

Article

Exogenous Glomalin-Related Soil Proteins Differentially Regulate Soil Properties in Trifoliolate Orange

Rui-Cheng Liu ¹, Ying-Ning Zou ¹, Kamil Kuča ² , Abeer Hashem ³, Elsayed Fathi Abd_Allah ⁴ 
and Qiang-Sheng Wu ^{1,2,*} 

¹ College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China; 202072809@yangtzeu.edu.cn (R.-C.L.); 500406@yangtzeu.edu.cn (Y.-N.Z.)

² Department of Chemistry, Faculty of Science, University of Hradec Králové, 50003 Hradec Králové, Czech Republic; kamil.kuca@uhk.cz

³ Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; habeer@ksu.edu.sa

⁴ Plant Production Department, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; eabdallah@ksu.edu.sa

* Correspondence: wuqiangsheng@yangtzeu.edu.cn

Abstract: Glomalin-related soil protein (GRSP) is a specific glycoprotein secreted into the soil by hyphae and spores of arbuscular mycorrhizal fungi that have many potential functions. It is not clear whether exogenous GRSP has an effect on plant growth and soil properties or whether the effects are related to the type of GRSP used. In this study, trifoliolate orange (*Poncirus trifoliata* L. Raf.) seedlings were used to analyze the effects of easily extractable GRSP (EE-GRSP) and difficultly extractable GRSP (DE-GRSP) at a quarter-, half-, and full-strength concentration on shoot and root biomass as well as soil properties. The results showed that, at different strengths, exogenous EE-GRSP significantly increased shoot and root biomass compared to the control, which displayed the most significant effects from the half-strength EE-GRSP. In contrast, DE-GRSP, at various strengths, significantly reduced shoot and root biomass. Furthermore, the application of exogenous EE-GRSP stimulated soil water-stable aggregate (WSA) content at 2–4 mm and 0.5–1 mm sizes, while DE-GRSP strongly reduced WSA content at the 2–4 mm, 1–2 mm, 0.5–1 mm, and 0.25–0.5 mm sizes, consequently leading to an increase or decrease in the WSA stability, according to the mean weight diameter. However, exogenous EE-GRSP decreased soil pH and DE-GRSP increased it, which was related to WSA stability. Exogenous EE-GRSP almost significantly increased soil acidic, neutral, and alkaline phosphatase activity at different strengths, while exogenous DE-GRSP, also at different strengths, significantly inhibited soil acidic phosphatase activity. The application of both exogenous EE-GRSP and DE-GRSP increased the organic carbon content of the soil. This study concluded that exogenous GRSP exerted differential effects on plant biomass and soil properties, and EE-GRSP can be considered as a soil stimulant for use in citrus plants. To our knowledge, this is the first report on the negative effects of exogenous DE-GRSP on plant biomass and soil properties.

Keywords: glycoprotein; mycorrhiza; phosphatase activity; soil aggregate; soil organic carbon



Citation: Liu, R.-C.; Zou, Y.-N.; Kuča, K.; Hashem, A.; Abd_Allah, E.F.; Wu, Q.-S. Exogenous Glomalin-Related Soil Proteins Differentially Regulate Soil Properties in Trifoliolate Orange. *Agronomy* **2021**, *11*, 1896. <https://doi.org/10.3390/agronomy11101896>

Academic Editor: Samantha C. Karunarathna

Received: 7 August 2021

Accepted: 8 September 2021

Published: 22 September 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with approximately 80% of terrestrial plants. After colonizing the roots of host plants, AMF develop mycorrhizal extraradical hyphae, which extend into the soil beyond the root, thus increasing the root surface area of the plant and its contact with the soil/rhizosphere [1,2]. As a result, arbuscular mycorrhizas promote the growth and development of the host plant by improving the fruit quality, water, and nutrient acquisition, in addition to increasing its stress resistance [2–7]. The beneficial effects of mycorrhizal symbiosis are partly attributed to the production of a specific glycoprotein, glomalin, which is secreted by the hyphae and

spores of AMF [8–10]. Rillig [11] proposed the term glomalin-related soil protein (GRSP) to describe the soil with glomalin, based on the Bradford [12] assay. For the extraction of GRSP, Wu et al. [13] divided GRSP into easily extractable GRSP (EE-GRSP) and the more difficult to extract GRSP (DE-GRSP). GRSP can attach to the outside of soil aggregates and reduce the loss of soil, water, and nutrients [14]. In addition, glomalin can enter the soil with the degradation of extraradical mycelium, which in turn plays a very important role in improving the organic carbon sequestration and soil properties [15]. It is documented that GRSP promotes the formation and stability of soil aggregates, in addition to improving soil and plant water relations [14,16].

GRSP is considered to be insoluble in water and difficult to decompose in its natural state; therefore, it is extremely stable with a turnover time of 6–42 years [17,18]. GRSP is mainly composed of protein bodies, carbohydrates, iron, and other ions; the iron content reaches 1%–9%, and the type and content of cations in GRSP vary greatly depending upon the nature of the soil [19]. Gadkar and Rillig [20] found that the sequence of GRSP was highly homologous to heat shock protein 60 (Hsp60) with >80% homology. This was found by sequencing with liquid chromatography–mass spectrometry.

In GRSP fractions, EE-GRSP is considered to be newly generated by AMF and relatively more labile [21]. Studies have attempted to use EE-GRSP as a plant/soil stimulant. It was demonstrated that exogenous EE-GRSP increased the organic carbon content and phosphatase activity of soil in citrus orchards [22] as well as the stability of soil aggregates and the content of water-stable aggregates [21]. However, the other GRSP fraction, DE-GRSP, originates from the turnover of EE-GRSP and thus an older glomalin [23]. To date, there remains no information regarding the effects of exogenous DE-GRSP application on plant growth and soil properties. Therefore, the aim of this study is to apply EE-GRSP and DE-GRSP at different concentrations on trifoliolate orange seedlings and evaluate the effects on biomass production, soil aggregate stability, SOC, phosphatase, and pH value.

