https://doi.org/10.17221/100/2020-PSE

Mycorrhiza-released glomalin-related soil protein fractions contribute to soil total nitrogen in trifoliate orange

Lu-Lu Meng[1], Jia-Dong He[1], Ying-Ning Zou[1], Qiang-Sheng Wu[1,2], Kamil Kuča[2]*

[1]*College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei, P.R. China*
[2]*Department of Chemistry, Faculty of Science, University of Hradec Králové, Hradec Králové, Czech Republic*
**Corresponding author: wuqiangsh@163.com*

Citation: Meng L.-L., He J.-D., Zou Y.-N., Wu Q.-S., Kuča K. (2020): Mycorrhiza-released glomalin-related soil protein fractions contribute to soil total nitrogen in trifoliate orange. Plant Soil Environ., 66: 183–189.

Abstract: Glomalin released from arbuscular mycorrhizal fungi (AMF) has important roles in soil nutrient cycles, whereas contributing to glomalin-related soil protein (GRSP) fractions to soil nitrogen (N) is unknown. In this study, a two-chambered root-box that was divided into root chamber (root and mycorrhizal fungi hypha) and hypha chamber (free of the root) was used, and three AMF species including *Diversispora epigaea*, *Paraglomus occultum*, and *Rhizoglomus intraradices* were separately inoculated into the root chamber. Plant growth, soil total N, N content of purified GRSP fractions, and its contribution to soil total N, and leaf and root N contents were analysed. After four months, total biomass and root total length, surface area, and volume were improved by all AMF inoculations. AMF inoculations dramatically increased soil total N content in two chambers. The N content of purified easily extractable GRSP (EE-GRSP) and difficultly extractable GRSP (DE-GRSP) was 0.10 ± 0.01 mg/g and 0.16 ± 0.02 mg/g, respectively, accounted for 15.6 ± 1.6% and 18.1 ± 1.8% of soil total N, respectively. AMF inoculations stimulated the N accumulation in EE-GRSP and DE-GRSP, especially in the hypha chamber. It concluded that GRSP, especially DE-GRSP, acts as a soil N pool accounting for 33.8 ± 1.9% of soil total N in orchards.

Keywords: arbuscular mycorrhizas fungi; citrus; glycoprotein; macronutrient; symbiosis

Arbuscular mycorrhizal fungi (AMF), a beneficial soil microorganism, can form a mutualistic symbiosis with roots of approximately 80% of terrestrial plants and are considered as a component of various ecosystems (Hodge et al. 2001, Zhang et al. 2020). AMF delivers nutrients, mainly phosphorus (P) and nitrogen (N), to host plants in exchange for carbon (C). In addition, mycorrhizal fungi confer many positive effects on host plants, including promoting plant growth, stabilising soil aggregation, maintaining soil moisture, improving tolerance of abiotic and biotic stress, and increasing plant biodiversity (Wu et al. 2013, 2019, He et al. 2019).

Glomalin is a glycoprotein secreted by hyphae and spores of AMF, which is defined as glomalin-related soil protein (GRSP) in soil, according to its extraction protocol and assay (Wright and Upadhyaya 1996, Zou et al. 2016). Wu et al. (2015) divided GRSP into easily extracted GRSP (EE-GRSP) and difficultly extractable GRSP (DE-GRSP). AMF hyphal turnover could imply the accumulative levels of GRSP in soil (Etcheverría et al. 2009). Earlier studies reported that GRSP was an organic carbon source in soil, thereby influencing the aggregate formation, stabilisation and contributes to soil carbon sequestration (Nautiyal et al. 2019, He et al. 2020). In alkaline soils of arid areas, GRSP was in a position to bond microelements, including Fe, Cu, Mn, Ni, and Pb, to increase nutrient availability for plant growth and to alleviate the damage of toxic metals (Emran et al. 2017). Rillig et al. (2001) reported about 5% contribution of GRSP to soil total nitrogen (N)

