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Plant performance of enhancing licorice with dual inoculating dark septate endophytes and *Trichoderma viride* mediated via effects on root development

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Abstract

This study aimed to assess whether licorice (*Glycyrrhiza uralensis*) can benefit from dual inoculation by *Trichoderma viride* and dark septate endophytes (DSE) isolated from other medicinal plants. We investigated the influences of three DSE (*Paraboeremia putaminum*, *Scytalidium lignicola*, and *Phoma herbarum*) isolated from other medicinal plants on the performance of licorice at different *T. viride* densities (1×10^6, 1×10^7, and 1×10^8 CFU/mL). Three DSE strains could colonize the roots of licorice, and they established a positive symbiosis with host plants depending on DSE species and *T. viride* densities. Inoculation of *Paraboeremia putaminum* increased the root biomass, length, surface area, and root:shoot ratio. *Scytalidium lignicola* increased the root length, diameter and surface area and decreased the root:shoot ratio. *Phoma herbarum* increased the root biomass and surface area. *T. viride* increased the root biomass, length, and surface area. Structural equation model (SEM) analysis showed that DSE associated with *T. viride* augmented plant biomass and height, shoot branching, and root surface area. Variations in root morphology and biomass were attributed to differences in DSE species and *T. viride* density among treatments. *Paraboeremia putaminum* or *Phoma herbarum* with low- or medium *T. viride* density and *S. lignicola* with low- or high *T. viride* density improved licorice root morphology and biomass. Our findings support the viewpoint that non-host DSE enhanced the root growth of the host plant under different densities *T. viride* conditions and may also be used to promote the cultivation of medicinal plants.

1. Introduction

Plant–microbe interactions drive plant health and the biogeochemical cycle in agricultural ecosystems (Lambers et al. 2009; Lugtenberg et al. 2016). Plants create important habitats for the microorganisms interacting with them and deliver photosynthate to them (Gutierrez-Garcia et al. 2019). In return, soil-borne microorganisms such as arbuscular mycorrhizal (AM) fungi, dark septate endophytes (DSE), and *Trichoderma* spp. improve plant productivity and maintain plant health. Thus, they are of particular interest in sustainable agriculture (Avis et al. 2008; Rai et al. 2014; Lugtenberg et al. 2016).

Dark septate endophytes (DSE) are diverse facultative biotrophic ascomycetes characterised by melanised septate hyphae and microsclerotia. They are found in the roots of > 600 different plant species (Xie et al. 2017). The relationships between host plants and DSE range from symbiotic to parasitic depending on the particular host-symbiont combination (Li et al. 2019). Previous research showed that DSE inoculation stimulates the growth of medicinal plants and improves medicinal compound yield (He et al. 2019a,b). Several DSE promote host plant growth by facilitating carbon, nitrogen, and phosphorus uptake (Della Monica et al. 2015; Surono and Narisawa 2017), and protecting host plants against biotic stress (pathogens) and abiotic stress (heavy metal, salt, and drought) (Su et al. 2013; Qin et al. 2017; Li et al. 2018). *Trichoderma* spp. are common rhizosphere inhabitants that have been investigated as biological control agents, biofertilisers, and soil amendments for application in agricultural and horticultural systems (Herrera-Parra et al. 2017). *Trichoderma* spp. improve plant growth mainly by solubilising soil nutrients (Bayoumi et al. 2019), and increasing root length and secondary root number,

and upregulating phytohormones such as indoleacetic acid, cytokinin, gibberellins, and zeatin (Gravel et al. 2007). Hence, further research is required on the use of these beneficial fungi for the improvement of the growth and quality of medicinal plants.

Licorice (*Glycyrrhiza uralensis* Fisch.) is a widely distributed herbaceous perennial medicinal plant. Its roots and rhizomes are important and commonly administered medicinal materials in China and other parts of the world. Itis included in the official Chinese Pharmacopoeia, a compendium of drugs compiled by the Pharmacopoeia Commission of the Ministry of Health of China. Ithas several pharmacological effects and biological functions because of its constituents such as glycyrrhizin and glycyrrhizic acid (He et al. 2019a). Italso conserves water and prevents wind erosion in arid agricultural ecosystems. Moreover, its leguminous roots fix atmospheric nitrogen. Interactions between at least two different fungi and licorice have been reported (Yuan et al. 2016; Berthelot et al. 2019), Nevertheless, the effects of DSE combined with *Trichoderma* spp. on licorice growth have not been investigated. In earlier studies, the major DSE groups coexisted with multiple plants in various ecosystems and showed no colonisation specificity (David et al. 2016; Li et al. 2018).

In a previous study using *Trichoderma viride* for cellulose degradation, our objective was to assess the effects of DSE (*Acrocalymma vagum, Paraboeremia putaminum)* combined with sterilised or unsterilised organic residues containing *Trichoderma viride* on licorice growth. The combination of DSE and *T. viride* residue enhanced plant growth more effectively either agent alone (He et al. 2019a). Here, we used *Trichoderma viride* as a growth promoter and investigated the effects of various DSE species both alone and in combination with *Trichoderma viride* on greenhouse-raised licoricein sandy soil. We isolated DSE from the roots of *O. japonicus* and *L. japonica* on the farmlands of northern China. We then inoculated licoriceeither with the aforementioned DSE alone or in combinationwith various densities of *T. viride.* In this research, we endeavoured to answer the following questions: (1) What are the characteristics of DSE in the roots of *O. japonicus* and *L. japonica* from the farmlands of northern China? (2) Can these DSE colonise and influence the growth of licorice plants under artificial culture conditions? (3) Does *T. viride* density affect DSE-related symbiosis?

2. Materials And Methods

2.1 DSE isolation and identification

The DSE strains were isolated from the roots of *O. japonicus* and *L. japonica* growing naturally at the Anguo Medicine Planting Site (115°20'E, 38°24'N), Hebei Province, China. The root samples were washed with deionised water, sterilised in 75% (v/v) ethanol for 5 min and in 10% (v/v) sodium hypochlorite for 5 min, rinsed thrice with deionised water, dried on sterile filter paper, and set in Petri dishes containing potato dextrose agar (PDA) supplemented with ampicillin and streptomycin sulphate. The plates were incubated at 27 °C in the dark and observed daily. To confirm that the isolates were true endophytes, 200 µL of the deionised water used to rinse the sterilised roots was applied to plain PDA medium. Mycelia growing from the cut ends of the root segments were transferred to fresh PDA plates and kept in the dark

at 27 °C before microscopic observations and measurements (He et al. 2019a,b). Twenty-six colonies were isolated from 90 root segments. Isolates with similar morphology and growth rate were grouped into the morphotypes DSE1, DSE2, DSE3, DSE4, DSE5, DSE6, and DSE7. Three replicates per isolate were cultured at 10 °C for 2 mo to induce sporogenesis (Knapp et al. 2015).

For the molecular identification of the seven endophytic fungi, fresh mycelia (~50 mg) were scraped from the surfaces of the PDA plates and their DNA was extracted with a genomic DNA extraction kit (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). The universal primers ITS4 (5´-TCCTCCGCTTATTGATATGC–3´) and ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG–3´) amplified the internal transcribed spacer (ITS) region by PCR (White et al. 1990). The reaction system (20 mL) consisted of 3.5 mL DNA template, 0.5 mL of each primer (10 mM), 10 mL of 2× Es Taq Master Mix (CoWin Biosciences, Beijing, China), and 5.5 mL ddH_2O. PCR was performed in a Life ECO™ thermocycler (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China) with an initial denaturing step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, 1 min of primer annealing at 55 °C, 1 min of extension at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were purified and sequenced. The sequences were deposited in GenBank under accession numbers MK583545 (DSE1), MK583546 (DSE2), MK583547 (DSE3), MK583549 (DSE4), MK601233 (DSE5), MK601234 (DSE6), and MK601236 (DSE7). Clustal X (v. 1.81) was used to align the sequences. The maximum likelihood tree was drawn with MEGA v. 6 (Tamura et al. 2013). Based on their growth status, DSE4, DSE5, and DSE6 were selected for use in the subsequent pot inoculation experiments.