2. Materials and Methods

2.1. Plant Culture

The seed of trifoliolate orange (*Poncirus trifoliata* L. Raf.) was soaked in 1 mol/L sodium hydroxide solution for 10 min, disinfected with 75% alcohol for 10 min, and washed with distilled water for 4 times. Subsequently, the seeds were placed in sterilized river sand ($\phi \leq 2$ mm) and incubated at 28 °C/20 °C (day/night) with 68% relative air humidity. A month later, the three seedlings with four leaves were transplanted into a 2.1 L plastic pot with a 4 mm diameter that was pre-loaded with soil and sterilized (0.11 MPa, 121 °C) for 2 h. The physicochemical characteristics of the soil are shown in Table 1. The plants were placed in a greenhouse, where the light intensity was 900 $\mu\text{mol}/\text{m}^2/\text{s}$, the temperature was 28 °C/20 °C (day/night), and the relative humidity was 68%.

Table 1. Physicochemical characteristics of the soil.

pH	Soil Organic Carbon (g/kg)	Alkaline N (mg/kg)	Olsen-P (mg/kg)	Available K (mg/kg)
6.4	9.6	11.1	15.7	21.3

2.2. Preparation and Application of GRSP and Experimental Design

The soil from the citrus orchard in Yangtze University was collected, air-dried, and sieved to remove stones and root segments, and then EE-GRSP and DE-GRSP were extracted by the method of Wu et al. [23]. A 0.5 g soil sample was mixed with 4 mL of 20 mmol/L citrate buffer (pH 7.0), extracted at 103 kPa, heated to 121 °C for 30 min, and centrifuged at 10,000 \times g/min for 5 min. The supernatant was intended to be the full-strength EE-GRSP (1 GRSP). The centrifugal residue continued to be mixed with 4 mL of 50 mmol/L sodium citrate buffer (pH 8), extracted at 103 kPa and heated to 121 °C for 60 min, and centrifuged at 10,000 \times g/min for 10 min. This supernatant was selected as

full-strength DE-GRSP. The protein concentration of full-strength EE-GRSP and DE-GRSP was 0.24 mg/L and 0.36 mg/L, respectively, according to Bradford's [12] method, with bovine serum protein as the standard.

Different strengths of EE-GRSP and DE-GRSP were prepared with dilutions of full-strength GRSP with 20 mmol/L citrate buffers (pH 7.0) and 50 mmol/L sodium citrate buffers (pH 8), respectively. After two weeks of seedling transplanting, 50 mL of different concentrations of GRSP was applied to the pot weekly, and the same amount of citrate buffer (pH 7.0) was applied to the control group. The experiment ended after 16 weeks of exogenous GRSP application.

In this experiment, a completely randomized block design was carried out, in which seven treatments, namely, the control (0 GRSP), quarter-strength EE-GRSP, half-strength EE-GRSP, full-strength EE-GRSP, quarter-strength DE-GRSP, half-strength DE-GRSP, and full-strength DE-GRSP, were arranged. Each treatment had four replicates, with two pots for each replicate, totaling 56 pots. The pots were arranged randomly, and their positions were changed regularly to decrease the impact of light on pot locations.

2.3. Variable Determinations

Plants were separated from the shoot and root parts at harvest, and then their fresh weight was measured. At harvest time, the soil sticking to the root surface was gently shaken off, air-dried, and collected for the analysis of particular soil properties.

Soil pH value was determined as per the protocol outlined by Cheng et al. [24] with a pH meter (PH828+, Smart Sensor, Arco Science & Technology Ltd., Dongguan, China).

Soil water-stable aggregates (WSAs) in the sizes of 2–4 mm, 1–2 mm, 0.5–1 mm, and 0.25–0.5 mm were assayed according to the wet-sieving method [25]. Soil WSA stability was measured according to the analysis of the mean weight diameter (MWD) with the following formula [25]:

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

where X_i is the mean diameter of the i sieve opening (mm), W_i is the proportion of the i size fraction in the total sample mass, and n is number of size fractions.

SOC was determined by the dichromate oxidation spectrophotometric method as outlined by Rowell [26]. Determination of soil acid phosphatase (ACP, extracted by 0.1 mmol/L sodium acetate buffer at pH 5.0), neutral phosphatase (NEP, extracted by 20 mmol/L citrate buffer at pH 7.0), and alkaline phosphatase (ALP, extracted by 0.2 mmol/L borate buffer at pH 10.0) activity was assayed by the method of Zhao and Jiang [27], using disodium phenyl phosphate as the substrate and redistilled phenol as the standard.

The extraction and analysis of EE-GRSP and DE-GRSP in soil was previously described in Section 2.2.

2.4. Statistical Analysis

The one-way analysis of variance (ANOVA) in SAS software (v8.1) was used to analyze the experimental data, and Duncan's multiple-range test was used to compare significant differences among treatments ($p < 0.05$). Figures were generated using SigmaPlot (v10.0).

3. Results

3.1. Changes in Biomass Production

After treatment with exogenous EE-GRSP, the EE-GRSP-treated plants were recorded as having significantly higher shoot and root biomass of trifoliolate orange seedlings. A total of 39% and 19% of the shoots and roots received a quarter-strength of EE-GRSP application, 52% and 53% received a half-strength EE-GRSP application, and 49% and 34% received a full-strength EE-GRSP application, respectively (Figure 1a). Evidently, the half-strength EE-GRSP treatment provided the best promotive effects. However, compared with the control (0 GRSP), all DE-GRSP treatments significantly reduced shoot and root biomass production with the increase in DE-GRSP concentrations (Figure 1b). Among them, the

quarter-strength DE-GRSP considerably reduced shoot and root biomass by 51% and 53%, the half-strength DE-GRSP by 56% and 56%, and the full-strength DE-GRSP by 71% and 72%, respectively.

