Exogenous easily extractable glomalin-related soil protein promotes soil aggregation, relevant soil enzyme activities and plant growth in trifoliate orange

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ABSTRACT

Studies on glomalin-related soil protein (GRSP) have focused on soil aggregation and fungal physiology, whereas it is not known how exogenous GRSP could positively impact on these processes, soil enzyme activity and plant growth. Easily extractable GRSP [EE-GRSP, 0.022 mg protein/mL citrate buffer (20 mmol, pH 7.0)] from a 26-year-old citrus orchard was exogenously applied into 5-month-old potted trifoliate orange (*Poncirus trifoliata*) for 3 months to evaluate effects on soil water-stable aggregate distribution, relevant soil enzyme activities and plant growth. Depending on the applied concentrations as 1/2, 1/4 or full strength, exogenous EE-GRSP generally significantly increased the distribution of soil water-stable aggregates and mean weight diameter (MWD, an aggregate stability indicator). Values of MWD and plant biomass production curvilinearly positively correlated with exogenous EE-GRSP applications. Exogenous EE-GRSP generally significantly increased the activity of rhizospheric polyphenol oxidase, peroxidase, acid and alkaline phosphatase. Both the 1/2-strength and 1/4-strength, but not the full-strength exogenous EE-GRSP, significantly stimulated plant growth performance. Our results firstly demonstrated the positive contribution of exogenous EE-GRSP to soil aggregation, relevant rhizospheric enzyme activities and/or plant growth, which has important implications for exploring GRSP in enhancing soil structure and/or plant performance.

Keywords: N-linked glycoprotein; binding agent; humic substances; arbuscular mycorrhizal fungi

Soil aggregation, a crucial soil ecosystem process, contributes to the improvement of soil porosity and water infiltration, development of root systems, increase of soil organic carbon, and resistance to erosion. Soil aggregation can be mediated by a variety of aggregate stabilizing agents such as soil organic matter, plant roots, soil microbial communities, and products of their activities (Rillig 2004, Rillig and Mummey 2006, Wu et al. 2012, Peng et al. 2013).

In soil, arbuscular mycorrhizal fungi (AMF) play a major role in macroaggregate (> 0.25 mm) formation through the production of extracellular compounds (Rillig and Mummey 2006) and physical enmeshment of soil particles by extensive hyphae networks (Peng et al. 2013). Among those released compounds from AMF, glomalin, an N-linked glycoprotein, has received ever-increasing attention in soil environment and fungal physiology (Purin and Rillig 2007). In soils, glomalin is defined as glomalin-related soil protein (GRSP) (Rillig 2004). GRSP is regarded as an important binding agent in soil aggregation, because GRSP binds soil particles and alters soil wetting behaviour, thus, representing a 'gluing' functioning on aggregate formation and stabilization (Wright and Upadhyaya 1998).

The understanding of GRSP functioning on soil aggregation is generally derived from a highly positive correlation between GRSP concentration and the percentage of water-stable aggregates (WSAs) or soil aggregate stability, in terms of regression analysis and/or path analysis (Wright and Upadhyaya 1998, Fokom et al. 2012, Wu et al. 2012). Moreover, GRSP showed a stronger effect on aggregate stability than root mycorrhizal colonization in root/hyphae chamber planted with *Funneliformis mosseae*-colonized trifoliate orange (Wu et al. 2014). Logarithmic or sigmoidal correlation analyses indicated that in a given soil GRSP did not improve soil aggregate stability if the GRSP concentration was over a 'saturation' level (Rillig 2004). Studies also indicated that exogenous humic substances extracted from poplar sawdust significantly improved the growth of maize (Eyheraguibel et al. 2008). Schindler et al. (2007) proposed that GRSPs exhibited a resemblance to humic substances. Therefore, there is the hypothesis that exogenous GRSPs, as analogues of humic substances, might also have potential effects on soil aggregation and plant growth. However, at present no direct evidence has addressed if exogenous GRSP could enhance soil aggregation and/or plant growth.

