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Agronomic Practices Alter Regulated Effects of Easily Extractable Glomalin-Related Soil Protein on Fruit Quality and Soil Properties of Satsuma Mandarin

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Abstract: Easily extractable glomalin-related soil protein (EEG) released from arbuscular mycorrhizal fungi exhibits many roles in soil and plants, while it is not clear whether the biostimulator alone or in combination with agronomic practices can improve soil characteristics and fruit quality in citrus. The objective of this study was to analyze the effects of foliar sprays of EEG, singly or in combination with fruit bagging (FB), reflective film mulching (RF), and grass-proof cloth mulching (GPC) on root mycorrhizal colonization rate, fruit internal and external quality, and soil structure and fertility on an early-ripening Satsuma mandarin "Oita 4" (*Citrus unshiu* cv. Oita 4). Exogenous EEG application increased root mycorrhizal colonization, while agronomic practices dramatically inhibited root mycorrhizal colonization in EEG-treated trees. Foliar EEG application significantly improved the internal and external quality of fruits, but the combination of three agronomic practices with EEG did not further enlarge the improved effect on the external quality of fruit and even produced an inhibitory effect. Also, the addition of RF further amplified the improved effect of EEG on the contents of fruit vitamin C and soluble solids. EEG application also significantly elevated sucrose in the fruit pith and juice sac, fructose in the fruit peel, fruit pith, segment membrane, and juice sac, and glucose in the fruit pith, segment membrane, and juice sac. Additional RF treatment could increase sucrose in the fruit peel and juice sac as well as glucose in the fruit peel to varying degrees in EEG-treated trees. EEG application also significantly increased soil organic carbon, mean weight diameter, nitrate nitrogen, ammonium nitrogen, available phosphorus, and available potassium levels, with additional agronomic practices almost reducing the promoting effect of EEG on these soil variables. It has been summarized that a single EEG application had positive effects on fruit quality and soil fertility, while the additional agronomic practices resulted in little change or even suppressed the positive effects of EEG.

Keywords: aggregate; glomalin; mycorrhiza; soil organic carbon; sugar

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Citation: Lei, A.-Q.; Yang, Q.-H.; Zhang, Y.; Liao, W.-Y.; Xie, Y.-C.; Srivastava, A.K.; Hashem, A.; Alqahtani, M.D.; Abd_Allah, E.F.; Wu, Q.-S.; et al. Agronomic Practices Alter Regulated Effects of Easily Extractable Glomalin-Related Soil Protein on Fruit Quality and Soil Properties of Satsuma Mandarin. *Agronomy* **2023**, *13*, 881. https://doi.org/10.3390/agronomy13030881

Academic Editor: Salvatore Camposeo

Received: 28 February 2023
Revised: 11 March 2023
Accepted: 13 March 2023
Published: 16 March 2023

1. Introduction

Citrus plants are cultivated in more than 140 countries around the world with an annual production of 124.3 million tons, and its fruits are rich in various ingredients such as carotenoids, vitamins, essential oils, coumarins, sugars, etc. [1]. Satsuma mandarin

(*Citrus unshiu* Marc.) is a very popular, fresh variety of citrus due to its easy peel, seedlessness, and high nutritional value [2]. The Wase Satsuma Oita 4 cultivar (*C. unshiu* cv. Oita 4) is an early-ripening Satsuma mandarin selected from 'Oita Wase'. This variety is harvested in mid to late August with a high yield, good stability, and beautiful fruit shape, but the quality of the fruit is relatively poor because of the early harvest [3]. Therefore, it is an urgent task to improve the fruit quality of "Oita 4".

Citrus roots can be colonized by soil arbuscular mycorrhizal fungi (AMF) to form mutualistic arbuscular mycorrhizae, which in turn help citrus obtain more water and nutrients from the soil [4,5]. AMF spores and hyphae release a recalcitrant, hydrophobic, and heat-stable glycoprotein called glomalin, which is deposited in the soil and defined as glomalin-related soil protein (GRSP). It is known that GRSP contains a large number of elements such as C, H, O, N, P, K, Fe, Ca, Mg, Si, and so on [6]. In addition, GRSP also contains several functional groups including carbohydrates (3–6%), proteins (30–40%), and aliphatic groups (20–70%) [6]. The presence of GRSP in the soil has attracted significant attention for its role in promoting the formation of soil aggregates, maintaining the stability of soil aggregates, increasing the soil organic carbon (SOC) pool, improving plant tolerance, and reducing the toxicity of heavy metals in the soil [7]. The C in GRSP contributes $23.26 \pm 2.67\%$ of the total SOC [8]. As a result, GRSP can be used as a gluing agent to cement microaggregates into macroaggregates, thus improving the soil structure [9]. On the other hand, GRSP is attached to the surface of soil aggregates, and its hydrophobicity reduces the loss of water within the aggregates, which is conducive to soil water retention [10]. In addition, the turnaround time of GRSP reaches 6–42 years, thus increasing the soil C sequestration and subsequent soil health [6].