Supported by the National Natural Science Foundation of China, Grant No. 31372017, and by the Plan in Scientific and Technological Innovation Team of Outstanding Young Scientists, Hubei Provincial Department of Education, Project No. T201604.

https://doi.org/10.17221/100/2020-PSE

in an ancient soil (> 4 million years). Lovelock et al. (2004) found that the amount of N in GRSP in tropical soil was estimated at 4%, representing 5% of soil N pools. Zhang et al. (2017) also revealed high N content (13.13%) in total GRSP extracted from a high-saline soil. As a result, GRSP can drive the distribution of soil N in a large scale. In addition, total GRSP (T-GRSP), in combination with T-GRSP/soil organic carbon, could reflect the qualitative changes in reactive N levels of forest soil (Rotter et al. 2017). These results suggest the important contribution of GRSP to soil nutrients, including N. However, the information is still scarce, for example, how much DE-GRSP and EE-GRSP contributes soil N. On the other hand, GRSP is a mixture of substances of mycorrhizal and non-mycorrhizal origin (Gillespie et al. 2011). If GRSP is not purified, the data concerning the contribution of GRSP on N are inaccurate.

Trifoliate orange (*Poncirus trifoliata* L. Raf.) is a citrus rootstock used in Southeast Asia, and it is dramatically dependent on mycorrhizal symbiosis in the field (He et al. 2019, Zhang et al. 2020). Our study was undertaken to explore the effects of three different AMF species on plant growth and the contribution of purified GRSP fractions to soil total N in trifoliate orange.

MATERIAL AND METHODS

Plant culture. The two-compartmented root-box (L 20 cm × W 12.5. cm × H 16 cm) was constructed of polymethylene methacrylate. The root-box had two chambers, including the root chamber and hypha chamber, which were separated by two sheets of 37-μm nylon meshes, in which a 0.5 cm air gap was designed to prevent the substance communication. The 37-μm nylon mesh allows AMF extraradical hyphae, but not roots, to pass through it (He et al. 2020).

Two trifoliate orange seedlings with four-leaf-old grown in autoclaved sand were planted in the root chamber, where 100 g of mycorrhizal inoculums were applied. Three AMF species, viz., *Rhizoglomus intraradices* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl, *Diversispora epigaea* (B.A. Daniels & Trappe) C. Walker & A. Schüßler, and *Paraglomus occultum* (C. Walker) J.B. Morton & D. Redecker were separately inoculated in root chamber, and the inoculums contained spores, infected root segments of white clover, AMF extraradical hyphae, and substrates. The non-AMF treatment gave 100 g of autoclaved inoculums in combination with 2 mL filtrate (25 μm filter) of inoculums. Hence, the experiment had four AMF inoculations, respectively, with *D. epigaea*, *P. occultum*, *R. intraradices*, and non-AMF control. Each treatment had six replicates, having 24 root boxes and 48 seedlings. Either root chamber or hypha chamber received 1.3 kg autoclaved soils with 4-mm size from the rhizosphere of 26-year-old Satsuma mandarin grafted on trifoliate orange, which had pH 6.2, available P content 15.1 mg/kg, and organic carbon content 9.9 mg/g. The treated seedlings grown in root-boxes were placed in a greenhouse for four months from March to July 2017 with 25/19°C day/night temperature, 68% average air humidity, and 721–967 μmol/m^2/s photon flux density.

Determination of plant growth. After four months of treatments, the seedlings were harvested, and total (shoot + root) biomass was determined. The root of each seedling was scanned by an Epson Flatbed Scanner (J221A, Jakarta Selatan, Indonesia), and the root image was analysed by a professional WinRHIZO software (Regent Instruments Inc., Quebec, Canada) for total root length, surface area, and volume.

