Direct and indirect effects of glomalin, mycorrhizal hyphae, and roots on aggregate stability in rhizosphere of trifoliate orange

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To test direct and indirect effects of glomalin, mycorrhizal hyphae, and roots on aggregate stability, perspex pots separated by 37-μm nylon mesh in the middle were used to form root-free hyphae and root/hyphae chambers, where trifoliate orange (Poncirus trifoliata) seedlings were colonized by Funneliformis mosseae or Paraglomus occultum in the root/hyphae chamber. Both fungal species induced significantly higher plant growth, root total length, easily-extractable glomalin-related soil protein (EE-GRSP) and total GRSP (T-GRSP), and mean weight diameter (an aggregate stability indicator). The Pearson correlation showed that root colonization or soil hyphal length significantly positively correlated with EE-GRSP, difficultly-extractable GRSP (DE-GRSP), T-GRSP, and water-stable aggregates in 2.00–4.00, 0.50–1.00, and 0.25–0.50 mm size fractions. The path analysis indicated that in the root/hyphae chamber, aggregate stability derived from a direct effect of root colonization, EE-GRSP or DE-GRSP. Meanwhile, the direct effect was stronger by EE-GRSP or DE-GRSP than by mycorrhizal colonization. In the root-free hyphae chamber, mycorrhizal-mediated aggregate stability was due to total effect but not direct effect of soil hyphal length, EE-GRSP and T-GRSP. Our results suggest that GRSP among these tested factors may be the primary contributor to aggregate stability in the citrus rhizosphere.

Soil structure, the three-dimensional arrangement of organic/mineral aggregates and pore spaces in soil on scales, has shown important roles in soil carbon (C) sequestration, nutrient and gas fluxes and water quality. Soil macroaggregates (>0.25 mm size) establish the bulk soil structure, whilst the microaggregates (<0.25 mm size) constitute part of the sediment load. Soil aggregation refers to soil microaggregates that are bound together by binding agents to form stable macroaggregates. Meanwhile, water-stable aggregates (WSAs) in macroaggregates, which are stable to the action of repeated soil wetting and drying cycles, are widely used to evaluate aggregate stability by means of the wet-sieving analysis. As an indicator of soil structure related to soil water regime, erodibility, and nutrient availability, aggregate stability is affected by soil physical, chemical, and/or microbial community properties, root systems, plant species and/or communities.

Among soil microbial communities, fungi usually present a more profound functioning on stabilizing macroaggregates than bacteria. The ubiquitous soil arbuscular mycorrhizal fungi (AMF) can contribute to soil aggregate stability directly by their extraradical fungal hyphae or indirectly by altering the biochemical and morphological properties of host plants. Such direct and indirect contributions are often intertwined together. For instance, a microcosm experiment showed that mean weight diameter (MWD, an indicator of aggregate stability) highly positively correlated with soil hyphal length but weakly with root volumes. The contribution to the formation of 2–5 and 1–2 mm WSA was greater in mycorrhizal hyphae than in maize roots. However, the AM fungus Glomus geosporum, G. mosseae (now Funneliformis mosseae), or G. intraradices did not, but the roots of Plantago lanceolata did, affect aggregate stability and aggregate size distribution in the rhizosphere of a sandy loam soil. Recently a hierarchical aggregation model has shown that organic matter, roots, and AMF are required for soil aggregate stability, whilst the contribution of roots to soil structure is further stabilized by AMF. In a plant-fungus symbiosis, aggregate stability significantly positively correlated with either root length or root mycorrhizal colonization, but the latter showed a stronger correlation.

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OPEN

SUBJECT AREAS:
FUNGAL HOST RESPONSE

ARBUSCULAR MYCORRHIZA

Received 2 May 2014
Accepted 30 June 2014
Published 25 July 2014

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SCIENTIFIC REPORTS | 4 : 5823 | DOI: 10.1038/srep05823
Glomalin, a fungal glycoprotein that has not been biochemically defined, but operationally quantified, from diverse soils as glomalin-related soil protein (GRSP)\(^{20,21}\), is only released by an AM fungus into defined, but operationally quantified, from diverse soils as glomalin-defined, but operationally quantified, from diverse soils as glomalin. Glomalin generally contains 3–5% N, 36–59% C, 4–6% H, 33–49% O, 0.03–0.1% P, and 2–5% Fe\(^{23–27}\). As a soil particle binding agent, this insoluble, hydrophobic and heat-resistant GRSP strongly relates to the amount of WSA\(^{20,21,26–28}\) and aggregate stability in various soils\(^{13,17,20,29,30}\). Recently, GRSP was divided into fraction 1 and fraction 2\(^{23}\). In general, the GRSP fraction 1, as a newly produced glo-