Because of its richness in various elements, easily extractable GRSP (EEG), an active component of GRSP, seem to be used as a biostimulant for plant growth and soil fertility promotion [11–14]. The foliar application of EEG on potted trifoliate orange plants could improve growth performance and drought resistance under drought conditions [12]. This improvement in growth is closely associated with the regulation of chlorophyll, auxins, and cytokinin concentrations by EEG [13]. In addition, in sweet oranges (Lane Late navel orange and Rhode Red Valencia), the foliar application of EEG elevated vitamin C and sugar concentrations and the solid–acid ratio in fruits, along with promoting a better coloration value in Rhode Red Valencia [14]. Nevertheless, another GRSP fraction, difficultly extractable GRSP (DEG) dramatically inhibited the plant growth of trifoliate orange seedlings [13]. It seems that among GRSP fractions, EEG has a certain improvement effect on citrus fruit quality, but existing evidence is still lacking.

In the field, some agronomic strategies, including fruit bagging (FB), reflective film mulching (RF), and grass-proof cloth mulching (GPC) on the ground can partly improve the fruit quality of citrus [15,16]. Wang et al. [17] found that FB pomelo (*C. grandis*) fruits were bright in fruit coloring with fine and smooth oil bubbles. As reported by Zhang [18], citrus orchard can use suitable reflective materials to cover the ground at the appropriate time, which can promote fruit coloring and sugar accumulation. In sweet orange (Navelina cultivar) trees, RF with kaolin and calcium carbonate increased tree yield, fresh weight, and total soluble solids of fruits [15]. GPC treatment has good air permeability and water permeability, which can effectively control weed growth in the field, reduce soil water evaporation, and improve crop yield [19].

Our previous studies have shown that the foliar application of EEG could partially improve the fruit quality of "Oita 4" [20]. It is unclear whether and how these agronomic practices in combination with the foliar application of EEG improve citrus fruit quality and soil properties. The aim of this study was to evaluate the effects of foliar spraying of EEG, combined with FB, GPC, and RF on the intrinsic and extrinsic fruit quality and soil fertility of "Oita 4", in order to screen a combination for the field application of "Oita 4".

2. Materials and Methods

2.1. Preparation of Exogenous EEG Solution

EEG was extracted using the protocol of Wu et al. [21]. The 4 g air-dried soil from the Experimental Statement, College of Horticulture and Gardening, Yangtze University, was extracted with 32 mL of 20 mmol/L sodium citrate buffer (pH 7.0) at 103 KPa and 121 °C for 30 min and centrifuged at 8500× g/min for 10 min. The supernatant was collected as the stock solution of exogenous EEG, which was stored at 4 °C for less than one month. Subsequently, the Bradford [22] assay was used to determine the protein concentration of the stock EEG solution, in which 17.5 mg protein/L was achieved. The original EEG solutions were diluted with an equal volume of 20 mmol/L sodium citrate buffer (pH 7.0) using exogenous EEG solutions.

2.2. Citrus Materials and Treatment Applications

The citrus materials, Satsuma mandarin "Oita 4" trees, were selected from an orchard (30°40′40″ N, 111°28′31″ E) in Yichang (Hubei, China) with contour terrace planting and eight-year-old trees, in which trifoliate orange was used as the rootstock. Forty trees with uniform growth vigor were selected and divided into five groups for the following treatments: (i) without any additional treatment (Control); (ii) foliar spray with EEG (EEG); (iii) foliar spray with EEG in combination with FB treatment (EEGP+FB); (iv) foliar spray with EEG in combination with RF treatment (EEG+RF); (v) foliar spray with EEG in combination with GPC treatment (EEG+GPC). Each treatment was replicated four times, with each replicate containing two trees in a randomized block arrangement.

