Postprint: Identification of Trichoderma harzianum T9131 and Analysis of Its Antagonistic Effects Against Pathogens and Induction of Disease Resistance in Astragalus membranaceus

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Abstract

Trichoderma is a common fungus in soil and plant rhizosphere that plays an important role in agricultural biological control. To investigate the role of Trichoderma in Astragalus resistance against root rot, this study isolated and identified Trichoderma from diseased Astragalus roots and examined its antagonistic effect against the pathogen and its influence on disease resistance physiological indicators of Astragalus. The Trichoderma species was determined through morphological observation, ITS sequence and tef1 sequence analysis; the antagonistic effect of the isolated Trichoderma against Fusarium solani was analyzed using plate confrontation assays; and physiological indicators of Astragalus including catalase (CAT), superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), peroxidase (POD) and proline (Pro) were measured to clarify the role of the isolated Trichoderma in inducing disease resistance in Astragalus. The results showed that: (1) The Trichoderma isolated and identified from Astragalus diseased roots was Trichoderma harzianum, designated as T9131. (2) When Trichoderma harzianum T9131 and Fusarium solani HYFS-1 were confronted on plates for 6 days, the inhibition rate of T9131 against HYFS-1 was $72\% \pm 1\%$. (3) Compared with inoculation with Fusarium solani HYFS-1 alone, T9131 significantly increased SOD activity when HYFS-1 infection was at 0 h; when HYFS-1 infection was at 24 h, T9131 significantly increased SOD and POD activities and Pro content; when HYFS-1 infection was at 48 h, T9131 significantly increased POD and PAL activities and Pro content. In conclusion, Trichoderma harzianum T9131 can control Astragalus root rot through directly inhibiting the growth of pathogen HYFS-1 and inducing disease resistance physiological indicators including SOD, POD, PAL activities and Pro content in Astragalus, laying a foundation for further investigation of the biocontrol function

of Trichoderma harzianum and its mechanism of action in Astragalus resistance against root rot.

Full Text

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Identification of *Trichoderma harzianum* T9131 and Its Antagonistic Activity Against Pathogens and Induction of Disease Resistance in *Astragalus membranaceus*

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Abstract: *Trichoderma* species are ubiquitous fungi in soil and plant rhizospheres that play crucial roles in agricultural biological control. To investigate the role of *Trichoderma* in enhancing resistance of *Astragalus membranaceus* against root rot, we isolated and identified a *Trichoderma* strain from diseased *A. membranaceus* roots and evaluated its antagonistic effects against pathogens and its impact on disease resistance-related physiological indicators in *A. membranaceus*. Strain identification was performed through morphological observation, ITS sequence analysis, and *tef1* sequence analysis. Antagonistic activity against *Fusarium solani* HYFS-1 was assessed using dual culture plate confrontation assays. The role of the *Trichoderma* isolate in inducing resistance was determined by measuring key physiological indicators including catalase (CAT), superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), peroxidase (POD), and proline (Pro). The results demonstrated: (1) The isolated *Trichoderma* strain was identified as *Trichoderma harzianum* and designated T9131. (2) After 6 days of co-cultivation, T9131 exhibited a 72% ± 1% inhibition rate against *F. solani* HYFS-1. (3) Compared to *F. solani* infection alone, T9131 pretreatment significantly enhanced SOD activity at 0 h post-infection; at 24 h post-infection, T9131 significantly increased SOD and POD activities and Pro content; and at 48 h post-infection, T9131 significantly elevated POD and PAL activities and Pro content. These findings indicate that *T. harzianum* T9131 can control *A. membranaceus* root rot through both direct inhibition of

pathogen growth and induction of host defense responses, as evidenced by enhanced SOD, POD, and PAL activities and increased Pro accumulation. This study provides a foundation for elucidating the biocontrol mechanisms of *T. harzianum* against *A. membranaceus* root rot.

