulated and down-regulated genes were marked with different colors.

Differential expression analysis revealed distinct transcriptional responses induced by different stresses and AMF treatments (Figure 3B). Compared with the control condition, salinity and drought stresses resulted in predominant downregulation of gene expression, with 1209 and 731 downregulated genes identified, respectively. In contrast, *O. cumana* parasitism induced a stronger transcriptional activation pattern, with more upregulated genes than downregulated genes (398 versus 152 genes). These results indicate that sunflower

roots exhibited stress-specific transcriptional response patterns depending on the type of environmental challenge.

AMF inoculation further reshaped the transcriptional profiles under stress conditions. Under salinity stress, AMF treatment induced a large number of differentially expressed genes, with 1396 upregulated genes identified in the AS vs. S comparison. In contrast, AMF regulation under *O. cumana* parasitism showed a more balanced expression pattern, with 213 upregulated and 227 downregulated genes. Only a limited number of DEGs were detected between AMF-treated and non-treated plants under drought conditions (AD vs. D), suggesting that AMF-mediated drought adaptation may involve regulatory mechanisms beyond transcriptional changes at the sampling stage.

To identify common transcriptional responses associated with AMF colonization across different environmental conditions, Mfuzz soft clustering analysis was performed based on gene expression dynamics (Figure 4). Among the identified clusters, Cluster 1 containing 4088 genes showed a highly consistent expression pattern. Genes within this module were consistently upregulated following AMF inoculation under control, salinity, drought, and *O. cumana* conditions, indicating that these genes represent a core AMF-responsive transcriptional module across different stress environments.

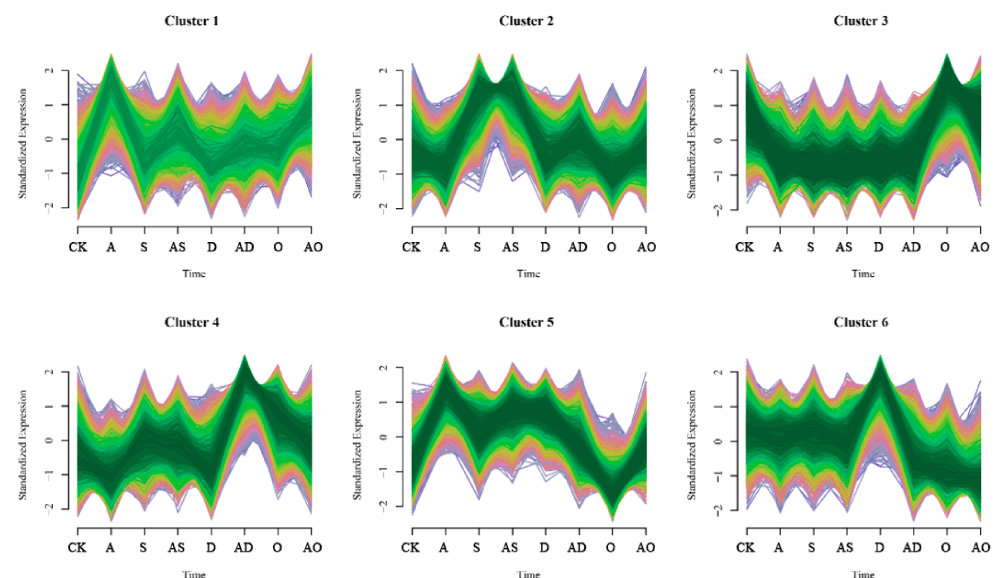


Figure 4. Soft clustering analysis of multiple conditional gene expression dynamics based on Mfuzz. Global gene expression trend patterns of sunflower roots under multiple stress and symbiotic treatment trajectories. The genes in the Cluster 1 module showed a highly consistent up-regulation trend after inoculation with AMF under normal (CK), salinity (Salt), drought (Drought) and *Orobanchae parasitism* (O) conditions, representing the core non-specific response gene group induced by AMF.

Functional enrichment analysis was subsequently performed to characterize the biological functions of Cluster 1 genes (Figure 5). GO enrichment analysis showed that AMF-responsive genes were mainly associated with structural reinforcement and secondary metabolism. The most significantly enriched biological processes included phenylpropanoid metabolic process and phenylpropanoid biosynthesis, followed by cell wall biogenesis, xylan biosynthetic process, and oxidoreductase activity. The tree plot in Figure 5 summarizes the functional relationships among these enriched GO terms, showing that terms related to cell wall organization, phenylpropanoid metabolism, and oxidoreductase activity cluster into distinct functional groups. These results suggest that AMF-induced transcriptional regulation contribute to enhanced structural protection and antioxidant capacity.

Collectively, transcriptomic analysis revealed that AMF induced a conserved transcriptional response across diverse stress conditions. This response was mainly associated with cell wall modification, phenylpropanoid metabolism, lipid remodeling, and secondary metabolite biosynthesis, providing a molecular basis for enhanced stress adaptation in sunflowers.