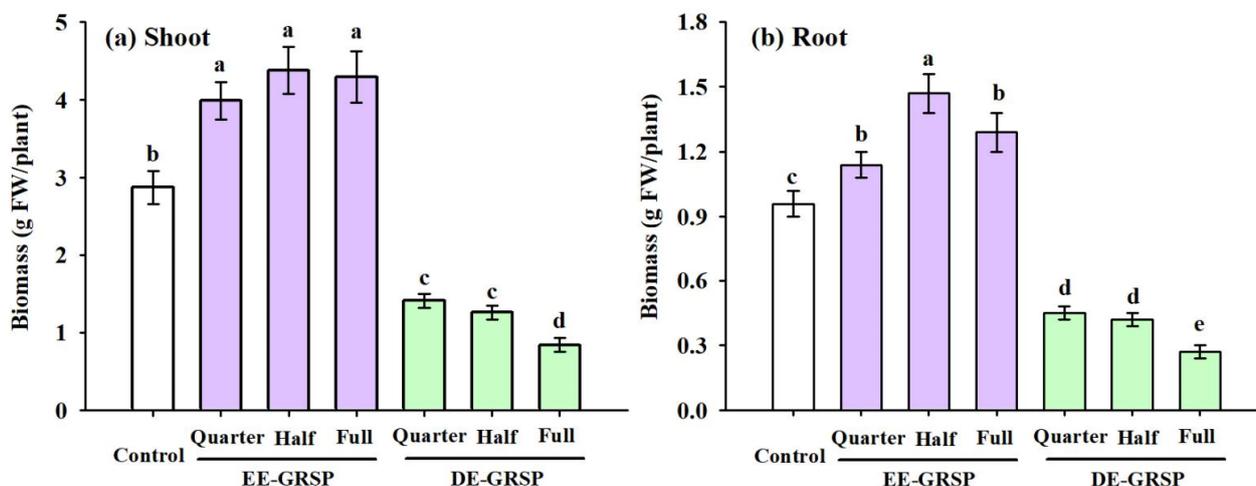


Figure 1. Effect of exogenous EE-GRSP and DE-GRSP on shoot (a) and root (b) biomass production of trifoliolate orange seedlings. Data (mean \pm SD, $n = 4$) followed by different letters above the bars indicate significant differences ($p < 0.05$) between treatments.

3.2. Changes in Soil WSA Distribution and Stability

Exogenous GRSP treatment strongly affected the distribution of WSAs in the rhizosphere of trifoliolate orange seedlings (Table 2). All EE-GRSP treatments significantly increased WSA percentages in the 2–4 mm and 0.5–1 mm sizes compared with the control (0 GRSP). In addition, the quarter- and full-strength EE-GRSP also significantly increased WSA percentages in the 1–2 mm size, and the half-strength EE-GRSP significantly increased WSA percentages in the 0.25–0.5 mm size. Three DE-GRSP treatments collectively reduced WSA percentages in the 2–4 mm, 1–2 mm, 0.5–1 mm, and 0.25–0.5 mm sizes. In addition, compared to the control, the quarter-, half-, and full-strength EE-GRSP significantly increased MWD by 19%, 20%, and 16%, respectively; quarter-, half-, and full-strength DE-GRSP significantly decreased MWD by 55%, 61%, and 65%, respectively.

Table 2. Effects of exogenous GRSP on water-stable aggregate (WSA) content and the mean weight diameter (MWD) of trifoliolate orange.

Treatments	Percentage of WSA (%)				MWD (mm)
	2–4 mm	1–2 mm	0.5–1 mm	0.25–0.5 mm	
Control	28.95 \pm 0.63d	4.28 \pm 0.26c	10.96 \pm 0.59b	17.66 \pm 0.88b	0.74 \pm 0.01b
Quarter-strength EE-GRSP	35.76 \pm 0.16b	3.35 \pm 0.33d	16.55 \pm 1.60a	11.36 \pm 0.32c	0.88 \pm 0.01a
Half-strength EE-GRSP	37.51 \pm 1.02a	7.44 \pm 0.25a	15.01 \pm 1.38a	22.57 \pm 1.60a	0.89 \pm 0.02a
Full-strength EE-GRSP	33.44 \pm 1.03c	5.09 \pm 0.07b	14.29 \pm 0.81a	17.04 \pm 0.78b	0.86 \pm 0.02a
Quarter-strength DE-GRSP	13.00 \pm 1.28f	2.38 \pm 0.14e	5.58 \pm 0.23d	9.33 \pm 0.29d	0.33 \pm 0.03c
Half-strength DE-GRSP	8.52 \pm 1.23g	3.33 \pm 0.24d	5.51 \pm 0.74d	10.94 \pm 0.96c	0.26 \pm 0.03d
Full-strength DE-GRSP	15.11 \pm 0.73e	3.07 \pm 0.19d	7.25 \pm 0.64c	10.58 \pm 0.64cd	0.29 \pm 0.03cd

Data (mean \pm SD, $n = 4$) followed by the different letters among treatments indicate significant differences at $p < 0.05$.

Regression analysis showed a significant curvilinear correlation between MWD and exogenous EE-GRSP concentrations, with a trend of increasing and then decreasing with a peak between 0.006–0.012 mg of protein/mL citrate buffer (Figure 2a). In addition, MWD values showed a significant curvilinear correlation in the concentration of DE-GRSP (Figure 2b), with a trend of decreasing and then slightly increasing to reach a trough between 0.009–0.018 mg of protein/mL citrate buffer.

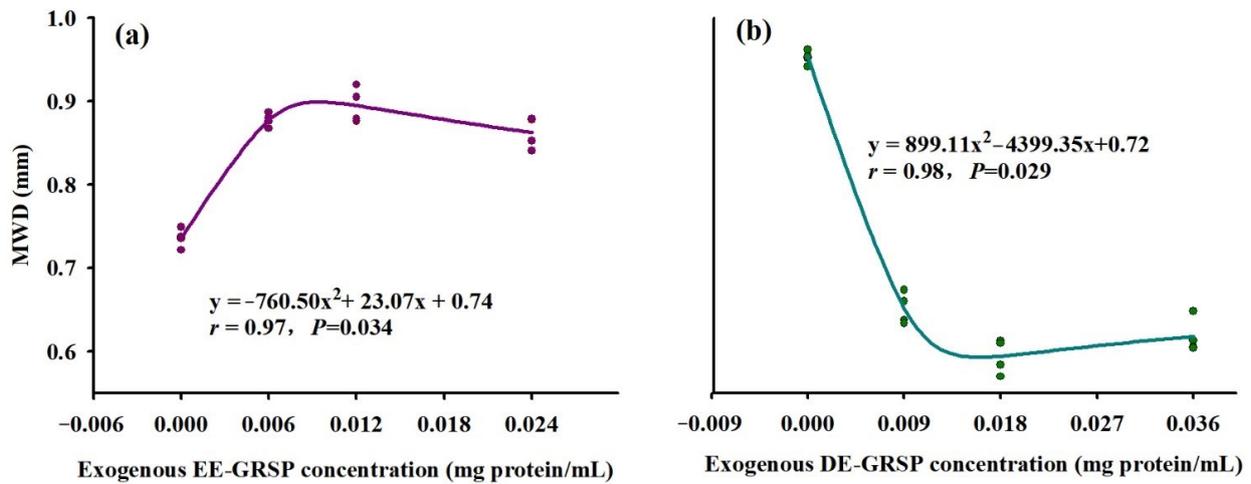


Figure 2. Correlation between exogenous EE-GRSP (a) and exogenous DE-GRSP (b) and the soil mean weight diameter ($n = 16$).