Keywords: *Astragalus membranaceus* root rot; *Trichoderma harzianum*; *Fusarium solani*; antagonistic activity; physiological indicators

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Astragalus membranaceus is a perennial herbaceous legume that includes two major varieties: *A. membranaceus* and *A. membranaceus* var. *mongholicus*. The dried roots are highly valued in traditional medicine (Ren et al., 2016). However, root rot disease has become a critical factor limiting the development of the *A. membranaceus* industry (Ma et al., 2019). Current management strategies primarily include agricultural and chemical control methods, but agricultural practices such as crop rotation are difficult to implement, while chemical control often leads to pesticide residues and environmental pollution (Ren et al., 2016; Gao et al., 2019).

Biocontrol agents have emerged as promising environmentally friendly alternatives to conventional chemical pesticides for disease management (Singh et al., 2023). In Hunyuan County, Datong City, Shanxi Province–a major production area for *A. membranaceus*–most biocontrol agents screened to date have been bacterial strains, including *Bacillus* G10 (Ren et al., 2016), *Bacillus atrophaeus* R88 (Gao et al., 2019), and *Bacillus velezensis* C44 (Ma, 2023). In contrast, few studies have reported the screening of biocontrol fungi.

Trichoderma species are widely distributed biocontrol fungi in nature, with over 370 recognized species. Among them, *Trichoderma harzianum* has attracted considerable attention for its ability to control diverse plant pathogens (Singh et al., 2023). *T. harzianum* is commonly found in temperate climates, inhabiting soil, other fungi, decaying plant material, and living plant tissues (Guzman-Guzman et al., 2023). As a biocontrol agent, it effectively suppresses plant pathogens

through competition, antibiosis, and induction of plant defense responses (Braun et al., 2018; Guzman-Guzman et al., 2023).

Previous studies have demonstrated that *T. harzianum* EMF910 can inhibit the growth of root rot pathogens and reduce disease incidence in *A. membranaceus* grown in saline-alkali regions of Ningxia (Zhang et al., 2024). Our research group has previously shown that *T. harzianum* T9131 significantly enhances resistance of *A. membranaceus* against the local pathogen HYFS-1 isolated from Hunyuan County, Datong (Niu et al., 2024), but the formal identification of this strain has not been reported. Furthermore, elucidating the defense mechanisms induced by biocontrol agents represents a novel approach to plant protection (Singh et al., 2014; Niu et al., 2024). *T. harzianum* T9131 has been reported to confer resistance against HYFS-1 infection by initially increasing and subsequently decreasing H_2O_2 content in *A. membranaceus* (Niu et al., 2024). H_2O_2 is a crucial reactive oxygen species (ROS), and ROS accumulation can promote programmed cell death (PCD) to limit pathogen invasion (Sehrish et al., 2020). However, excessive ROS accumulation can damage plant tissues (Singh et al., 2023), necessitating strict regulation of ROS production and scavenging. Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are key enzymes that regulate H_2O_2 levels (Sharma et al., 2012). Proline (Pro) also functions as an antioxidant that modulates ROS balance (Gratao et al., 2012; Sarker et al., 2018) and can be induced by *Trichoderma* inoculation (Batool et al., 2020; Zhang et al., 2023; Lian et al., 2023). Whether *T. harzianum* T9131 regulates H_2O_2 content through SOD, POD, CAT, and Pro remains to be investigated. Our previous research also demonstrated that total flavonoids extracted from *A. membranaceus* significantly inhibit HYFS-1 growth (Niu et al., 2023). Phenylalanine ammonia-lyase (PAL) is a key enzyme in flavonoid biosynthesis and plays an important role in plant defense against various abiotic and biotic stresses (Macdonald et al., 2007). Whether PAL participates in *T. harzianum* T9131-induced disease resistance in *A. membranaceus* requires further investigation.

Biocontrol fungi primarily protect plants from disease by inhibiting pathogen growth and enhancing host resistance (Shoresh et al., 2010). Although previous studies have shown that T9131 induces resistance in *A. membranaceus* against HYFS-1, no reports have documented the identification of biocontrol fungi from Hunyuan County or evaluated their antimicrobial capacity and ability to induce disease resistance-related enzyme activities and physiological indicators. The present study aims to identify biocontrol fungi from *A. membranaceus* root rot in Hunyuan County, Datong, using morphological observation, molecular identification, plate confrontation assays, and physiological indicator measurements. Specifically, we address three questions: (1) identification of the biocontrol fungal species; (2) clarification of its efficacy against *A. membranaceus* root rot; and (3) elucidation of the physiological and biochemical mechanisms underlying induced disease resistance. This research will provide a theoretical foundation for controlling root rot in *A. membranaceus* from Hunyuan County.

