



Exogenous application of EE-GRSP and changes in citrus rhizosphere properties

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Received: 8 January 2015; Accepted: 13 February 2015

ABSTRACT

Mycorrhization-induced secretion of glomalin-related soil protein (GRSP) is known to contribute in improving soil fertility and aggregate stability. But, there is hardly any evidence that exogenous GRSP could produce the similar type of response. The present study was, therefore, carried out to extract easily extractable GRSP (EE-GRSP) from citrus rhizosphere soils and evaluate the response at 1/4, 1/2 and full strength EE-GRSP solutions watered into the rhizosphere of 27-year-old Satsuma mandarin (*Citrus unshiu*) grafted on the rootstock of *Poncirus trifoliata*. After ~5 months, exogenous EE-GRSP treatments produced significantly positive responses on endogenous EE-GRSP, difficultly-extractable GRSP (DE-GRSP), total GRSP production and soil organic carbon (SOC) content. A significantly positive correlation of these endogenous GRSP fractions with SOC indicated an active participation of GRSP on soil C pool. Exogenous GRSP showed a key role in binding water-stable aggregate (WSA) at 2–4, 1–2, 0.5–1, and 0.25–0.5 mm size and enhancing WSA stability through changes in mean weight diameter (MWD). A stronger correlation of EE-GRSP and total GRSP with MWD was observed than DE-GRSP fraction. On the other hand, exogenous EE-GRSP showed a positive effect on soil phosphatase activity, and soil endogenous EE-GRSP was significantly and positively correlated only with neutral phosphatase. Our studies suggest that exogenous EE-GRSP could be used as an effective regulator to affect soil fertility and soil structure within citrus rhizosphere.

Key words: Aggregate stability, Carbon, Glomalin, Water-stable aggregate

Arbuscular mycorrhizal fungi (AMF), one of the soil inhabited symbiotic fungi, have shown significant response on plant growth, nutrient and water absorption, tolerance to abiotic and biotic stresses, and ecological systems. In addition, lots of studies kept a watchful eye on the role of AMF-regulated soil aggregation. An extraradical mycelium of arbuscular mycorrhizas (AMs) possess the ability to physically enmesh the soil particles for formation of different macroaggregate (>0.25 mm size) (Peng *et al.* 2013). On the other hand, spores and hyphae of AMF can produce a special N-linked glycoprotein compound, called glomalin, known as glomalin-related soil protein (GRSP) (Rillig 2004). This GRSP is a hydrophobin bound soil particles and can modulate soil wetting behavior, thereby, regulating water-stable aggregate (WSA) formation and its stabilization as an important binding agent (Fokom *et al.* 2012, Wright and Upadhyaya 1998, Wang *et al.* 2014, Wu *et al.* 2012, 2014). The direct contribution of GRSP on stability of WSA was much stronger than root mycorrhizal colonization or

mycorrhizal extraradical mycelium (Rillig *et al.* 2001, Wu *et al.* 2014). However, there is no direct evidence available about the role of exogenous GRSP on the magnitude of soil aggregation.

Besides soil aggregation, GRSP also accounts for enriching rhizosphere soil in terms of organic carbon in undisturbed soil, thereby, showing a potentially important soil C storage pool (Rillig *et al.* 2001, Walley *et al.* 2014). Besides C, GRSP is rich source of nutrients (2.5–3.1% N, 31.4% Fe and 2.8% Al) which could potentially contribute in improving soil fertility (Borie *et al.* 2010). The nutrients in GRSP could be soil enzymatic substrates for triggering the crop growth.

Previous studies reported that exogenous humic substances extracted from poplar sawdust improved growth and rhizospheric traits of maize plant (Eyheraguibel *et al.* 2008). It is not clear if exogenous GRSP similar to humic substances hold the same role. The aims of the present study were to: i. evaluate the effects of EE-GRSP on WSA aggregate stability, SOC, and soil phosphatase activity in citrus rhizosphere and ii. establish the functioning of exogenous EE-GRSP in plant growth regulation.

MATERIALS AND METHODS

The field trial was conducted in a 27-year-old citrus

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orchard on the Yangtze University campus (30°36' N, 112°14' E, 36 m above sea level). The citrus orchard was cultivated with *Citrus unshiu* cv. Guoqing 1 grafted on *Poncirus trifoliata* at a spacing of 3×4 m on 0.4 m high raised bed to allow adequate drainage. The soil of the orchard had pH 6.1, available nitrogen 11.8 mg/kg, Olsen-P 15.3 mg/kg and available potassium 21.5 mg/kg. The soil was taxonomically classified as the Xanthi-udic ferralsol soil (FAO system). The annual mean temperatures and annual rainfall ranged from 15.9 to 16.6°C and 1100 to 1300 mm, respectively.