On 22 June 2021, the EEG treatment was performed, because the physiological fruit fall of "Oita 4" was over. Based on the results of Meng et al. [14], the EEG application was performed by foliar spraying 1 L of EEG solution per tree in the canopy, and this was repeated weekly for a total of three times. After the initial EEG spraying was completed, RF and GPC were implemented. Among them, the RF is silver-black, provided by Shandong Linyi Xiantong Plastic Products Co., Ltd. (Linyi, China), PE material, 125 × 1.2 m in length and width, and 0.012 mm in thickness; the GPC is black, made of a PLA fiber material, 125 × 1.2 m in length and width, and provided by Jiangxi Weijun Technology Co., Ltd. (Ganzhou, China). FB was carried out on 28 July 2021. The FB was purchased from Shandong Yipin Jinnong Packaging Co., Ltd. (Yantai, China). A paper bag with yellow on the outside and black on the inside was obtained, each containing 36 g and length and width of 160 × 198 mm. No additional fertilizer was added after the use of EEG and these agronomic measures.

At the commercial maturity stage of "Oita 4" fruits (12 September 2021), 20 fruits were harvested from each treated tree. At the same time, the 0–5 cm soil under the tree canopy was removed, and fine roots and soils at 5–15 cm soil depth were collected and brought back to the laboratory for determination of relevant variables on the same day.

2.3. Determinations of Variables

A vernier caliper (Guanglu 0–150, Guilin, China) was used to determine the longitudinal and transverse diameter of the fruit and the thickness of the peel. Individual fruit weight as well as pulp weight were weighed by an electronic balance (ASC-30, Jinhua, China). Fruit coloration values were determined by a colorimeter (CR10, Minolta, Japan), and fruit hardness was determined by a sclerometer (GY-B, Hangzhou, China). The soluble solids content of fruits was assayed using a portable refractometer (WYT-4, Quanzhou, China). Fruit titratable acids content was measured by the titration method [23].

The fruit was divided into the fruit peel, fruit pith, segment membrane, and juice sac, and their glucose, fructose, and sucrose concentrations were carried out as per the method described by Wu et al. [23].

Root AMF colonization was determined according to the method by Phillips and Hayman [24] with the staining with 0.05% trypan blue, and then root AMF colonization rate was calculated using the formula described by Liu et al. [25]. EEG and difficultly extractable glomalin-related soil protein (DEG) concentrations were determined according to the method by Wu et al. [23], as described above. Soil water–stable aggregate distribution in the size of 0.25–0.5, 0.5–1, 1–2, and 2–4 mm was analyzed according to the wet sieve method [26] using a Soil Aggregate Analyzer (DM200, Shanghai, China), and then the mean weight diameter (MWD) was calculated as an indicator of soil aggregate stability according to the method by Kemper and Rosenau [27].

SOC content was determined based on the potassium dichromate external heating method [28]. Soil NH_4^+-N, NO_3^--N, Olsen P, and available K contents were analyzed using a Soil Nutrient Detector (HM-TYD; Weifang, China), based on the user's manual.

2.4. Data Analysis

The data were statistically analyzed using an analysis of variance (ANOVA) with SAS software. Significant ($p < 0.05$) differences were compared using the Duncan's new multiple range test. Percentage data including root AMF colonization rate, distribution of WSAs, titratable acids content, and soluble solids content were subjected to the arcsine transformation before analysis.

3. Results

3.1. Changes in Root AMF Colonization

Native AMF colonization was observed in the roots of "Oita 4" trees (Figure 1a). The control trees demonstrated 17.84% of the root AMF colonization rate, while exogenous EEG application significantly increased the root AMF colonization rate by 73.65% (Figure 1b). Although EEG+FB, EEG+RF, and EEG+GPC treatments also increased the root AMF colonization by 48.32%, 34.53%, and 39.18% compared to the Control, the application of FB, RF, and GPC dramatically inhibited the root AMF colonization of EEG-treated trees, along with there being no differences among the three treatments.