2.2 Plant and *Trichoderma viride* materials

Licoricewas selected as the host plant as it is valuable in pharmacology and vegetation restoration. The licorice seeds were provided by the China National Traditional Chinese Medicine Corporation of Beijing, China and stored at 4 °C. The *Trichoderma viride* strain was provided by the Institute of Medicinal Plant Development, the Chinese Academy of Medical Sciences and Peking Union Medical College, China and incubated at 28 °C in PDA culture medium. The *Trichoderma* isolates were identified at the species level using both morphological (Supplementary Fig. S1) (Chaverri et al. 2015) and molecular (Supplementary Fig. S2) analysis (Sun et al. 2012). Their GenBank accession number is MK396066. Five millilitres sterile water was placed in a Petri dish containing mature *T. viride.* The suspension was thoroughly mixed and transferred to a sterile conical flask on an ultraclean workbench (Al-Hazmi et al. 2016). A haemocytometer was used to measure the *T. viride* spore inoculum density. Three *T. viride* spore concentrations were used in the subsequent pot inoculation experiments: low density (LT; 1×10^6 CFU mL^{-1}), medium density (MT; 1×10^7 CFU mL^{-1}), and high density (HT; 1×10^8 CFU mL^{-1}).

2.3 Experimental design

The experiment was conducted in a growth chamber. A completely randomised factorial design was used. It comprised four DSE inoculations × three *T. viride* densities and there were five replicates. The DSE

treatments included *Paraboeremia putaminum* (PP), *Phoma herbarum* (PH), *Scytalidium lignicola* (SL), and an uninoculated control. The three *T. viride* densities were low (LT), medium (MT), and high (HT). Sterilised *T. viride* spore fluidserved as a control.Eighty experimental bottles were prepared.

The licoriceseeds were surface-sterilised by dipping in 70% (v/v) ethanol for 3 min followed by 2.5% (v/v) sodium hypochlorite for 10 min with stirring. The sterilised seeds were washed several times with sterile water, aseptically planted in Petri dishes containing water agar (10 g L^{-1}) medium, and germinated at 27 °C. Each seedling was then transferred to a bottle (base diameter, 5.5 cm; aperture, 8.5 cm; height, 11.5 cm) containing 500 g sandy soil collected from the natural habitats of licorice. The bottlesand soil were pre-sterilised by autoclaving for 120 min at 121 °C. Two seedlings were planted per sterile microcosm. The physicochemical properties of the sandy soil were as follows: organic matter, 25.38 mg g^{-1}; available nitrogen, 22.50 μg g^{-1}; and available phosphorus, 1.68 μg g^{-1}. After 1 month, the seedlings were irrigated with 30 mL of various densities of *T. viride* spore fluid. The control seedlings were irrigated with 30 mL sterilised *T. viride* spore fluid.

The DSE inocula were prepared by aseptically growing DSE isolates in Petri dishes containing PDA culture medium. Two 5-mm plugs were excised from the edge of an actively growing DSE colony and inoculated within 1 cm of the roots of the licorice seedlings. The uninoculated controls were treated with plugs excised from sterile fungus-free medium. All inoculation procedures were conducted on a clean workbench. All experimental bottles were maintained in a growth chamber under a 14 h/10 h photoperiod at 27 °C/22 °C (day/night) and 60% mean air RH. The duration of the experiment was 3 months.

2.4 Plant growth parameters

At the end of the experiment, the plant height (cm), number of branches, and number of leaves were recorded. The shoots and roots were separately harvested from each bottle. The roots were washed with tap water to remove the soil. Individual root sections were floated in water in a plexiglass tray to a depth of ~1 cm and scanned with a desktop scanner (EPSON Perfection V800 Photo; Epson, Nagano, Japan). Root length, mean root diameter, and root surface area were measured by a WinRHIZO image analysis system (Chen et al. 2012). Shoot and root biomass were measured after drying the samples in an oven at 70 °C for 48 h.

2.5 DSE root colonization

Root colonization by the DSE isolates was evaluated using the method of Phillips and Hayman (1970). The roots were cleared with 10% (w/v) potassium hydroxide (KOH) in a water bath at 100 °C for 60 min and stained with 0.5% (w/v) acid fuchsin at 90 °C for 20 min. Thirty randomly selected 0.5-cm root segments per sample were mounted on slides and observed under an optical microscope.

2.6 Statistical analysis

The effects of DSE, *T. viride,* and their interactions on the measured variables were evaluated by two-way ANOVA ($P < 0.05$). The statistical significance of the results was determined by Duncan's multiple-range tests ($P < 0.05$). Nonmetric multidimensional scaling (NMDS) and an analysis of similarities (ANOSIM) test were conducted using the 'vegan' package in R v. 3.5.3 (Oksanen et al. 2007) to establish the effects of DSE and *T. viride* on licorice root morphology and biomass. A Mantel test and a structural equation model (SEM) tested the effects of DSE species, *T. viride,* and their interactions on licorice morphology and biomass in the 'ecodist' package of R v. 3.2.2 (Goslee and Urban 2007). Maximum likelihood was calculated with AMOS v. 21.0. Variance partitioning was performed to estimate the magnitude of influence of each factor on the plant growth parameters and biomass production. SPSS v. 21.0 for Windows (IBM Corp., Armonk, NY, USA), Canoco v. 4.5, RStudio 'vegan' package (Borcard et al. 2011), and KaleidaGraph v. 4.5 were used for the statistical analyses and graph plotting.

3. Results

3.1 Characterization and identification of DSE

Typical DSE hyphae and microsclerotia were observed in the roots of *O. japonicus* and *L. japonica* (Supplementary Fig. S3). Brown to yellow-brown septate hyphae with thick lateral walls invaded the epidermal and/or cortical cells (Supplementary Fig. S3A, S3C). Chainlike and conglomerated microsclerotia filled single cortical cells and/or colonised > 1 cell (Supplementary Fig. S3B, S3D).

Five and two DSE colonies isolated from *O. japonicus* and *L. japonica,* respectively,were ashen grey to dark brown (Supplementary Fig. S4). DSE1 and DSE4 produced spores but neither conidia nor reproductive structures were observed in the other isolates. A comparative analysis of the fungal sequences in the GenBank database identified *Acrocalymma vagum (*DSE1), *Paraphoma radicina (*DSE2), *Curvularia pallescens (*DSE3), *Scytalidum lignicola (*DSE4), *Paraboeremia putaminum (*DSE5),and *Phoma herbarum (*DSE6, DSE7) (Supplementary Fig. S5). Based on their growth status, we selected *S. lignicola* (SL, DSE4), *P. putaminum (*PP, DSE5), and *P. herbarum (*PH, DSE6) for the pot inoculation experiments.

3.2 Shoot morphological traits of *G. uralensis* seedlings

After 3 months of growth, all inoculated licorice seedlings were alive, green, and healthy. The roots of all inoculated plants were colonised by DSE4, DSE5, and DSE6 (Supplementary Fig. S6). Relative to the control plants, DSE or MT inoculation significantly increased plantheight whereas HT inoculation decreased it. In contrast, LT inoculation did not significantly modify plant height compared with the control plants (Fig. 1A). Only *S. lignicola* orHT inoculation significantly increased shoot branch number relative to the control plants. However, no significant differences in shoot branch number were observed between the other inoculated plants and the control plants (Fig. 1B). Compared with the control plants, *P. herbarum,* LT, and MT inoculation increased the leaf number whereas the other inoculants had no significant effect on this trait (Fig. 1C).

Interactions between DSE species and *T. viride* density were significant for plant height and shoot branch number (Table 1). Under LT conditions, *P. putaminum* inoculation increased plant height, *S. lignicola* decreased it, and *P. herbarum* had no significant effect on this characteristic compared with the control plants (Fig. 1A). Under LT conditions, only *P. herbarum* increased shoot branch and leaf numbers relative to the control plants. The other isolates had no significant impact on shoot branch or leaf number compared with the control plants (Fig. 1B, 1C). Under MT conditions, *P. putaminum* and *S. lignicola* increased plant height and leaf number, *S. lignicola* increased shoot branch number, and *P. herbarum* had no significant influence on plant height and shoot branch or leaf number relative to the control plants (Fig. 1A–1C). Under HT conditions, compared with the control plants, *P. herbarum* increased plant height and shoot branch and leaf numbers, *S. lignicola* increased both shoot branch and leaf numbers and decreased plant height, and *P. putaminum* decreased plant height but had no effect on shoot branch or leaf numbers (Fig. 1A–1C).