### Table 1 | Effects of *Funneliformis mosseae* and *Paraglomus occultum* on root colonization, plant growth performance, and root total length of 5-month-old trifoliate orange seedlings grown in 37 µm nylon-mesh separated root/hyphae chambers

| Treatment          | Root colonization (%) | Plant height (cm) | Stem diameter (cm) | Leaf number per plant | Root total length (cm) | Dry weight (g) |
|--------------------|-----------------------|-------------------|-------------------|----------------------|------------------------|---------------|
| Non-AMF           | 0.0 ± 0.0c            | 9.6 ± 1.5c        | 0.28 ± 0.03b      | 12 ± 2c              | 242 ± 18b              | 0.89 ± 0.19c  |
| *F. mosseae*      | 34.7 ± 4.5b           | 27.7 ± 3.9a       | 0.37 ± 0.03a      | 25 ± 2a              | 374 ± 42a              | 2.57 ± 0.43a  |
| *P. occultum*     | 43.7 ± 3.4a           | 21.4 ± 3.6b       | 0.33 ± 0.03a      | 21 ± 3b              | 320 ± 39a              | 1.82 ± 0.24b  |

**Note:** Data [means ± SE, n = 4] followed by different letters indicate significant differences (P < 0.05) among mycorrhizal treatments.

### Results

**Root colonization and plant growth performance.** Root coloniza-

### Hyphal length and GRSPs in root/hyphae or root-free hyphae chambers.

### Distribution of WSA fractions and MWD in root/hyphae or root-

### Correlation analyses.** Pearson correlation analyses indicated that root AM colonization in the root/hyphae chamber positively
Table 2 | Effects of *Funneliformis mosseae* and *Paraglomus occultum* on soil hyphal length and GRSP in 37 μm nylon-mesh separated root/hyphae and root-free hyphae chambers

| Treatment       | Hyphal length (m g⁻¹ FW) | EE-GRSP | DE-GRSP | T-GRSP | Ratio of EE-GRSP/DE-GRSP |
|-----------------|--------------------------|---------|---------|--------|--------------------------|
| Non-AMF        | NA                       | NA      | 0.34 ± 0.03b,x 0.30 ± 0.02b,y | 0.53 ± 0.03b,x 0.53 ± 0.02b,y | 0.87 ± 0.01c,x 0.82 ± 0.01c,y | 0.63 ± 0.09b,x 0.56 ± 0.05b,x |
| *F. mosseae*   | 0.26 ± 0.02b,x 0.20 ± 0.01b,y | 0.44 ± 0.03a,x 0.35 ± 0.02a,y | 0.53 ± 0.03b,x 0.52 ± 0.02b,y | 0.97 ± 0.04b,x 0.88 ± 0.04b,y | 0.83 ± 0.05a,x 0.69 ± 0.04a,y |
| *P. occultum*  | 0.88 ± 0.02a,x 0.60 ± 0.03a,y | 0.44 ± 0.03a,x 0.36 ± 0.02a,y | 0.64 ± 0.02a,x 0.64 ± 0.03a,x | 1.08 ± 0.03a,x 1.00 ± 0.03a,y | 0.70 ± 0.04b,x 0.56 ± 0.05b,y |

Note: Trifoliate orange seedlings were 5-month-old and grown in 37 μm nylon-mesh separated two-chambered pots. Data (means ± SE, n = 4) followed by different letters indicate significant differences (P < 0.05) between mycorrhizal treatments for the same chamber (a, b, c) or between root chamber and root-free hyphal chamber for the same mycorrhizal treatment (x, y). Abbreviations: DE-GRSP, difficultly extractable glomalin-related soil protein; EE-GRSP, easily extractable glomalin-related soil protein; NA, not available; T-GRSP, total (EE-GRSP + DE-GRSP) glomalin-related soil protein.