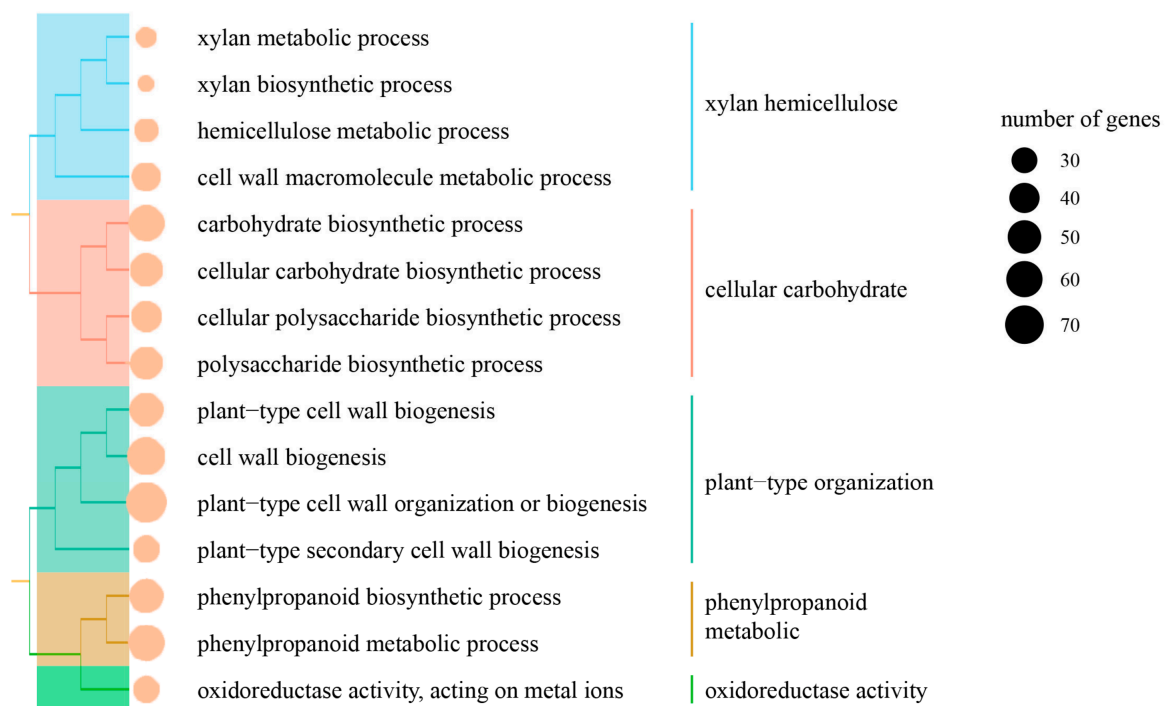


Figure 5. Functional enrichment analysis of AMF core up-regulated gene cluster (Cluster 1). The figure mainly shows the significant enrichment processes related to structural reinforcement and secondary metabolism. The key items included phenylpropanoid metabolism and its biosynthetic pathway, cell wall construction, and oxidoreductase activity. The tree was generated using the treeplot () function to visualize functional relationships among 10 representative enriched GO terms. These terms were manually selected from significantly enriched GO terms based on their biological relevance to major functional categories. Branch lengths and clustering indicate functional similarity among GO terms. The colors denote major functional categories, and the circle sizes represent the number of genes annotated to each term.

3.3. Metabolomic Analysis Reveals AMF-Induced Metabolic Reprogramming

To investigate the global metabolic changes induced by arbuscular mycorrhizal fungus (AMF) colonization, an overall orthogonal partial least squares discriminant analysis (OPLS-DA) model was constructed using all root metabolome samples. The OPLS-DA score plot showed a clear separation between AMF-inoculated and non-inoculated samples, indicating that AMF colonization substantially altered the metabolic profiles of sunflower roots (Figure S3). The model showed high explanatory and predictive performance, with R^2Y and Q^2 values of 0.986 and 0.599, respectively. Furthermore, a 200-time permutation test confirmed the robustness of the model, with low intercept values for pR^2Y (0.01) and pQ^2 (0.005), suggesting that the observed metabolic separation was reliable and not caused by model overfitting.

Based on the overall comparison between AMF-inoculated and non-inoculated samples, differential metabolites (DMs) were identified to characterize the metabolic response associated with AMF colonization (Figure 6). The results showed that AMF induced extensive metabolic remodeling in sunflower roots, indicating that fungal symbiosis strongly influenced host metabolic profiles. Since different stress conditions may impose additional effects on metabolic regulation, subsequent analyses further focused on specific stress

backgrounds to characterize AMF-associated metabolic responses under salinity, drought, and *Orobanch* parasitism.

