BMC Microbiology

RESEARCH Open Access

Arbuscular mycorrhizal fungi and *Trichoderma longibrachiatum* alter the transcriptome of *Vicia villosa* in response to infection by the fungal pathogen *Stemphylium vesicarium*

Tingting Ding[1], Wei Feng[3*], Meiting Bai[4], Lijun Gu[2] and Tingyu Duan[2,5*]

Abstract

Background Leaf spot caused by *Stemphylium vesicarium* is a severe disease of *Vicia villosa* and first reported in 2019. Arbuscular mycorrhizal fungi (AMF) and *Trichoderma* are common beneficial microorganisms in soil that enhance plant resistance to pathogens. This study established a greenhouse experiment to examine the physiological and transcriptomic changes of *V. villosa* that were co-inoculated with the AMF *Sieverdingia tortuosa* and *Trichoderma longibrachiatum* to determine their effects on the development of resistance to disease.

Results Infection by the pathogen reduced the shoot biomass of *V. villosa*. Individual inoculation or co-inoculation with AMF and *T. longibrachiatum* reduced the severity of disease and promoted defense-related reactions, such as the production of salicylic acid (SA), activity of phenylalanine ammonia lyase and chitinase. Inoculation of *Trichoderma* alone or in combination with AMF significantly increased the content of SA of the diseased *V. villosa* by 12.23% and 12.80%, respectively. Treatment with AMF alone significantly increased the chitinase activity of susceptible *V. villosa* by 6.4% compared with *V. villosa* only infected with *S. vesicarium*. Gene ontology terms that related to plant disease resistance, such as upregulated "Defense response", "Peroxidase activity", and "Signal acceptor activity", were significantly enriched in diseased plants that had been inoculated with *S. tortuosa* and *T. longibrachiatum*. However, they were not significantly enriched in susceptible plants that had not been inoculated with *S. tortuosa* and *T. longibrachiatum*. The expression of the genes that were involved in the Kyoto Encyclopedia of Genes and Genomes pathways "Isoflavonoid biosynthesis" and "Flavone and flavonol biosynthesis" and were related to disease defense was upregulated.

Conclusion Both of *T. longibrachiatum* and AMF exhibit significant potential in managing leaf spot disease caused by *S. vesicarium* in *V. villosa*. The mechanism includes the increased SA content as well as the expression of pathogen defense-related genes in plant. *T. longibrachiatum* alone and combined with AMF resulted in a significant increase in SA levels. Furthermore, AMF also significantly up-regulated the expression of NPR1-related genes, which are integral

*Correspondence:
Wei Feng
fwnpw@163.com
Tingyu Duan
duanty@lzu.edu.cn

Full list of author information is available at the end of the article

to systemic acquired resistance. Our findings underscore the efficacy of *T. longibrachiatum* and AMF as potential biological control agents, providing a promising strategy for the management of leaf spot disease in *V. villosa*.

Keywords *Trichoderma longibrachiatum*, *Sieverdingia tortuosa*, *Stemphylium vesicarium*, RNA-seq

Background

Vicia villosa Roth is widely planted as a forage crop and green manure in orchard and rice (*Oryza sativa*) fields worldwide, including China [1], Japan [2] and Argentina [3]. Rotation between *V. villosa* and crops in farming systems can reduce the need to utilize nitrogen fertilizer by more than 45 $kg.N^{-1}$ [4]. In addition, *V. villosa* reduces the spread of invasive weeds [5] and alters the soil microbial community to increase the probability of plant survival through allelopathy [2]. Leaf spot disease caused by *Stemphylium vesicarium* is usually accompanied by a high disease incidence that can cause defoliation and occur widely on the plant [1, 6]. Leaf spot of *V. villosa* caused by *S. vesicarium* was first reported in China in 2019. The pathogen causes spot disease on fruits [7], vegetables [8, 9]and forage crops [10]. The prevalence of disease increases the input of fungicides and decreases the yield of plants. Despite the effectiveness of chemical control measures against plant diseases, fungicides have direct and indirect risks for microorganisms, macrophytes, invertebrates and vertebrates [11]. The long-term use of chemicals has been shown to cause fungicide resistance in *S. vesicarium* [12, 13]. Biological methods and the planting of resistant cultivars/varieties have been approved as efficient methods for disease management [14]. However, biocontrol products are also applied for disease management, and biocontrol only accounts for approximately 1% of the sales of agricultural chemicals [15]. Environmentally friendly methods, such as biocontrols, should be developed for disease management.

Arbuscular mycorrhizal fungi (AMF) are a group of soil microorganisms that coexist with plant roots, improve plant disease resistance and reduce the occurrence of diseases [16]. More than 15 genera of plants that form symbioses with AMF to resist diseases caused by 28 pathogens were reported from 2018 to 2020 [17]. Studies indicate that the mechanisms of AMF to reduce the disease incidence include promoting plant growth; increasing the activities of defense enzymes related to plant disease resistance [18]; enhancing the expression of genes involved in pathogenesis-related (PR) proteins, chitinase activity, phenylalanine metabolism, and photosynthesis [19]; inhibiting the multiplication of pathogens and competing with the pathogen for nutrients [20]. In particular, the antioxidant system and the signaling pathway of salicylic acid (SA) involved in plant resistance to pathogen infection were significantly enhanced by the presence of AMF [21]. Alternatively, *Trichoderma* species are widely used for the biocontrol of plant diseases, and they produce compounds that are highly effective at inhibiting pathogens [22]. *Trichoderma viride* and *T. harzianum*, among other *Trichoderma* species, have varying degrees of inhibition on 29 plant pathogenic fungi that are members of 18 genera [23]. For example, the secondary metabolites produced by *T. atroviride* and the volatile organic compounds that it releases inhibited the survival and growth of pathogens [23, 24]. *T. asperellum* inhibits the proliferation of pathogens in cucumber (*Cucumis sativus*) and activates independent metabolic pathways involved in the transduction and biosynthesis of plant signals, which thereby induced systemic responses to infection by the pathogen *Pseudomonas syringae* pv. *lachrymans*, the causal agent of cucumber angular leaf spot [25].

The co-inoculation of AMF and *Trichoderma* improves plant disease resistance more than a single inoculation with either organism, and the disease incidence and severity have been shown to be significantly reduced [26]. For example, *T. viride* along with *Funneliformis mosseae* or *Acaulospora laevis* significantly reduced the incidence of *Fusarium* wilt in tomato (*Solanum lycopersicum*) plants by 50%, and *T. viride* along with *F. mosseae* and *A. laevis* can completely control the disease [26]. However, the control of diseases depends on the particulars of AMF and *Trichoderma*. In the field, inoculation with AMF and *Trichoderma* separately decreased the incidence of wilt disease in muskmelon (*Cucumis melo*) by 25-60%, but co-inoculation with *Glomus claroideum* and *T. harzianum* had synergistic effects in a negative manner [27]. Moreover, the effects of the combination of AMF and *Trichoderma* on different plant varieties also differ. The co-inoculation of AMF and *Trichoderma* had the greatest effect at controlling stem rot disease in Jerusalem artichoke (*Helianthus tuberosus*) variety HEL246 but not in variety JA37 [28].

The infection of a plant with a pathogen usually causes a series of physiological reactions in the host. Large amounts of reactive oxygen species (ROS) accumulate in the early stage of infection and activate the defense responses of plants against pathogens [29]. Many types of ROS can damage plants, and enzymes, such as superoxide dismutase (SOD) and peroxidase (POD), clear the ROS to avoid serious damage to the plant cells. In addition, plants release chitinase, which reduces growth and sporulation at the later stages of disease development, and prevents the fungal pathogens from further infecting the plants [30]. Phenylalanine ammonia-lyase (PAL) is involved in complex defense response programs,

including flavonoid and isoflavone biosynthesis [31] and the biosynthesis of SA [32]. SA and jasmonic acid (JA) are important hormones that transmit defensive signals and play important roles in plant disease resistance [33]. Changes in the levels of expression of the genes for these hormones play an indispensable role in the physiological processes described above [19].

Previous research on the co-inoculation of AMF and *Trichoderma* in relation to plant diseases provided controversial results. Moreover, these studies mainly focus on the disease severity and the physiological mechanisms. For example, AMF and *T. harzianum* regulate chlorophyll, phenolic compounds content and peroxidase activity against tomato root rot caused by *Fusarium oxysporum* [34]. Both AMF and *Trichoderma* can activate plant resistance to pathogen infection by upregulating systemic acquired resistance (SAR) genes [35]. However, the relationship between pathogen induced defense enzyme activity and their associated genes has been largely overlooked. Specifically, there is a lack of understanding regarding the mechanisms by which the co-inoculation of AMF and *Trichoderma* impacts the physiological processes regulated by plant genes to enhance resistance against pathogen infection. In particular, even less information is available on the molecular mechanisms revealed by a transcriptome analysis, such as RNA-seq.

Strategies for plant pathogens have emphasized the importance of upcoming methodologies, such as microbiome management, phage cocktails, biocontrol agents and microbial volatilomes, as sustainable practices for disease management [36]. The inhibitory effects of AMF and *Trichoderma* on pathogens in vitro and the occurrence of disease in vivo were identified [24]. However, few studies have reported the regulatory effects of AMF and *Trichoderma* on the responses of plants to pathogens at the levels of physiology and gene expression. This study aimed to determine the regulatory effects of AMF and *T. longibrachiatum* on *V. villosa* by analyzing the activities of enzymes, contents of hormones and the transcriptome. This study addressed the following hypotheses: (1) AMF and *T. longibrachiatum* will decrease the incidence of disease on *V. villosa* caused by *S. vesicarium*; (2) AMF and *T. longibrachiatum* will increase the activities of enzymes, content of hormones, and level of gene expression related to disease resistance in the plant; and (3) The co-inoculation of AMF and *T. longibrachiatum* will have synergistic effects on the inhibition of leaf spot disease compared to separate inoculations.

