# Systematicness of glomalin in roots and mycorrhizosphere of a split-root trifoliate orange

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**ABSTRACT**

Understanding the behavior of mycorrhiza-originated glomalin, either of plant or soil origin, is anticipated to facilitate better opportunities of modulating antioxidants and carbon distribution in plants. In this study, trifoliate orange seedlings with half of roots were colonized by *Acaulospora scrobiculata* and *Funneliformis mosseae* in a split-root rootbox. Mycorrhizal inoculation showed a significantly higher plant biomass of trifoliate orange, regardless of mycorrhizal species. Glomalin-related root protein showed a systematic increase in non-mycorrhiza-inoculated chamber under inoculation with *A. scrobiculata* and *F. mosseae* than under non-mycorrhizal inoculation. Similar increase was also observed in easily extractable glomalin-related soil protein and total glomalin-related soil protein as a result of *F. mosseae* colonization only. Mean weight diameter and soil organic carbon were significantly higher under mycorrhization than non-mycorrhization, irrespective of mycorrhized or non-mycorrhized chamber. Mycorrhizal inoculation stimulated an increase in soil protease activity in the mycorrhized chamber, without any distinctive change in the non-mycorrhized chamber. These results, hence, suggested that mycorrhization conferred a systematic increase in glomalin synthesis in roots and soils, collectively, aiding in better aggregate stability and soil carbon sequestration.

**Keywords**: Glomeromycota; glycoprotein; hyphae; mineral nutrients; mutualistic symbiosis

Arbuscular mycorrhizal fungi (AMF), the phylum Glomeromycota, inhabit under diverse soils of terrestrial ecosystems, to establish the mutualistic symbiosis with roots of more than 80% terrestrial plants (Smith and Read 2008). AMF provide mutualistic benefits including uptake and transport of mineral nutrients and water from soils to AMF and subsequently the host plant, in exchange of substantial carbon from the host plants to the AMF for their sustenance (Walder et al. 2012).

Hyphae and spores of AMF release a stable and persistent glycoprotein, called glomalin, bound to soils as glomalin-related soil protein (GRSP). GRSP is characterized by the presence of iron and N-linked oligosaccharides, a stable compound, insoluble in water, and resistant to heat degradation in its native state (Rillig 2004). Stabilizing ability of GRSP in soil aggregates in an ecosystem is well known through sloughing off hyphae onto the surrounding organic matter, further binding the clay particles via cation bridging by iron, in form of hydrophobic coating (Yadav and Pandey 2014, Zou et al. 2016). Such process of soil aggregation also contributes towards an increase of the soil organic carbon (SOC) pool (Wu et al. 2015b).

The split-root experiment is commonly used to study the change in bacterial community, carbohydrates, plant biomass, antioxidant enzyme activities

and root morphology due to AMF colonization in various plants (Zhang et al. 2011, Wu et al. 2016). In the split root technique, the root system of a plant is divided into two parts; one part is inoculated with AMF only, and the other part remains uninoculated, providing a comparison of C-sink strength between AMF and non-AMF root halves of the same plant (Lerat et al. 2003).

Considering the presence of glomalin in the root (Rosier et al. 2008), dual objectives were hypothesized viz., (i) Root-produced glomalin has the systematicness (communication ability) in soils and roots from AM root side to non-AM root side, and (ii) Root-produced glomalin (as a part of soil organic matter) is released into the soil to stabilize soil aggregates. In this background, we tested these objectives using a split-root rootbox with only one half of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] roots colonized by AMF.

## MATERIAL AND METHODS

**Plant culture**. Seeds of trifoliate orange were surface-sterilized with 70% ethanol solution for 10 min, and germinated in the autoclaved sand at 28/20°C (day/night) for three weeks, under air relative humidity of 80%, photoperiod 16:8, and photosynthetic photon flux density of 1200 µmol/m$^2$/s. After three weeks, four-leaf-old trifoliate orange seedlings were selected and a great mass of tap root was cut, leaving only 3-cm long to induce lateral roots in the sand. After seven weeks, the treated seedlings produced lots of lateral roots in the reserved taproot.