In March 2014, the soil was collected, mixed well, air-dried, and sieved through 4 mm mesh. The subsamples of soil (1 g) were extracted with 8 mL 20 mM citrate buffer (pH 7.0), autoclaved for 30 min at 121°C (0.11 MPa), and centrifuged at 10 000×g for 5 min, as per the procedure of Koide and Peoples (2013). The supernatants were transferred to a clean bottle and stored at 4°C. The EE-GRSP level of the supernatants was 0.022 mg protein/ml citrate buffer on the basis of the Bradford assay (Bardford 1976).

On 9 April and 9 May 2014, 500 mL of exogenous EE-GRSP solutions with the designed levels was applied into the rhizosphere (0.5×0.5 m²) of the test citrus plants. As treatments, four EE-GRSP strengths were used comprising full-strength EE-GRSP (full EE-GRSP), half-strength EE-GRSP (1/2 EE-GRSP), quarter-strength EE-GRSP (1/4 EE-GRSP), and zero-strength EE-GRSP (0 EE-GRSP). The 1/2 and 1/4 EE-GRSP were prepared using full-strength EE-GRSP by diluting 20 mM citrate buffer (pH 7.0). Each treatment was replicated four times using the completely randomized arrangement.

On 29 August 2014, the soils were collected from 10–15 cm depth of the treated citrus rhizosphere, mixed well, air-dried, and sieved (4 mm). Distribution of WSA in 2–4, 1–2, 0.5–1 and 0.5–0.25 mm size was determined by wetting sieve method described by Wu *et al.* (2008). Mean weight diameter (MWD) of WSA as an indicator of aggregate stability was calculated on the basis of Kemper and Rosenau (1986). SOC was determined by the dichromate oxidation spectrophotometric method (Rowell 1994). While, soil phosphatase activity was measured by the protocol outlined by Zhao and Jiang (1986) using redistilled phenol as the standard. Various forms of phosphatase enzyme, viz. acid phosphatase (ACP), neutral phosphatase (NEP) and alkaline phosphatase (ALP) were extracted with 0.1 M sodium acetate buffer (pH 5.0), 20 mM citrate buffer (pH 7.0) and 0.2 mM

borate buffer (pH 10.0). Total phosphatase (TP) is the sum of ACP, NEP and ALP. Two GRSP fractions, EE-GRSP and difficultly-extractable GRSP (DE-GRSP) were determined by the protocol described by Wu *et al.* (2014). T-GRSP was calculated as the sum of EE-GRSP and DE-GRSP.

The generated data (means ± SE, *n* = 4) were statistically analyzed with one-way variance (ANOVA) on the basis of the SAS software (v 8.1). The significant differences between treatments were compared with the Duncan's multiple range test at *P* < 0.05. The Pearson's correlation coefficients between variables were performed by the Proc Corr's procedure of SAS.

RESULTS AND DISCUSSION

All the treatments involving various levels of EE-GRSP significantly increased all the three endogenous GRSP fractions, viz. EE-GRSP, DE-GRSP and T-GRSP in citrus rhizosphere by 15.7–62.9, 4.8–9.6 and 10.0–34.1%, respectively (Table 1). Moreover, the three endogenous GRSP fraction concentration increased with the increase concentration of exogenous EE-GRSP. In addition, 1/2 and full strength exogenous EE-GRSP stimulate the significant increase of EE-GRSP/DE-GRSP value. These observations supported the fact that an increasingly higher magnitude of exogenous EE-GRSP on these endogenous GRSP fraction levels. The result also revealed that EE-GRSP is new most juvenile form of glomalin and relatively labile, and EE-GRSP later turns over DE-GRSP (Koide and Peoples 2013). It is hence, anticipated that the application of exogenous EE-GRSP could stimulate the procession of EE-GRSP turnover.

