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Field Inoculation of Arbuscular Mycorrhizal Fungi Improves Fruit Quality and Root Physiological Activity of Citrus

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Abstract: Soil arbuscular mycorrhizal (AM) fungi form a mutualistic symbiosis with plant roots and produce many benefits on host plants under potted conditions, while field inoculation of AM fungi on citrus (a woody plant) has been rarely reported. The present study aimed to analyze the changes in mycorrhizal growth, root vitality, and fruit quality of *Citrus reticulata* Blanco var. Ponkan mandarin cv. Jinshuigan grafted on *Poncirus trifoliata* L. after inoculation with a mix of AM fungi (*Diversispora versiformis*, *Funneliformis mosseae*, and *Rhizophagus intraradices*) and single *F. mosseae*. After the second year of AM fungal inoculations, root mycorrhizal colonization (%), root vitality, hyphal length in soil, and easily extractable glomalin-related soil protein content were significantly increased, while difficult-to-extract glomalin-related soil protein content was decreased. Two mycorrhizal fungal inoculation treatments collectively improved fruit quality parameters such as polar diameter, equatorial diameter, the weight of single fruits, fruit peel, and sarcocarp, coloration value, and soluble solids content. Our study, therefore, suggested that field inoculation with AM fungi improved root physiological activities in terms of mycorrhizal growth and root vitality and thus improved fruit quality. The effect of mixed-AM treatment was more significant than that of *F. mosseae* alone.

Keywords: citrus; fruit; glomalin; mycorrhizal inoculation; nutrient acquisition

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1. Introduction

Arbuscular mycorrhizal (AM) fungi are beneficial soil microorganisms widely existing in nature colonizing the roots of about 80% of land plants including cereals, vegetables, and fruit trees [1]. Arbuscular mycorrhiza is a mutual–reciprocal symbiosis between AM fungi and host plants [2], where the host plant provides photosynthates for the growth of AM fungi, and AM fungi expand the absorption area of roots through extraradical hyphae and deliver water and nutrients to the host plant [3,4]. AM hyphae also produce a water-insoluble protein (glomalin) in soil (defined as glomalin-related soil protein GRSP), generally associated with insoluble humus or mineral fractions and contributing to stabilize soil aggregates [5]. Moreover, GRSP is considered to contribute to pools of soil organic carbon and providing positive impacts on plant growth [6,7]. The benefits that such AM fungi bring to host plants provide new pathways for the improvement of plant growth as well as sustainable agriculture and environmental management [8].

Citrus is a widely grown fruit tree globally with rare and short root hairs, heavily dependent upon the AM symbiosis to promote nutrient acquisition [9,10]. Generally, the

AM fungal colonization rate of citrus roots in the field is quite low, less than 20%, due to poor soil texture, environmental factors, and genetic factors, which may be a limiting factor affecting the high yield and good fruit quality of citrus [11]. Earlier studies have demonstrated that AM fungi had a mitigating effect on potted plants under soil moisture deficit [12,13] or under heavy metal pollution of soil [14,15]. Regvar et al. [16] applied AM fungi in several industrial crops in the field. They found that AM fungi could increase the carotenoid content of carrot and tomato fruits, but also increase the chlorophyll content of coriander leaves. Similarly, Laura et al. [17] found that AM fungi effectively increased iron (Fe) and zinc (Zn) content of wheat grains. Other studies showed that AM fungi inoculation increased the content of starch and soluble carbohydrates in the leaves and roots of palm [18]. Paskovic et al. [19] analyzed the field response of AM fungal inoculation to fruit quality of tomato. They found an increase in total nitrogen (N), magnesium (Mg), and hydrophilic phenol contents in fruit. However, the application of AM fungi to plants in the field and further evaluation of its benefits are limited, especially in citrus plants [20].

The present study aimed to analyze the effect of AM fungi on the fruit quality of citrus in the field, to provide data-supported evidence for the applicability of AM fungi inoculation in citriculture.