3.3 Root morphological traits of *G. uralensis* seedlings

P. putaminum and *S. lignicola* inoculation significantly increased licorice root length, diameter, and surface area relative to the control plants. However, *P. herbarum* only increased the root surface area compared with the control plants (Fig. 1D–1F). Root diameter and surface area wereincreased at various *T. viride* densities (Fig. 1E, 1F). The LT and HT treatments increased the root length whereas the MT treatment had no significant impact on this trait compared with the control plants (Fig. 1D).

Under LT conditions, DSE increased the root length, diameter, and surface arearelative to the control plants.Under MT conditions,DSE increased the root diameter and surface area, *S. lignicola* and *P. herbarum* increased the root length, and *P. putaminum* had no significant influence on root length compared with the control plants.Under HT conditions, DSE increased the root surface area but *S. lignicola* and *P. herbarum* onlyincreased the root length and diameter relative to the control plants (Fig. 1D–F). The interactions between DSE species and *T. viride* density were significant only for root length and surface area (Table 1).

3. 4 Biomass production of *G. uralensis* seedlings

The shoot and root biomass and the root:shoot ratio of licoriceweresignificantly and separately influenced by DSE species and *T. viride* density (Fig. 2A). *P. putaminum* increased the root biomass and the root:shoot ratio, *S. lignicola* increased the shoot biomass anddecreased theroot:shoot ratio, and *P. herbarum* only increased the root biomass compared with the control plants. The LT and MT conditions increased the shoot and root biomass whereas HT increased the root biomass and the root:shoot ratio and decreased the shoot biomass relative to the control plants (Fig. 2A). There were significant positive interactions between DSE and *T. viride* with respect to shoot and root biomass. However, the interactions between DSE and *T. viride* on the root:shoot ratio varied with DSE species and *T. viride* density (Fig. 2B, Table 1).

Nonmetric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) indicated that licorice root morphology and biomass were significantly separated by DSE species (R = 0.0505, *P* = 0.046) and *T. viride* density (R = 0.1223, *P* = 0.001) (Fig. 3).

3. 5 Correlation analyses

A Mantel test and a structural equation model (SEM) were used to illustrate the effects of DSE, *T. viride,* and their interaction on the growth parameters of licorice. The Mantel test disclosed significant relationships among DSE, *T. viride,* plant biomass, root length, diameter, and surface area, leaf number, plant height, and shoot branching (Supplementary Table S1). We used correlation coefficients (R-values) and the SEM to quantify the relative effects of DSE, *T. viride,* the combination of DSE and *T. viride* on total root length, diameter, and surface area on plant biomass, plant height, leaf number, and shoot branching (χ^2 = 136.933, degrees of freedom (df) = 12, *P* = 0.005, root mean square error of approximation (RMSEA) = 0.407, goodness-of fit index (GFI) = 0.750, Akaike information criterion (AIC) = 222.933). DSE had significant direct effects on root length and diameter, plant biomass, and shoot branching. *T. viride* had significant direct effects on root length, surface area, and diameter, plant biomass and height, and leaf number. The combination of DSE and *T. viride* significantly positively influenced root surface area, plant biomass and height, and shoot branching (Fig. 4).

3. 6 Variation partitioning of plant growth parameters and biomass production

A variance partitioning analysis was performed to quantify the contributions of DSE species and *T. viride* density to the plant growth parameters and biomass production (Figs. 5–7). The combination of PPandLT explained 38.2% of the variance in shoot biomass (Fig. 5A), 61.2% of the variance in root biomass (Fig. 5B), 16.9% of the variance in shoot growth traits (Fig. 5C), and 58.0% of the variance in root growth traits (Fig. 5D). The pure variances in root biomass and growth traits explained by PP were 45.3% and 39.5%, respectively, whilst LT explained 15.9% and 18.5%, respectively. The simultaneous influence of PP combined with LT on root biomass and growth traits explained 9.9% and 6.7% of the variance, respectively. The combination of PPandMT explained 55.9% of the variance in shoot biomass (Fig. 5E), 70.8% of the variance in root biomass (Fig. 5F), 34.6% of the variance in shoot growth traits (Fig. 5G), and 16.1% of the variance in root growth traits (Fig. 5H). The pure variances in root biomass and growth traits explained by PP were 20.0% and 13.9%, respectively, whilst MT explained 32.7% and 2.2%, respectively. The simultaneous influence of PPcombined with MT on root biomass and growth traits explained 18.1% and 4.3% of the variance, respectively. The combination of PP andHT explained 47.1% of the variance in shoot biomass (Fig. 5I), 13.0% of the variance in root biomass (Fig. 5J), 14.5% of the variance in shoot growth traits (Fig. 5K), and 19.7% of the variance in root growth traits (Fig. 5L). The pure variances in root biomass and growth traits explained by PP were 8.1% and 6.0%, respectively, whilst HT explained 4.9%

and 13.7%, respectively. The combination of PP andHT explained 22.1% and 7.1% of the variance in root biomass and growth traits, respectively.

The combination of SLandLT explained 50.2% of the variance in shoot biomass (Fig. 6A), 29.5% of the variance in root biomass (Fig. 6B), 23.3% of the variance in shoot growth traits (Fig. 6C), and 48.2% of the variance in root growth traits (Fig. 6D). The pure variances in root biomass and growth traits explained by SL were 8.7% and 22.1%, respectively, whilst LT explained 20.8% and 26.1%, respectively. The combination of SLand LT explained 5% and 11.1% of the variance in root biomass and growth traits, respectively. The combination of SLandMT explained 12.2% of the variance in shoot biomass (Fig. 6E), 12.6% of the variance in root biomass (Fig. 6F), 59.5% of the variance in shoot growth traits (Fig. 6G), and 12.4% of the variance in root growth traits (Fig. 6H). The pure variance in the root biomass explained by SL was 4.4% whilst MT explained 8.2% and 12.4% of the variance in root biomass and growth traits, respectively. The combination of SLand MT explained 18.9% and 16.6% of the variance in root biomass and growth traits, respectively. The combination of SLandHT explained 77.3% of the variance in shoot biomass (Fig. 6I), 44.1% of the variance in root biomass (Fig. 6J), 24.8% of the variance in shoot growth traits (Fig. 6K), and 26.2% of the variance in root growth traits (Fig. 6L). The pure variances in root biomass and growth traits explained by SL were 16.7% and 5.2%, respectively, whilst HT explained 27.4% and 21.0%, respectively. The combination of SL and HT explained 8.6% of the variance in shoot biomass.

The combination of PHandLT explained 49.0% of the variance in shoot biomass (Fig. 7A), 47.3% of the variance in root biomass (Fig. 7B), 23.3% of the variance in shoot growth traits (Fig. 7C), and 43.4% of the variance in root growth traits (Fig. 7D). The pure variances in root biomass and growth traits explained by PH were 23.2% and 0.9%, respectively, whilst LT explained 24.1% and 42.5%, respectively. The combination of PHand LT explained 9.4% and 11.7% of the variance in root biomass and growth traits, respectively. The combination of PHandMT explained 83.0% of the variance in shoot biomass (Fig. 7E), 20.9% of the variance in root biomass (Fig. 7F), 2.3% of the variance in shoot growth traits (Fig. 7G), and 14.2% of the variance in root growth traits (Fig. 7H). The pure variances in the root biomass and growth traits explained by PH were 9.4% and 4.3%, respectively. MT explained 11.5% and 9.9% of the variance in the root biomass and growth traits, respectively. The combination of PHand MT explained 11.4% and 0.5% of the variance in the root biomass and growth traits, respectively. The combination of PHandHT explained 59.0% of the variance in shoot biomass (Fig. 7I), 15.1% of the variance in root biomass (Fig. 7J), 40.6% of the variance in shoot growth traits (Fig. 7K), and 39.1% of the variance in root growth traits (Fig. 7L). The pure variances in the root biomass and growth traits explained by PH were 0.7% and 0.9%, respectively. HT explained 7.4% and 38.2% of the variance in root biomass and growth traits, respectively. The combination of PHand MT explained 10.0% and 20.4% of the variance in the root biomass and growth traits, respectively.