Table 1 AMF, *T. longibrachiatum* and pathogen treatments

Treatments	AMF	T. longibrachiatum	S. vesicarium
NMNTP-	×	×	×
AMNTP-	√	×	×
NMTLP-	×	√	×
AMTLP-	√	√	×
NMNTP+	×	×	√
AMNTP+	√	×	√
NMTLP+	×	√	√
AMTLP+	√	√	√

Methods

Source of plant seeds, AMF and fungal isolates

Vicia villosa seeds were obtained from Jiangsu Aoqi Landscaping Engineering Co., Ltd. (Jiangsu, China). *Sieverdingia tortuosa* (N.C. Schenck & G.S. Sm.) Błaszk., Niezgoda, & B.T. Goto, previously known as *Glomus tortuosum* [37], was purchased from the Bank of Glomeromycota in China (Beijing, China) for use as an AMF. The AMF inoculum was composed of dry soil that contained AMF spores (>50 spores·g^{-1}), mycelia and fragments of white clover (*Trifolium subterraneum*) roots, which was prepared in potted cultures in a greenhouse. A total of 100 g of an aliquot of inoculum that contained approximately 100 spores·g^{-1} was added to each pot for the mycorrhizal treatment (AM), whereas 100 g of sterilized river sand was added to each pot for the other treatments (NM). *Trichoderma longibrachiatum* was isolated from the rhizosphere soil collected from fields of *V. villosa*. The fungus *Stemphylium vesicarium* was isolated from infected *V. villosa* in the field [1].

Growth medium

Black loam soil was purchased from a local market and autoclaved at 121 °C for 2 h for sterilization. Similarly, the sand was sieved through a 2 mm sieve and oven-sterilized at 170 °C for 4 h. The sterile soil and sand were then mixed at a ratio of 1:3 and used as the growth media.

Greenhouse study

The experiments were conducted in pots from October to December 2020 in a greenhouse at the College of Pastoral Agriculture Science and Technology, Lanzhou University (35.96 ° N, 104.17 ° E). The pathogen *S. vesicarium* (SV) was used in the pathogen inoculation treatments. The AMF and *T. longibrachiatum* were used to control the disease. The experiment involved three factors and a total of eight treatments (Table 1). *Vicia villosa* seeds were soaked in 10% hydrogen peroxide (H_2O_2) for 5 min, rinsed three times with sterilized distilled water, and subsequently germinated on moist filter paper in the dark at 25 °C for 2 days. *Vicia villosa* seedlings were sown in soil inoculated with AMF *Sieverdingia tortuosa* (AM) or non-inoculated (NM) soil as described by Gao et al. [38]. A total of 13 seedlings were transplanted into each pot, and 10 plants of similar sizes were maintained after 1 week. Plates that contained sterile potato dextrose agar (PDA) were prepared by dissolving 200 g of potato, 20 g

of dextrose and 18 g of agar in 1000 mL of water. They were then inoculated with *T. longibrachiatum* and incubated at 28 °C for 2 weeks. The hyphae and spores were then scraped in sterile glass slides to form a spore suspension of *T. longibrachiatum* in sterile water. A 2.5 mL suspension of *T. longibrachiatum* (10^7 conidia·mL^{-1}) per plant was subsequently poured into the soil close to *the V. villosa* roots 1 week after the sprouts had emerged. Suspensions of *S. vesicarium* that contained ~ 10^6 CFU·mL^{-1} conidia with two drops of Tween-20 were used to inoculate the *V. villosa* plants 7 weeks after emergence by individual spraying with 5 mL of inoculum per pot. Equal amounts of sterile water were sprayed onto the uninoculated plants. The plants were covered with black plastic bags for 48 h after spraying to maintain moisture and facilitate infection. The disease incidence and disease index (DI) were calculated every 3 days [38].

Plant harvest and measurement of the samples

At harvest, 0.2 g of fresh shoot material from each pot was used to measure the activities of SOD and POD [39], content of malondialdehyde (MDA) and the activity of PAL [40]. Another set of 0.2 g fresh shoots was used to determine the contents of jasmonic acid (JA) [40] and SA and the activity of chitinase [41]. Approximately 0.2 g of fresh shoot tissue was used to extract the total RNA. All the tissues sampled were stored in liquid nitrogen and transferred to the laboratory for subsequent experiments. The extent of AM colonization was determined using 0.2 g of fresh roots [42]. The remaining portions of the shoots and roots were used to determine the total shoot and root dry weights based on the fresh: dry weight ratios. The disease incidence and disease index were calculated using 20 randomly selected leaves from each pot as shown below:

$$\text{Disease incidence} = \text{number of diseased leaves} / \text{the total number of leaves} \times 100\%$$

The disease index was determined as Ren described [43].

Construction and sequencing of the cDNA library

The leaf tissues were flash-frozen in liquid nitrogen and used to extract the total RNA. The total RNA was isolated from the harvested tissues using the TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). DNase I was used to remove the DNA from the total RNA, and a Qubit RNA analysis kit (Thermo Fisher Scientific) was used to determine the purity of total RNA. Finally, an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) was used to evaluate the RNA integrity. The treated RNA was amplified by PCR with specific primers, and a cDNA library was obtained after treatment. The 150 bp paired-end original reads were generated after sequencing by a BGI DNBseq 500 sequencing platform (BGI Genomics, Shenzhen, China). The original transcriptome data were uploaded to the Sequence Read Archive of the NCBI (BioProject ID: PRJNA960699).

The total reads were filtered to obtain clean reads using SOAPnuke software (v. 1.4.0) [44]. Trinity was used to assemble the clean reads *de novo*. All the unigenes were obtained after clustering and reducing the assembled transcripts using TGICL, which was assembled into the reference genome. Its quality was evaluated using the BUSCO database. Bowtie2 (v. 2.2.5) was used to map the clean reads to the reference genome, and the levels of expression of the genes and transcripts were calculated using RSEM [45]. Transcoder software was used to predict the CDS [46], and Primer3 was used to detect the simple sequence repeats (SSR) [47]. All the unigenes were annotated using the NR, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [48].

The levels of expression of the genes and an analysis of the differentially expressed genes (DEG) were performed with Bowtie2, which was subsequently used to align the clean reads to the genome sequences. RSEM was used to calculate the levels of expression of the genes in each sample [45, 49]. Significant differences in gene expression were determined using an adjusted p-value (Q-value) < 0.001 and an absolute value of |log2 (fold change)| ≥ 1 [50, 51]. The Q-value was obtained by correcting the p-value with the false discovery rate (FDR) [52, 53]. The GO database and KEGG were used to determine the biological functions and pathways of the DEGs, and the pHYPER function in R software was used for the enrichment analysis [54]. The GO terms or KEGG pathways were considered significantly enriched if the Q-value was ≤ 0.05.

Quantitative Real-time polymerase chain reaction (qRT-PCR): Six genes were selected for qRT-PCR to validate the results of transcriptome analysis. Four independent biological replicates and three technical replicates were involved in each treatment to eliminate the effects of machine equipment, experimental operations. Total RNA of RNA-SEQ was reverse-transcribed into cDNA using TUREscript 1st Strand cDNA SYNTHESIS kit (Aidlab, Beijing, China). qRT-PCR reaction was performed on BIO-RAD C1000TouchTMThermal Cycler (USA). The qRT-PCR reaction system consisted of 5 μL TIANGEN®SYBR Green, 1 μL cDNA, 0.5 μL forward primer, 0.5 μL reverse primers, and 3 μL ddH_2O. $2^{-\triangle\triangle Ct}$ were calculated to indicate the relative gene expression [55].

Statistical analysis

The data are presented as the mean ± standard error of the mean of four replicates. An analysis of variance (ANOVA) was performed using JMP 4 statistical software at a probability level of $P < 0.05$, and the results are displayed as column plots that were generated using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). The GO enrichment analysis was also performed using GraphPad Prism 8. Heatmaps were generated using the *pheatmap* package of R software (4.2.3), and the Bubble plots were generated using the ggplot2 package of this software. The Pearson correlations between qRT-PCR results and RNA-seq data were calculated using the *ggplot2* package of R software (4.2.3) [56].

Results

Plant growth and disease severity

The AMF *Sieverdingia tortuosa* effectively colonized the roots of *V. villosa* (Fig. S1A). The colonization by the AMF was not significantly affected by *T. longibrachiatum* and *S. vesicarium* (Fig. S1B). The shoot biomass of *V. villosa* significantly decreased by 24.56% after 9 days of infection by *S. vesicarium* (Fig. 1A). Inoculation with *S. tortuosa* and *T. longibrachiatum* (AMTLP+) had no significant effect on the shoot biomass of *V. villosa* infected with *S. vesicarium* (NMNTP+) or uninfected (NMNTP-). Infection with *S. vesicarium* and inoculation with *S. tortuosa* and also *T. longibrachiatum* (NMTLP+, AMNTP+, and AMTLP+) had no significant effects on the root biomass of *V. villosa* (NMNTP+) (Fig. 1B).

Vicia villosa infected by *S. vesicarium* showed typical symptoms on the third day after inoculation (Fig. S1C). The uninfected plants did not show any symptoms. Notably, there were no differences in the time that the typical symptoms appeared among the treatments. The disease incidence and disease index also increased with time. However, the severity of the disease of the plants that had been inoculated with *T. longibrachiatum* and *S. tortuosa* (AMTLP+) decreased compared to the plants that were only infected by the pathogen (NMNTP+). On the ninth day after inoculation, the disease index and incidence of AMNTP+, NMTLP+, and AMTLP + were 14.72-33.38% and 20.68-36.55% lower than those of the plants only infected by *S. vesicarium* (NMNTP+), respectively (Fig. 2A, B).