3.3. Changes in Soil pH Value

The application of exogenous GRSP significantly changed the soil pH value. The reduction in the soil pH value was caused by exogenous EE-GRSP and the increase by exogenous DE-GRSP (Figure 3). There were no significant differences in soil pH value among the three EE-GRSP treatments or the three DE-GRSP treatments. In addition, the soil pH value was substantially and negatively correlated with MWD (Figure 4).

3.4. Changes in SOC Content

Exogenous EE-GRSP and DE-GRSP application considerably increased the SOC content (Figure 5). Among the treatments, the SOC quantity rose with the increase in exogenous DE-GRSP concentration. Between the three EE-GRSP treatments, the half-strength EE-GRSP application showed a relatively better effect of increasing the SOC. A significantly and positively linear relationship was observed between the SOC and the exogenous EE-GRSP concentration (Figure 6a) and the DE-GRSP concentration (Figure 6b), respectively.

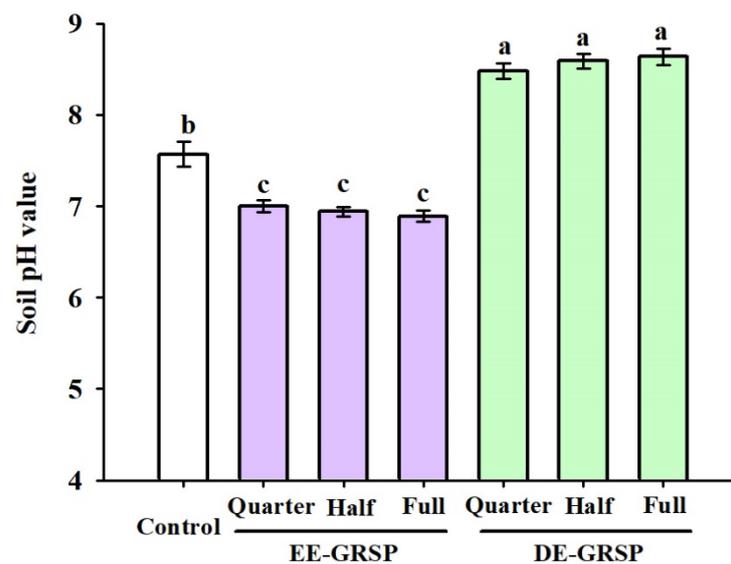


Figure 3. Effects of exogenous EE-GRSP and DE-GRSP on the soil pH value in the rhizosphere of trifoliolate orange seedlings. Data (means \pm SD, $n = 4$) followed by different letters above the bars indicate significant differences ($p < 0.05$) between treatments.

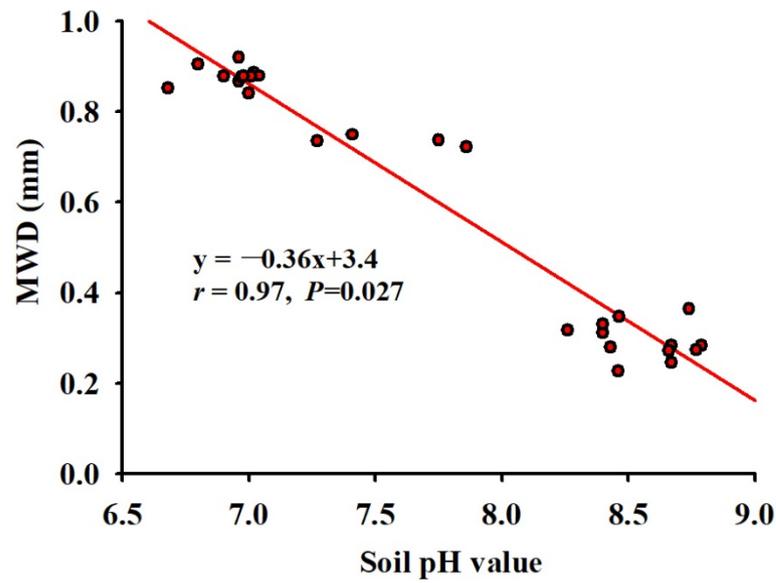


Figure 4. Correlation between the soil pH value and the soil MWD ($n = 28$).

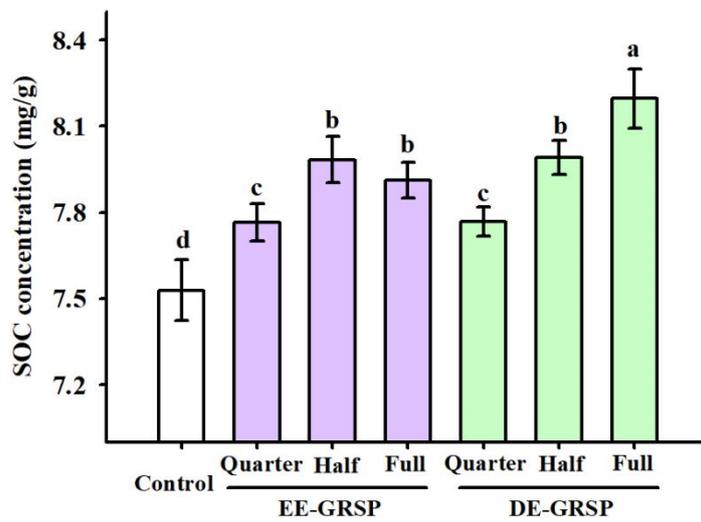


Figure 5. Effects of exogenous EE-GRSP and DE-GRSP on soil organic carbon of trifoliolate orange seedlings. Data (mean \pm SD, $n = 4$) followed by different letters above the bars indicate significant differences ($p < 0.05$) between treatments.