Table 3 | Effects of *Funneliformis mosseae* and *Paraglomus occultum* on water-stable aggregate (WSA) size distribution and mean weight diameter (MWD) in 37 μm nylon-mesh separated root/hyphae and root-free hyphae chambers

| Distribution of WSA fraction (%) | 2.00–4.00 mm | 1.00–2.00 mm | 0.50–1.00 mm | 0.25–0.50 mm | MWD [mm] |
|---------------------------------|--------------|--------------|--------------|--------------|---------|
| Treatment                       | Root/hyphae chamber | Hyphae only chamber | Root/hyphae chamber | Hyphae only chamber | Root/hyphae chamber | Hyphae only chamber | Root/hyphae chamber | Hyphae only chamber | Root/hyphae chamber | Hyphae only chamber |
| Non-AMF                         | 1.77 ± 0.31b,y | 2.67 ± 0.31a,x | 1.43 ± 0.18b,x | 0.95 ± 0.21b,y | 2.51 ± 0.56b,x | 1.72 ± 0.22b,y | 3.71 ± 0.33b,x | 2.43 ± 0.38b,y | 0.07 ± 0.01c,x | 0.08 ± 0.01b,x |
| *F. mosseae*                    | 4.52 ± 0.08a,y | 4.80 ± 0.21a,x | 2.01 ± 0.30a,x | 2.69 ± 0.33a,x | 2.06 ± 0.45b,x | 4.06 ± 0.17b,x | 5.15 ± 0.83a,y | 0.14 ± 0.00b,x | 0.14 ± 0.01a,x |
| *P. occultum*                   | 4.47 ± 0.34a,x | 4.94 ± 0.55a,x | 1.55 ± 0.29b,x | 1.02 ± 0.08b,y | 10.80 ± 0.87a,x | 3.84 ± 1.53a,y | 5.34 ± 0.78a,x | 5.42 ± 0.34a,x | 0.17 ± 0.02a,x | 0.14 ± 0.02a,y |

Note: Trifoliate orange seedlings were 5-month-old and grown in 37 μm nylon-mesh separated two-chambered-root perspex pots. Data (means ± SE, n = 4) followed by different letters indicate significant differences (P < 0.05) between mycorrhizal treatments for the same chamber (a, b, c) or between root chamber and root-free hyphal chamber for the same mycorrhizal treatment (x, y).
correlated with root total length, hyphal length, EE-GRSP, DE-GRSP, and T-GRSP (Table 4). The hyphal length in both the root/hyphae and root-free hyphae chamber positively correlated with these three GRSP fractions. The WSA$_{0.25-0.50}$ mm, WSA$_{0.50-1.00}$ mm, and WSA$_{2.00-4.00}$ mm fraction in both the root/hyphae and root-free hyphae chamber generally positively correlated with root colonization, hyphal length, EE-GRSP, DE-GRSP and T-GRSP, except EE-GRSP for WSA$_{0.50-1.00}$ mm and DE-GRSP for WSA$_{0.25-0.50}$ mm fraction (Table 4). Interestingly, the WSA$_{0.50-1.00}$ mm fraction did not correlate with any tested variable, and almost all WSA$_{0.50-1.00}$ mm did not correlate with root total length, except the WSA$_{0.25-0.50}$ mm fraction. In addition, MWD significantly positively correlated with both the root mycorrhizal colonization and root total length (Fig. 1), and also with hyphal length (Fig. 2a), and EE-GRSP, DE-GRSP and T-GRSP (Fig. 2b).

Path analyses. Six independent variables (root AM colonization, root total length, hyphal length, and EE-GRSP, DE-GRSP, and T-GRSP) were considered in the path analysis (Table 5). In the root/hyphae chamber, either root AM colonization, EE-GRSP or DE-GRSP showed a significantly direct and total (direct plus indirect) effect on MWD, and the direct contribution of these three variables ranked as DE-GRSP = EE-GRSP > root AM colonization (Table 5). On the other hand, although root total length, soil hyphal length, or T-GRSP did not show a direct effect on MWD in the root/hyphae chamber, these three independent variables still showed a strongly total effect on MWD.

In the root-free hyphae chamber, the following four independent variables, hyphal length, EE-GRSP, DE-GRSP and T-GRSP showed no direct effects on MWD, whilst the hyphal length, EE-GRSP and T-GRSP exhibited a significantly total effect on MWD (Table 5).