MDA, SA, JA and enzyme activity of *V. villosa*

The pathogen had no effect on the content of MDA across the treatments. Colonization with AMF and *T. longibrachiatum* significantly reduced the negative effects by *S. vesicarium*. Inoculation with *T. longibrachiatum* resulted in a significant increase in the content of SA by 12.24-16.65% of the healthy plant (NMTLP-) in contrast to the other three treatments (NMNTP-, AMNTP-, and AMTLP-) (Fig. 3A). Inoculation with *T. longibrachiatum* (NMTLP+) and the co-inoculation of AMF and *T. longibrachiatum* (AMTLP+) significantly increased the content of SA of the susceptible plant compared to the NMNTP + and AMNTP + treatments (Fig. 3B). The inoculation with AMF and inoculation with a combination of AMF and *T. longibrachiatum* increased the content of JA in the healthy *V. villosa* compared to the plants that had not been inoculated with beneficial fungi. The diseased plants that had been separately inoculated with AMF and *T. longibrachiatum* (NMTLP + and AMNTP+) and co-inoculated with AMF and *T. longibrachiatum* (AMTLP+) had significantly lower contents of JA than *V. villosa* that was only infected with *S. vesicarium* (NMNTP+) by 7.33-26.93% (Fig. 3C). Infection with the pathogen (NMNTP+) increased the content of JA in *V. villosa* compared to the plants that had not been inoculated with AMF and *T. longibrachiatum.* The inoculation

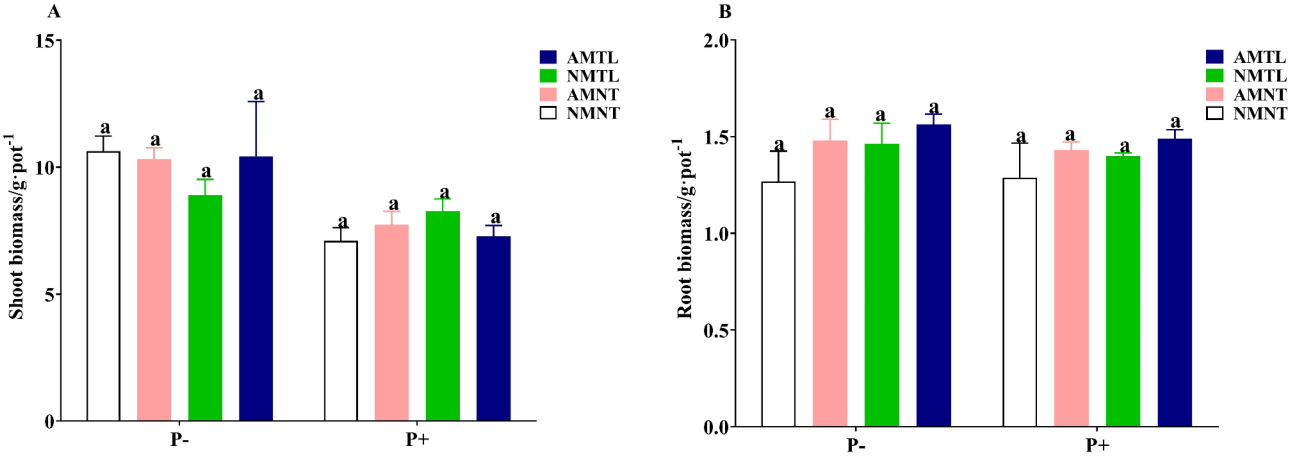

Fig. 1 Shoot biomass (**A**), root biomass (**B**) of *Vicia villosa*. NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*. Different lowercase letters on the bars means there is significant difference across treatments at $P < 0.05$ by Tukey's HSD test

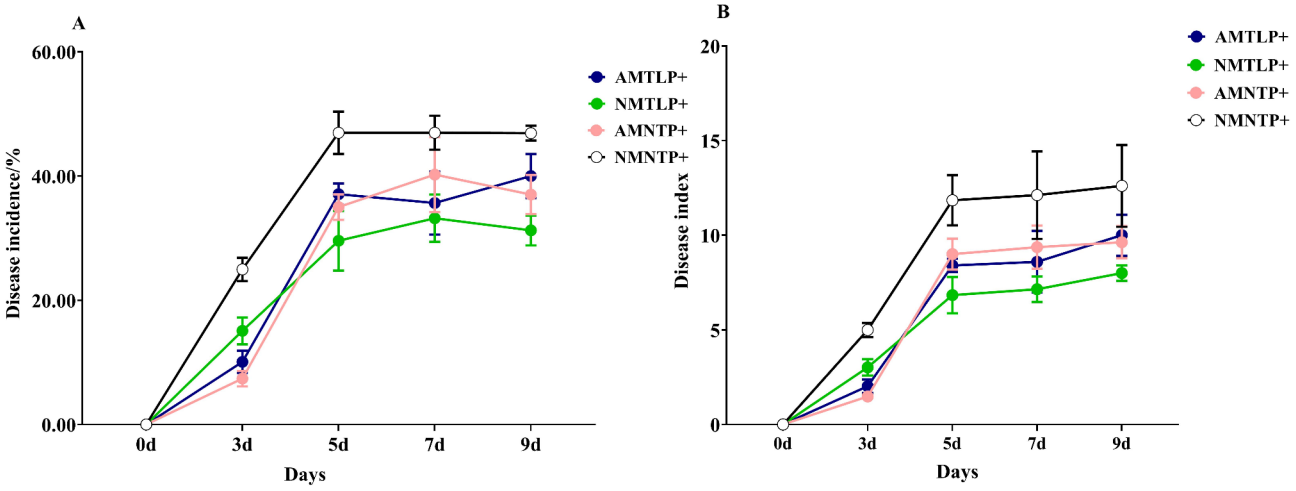

Fig. 2 Disease incidence (**A**) and disease index (**B**) of *V. villosa*. NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*

of AMF and co-inoculation of AMF and *T. longibrachiatum* decreased the content of JA of the susceptible plants (AMNTP + and AMTLP+) compared to the healthy plants (AMNTP- and AMTLP-).

The inoculation of AMF and *T. longibrachiatum* separately or combined resulted in a significant decrease in the activity of SOD in the healthy plants compared to the lack of inoculation with AMF and *T. longibrachiatum* (Fig. 4A). The inoculation of healthy *V. villosa* with AMF resulted in lower levels of POD activity than that in the plants that were co-inoculated with AMF and *T. longibrachiatum*. However, the inoculation of *V. villosa* infected with *S. vesicarium* (NMTLP+) with *T. longibrachiatum* resulted in plants with lower POD activity than that of the susceptible plants co-inoculated with AMF and *T. longibrachiatum* (AMTLP+) (Fig. 4B). The separate inoculation with AMF and *T. longibrachiatum* (AMNTP- and NMTLP-) increased the chitinase activity of the healthy plants by 9.45% and 8.35%, respectively, compared to the *V. villosa* plants that had not been inoculated (NMNTP-) (Fig. 4C). However, treatment with AMF alone significantly increased the chitinase activity of *V. villosa* infected with *S. vesicarium* (AMNTP+) compared to *V. villosa* infected with *S. vesicarium* (NMNTP+). Compared to *V. villosa* infected with *S. vesicarium* (NMNTP+), the co-inoculation of AMF and *T. longibrachiatum* had a negative effect on the chitinase activity of the susceptible plant (AMTLP+), which was reduced by 12.36%. Compared to the healthy *V. villosa* inoculated with AMF and *T. longibrachiatum* (AMNTP-, NMTLP-, and AMTLP-), the separate or combined inoculation of diseased *V. villosa* with AMF and *T. longibrachiatum* (AMNTP+, NMTLP+, and AMTLP+) significantly increased the activity of PAL by 22.5-46.70% (Fig. 4D).

RNA-seq and mapping

An average of 45.27 M raw reads were generated, and 42.37 M clean reads were obtained after filtering. The proportion of clean reads was 93.71% on average; the lowest proportion was 88.56%, and the highest was 96.38%. The percentages of Q20 and Q30 were more than 96% and 90%, respectively. The GC content of the 12 treatments was 40.87% on average. The clean reads were aligned to the reference genome with an average mapping ratio of 83.91% (Table S1). The mapping ratio of *V. villosa* infected by *S. vesicarium* was approximately 83.36-83.67%, while that of heathy *V. villosa* ranged from approximately 84.61–85.23%.

DEGs of the different treatments

The analysis of DEGs indicated that the response of *V. villosa* to infection by *S. vesicarium* was stronger than that of the inoculation with AMF and *T. longibrachiatum*. There were 458, 482, and 485 DEGs in the NMNTP- - AMNTP-, NMNTP- - NMTLP- and NMNTP- - AMTLP-, respectively. However, there were 20,856 DEGs in NMNTP- - NMNTP+. There were 18,580, 16,623, and 14,603 DEGs in AMNTP- - AMNTP+, NMTLP- - NMTLP+, and AMTLP- - AMTLP+, respectively. In addition, after infection with *S. vesicarium*, the number of DEGs with upregulated expression was higher than that of the DEGs with downregulated expression (Fig. S2).

GO analysis

A GO enrichment analysis was used to elucidate the biological function of the DEGs. The partial GO terms that were significantly enriched in AMNTP- - AMNTP+, NMTLP- - NMTLP+, and AMTLP- -AMTLP + and associated with the occurrence of plant disease and disease resistance are shown (Fig. 5).