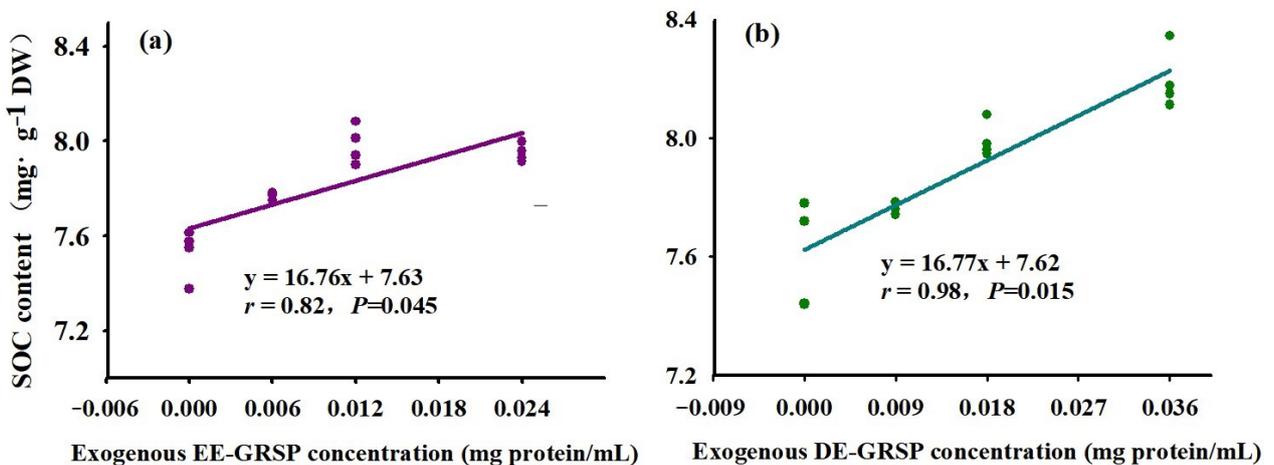


Figure 6. Correlation between exogenous EE-GRSP (a) and exogenous DE-GRSP (b) and soil organic carbon ($n = 16$).

3.5. Changes in Soil Phosphatase Activity

The control, quarter-, half-, and full-strength EE-GRSP treatments significantly increased the activity of soil acid phosphatase by 20%, 29%, and 10%, respectively, and that of neutral phosphatase by 21%, 28%, and 86%, respectively (Figure 7a,b). The application of the half- and full-strength EE-GRSP also significantly increased soil alkaline phosphatase activity by 26% and 13%, respectively, with no significant effect caused by the application of the quarter-strength EE-GRSP (Figure 7c). In addition, compared with the control, the quarter-, half-, and full-strength DE-GRSP treatments had no significant effect on the activity of soil neutral and alkaline phosphatase, but the treatments significantly decreased soil acid phosphatase activity by 11%, 13%, and 31%, respectively (Figure 7a–c).

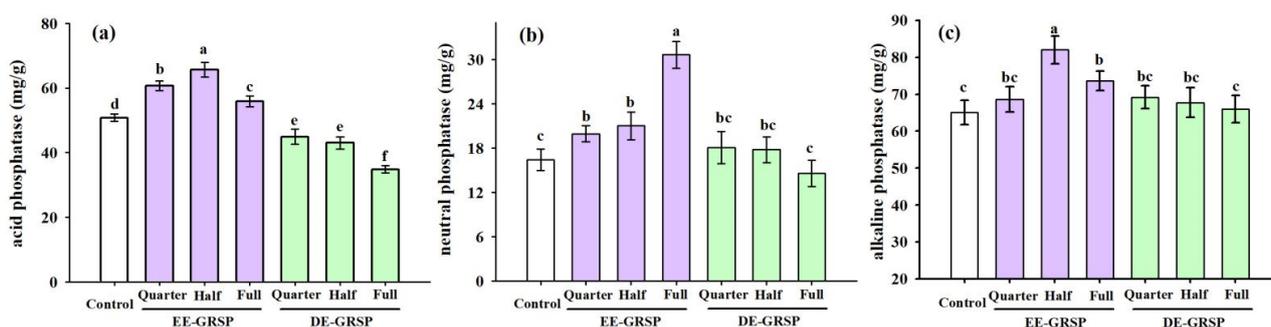


Figure 7. Effects of exogenous GRSP on soil acid (a), neutral (b), and alkaline (c) phosphatase activity in trifoliolate orange seedlings. Data (mean \pm SD, $n = 4$) followed by different letters above the bars indicate significant differences ($p < 0.05$) between treatments.

3.6. Changes in Soil GRSP Concentrations

All exogenous GRSP applications significantly increased the concentration of soil GRSP components, followed by increased concentrations of EE-GRSP and DE-GRSP. The control, quarter-, half- and full-strength EE-GRSP treatments significantly increased the soil EE-GRSP content by 24%, 25%, and 31% (Figure 8a); soil DE-GRSP by 4%, 4%, and 10% (Figure 8b); and T-GRSP by 13%, 14%, and 19% (Figure 8c), respectively. Similarly, the control, quarter-, half-, and full-strength DE-GRSP treatment significantly increased the soil EE-GRSP content by 5%, 7%, and 14% (Figure 8a); DE-GRSP by 44%, 53%, and 69% (Figure 8b); and T-GRSP by 26%, 31%, and 44% (Figure 8c), respectively.

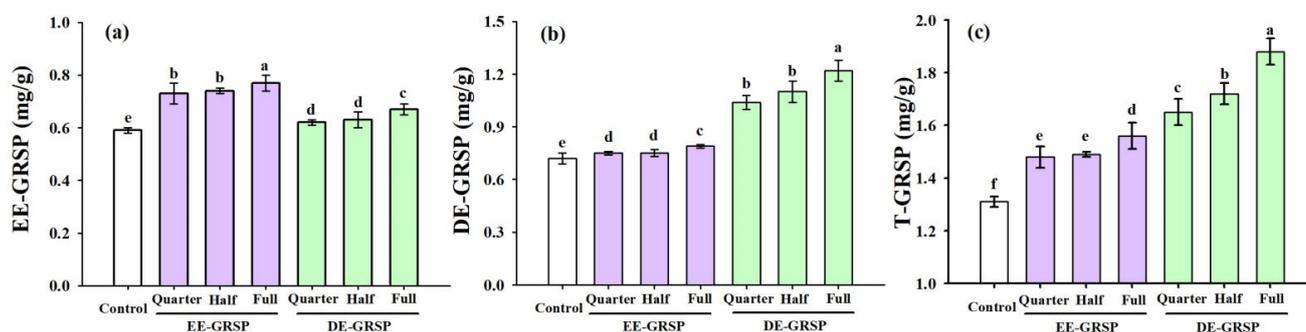


Figure 8. Effects of exogenous EE-GRSP and DE-GRSP on endogenous EE-GRSP (a), DE-GRSP (b), and T-GRSP (c) content in the rhizosphere of trifoliolate orange seedlings. Data (mean \pm SD, $n = 4$) followed by different letters above the bars indicate significant differences ($p < 0.05$) between treatments.