Discussion

Our study showed a significantly positive Pearson correlation between root AM colonization and WSA$_{0.25-0.50}$ mm, WSA$_{0.50-1.00}$ mm, WSA$_{0.25-0.50}$ mm or MWD in the root/hyphae chamber (Table 4; Fig. 1a). These results agreed with a previous study in amaranth, Bermuda grass, maize, and sunflower plants. Moreover, the contribution of root AM colonization to MWD was direct (Table 5), implying that root colonization, as a direct component, could confer upon a primary role in aggregate stability. However, in both the salt-stressed _Lactuca sativa_ and the drought-stressed _Phaseolus vulgaris_ plants, AMF colonization did not correlate with aggregate stability. It seemed that abiotic stresses might interfere with such a direct contribution of root colonization.

Mycorrhizal hyphae have been confirmed primarily to stabilize macroaggregates in various soils by enmeshing soil particles and binding microaggregates into macroaggregates. Our study also showed that soil hyphal length was significantly positively correlated with WSA$_{0.25-0.50}$ mm, WSA$_{0.50-1.00}$ mm, and WSA$_{2.00-4.00}$ mm (Table 4), as well as MWD (Fig. 1b, 2a), in both the root/hyphae and root-free hyphae chamber. These results indicated that mycorrhizal hyphae involved in the distribution of WSA size in 2.00–4.00, 0.50–1.00, and 0.25–0.50 mm and aggregate stability. This is in accordance with a strong relationship between soil hyphal length and MWD in _F. mossea_ and _G. intraradices_-infected _Medicago sativa_. Moreover, the path analysis indicated that the Pearson correlation between soil hyphal length and MWD was not from a direct effect but from a total effect (Table 5). Here we do not argue that soil mycorrhizal hyphae could physically entangle primary soil particles, enmesh and bind macroaggregates and small macroaggregates into larger aggregates. However, a 0.20–0.88 m g$^{-1}$ hyphal length (Table 2) was obviously lower than 4.0–6.2 m g$^{-1}$ from soil growing _Lolium rigidum_ with _Scutellospora calospora_. A shorter length of hyphae in the present study might confer a weaker enmeshment between soil WSA’s, and the slaking forces during the wet-sieving process might have disrupted the enmeshing role of mycorrhizal hyphae in aggregates. Such shorter hyphal length was only easier to stabilize sandy soil with a smaller specific surface area. On the other hand, the hyphae also released glomalin into soils as GRSP, which could give a direct effect on MWD (Table 5). Even so, mycorrhizal hyphae in lower length still showed a significantly Pearson correlation with MWD (Fig. 1b, 2a) through a total effect. Moreover, Tisdall et al. found that besides hyphal length, hyphal surface area might also determine the role of hyphae-mediated aggregate stability. Graf and Frei further proposed that mycorrhizal hyphae might act as “flexible string bags” due to their tensile strength and release of GRSP, thereby exhibiting a certain plasticity to stabilize aggregates. Mycorrhizal hyphae might thus play a key role in aggregate stability, which would depend on hyphal length, plant and/or mycorrhizal species.

Besides mycorrhizal hyphae, soil aggregate stability also involves physical entanglement of fine roots of the host plants. In the present study, AMF inoculation significantly increased root total length, irrespective of AMF source (Table 1), which might modulate the distribution of WSA$_{0.25-0.50}$ mm, thereby positively stabilizing WSA (e.g., MWD) (Table 4; Fig. 1a). However, the direct effect of root total length on aggregate stability was not significant on the basis of the path analysis in the root/hyphae chamber (Table 5), which is in agreement with the results of Willer and Jastrow. The Pearson correlation between root total length and MWD (Fig. 1a) was due to an indirect effect of EE-GRSP and/or T-GRSP, but not a direct effect, according to the path analysis in our study (Table 5).

In addition, the root total length exhibited lower direct and total effects on MWD than did the hyphae in the root/hyphae chamber (Table 5), which is in agreement with previous studies. Possibly, in a relatively smaller root/hyphae chamber, the development of hyphal network throughout the whole soil was obviously faster than...
Greater root systems could provide more chances to be colonized by AMF, thereby increasing the production of hyphae, finally more GRSP production. On the other hand, a better root system could also release more root exudates into the rhizosphere, which could directly affect aggregate stability. However, no information is available if a direct effect of roots on aggregate stability in a larger growth container could be stronger than that of mycorrhizal hyphae.