A split-root perspex rootbox (20 cm length, 10 cm width, and 18 cm height) was separated by a 15-cm-height Perspex in the middle to form two equal chambers, as described schematically in Figure 1. Each chamber was filled with 1550 g of autoclaved (121°C, 0.11 Mpa, 2 h) soil (Xanthi-Udic Ferralsols, FAO system) collected from a citrus orchard of the Yangtze University campus. To facilitate the separation of lateral roots, we made a semicircle of 1 cm diameter at the top of the separated perspex. To fix the split-root trifoliate orange seedlings, we used a 5 × 3 × 2 cm (length × width × height) perlite board at the bottom of the seedlings to ensure an upright growth.

At the time of seedlings transplanting, ~1000 spores of *Acaulospora scrobiculata* Trappe and

*Funneliformis mosseae* (Nicol. and Gerd.) Schüßler and Walker were inoculated into the AMF-treated (M) chamber, while the non-AMF-treated (NM) chamber was supplied with the same quantity of autoclaved inoculum plus 2 mL filtrate (25 µm) of mycorrhizal inocula to keep similar type of microorganisms, except the AMF.

All the treated seedlings were placed in a greenhouse from April to September 2013 (965 µmol/m$^2$/s photosynthetic photon flux density, 28/21°C day/night temperature, and 85% relative humidity). The seedlings were watered at three-day intervals to maintain soil moisture optimum for plant growth.

**Experimental design**. The experiment consisted of three treatments with four replicates in a completely randomized design, for a total of 12 rootboxes. The three treatments included: (i) two non-AMF root halves of trifoliate orange (NM/NM); (ii) one half of roots was inoculated with *A. scrobiculata*, keeping the other half without AMF as non-AMF (As/NM), and (iii) one half of roots was inoculated with *F. mosseae*, keeping the other half without AMF as non-AMF (Fm/NM).

**Determination of variables**. All the treated seedlings were harvested after five months of AMF inoculation and separated into leaf, stem, and root for measurement of their fresh weight. The soil from both the M and NM chamber of root box was collected, air-dried, and sieved through 4-mm mesh.
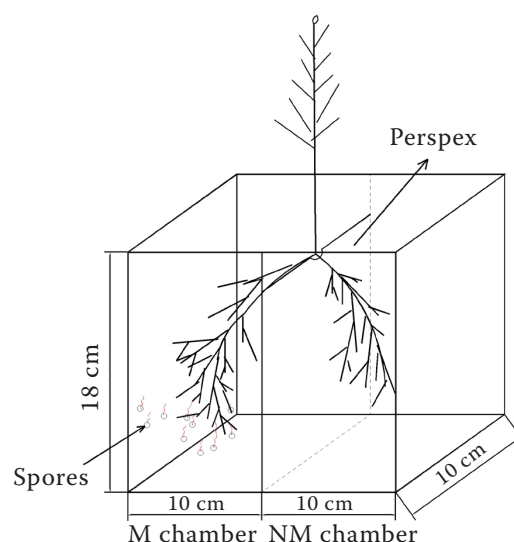


Figure 1. Schematic representation of a split-root trifoliate orange seedlings with half of root volume inoculated with *Acaulospora scrobiculata* or *Funneliformis mosseae*. M chamber and NM chamber refer to inoculation with or without mycorrhizal fungi, respectively

Root mycorrhizas were stained using trypan protocol as described by Phillips and Hayman (1970), and root mycorrhizal colonization was calculated as the percentage of AMF colonized root lengths against total root lengths. Soil hyphal length was determined using the protocol as described by Bethlenfalvay and Ames (1987).

Glomalin-related root protein (GRRP) in AMF and non-AMF root halves was determined as per the method proposed by Rosier et al. (2008). A 10-mg root sample was autoclaved with 50 mmol sodium citrate (pH 8.0) at 121°C and 0.11 Mpa for 60 min and subsequently centrifuged at 5000 g for 15 min. The Bradford (1976) assay was used to determine the concentration of GRRP in the supernatant.