Determination of root mycorrhizal colonisation and AMF hyphae in soil. Root mycorrhizal structures were stained as per the protocol described by Phillips and Hayman (1970) with 0.05% (*w*/*v*) trypan blue. Root mycorrhizal colonisation was expressed with the percentage of mycorrhizal hyphae-colonised lengths against total lengths. AMF hyphae length in the soil was measured by the protocol described by Bethlenfalvay and Ames (1987).

Determination of N content of GRSPs, soil, leaf, and root. Soil EE-GRSP and DE-GRSP were extracted by the protocol of Wu et al. (2015). The solution of EE-GRSP and DE-GRSP was purified by the method outlined by Rillig et al. (2001) after precipitated by 2 mol/L HCl, dissolved by 0.5 mol/L NaOH, dialysed by ddH_2O in the dialysis bag (MD: 25 mm) at 25 °C for 60 h, and freeze-dried. The N content in purified EE-GRSP and DE-GRSP that was expressed as EE-GRSP (N) and DE-GRSP (N) was measured using a TruSpec CN Elemental Analyser (Leco Corporation, St. Joseph, USA). Soil total N content was monitored with a Kjeltec TM2300 Azotometer (FOSS, Hoganas, Sweden). Leaf and dry root samples (0.25 g) were digested by H_2SO_4-H_2O_2, and the N content was determined by the Kjeldahl method.

Statistical analysis. Data were analysed by one-factor analysis of variance with SAS software (v8.1, USA). Duncan's multiple range tests at 0.05 levels were utilised to compare the significant differences between treatments.

https://doi.org/10.17221/100/2020-PSE

RESULTS AND DISCUSSION

Changes in mycorrhizas. In this study, root colonisation of AMF-inoculated seedlings ranged from 25.7% to 34.8%, and root AMF colonisation by *P. occultum* was significantly higher than that by *R. intraradices* and *D. epigaea* (Figure 1A). AMF extraradical hyphae were found in the two chambers and were significantly higher in the root chamber than in the hypha chamber (Figure 1B). AMF extraradical hyphal length among three AMF treatments ranked as *P. occultum* > *D. epigaea* > *R. intraradices* in the two chambers. This suggests that the inoculation of *P. occultum* conferred a greater mycorrhizal status in trifoliate orange than *D. epigaea* and *R. intraradices*, which is in accordance with the findings of Heidari and Karami (2014) in sunflower plants, based on the compatibility of both mycorrhizal fungi and host plants.

Changes in plant growth responses to mycorrhizal inoculation. Three AMF inoculations were observed to display a higher magnitude of plant growth response than non-AMF inoculation (Figure 2). Compared with non-AMF inoculation, all the AMF treatments significantly increased total (shoot + root) biomass (Figure 2A), total root length (Figure 2B), root surface area (Figure 2C), and root volume (Figure 2D),while inoculation with *P. occultum* exhibited a relatively greater effect on plant biomass than inoculation with *D. epigaea* and *R. intraradices*. The positive effect of AMF on trifoliate orange was independent of AMF species used. The growth improvement under AMF inoculation is due to soil AMF extraradical mycelium extended to where the root system is ineffective to absorb nutrients (Trouvelot et al. 2015). In addition, mycorrhizal plants represented greater root morphology, which is associated with changes in root auxins and polyamines by mycorrhisation (Zhang et al. 2019, 2020).

Changes in soil total N content. GRSP is an important pool of organic N in the soil to link aggregate stability, long-term N storage, and responds of land use (Rillig et al. 2003, Gillespie et al. 2011). In the present study, inoculations with *R. intraradices*, *D. epigaea*, and *P. occultum* gave 21, 40, and 20% significantly higher soil total N concentrations in the root chamber, as compared with non-AMF control. In the hypha chamber, treatments by *R. intraradices* and *P. occultum*, but not by *D. epigaea*, significantly increased soil total N content than non-AMF treatment by 24% and 13%, respectively. Figure 3 also showed that soil total N content was higher in the root chamber than in the hypha chamber under inoculation with *D. epigaea* and *P. occultum*. As reported by Hodge et al. (2001), hyphae of an AM fungus *Glomus hoi* could accelerate the decomposition of organic patches and also acquire N directly from organic patches. In addition, AMF also accelerated N releasing and transforming by other microbes in organic matters (Bukovská et al. 2018).