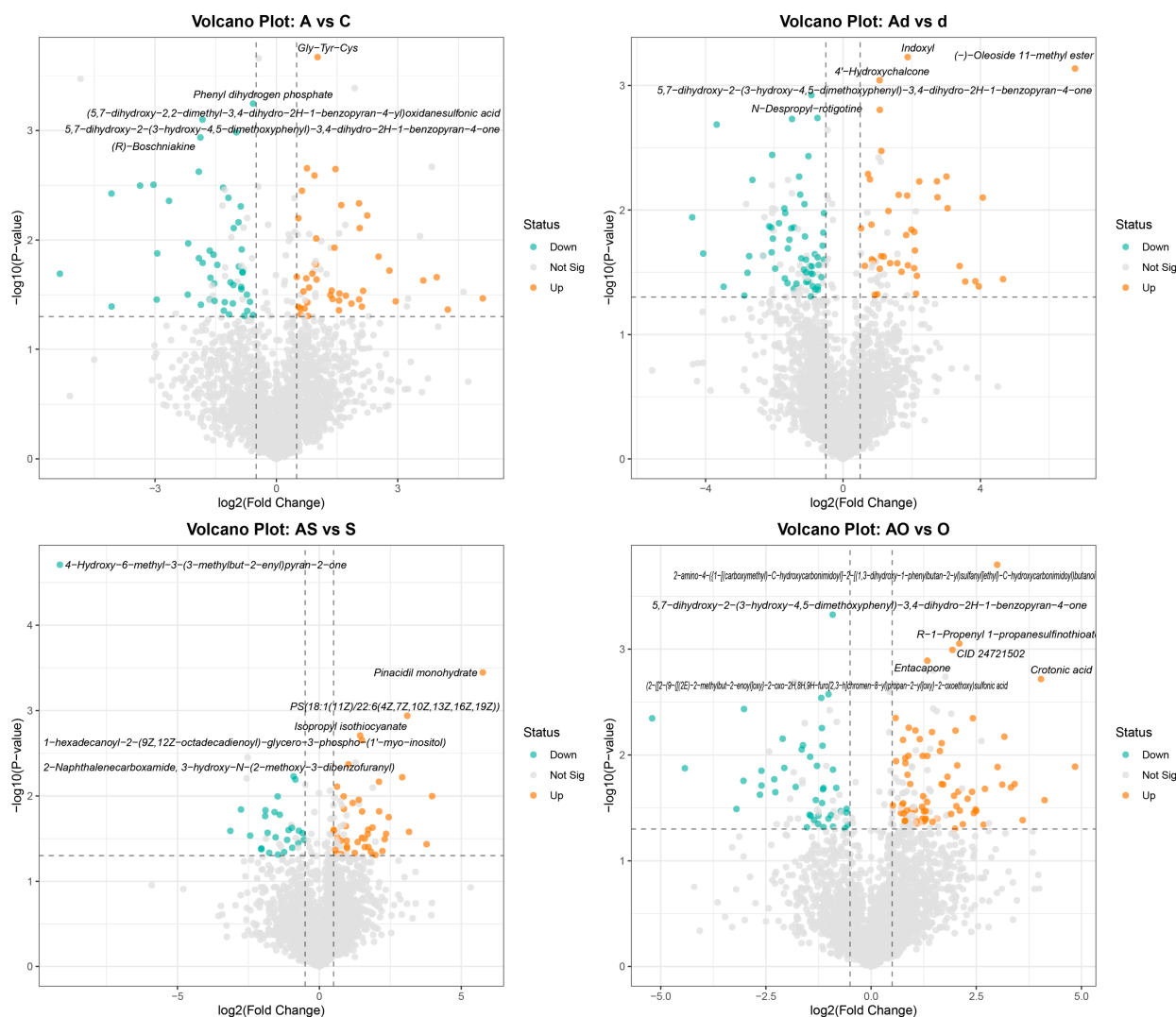


Figure 6. Effects of AMF inoculation on global metabolite expression in sunflower roots. Screening and distribution of differential metabolites (DMs) between AMF-inoculated and non-inoculated groups.

To further classify the chemical characteristics of AMF-responsive metabolites, all identified DMs were categorized according to metabolite ontology (Figure 7). A total of 14 major chemical classes were identified. Among them, organic acids (60 metabolites), amino acids and derivatives (44 metabolites), benzene derivatives (55 metabolites), and phenolic acids (15 metabolites) represented the major proportion of altered metabolites. In addition, several important secondary metabolite groups, including flavonoids (7 metabolites), lignans and coumarins (9 metabolites), and alkaloids (18 metabolites), also exhibited significant changes after AMF inoculation. Lipid-related compounds, including lipids (9 metabolites) and glycerophospholipids (11 metabolites), were also affected by AMF treatment.

The distribution pattern of differential metabolites suggested that AMF-mediated metabolic regulation mainly involved three functional categories. First, the strong alteration of organic acids and amino acid derivatives indicated changes in primary carbon and nitrogen metabolism. Second, the enrichment of phenolic acids, flavonoids, and alkaloids suggested that AMF was associated with changes in the accumulation of defense-associated secondary metabolites. Third, the alteration of lipid and glycerophospholipid metabolites

was consistent with transcriptomic evidence showing the involvement of lipid-related metabolic pathways.