2. Materials and Methods

2.1. Experimental Setup

The three-year-old *Citrus reticulata* Blanco var. Ponkan mandarin cv. Jinshuigan grafted on *Poncirus trifoliata* L. Raf. was selected and grown in a citrus orchard located in Jingzhou (30°21′27″ N, 112°3′5″ E), Hubei, China. The trees in the citrus orchard had an inter-planting spacing of 3 × 4 m. The soil in the orchard is classified as Xanthi-Udic-Ferralsol according to the FAO system, and characterized by contents of available N 70.10 mg/kg, Bray-extractable P 40.47 mg /kg, available K 271.12 mg/kg, and organic carbon 9.88 g/kg. In March 2018, the inoculation of exogenous AM fungi was carried out. Two inoculations, including single *Funneliformis mosseae* and a mixture of *Diversispora versiformis*, *F. mosseae*, and *Rhizophagus intraradices* were carried out. These AM fungi were provided by the Bank of Glomeromycota in China (BGC; Beijing, China). Meanwhile, the *F. mosseae* strain (BGC XJ02A), *D. versiformis* strain (BGC BJ08), and *R. intraradices* strain (BGC HUN02B) were isolated from the rhizosphere of *Allium flavidum* Ledeb. in Kangsu (Xinjiang, China), *Cynodon dactylon* (L.) Pers. in Guiyang (Hunan, China), and *Lycopersicon esculentum* Miller in Beijing (China), respectively. The identified spores of AM fungi were propagated by *Trifolium repens* L. in a trap culture under greenhouse conditions (880 μmol/m^2/s photo flux density, 28 °C/20 °C day/night temperature, and 68% relative air humidity). Three months later, the plants were harvested. The shoots were removed, and the mycorrhizal inoculum provided spores (20 spores/g for *F. mosseae* and 26 spores/g for mixed AM fungi), AM fungi-colonized root segments (82% for *F. mosseae* and 90% for mixed AM fungi), and soil mycelium. These mycorrhizal inocula were stored at 4 °C for no more than six months prior to use.

Fifteen citrus trees with similar vigor growth (plant height: 168.8 ± 5.6 cm; trunk diameter: 18.9 ± 0.6 mm) were divided into three groups: non-AM fungal inoculation (non-AMF), *F. mosseae* inoculation (FM), and mixed AM fungal inoculation (mixed-AMF). Each fungal inoculation was applied to five trees. In the selected tree trunk of a horizontal distance of 40 cm in north–south direction and east–west direction, a trench with 40 cm × 15 cm × 30 cm was dug. A total of 600 g mycorrhizal inoculum was applied into the trenches adjacent to citrus roots. After the soil was backfilled, seeds (300 seeds per tree) of white clover were sown on top of the soil filling the trenches to further promote the AM fungal development. For non-AM fungal treatments, the same amount of autoclaved mycorrhizal inocula were applied as a control. No organic amendment was supplied at the time of AM fungal inoculation. After AM fungal inoculations, all the treated trees received consistent field management.

2.2. Determinations of Variables

The fruit sampling was carried out during the second year (2019) of fruit ripening in November. The twenty-five fruits were collected from different directions on each tree and immediately returned to the laboratory. The horizontal and vertical diameters of the fruit were determined with a digital vernier caliper. The weight of individual fruits was measured by an electronic balance. The color value of the fruit was calculated by a colorimeter (CR-400, Konica Minolta Sensing Inc., Osaka, Japan). The solid soluble content of the fruit was monitored by a digital refractometer (PAL-1, ATAGO Co., Tokyo, Japan). Fruit peel and sarcocarp weight was measured on the same day using a digital electronic balance.

The surface soil (0–5 cm) was removed and fine roots with a diameter of 0.5–1.0 mm of the current year were sampled in 5–15 cm of soil depth, gently shaking off the soil attached to the root being considered as rhizospheric soils. The soil and root samples were enclosed in sealed bags and taken back to the laboratory. Parts of root samples were used to analyze mycorrhizal colonization, and other root samples were utilized to determine nutrient element content.

The root samples were cut into 1-cm-long root segments, and mycorrhiza in the root segments was stained according to the protocol described by [21]. Arbuscular mycorrhiza were observed under a biomicroscope (NE610, Ningbo Yongxin Optics Co., Ltd., Ningbo, China). The root mycorrhizal colonization rate was calculated as the percentage of the colonized root-segment length versus the total observed root-segment length. Mycorrhizal hyphal length in the soil was determined according to the method described by [22]. The root vitality was determined by triphenyltetrazole chloride staining method as described by [23]. Two fractions of glomalin-related soil protein, including easily-extractable glomalin-related soil protein (EE-GRSP), and difficult-to-extract glomalin-related soil protein (DE-GRSP), were extracted and assayed collectively according to the method of [24].