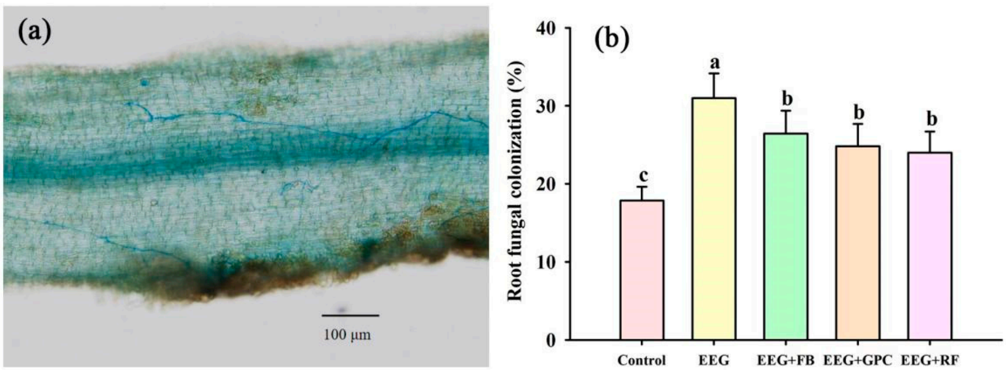

Figure 1. Root AMF colonization (**a**) and effects of foliar spraying easily extractable glomalin-related soil protein in combination with agronomic strategies on the root AMF colonization rate (**b**) of Satsuma mandarin "Oita 4" trees. Data (means ± SD, $n = 4$) are significantly ($p < 0.05$) different if followed by different letters above the bars. Abbreviations: Control, the trees in which no external treatments were applied; EEG, the trees in which easily extractable glomalin-related soil protein was applied; EEG+FB, the trees in which easily extractable glomalin-related soil protein was applied in combination with fruit bagging; EEG+GPC, the trees in which easily extractable glomalin-related soil protein was applied in combination with grass-proof cloth mulching; EEG+RF, the trees in which easily extractable glomalin-related soil protein was applied in combination with reflective film mulching.

3.2. Changes in the External Quality of Fruits

Foliar spraying EEG, singly or in combination with agronomic practices, produced significant changes in fruit appearance (Figure 2). Among the treatments, EEG and EEG+FB treatments significantly improved the fruit coloration value by 9.16% and 18.47%, respectively, compared to the Control (Table 1). EEG+RF, EEG+GPC, EEG, and EEG+FB treatments all significantly reduced fruit hardness by 49.73%, 48.15%, 38.05%, and 30.11%, respectively, compared to the Control. The single EEG treatment combined with agronomic practices also increased fruit size, where EEG and EEG+GPC both significantly increased longitudinal diameter by 8.10% and 5.02% and transverse diameter by 9.56% and 6.55%, respectively. Compared with the Control, EEG, single or in combination with FB, RF, and GPC, distinctly increased fruit peel, fruit pulp, and single fruit weight by: 33.14%, 27.71%, and 20.94% under EEG conditions; 10.44%, 14.43%, and 5.88% under EEG+FB conditions; 29.05%, 28.35%, and 23.53% under EEG+RF conditions; and 27.00%, 19.44%, and 12.93% under EEG+GPC conditions, respectively. Overall, the EEG+RF and EEG treatments represented relatively stronger effects in improving the external quality of fruits than other treatments.

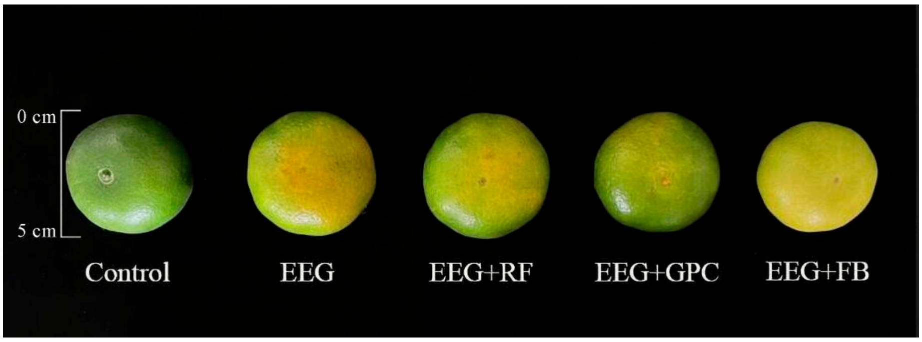

Figure 2. Changes in the fruit appearance of Satsuma mandarin "Oita 4" trees treated by foliar spraying easily extractable glomalin-related soil protein in combination with agronomic strategies. The abbreviations are shown in Figure 1.

Table 1. Effects of foliar spraying easily extractable glomalin-related soil protein in combination with agronomic strategies on the external quality of fruits in Satsuma mandarin "Oita 4".