Soil enzymes play vital roles in element biogeochemical cycling (Bowles et al. 2014). The activity of peroxidase (POD), phosphatase, and polyphenol oxidase (PPO) directly relates to carbon (C), nitrogen (N), and phosphorus (P) availability to plant growth (Finkenbein et al. 2013). Since GRSP contained ~37% C, 3–5% N, and 0.03–0.1% P, it seems that any addition of exogenous EE-GRSP might affect soil enzyme activities and/or plant growth. However, the production of GRSP in citrus rhizosphere mediated by AMF did not depend on external P concentrations (Wu et al. 2015). Since GRSPs contain a certain amount of organic carbon and nitrogen, there is the hypothesis that exogenous EE-GRSP also influenced soil enzyme activities.

To address the two above-mentioned hypotheses, easily extractable glomalin-related soil protein (EE-GRSP) was firstly extracted from a citrus rhizosphere soil, then externally applied the extracted EE-GRSP under three strengths to potted citrus seedlings, and finally evaluated if the applied exogenous EE-GRSP could affect soil aggregation, relevant soil enzyme activities and plant growth. Another aim of the present study was to screen an efficient strength of exogenous EE-GRSP for a possible application to citrus plants. The expected results could further reveal potentially functional significances of exogenous GRSP in soil structure and plant performance.

MATERIAL AND METHODS

Preparation of exogenous EE-GRSP solution. In March 2013, soil (Xanthi-udic Ferralsol, FAO system) was collected from 0–20 cm depth in the rhizosphere of a 26-year-old citrus orchard on the Yangtze University campus (30°36'N, 112°14'E, 36 m a.s.l.), where *Citrus unshiu* cv. Guoqing 1 (grafted on *Poncirus trifoliata*) has been growing since 1987. The soil had a pH of 6.1, organic carbon 9.7 g/kg, available nitrogen 11.8 mg/kg, Oslen-P 15.3 mg/kg, and available potassium 21.5 mg/kg. The collected soils were well mixed, air-dried, and sieved (4 mm) after the removal of roots and debris. Extraction of EE-GRSP [soil:20 mmol citrate buffer (pH 7.0) = 1:8, w/v] was at 121°C under 0.11 MPa for 30 min in an autoclave, and then centrifuged at 10 000 × *g* for 5 min (Koide and Peoples 2013). The EE-GRSP concentration was 0.022 mg protein/mL citrate buffer (corresponding to 0.51 mg GRSP/g soil), which was determined using the Bradford (1976) assay with bovine serum albumin as the standard.

Experimental design. In a completely randomized design, the experiment consisted of four treatments or EE-GRSP strengths: (1) zero-strength EE-GRSP with citrate buffer (20 mmol, pH 7.0) as the control; (2) quarter-strength (1/4 EE-GRSP); (3) half-strength (1/2 EE-GRSP), and (4) full-strength (full EE-GRSP), respectively. Meanwhile, the 1/2 and 1/4 strength EE-GRSP was diluted by the same citrate buffer. Each treatment had four replicates or pots, for a total of 16 pots.

Plant culture. Two non-mycorrhizal 20-day-old trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seedlings with similar size and three leaves were transplanted into a 2 L plastic pot containing 1.5 kg soil (Xanthi-udic Ferralsol) from the same citrus orchard. After transplanting, each pot was watered once every two days with 50 mL distilled water for two months (30 March to 29 May 2013) and then with 50 mL designed EE-GRSP solution for another period of three months (30 May to 30 August 2013). Seedlings were grown in an environment-controlled glasshouse in the Yangtze University campus, where the photosynthetic photon flux density was 728–965 µmol/m^2/s, day/night temperature

28/21°C, and relative humidity 85%. The position of pots in the glasshouse was weekly relocated.

Variable measurements. Plant height and stem diameter were recorded before harvest. Shoots and roots were dried at 75°C for 48 h to consistent weights. Soil adhered in the roots was collected, air-dried and sieved through 4-mm mesh screen.