Changes in SOC

Glomalin molecules generally contain 30–40% C on the basis of its protein and sugar subunits (Rillig *et al.* 2003). After the mycorrhizal hyphae senesce, glomalin is deposited within the soil, thereby, representing the part of the soil SOC pool (Treseder and Turner 2007). The present study showed that SOC concentration increased by 12.6 to 33.4% with 1/2 EE-GRSP and full EE-GRSP, respectively compared with 0 EE-GRSP treatments (Fig 1). These results indicated that EE-GRSP has the potential ability to regulate soil C pool. Correlation studies also revealed that SOC was strongly and positively correlated with endogenous EE-GRSP, DE-GRSP and T-GRSP concentrations (Fig 2). The percentage increase in T-GRSP versus SOC was 5.6–6.0%,

Table 1 Effect of exogenous EE-GRSP on soil endogenous GRSP fraction production of citrus grown in the field

Treatment	GRSP fraction (mg/g DW)			EE-GRSP/ DE-GRSP
	EE-GRSP	DE-GRSP	T-GRSP	
0 EE-GRSP	0.229±0.015d	0.270±0.005c	0.499±0.017d	0.85±0.05c
1/4 EE-GRSP	0.265±0.020c	0.283±0.006b	0.549±0.018c	0.94±0.08c
1/2 EE-GRSP	0.312±0.011b	0.299±0.007a	0.611±0.018b	1.04±0.02b
Full EE-GRSP	0.373±0.008a	0.296±0.009a	0.669±0.009a	1.26±0.06a

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

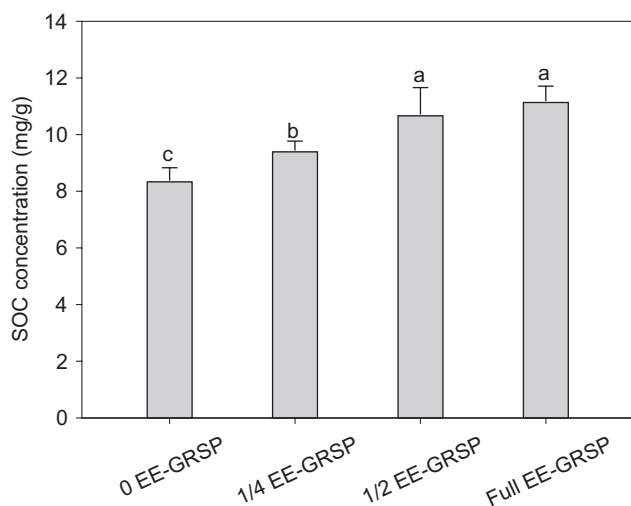


Fig 1 Effects of exogenous EE-GRSP on soil organic carbon (SOC) concentration of citrus grown in the field

which is in agreement with the results (as much as 5%) of Rillig (2001) in tropical forest soils. In three land use systems of humid forest zone of southern Cameroon, soil endogenously secreted easily-extracted GRSP (EE-GRSP) concentration was positively ($r^2=0.76$, $P<0.01$) correlated with soil total C (Fokom *et al.* 2013), thus, favoring the view that GRSP contributes towards improving soil stock of C. Exogenous EE-GRSP can, hence, be considered as a soil fertility modulator in citrus rhizosphere.

Aggregate distribution and stability

Compared with no-EE-GRSP treatment, all the EE-GRSP applications significantly increased the percentages of WSA in 2–4, 1–2, 0.5–1 and 0.25–0.5 mm size. However, these differences were non-significant with respect to

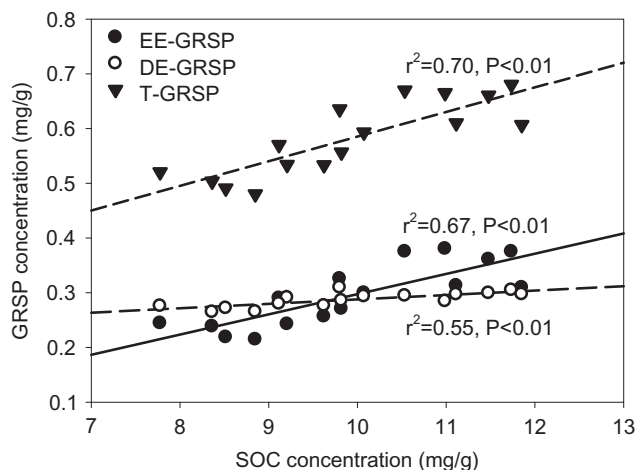


Fig 2 Linear regression between soil organic carbon (SOC) concentration and glomalin-related soil protein (GRSP) concentration in citrus grown in field ($n=16$)

WSA_{2-4mm} between 1/4 EE-GRSP and 0 EE-GRSP treatments and $WSA_{0.25-0.5mm}$ between 1/2 EE-GRSP and 0 EE-GRSP treatments (Table 2). These results clearly indicated that exogenous GRSP played a key role in binding different WSAs. The exogenous GRSP-mediated in WSA formation, consistent with the endogenous GRSP-mediated WSA formation as reported by many previous studies (Fokom *et al.* 2012).