4. Discussion

The present study showed that different concentrations of EE-GRSP dramatically increased shoot and root biomass production, while half-strength EE-GRSP had the most significant effect, which was consistent with the results of Wang et al. [28] and Chi et al. [29] in trifoliolate orange seedlings. The EE-GRSP contained high amounts of humic acids,

which had functions in improving plant growth [30]. However, different concentrations of DE-GRSP all significantly reduced shoot and root biomass production with increased concentrations, implying that exogenous DE-GRSP negatively affects the growth of trifoliolate orange seedlings. The different growth responses for exogenous EE-GRSP and DE-GRSP applications could be derived from two facts: (i) the difference in the intrinsic elemental composition between EE-GRSP and DE-GRSP (e.g., higher P, Mo, and O contents in purified EE-GRSP versus purified DE-GRSP; (ii) changes in the synthesis and transport of auxins in plants with higher root indole acetic acid and indole butyric acid in EE-GRSP-treated plants than in DE-GRSP-treated plants [31]. The variation in the intrinsic elemental contents of the two GRSP fractions consisted of different organic substances, such as tyrosine, tryptophan, fulvic acid, humic acid, nitrobenzoxadidole, and calcofluor [32], thus triggers differential plant growth responses. In addition, GRSP is known to contain many impurities as well as proteins of mycorrhizal fungal origin [30,33]. It is possible that EE-GRSP and DE-GRSP always contain different impurities, and these impurities have differential effects on plants; however, the applied EE-GRSP solution (pH 7.0) had a lower pH value than that of DE-GRSP (pH 8.0), and citrus prefers slightly acidic soil.

Soil aggregates are an important constituent of soil structure, and their quantity and stability are the essential criteria for evaluating soil quality [34]. During the formation of soil aggregates, GRSPs, similarly to glue, are involved in binding small soil particles into microaggregates (<0.25 mm in diameter) to large aggregates (> 0.25 mm in diameter), ultimately forming a stable soil aggregate structure [35]. Compared with the control treatment, the quarter-strength EE-GRSP application significantly increased WSA in the sizes of 2–4 mm and 0.5–1.0 mm but reduced WSA in the 1–2 mm and 0.25–0.5 mm sizes. The half-strength EE-GRSP treatment considerably increased WSA in all sizes. The full-strength EE-GRSP significantly increased WSA in the 2–4 mm, 1–2 mm, and 0.5–1 mm sizes. Wu et al. [23] also reported that exogenous EE-GRSP significantly increased WSA in the sizes of 1–2 mm and 0.5–1 mm in field citrus. Contrastingly, the present study found that treatments with the quarter-, half-, and full-strength DE-GRSP dramatically decreased WSA in all sizes. It was proposed that in GRSP fractions, only EE-GRSP represented the positive effect on WSA formation, whereas DE-GRSP had a negative effect. In general, soil WSA formation is involved in mycorrhizal hyphae, SOC, roots, GRSP, etc. [36]. Wu et al. [37] reported that in the presence of roots and mycorrhizal hyphae, root biomass, and mycorrhizal colonization are the main mechanisms in WSA formation, and total GRSP concentration (the sum of EE-GRSP and DE-GRSP) is of paramount importance in WSA formation.

In this study, we also observed differential effects of exogenous EE-GRSP and DE-GRSP on MWD: the increase caused by EE-GRSP and the decrease caused by DE-GRSP. Correlation analysis indicated that there was a significant curvilinear correlation between MWD and the concentration of exogenous EE-GRSP and DE-GRSP. This suggested that EE-GRSP application had positive effects on WSA stability, whereas DE-GRSP had deleterious effects on WSA stability. Thus, in GRSP fractions, only EE-GRSP contributed to the aggregate stability, as previously reported by Wu et al. [38] under the presence of only mycorrhizal extraradical hyphae conditions. Chi et al. [39] reported that higher soil pH in saline–alkali regions reduced soil aggregate contents and stability. In our study, soil pH value was increased with the increase in exogenous DE-GRSP concentrations and decreased with the increase in exogenous EE-GRSP concentrations. In addition, soil pH value was significantly and negatively correlated with MWD, implying that exogenous GRSP could modulate soil pH value to affect WSA formation and stability, which still needs to be researched in future studies.

SOC content affects the physical and chemical properties of soil, as well as its fertility characteristics, and, thus, it is considered as one of the decisive factors in evaluating soil quality [40]. The present work indicates that the control, quarter-, half-, and full-strength EE-GRSP and DE-GRSP treatments increased the SOC content, which is consistent with our earlier results in a 27-year-old Satsuma mandarin grafted onto a trifoliolate orange

after being applied with exogenous EE-GRSP [23]. Additionally, the stimulated effect on SOC was observed under exogenous DE-GRSP application conditions. Studies showed that purified GRSP contributed to $23.26 \pm 2.67\%$ of the total SOC [4]. In this study, DE-GRSP had a relatively higher contribution to the SOC than EE-GRSP in the full-strength concentration. This further suggests that DE-GRSP in the full-strength concentration had a stronger contribution to SOC than EE-GRSP. Our previous data also revealed a higher contribution of purified DE-GRSP ($14.59 \pm 2.21\%$) versus purified EE-GRSP ($8.67 \pm 0.95\%$) to SOC [4].