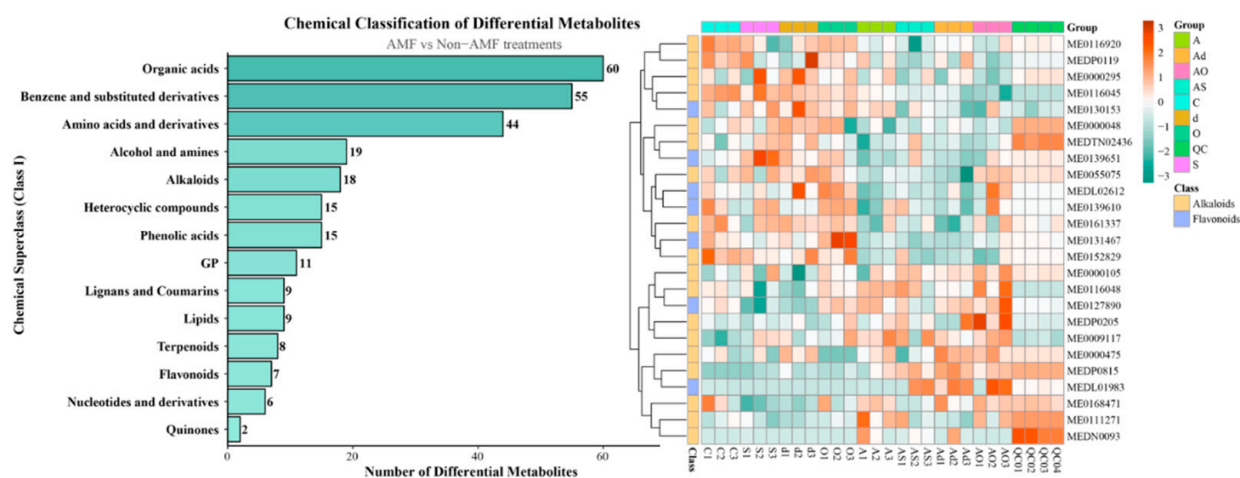


Figure 7. The fine classification of global differential metabolites Ontology. Based on the chemical classification ontology attributes, the global differential metabolites significantly induced by AMF were finely divided into 14 major chemical components. The column diagram highlights the proportion and expression fluctuations of core components such as organic acids, amino acids and derivatives, benzene and its derivatives, phenolic acids, flavonoids, alkaloids, and lipids in the total differential metabolites.

To further evaluate metabolic pathway-level changes, gene set enrichment analysis based on metabolite ranking (fgsea) was performed using the global differential metabolite dataset (Figure 8). Due to the limited annotation coverage of plant-specific secondary metabolites in KEGG databases, most pathways did not remain significant after multiple testing correction. However, several biologically relevant metabolic trends were identified based on normalized enrichment scores (NES).

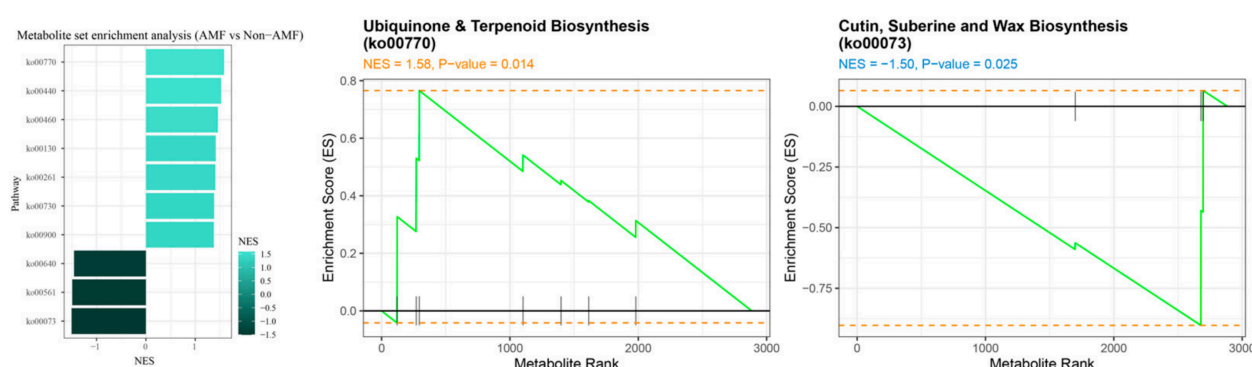


Figure 8. The fgsea trend distribution of the global network of differential metabolites. The figure shows the trend of normalized enrichment score (NES) of the sunflower root metabolic network mediated by AMF. Positive enrichment (NES > 0) highlighted the synergistically upregulated defense and signal transduction pathways (such as phosphoinositide metabolism, amino acid metabolism, and secondary metabolic pathways). Negative enrichment (NES < 0) showed down-regulated physical barriers and lipid pathways (such as cutin/cork/wax biosynthesis and glyceride metabolism).

Several defense and signaling-related pathways showed positive enrichment trends following AMF inoculation, including phosphoinositide metabolism (ko00440), amino acid-related metabolism (ko00460), vitamin B6 metabolism (ko00770), and secondary metabolism-associated pathways (ko00261), with NES values ranging from approximately

1.3 to 1.6. In contrast, pathways associated with cutin, suberin, and wax biosynthesis (ko00073) and glycerolipid metabolism (ko00561) showed negative enrichment trends.