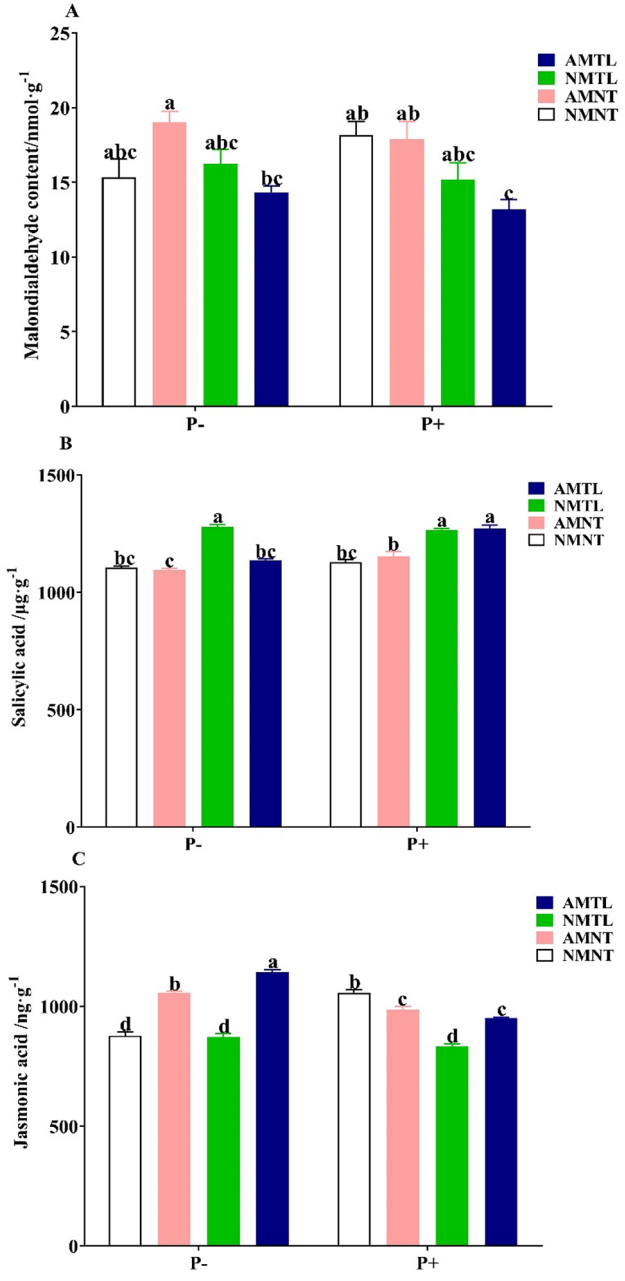

Fig. 3 Malondialdehyde (**a**), salicylic acid (**b**) jasmonic acid (**c**) content of *V. villosa*. NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*. Different lowercase letters on the bars means there is significant difference across treatments at $P < 0.05$ by Tukey's HSD test

A total of 290 GO terms were significantly enriched in the NMNTP- - NMNTP + comparison. The GO terms, such as flavonoid biosynthetic process (GO:0006559), cell surface receptor signaling pathway (GO:0007166), superoxide metabolic process (GO:0006801), and phenylalanine ammonia-lyase activity (GO:0045548), were significantly enriched, and the expression of most of the genes involved in these GO terms was upregulated (Fig. 5A).

In the AMNTP- - AMNTP + comparison, 303 GO terms were significantly enriched, including 125 biological process, 29 cellular component, and 149 molecular functions. In particular, many of the genes involved in defense response (GO:0006952), defense response to other organism (GO:0098542), abscisic acid-activated signaling pathway (GO:0009738), and response to oxidative stress (GO:0006979) were upregulated (Fig. 5B). However, the genes that were categorized into the GO terms, such as regulation of response to reactive oxygen species (GO:1901031), response to biotic stimulus (GO:0009607), phenylpropanoid biosynthetic process (GO:0009699), negative regulation of translation in response to stress (GO:0032055), photosystem II repair (GO:0010206), plasmodesma (GO:0009506), and pectinesterase inhibitor activity (GO:0046910) were downregulated in *V. villosa* that had been inoculated with AMF.

Out of the total of the 248 GO terms, 98 were classified to biological process; 26 were presented in cellular component, and 124 were classified to molecular function in the NMTLP- - AMTLP + comparison group. It is worth noting that many of the genes enriched in the abscisic acid-activated signaling pathway (GO:0009738), defense response to other organism (GO:0098542), calcium-mediated signaling (GO:0019722), phosphorelay signal transduction system (GO:0000160), defense response (GO:0006952), extracellular space (GO:0005615), and peroxidase activity (GO:0004601) were upregulated. In contrast, the levels of expression of the genes involved in the regulation of response to reactive oxygen species (GO:1901031), negative regulation of translation in response to stress (GO:0032055), photosystem I reaction center (GO:0009538), and signaling receptor activity (GO:0038023) were downregulated (Fig. 5C).

In the AMTLP- AMTLP + comparison group except for the genes involved in regulation of response to reactive oxygen species (GO:1901031) that were totally downregulated, but most of the genes that were categorized into other GO terms were upregulated. In particular, all the genes in calcium-mediated signaling (GO:0019722) and killing of cells of other organism (GO:0031640) were upregulated (Fig. 5D).

KEGG pathway analysis

A KEGG pathway analysis was utilized to determine the biological pathways that involved DEGs. There were 137, 136, 136, and 136 pathways in which 27, 32, 22, and 28 KEGG pathways were significantly enriched, respectively (Q-value < 0.05) in the NMNTP- - NMNTP+, AMNTP- - AMNTP+, NMTLP- - NMTLP+, and

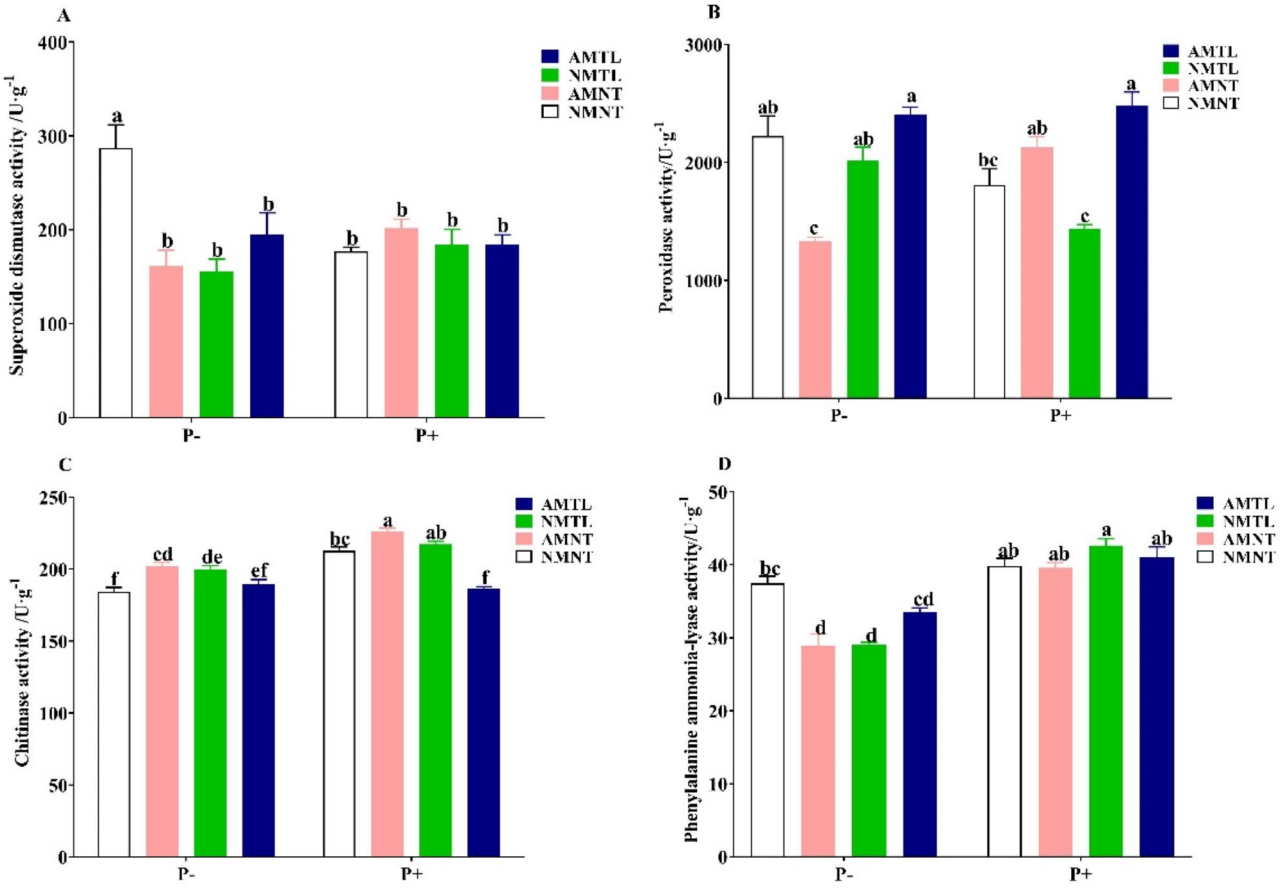

Fig. 4 Superoxide dismutase (**a**), peroxidase (**b**), chitinase (**c**) and phenylalanine ammonia-lyase (**d**) activity of *V. villosa*. NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*. Different lowercase letters on the bars means there is significant difference across treatments at $P < 0.05$ by Tukey's HSD test

AMTLP- - AMTLP + comparison group, respectively. Ten KEGG pathways with the highest rich ratio are shown in Fig. 6.

The pathway connected with flavonoid biosynthesis was influenced by the infection with *S. vesicarium* compared to that in the healthy plants. The genes of *V. villosa* that were infected with *S. vesicarium* were clearly shown to be involved in isoflavonoid biosynthesis (ko00943) and flavone and flavonol biosynthesis (ko00944) and were upregulated compared with the genes of plants in the NMNTP- treatment. A similar pattern of gene expression occurred in the AMNTP- - AMNTP + and AMTLP- -AMTLP + comparison groups. Interestingly, the genes of photosynthesis - antenna proteins (ko00196) in NMNTP+, AMNTP+, NMTLP + and AMTLP + were downregulated compared to the genes in NMNTP-, AMNTP-, NMTLP- and AMTLP-.