Our results showed that except a similar concentration of DE-GRSP between non-AMF and *P. occultum* treatment, *F. mossea* or *P. occultum* colonization significantly increased the concentrations of EE-GRSP, DE-GRSP, and T-GRSP, irrespective of the root/hyphae or root-free hyphae chamber, which is in coincidence with previous studies. Moreover, the inoculation with AMF also increased the ratio of EE-GRSP versus DE-GRSP in both the root/hyphae and root-free hyphae chamber (Table 3), but the significant differences occurred only between non-AMF and *F. mossea*. Since EE-GRSP is recently produced, and DE-GRSP is relatively older and also from EE-GRSP, a higher ratio of EE-GRSP versus DE-GRSP in the mycorrhizosphere might suggest that AMF colonization induced more new GRSP production, and EE-GRSP had also partly given rise to DE-GRSP. In addition, root colonization and soil hyphal length highly positively correlated with these three GRSP fractions (Table 4), suggesting that GRSP production depended on mycorrhizal hyphae production, since GRSP originated from AM hyphae and spore walls.

Our results also indicated that EE-GRSP significantly positively correlated with WSA2.00–4.00 mm, WSA0.25–0.50 mm, and MWD, whilst DE-GRSP significantly positively correlated with WSA0.50–1.00 mm and T-GRSP with WSA2.00–4.00 mm, WSA0.50–1.00 mm, and WSA0.25–0.50 mm regardless of the presence of roots or not (Table 4). The positive effect...
of GRSP thereby, stabilized the aggregates, resulting in a positive Pearson correlation between EE-GRSP or T-GRSP and MWD in both the root/hyphae and root-free hyphae chamber, though there was a positive Pearson correlation between the DE-GRSP and MWD in the root/hyphae/chamber (Fig. 1c, 2b). Meanwhile, EE-GRSP and DE-GRSP showed a significantly direct effect on MWD under the presence of both roots and hyphae, whilst the direct effect of DE-GRSP was slightly greater than that of the EE-GRSP (Table 5). Peng et al. also found that GRSP presented a direct effect on MWD in a neutral purple soil. These results suggested that although the two GRSP fractions could give direct effect on MWD, new glomalin, e.g. the DE-GRSP, could characterize relatively more labile, and the older glomalin, e.g., the DE-GRSP, could be more stable, thereby contributing more to stabilize aggregates than the labile EE-GRSP. As reported by Daynes et al., formation and stabilization of the macro-aggregates (>0.25 mm) mainly depended on diverse factors including fine roots and AMF. The results in the present study confirmed that macroaggregate stability in root/hyphae chamber of trifoliate orange was directly due to mycorrhizal colonization, EE-GRSP and DE-GRSP, whilst GRSP played a primary role in aggregate stability. Using the fluoresecently labeled lectin, Caesar-Tonthat revealed that fungal-derived materials such as fucosyl residues played a vital role in soil aggregation. It is possible that GRSP as an important AM-released material has the key function on aggregation stabilization under the presence of root and hyphae.

However, under the root-free hyphae condition, the two GRSP fractions of EE-GRSP and DE-GRSP did not show a direct effect on MWD, but the EE-GRSP showed a significantly total effect (Table 5). These results thus suggested that under a root-free hyphae condition, an active EE-GRSP, T-GRSP, and hyphal length might undertake a total effect, but not a direct effect, on aggregation. The distinct function of EE-GRSP and DE-GRSP on aggregate stability between the root/hyphae and root-free hyphae chamber might relate with the presence or absence of roots. In the presence of roots, AM would have already formed and more AMF spores were produced, leading to a steady GRSP production. In contrast, in the absence of roots, the GRSP production is exclusively dependent on extraradical hyphae. In the present study, the root-free hyphae chamber possessed lower hyphal length, indicating a less GRSP production. It is reasonable that the functioning of GRSP on aggregate stability might thus be greater in the root/hyphae chamber than in the root-free chamber. As a result, the respective functioning of EE-GRSP or DE-GRSP might be ascertained if the exact component of these two GRSP fractions could be distinguished.