4. Discussion

4.1 Investigation and identification of DSE

DSE are common root endophytic fungi with wide host and ecogeographical ranges (Li et al. 2015; Qin et al. 2017). The melanised DSE mycelia and microsclerotia characteristic in the roots of *O. japonicus* and *L. japonica* were observed in almost all root samples collected from a medicinal plant cultivation area in northern China. DSE mycelia have melanin-enriched cell walls that provide structural rigidity and protect the cells against abiotic and biotic stress (Gessler et al. 2014; Berthelot et al. 2017). Microsclerotia resemble the vesicles of arbuscular mycorrhizae and are propagules or dormant structures (Peterson et al. 2008). Aaltonen (2001) suggested that the melanised hyphae and microsclerotia of DSE may be essential for plant growth and survival in high-stress environments. Morphological and molecular methods identified the DSE strains isolated from *O. japonicus* and *L. japonica* roots as *Acrocalymma vagum, Paraphoma radicina, Curvularia pallescens, Scytalidum lignicola, Paraboeremia putaminum,* and *Phoma herbarum. A. vagum* and *P. putaminum* have been detected intobacco and licorice roots (Jin et al. 2017; He et al. 2019a,b). *P. radicina* was isolatedin the root surfaces of soybean (Morganjones and White 1983). *C. pallescens* was foundon the surfaces of banana fruits (Alvindia and Natsuaki 2008), in cereals (Elgorban et al. 2017), and on the leaves of Lippia sidoides and *Humboldtia brunonis (*de Siqueira et al. 2011; Sheik and Chandrashekar 2018). *P. herbarum* was observedin the roots of salt-stressed soybean plants (Hamayun et al. 2009). These findings indicate that DSE are neither host- nor tissue-specific and may be functional in plants other than the original host (Li et al. 2018).

4. 2 Effects of DSE or *T. viride* aloneon the growth of licorice seedlings

Related studies showed that DSE inoculation had negative, neutral, or positive effects on plant growth (Li et al. 2019; He et al. 2019b). Here, all three DSE species acted as host colonisers. The DSE had significant direct effects on total root length, root diameter, plant biomass, and shoot branching in licorice. Relative to the control plants, *P. putaminum* increased the root biomass and root:shoot ratio, *S. lignicola* increased the shoot biomass and decreased the root:shoot ratio, and *P. herbarum* increased the root biomass. We propose that the growth-promoting efficacy of the DSE in their host plants varies with DSE species (Dolatabadi et al. 2011; Zhu et al. 2015). Previous studies reported that *P. herbarum* promotes plant growth byproducing gibberellins (Hamayun et al. 2009). However, it also a causative agent of leaf spot disease in *Camellia sinensis* (Thangaraj et al. 2018) and *Rhizoma atractylodis* (You et al. 2018). *Paraboeremia* spp. (including *P. putaminum)*were recently distinguished from *Phoma* spp. *S. lignicola* was identifiedas a causative agent of root rot (Shivanna et al. 2014). It was confirmed that the pathogenic-endophytic lifestyles of certain fungi are interchangeable (Lana et al. 2011). There is no published information on the positive influences of *P. putaminum* and *S. lignicola* on plant growth. However, Goh et al. (2016) reported that *Scytalidium parasiticum* increased plant height and leaf area in *Elaeis guineensis.* Our results corroborate the hypothesis that the pathogenic or mutualistic effects of DSE in plants may vary with DSE species and growth conditions (Reininger et al. 2012; Hengodage et al. 2017).

It was reported that *Trichoderma* spp. can be applied as a biofertiliser and plant growth promoter (Velmourougane et al. 2017). Here, nonmetric multidimensional scaling (NMDS) and a structural equation model (SEM) showed that *T. viride* exhibited significant direct effects on total root length, root diameter and surface area, plant biomass and height, and leaf number. Low- and medium-density *T. viride* increasedshoot and root biomass. High-density *T. viride* increased root biomass and root:shoot ratio and decreased shoot biomass. Our results are consistent with those reported by Al-Hazmi and TariqJaveed (2015). They discovered that all four *Trichoderma harzianum* and *T. viride* inoculum densities increased tomato plant growth especially at the maximum density of 10^{10} spores g^{-1} soil. Rosmana et al. (2016) found that treatment with various concentrations of *Trichoderma asperellum* increased the numbers of *Theobroma cacao* buds and branchesby 90.7% and 21.7%, respectively, relative to the control. Earlier research demonstrated inconsistent results for the effects of *T. harzianum* and *T. viride* on tomato plant growth (Mouria et al. 2007). Thus, the species and inoculum density of *Trichoderma* used may be key factors determining whether the relationship between this fungus and its plant host is mutualistic.

4. 3 Interactions of DSE and *T. viride* on the growth of licorice seedlings

In a previous study, we assessed the effects of combinations of DSE and organic residue on licorice. *Trichoderma viride* was only used for cellulose degradation (He et al. 2019a). Here, we investigated the effects of combination of DSE and *T. viride* at various densities on licorice. *Trichoderma viride* was used here as a growth promoter. Compared with the standalone DSE inoculant, *P. putaminum+ T. viride* at low- and medium density increased root biomass. *P. putaminum + T. viride* at high density increased shoot biomass and the root:shoot ratio. *S. lignicola + T. viride* at low density increased shoot and root biomass and the root:shoot ratio. *S. lignicola + T. viride* at medium- and high density increased root biomass and the root:shoot ratio. *P. herbarum + T. viride* at lowdensity increased root biomass and the root:shoot ratio. *P. herbarum + T. viride* at highdensity increased root biomass. *P. herbarum + T. viride* at medium density increased the root:shoot ratio and decreased shoot biomass. A structural equation model (SEM) revealed that combinations of DSE and *T. viride* significantly positively influenced root surface area, plant biomass and height, and shoot branching. A variance partitioning analysis indicated that 45.3% and 39.5% of the variance in root biomass and morphology, respectively, under low-density *T. viride,* 20.0% and 13.9% of the variance in root biomass and morphology, respectively, under medium-density *T. viride,* and 47.1% of the variance in shoot biomass under high-density *T. viride* were attributable to the presence of *P. putaminum.* Moreover,30.7% and 22.1% of the variance in root biomass and morphology, respectively, under low-density *T. viride,* and 77.3%, 16.7%, and 16.1% of the variance in shoot biomass, root biomass, and shoot morphology, respectively, under high-density *T. viride* were attributable to the presence of *S. lignicola.* In addition,23.2% and 15.1% of the variance in root biomass and shoot morphology, respectively, under low-density *T. viride,* 8.1% and 9.4% of the variance in shoot and root biomass, respectively, under medium-density *T. viride,* and 59.0%, 7.7%, and 40.6% of the variance in shoot biomass, root biomass, and shoot morphology, respectively, under high-density *T. viride* were attributable to the presence of *P. herbarum.* Fungi only colonise plant roots. Nevertheless, certain fungal taxa interact

with plant leaves and stems (Harman 2011). Two possible factors may explain the enhanced growth observed in licorice inoculated with DSE and *T. viride.* DSE and *T. viride* increase plant root growth and nutrient uptake (Paal and Banner 2003; Vergara et al. 2017). Comparatively greater root biomass is probably associated with larger numbers of lateral and secondary roots and root hairs. This adaptive root morphology augments plant nutrient uptake efficiency (Narisawa et al. 2007). DSE and/or *T. viride* induced positive or neutral root system architecture responses. The production of unique bioactive compounds such as phytohormones by DSE (Sirrenberg et al. 2007; Priyadharsini and Muthukumar 2017) and *Trichoderma* strains (Vinale et al. 2008; Contreras-Cornejo et al. 2009), or the indirect regulation of these compounds affects growth regulator production in host plants (Vadassery et al. 2008; Wani et al. 2016; Nieto-Jacobo et al. 2017; Zhang et al. 2019). Host plant phytohormone levels were not measured in the present study. However, interactions between DSE and *T. viride* significantly increased root biomass and surface area compared with the control plants. We speculate that the beneficial effects of DSE and *T. viride* on plant roots may explained by the increases in phytohormone production they promote (Contreras-Cornejo et al. 2009; Terhonen et al. 2019). The variance partitioning analysis demonstrated persistent variance in plant biomass and growth traits. Thus, certain factors and parameters that were not measured such as DSE inoculation volume and plant culture time may have also significantly affected licorice growth.