The defense responses of plants

The profiles of the genes that were expressed for phenylpropanoid biosynthesis and signal transduction associated with SA are illustrated in Fig. 7. The levels of expression of the genes associated with PAL, cinnamate-4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL) that were upregulated in the diseased plants inoculated with AMF and *T. longibrachiatum* separately or combined were the same when compared to those in healthy *V. villosa*. Similar patterns of gene expression also occurred in NPR1 and PR1, which along with SA, are involved in the signal transduction for plant disease resistance. More genes related to the biosynthesis of PAL in the AMNTP- - AMNTP + and NMTLP- - NMTLP + comparison groups were upregulated compared with the CK vs. SV comparison group. Interestingly, *CL758.Contig3_All* and *Unigene2456_All*. associated with NPR1 are involved in signal transduction were significantly upregulated in the plants of the AMNTP + treatment compared to the plants of the AM treatment, but similar results did not occur in the NMNTP- - NMNTP+, NMTLP- -NMTLP + and AMTLP- - AMTLP + comparison group. The levels of gene expression associated with PR1 were higher in AMNTP- - AMNTP+, NMTLP- - NMTLP + and AMTLP- - AMTLP + than in the NMNTP- - NMNTP + group.

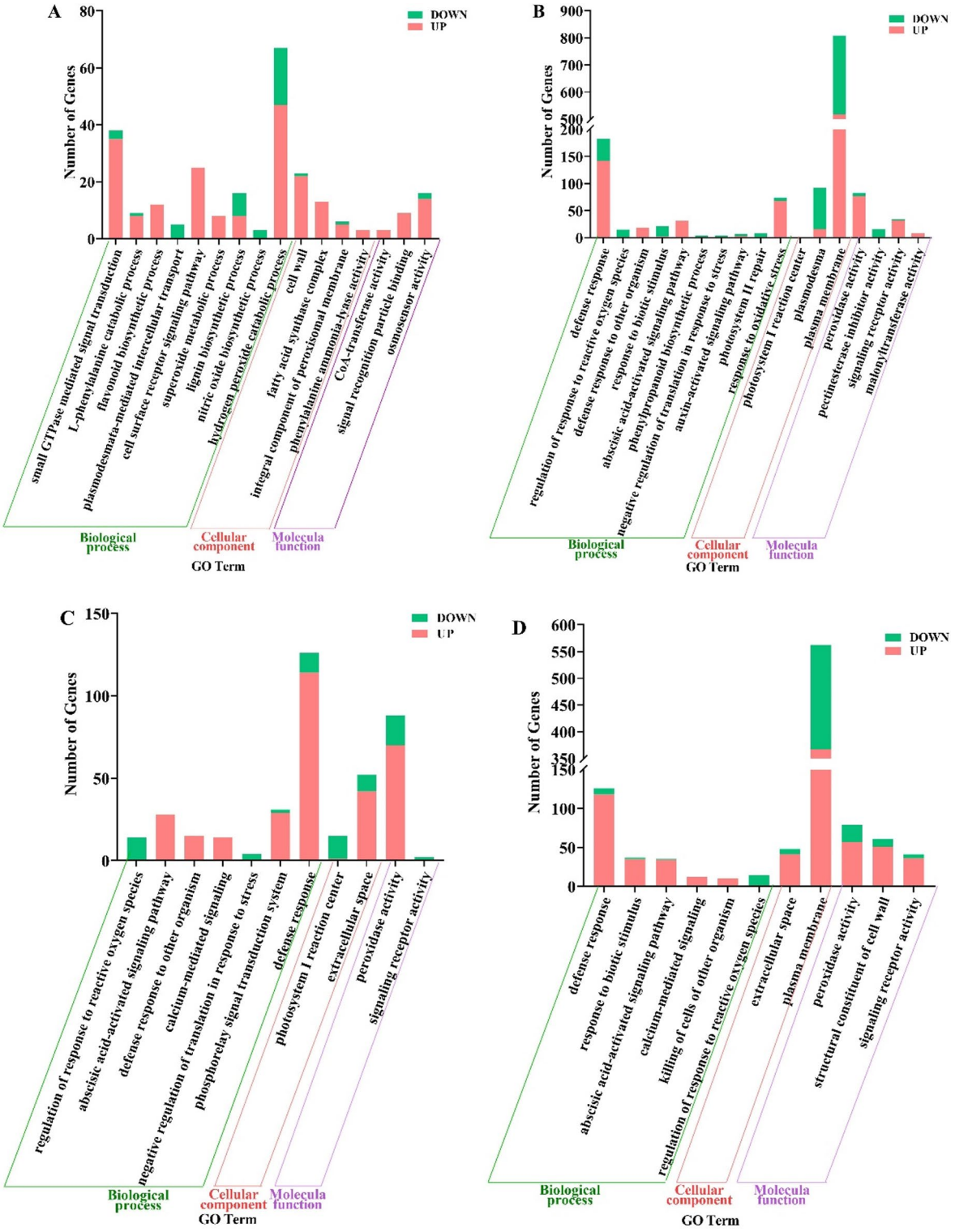

Fig. 5 GO enrichment analysis in NMNTP- - NMNTP+ (**A**), AMNTP- - AMNTP+ (**B**), NMTLP- - NMTLP+ (**C**) and AMTLP- - AMTLP+ (**D**). NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*

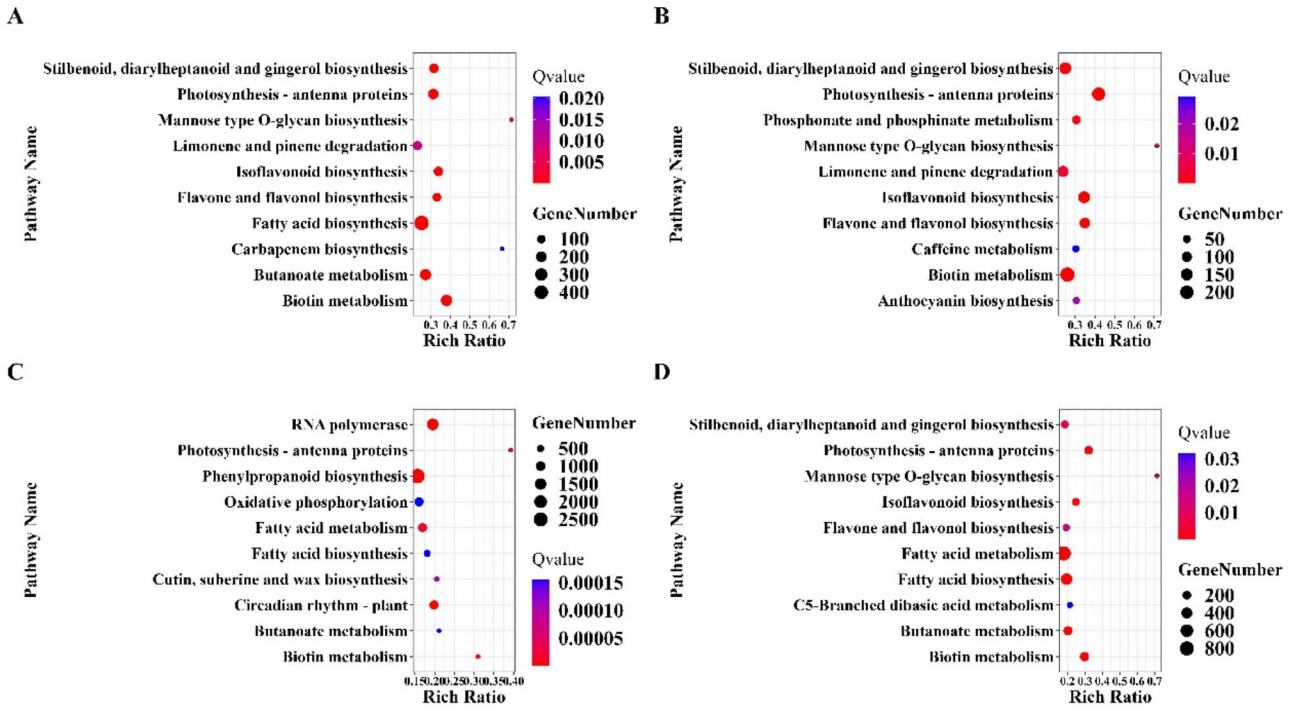

Fig. 6 KEGG analysis in NMNTP- - NMNTP+ (**A**), AMNTP- - AMNTP+ (**B**), NMTLP- - NMTLP+ (**C**) and AMTLP- - AMTLP+ (**D**). NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*

qRT-PCR

Six DEGs were selected for real-time quantitative reverse transcription PCR (qRT-PCR) (Fig. S3). The Pearson correlation of qRT-PCR results for *CL4130. Contig3_All*, *CL5979. Contig3_All*, *CL6827. Contig6_All*, and *CL9091. Contig6_All* with the fragments of kilobase of transcript per million mapped reads (FPKM) values were more than 0.8. The result indicated that the RNA-seq data are reliable.

Discussion

This study revealed the effects of the inoculation of AMF and *Trichoderma longibrachiatum* on the defense response of *Vicia villosa* to infection by *Stemphylium vesicarium*. Infection with *S. vesicarium* decreased the shoot biomass of *V. villosa*. Inoculation with AMF and *T. longibrachiatum* separately or in combination decreased the severity of *S. vesicarium* leaf spots and increased the content of SA as well as POD and chitinase activity, to resist infection by *S. vesicarium*. In particular, inoculation with AMF and *T. longibrachiatum* increased the levels of expression of the genes for the GO terms associated with disease defense, such as defense response to other organisms, calcium-mediated signaling and peroxidase activity. Inoculation with AMF increased the expression of NPR1-related genes. In addition, inoculation with *T. longibrachiatum* both alone and in combination with AMF mainly increased the SA content and enhanced the resistance of *V. villosa* to *S. vesicarium*. The hypotheses that were tested in this study included the following: (1) AMF and *T. longibrachiatum* will decrease the incidence of disease on *V. villosa* caused by *S. vesicarium*; and (2) AMF and *T. longibrachiatum* will increase the activity of plant enzymes, contents of hormones and the level of gene expression related to disease resistance. The results confirmed both of these hypotheses.