In short, under the root plus mycorrhizal hyphae condition, aggregate stability mainly depended on the direct effect of root colonization, EE-GRSP and DE-GRSP, combining with the total effect of T-GRSP, hyphal length and root total length. On the other hand, under the root-free hyphae conditions, aggregate stability mainly relied on the total effect of soil hyphal length, EE-GRSP and T-GRSP.

### Methods

#### Experimental design

The experiment had three inoculations or treatments: (1) *Funnelliformis mosseae*, (2) *Paraglomus occultum*, and (3) non-AMF (control). Each treatment had four replicates, for a total of 12 experimental per pots in a completely randomized arrangement.

#### Mycorrhizal inocula

The fungal isolates, *Funnelliformis mosseae* (Nicol. & Gerl.) Schüttler & Walker and *Paraglomus occultum* (Walker) Morton & Redecker, were from the rhizosphere of *Incarvillea youngusbandii* in Dangxiong (90°45'E and 29°31'N, 4.300 m above the sea level), Tibet, and of *Pramus persica* in Pinggu (116°55'E and 40°02'N, ~700 m above the sea level), Beijing, China, respectively. Through identified fungal spores, the mycorrhizal inocula, propagated with white clover (*Trifolium repens*) for 16 weeks, were a mixture of sands, fine root segments and spores (23 or 28 spores g⁻¹ for *F. mosseae* or *P. occultum*).

#### Experimental pots and plant growth conditions

Pots were incubated for 16 weeks, were a mixture of sands, fine root segments and spores (23 or 28 spores g⁻¹ for *F. mosseae* or *P. occultum*).

### Table 5: Path analyses between mean weight diameter (MWD) and arbuscular mycorrhizal (AM) colonization, root total length, hyphal length, or soil GRSP fractions in 37 μm nylon-mesh separated root/hyphae and root-free hyphae chambers

| Independent variable | Root/hyphae chamber | Hyphae only chamber | Root/hyphae chamber | Root/hyphae chamber | Hyphae only chamber |
|----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| AM colonization      | 0.63*               | NA                  | 0.35                | NA                  | 0.98**              |
| Root total length    | 0.08                | NA                  | 0.56                | NA                  | 0.64*               |
| Hyphal length        | 0.16                | 0.30                | 0.73                | 0.40                | 0.89**              |
| EE-GRSP              | 13.41*              | 7.70                | -12.55              | -6.76               | 0.86**              | 0.94**              |
| DE-GRSP              | 13.60*              | 11.29               | -12.93              | -10.89              | 0.67                | 0.40                |
| T-GRSP               | -22.21              | -15.69              | 23.14               | 16.39               | 0.93**              | 0.70*               |

Note: Trifoliate orange seedlings were 5-month-old and colonized with *Funnelliformis mosseae* and *Paraglomus occultum* and grown in 37 μm nylon-mesh separated two-chambered perspex pots. * P < 0.05; ** P < 0.01. Abbreviations: DE-GRSP, difficultly-extractable glomalin-related soil protein (DE-GRSP); EE-GRSP, easily-extractable glomalin-related soil protein; NA, not available; T-GRSP, total (EE-GRSP + DE-GRSP) glomalin-related soil protein.

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hyphal length was based on Bethlenfalvay and Ames'41. Soil organic carbon (SOC) was determined by the dichromate oxidation spectrophotometric method42.

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Acknowledgments

This study was supported by the National Natural Science Foundation of China (31372017), the Key Project of Chinese Ministry of Education (2111107), the Key Project of Natural Science Foundation of Hubei Province (2012FFA001), the Science-Technology Research Project for the Excellent Middle-aged and Young Talents of Hubei Provincial Department of Education (Q20111301), and the Excellent Young Teacher Research Support Program of Yangtze University (cyq201324).

Author contributions

Q.S.W. planned the study, conducted statistical analyses and wrote the manuscript. M.Q.C. conducted greenhouse and laboratory work. Y.N.Z. planned the study and made all tables and figures. X.H.H. planned the study and wrote the manuscript. All authors approved the manuscript submission.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Wu, Q.-S., Cao, M.-Q., Zou, Y.-N. & He, X.-H. Direct and indirect effects of glomalin, mycorrhizal hyphae, and roots on aggregate stability in rhizosphere of trifoliate orange. Sci. Rep. 4, 5823; DOI:10.1038/srep05823 (2014).

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