1.1 Materials

Diseased *Astragalus membranaceus* plants showing root rot symptoms were collected from Hunyuan County, Datong City, Shanxi Province in 2021 for *Trichoderma* isolation. The pathogenic *Fusarium solani* strain HYFS-1 was previously isolated in our laboratory and maintained for experimental use.

1.2.1 Morphological Identification of *Trichoderma*

Trichoderma isolation and purification were performed following the method described by Niu et al. (2023). The fungus was cultured on potato dextrose agar (PDA) medium for 6–7 days, after which colony characteristics and conidial morphology were examined. Conidia were observed using a fluorescence microscope (Leica DM6B). Morphological identification was conducted according to the criteria established by Chaverri et al. (2015) and Yang (2009).

1.2.2 Molecular Identification of *Trichoderma*

Mycelia were harvested from 4–5-day-old cultures, and genomic DNA was extracted using a fungal genomic DNA extraction kit (Sangon Biotech, Shanghai). The internal transcribed spacer (ITS) region and the translation elongation factor 1-alpha (*tef1*) gene were amplified using the fungal identification primers ITS1/ITS4 and EF1–728F/tef1rev, respectively (Table 1), following the PCR protocol described by Niu et al. (2023). Amplification products were verified by 1% agarose gel electrophoresis and sequenced by Sangon Biotech. Phylogenetic trees were constructed using the neighbor-joining method (bootstrap = 1,000) in MEGA 7.0 software, incorporating sequence information from Liu et al. (2019) and Sadfi-Zouaoui et al. (2009).

Table 1 Primers used for amplification of ITS sequences

Primer name	Primer Sequence (5 to 3)
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
EF1–728F	CATCGAGAAGTTCGAGAAGG
tef1rev	GCCATCCTTGGAGACCAGC

1.2.3 Dual Culture Confrontation Assay Between *T. harzianum* and *F. solani*

In 90 mm Petri dishes containing PDA medium, 6 mm mycelial plugs of *T. harzianum* T9131 and *F. solani* HYFS-1 were inoculated approximately 20 mm from opposite ends of the plate. Control plates were inoculated with HYFS-1 alone. Each treatment consisted of three replicates. All plates were incubated at 27 °C for 6 days. The growth radius of HYFS-1 was measured daily, and the inhibition rate was calculated according to Ma et al. (2021) using the formula:

Inhibition rate (%) = (radius of control colony -radius of treated colony) / radius of control colony × 100.

1.2.4 Preparation of *T. harzianum* Spore Suspension

After 7 days of growth, 5 mL of sterile distilled water was added to each plate, and spores were dislodged by repeated pipetting. The resulting spore suspension was collected in centrifuge tubes and diluted 100-fold. After thorough mixing, 6 µL of the suspension was loaded onto a Watson 177-212C hemocytometer for counting under a microscope. The final spore concentration was adjusted to 1 × 10^8 spores/mL.

1.2.5 Physiological Indicator Measurements

Healthy, uniform seeds of *Astragalus membranaceus* var. *mongholicus* were sown in pots (14 cm diameter) containing a substrate mixture of nutrient soil and vermiculite (1:2 ratio), with approximately 20 seeds per pot. The pots were placed in a growth chamber at 25 °C with a 16 h light/8 h dark photoperiod. When seedlings reached the 4–5 leaf stage, the treatment group was root-drenched with 150 mL of *T. harzianum* T9131 spore suspension (T), while the control group received 150 mL of sterile water. After 48 h of treatment, all plants were inoculated with 5-day-old HYFS-1 using the hypocotyl inoculation method (Niu et al., 2023). Root samples were collected at 0, 24, and 48 h post-inoculation for determination of PAL, POD, SOD, CAT, and Pro contents, with three replicates per indicator. All physiological measurements were performed using commercial assay kits from Sangon Biotech (Shanghai): PAL activity (D799599-0050), POD activity (D799591-0050), SOD activity (D799593-0050), CAT activity (D799597-0050), and Pro content (D799575-0050).