Two soil GRSP fractions viz., easily-extractable GRSP (EE-GRSP) and difficultly-extractable GRSP (DE-GRSP) were determined as per procedures suggested by Wu et al. (2015a). A 1-g soil sample was incubated with 8 mL 20 mmol citrate buffer (pH 7.0) at 121°C and 0.11 Mpa for 30 min, centrifuged at 10 000 g for 3 min, and determined using the Bradford (1976) assay in the supernatant for EE-GRSP. The centrifugal residue after EE-GRSP extraction was mixed with 8 mL 50 mmol citrate (pH 8.0) for 60 min, centrifuged at 10 000 g for 3 min, and then determined according to the Bradford (1976) assay in the supernatant for DE-GRSP. Total GRSP (T-GRSP) is the total of EE-GRSP and DE-GRSP.

Mean weight diameter (MWD) was used to calculate soil aggregate stability in 0.25–4 mm water-stable aggregates (Kemper and Rosenau 1986). Determination of MWD was carried out using the procedure as outlined by Zou et al. (2016). Determination of soil organic carbon (SOC) was followed as per the procedure of Rowell (1994). Soil active carbon (SAC) was determined using the protocol as described by Matsumoto et al. (2001). Hot water-extractable carbohydrate (HWC) and hydrolyzable carbohydrate (HC) concentrations

were determined following the procedure as described by Wu et al. (2008). Soil protease activity was measured according to Cao et al. (1982).

**Statistical analysis**. Data (means ± SD, $n$ = 4) were subjected to the analysis of variance (ANOVA). Means were compared using the Duncan's multiple range test at the 5% level.

## RESULTS AND DISCUSSION

**Mycorrhizal status and plant growth**. The split-root trifoliate orange seedlings inoculated with *A. scrobiculata* and *F. mosseae* showed 15.8% and 38.9% root colonization, respectively, corresponding to 0.22 and 0.55 m/g of soil hyphal length in the M chamber (Table 1). No mycorrhizas were discovered either in roots or in soils of the NM chamber. A considerably higher mycorrhizal status was observed in roots and soils in Fm/NM than in As/NM seedlings.

AMF-seedlings likewise showed significantly higher plant biomass in leaf, stem, and root than non-AMF seedlings (Table 1). Moreover, the effect of AMF on plant biomass was significantly higher with Fm/NM than with As/NM treatment. Ortas and Ustuner (2014) earlier reported an improvement in plant biomass as a result of AMF inoculation in trifoliate orange seedlings without split-root treatments.

**Glomalin-related root protein (GRRP)**. The presence of GRRP in the roots of *Bromus inermis* colonized by different AMF isolates according to the Bradford (1976) assay, was observed as a result of uptake and storage of arginine within intraradical hyphae of AMF (Rosier et al. 2008). They also proposed the use of GRRP to estimate root AMF colonization. In our study, GRRP varied from 0.050 to 0.063 mg/g DW in roots of the split-root seedlings (Figure 2a). Interestingly, M root side

Table 1. Mycorrhizal status (root colonization and soil hyphal length) and plant biomass of *Acaulospora scrobiculata* and *Funneliformis mosseae*-colonized trifoliate orange seedlings grown in a split-root rootbox

| Treatment | Root colonization (%) | | Soil hyphal length (m/g soil) | | Plant biomass (g FW/plant) | | |
|---|---|---|---|---|---|---|---|
| | M chamber | NM chamber | M chamber | NM chamber | leaf | stem | root |
| NM/NM | 0[c] | 0 | 0[c] | 0 | 0.34 ± 0.09[c] | 0.65 ± 0.14[c] | 1.06 ± 0.09[c] |
| As/NM | 15.8 ± 1.2[b] | 0 | 0.22 ± 0.12[b] | 0 | 0.50 ± 0.08[b] | 0.98 ± 0.11[b] | 1.63 ± 0.28[b] |
| Fm/NM | 38.9 ± 3.5[a] | 0 | 0.55 ± 0.02[a] | 0 | 1.41 ± 0.06[a] | 2.68 ± 0.18[a] | 3.41 ± 0.33[a] |