Together, metabolomic analyses demonstrated that AMF colonization resulted in extensive metabolic reprogramming in sunflower roots, involving primary metabolism adjustment, secondary defense compound accumulation, and lipid-associated metabolic changes. These metabolic alterations, together with transcriptomic responses, provided a foundation for further integrated analysis of gene–metabolite regulatory relationships.

3.4. Integrated Transcriptomic and Metabolomic Analysis Reveals Key Molecular Associations Underlying AMF-Mediated Stress Adaptation

To further investigate the molecular interactions between transcriptional regulation and metabolic reprogramming during AMF-mediated stress adaptation, an integrated transcriptomic and metabolomic analysis was performed based on the AO vs. O comparison. A nine-quadrant plot was constructed by combining the fold changes in differentially expressed genes (DEGs) and differential metabolites (DMs), allowing the identification of molecular pairs showing coordinated changes at both transcriptional and metabolic levels (Figure 9).

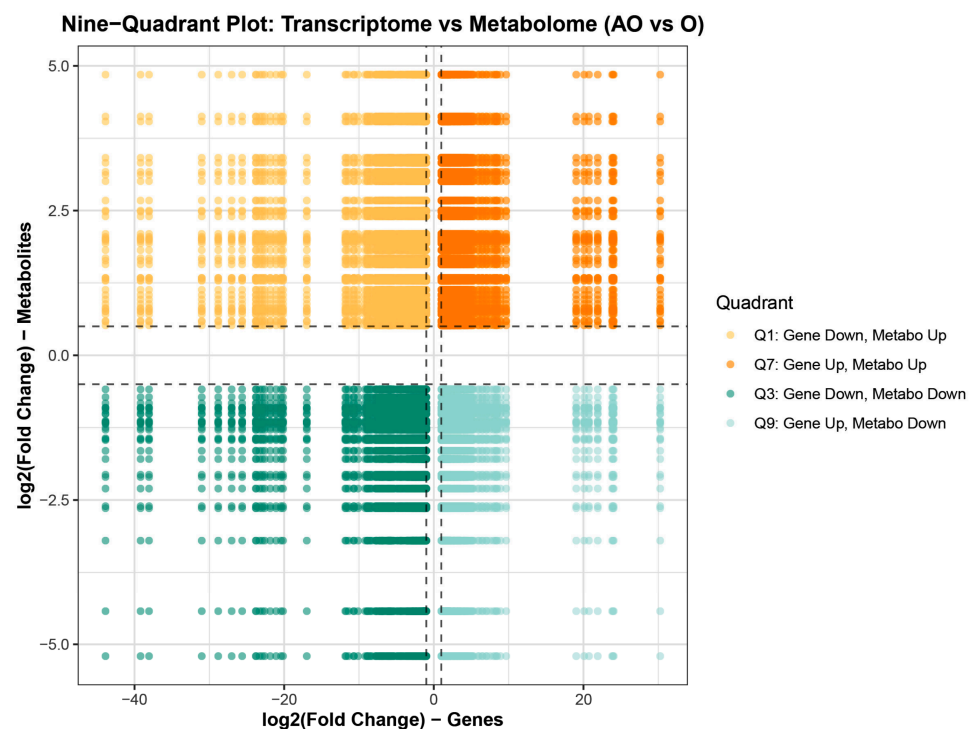


Figure 9. Nine-quadrant plot of co-regulation of transcription and metabolism under AO and O treatment. Scatter points represent gene and metabolite pairs with quantitative information in the comparison of *O.cumana* parasitism combined with AMF treatment (AO) and *O.cumana* parasitism only (O). The horizontal and vertical coordinates are the $\log_2$ Fold Change values of genes and metabolites, respectively. The third and seventh quadrants represent synergistic down-regulated and synergistic up-regulated molecules, respectively. High-confidence combinations filtered by Spearman correlation ($|r| > 0.7$) were highlighted in the plot.

The nine-quadrant analysis revealed that numerous gene–metabolite pairs were distributed mainly in quadrant III (co-downregulated) and quadrant VII (co-upregulated), suggesting coordinated changes between transcript accumulation and metabolite variation after AMF inoculation under *Orobancha* parasitism. To further identify high-confidence associations, candidate gene–metabolite pairs were subjected to Spearman correlation analysis across all samples. By integrating correlation coefficients and statistical significance,

a total of 40 highly correlated gene–metabolite pairs were identified, and the detailed information for these pairs is provided in Table S4.

Among these associations, several genes involved in primary metabolism and symbiotic responses showed strong relationships with corresponding metabolites. For example, HanXRQr2_Chr09g0408921, annotated as malate dehydrogenase (MDH), showed significant upregulation in the AO treatment compared with *Orobanche* infection alone ($\log_2FC = 2.19$). Meanwhile, its associated metabolite, stearyl citrate, exhibited a similar increasing trend. As MDH is an important enzyme involved in the tricarboxylic acid cycle, the coordinated alteration of MDH expression and organic acid-related metabolites suggested a potential adjustment of carbon metabolism during AMF-mediated adaptation.