Changes in GRSP (N). Glomalin is believed to be an N-linked glycoprotein with oligosaccharides (Wright and Upadhyaya 1998) and thus contains 3–5% N in combination with about 37% C (Lovelock et al. 2004). Our study found that the N content in

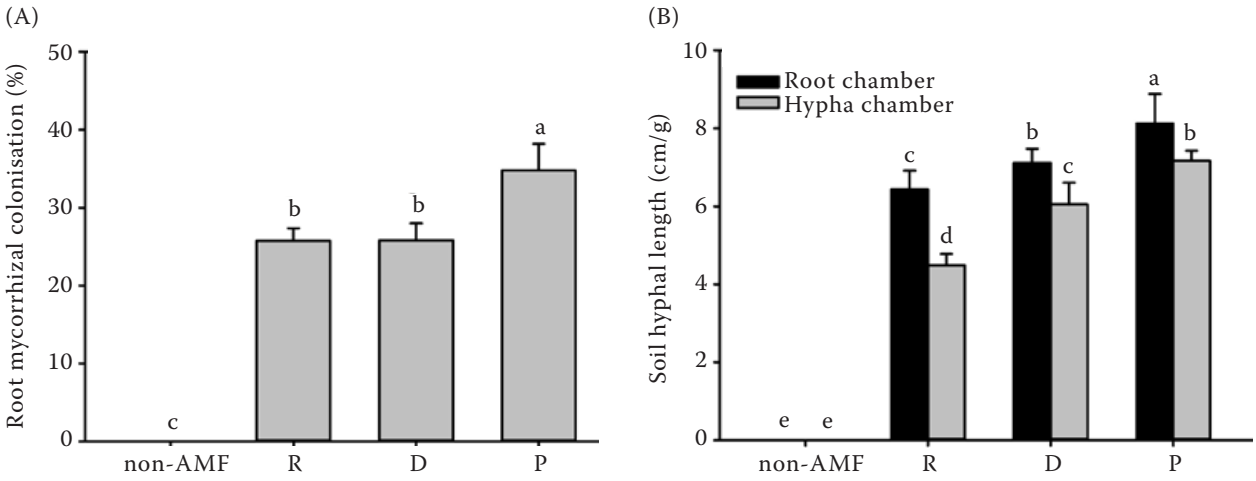

Figure 1. Effects of arbuscular mycorrhizal fungi (AMF) inoculations on (A) root mycorrhizal colonization and (B) soil AMF hyphal length of trifoliate orange seedlings grown in a two-chambered root-box. Means ± standard deviation (n = 6) followed by different letters above the bars indicate significant (P < 0.05) differences between mycorrhizal treatments. R – *Rhizoglomus intraradices*; D – *Diversispora epigaea*; P – *Paraglomus occultum*