1.2.6 Data Analysis

Experimental data were processed using WPS Office Excel. Graphs were generated using GraphPad Prism 9. Statistical significance was analyzed using SPSS 23 software, with $P < 0.05$ considered statistically significant.

2.1 Morphological Identification of *Trichoderma*

From our previous pathogen isolation and purification of diseased *A. membranaceus* plants, five fungal isolates were obtained (Niu et al., 2023). One isolate exhibited rapid growth on PDA medium, initially forming white, fluffy mycelial colonies (Figure 1 [Figure 1: see original paper]: A). After several days, the colonies turned green (Figure 1: B). Conidia were spherical to oval, smooth-edged, and green-colored (Figure 1: C), measuring (3.6–5.4) µm × (3.2–5.1) µm. These morphological characteristics are consistent with those of *T. harzianum*.

Figure 1 Morphological characteristics of isolated strain

A. Colony morphology on PDA medium for 3 d; B. Colony morphology on PDA medium for 7 d; C. Conidia.

2.2 Molecular Identification of *Trichoderma*

The ITS region (GenBank: PQ465589) and *tef1* gene (GenBank: PQ490419) were successfully amplified using fungal identification primers. Sequencing revealed an ITS fragment of 595 bp (Figure 2 [Figure 2: see original paper]: A) and a *tef1* fragment of 626 bp (Figure 3 [Figure 3: see original paper]: A). Phylogenetic trees constructed for both sequences, using *Bacillus siamensis* as the outgroup, showed highest homology with *T. harzianum* reference strains (Figure 2: B; Figure 3: B). Based on these molecular data and morphological characteristics, the isolate was identified as *Trichoderma harzianum* and designated T9131.

Figure 2 Molecular identification of ITS1 and ITS4 primers for *Trichoderma harzianum*

A. Agarose gel electrophoresis for amplification product; B. Phylogenetic tree of amplification sequences; * indicates the reference strains.

Figure 3 Molecular identification of EF1-728F and tef1rev primers for *Trichoderma harzianum*

A. Agarose gel electrophoresis for amplification product; B. Phylogenetic tree of amplification sequences.

2.3 Antagonistic Activity of *T. harzianum* Against *F. solani*

To evaluate the inhibitory effect of *T. harzianum* T9131 on *F. solani* HYFS-1, a dual culture confrontation assay was performed. T9131 grew rapidly, making contact with HYFS-1 after 1 day of co-cultivation (Figure 4 [Figure 4: see original paper]: A). By day 2, T9131 had produced green spores and began to overgrow HYFS-1, which concurrently produced orange-yellow pigments (Figure 4: B). After 3 days, T9131 had completely covered HYFS-1, which ceased further growth, yielding an inhibition rate of 47% ± 1%. By day 6, the inhibition rate reached 72% ± 1% (Figure 4: C).

Figure 4 A plate confrontation experiment of *Trichoderma harzianum* T9131 and *Fusarium solani* HYFS-1

A. Confrontation for 1 d; B. Confrontation for 2 d; C. Confrontation for 6 d.

2.4 Physiological Indicators Induced by *T. harzianum* in Astragalus

Previous studies showed that *A. membranaceus* plants begin to wilt 48 h after HYFS-1 infection (Niu et al., 2023), and that root drenching with *T. harzianum*

T9131 prior to HYFS-1 inoculation alleviates wilting symptoms (Niu et al., 2024). To investigate the role of T9131 in disease resistance, we measured the activities of SOD (Figure 5 [Figure 5: see original paper]: A), POD (Figure 5: B), CAT (Figure 5: C), PAL (Figure 5: D), and Pro content (Figure 5: E). Compared to HYFS-1 infection alone (H_0 h), T9131 pretreatment (T+H_0 h) significantly increased SOD activity while significantly decreasing POD, CAT, and PAL activities, with Pro content showing an upward trend. At 24 h post-infection, T+H_{24} h showed significant upregulation of SOD, POD, and Pro compared to H_{24} h, with no significant change in CAT and significant down-regulation of PAL. At 48 h post-infection, T+H_{48} h exhibited significant increases in POD, PAL, and Pro compared to H_{48} h, with SOD also increasing and CAT remaining unchanged (Figure 5). These results demonstrate that *T. harzianum* T9131 primarily induces POD, SOD, and PAL activities and promotes Pro accumulation.