Another candidate gene, HanXRQr2_Chr08g0317511, was annotated as a Nodulin MtN21/EamA-like amino acid transporter. This gene was significantly induced under AO treatment ($\log_2FC = 1.68$) and showed a significant association with amino acid derivatives, including N-methyl-D-aspartic acid. Nodulin-related transport proteins have been reported to participate in metabolite transport and plant–microbe symbiotic processes. Therefore, the coordinated changes between this transporter gene and amino acid-related metabolites may reflect the regulation of nutrient exchange and nitrogen-associated metabolism during AMF-associated stress responses.

In addition, HanXRQr2_Chr01g0042921, encoding a flavin-containing monooxygenase belonging to the YUCCA family (YUC6), exhibited significant induction in the AO treatment ($\log_2FC = 2.10$). This gene showed a significant negative correlation with phosphatidylglycerol (PG)-related metabolites. Given that YUCCA family members are involved in auxin biosynthesis and auxin signaling contributes to root development and plant–microbe interactions, this association suggested a potential link between hormone-related regulation and membrane lipid remodeling during AMF-mediated adaptation.

Collectively, the integrated transcriptomic and metabolomic analysis identified coordinated molecular changes associated with carbon metabolism, nitrogen transport, hormone signaling, and lipid metabolism under AMF and *Orobanche* interactions. These gene–metabolite associations provide potential molecular candidates for understanding how AMF enhances sunflower stress adaptation through coordinated changes at transcriptional and metabolic levels.

4. Discussion

Arbuscular mycorrhizal fungi (AMF) are increasingly recognized as important regulators of plant stress adaptation rather than simple nutrient providers. AMF inoculation enhanced sunflower tolerance to multiple stresses, including salinity, drought, and *Orobanche* parasitism. A notable finding was that AMF alone induced the activation of antioxidant-related responses, as indicated by increased CAT and POD activities even under non-stress conditions. This suggests that AMF may establish a systemic priming state in sunflower, enabling plants to respond more rapidly when exposed to subsequent environmental challenges [30,34–39].

This concept is consistent with previous studies showing that AMF colonization can prepare plants for future stress by modifying defense signaling pathways while maintaining relatively low metabolic costs under favorable conditions [34–38]. Unlike direct stress responses, which often require extensive resource allocation, AMF-mediated priming provides a more balanced strategy by maintaining growth while enhancing stress responsiveness [34,36,37]. The identification of a common AMF-responsive gene module (Cluster 1) across different stress conditions further supports this idea, indicating that AMF may activate a conserved molecular program in addition to stress-specific responses [30,34–38].

The improved stress tolerance of AMF-colonized sunflower was closely related to changes in root architecture and physiological responses. Roots are important for plant adaptation because they directly interact with soil water, nutrients, and microorganisms. Across different sunflower varieties, AMF consistently promoted lateral root development across different genetic backgrounds. This suggests that AMF-induced changes in root architecture may help plants acquire more water and nutrients. Previous experimental and meta-analysis studies support this view. AMF colonization can change root system architecture and promote lateral root formation. It can also increase root biomass, improve root traits, and enhance nutrient uptake under drought conditions [10,40–42]. Gutjahr et al. reported that *Glomus intraradices* significantly increased lateral root formation in rice, showing that AMF can directly affect root system architecture [40]. These effects may be related to changes in plant hormone signaling, nutrient status, and fungal signals during AMF symbiosis [10,41].

In addition to root changes, AMF also affected several physiological traits related to stress tolerance. Under salinity stress, AMF increased phenolic and flavonoid accumulation in roots and reduced H₂O₂ accumulation in leaves. These changes suggest stronger antioxidant protection and less oxidative damage. Under drought stress, AMF increased proline accumulation and antioxidant enzyme activity. These changes may help maintain water balance and protect photosynthetic tissues. These results are consistent with previous studies showing that AMF can improve drought and salinity tolerance by regulating water status, antioxidant systems, osmotic adjustment, and mineral nutrition [42–46]. AMF can also improve salinity tolerance by maintaining ion balance and enhancing antioxidant defense [43]. Meta-analysis studies have shown that AMF inoculation can reduce oxidative stress indicators such as H₂O₂ and MDA under drought conditions [44,45]. AMF can also increase antioxidant enzyme activities, including CAT and POD, across different plant species [45]. However, the effects on osmolytes such as proline may vary among plant species and experimental conditions [44]. Therefore, AMF-mediated drought tolerance is likely controlled by several physiological processes rather than by osmotic adjustment alone.

Salt, drought, and *Orobanch*e parasitism are different types of stress. However, plants may use some common defense pathways to maintain cellular stability. Our multi-omics analysis showed that AMF activated several molecular processes shared across different stress conditions. This result is consistent with the concept of AMF-induced resistance, in which AMF can regulate several defense pathways and improve plant responses to different stresses [30,34–39,46]. The common AMF-responsive gene module identified in our study further suggests that AMF may establish a shared physiological and transcriptional state. This state may then be adjusted according to the specific stress.