Soil enzymatic activity is a key indicator of soil biochemical characteristics [41]. Among them, soil phosphatases catalyze the mineralization of soil organophosphorus compounds, and phosphatase activities directly affect the decomposition and transformation of soil organophosphorus [42]. Exogenous EE-GRSP treatments significantly increased soil acidic, neutral, and alkaline phosphatase activity, which was consistent with previous works on Satsuma mandarin trees grown in fields [22]. In contrast, exogenous DE-GRSP mainly reduced soil acid phosphatase activity but did not alter soil neutral and alkaline phosphatase activity. This means that the rhizosphere of EE-GRSP-treated plants possessed stronger acid, neutral and alkaline phosphatase activity, while the rhizosphere of DE-GRSP-applied plants had lower acid phosphatase activity; this change may correlate with differential plant growth triggered by GRSP applications.

5. Conclusions

In conclusion, the application of exogenous EE-GRSP considerably increased plant biomass, stimulated the formation and stability of soil WSAs, reduced soil pH value, and elevated SOC content and soil phosphatase activity. This indicates that exogenous EE-GRSP can be considered as a biostimulator for the improvement of both plant growth and soil properties. Furthermore, we also observed that exogenous DE-GRSP exhibited adverse effects on plant biomass, WSA formation and stability, soil pH, and soil acid phosphatase activity. Such results guide the management of soil GRSP in citrus orchards for reducing DE-GRSP and increasing EE-GRSP.

Author Contributions: Conceptualization, R.-C.L.; methodology, Y.-N.Z., Q.-S.W., and K.K.; data collection and statistical analysis, R.-C.L.; writing—original draft preparation, R.-C.L.; writing—review and editing, A.H., E.F.A., K.K., and Q.-S.W.; supervision, Q.-S.W. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Natural Science Foundation of China (31372017) and the 2020 Joint Projects between Chinese and CEECs' Universities (202019). The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP-2021/356), King Saud University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data supporting the findings of this study are included in this article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Walder, F.; Heijden, M.G.V.A. Regulation of resource exchange in the arbuscular mycorrhizal symbiosis. *Nat. Plants* **2015**, *1*, 15159. [[CrossRef](#)]
2. Wu, Q.S.; He, J.D.; Srivastava, A.K.; Zhang, F.; Zou, Y.N. Development of propagation technique of indigenous AMF and their inoculation response in citrus. *Ind. J. Agric. Sci.* **2019**, *89*, 1190–1194.
3. Gao, W.Q.; Wang, P.; Wu, Q.S. Functions and application of glomalin-related soil proteins: A review. *Sains Malays.* **2019**, *48*, 111–119. [[CrossRef](#)]
4. He, J.D.; Chi, G.G.; Zou, Y.N.; Shu, B.; Wu, Q.S.; Srivastava, A.K.; Kuča, K. Contribution of glomalin-related soil proteins to soil organic carbon in trifoliolate orange. *Appl. Soil Ecol.* **2020**, *154*, 103592. [[CrossRef](#)]