Distributions of soil water-stable aggregate (WSA) at 2.00–4.00, 1.00–2.00, 0.50–1.00, and 0.25–0.50 mm size were determined using the wet-sieving method (Kemper and Rosenau 1986) with a soil aggregate analyzer (DM200–IV, Shanghai, China). Soil WSA stability as mean weight diameter (MWD) was calculated by the following formula (Kemper and Rosenau 1986):

$$MWD = \sum_{i=1}^{n} X_i W_i$$

Where: X_i – mean diameter of the i sieve opening (mm); W_i – proportion of the i size fraction in the total sample mass; n – number of size fractions.

Determination of soil PPO and POD activity (mg purpurogallin/g soil/h) was accorded to Yan (1988) using pyrogallol as the substrate and purpurogallin as the standard. Determination of soil acid phosphatase (ACP, extracted by 0.1 mmol/L sodium acetate buffer at pH 5.0) and alkaline phosphatase (ALP, extracted by 0.2 mmol/L borate buffer at pH 10.0) activity (mg redistilled phenol/g soil/h) was accorded to Zhao and Jiang (1986) using disodium phenyl phosphate as the substrate and redistilled phenol as the standard.

Statistical analysis. Data (means ± SE, $n = 4$) were subjected to one-way variance (ANOVA) with the SAS v8.1 (SAS Institute Inc., Cary, USA), and the significant differences in means between treatments were compared with the Duncan's multiple range test at $P < 0.05$.

RESULTS AND DISCUSSION

Distribution and stability of WSAs. Compared with the no-EE-GRSP citrate buffer control, the application of 1/2 strength extracted EE-GRSP from the citrus rhizosphere soil significantly increased $WSA_{2.00-4.00\,mm}$, $WSA_{1.00-2.00\,mm}$ and $WSA_{0.50-1.00\,mm}$, while the 1/4 strength EE-GRSP significantly increased $WSA_{1.00-2.00\,mm}$ and $WSA_{0.25-0.50\,mm}$ only (Table 1), and the full strength EE-GRSP significantly increased $WSA_{2.00-4.00\,mm}$ and $WSA_{1.00-2.00\,mm}$. Moreover, all EE-GRSP treatments significantly increased MWD (an aggregate stability indicator), and the MWD value was significantly higher under 1/2 EE-GRSP treatment than under other EE-GRSP treatments (Table 1). These results provided the direct evidence that exogenously applied EE-GRSP could generally enhance soil WSA aggregation, though previous studies found that GRSP correlated with soil aggregation in terms of regression analysis (Wright and Upadhyaya 1998, Fokom et al. 2012). Therefore, exogenous EE-GRSP could be regarded as a soil 'glue' agent on WSA formation and stabilization. The EE-GRSP-mediated % of WSA was generally higher in the 0.25–0.50 mm > 0.50–1.00 mm > 1.00–2.00 mm > 2.00–4.00 mm fraction, except between 0.25–0.50 mm and 0.50–1.00 mm under 1/2 EE-GRSP (Table 1). As a result, the present study confirmed our hypothesis that exogenous GRSPs as analogues of humic substances could have positive effects on soil aggregation.

In addition, MWD was curvilinearly positively increased with GRSP with two phases (Figure 1). The first phase was a gradual MWD increase within 0 and 0.011 mg protein/mL citrate buffer (till 1/2 strength EE-GRSP), while the second phase was a gradual MWD decrease within 0.011 and

Table 1. Effect of exogenous easily extractable glomalin-related soil protein (EE-GRSP) on distribution of water-stable aggregate (WSA) at the size of 2.00–4.00, 1.00–2.00, 0.50–1.00, and 0.25–0.50 mm and mean weight diameter (MWD) in the rhizosphere of 5-month-old trifoliate orange (*Poncirus trifoliata*) seedlings