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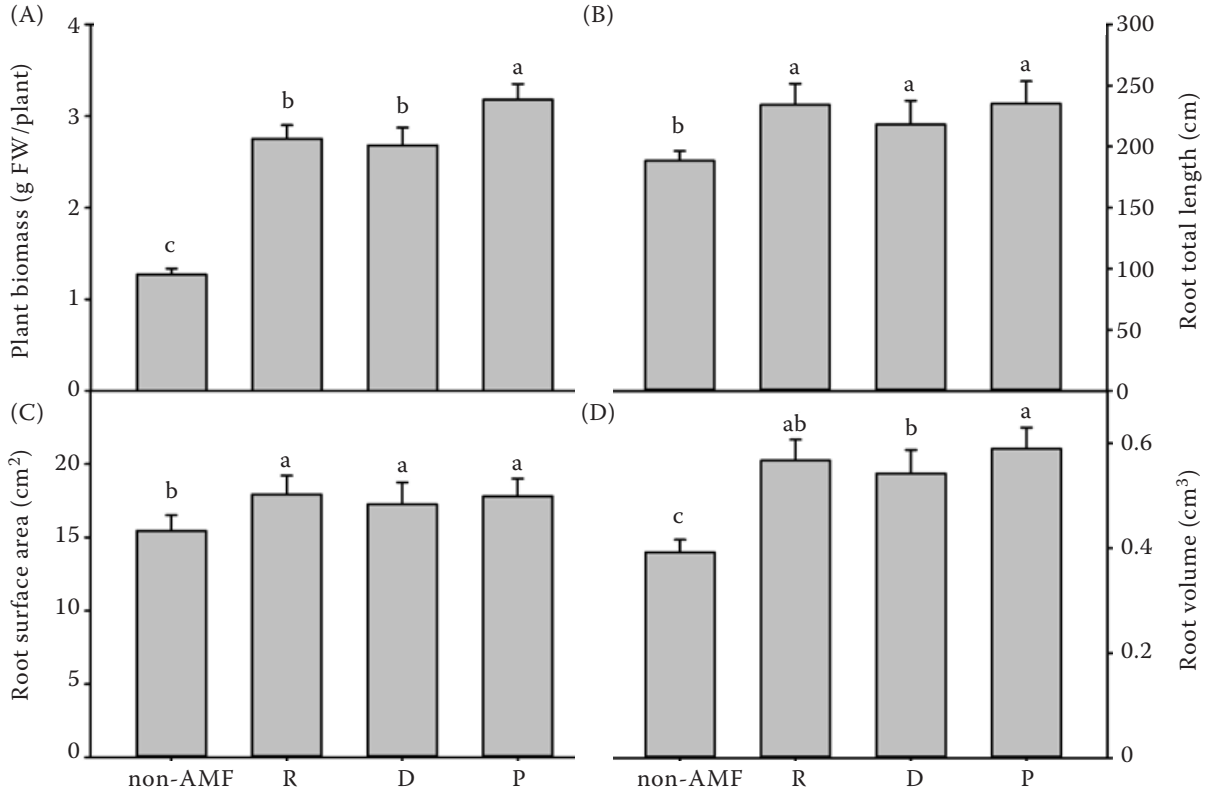

Figure 2. Effects of arbuscular mycorrhizal fungi (AMF) inoculations on (A) plant biomass, (B) root total length, (C) root surface area, and (D) root volume of trifoliate orange seedlings grown in a two-chambered root-box. Means ± standard deviation (n = 6) followed by different letters above the bars indicate significant (P < 0.05) differences between mycorrhizal treatments. R – *Rhizoglomus intraradices*; D – *Diversispora epigaea*; P – *Paraglomus occultum*; FW – fresh weight

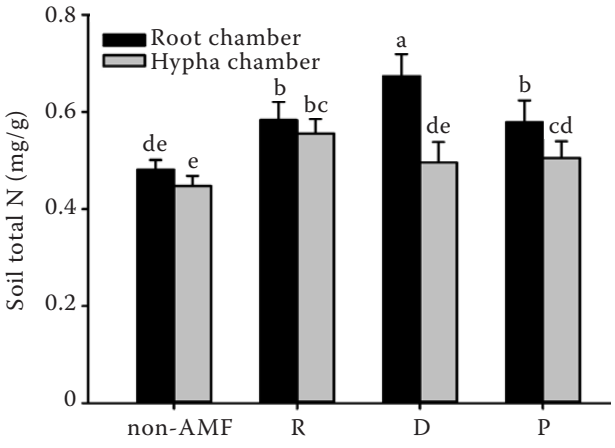

Figure 3. Effects of arbuscular mycorrhizal fungi (AMF) inoculations on soil total nitrogen (N) concentrations of trifoliate orange seedlings grown in a two-chambered root-box. Means ± standard deviation (n = 4) followed by different letters above the bars indicate significant (P < 0.05) differences between mycorrhizal treatments. R – *Rhizoglomus intraradices*; D – *Diversispora epigaea*; P – *Paraglomus occultum*