Typical mycorrhizal structures were observed when *V. villosa* was inoculated with AMF, which indicated that the AMF had successfully established symbiotic relationships with the plants. Infection with the pathogen had no significant effect on the colonization of AMF but decreased the shoot biomass of *V. villosa*. *S. vesicarium* causes brown lesions, which generally affect the photosynthesis of plants, and decreases in photosynthate and shoot biomass were the most obvious manifestation of infection [57]. AMF inhibited the development of the leaf spot disease caused by *S. vesicarium*.

The accumulation of ROS in large quantities and peroxidation of membrane lipids occur when pathogens attack plants. Malondialdehyde, a product of the peroxidation, is regarded as the indicator of the degree of damage from the pathogen to the plant [58]. After inoculation with *T. longibrachiatum* in combination with AMF and *T. longibrachiatum* in combination after infection, the MDA content of *V. villosa* decreased compared with that of the diseased plants that had not been inoculated with AMF

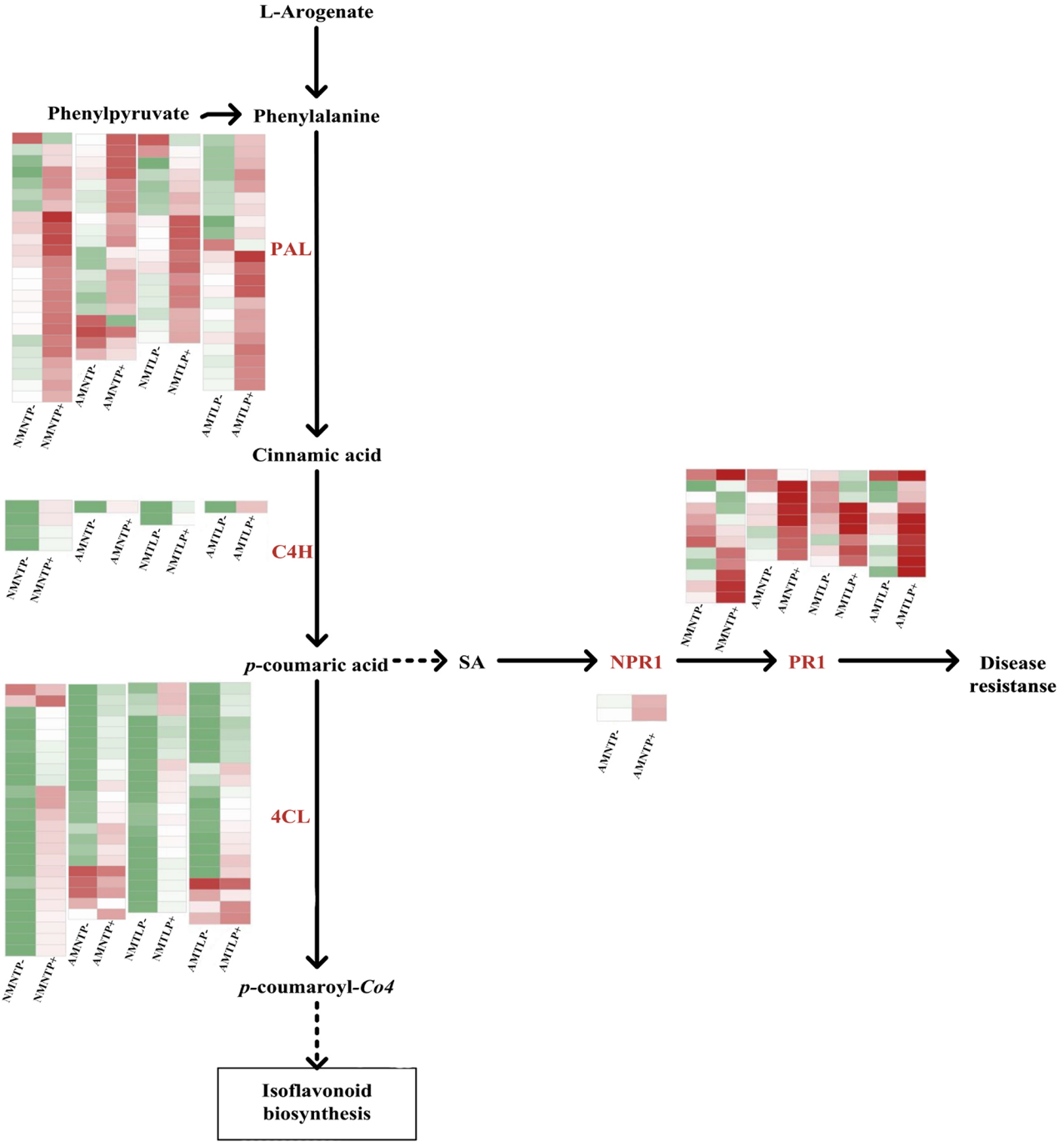

Fig. 7 Expression of regulatory genes in phenylpropane metabolism and the defense pathway of SA involved. NM = uninoculated with *S. tortuosa*, AM = inoculated with *S. tortuosa*, NT = uninoculated with *T. longibrachiatum*, TL = inoculated with *T. longibrachiatum*, P-=uninfected by *S. vesicarium*, P + = infected by *S. vesicarium*

and *Trichoderma*. These results indicate that *T. longibrachiatum* and AMF decrease the amount of stress to the plants caused by the pathogen. The accumulation of ROS in plants caused by pathogen infection activates signaling pathway to produce pathogenesis-related (PR) proteins. These proteins help mitigate the damage caused by ROS to cells or induce hypersensitivity responses to limit the spread of pathogens [59]. Chitinase is a PR protein that is involved in the defense of higher plants against pathogens [60, 61]. The inoculation with AMF enhanced the ability of plants to fight pathogens by increasing the activity of this enzyme. In addition to chitinase, POD is also a highly important member of the PR protein family. POD and SOD prevent the accumulation of active oxygen [62],

which severely damages plant cells. These results showed that the inoculation with AMF combined with *T. longibrachiatum* increased the activity of POD and thus, was a positive regulator of the resistance of plants to pathogens. El-Sharkawy et al. [24] also reported that inoculation with AMF and *Trichoderma* increased the activities of POD and polyphenol oxidase (PPO) in wheat (*Triticum aestivum*) and decreased the severity of stem rust on wheat caused by the fungal pathogen *Puccinia graminis*.

SA is an important regulatory compound for the plant defense response to pathogens [32]. These results also showed that inoculation with *T. longibrachiatum* and the combination of AMF and *T. longibrachiatum* significantly increased the content of SA, which contrasted with the trend of MDA content. This finding indicates that SA is an important compound that adjusts the plant's response to infection [63]. Previous research had shown that SA and JA inhibit each other in their role in defending the plants against pathogens [64]. This study found that AMF and *T. longibrachiatum* decreased the content of JA in the plants infected by *S. vesicarium*. The JA content of the diseased plants that were not inoculated with AMF or *T. longibrachiatum* increased significantly compared with the healthy plants, but the content of SA did not. These results indicate that the *V. villosa* responded pathogen infection through a JA-mediated defense signaling pathway. In contrast, *V. villosa* inoculated with *T. longibrachiatum*, either alone or in combination with AMF activates a SA-mediated defense signaling pathway when infected by *S. vesicarium*. PAL plays an essential role in SA biosynthesis [32], cell wall composition [65] and plant defense and has been demonstrated in previous studies [66]. After infection by *S. vesicarium*, the PAL activity of the diseased plants increased compared with that of the healthy plants. Interestingly, *T. longibrachiatum* increased the activity of PAL in the diseased plants. However, the AMF combined with *T. longibrachiatum* did not significantly change the activity of PAL compared with the diseased plants that were not inoculated with either treatment. The results indicated that infection by *S. vesicarium* promoted the defense pathway to the pathogen that utilized the activity of PAL, and *T. longibrachiatum* enhanced this effect. Hypothesis 3 that the co-inoculation of AMF and *T. longibrachiatum* will have a synergistic effect on the inhibition of disease compared with the plants that had been inoculated separately was partially upheld. A previous study showed that inoculation with AMF and other beneficial microorganisms promoted the production of antibacterial compounds, such as antibiotics and phenolics, while also inducing systemic resistance and producing defense enzymes [67].

Infection by *S. vesicarium* increased the number of DEGs, particularly the number of upregulated genes. An analysis of GO enrichment showed that the activity of peroxidase (GO:0004601) was significantly enriched in the diseased plant inoculated with AMF and *T. longibrachiatum* separately or together compared with the healthy plant, and this result was identical to the activity of POD. The number of DEGs increased with the degree of infection by the pathogen, and most of the DEGs were involved in the metabolic processes and signal transduction in response to infection by the pathogen [68]. The regulation of plant physiological processes, such as scavenging active oxygen, is not the only way for plants to defend themselves against biological stress. Signal transduction and plant–pathogen interactions are also important in the resistance of plants to infection [69]. The results of this study showed that defense response (GO:0006952), abscisic acid-activated signaling pathway (GO:0009738) and signaling receptor activity (GO:0038023) were significantly enriched in the AMNTP- - AMNTP+, NMTLP- - NMTLP + and AMTLP- - AMTLP + treatment groups but were not significantly enriched in the NMNTP- - NMNTP + treatment group. These GO terms are involved in plant disease resistance. For example, abscisic acid enhances the resistance of tobacco (*Nicotiana tabacum*) to white mold (*Sclerotinia sclerotiorum*) [70], and the defense response (GO:0006952) is also associated with disease resistance in *V. sativa* [18]. Compared to the individual inoculation of AMF and *T. longibrachiatum*, the combined treatment of AMF and *T. longibrachiatum* did not reveal specific GO terms involved in plant disease resistance.