Transcriptomic analysis showed that AMF-responsive genes were mainly enriched in phenylpropanoid metabolism, cell wall biogenesis, oxidoreductase activity, and lipid-related pathways. These pathways are important for plant stress responses. Phenylpropanoid compounds, including phenolic acids and flavonoids, can act as antioxidants and also participate in defense signaling. Changes in cell wall-related processes may also strengthen physical barriers and affect plant–microbe interactions [35,38,46,47]. Previous studies have shown that AMF can regulate phenylpropanoid and flavonoid biosynthesis and increase secondary metabolite accumulation in host plants [47,48]. AMF-induced changes in cell wall processes may also affect plant defense and interactions between roots and surrounding microorganisms [38]. These findings may help explain the enrichment of phenylpropanoid and cell wall-related pathways observed in our transcriptomic data.

Metabolomic analysis also supported the transcriptomic results. AMF caused significant changes in phenolic compounds, flavonoids, alkaloids, amino acid derivatives, and lipid-related metabolites. These results suggest that AMF-mediated stress tolerance

involves changes in several metabolic pathways rather than a single defense pathway. Specifically, the increased accumulation of organic acids such as malate and citrate, amino acid derivatives including N-methyl-D-aspartic acid, and phenolic compounds such as chlorogenic acid and caffeic acid suggests a coordinated reprogramming of primary and secondary metabolism. These metabolites are involved in carbon and nitrogen metabolism, antioxidant defense, and cell wall strengthening, which are consistent with the observed physiological improvements in stress tolerance. Previous studies have shown that AMF can affect both primary and secondary metabolism, including sugars, organic acids, amino acids, phenolics, flavonoids, alkaloids, and other specialized metabolites [47,49–51]. The extent and direction of these changes can depend on the host genotype, AMF species or strain, and environmental conditions [47,49]. Lipid metabolism is also important for AMF symbiosis because host plants provide carbon to AMF and undergo changes in lipid metabolism during fungal colonization [50]. Therefore, the metabolic changes observed in our study may result from both AMF symbiosis and stress responses. Lipid metabolism may also contribute to AMF-associated stress responses, as fatty acids serve as important carbon resources transferred from plants to AMF and participate in plant–microbe interactions. The enrichment of lipid-related pathways observed in this study therefore suggests that AMF inoculation may involve changes in carbon allocation and membrane lipid metabolism under stress. In addition, phenylpropanoid and flavonoid metabolism is closely associated with plant defense and interactions with beneficial microorganisms. These metabolic changes may contribute to the establishment and maintenance of AMF-associated microbial interactions.

Orobanch parasitism is a special type of biotic stress because the parasite directly removes water and nutrients from host roots and causes strong metabolic changes. Compared with abiotic stress, plant responses to parasitic plants require both defense activation and regulation of resource allocation and root signaling. Parasitic plants form specialized haustoria that penetrate host tissues and connect with the host vascular system. This allows water, nutrients, and other compounds to move from the host to the parasite [52,53]. Studies of *Orobanch* species have also shown that host-derived carbon and nitrogen compounds contribute to parasite growth and development [54]. Therefore, resistance to *Orobanch* may require both defense responses and regulation of host resource use.

AMF also strongly affected the transcriptional and metabolic responses of sunflower under *Orobanch* parasitism. Integrated analysis identified several strong gene–metabolite associations related to carbon metabolism, amino acid transport, hormone regulation, and lipid metabolism. For example, the coordinated increase in the MDH gene and organic acid-related metabolites suggests that AMF may affect carbon metabolism during parasite infection. The association between the Nodulin MtN21/EamA-like transporter and amino acid derivatives also suggests that nutrient transport may be involved in AMF-mediated adaptation. These associations are biologically reasonable because parasitic plants depend strongly on host-derived carbon and nitrogen, and changes in amino acid and organic acid metabolism have been reported in host–*Orobanch* interactions [54,55]. However, these gene–metabolite associations should be considered candidate relationships rather than direct evidence of gene function. Further experiments are needed to confirm their roles.

These findings extend current knowledge of AMF-induced resistance beyond pathogen and insect interactions. They suggest that AMF may also help plants respond to parasitic plants through coordinated metabolic regulation. Previous studies have suggested that mycorrhiza-induced resistance can affect interactions with parasitic plants, although the mechanisms are less well understood than those involved in pathogen and insect interactions [34,35]. *Orobanch* infection involves both tissue invasion and continuous loss of host resources. Therefore, AMF-induced changes in root architecture, defense signaling,

and primary metabolism may contribute to improved host tolerance during parasite infection. Our integrated transcriptomic and metabolomic results provide initial support for this possibility.

In addition to stress tolerance, AMF-induced metabolic changes may also improve crop quality. AMF increased several groups of bioactive metabolites, including phenolic acids, flavonoids, and other secondary metabolites. These compounds are associated with antioxidant activity and nutritional value. Therefore, AMF inoculation may provide benefits beyond stress protection. Previous studies have shown that AMF can increase the production and accumulation of phenolics, flavonoids, alkaloids, and other specialized metabolites in plants [47,49,51]. Zhao et al. showed that AMF can increase secondary metabolite accumulation through improved plant growth and regulation of secondary metabolism. However, these effects can vary with host genotype, AMF strain, and environmental conditions [47]. Therefore, the increased bioactive metabolites observed in sunflower may be another benefit of AMF inoculation. Their effects on seed nutritional quality and crop value need further study.