EE-GRSP and DE-GRSP was 0.10 ± 0.01 mg/g and 0.16 ± 0.02 mg/g, respectively, accounting for 0.26 ± 0.03 mg N/g T-GRSP (Figure 4). However, Zhang et al. (2017) reported high N content (13.13%) in T-GRSP extracted from a high-saline soil. It suggests that GRSP (N) might be affected by the soil environment. Our study also observed that DE-GRSP contained relatively higher N content than EE-GRSP, no matter which chambers. In root chamber, inoculation with *R. intraradices*, *D. epigaea*, and *P. occultum* significantly increased EE-GRSP (N) content by 33, 33, and 44%, and DE-GRSP (N) content by 38, 31, and 54%, respectively, compared with non-AMF inoculation (Figure 4). Compared with non-AMF, *R. intraradices*, *D. epigaea*, and *P. occultum* represented 57, 43, and 57% significantly higher EE-GRSP (N) content and 36, 36, and 55% significantly higher DE-GRSP (N) content in hypha chamber (Figure 4). It seems that AMF stimulated accumulation of EE-GRSP (N) and DE-GRSP (N), which is more effectively under the condition of hypha only, free of the root, than under the condition of both root and AMF hypha. AMF-

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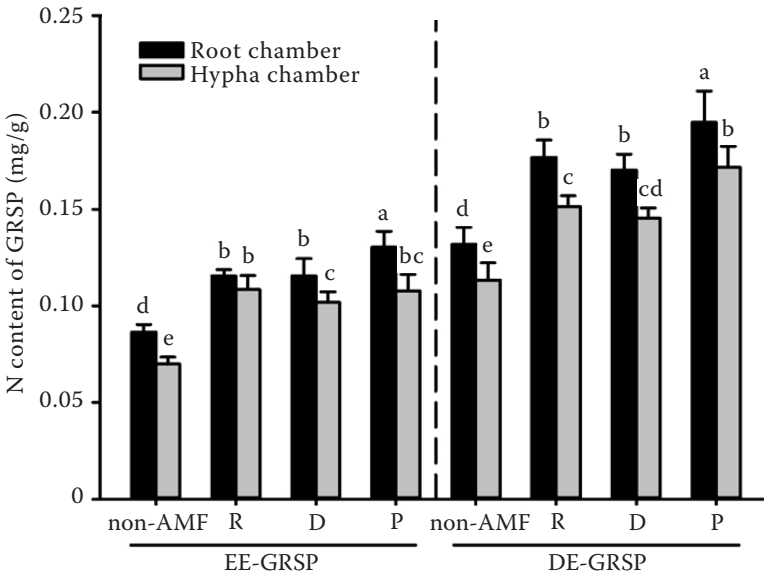

Figure 4. Effects of arbuscular mycorrhizal fungi (AMF) inoculations on nitrogen (N) content of easily extractable glomalin-related soil protein (EE-GRSP) and difficultly extractable GRSP (DE-GRSP) fraction [EE-GRSP (N) and DE-GRSP (N)] to soil total N of trifoliate orange seedlings grown in a two-chambered root-box. Means ± standard deviation (n = 4) followed by different letters above the bars indicate significant (P < 0.05) differences between mycorrhizal treatments. R – *Rhizoglomus intraradices*; D – *Diversispora epigaea*; P – *Paraglomus occultum*

stimulated increase of GRSP (N) may be due to the increase in the N-associated substances of mycorrhizas (Govindarajulu et al. 2005), which needs to be studied in the future. As proposed by Gillespie et al. (2011), GRSP is a mixture of proteinaceous, humic, lipidic, and inorganic substances originated from the mycorrhizal and non-mycorrhizal origin, which contains a large amount of N. Hence, GRSP is an important soil N pool, as evidenced by the significant and positive correlation of GRSP (N) with leaf and root N content and soil total N and in the two chambers (Table 1).