5. Conclusion

Here, we explored the associations between licorice roots and dark septate endophytes derived from the roots of *O. japonicus* and *L. japonica* grown on the farmlands of northern China. Three DSE species were effective colonizers of *O. japonicus, L. japonica,* and licorice roots. They had various DSE species-dependent positive effects on host plant root growth. *P. putaminum* significantly increased root biomass and root:shoot ratio and improved root morphology, *S. lignicola* improved root morphology, and *P. herbarum* increased root biomass and surface area. *T. viride* at low- and medium density increased root biomass and improved root morphology. *T. viride* at high density increased root biomass and improved root morphology. The combination of DSE and *T. viride* significantly and positively influenced root biomass, length, and surface area. Our findings substantiate the hypothesis that DSE-*T. viride* co-inoculation improves root development and nutrient absorption in host plants and could, therefore, enhance plant growth and biomass production. Licorice root is an important and valuable herbal medicine. Moreover, the whole plant reclaims drought-affected soils. Microbes living in the licorice rhizosphere form mutualistic associations with the host and help regulate its growth and stress adaptation. Future research should investigate the functions of other DSE species and evaluate the application of dual DSE-*Trichoderma* spp. inoculants in medicinal plant cultivation.

Declarations

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Author Contributions

Data curation, CH and JH; Formal analysis, CH; Funding acquisition, WW; Investigation, CH and JH; Methodology, CH, WW and JH; Project administration, WW; Resources, CH; Supervision, WW; Visualization, WW; Writing—original draft, CH and WW.

Compliance with Ethical Standards

Conflict of interest

The authors declare no conflict of interest.

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Table

Table 1 Two-way analysis of variance for the effects of dark septate endophyte (DSE) and *Trichoderma viride* (TV) inoculation on plant biomass and morphological traits of *Glycyrrhiza uralensis*

	DSE		TV		DSE × TV	
	F	*P*	*F*	*P*	*F*	*P*
Shoot biomass (g/pot)	9.31	**<0.001**	8.88	**<0.001**	4.35	**<0.001**
Root biomass (g/pot)	4.25	**0.010**	6.69	**0.001**	2.86	**0.009**
Root: shoot ratio	2.42	0.077	0.98	0.41	5.50	**<0.001**
Plant height (cm)	0.79	0.508	4.29	**0.009**	3.04	**0.006**
Shoot branch (No.)	4.60	**0.007**	1.08	0.367	2.24	**0.034**
Leaf number (No.)	3.10	**0.035**	2.92	**0.043**	1.96	0.065
Root length (cm)	7.23	**<0.001**	9.38	**<0.001**	2.09	**0.048**
Root diameter (mm)	4.84	**0.006**	7.36	**<0.001**	1.89	0.075
Root surface area (cm^2)	1.21	0.315	4.56	**0.007**	2.39	**0.025**

Significant *P*-values are in bold

Figures

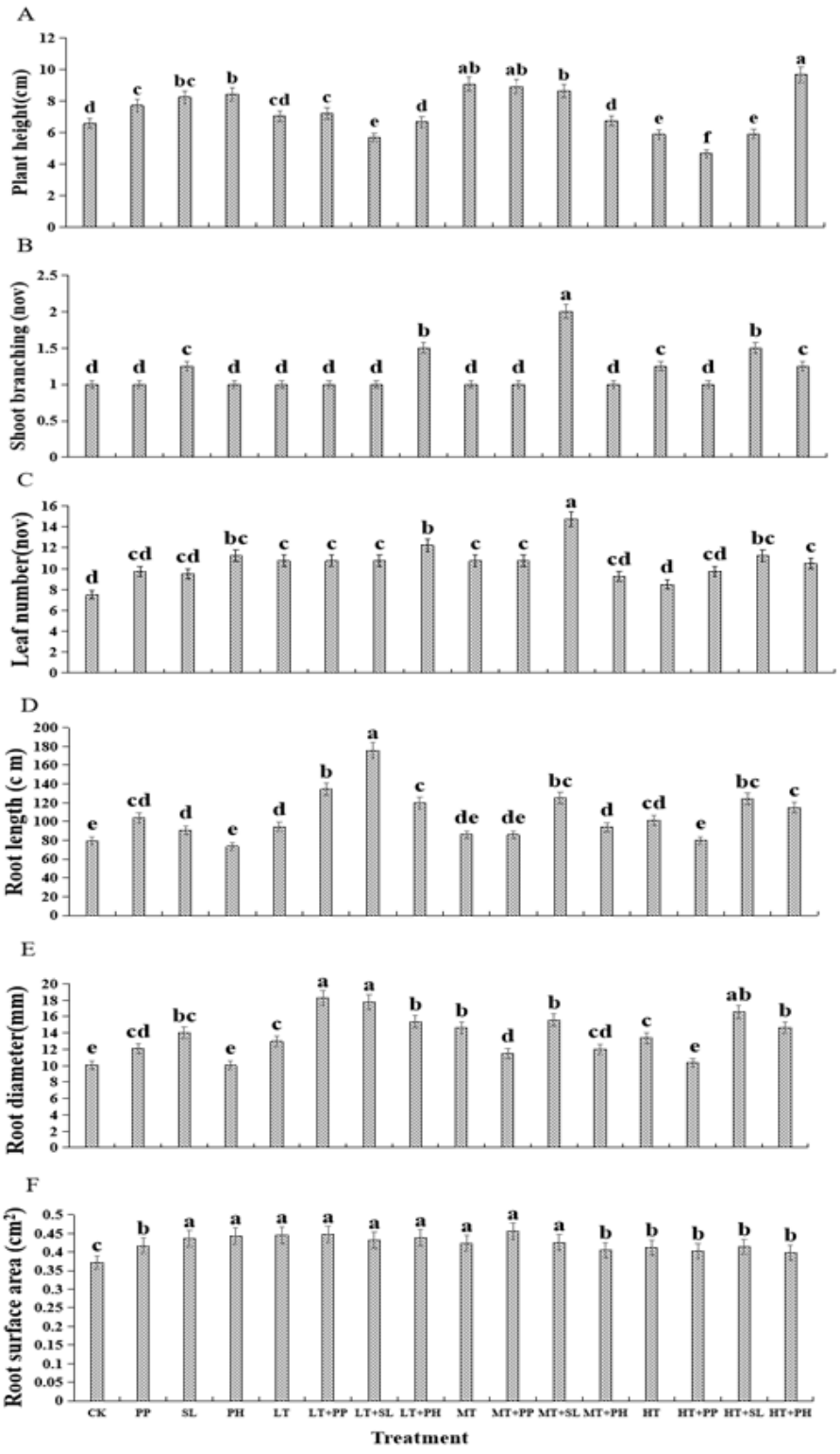

Figure 1

Effects of dark septate endophyte (DSE) and Trichoderma viride on the shoot and root morphological traits of G. uralensis seedlings. CK indicates non-inoculated plants and no T. viride. PP, P. putaminum; SL, S. lignicola; PH, P. herbarum; LT, low T. viride density; MT, medium T. viride density; HT, high T. viride density; LT+PP→HT+PH, different combinations of DSE species and T. viride density. Different letters indicate significant differences at $P < 0.05$.