Treatments	Coloration Value	Hardness (kg $\times 10^5$/cm^3)	Fruit Size (mm)		Weight (g/Fruit)		
			Longitudinal Diameter	Transverse Diameter	Pulp	Peel	Single Fruit
Control	53.70 ± 0.88 c	31.09 ± 3.91 a	44.84 ± 1.39 c	60.47 ± 2.26 c	75.06 ± 7.19 c	15.42 ± 1.23 b	96.82 ± 8.45 d
EEG	58.62 ± 2.78 b	16.12 ± 1.30 d	48.47 ± 1.45 a	66.25 ± 2.35 a	95.86 ± 7.98 a	20.53 ± 2.34 a	117.09 ± 13.19 ab
EEG+FB	63.62 ± 2.57 a	21.73 ± 0.99 b	46.29 ± 1.02 bc	61.27 ± 3.27 c	85.89 ± 12.11 b	17.03 ± 3.12 b	102.51 ± 7.76 cd
EEG+GPC	53.92 ± 0.73 c	19.26 ± 1.11 c	47.09 ± 0.96 ab	64.43 ± 1.16 ab	89.65 ± 4.36 ab	19.58 ± 1.47 a	109.34 ± 2.20 bc
EEG+RF	55.45 ± 1.68 c	15.63 ± 2.13 d	47.13 ± 1.33 ab	62.16 ± 2.05 bc	96.34 ± 4.62 a	19.90 ± 1.65 a	119.60 ± 9.48 a

Note: Different letters indicate significant differences at the $p < 0.05$ level. The abbreviations are shown in Figure 1.

3.3. Changes in the Internal Quality of Fruits

Compared with the Control, treatments with EEG+RF, EEG, EEG+GPC, and EEG+FB significantly increased fruit vitamin C content by 86.30%, 56.16%, 23.29%, and 47.95%, respectively, while they reduced fruit titratable acids content by 46.47%, 45.00%, 26.67%, and 40.00%, respectively (Table 2). In addition, EEG+RF, EEG, and EEG+FB all increased fruit soluble solid content by 22.22%, 13.38%, and 11.9%, respectively, although there was no change under EEG+GPC conditions. Similarly, compared with the Control, treatments with EEG+RF, EEG+GPC, EEG, and EEG+FB significantly increased fruit soluble solids/titratable acids content by 112.46%, 33.78%, 94.18%, and 64.20%, respectively.

3.4. Changes in Fruit Sucrose Concentrations

In the fruit peel, a single EEG application did not significantly affect sucrose concentrations, and only EEG+RF treatment significantly increased sucrose concentrations of fruit peels by 31.21% compared with the Control (Figure 3a). In the fruit pith, foliar application of EEG significantly increased sucrose concentrations by 21.49%, while the combination of exogenous agronomic measures slightly reduced sucrose concentrations, along with no difference being observed compared with the Control. Foliar application of EEG, singly or in combination with agronomic strategies, collectively did not affect sucrose concentrations when compared with the Control. In the juice sac, EEG+RF and EEG significantly increased sucrose concentrations by 34.48% and 20.50%, respectively, compared with the Control.

Table 2. Effects of foliar spraying easily extractable glomalin-related soil protein in combination with agronomic strategies on the internal quality of fruits in Satsuma mandarin "Oita 4".

Treatments	Vitamin C (mg/g)	Titratable Acids (%)	Soluble Solids Content (%)	Soluble Solids/ Titratable Acids
Control	10.95 ± 1.02 d	0.60 ± 0.01 a	8.82 ± 0.20 c	15.81 ± 1.33 d
EEG	17.1 ± 1.43 b	0.33 ± 0.04 cd	10.00 ± 0.21 b	30.70 ± 2.16 a
EEG+FB	16.2 ± 0.49 b	0.36 ± 0.01 c	9.87 ± 0.77 b	25.96 ± 1.43 b
EEG+GPC	13.5 ± 0.77 c	0.44 ± 0.01 b	9.15 ± 0.66 c	21.15 ± 1.53 c
EEG+RF	20.4 ± 0.49 a	0.32 ± 0.20 d	10.78 ± 0.44 a	33.59 ± 2.89 a

Note: Different letters indicate significant differences at the $p < 0.05$ level. The abbreviations are shown in Figure 1.