The present work showed that MWD, an indicator of WSA stability, was 23.2–51.8% significantly higher under exogenous EE-GRSP conditions than no-EE-GRSP conditions (Table 2). Wang *et al.* (2014) reported that MWD in rhizosphere of potted trifoliate orange seedlings was significantly higher under mycorrhization than non-mycorrhization. Furthermore, MWD increased with the

Table 2 Effects of exogenous EE-GRSP on percentage of water-stable aggregate (WSA) and mean weight diameter (MWD) of citrus grown in the field

Treatment	Percentage of WSAs (%)				MWD (mm)
	2–4 mm	1–2 mm	0.5–1 mm	0.25–0.5 mm	
0 EE-GRSP	5.45±0.37c	11.62±0.96c	21.76±0.77b	17.04±1.49b	0.56±0.02d
1/4 EE-GRSP	6.14±0.23c	14.86±0.47b	25.36±1.26a	25.97±0.34a	0.69±0.01c
1/2 EE-GRSP	7.55±0.61b	19.79±0.69a	25.65±1.25a	19.23±2.19b	0.79±0.03b
Full EE-GRSP	8.98±0.66a	20.07±1.34a	25.12±0.98a	23.62±1.54a	0.85±0.03a

Data (means ± SE, $n=4$) followed by the different letters among treatments indicate significant differences at $P<0.05$.

Table 3 Effect of exogenous EE-GRSP on the soil phosphatase activity of citrus grown in the field

Treatment	Acid phosphatase (mg hydroxybenzene/g)	Neutral phosphatase (mg hydroxybenzene/g)	Alkaline phosphatase (mg hydroxybenzene/g)	Total phosphatase (mg hydroxybenzene/g)
0 EE-GRSP	1.53±0.10c	1.65±0.13d	2.74±0.21c	5.92±0.29b
1/4 EE-GRSP	1.66±0.06c	2.00±0.07c	4.23±0.22a	7.90±0.19a
1/2 EE-GRSP	2.95±0.09a	2.47±0.32b	2.77±0.23c	8.19±0.26a
Full EE-GRSP	1.92±0.15b	3.04±0.18a	3.39±0.19b	8.35±0.48a

Data (means ± SE, $n=4$) followed by the different letters among treatments indicate significant differences at $P < 0.05$.

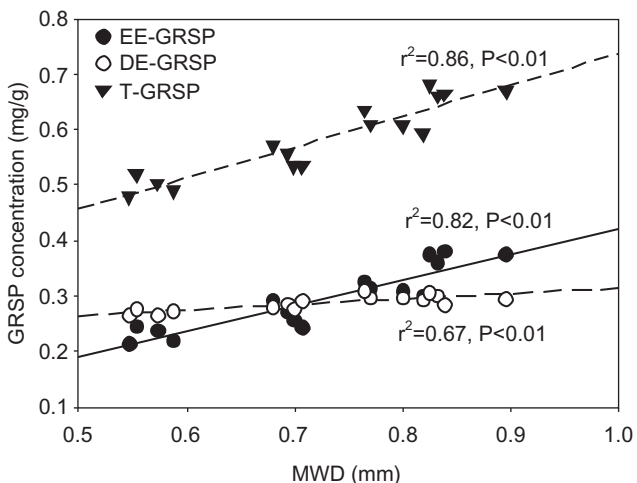


Fig 3 Linear regression between mean weight diameter (MWD) and glomalin-related soil protein (GRSP) concentration in citrus grown in the field ($n=16$)

increase of exogenous EE-GRSP concentration, suggesting that the MWD change was highly dependent on exogenous EE-GRSP concentration. Correlation analyses showed that MWD was positively correlated with all the three GRSP fractions (Fig 3). The correlation of EE-GRSP and T-GRSP with MWD was much stronger than with DE-GRSP, since EE-GRSP and T-GRSP participated more strongly to undertake a more important function on stabilizing WSA than DE-GRSP. After all, EE-GRSP was more active, whereas DE-GRSP was more passivate in respective changes (Wu *et al.* 2014).