After inoculation with the biocontrol microorganisms, the plant responses to biotic stimuli improved [71, 72]. The results of this study confirmed the phenomenon that AMF and *T. longibrachiatum* help plants to improve their resistance to infection. Phenylalanine metabolism (ko00360), isoflavonoid biosynthesis (ko00943) and flavone and flavonol biosynthesis (ko00944) have been proven to play positive roles in plant defense processes according to analyses of the KEGG pathway [73, 74]. The KEGG pathways associated with the top ten pathways according to the enrichment ratio in the NMNTP- - NMNTP+, AMNTP- - AMNTP+, NMTLP- - NMTLP + and AMTLP- - AMTLP + treatments were also analyzed. All or part of phenylalanine metabolism (ko00360), isoflavonoid biosynthesis (ko00943) and flavone and flavonol biosynthesis (ko00944) were significantly enriched with a high rich ratio. Thus, phenylpropanoid biosynthesis and signal transduction were examined in more detail. C4H is involved in the synthesis of SA [75], and C4L-related genes were higher up-regulated expression in AMTLP- -AMTLP + than that in other treatment groups. This resulted in an increased SA content in plants infected by *S. vesicarium*. Although there was no more significant up-regulated gene

expression in NMTLP- - NMTLP + than in NMNTP- - NMNTP+, this may be attributed to *T. longibrachiatum* colonizing plant roots during the early stage of plant growth, which enhances increase SA content. The expression of NPR1-related genes was significantly upregulated only in AMNTP- - AMNTP+, indicating that AMF enhanced the downstream defense process of the SA signaling pathway rather than increased the SA content directly. Both *T. longibrachiatum* and the combination of *T. longibrachiatum* and AMF increased the resistance of *V. villosa* to *S. vesicarium* by increasing the SA content. However, the combination of AMF and *T. longibrachiatum* did not show a significant up-regulation expression of genes involved in SA signaling pathway compared to the separate treatment, supporting the finding that the combination did not reduce the disease severity more than either treatment alone. While both AMF and *Trichoderma* positively influence plant growth, development, resistance to biotic and abiotic stress, their effects are not always additive [76].

In conclusion, the AMF increased the activities of chitinase in the plant and *T. longibrachiatum* increased the content of SA and the levels of genes expression related to pathogen defense, which reduced the severity of plant disease. SA-mediated signaling pathway plays a crucial role in the resistance of *V. villosa* to pathogens. Inoculation with *T. longibrachiatum* both alone and in combination with AMF, mainly increased the SA content and enhanced the resistance of *V. villosa* to *S. vesicarium*. Additionally, the inoculation with AMF increased the expression of NPR1-related genes in SA signaling pathway. These results expand our understanding of how muti-microbial mutualisms affect the impacts of disease. In future studies, it is essential to consider the identification of gene functions and analysis of metabolites that are involved in plant resistance.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03778-y.

Supplementary Material 1

Author contributions

Tingting Ding wrote the main manuscript text. Wei Feng, Meiting Bai and Tingyu Duan revised the manuscript. Lijun Gu provided the Trichoderma longibrachiatum. Tingyu Duan Provided funding for the study.

Funding

This research was supported by China Modern Agriculture Research System (CARS-22 Green Manure) and Gansu Forestry and Grassland Administration-Grassland monitoring and evaluation technology support project of Gansu Province (2021 – 794).

Data availability

The original transcriptome data were uploaded to the Sequence Read Archive of the NCBI (BioProject ID: PRJNA960699), and the data have provided within the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Author details

[1]Grassland Research Center of National Forestry and Grassland Administration, Chinese Academy of Forestry, Beijing 100091, China
[2]State Key Laboratory of Herbage Improvement and Grassland Agro-Ecosystems, Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730020, China
[3]Institute of Agro-Resources and Environment, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050051, China
[4]Gansu Open University, Lanzhou 730000, China
[5]College of Pastoral Agriculture Science and Technology, Lanzhou University, NO. 768 Jiayuguan West Road, Lanzhou 730020, China

Received: 22 February 2024 / Accepted: 22 January 2025
Published online: 25 February 2025

References

1. Yan ZC, Zhang WZ, Duan TY. First Report of Leaf Spot caused by *Stemphylium vesicarium* on *Vicia villosa* in China. Plant Dis. 2019;103:1039–1039.
2. Mardani-Korrani H, Nakayasu M, Yamazaki S, Aoki Y, Kaida R, Motobayashi T, Kobayashi M, et al. L-Canavanine, a root exudate from hairy vetch (*Vicia villosa*) drastically affecting the soil microbial community and metabolite pathways. Front Microbiol. 2021;12:701796.
3. Ibanez SG, Travaglia CN, Medina MI, Agostini E. *Vicia villosa* Roth: a cover crop to phytoremediate arsenic polluted environments. Environ Sci Pollut Res. 2021;28:38604–12.
4. Pott LP, Carneiro Amado TJ, Schwalbert RA, Gebert FH, Reimche GB, et al. Effect of hairy vetch cover crop on maize nitrogen supply and productivity at varying yield environments in Southern Brazil. Sci Total Environ. 2021;759:144313.
5. Fogliatto S, Patrucco L, De Palo F, Moretti B, Milan M, Vidotto F. Cover crops as green mulching for weed management in rice. Italian J Agron. 2021;16:1850.
6. Shi M, Li YZ. First Report of Leaf Spot caused by *Ramularia sphaeroidea* on *Vicia villosa* var. glabrescens in China. Plant Dis. 2021;105:4159.
7. Tanahashi M, Okuda S, Miyazaki E, Parada RY, Ishihara A, Otani H, Osaki-Oka K. Production of host-selective sv-toxins by *Stemphylium* sp. causing brown spot of European pear in Japan. J Phytopathol. 2017;165:189–94.
8. Spawton KA, Stein L, du Toit LJ. Susceptibility of spinach cultivars to Stemphylium leaf spot (*Stemphylium Vesicarium*) and white rust (*Albugo Occidentalis*) in Texas. Phytopathology. 2021;111:50.
9. Wang CH, Tsai YC, Tsai I, Chung CL, Lin YC, Hung TH, Suwannarach N, Cheewangkoon R, Elgorban AM, Ariyawansa HA. *Stemphylium* leaf bligWelsh welsh oallium*Allium fistulosum*): an emerging disease in Sanxing, Taiwan. Plant Dis. 2021;105:4121.
10. Diaz-Valderrama JR, Casa-Coila VH, Sencia-Torres V, Macedo-Valdivia D, Zanabria-Galvez SJ, Baributsa D, Woloshuk C. First Report of *Stemphylium* leaf spot caused by *Stemphylium vesicarium* on alfalfa (*Medicago sativa*) in Peru. Plant Dis. 2021;105:1196.
11. Zubrod JP, Bundschuh M, Arts G, Bruehl CA, Imfeld G, Knaebel A, et al. Fungicides: an overlooked pesticide class? Environ Sci Technol. 2019;53:3347–65.
12. Foster JM, Tayviah CS, Stricker SM, Gossen BD, McDonald MR. Susceptibility to *Stemphylium vesicarium* of asparagus, onion, pear, and rye in Canada. Can J Plant Pathol. 2019;41:228–41.
13. Hay FS, Sharma S, Hoepting C, Strickland D, Luong K, Pethybridge SJ. Emergence of Stemphylium leaf blight of onion in New York associated with fungicide resistance. Plant Dis. 2019;103:3083–92.
14. Panth M, Hassler SC, Baysal-Gurel F. Methods for management of soilborne diseases in crop production. Agriculture-Basel. 2020;10:16.
15. Fravel DR. Commercialization and implementation of biocontrol. Annu Rev Phytopathol. 2005;43:337–59.
16. Smith S, David RF, Mycorrhizal Symbiosis. (Third Edition). New York: Academic, 2008. pp. 769–787.
17. Chen T, Duan T. Research progress of arbuscular mycorrhizal fungi affecting plant diseases. Pratacultural Sci. 2021;38:1097–109.