Treatment	Distribution of WSA (%)				MWD (mm)
	2.00–4.00	1.00–2.00	0.50–1.00	0.25–0.50	
Citrate buffer	3.37 ± 0.28[c,δ]	9.64 ± 0.68[c,γ]	21.08 ± 0.78[b,β]	24.15 ± 1.07[b,α]	0.49 ± 0.02[c]
1/4 EE-GRSP	3.42 ± 0.20[c,δ]	12.58 ± 0.65[b,γ]	23.95 ± 4.74[b,β]	30.45 ± 2.19[a,α]	0.59 ± 0.03[b]
1/2 EE-GRSP	4.20 ± 0.14[b,δ]	14.69 ± 0.90[a,γ]	32.05 ± 5.91[a,α]	24.92 ± 0.60[b,β]	0.68 ± 0.04[a]
Full EE-GRSP	4.63 ± 0.11[a,γ]	12.52 ± 0.58[b,β]	25.74 ± 1.28[b,α]	26.14 ± 2.18[b,α]	0.62 ± 0.01[b]

Data (means ± SE, $n = 4$) followed by the different letters among treatments for the same particle size within a column (a, b) or among particle sizes for the same treatment within a row (α, β, γ, δ) indicate significant differences at $P < 0.05$

0.022 mg protein/mL citrate buffer (till full EE-GRSP strength). Previous studies also showed that aggregate stability was linearly or curvilinearly positively related to GRSP concentrations in soil (Wright and Anderson 2000, Spohn and Giani 2010). Piccolo et al. (1997) found that humic substances at medium (0.10 g/kg soil) but not at high (10 g/kg soil) concentrations had the highest aggregate-stabilizing effect. GRSP as a humic-like substance (Schindler et al. 2007) might also exhibit such a similar effect. In addition, the exogenously applied full strength EE-GRSP could saturate soil pores. Zou et al. (2014) proposed that GRSP could form a hydrophobic layer on the surface of WSA and fungal hyphae to slow down water penetration into WSAs, which might interfere with the function of exogenous full-strength EE-GRSP on soil aggregation (Rillig 2004). It would be therefore interesting and useful to determine the functioning of exogenous EE-GRSP on soil water relation. Moreover, GRSP contained larger amounts of hot-water extracted carbohydrates (Wright and Upadhyaya 1998), which positively correlated with aggregate stability in an Oxisol under legume-based and pure grass pastures in the Eastern Colombian savannas (Gijsman and Thomas 1995). As a result, soil aggregation could not be enhanced when the exogenously applied GRSP concentration was beyond a threshold. As stated above, exogenous EE-GRSP-mediated soil aggregation might depend on EE-GRSP strength.

Soil enzyme activities. The present results displayed that all the three strengths of EE-GRSP application significantly increased the ALP and POD activity, both the 1/4 and 1/2 strength of EE-GRSP significantly increased the PPO activity, and the 1/2 and full strength of EE-GRSP significantly
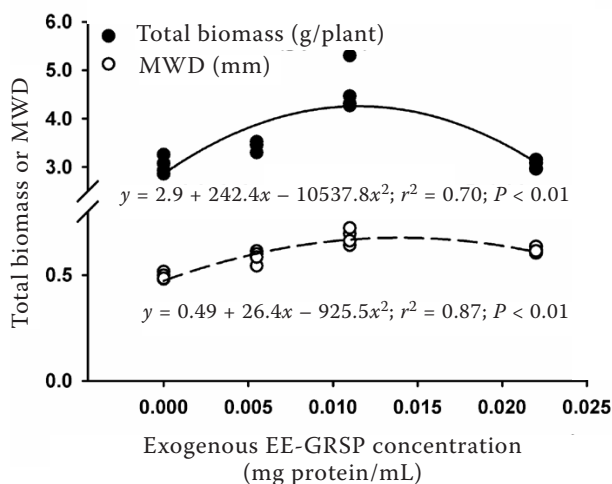