Data (means ± SD, $n$ = 4) followed by different letters indicate significant differences among treatments after using the Duncan's multiple range test ($P$ < 0.05). M chamber and NM chamber stand for AMF-inoculated and AMF-noninoculated chamber

showed significantly higher GRRP concentration (22% and 26%, respectively) with Fm/NM and As/NM than with NM/NM seedlings, suggesting that AMF inoculation stimulated the production of GRRP. Rosier et al. (2008) earlier reported that AMF colonization significantly increased GRRP concentrations within roots of *Bromus inermis* colonized by several AMF isolates. The GRRP was also increased in *Daucus carota* under sterile *in vitro* conditions and *Plantago lanceolata* and *Sorghum bicolor* under greenhouse conditions (Rosier et al. 2008). In the NM root side, significantly higher GRRP concentration ranked in the order: As/NM > Fm/NM > NM/NM. Since the NM root side was not inoculated with AMF, such increases in GRRP could be explained through systematicness of GRRP production in split-root seedlings. These observations also suggested that GRRP produced in the M root side also stimulated the production of GRRP in the NM root side. The systematicness of GRRP production in the whole root system would thus help the non-AMF-colonized roots to produce more GRRP for

eventual carbon turnover. However, the extraction protocol of GRRP does not absolutely eliminate non-AMF-derived proteins either from roots alone or associated with plant roots according to the Bradford assay (Wu et al. 2014b).

**Glomalin-related soil protein (GRSP)**. GRSP is considered as a glue agent of soil aggregate stability (Wu et al. 2014a, 2015a, b, c). In the M chamber, significantly higher EE-GRSP ranked in the order: *F. mosseae* ≥ *A. scrobiculata* > non-AMF, indicating that AMF inoculation stimulated greater synthesis in EE-GRSP, since EE-GRSP is comparatively newly produced by hyphae and spores of AMF (Wu et al. 2015a). While, in the NM chamber, the maximum concentration of EE-GRSP was observed by inoculation with *F. mosseae* followed by *A. scrobiculata* and non-AMF (Figure 2b) in the decreasing order, suggesting that *F. mosseae* possessed greater ability to synthesize EE-GRSP than *A. scrobiculata* in the NM chamber. Higher GRSP was observed with the treatment Fm/NM and As/NM than NM/NM, providing a strong clue, that root glomalin is deposited into the soil, resulting in an increase
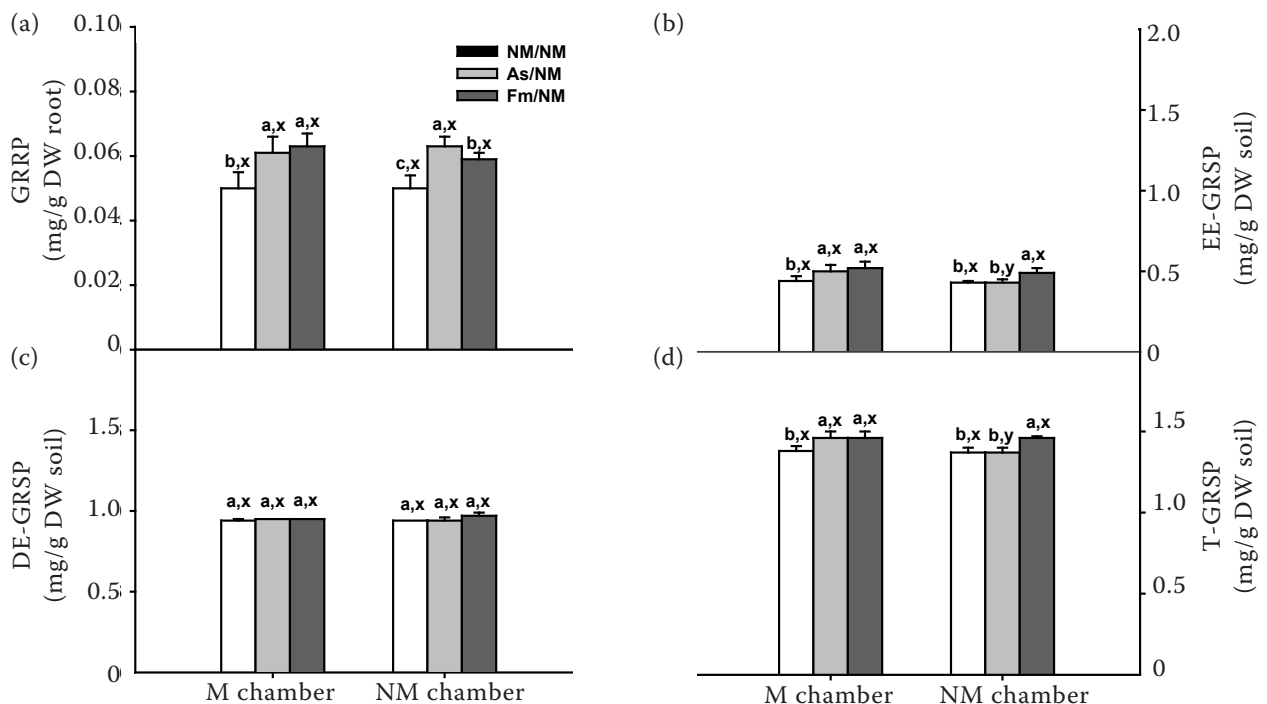