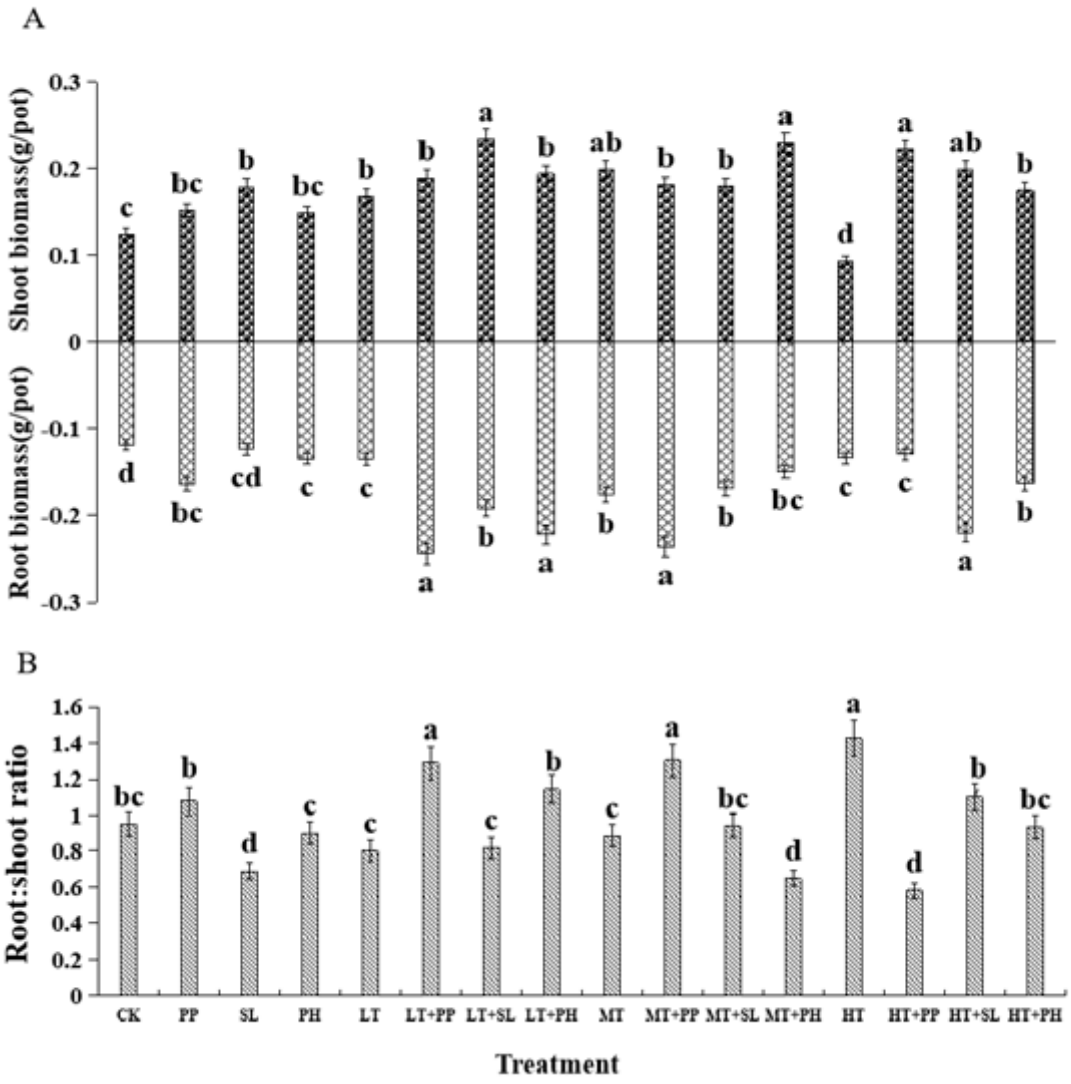

Figure 2

Effects of dark septate endophyte (DSE) and Trichoderma viride on the biomass production of G. uralensis seedlings. CK indicates non-inoculated plants and no T. viride. PP, P. putaminum; SL, S. lignicola; PH, P. herbarum; LT, low T. viride density; MT, medium T. viride density; HT, high T. viride density. LT+PP→HT+PH, different combinations of DSE species and T. viride density. Different letters indicate significant differences at $P < 0.05$.

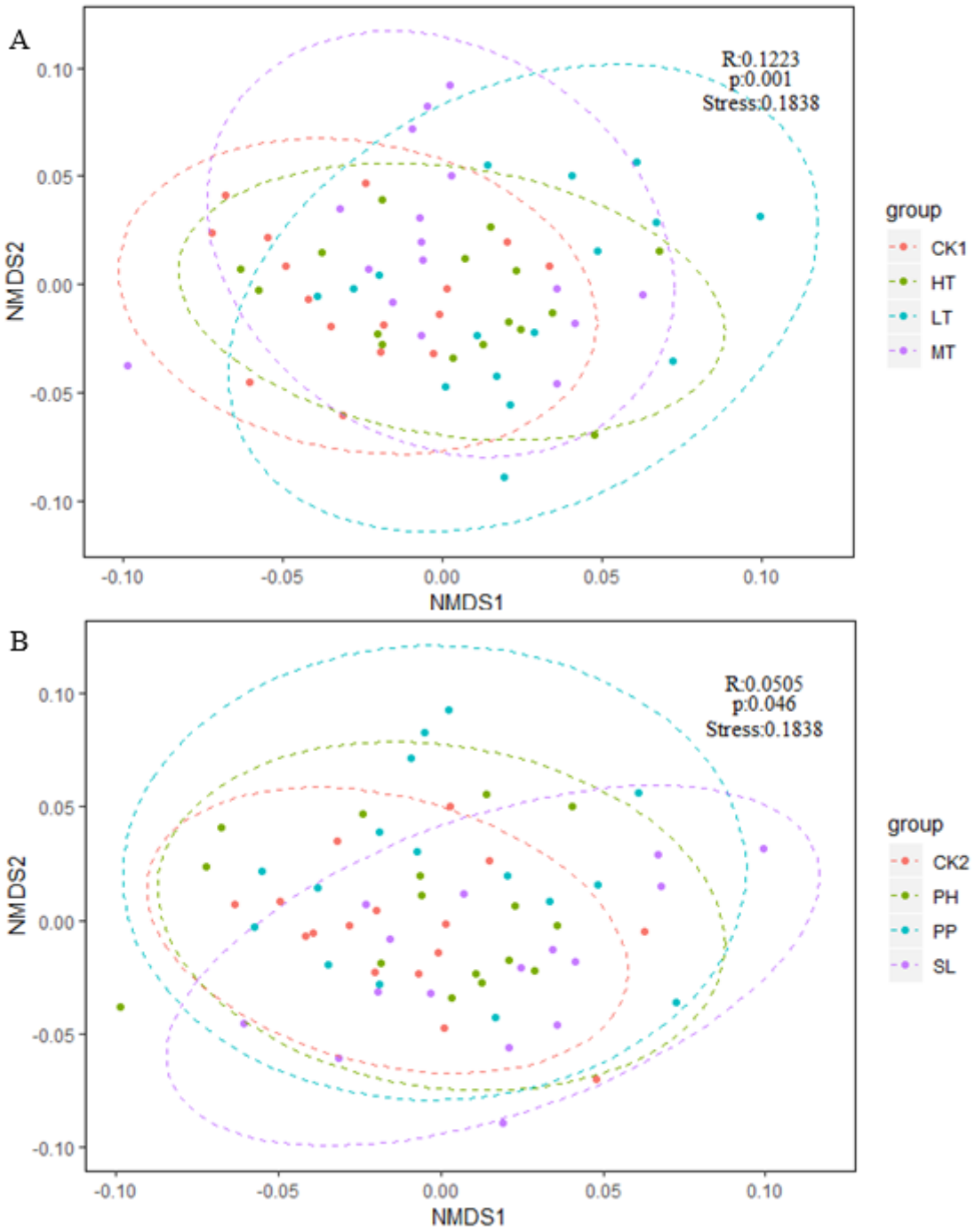

Figure 3

Non-metric multidimensional scaling (NMDS) ordination of the root morphological architecture of G. uralensis under the interaction between DSE and T. viride density. Ellipses in the plots denote 95% confidence intervals for the centroids of root morphological architecture with T. viride density (A) and DSE species (B). CK1: treatments without T. viride with DSE; LT: low density of T. viride with DSE interaction; MT: moderate density of T. viride with DSE interaction; HT: high density of T. viride with DSE interaction. CK2: treatments without DSE with T. viride; PH: P. herbarum with T. viride interaction; PP: P. putaminum with T. viride interaction; SL: S. lignicola with T. viride interaction.