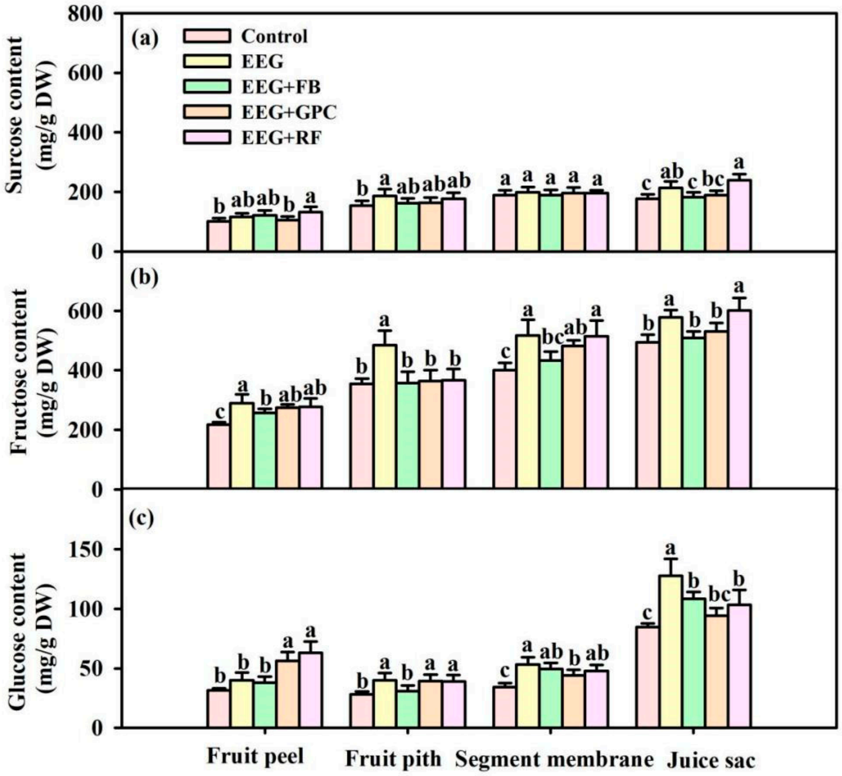

Figure 3. Effects of foliar spraying easily extractable glomalin-related soil protein in combination with agronomic strategies on sucrose (**a**), fructose (**b**), and glucose concentrations (**c**) of fruits in Satsuma mandarin "Oita 4". Data (means ± SD, $n = 4$) are significantly ($p < 0.05$) different if followed by different letters above the bars. The abbreviations are shown in Figure 1.

3.5. Changes in Fruit Fructose Concentrations

In the fruit peel, EEG, applied singly or in combination with three agronomic strategies, collectively elevated fructose concentrations by 17.94–33.21% compared with the Control, with the highest positive effect being with single EEG treatment (Figure 3b). In the fruit pith, only EEG among the other treatments significantly increased fructose concentrations by 36.55% compared with the Control. In the segment membrane, EEG, EEG+RF, and EEG+GPC distinctly elevated fructose concentrations by 29.28%, 28.55%, and 20.28% compared with the Control. In the juice sac, EEG+GPC and EEG+FB did not alter fructose concentrations, while EEG and EEG+RF dramatically increased fructose concentrations by 17.08% and 21.66%, respectively, compared with the Control.

3.6. Changes in Fruit Glucose Concentrations

In the fruit peel, EEG and EEG+FB treatments did not significantly affect glucose concentrations, but EEG+RF and EEG+GPC treatments significantly increased glucose concentrations by 99.84% and 78.19%, respectively, compared to the Control (Figure 3c). In the fruit pith, EEG+FB treatment did not change the glucose concentration but EEG, EEG+GPC, and EEG+RF all significantly increased the glucose concentration by 41.72%, 37.44%, and 38.86%, respectively, compared with the Control. In the segment membrane, EEG, alone or in combination with three agronomic strategies, distinctly elevated glucose concentrations by 28.29–55.22%, while EEG presented the greatest impact. In the juice sac, EEG alone or in combination with agronomic strategies, to some extent, increased glucose concentrations by 11.00–50.55%, with the best effect being demonstrated by single EEG application.

3.7. Changes in Soil Properties

EEG, alone or in combination with agronomic strategies, improved soil fertility and structure to some extent (Table 3). EEG, alone or in combination with agronomic strategies, significantly increased SOC by 29.69–62.14%, with EEG showing the best effect; these treatments also significantly increased EEG concentrations by 65.52–131.03% and DEG concentrations by 59.68–82.26%, with exogenous EEG treatment showing the most prominent effect. Among these treatments, only the EEG treatment significantly elevated the MWD by 35.86% compared with the Control. In addition, EEG alone or in combination with agronomic measures significantly increased NH_4^+-N, NO_3^--N, Olsen P, and available K concentrations by 68.10–167.62%, 57.19–139.94%, 18.51–131.87%, and 126.75–412.40%, respectively, all of which experienced the most prominent effects with the single EEG treatment. The addition of agronomic practices reduced the positive effect of EEG on improvements to soil fertility to varying degrees.

Table 3. Effects of foliar spraying easily extractable glomalin-related soil protein in combination with agronomic strategies on soil fertility and structure in Satsuma mandarin "Oita 4" trees.