Figure 2. Effects of arbuscular mycorrhizal fungi (AMF) on glomalin-related root protein (GRRP), easily-extractable glomalin-related soil protein (EE-GRSP), difficultly-extractable glomalin-related soil protein (DE-GRSP), and total glomalin-related soil protein (T-GRSP) concentrations of trifoliate orange seedlings colonized with (M) or without (NM) *Acaulospora scrobiculata* or *Funneliformis mosseae* and grown in a two-chambered split-root system. Data (means ± SD, *n* = 4) followed by different letters among AMF treatments for the same split root compartment or between the M and NM split root compartment for the same AMF treatment (x, y) indicate significant differences at *P* < 0.05

in EE-GRSP in the NM chamber. The result on EE-GRSP changes in M and NM chambers in response to different treatments was analogous to changes in concentration of T-GRSP (Figure 2d). However, DE-GRSP levels remained unaffected in response to different treatments (Figure 2c). It is well known that DE-GRSP is a comparatively older form of soil glomalin, originated from EE-GRSP, primarily accountable for slow turnover of GRSP consuming as much as 6–42 years (Rillig et al. 2001, Wu et al. 2015a). In our study, five-month period may be not sufficient to induce some visible changes in turnover of EE-GRSP into DE-GRPS, accountable for non-significant changes in DE-GRSP. Any systematicness of GRRP would, therefore, induce the cascading systematicness in EE-GRSP and T-GRSP production, excluding DE-GRSP changes. In split-root white clover plants, antioxidant enzyme activities were increased in the non-AMF root side after the other root sides were colonized by *F. mosseae* and *Rhizophagus intraradices* (Zhang et al. 2011), providing a strong clue about the systematicness in antioxidant defense system of whole roots by mycorrhization.

**Soil aggregate stability**. A functional role of GRSP is associated with aggregate stability of soils (Yadav and Pandey 2014, Zou et al. 2016). The present study indicated a distinctively greater MWD with treatment Fm/NM and As/NM than NM/NM split-root seedlings, irrespective of AMF species and M or NM chamber (Figure 3). The improvement of soil aggregate stability under mycorrhization resulted from the integrative effect of mycorrhizal hyphae, GRSP, SOC, and root volume (Wu et al. 2014a). In our study, a significantly positive correlation of MWD with EE-GRSP ($r = 0.70$, $P < 0.01$) and T-GRSP ($r = 0.67$, $P < 0.01$) confirmed the functional impact of GRSPs in soil aggregate stability.

**Changes in soil carbon**. A significantly higher SOC was observed in both, M as well as NM chambers, involving treatments such as Fm/NM and As/NM seedlings compared to NM/NM split-root seedlings (Figure 4a). The effectiveness of various treatments with regard to magnitude of changes in SOC was observed in the decreasing order of *F. mosseae* followed by *A. scrobiculata* and non-AMF. Glomalin is reported to contain nearly 37% C (Lovelock et al. 2004), which contributes 4–5% of soil total C (Rillig et al. 2001). In this background, GRSP could be part of the major contributory factors of SOC sequestration. SOC

can also be utilized as a cementing agent for soil aggregate stabilization, thus, providing a cushion against microbial decomposition of glomalin (Wu et al. 2015c). Hence, the increase of soil SOC as a result of mycorrhization could accelerate a concurrent improvement in soil aggregate stability, as confirmed in our study through a significantly positive correlation between MWD and SOC ($r = 0.57$, $P < 0.01$).