Changes in soil phosphatases

Soil enzymes play important role in nutrient cycling, and are considered as an index of soil fertility. Soil phosphatase hydrolyzes the ester bonds binding P to C in organic matter, resulting in release of inorganic P from organically bound P. In this study, ACP activity was significantly higher under 1/2 EE-GRSP and full EE-GRSP conditions than either under 1/4 EE-GRSP or 0 EE-GRSP conditions (Table 3). Exogenous EE-GRSP applications induced an increase in NEP and TP activity, irrespective of EE-GRSP concentration. The treatments with 1/4 EE-GRSP and full EE-GRSP significantly increased ALP activity, compared with 1/2 EE-GRSP and 0 EE-GRSP treatments. These results warranted that exogenous EE-GRSP conferred a positive effect on soil phosphatase activity, dependent on exogenous EE-GRSP concentration and phosphatase species.

Gispert *et al.* (2013) reported a significantly positive correlation between T-GRSP and soil TP activity in 0.25–2.00 and 2.00–5.60 mm soil aggregates. Our study indicated that soil EE-GRSP, DE-GRSP and T-GRSP were significantly and positively correlated with NEP and TP activity, but not with ALP and ACP. However, there was significant positive correlation between DE-GRSP and ACP (Fig 4a–4d), since EE-GRSP here was extracted with 20 mM citrate buffer (pH 7.0), whilst exogenous EE-GRSP solution was diluted by the citrate buffer (pH 7.0). As a result, this exogenous EE-

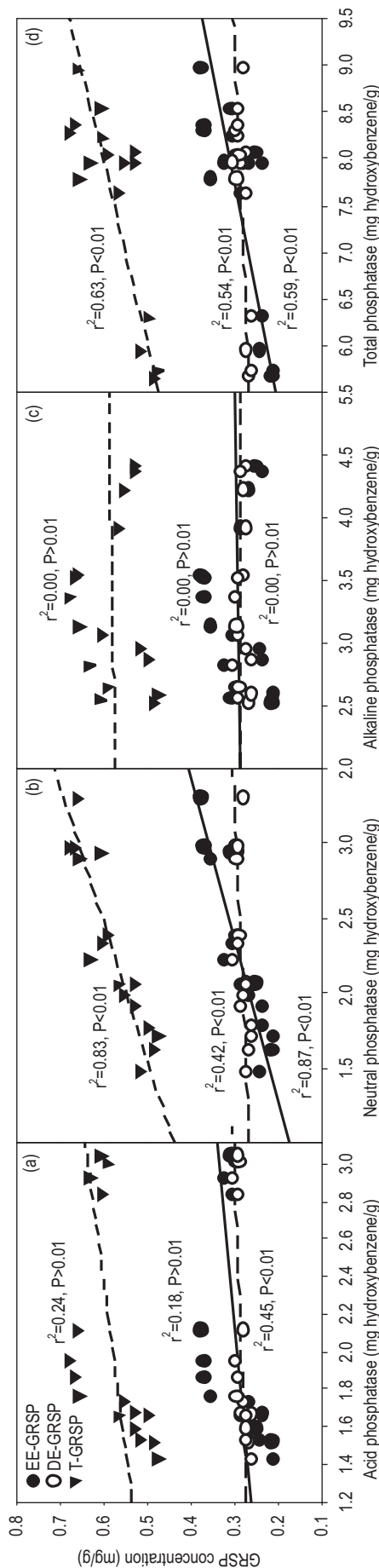


Fig 4 Linear regression between acid (a), neutral (b), alkaline (c), and total (d) phosphatase activity and glomalin-related soil protein (GRSP) concentration in citrus grown in field ($n=16$)

GRSP solution represented a neutral environment, which would stimulate NEP activity instead of ACP or ALP activity.

Exogenous EE-GRSP applications had the significantly positive effects on inducing endogenous EE-GRSP, DE-GRSP and T-GRSP production and SOC concentration. A significantly positive correlation between SOC and these GRSP fractions indicated a potential contribution of GRSP on enriching soil C sink. Exogenous GRSP had a key role in stabilizing WSA stability and a positive effect on soil phosphatase activity. Meanwhile, the correlation of EE-GRSP and T-GRSP on MWD was much stronger than the DE-GRSP. Further ability of GRSP in altering dynamics of soil phosphatase activity and modulating the labile pool of soil nutrients is towards better crop performance. To our knowledge, it is the first report indicating the potential contributions of exogenous EE-GRSP as a soil regulator on soil fertility and soil structure.

ACKNOWLEDGEMENT

This study was supported by the National Natural Science Foundation of China (31372017).

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