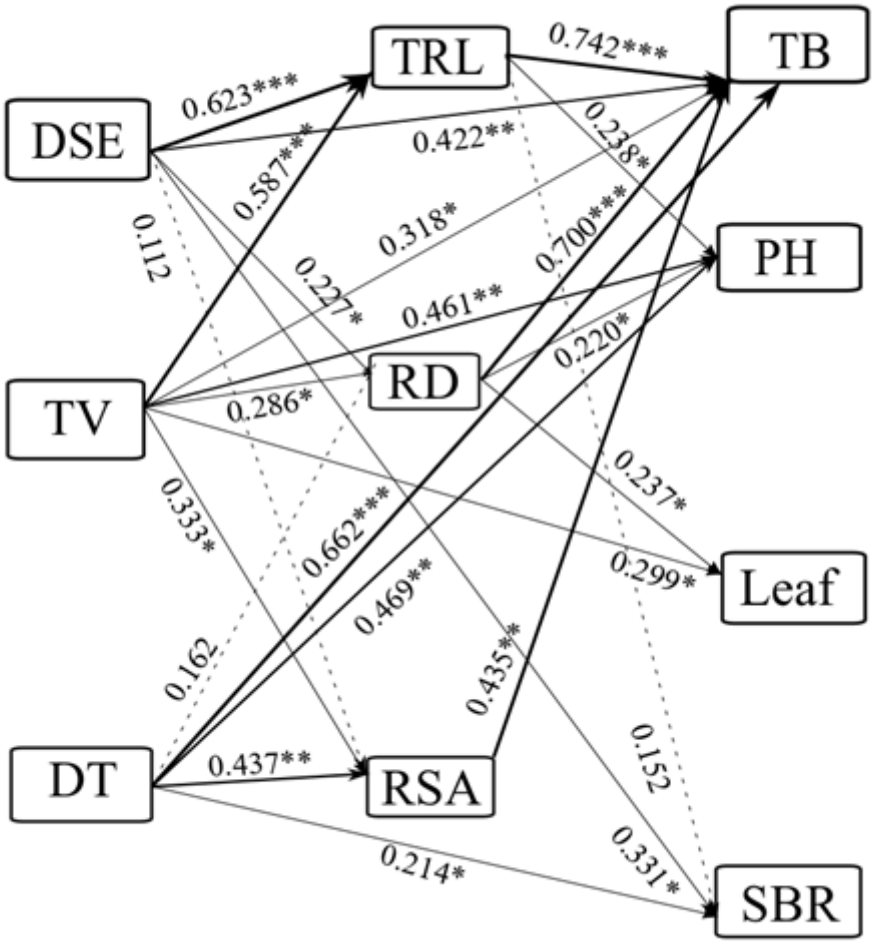

Figure 4

Structural equation model (SEM) showing the causal relationships among DSE species, T. viride and the root morphology on the plant aboveground morphology. The final model fitted the data well: maximum likelihood, X2=136.933, df=12, P=0.005, goodness-of-fit index=0.750, Akaike information criteria=222.933, and root mean square error of approximation=0.407. Solid lines and dashed lines indicate significant and non-significant pathways, respectively. The width of the solid lines indicates the strength of the causal effect, and the numbers near the arrows indicate the standardized path coefficients (*P<0.05, **P<0.01, ***P<0.001). TV=T. viride. DT=combination of DSE and T. viride. TRL=total root length. RD=root diameter. RSA=root surface area. TB=total biomass. PH=plant height. leaf=number of leaf. SBR=shoot branching.

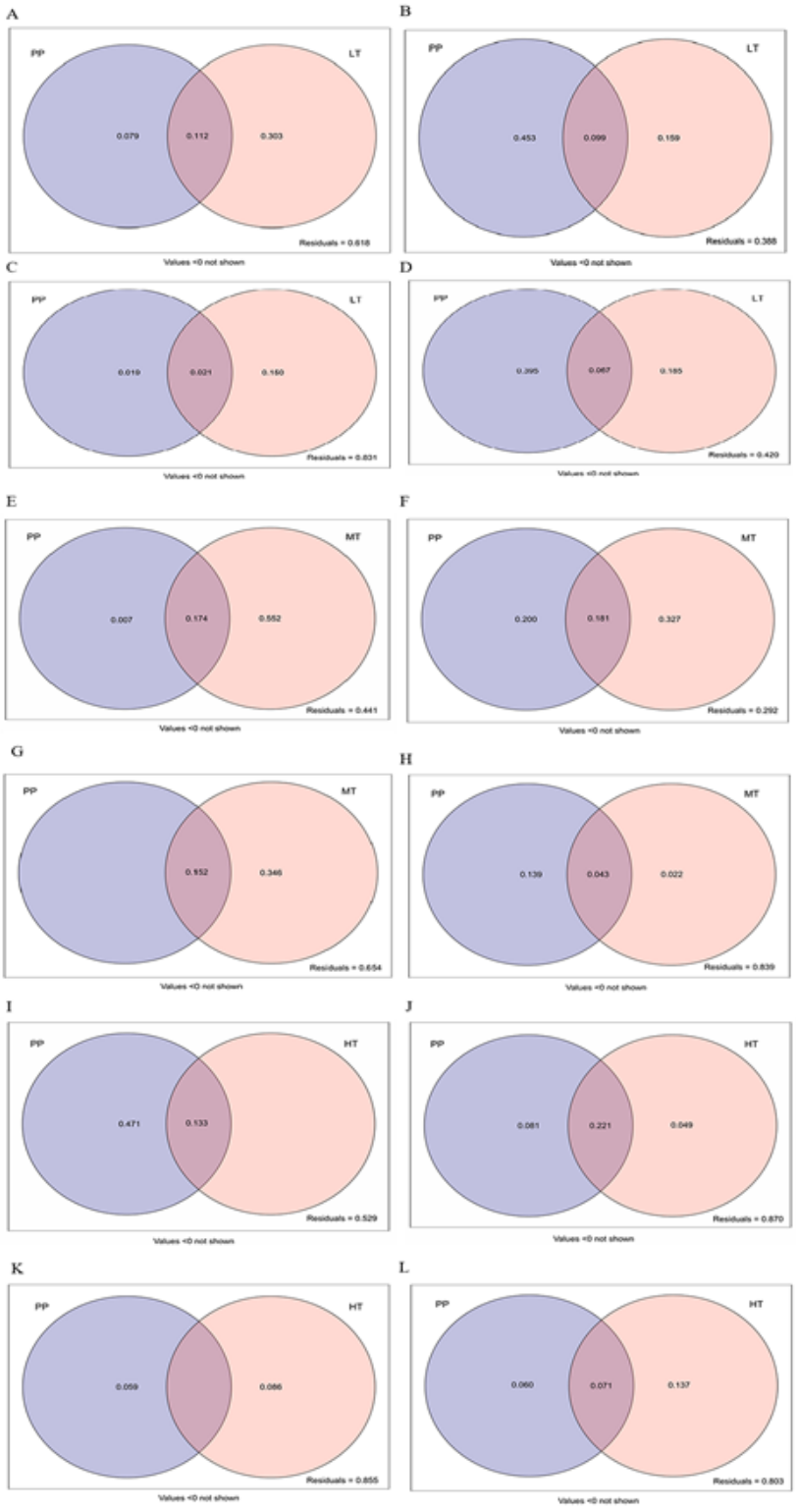

Figure 5

Variation partitioning of P. putaminum and T.viride of low density on plant biomass and growth traits (A-D). Variation partitioning of P. putaminum and T.viride of medium density on plant biomass and growth traits (E-H). Variation partitioning of P. putaminum and T.viride of high density on plant biomass and growth traits (I-L). PP=P. putaminum, A, E, I. shoot biomass; B, F, J. root biomass; C, G, K. shoot growth

traits (including plant height, leaf number and branching number); D, H, L. root growth traits (including root length, root diameter and root surface area). Values below 0 are not shown.