18. Ding T, Zhang W, Li Y, Duan T. Effect of the am fungus *Sieverdingia tortuosa* on common vetch responses to an anthracnose pathogen. Front Microbiol. 2020;11:542623.
19. Li Y, Duan T, Nan Z, Li Y. Arbuscular mycorrhizal fungus alleviates alfalfa leaf spots caused by *Phoma medicaginis* revealed by RNA-seq analysis. J Appl Microbiol. 2021;130:547–60.
20. Abdel-Fattah GM, Shabana YM. Efficacy of the arbuscular mycorrhizal fungus *Glomus clarum* in protection of cowpea plants against root rot pathogen *Rhizoctonia Solani*. Z Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal Plant Dis Prot. 2002;109:207–15.
21. Hirpara DG, Gajera HP, Hirpara HZ, Golakiya BA. Antipathy of trichoderma against sclerotium rolfsii sacc.: evaluation of cell wall-degrading enzymatic activities and molecular diversity analysis of antagonists. J Mol Microbiol Biotechnol. 2017;27:22–8.
22. Van Jaarsveld WJ, Halleen F, Mostert L. In vitro screening of *Trichoderma* isolates for biocontrol of black foot disease pathogens. Phytopathologia Mediterranea. 2020;59:465–71.
23. Ruan Y, Liu F. Research progress of biological control mechanism and application of *Trichoderma*. J Zhejiang Agricultural Sci. 2020;61:2290–94.
24. El-Sharkawy HHA, Rashad YM, Ibrahim SA. Biocontrol of stem rust disease of wheat using arbuscular mycorrhizal fungi and *Trichoderma* spp. Physiol Mol Plant Pathol. 2018;103:84–91.
25. Yedidia I, Shoresh M, Kerem Z, Benhamou N, Kapulnik Y, Chet I. Concomitant induction of systemic resistance to *Pseudomonas Spingae* Pv. Lachrymans in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. Appl Environ Microbiol. 2003;69:7343–53.
26. Tanwar A, Aggarwal A, Panwar V. Arbuscular mycorrhizal fungi and *Trichoderma viride* mediated *Fusarium* wilt control in tomato. Biocontrol Sci Technol. 2013;23:485–98.
27. Martinez-Medina A, Roldan A, Pascual JA. Interaction between arbuscular mycorrhizal fungi and *Trichoderma harzianum* under conventional and low input fertilization field condition in melon crops: growth response and *Fusarium* wilt Biocontrol. Appl Soil Ecol. 2011;47:98–105.
28. Sennoi R, Singkham N, Jogloy S, Boonlue S, Saksirirat W, Kesmala T, et al. Biological control of southern stem rot caused by *Sclerotium rolfsii* using *Trichoderma Harzianum* and arbuscular mycorrhizal fungi on *Jerusalem artichoke* (*Helianthus tuberosus* L). Crop Prot. 2013;54:148–53.
29. Kumar V, Chaudhary P, Prasad A, Dogra V, Kumar A. Jasmonic acid limits *Rhizoctonia Solani* AG1-IA infection in rice by modulating reactive oxygen species homeostasis. Plant Physiol Biochem. 2023;196:520–30.
30. Lin P, Xia L, Wong JH, Ng TB, Ye X, Wang S, Shi X. Lipid transfer proteins from *Brassica campestris* and mung bean surpass mung bean chitinase in exploitability. J Pept Sci. 2007;13:642–8.
31. Saunders JA, O'Neill NR. The characterization of defense responses to fungal infection in alfalfa. Biocontrol. 2004;49:715–28.
32. Shine MB, Yang JW, El-Habbak M, Nagyabhyru P, Fu DQ, Navarre D, et al. Cooperative functioning between phenylalanine ammonia lyase and isochorismate synthase activities contributes to salicylic acid biosynthesis in soybean. New Phytol. 2016;212:627–36.
33. Zhao D, Wang H, Chen S, Yu D, Reiter RJ. Phytomelatonin: an emerging regulator of plant biotic stress resistance. Trends Plant Sci. 2021;26:70–82.
34. Alshammari NI, Bairum RS, Sulieman AME, Jamal A, Alamoudi M, et al. Control of tomato wilt disease fungus *fusarium oxysporum* f.sp. *Lycopersicon* by single or combine interaction of mycorrhiza, *Trichoderma Harzianum*, and effective microorganisms (Microbial Blend). J Pure Appl Microbiol. 2022;16:1362–9.
35. Hoenig M, Roeber VM, Schmuelling T, Cortleven A. Chemical priming of plant defense responses to pathogen attacks. Front Plant Sci. 2023;14:1146577.
36. Pandit MA, Kumar J, Gulati S, Bhandari N, Mehta P, Katyal R, et al. Major biological control strategies for plant pathogens. Pathogens. 2022;11:273.
37. Blaszkowski J, Niezgoda P, de Paiva JN, Galdino da Silva KJ, Theodoro RC, Jobim K et al. *Sieverdingia* gen. nov., *S. tortuosa* comb. nov., and *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota). Mycological Progress. 2019; 18: 1363-82.
38. Gao P, Li Y, Guo Y, Duan T. Coinoculation of Lucerne (*Medicago sativa*) with an AM fungus and a rhizobium reduces occurrence of spring black stem and leaf spot caused by *Phoma Medicaginis*. Pasture Sci. 2018;69:933–43. Crop.
39. Li H. Principles and techniques of Plant Physiology and Biochemistry Experiment. Beijing: Higher Education Press; 2000.
40. Wang X. Principles and Techniques of Plant Physiology and Biochemistry Experiment. 2nd Edn. Beijing: Higher Education Press, 2006.
41. El-Beltagi HS, Ahmed SH, Namich AAM, Abdel-Sattar RR. Effect of salicylic acid and potassium citrate on cotton plant under salt stress. Fresenius Environ Bull. 2017;26:1091–100.
42. Giovannetti M, Mosse B. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 1980;84:489–500.
43. Ren JZ. Research methods of grassland science. Beijing: China Agriculture; 1998.
44. Chen Y, Chen Y, Shi C, Huang Z, Zhang Y, Li S, et al. SOAPnuke: a MapReduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. Gigascience. 2018;7:120.
45. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.
46. Kim HS, Lee BY, Won EJ, Han J, Hwang DS, Park HG, et al. Identification of xenobiotic biodegradation and metabolism-related genes in the copepod *Tigriopus japonicus* whole transcriptome analysis. Mar Genom. 2015;24:207–8.
47. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012;40:e115.
48. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;12:59–60.
49. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323.
50. Love MI, Huber W, Anders S. Moderated estimation of Fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.
51. Zhuang H, Li J, Song J, Hettenhausen C, Schuman MC, Sun G, et al. Aphid (*Myzus persicae*) feeding on the parasitic plant dodder (*Cuscuta australis*) activates defense responses in both the parasite and soybean host. New Phytol. 2018;218:1586–96.
52. Benjamini Y, Hochberg Y. On the adaptive control of the false discovery fate in multiple testing with independent statistics. J Educational Behav Stat. 2000;25:60–83.
53. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci USA. 2003;100:9440–45.
54. Fu JJ, Geng JC, Miao YJ, Xu YM, Hu TM, Yang PZ. Transcriptomic analyses reveal genotype- and organ-specific molecular responses to cold stress in *Elymus nutans*. Biol Plant. 2018;62:671–83.
55. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402–8.
56. Congcong W, Liu H, Yu S, Chen H, Hu F, et al. Identification of genes involved in low temperature-induced senescence of panicle leaf in *Litchi chinensis*. Genes. 2019;10:111.
57. Yang B, Li YZ. Effects of Timothy *Cladosporium eyespot* on photosynthesis and biomass. Arch Microbiol. 2022;204:568.
58. Yusuf CS, Chand R, Mishra VK, Joshi AK. The Association between leaf malondialdehyde and lignin content and resistance to spot blotch in wheat. J Phytopathol. 2016;164:896–903.
59. Peleg-Grossman S, Melamed-Book N, Levine A. ROS production during symbiotic infection suppresses pathogenesis-related gene expression. Plant Signal Behav. 2012;7:409–15.
60. Vanloon LC. Pathogenesis-related proteins. Plant Mol Biol. 1985;4:111–6.
61. Grover A. Plant chitinases: genetic diversity and physiological roles. CRC Crit Rev Plant Sci. 2012;31:57–73.
62. Wei G, Zhu Z, Li J, Yao Q. Effects of silicon supply and *Sphaerotheca Fuliginea* inoculation on resistance of cucumber seedlings against powdery mildew. Can J Appl Ecol. 2004;15:2147–51.
63. Liu N, Wang Y, Li K, Li C, Liu B, Zhao L, Zhang X, Qu F, Gao L, Xia T, et al. Transcriptional Analysis of Tea Plants (Camellia sinensis) in response to salicylic acid treatment. J Agric Food Chem. 2023;71:2377–89.
64. Yuan H-M, Liu W-C, Lu Y-T. CATALASE2 coordinates SA-Mediated repression of both Auxin Accumulation and JA Biosynthesis in Plant defenses. Cell Host Microbe. 2017;21:143–55.
65. Cass CL, Peraldi A, Dowd PF, Mottiar Y, Santoro N, Karlen SD, et al. Effects of phenylalanine ammonia lyase (PAL) knockdown on cell wall composition, biomass digestibility, and biotic and abiotic stress responses in Brachypodium. J Exp Bot. 2015;66:4317–35.
66. Wang R, Wang G-L, Ning Y, PALs. Emerging Key players in Broad-Spectrum Disease Resistance. Trends Plant Sci. 2019;24:785–7.
67. Li F, Duan T. Research progress in the control of plant diseases by the combination of fungi and beneficial microorganisms. Chin J Grassland. 2021;43:93–105.
68. Sun Y, Fan M, He Y. Transcriptome analysis of watermelon leaves reveals candidate genes responsive to cucumber green mottle mosaic virus infection. Int J Mol Sci. 2019;20:610.

69. Xie X, Han Y, Yuan X, Zhang M, Li P, Ding A, et al. Transcriptome analysis reveals that exogenous melatonin confers lilium disease resistance to *Botrytis Elliptica*. Front Genet. 2022;13:674.
70. Ma LG, Zhang YH, Meng QL, Shi FM, Liu J, Li YC. Enhancement of *Sclerotina sclerotiorum* and oxalic acid resistance in tobacco by a novel pathogen-induced gst gene from sunflower. Crop Sci. 2018;58:1318–27.
71. Zitlalpopoca-Hernandez G, Pozo MJ, Hauser TP, Meyling NV. Combined effects of root-associated entomopathogenic and mycorrhizal fungi on the foliar pathogen *Botrytis Cinerea* in tomato. Biol Control. 2022;175:105034.
72. Santos M, Dianez F, Sanchez-Montesinos B, Huertas V, Moreno-Gavira A, Esteban Garcia B, Garrido-Cardenas JA, et al. Biocontrol of diseases caused by *Phytophthora Capsici* and *P. Parasitica* in pepper plants. J Fungi. 2023;9:360.
73. Zhan C, Li Y, Li H, Wang M, Gong S, Ma D, et al. Phylogenomic analysis of phenylalanine ammonia-lyase (PAL) multigene family and their differential expression analysis in wheat (*Triticum aestivum* L.) suggested their roles during different stress responses. Front Plant Sci. 2022;13:982457.
74. Saxena S, Pal L, Naik J, Singh Y, Verma PK, Chattopadhyay D, et al. The R2R3-MYB-SG7 transcription factor CaMYB39 orchestrates surface phenylpropanoid metabolism and pathogen resistance in chickpea. New Phytol. 2023;238:798–816.
75. Augustine L, Varghese L, Kappachery S, Ramaswami VM, Surendrababu SP, et al. Comparative analyses reveal a phenylalanine ammonia lyase dependent and salicylic acid mediated host resistance in *Zingiber zerumbet* against the necrotrophic soft rot pathogen *Pythium myriotylum*. Plant Sci. 2024;340:111972.
76. Meddad-Hamza A, Benzina F, Meddad C, Hamza N, Reghmit A, Ziane H, Ksentini H. Biological control of arbuscular mycorrhizal fungi and *Trichoderma harzianum* against *Fusarium oxysporum* and *verticillium dahliae* induced wilt in tomato plants. Egypt J Biol Pest Control. 2023;33:91.

Publisher's note