2.3. Statistical Analysis

The experimental data were analyzed using the one-factor analysis of variation by SAS software. The Duncan multiple range test was used to compare significant differences between treatments ($p < 0.05$; Duncan test).

3. Results

3.1. Changes in Degree of Root Mycorrhizal Colonization, Soil Hyphal Length, and Root Vitality

In the roots of 5–15 cm soil depth, a large extent of root mycorrhizal colonization was observed in the inoculated and uninoculated citrus trees (Figure 1a–c). External AM fungal inoculation into the rhizosphere of Ponkan mandarin, to some extent, increased the degree of root mycorrhizal fungal colonization, along with the increase of 62.0% in the mixed-AMF treatment versus the non-AMF treatment (Figure 2a). On the other hand, both the FM treatment and the mixed-AMF treatment significantly increased hyphal length in soil by 60.6% and 109.2% (Figure 2b) and root vitality by 55.3% and 60.5% (Figure 2c), respectively.

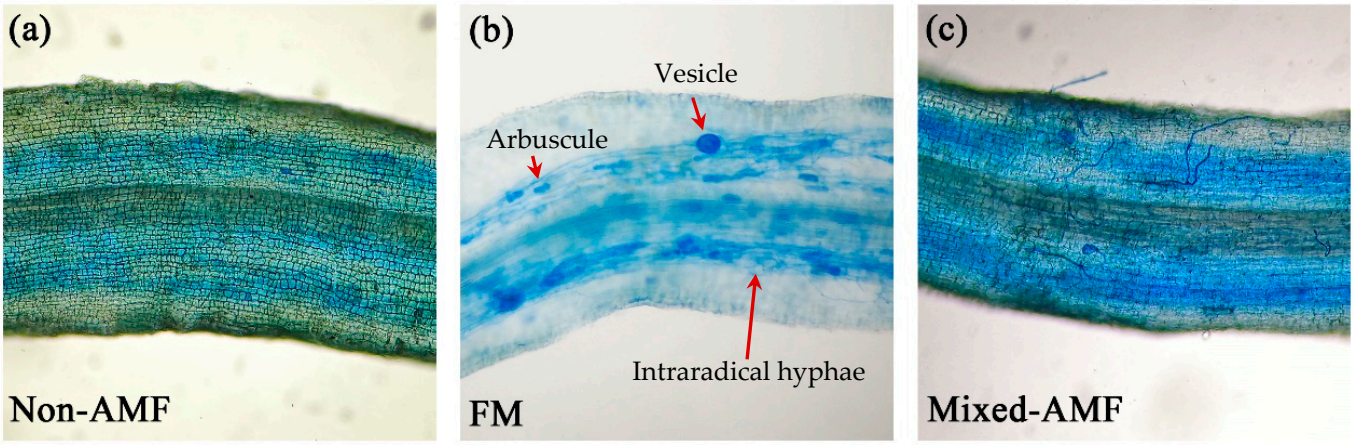

Figure 1. Mycorrhizal colonization of Ponkan mandarin roots grafted on trifoliate orange under field inoculations with non-AMF (**a**), *F. mosseae* (**b**), and mixed-AMF (**c**), respectively. The fine roots of the current year were collected from 5–15 cm soil depth and stained with trypan blue in lactoglycerol. These figures of mycorrhizal colonization were taken and used to calculate the degree of root mycorrhizal fungal colonization.