Figure 1. Quadratic regression between exogenous exogenous easily extractable glomalin-related soil protein (EE-GRSP) concentrations and total biomass (solid dots) or mean weight diameter (MWD) (open dots) in the rhizosphere of 5-month-old trifoliate orange (*Poncirus trifoliata*) seedlings ($n = 16$)

$y = 2.9 + 242.4x - 10537.8x^2$; $r^2 = 0.70$; $P < 0.01$

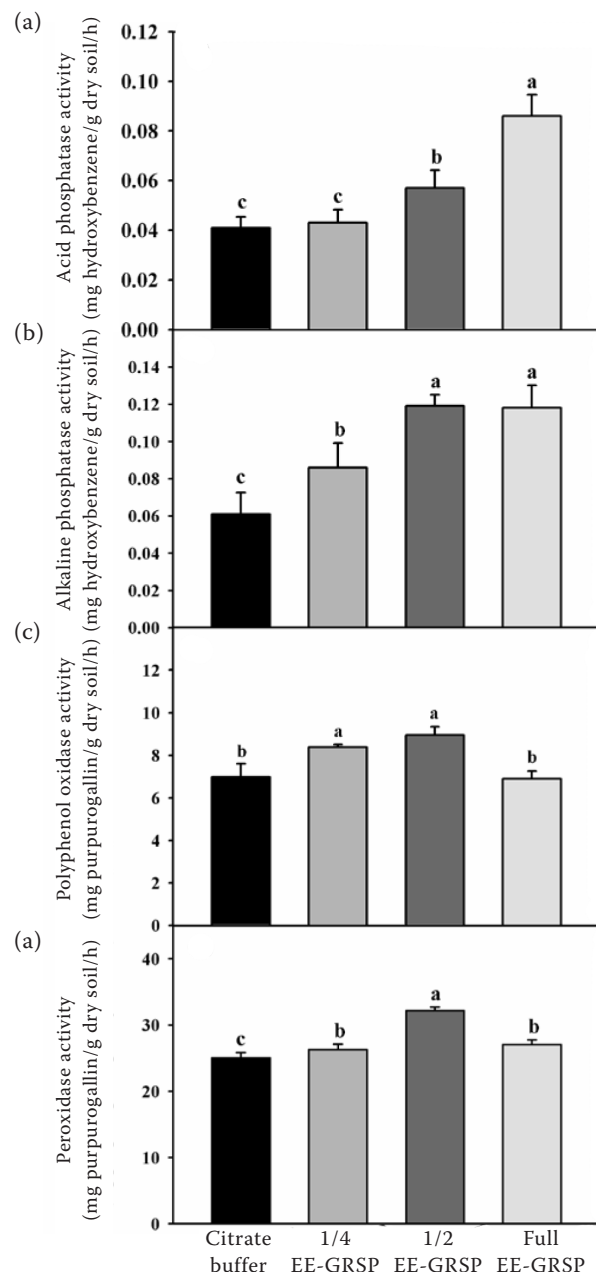$y = 0.49 + 26.4x - 925.5x^2$; $r^2 = 0.87$; $P < 0.01$

Figure 2. Effects of exogenous easily extractable glomalin-related soil protein (EE-GRSP) on activity of acid phosphatase (a), alkaline phosphatase (b), polyphenol oxidase (c) and peroxidase (d) in rhizosphere of 5-month-old trifoliate orange (*Poncirus trifoliata*) seedlings. Data (means ± SE, $n = 4$) followed by different letters above bars indicate significant differences between treatments at $P < 0.05$

increased the ACP activity (Figure 2). Among the three EE-GRSP applications, the greatest activity of PPO and POD was under the 1/2 strength and of ACP and ALP under the full strength of EE-GRSP application. This result is in agreement with our above hypothesis, viz., exogenous EE-GRSP positively affected soil enzyme activities. It is well known that GRSP contains ~37% C, 3–5% N, and 0.03–0.1% P (Rillig et al. 2001, Lovelock et al. 2004, Schindler et al. 2007), which are the important components and/or substrates of these soil enzymes. As a result, in addition to a small amount of N and P availability, the application of exogenous EE-GRSP would not only promote soil enzyme activities but also stimulate element biogeochemical cycling, which could benefit plant nutrition. Hence, further studies are needed to address changes of soil nutrient pools deriving from an exogenous EE-GRSP application.