- indicates significant differences ($P < 0.05$); ** indicates extremely significant differences ($P < 0.01$).

Figure 5 Root enzyme activity and Pro content of *Astragalus membranaceus* induced by *Trichoderma harzianum* T9131

3 Discussion and Conclusion

Fusarium solani is one of the primary pathogens causing root rot in *Astragalus membranaceus* and has been isolated and identified in various production regions including Shanxi (Ren et al., 2016; Niu et al., 2023), Qinghai (Ma et al., 2022), Longxi in Gansu (Niu et al., 2016), Weiyuan in Gansu (Chen et al., 2020), and Wuzhong in Ningxia (Zhang et al., 2024). The *F. solani* strain X12 isolated from Wuzhong, Ningxia was shown to be inhibited by *T. harzianum* EMF910 (Zhang et al., 2024). Our study demonstrates that *T. harzianum* T9131 isolated from Hunyuan County, Datong can also inhibit the growth of *F. solani* HYFS-1, indicating the potential value of *T. harzianum* for controlling *F. solani* in *A. membranaceus*. Root rot is a soil-borne disease (Niu et al., 2023). While *T. harzianum* EMF910 was isolated from soil (Zhang et al., 2024), our strain T9131 was isolated from diseased *A. membranaceus* roots. Previous reports have documented that *T. harzianum* can exist in decaying roots or as plant endophytes (Guzman-Guzman et al., 2023); therefore, whether T9131 functions as an endophyte in *A. membranaceus* warrants further investigation.

Under pathogen stress, *Trichoderma* can induce plant defense enzyme activities (Pang et al., 2023). For instance, *T. harzianum* NBRI-1055 enhanced SOD and CAT activities in sunflower under *Rhizoctonia solani* stress (Singh et al., 2011); *T. harzianum* T-aloe significantly increased POD and CAT activities in wheat challenged with *Fusarium graminearum* PH-1 (Pang et al., 2023); and *T. harzianum* Th elevated SOD, POD, CAT, and PAL activities in tomato under *Fusarium oxysporum* Fol stress (Zehra et al., 2023). Our study shows that under HYFS-1 stress, *T. harzianum* T9131 significantly increased SOD, POD,

and PAL activities, consistent with previous findings. Previous studies have reported positive correlations between SOD and POD activities and Pro accumulation (Han, 2006), and Pro can be induced by *Trichoderma* inoculation (Batool et al., 2020). Our results show that Pro content was generally higher in T9131-treated plants under HYFS-1 stress compared to controls, suggesting that T9131 may enhance SOD and POD activities by inducing Pro accumulation. In the oxidative defense system, SOD converts $O_2 \bullet^-$ to H_2O_2, which can then be detoxified to water and O_2 by POD and CAT (Sharma et al., 2012). The changes in SOD and POD activities induced by T9131 in our study corresponded well with the previously reported H_2O_2 dynamics (Niu et al., 2024), indicating that SOD and POD may participate in regulating H_2O_2 homeostasis. Furthermore, Pro has been shown to enhance SOD and POD activities through its own accumulation, thereby scavenging ROS (Jiao et al., 2011). We hypothesize that under *F. solani* HYFS-1 stress, *T. harzianum* T9131 first induces Pro accumulation, which subsequently enhances SOD and POD activities. These enzymes then modulate H_2O_2 levels, ultimately improving resistance of *A. membranaceus* against HYFS-1 (Figure 6 [Figure 6: see original paper]).

Figure 6 A model of *Trichoderma harzianum* T9131 inducing resistance under *Fusarium solani* HYFS-1 stress

In summary, the biocontrol fungus isolated from diseased roots in Hunyuan County, Datong was identified as *Trichoderma harzianum* T9131. This strain can directly inhibit the growth of the pathogenic *F. solani* HYFS-1 while simultaneously enhancing resistance in *A. membranaceus* by inducing key physiological indicators including SOD, POD, PAL activities, and Pro accumulation. These findings provide a theoretical basis for the application of *T. harzianum* as a biocontrol agent.

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Note: Figure translations are in progress. See original paper for figures.