5. Wu, Q.S.; Gao, W.Q.; Srivastava, A.K.; Zhang, F.; Zou, Y.N. Nutrient acquisition and fruit quality of Ponkan mandarin in response to AMF inoculation. *Ind. J. Agric. Sci.* **2020**, *90*, 1563–1567.
6. Yang, L.; Zou, Y.N.; Tian, Z.H.; Wu, Q.S.; Kuča, K. Effects of beneficial endophytic fungal inoculants on plant growth and nutrient absorption of trifoliolate orange seedlings. *Sci. Hort.* **2021**, *277*, 109815. [[CrossRef](#)]
7. Cheng, H.Q.; Giri, B.; Wu, Q.S.; Zou, Y.N.; Kuča, K. Arbuscular mycorrhizal fungi mitigate drought stress in citrus by modulating root microenvironment. *Arch. Agron. Soil Sci.* **2021**. [[CrossRef](#)]
8. Gałazka, A.; Niedźwiecki, J.; Grządziel, J.; Gawryjolek, K. Evaluation of changes in glomalin-related soil proteins (GRSP) content, microbial diversity and physical properties depending on the type of soil as the important biotic determinants of soil quality. *Agronomy* **2020**, *10*, 1279. [[CrossRef](#)]
9. Ortas, I.; Çağdas, A.; Ahmet, D. Effect of mycorrhizal species on growth and nutrient uptake by seedlings of citrus (*Citrus sinensis*) under three soil growth conditions. *Curr. Hort.* **2015**, *3*, 61–64.
10. Ortas, I. Effect of mycorrhizal inoculation on P and Zn uptake in citrus. *Curr. Hort.* **2017**, *5*, 59–61.
11. Rillig, M.C. Arbuscular mycorrhizae, glomalin, and soil aggregation. *Can. J. Soil Sci.* **2004**, *84*, 355–363. [[CrossRef](#)]
12. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [[CrossRef](#)]
13. 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 trifoliolate orange. *Sci. Rep.* **2014**, *4*, 5823. [[CrossRef](#)]
14. Meng, L.L.; He, J.D.; Zou, Y.N.; Wu, Q.S.; Kuča, K. Mycorrhiza-released glomalin-related soil protein fractions contribute to soil total nitrogen in trifoliolate orange. *Plant Soil Environ.* **2020**, *66*, 183–189. [[CrossRef](#)]
15. Barna, G.; Makó, A.; Takács, T.; Füzy, A.; Horel, A.; Skic, K. Biochar alters soil physical characteristics, arbuscular mycorrhizal fungi colonization, and glomalin production. *Agronomy* **2020**, *10*, 1933. [[CrossRef](#)]
16. Bertagnoli, B.; Oliveira, J.F.; Barbosa, G.M.; Filho, A.C. Poultry litter and liquid swine slurry applications stimulate glomalin, extraradicular mycelium production, and aggregation in soils. *Soil Till. Res.* **2020**, *202*, 104657. [[CrossRef](#)]
17. Rillig, M.C.; Wright, S.F.; Allen, M.F.; Field, C.B. Rise in carbon dioxide changes soil structure. *Nature* **1999**, *400*, 628–629. [[CrossRef](#)]
18. Wang, W.J.; Zhong, Z.L.; Wang, Q.; Wang, H.M.; Fu, Y.J.; He, X.Y. Glomalin contributed more to carbon, nutrients in deeper soils, and differently associated with climates and soil properties in vertical profiles. *Sci. Rep.* **2017**, *7*, 13003. [[CrossRef](#)] [[PubMed](#)]
19. Tian, H.; Liu, X.L.; Gai, J.P.; Zhang, J.L.; Li, X.L. Review of glomalin-related soil protein and its function. *Chin. J. Soil Sci.* **2009**, *40*, 1215–1220.
20. Gadkar, V.; Rillig, M.C. The arbuscular mycorrhizal fungal protein glomalin is a putative homolog of heat shock protein 60. *FEMS Microbiol. Lett.* **2006**, *263*, 93–101. [[CrossRef](#)]
21. Koide, R.T.; Peoples, M.S. Behavior of Bradford-reactive substances is consistent with predictions for glomalin. *Appl. Soil Ecol.* **2013**, *63*, 8–14. [[CrossRef](#)]
22. Wu, Q.S.; Li, Y.; Zou, Y.N.; He, X.H. Arbuscular mycorrhiza mediates glomalin-related soil protein production and soil enzyme activities in the rhizosphere of trifoliolate orange grown under different P levels. *Mycorrhiza* **2015**, *25*, 121–130. [[CrossRef](#)]
23. Wu, Q.S.; Srivastava, A.K.; Wang, S.; Zeng, J.X. Exogenous application of EE-GRSP and changes in citrus rhizosphere properties. *Ind. J. Agric. Sci.* **2015**, *85*, 58–62.
24. Cheng, H.Q.; Zou, Y.N.; Wu, Q.S.; Kuča, K. Arbuscular mycorrhizal fungi alleviate drought stress in trifoliolate orange by regulating H⁺-ATPase activity and gene expression. *Front. Plant Sci.* **2021**, *12*, 659694. [[CrossRef](#)] [[PubMed](#)]
25. Kemper, W.D.; Rosenau, R.C. Aggregate Stability and Size Distribution. In *Methods of Soil Analysis. Part 1. Physical and Mineralogical Methods*, 2nd ed.; Klute, A., Ed.; Soil Science Society of America Agronomy Monograph No. 9; Soil Science Society of America: Madison, WI, USA, 1986; pp. 425–442.
26. Rowell, D.L. *Soil Science: Methods and Applications*; Routledge: New York, NY, USA, 2014.
27. Zhao, L.P.; Jiang, Y. Research of determination of soil phosphatase activity. *Chin. J. Soil Sci.* **1986**, *17*, 138–141.
28. Wang, S.; Wu, Q.S.; He, X.H. Exogenous easily extractable glomalin-related soil protein promotes soil aggregation, relevant soil enzyme activities and plant growth in trifoliolate orange. *Plant Soil Environ.* **2015**, *2*, 66–71. [[CrossRef](#)]
29. Chi, G.G.; Srivastava, A.K.; Wu, Q.S. Exogenous easily extractable glomalin-related soil protein improves drought tolerance of trifoliolate orange. *Arch. Agron. Soil Sci.* **2018**, *64*, 1341–1350. [[CrossRef](#)]
30. Gillespie, A.W.; Farrell, R.E.; Walley, F.L.; Ross, A.R.S.; Leinweber, P.; Eckhardt, K.U. Glomalin-related soil protein contains non-mycorrhizal related heat-stable proteins, lipids and humic materials. *Soil Biol. Biochem.* **2011**, *43*, 766–777. [[CrossRef](#)]
31. Liu, R.C.; Gao, W.Q.; Srivastava, A.K.; Zou, Y.N.; Kuča, K.; Hashem, A.; Abd_Allah, E.F.; Wu, Q.S. Differential effects of exogenous glomalin-related soil proteins on plant growth of trifoliolate orange through regulating auxin changes. *Front. Plant Sci.* **2021**, *12*, 745402. [[CrossRef](#)]
32. Zhang, Z.L.; Wang, W.J.; Wang, Q.; Wu, Y.; Wang, H.M.; Pei, Z.X. Glomalin amount and compositional variation, and their associations with soil properties in farmland, northeastern China. *J. Plant Nutr. Soil Sci.* **2017**, *180*, 563–575. [[CrossRef](#)]
33. Schindler, F.V.; Mercer, E.J.; Rice, J.A. Chemical characteristics of glomalin-related soil protein (GRSP) extracted from soils of varying organic matter content. *Soil Biol. Biochem.* **2007**, *39*, 320–329. [[CrossRef](#)]
34. Bronick, C.J.; Lal, R. Soil structure and management: A review. *Geoderma* **2005**, *124*, 3–22. [[CrossRef](#)]

35. Rillig, M.C.; Wright, S.F.; Eviner, V.T. The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: Comparing effects of five plant species. *Plant Soil* **2002**, *238*, 325–333. [[CrossRef](#)]
36. Zou, Y.N.; Srivastava, A.K.; Wu, Q.S. Glomalin: A potential soil conditioner for perennial fruits. *Int. J. Agric. Biol.* **2015**, *18*, 293–297. [[CrossRef](#)]
37. Wu, Q.S.; Srivastava, A.K.; Cao, M.Q.; Wang, J. Mycorrhizal function on soil aggregate stability in root zone and root-free hyphae zone of trifoliolate orange. *Arch. Agron. Soil Sci.* **2014**, *61*, 813–825. [[CrossRef](#)]
38. Wu, Q.S.; Wang, S.; Srivastava, A.K. Mycorrhizal hyphal disruption induces changes in plant growth, glomalin-related soil protein and soil aggregation of trifoliolate orange in a core system. *Soil Till. Res.* **2016**, *160*, 82–91. [[CrossRef](#)]
39. Chi, C.M.; Zhao, C.W.; Sun, X.J.; Wang, Z.C. Estimating exchangeable sodium percentage from sodium adsorption ratio of salt-affected soil in the Songnen plain of northeast China. *Pedosphere* **2011**, *21*, 271–276. [[CrossRef](#)]
40. Bhogal, A.; Nicholson, F.A.; Chambers, B.J. Organic carbon additions: Effects on soil bio-physical and physico-chemical properties. *Eur. J. Soil Sci.* **2010**, *60*, 276–286. [[CrossRef](#)]
41. Cao, X.Y.; Li, J.P. Contents of soil organic carbon and its relations with soil physicochemical properties in Chinese fir plantations. *For. Res. Manag.* **2014**, *6*, 104–109.
42. Liu, S.J.; Xia, X.; Chen, G.M.; Mao, D.; Che, S.G.; Li, Y.X. Study progress on functions and affecting factors of soil enzymes. *Chin. Agric. Sci. Bull.* **2011**, *27*, 1–7.