Plant growth. Our study indicated that exogenous EE-GRSPs had the positive effects on plant growth of trifoliate orange seedlings. The significantly positive effects of external EE-GRSP on plant height and stem diameter among treatments generally ranked in the order of 1/2 strength ≥ 1/4 strength ≥ full strength EE-GRSP ≥ the citrate buffer treatment, while significantly higher biomass (shoot, root and total) production ranked as 1/2 strength > 1/4 strength > full strength EE-GRSP ≥ the citrate buffer (Table 2). Interestingly, these growth traits were not affected by the full strength EE-GRSP (Table 2). Quadratic regression analyses showed that the total biomass production increased with the increase of exogenous EE-GRSP ranging from 0–0.011 mg protein/mL citrate buffer while decreased in the range of 0.011–0.022 mg

protein/mL citrate buffer (Figure 1). These results might have confirmed our hyphothesis that EE-GRSP might serve as a humic-like substance to exert positive effect on plant growth, though depending on the strength of EE-GRSP (Table 2, Figure 1). Quantitative solid-state ^{13}C DPMAS NMR (direct-polarization magic angle spinning nuclear magnetic resonance) demonstrated that the NMR spectra of glomalin and humic acid closely resembled (Schindler et al. 2007), while synchrotron-based X-ray absorption near-edge structure (XANES) spectroscopy and pyrolysis field-ionization mass spectrometry (Py-FIMS) did characterize that GRSP contained non-mycorrhizal-related heat-stable protein and humic materials (Gillespie et al. 2011).

In conclusion, to our knowledge, this is the first report to address the potential role of exogenous EE-GRSP in soil aggregation, relevant soil enzyme activities and plant growth. Our study demonstrated that the application of exogenous EE-GRSP to some extent, increased distribution of WSAs at 0.25–4.00 mm size, thus enhancing WSA stability. On the other hand, exogenous EE-GRSP generally significantly increased rhizospheric ACP, ALP, POD, and PPO activity. Both the 1/2-strength and 1/4-strength, but not the full-strength exogenous EE-GRSP, significantly stimulated plant growth. Importantly, the 1/2 strength of rhizosphere extracted EE-GRSP presented the best effects on these roles, though varied with exogenous EE-GRSP concentrations. Our results could provide new insights into exploring GRSP as an analogue to a soil conditioner and/or plant growth substance in enhancing plant performance considering that ~80% higher plants are associated with AMF.

Table 2. Effects of exogenous easily extractable glomalin-related soil protein (EE-GRSP) on plant height, stem diameter, and shoot, root and total (shoot + root) dry weight of 5-month-old trifoliate orange (*Poncirus trifoliata*) seedlings

Treatment	Plant height (cm)	Stem diameter (mm)	Dry weight (g/plant)		
			shoot	root	total
Citrate buffer	32.2 ± 2.4[c]	4.00 ± 0.12[b]	2.28 ± 0.10[b]	0.75 ± 0.09[c]	3.03 ± 0.18[c]
1/4 EE-GRSP	35.7 ± 1.3[ab]	4.29 ± 0.14[a]	2.44 ± 0.09[b]	0.99 ± 0.02[b]	3.43 ± 0.10[b]
1/2 EE-GRSP	38.5 ± 2.2[a]	4.35 ± 0.24[a]	3.30 ± 0.19[a]	1.29 ± 0.05[a]	4.59 ± 0.23[a]
Full EE-GRSP	34.1 ± 1.7[bc]	4.13 ± 0.16[ab]	2.34 ± 0.08[b]	0.78 ± 0.02[c]	3.10 ± 0.10[c]

Data (means ± SE, *n* = 4) followed by the different letters with a column indicate significant differences between treatments at *P* < 0.05

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