Soil inorganic carbon concentration was also observed to be influenced by AMF inoculation, displaying a considerably higher SAC and lower HC concentration in M chamber with treatments like Fm/NM and As/NM compared to NM/NM treatment (Figure 4b, d). In the NM chamber, a significantly higher HWC concentration was observed with As/NM treatment than in NM/NM or Fm/NM treatment while lower HC concentration was observed with As/NM or Fm/NM treatment than with NM/NM treatment (Figure 4c, d). Such changes in soil inorganic carbon fractions could be attributed to C storage capacity of GRSP and the consequent increase in SOC (Wu et al. 2008).

**Soil protease activity**. Soil proteases participate in the decomposition of soil protein, and are closely related to available N in soils (Zhang et al. 2015). In our study, a significantly higher soil protease
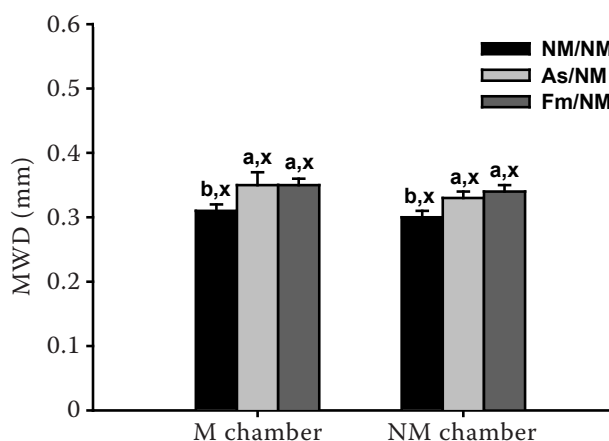


Figure 3. Effects of arbuscular mycorrhizal fungi (AMF) on soil mean weight diameter (MWD) of trifoliate orange seedlings colonized with (M) or without (NM) *Acaulospora scrobiculata* or *Funneliformis mosseae* and grown in a two-chambered split-root system. Data (means ± SD, *n* = 4) followed by different letters among AMF treatments for the same split root compartment or between the M and NM split root compartment for the same AMF treatment (x, y) indicate significant differences at *P* < 0.05
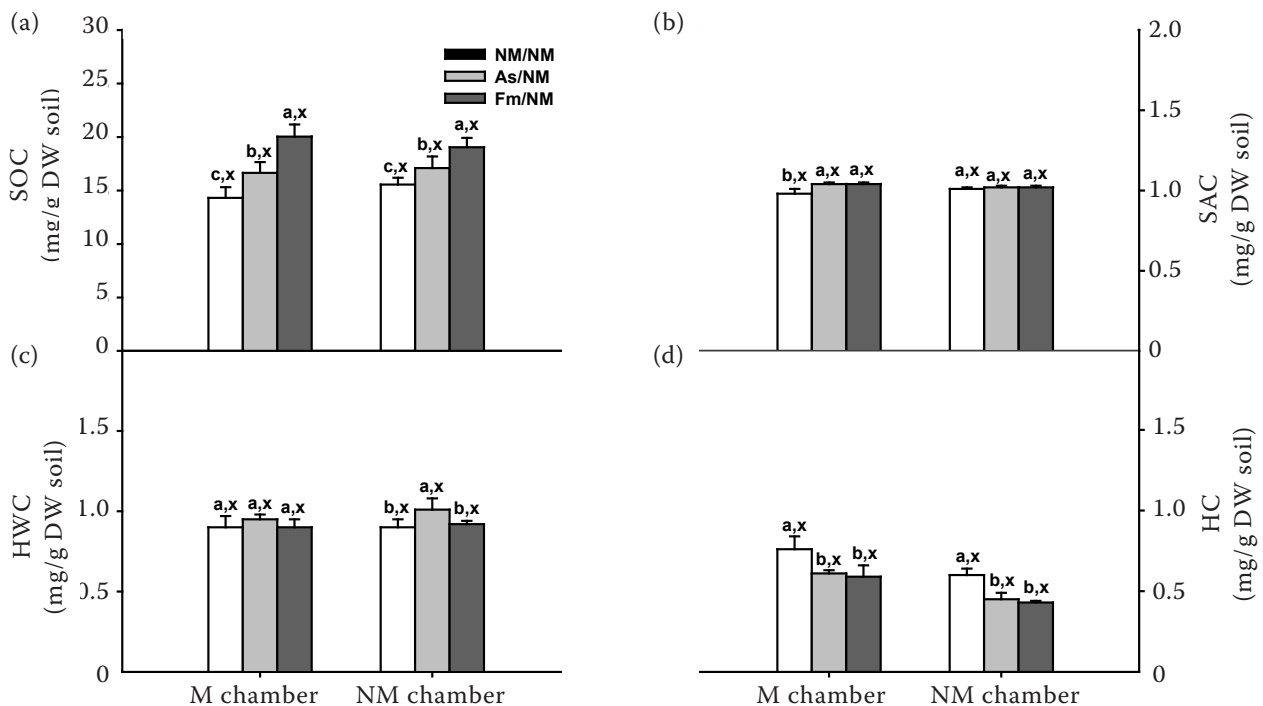
Figure 4. Effects of arbuscular mycorrhizal fungi (AMF) on soil organic carbon (SOC), soil active carbon (SAC), hot water-extractable carbohydrate (HWC), and hydrolyzable carbohydrate (HC) concentrations of trifoliate orange seedlings colonized with (M) or without (NM) *Acaulospora scrobiculata* or *Funneliformis mosseae* and grown in a two-chambered split-root system. Data (means ± SD, *n* = 4) followed by different letters among AMF treatments for the same split root compartment or between the M and NM split root compartment for the same AMF treatment (x, y) indicate significant differences at *P* < 0.05