Changes in the contribution of GRSP (N) to soil total N. The present study also estimated the contribution of EE-GRSP (N) and DE-GRSP (N) to soil total N. EE-GRSP (N) and DE-GRSP (N) accounted for 15.6 ± 1.6% and 18.1 ± 1.8% in soil total N, respectively (Figure 5). As a result, T-GRSP (N) contributed 33.8 ± 1.9% in soil total N, which is considerably higher than earlier studies in an ancient soil (~5%) and in a tropical soil (5%) (Rillig et al. 2001, Lovelock et al. 2004). Maybe, the age of soil and soil environment strongly affects the contribution of GRSP.

Mycorrhizal inoculations, to some extent, altered the contribution of GRSP (N) to soil total N. In root chamber, inoculation with *R. intraradices* significantly increased EE-GRSP (N)/soil total N by 13.5% and reduced DE-GRSP (N)/soil total N by 22.7%; inoculation with *D. epigaea* in root chamber showed no significant difference in GRSP (N)/soil total N; inoculated with *P. occultum* only significantly stimulated DE-GRSP (N)/soil total N by 25.2%. In hypha chamber, inoculation with *R. intraradices* significantly increased DE-GRSP (N)/soil total N and EE-GRSP (N)/soil total N by 12.9% and 13.1%, respectively; inoculation with *D. epigaea* significantly increased DE-GRSP (N)/soil total N by 19.4%, while reduced EE-GRSP (N)/soil total N by 14.0%; treatment with *P. occultum* increased EE-GRSP (N)/soil total N by 30.0%, while did not alter DE-GRSP (N)/soil total N. It concluded that mycorrhiza-mediated contribution of DE-GRSP (N) to soil total N is obviously dependent of with or without roots, GRSP types, and AMF species. Correlation studies also showed a significantly positive correlation between GRSP (N) and soil total N in the two chambers (Table 1). Therefore, the increase of GRSP (N) to soil

Table 1. Pearson's correlations (r) between variables

	Soil total nitrogen		GRSP (N)		Nitrogen	
	root chamber	hypha chamber	root chamber	hypha chamber	leaf	root
GRSP (N) in root chamber	0.54*	0.54*	1			
GRSP (N) in hypha chamber	0.51*	0.77**	0.88**	1		
Leaf nitrogen	0.45^{ns}	0.52*	0.77**	0.80**	1	
Root nitrogen	0.68**	0.52*	0.84**	0.85**	0.81**	1

*P < 0.05; **P < 0.01; ns – not significant at P = 0.05; GRSP – glomalin-related soil protein

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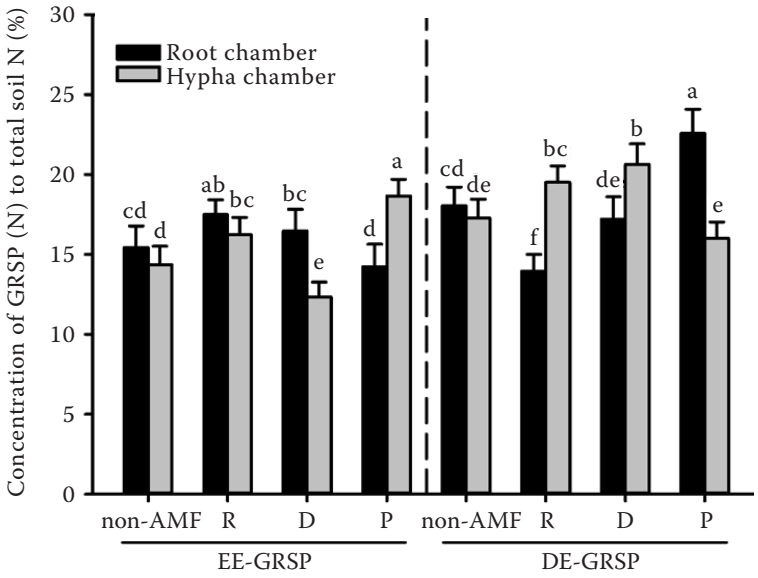