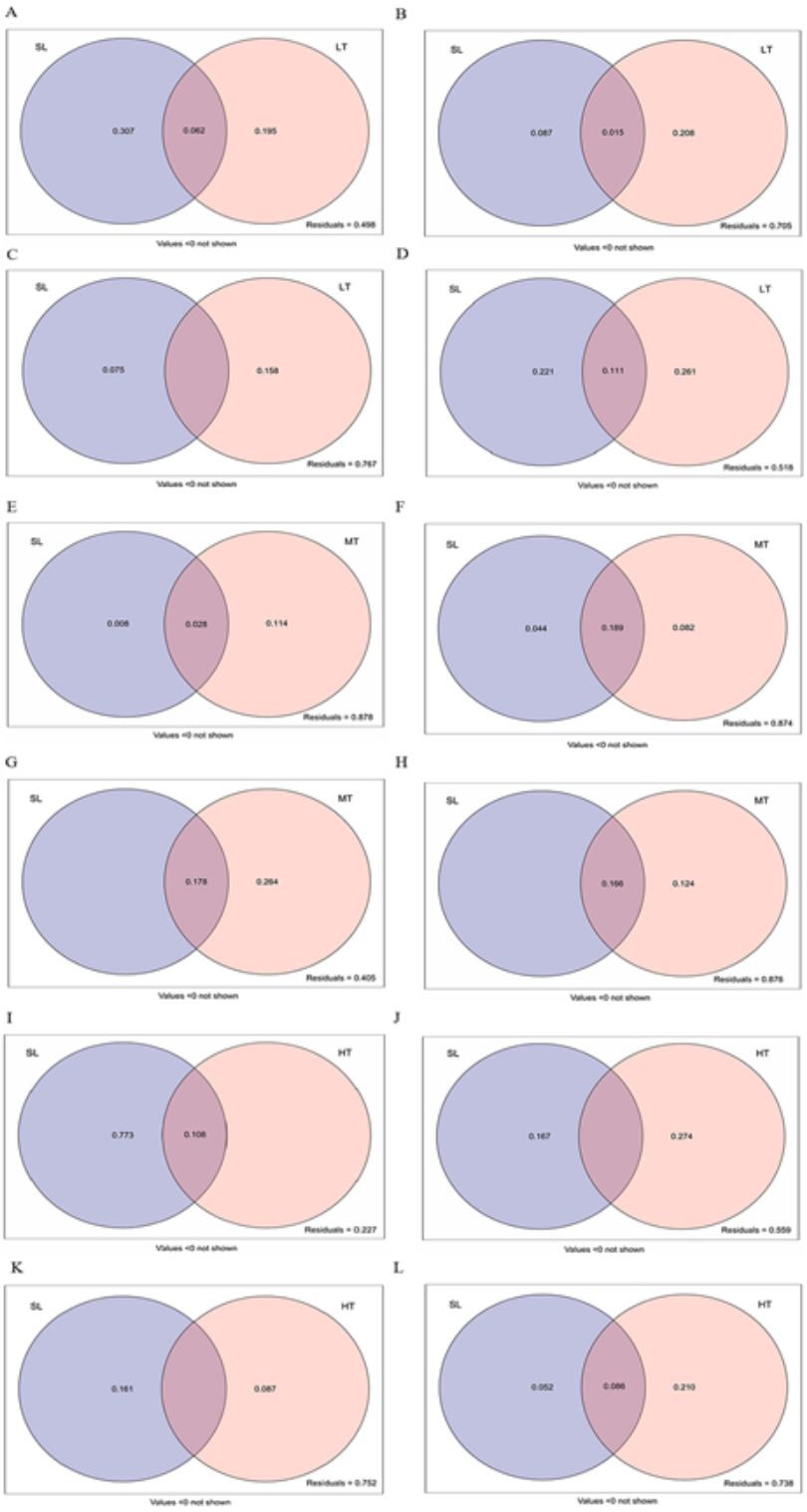

Figure 6

Variation partitioning of S. lignicola and T.viride of low density on plant biomass and growth traits (A-D). Variation partitioning of S. lignicola and T.viride of medium density on plant biomass and growth traits (E-H). Variation partitioning of S. lignicola and T.viride of high density on plant biomass and growth traits

(I-L). SL=S. lignicola, A, E, I. shoot biomass; B, F, J. root biomass; C, G, K. shoot growth traits (including plant height, leaf number and branching number); D, H, L. root growth traits (including root length, root diameter and root surface area). Values below 0 are not shown.

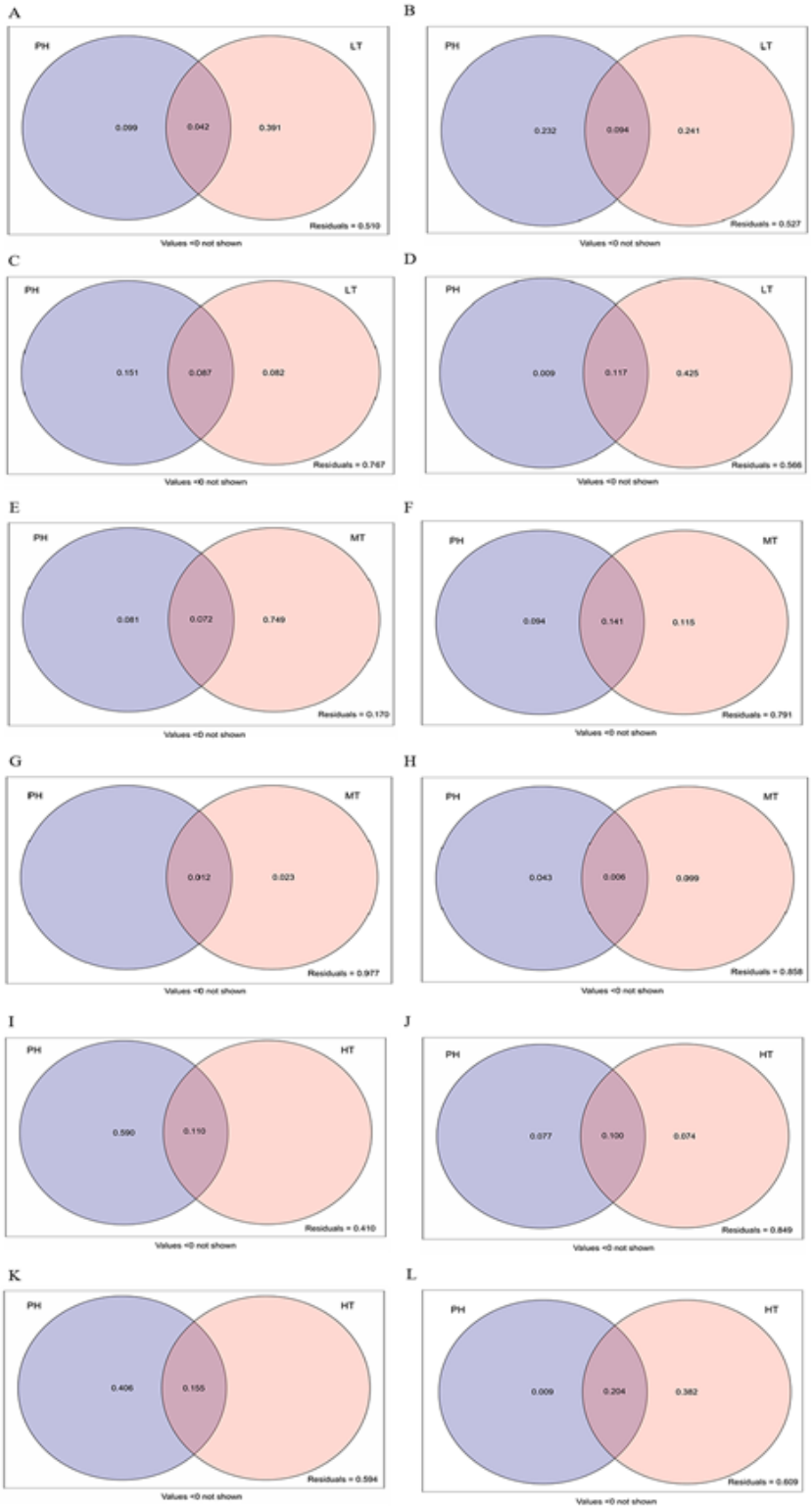

Figure 7

Variation partitioning of P. herbarum and T.viride of low density on plant biomass and growth traits (A-D). Variation partitioning of P. herbarum and T.viride of medium density on plant biomass and growth traits

(E-H). Variation partitioning of P. herbarum and T.viride of high density on plant biomass and growth traits (I-L). PH=P. herbarum, A, E, I. shoot biomass; B, F, J. root biomass; C, G, K. shoot growth traits (including plant height, leaf number and branching number); D, H, L. root growth traits (including root length, root diameter and root surface area). Values below 0 are not shown.

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- Supplementarymaterial.doc