activity was observed with Fm/NM and As/NM treatment than with NM/NM treatment in the M chamber, but not in the NM chamber (Figure 5). Soil protease activity was significantly and positively correlated with EE-GRSP (*r* = 0.62, *P* < 0.01), T-GRSP (*r* = 0.65, *P* < 0.01) and GRRP (*r* = 0.58, *P* < 0.01), respectively. Higher soil protease activity in the M chamber would confer a greater vulnerability towards decomposition of GRSPs, since mycorrhizosphere had higher EE-GRSP and T-GRSP content, thereby potentially aiding in the acceleration of soil carbon sequestration.

In conclusion, in split-root trifoliate orange seedlings, AMF inoculation within half of their roots significantly increased leaf, stem, and root biomass, irrespective of AMF species. A significantly higher GRRP, EE-GRSP, and T-GRSP concentrations under mycorrhization provided an evidence of systematicness of glomalin in soils and roots. This phenomenon of systematicness would later pave the way for split-root plant to enforce better soil aggregate stability and, hence, expand the sink capacity for carbon storage in mycorrhizosphere.
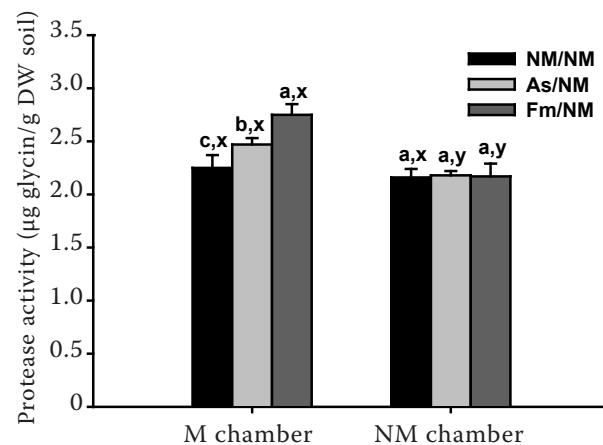


Figure 5. Effects of arbuscular mycorrhizal fungi (AMF) on soil protease activity of trifoliate orange seedlings colonized with (M) or without (NM) *Acaulospora scrobiculata* or *Funneliformis mosseae* and grown in a two-chambered split-root system. Data (means ± SD, *n* = 4) followed by different letters among AMF treatments for the same split root compartment or between the M and NM split root compartment for the same AMF treatment (x, y) indicate significant differences at *P* < 0.05

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