Treatments	SOC (mg/g)	MWD (mm)	EEG (mg/g)	DEG (mg/g)	NH_4^--N (mg/kg)	NO_3^--N (mg/kg)	Olsen P (mg/kg)	Available K (mg/kg)
Control	12.73 ± 0.4 d	1.98 ± 0.19 b	0.29 ± 0.04 c	0.62 ± 0.09 c	56.20 ± 1.86 d	83.77 ± 3.27 e	80.92 ± 4.20 e	108.68 ± 4.47 e
EEG	20.64 ± 0.79 a	2.69 ± 0.42 a	0.67 ± 0.07 a	1.13 ± 0.11 a	150.40 ± 6.20 a	201.00 ± 7.04 a	187.63 ± 8.07 a	556.88 ± 9.36 a
EEG+FB	17.54 ± 0.69 b	2.16 ± 0.52 ab	0.52 ± 0.05 b	1.06 ± 0.13 ab	104.50 ± 2.63 b	149.95 ± 6.49 c	108.53 ± 4.71 c	490.03 ± 3.34 b
EEG+GPC	17.02 ± 0.34 bc	2.01 ± 0.50 b	0.54 ± 0.04 b	0.91 ± 0.18 b	94.47 ± 5.46 c	168.58 ± 3.95 b	95.90 ± 3.45 d	246.43 ± 5.50 d
EEG+RF	16.51 ± 0.56 c	2.24 ± 0.36 ab	0.48 ± 0.05 b	0.99 ± 0.14 ab	100.77 ± 4.15 b	131.68 ± 6.78 d	141.98 ± 4.08 b	366.30 ± 10.53 c

Note: Different letters indicate significant differences at the $p < 0.05$ level.

3.8. Correlation Analysis

Root AMF colonization was significantly and positively correlated with soil EEG, DEG, MWD, SOC, NH_4^+-N, NO_3^--N, Olsen P, and available K, respectively (Table 4). MWD was significantly and positively correlated with EEG, SOC, NH_4^+-N, NO_3^--N, Olsen P, and available K, respectively. SOC was significantly and positively correlated with EEG and DEG.

Table 4. The correlation between AMF colonization and soil properties ($n = 20$).

	AMF Colonization	EEG	DEG	MWD	SOC
AMF colonization	1	0.81 **	0.66 **	0.49 *	0.83 **
MWD	0.49 *	0.82 **	0.31	1	0.46 *
SOC	0.83 **	0.92 **	0.77 **	0.46 *	1
NH_4^+-N	0.85 **	0.85 **	0.79 **	0.51 *	0.95 **
NO_3^--N	0.81 **	0.92 **	0.72 **	0.49 *	0.94 **
Olsen P	0.70 **	0.73 **	0.63 **	0.54 *	0.80 **
Available K	0.80 **	0.79 **	0.79 **	0.47	0.88 **

Note: *, $p < 0.05$; **, $p < 0.01$.

4. Discussion

In this study, foliar application of EEG alone significantly improved the root AMF colonization rate in Satsuma mandarin "Oita 4" trees. Liu et al. [20] also reported that EEG application significantly improved the root mycorrhizal colonization rate and soil mycelial length in Newhall orange and "Oita 4", but not Cocktail grapefruit, suggesting that the improvement of the root mycorrhizal colonization rate by EEG is specific to citrus species. EEG had been detected to contain 0.94−1.26 mg organic C/g EEG [5,29], thus providing the C source for the root mycorrhizae. In addition, EEG is rich in mineral elements, such as Fe and Mg [13], which contribute to the formation of chlorophyll and subsequently, the synthesis of photosynthates, thus promoting mycorrhizal formation in roots and soil. In contrast, the addition of agronomic practices including FB, RF, and GPC significantly suppressed the improved effect of EEG on root mycorrhizal colonization rate, because these measures reduced the carbohydrates allocated by host plants to the root for mycorrhizal fungi. Likewise, they may have negatively affected root growth, thus reducing root mycorrhizal colonization. However, the exact mechanism is unclear and needs to be investigated.

The results of this study also showed that foliar spraying of EEG significantly increased fruit size, weight, and coloration value, but reduced fruit hardness, which is consistent with previous studies on navel oranges such as Newhall and Lane Late [14,20]. This is because the EEG is rich in elements that are beneficial to fruit growth, such as K [13]. In addition, FB addition further increased the fruit coloration value, because FB can make the citrus peel delicate, smooth, and brightly colored [30]. FB, GPC, and RF combined with EEG reduced fruit size and weight to varying degrees compared with the EEG treatment alone. Feng et al. [31] reported no significant effects of FB on the fruit weight of Malyasia Sweet Pummelo. RF application did not significantly alter fruit weight, which is in agreement with Gao et al. [32] who studied Satsuma mandarin Miyagawa treated with vapor-permeable reflective film mulching.