From an agricultural perspective, beneficial microorganisms are a promising tool for sustainable crop production. By improving stress tolerance and regulating quality-related metabolites, AMF may be useful as a biological input for sunflower production in stress-prone environments [10,46,47,49]. However, future studies should test the stability of these effects under field conditions. It is also important to determine how different AMF species, soil conditions, and sunflower genotypes affect these responses. This consideration is important because the effects of AMF symbiosis can vary greatly with both the fungal and plant partners and with environmental conditions [47,49]. Field experiments, comparisons of different AMF species, and tests across different sunflower genotypes will therefore be needed before AMF-based approaches can be widely adopted in sunflower production.

5. Conclusions

In this study, we demonstrated that arbuscular mycorrhizal fungus (AMF) colonization enhanced sunflower adaptation to multiple environmental stresses, including salinity, drought, and *Orobanche* parasitism, through coordinated regulation of root architecture, physiological responses, and molecular processes. AMF promoted lateral root development across different sunflower varieties and induced a primed defense state characterized by enhanced antioxidant capacity even before stress exposure. Under stress conditions, AMF further improved physiological homeostasis by regulating reactive oxygen species metabolism, osmotic adjustment, and the accumulation of defense-related secondary metabolites.

Multi-omics analyses revealed that AMF-mediated stress adaptation was associated with extensive transcriptional and metabolic reprogramming. Transcriptomic analysis identified a conserved AMF-responsive gene module enriched in phenylpropanoid metabolism, cell wall organization, redox regulation, and lipid-associated pathways. Metabolomic analysis further demonstrated that AMF reshaped root metabolic profiles by altering primary metabolites, phenolic compounds, flavonoids, alkaloids, and lipid-related metabolites. Integrated transcriptomic–metabolomic analysis uncovered key gene–metabolite associations involved in carbon metabolism, nutrient transport, hormone regulation, and membrane remodeling, providing new insights into the molecular basis of AMF-mediated stress adaptation.

Overall, our findings highlight AMF as a promising biological strategy for improving sunflower resilience under complex environmental challenges. The simultaneous enhancement of stress tolerance and bioactive metabolite regulation suggests that AMF application has potential value for sustainable crop production and quality improvement. Further

field-based studies are needed to evaluate the stability and practical application potential of AMF-mediated benefits under diverse agricultural environments.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16202040/s1>, Table S1: AMF colonization rates of sunflower cultivars under different treatment conditions; Table S2: Detailed conditions of stress treatments used in this study; Table S3. Gene-Specific Primers Used in qPCR; Table S4. Gene-metabolite pairs identified by nine-quadrant analysis and Spearman correlation between the AO and O treatments; Figure S1. Validation of RNA-seq expression patterns of representative genes by qRT-PCR. Bars represent the relative expression levels determined by qRT-PCR, and lines indicate the corresponding RNA-seq expression values; Figure S2. Mantel analysis of the associations between AMF/*O. cumana* treatments and physiological traits in three susceptible sunflower cultivars; Figure S3. Root fresh weight of four sunflower varieties with or without AMF inoculation. CK, non-inoculated control; AMF, AMF-inoculated treatment. Data are presented as mean $\pm$ SD (n = 3). Statistical comparisons were performed between CK and AMF treatments within each sunflower variety using an independent-samples t-test. Asterisks indicate significant differences between CK and AMF within the same variety (* < 0.05, ** < 0.01); Figure S4. Main-effect OPLS-DA score plot and model validation of the sunflower root metabolome. OPLS-DA score plot comparing AMF-inoculated samples (A, AS, Ad, and AO; AMF_Yes) with non-inoculated samples (C, S, d, and O; AMF_No). Each point represents an individual sample. Permutation test of the OPLS-DA model based on 200 permutations. The permutation test was used to assess the robustness and reliability of the classification model. Global VIP values of individual metabolites obtained from the main-effect OPLS-DA model. The threshold line indicates the VIP criterion used for metabolite screening. Global VIP values were subsequently considered together with fold-change and statistical significance from pairwise comparisons to identify differential metabolites for downstream analyses..

Author Contributions: Y.Z.: Conceptualization, Methodology, Investigation, Formal Analysis, Data Curation, Writing—Original Draft Preparation. W.X., Q.H. and F.H.: Investigation, Validation. J.L., I.B. and A.A.: Methodology, Visualization. W.Z. and L.X.: Conceptualization, Resources, Supervision, Project Administration, Writing—Review and Editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by National Natural Science Foundation of China (32372566), and the Collaborative Innovation Center for Modern Crop Production co-sponsored by the Province and Ministry (CIC-MCP).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Transcriptome data are available in the NCBI SRA under BioProject PRJNA1544236. Further inquiries can be directed to the corresponding authors.

Acknowledgments: We acknowledge the Ministry of Agriculture and Rural Affairs Key Laboratory of Spectroscopy Sensing, and Rui Sun from the Agricultural Experiment Station of Zhejiang University for his assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

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