Figure 5. Effects of arbuscular mycorrhizal fungi (AMF) inoculations on the contribution of easily extractable glomalin-related soil protein (EE-GRSP) (N) and difficultly extractable GRSP (DE-GRSP) (N) to soil total nitrogen (N) of trifoliate orange seedlings grown in a two-chamber root-box. Means ± standard deviation (n = 4) followed by different letters above the bars indicate significant (P < 0.05) differences between mycorrhizal treatments. R – *Rhizoglomus intraradices*; D – *Diversispora epigaea*; P – *Paraglomus occultum*

total N by AMF inoculations may be attributed to the GRSP turnover as a soil N pool (Rotter et al. 2017), providing the implication of GRSP in stocking soil N. Such results provide more information about GRSP contribution to soil N, which guides N management in the soil of citrus orchards.

Changes in leaf and root N levels. It is estimated that in mycorrhizal plants, the contribution rate of N absorbed by external hyphae was 25% in total plant N Marschner and Dell 1994). Our study showed a strong improvement of leaf and root N contents by AMF inoculations (Figure 6). Compared with non-AMF-inoculated seedlings, the inoculated seedlings with *R. intraradices*, *D. epigaea* and *P. occultum* possessed 11.1, 8.1, and 13.6% significantly higher leaf N contents and 47.1, 41.7, and 49.1% significantly higher root N contents, respectively. This is in agreement with previous studies in tomato (Balliu et al. 2015), maize (Debeljak et al. 2018), and peach (Lü et al. 2019). The increase of leaf and root N contents in AMF-inoculated seedlings may result from the increasing absorption of soil N by AMF extraradical hyphae (Govindarajulu et al. 2005). In addition, AMF-increase N contents of plants were likely associated with the activity of soil saprotrophs and the N releasing and transforming by other microbes in organic matters (Bukovská et al. 2018). Our study also indicated that leaf and root N contents were significantly and positively correlated with soil total N and GRSP (N) in the two chambers, except for no significant correlation between leaf N and soil total N in root chamber (Table 1). It further suggests that in mycorrhizal plants, N content in plants, soil, and GRSP was closely related to each other.

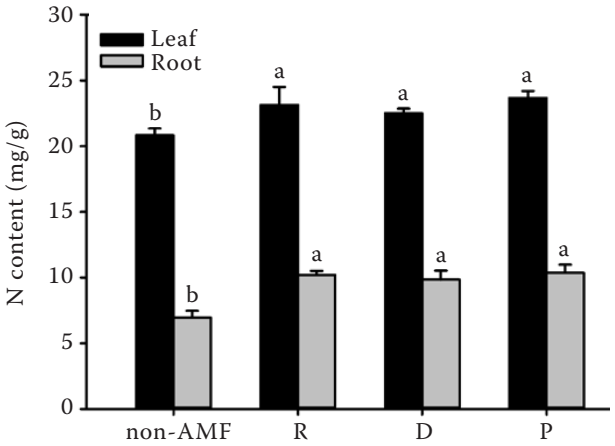

Figure 6. Effects of arbuscular mycorrhizal fungi (AMF) inoculations on leaf and root nitrogen (N) contents of trifoliate orange seedlings grown in a two-chambered root-box. Means ± standard deviation (n = 4) followed by different letters above the bars indicate significant (P < 0.05) differences between mycorrhizal treatments. R – *Rhizoglomus intraradices*; D – *Diversispora epigaea*; P – *Paraglomus occultum*

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Received: February 24, 2020
Accepted: April 23, 2020
Published online: April 28, 2020