EEG as a biostimulant has shown positive effects on fruit quality [14,20]. The results of this study showed that exogenous EEG significantly increased fruit vitamin C and soluble solids content and reduced titratable acids content, thus maintaining a higher soluble solids/titratable acids content compared to the Control. In addition, EEG-treated fruits of "Oita 4" recorded relatively higher sucrose, glucose, and fructose concentrations in the juice sac. Liu et al. [20] reported positive effects of foliar spraying EEG on soluble solids contents of cocktail grapefruit. Meng et al. [14] also observed the increase in vitamin C, sucrose, fructose, glucose, and soluble solids/titratable acids in Lane Late navel orange fruits after EEG application. In addition, RF combination with EEG further elevated vitamin C and soluble solids content more than single EEG treatments. Similar results were also reported by Gao et al. [32] in Satsuma mandarin Miyagawa after vapor-permeable reflective film mulching. In fact, the mulching of reflective film in citrus trees provides diffuse reflection so that the fruit surface receives uniform light, promoting sugar accumulation, and thus fruit coloring in advance [32,33]. EEG+FB distinctly reduced soluble solids/titratable acids because FB reduces the fruit's chance of receiving external light, which inhibits sugar accumulation in fruits [20,34,35]. By comprehensive analysis, it can be concluded that EEG in combination with RF has a relatively prominent effect on the improved fruit quality of "Oita 4", which can be considered in the cultivation of citrus, or at least "Oita 4".

EEG has many potential functions in soil, playing a particularly important role in C and N nutrient cycling of soil [8,20,29]. SOC is one of the decisive factors in evaluating soil quality, and MWD is an essential indicator of the stability of soil aggregates [36]. The results of this study showed that the EEG-treated soil of citrus trees recorded a significantly higher SOC compared to the Control. Liu et al. [13] reported higher total leaf chlorophyll levels in trifoliate orange seedlings after application of EEG. As a result, EEG application promoted root mycorrhizal colonization rate. Therefore, the enhancement of root mycorrhizal colonization would enable more plant C to be transferred to the mycorrhizal mycelium, after which it is then released into the soil using the GRSP form. Foliar spraying EEG dramatically increased soil EEG concentrations, resulting in an increase in SOC, with EEG being an important source of SOC [6,9]. Similarly, foliar application of EEG also increased MWD, NH_4^+-N, NO_3^--N, Olsen P, and available K concentrations. The developed mycorrhizae in EEG-treated plants could secrete more organic acids or catabolic enzymes into the rhizosphere soil, which could then accelerate soil mineralization and allow more release of soil nutrients from insoluble conditions [37], as seen in the significantly ($p < 0.01$) positive correlation between root AMF colonization and NH_4^+-N, NO_3^--N, Olsen P, and available K concentrations. However, the combination of EEG with RF, GPC, and FB significantly reduced the effect of a single EEG application on improvements in soil properties. This may be because these agronomic practices reduce root mycorrhizal colonization rate, and on the other hand, they may slow nutrient mineralization. More experiments are needed to decipher this effect.

5. Conclusions

EEG as a foliar sprayed biostimulator showed a strong positive effect on the improvement of the internal and external quality of Satsuma mandarin "Oita 4" fruits. The improvement was associated with an increase in the root mycorrhizal colonization rate and soil structure and fertility by EEG. In general, the additional agronomic practices made little change or even suppressed the positive effects of EEG, though RF combined with EEG could further increase the internal fruit quality such as vitamin c, soluble solids, and soluble solids/titratable acids. There are still some limitations to the conclusion of this study, as the experiment was only conducted for one year. Many years of experiments will need to be performed to better understand the effects of EEG in combination with agronomic practices.

Author Contributions: Conceptualization, Q.-S.W.; methodology, A.-Q.L.; Material preparation, A.-Q.L., Q.-H.Y., Y.Z. (Yi Zhang), W.-Y.L., Y.Z. (Ying Zhang) and Y.-C.X.; data determination and statistical analysis, A.-Q.L.; writing—original draft preparation, A.-Q.L.; writing—review and editing, A.K.S., A.H., Y.Z. (Yi Zhang), M.D.A., E.F.A. and Q.-S.W.; supervision, Q.-S.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Hubei Agricultural Science and Technology Innovation Action Project (Hubei Nongfa [2018] No. 1). The authors extend their appreciation to the Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2023R355), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

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

Acknowledgments: The authors extend their appreciation to the Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2023R355), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

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

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