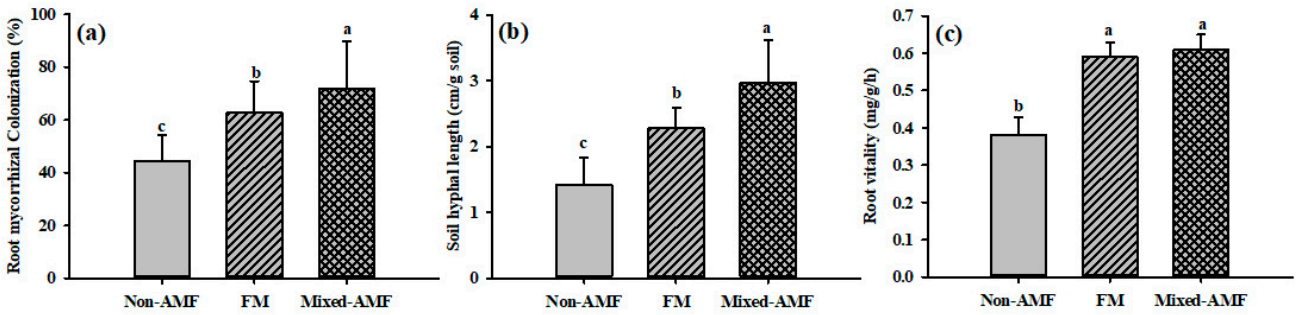

Figure 2. Effects of AMF inoculation on the degree of root mycorrhizal colonization (**a**), mycorrhizal hyphal length in soil (**b**), and root vitality (**c**) of Ponkan mandarin, grafted on trifoliate orange under field condition. Means ± SD ($n = 5$) followed by different letters at the bar indicate significant differences ($p < 0.05$) among treatments. The data were collected in 2019, the second year after AMF inoculation.

3.2. Changes in Soil GRSP Levels

Compared with non-AMF treatment, the mixed-AMF and FM inoculations significantly increased easily extractable glomalin-related soil protein (EE-GRSP) concentrations by 41.1% and 40.3%, respectively, while dramatically decreased difficult-to-extract glomalin-related soil protein (DE-GRSP) concentrations by 27.5% and 37.2%, respectively (Figure 3). There was no significant difference in EE-GRSP and DE-GRSP concentrations between mixed-AMF and FM treatments.

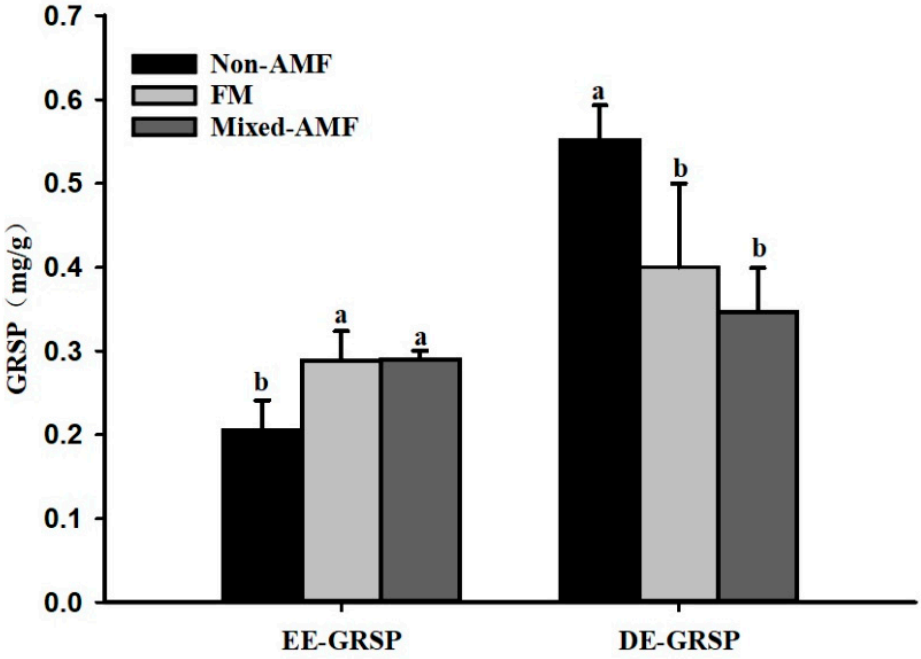

Figure 3. Effects of AMF inoculation on easily extractable glomalin-related soil protein (EE-GRSP) and difficult-to-extract glomalin-related soil protein (DE-GRSP) concentrations of Ponkan mandarin grafted on trifoliate orange under field conditions. Means ± SD (n = 5) followed by different letters at the bar indicate significant differences ($p < 0.05$) among treatments. The data were collected in 2019, the second year after AMF inoculation.

3.3. Changes in Internal and External Quality Parameters of Fruits

In the second year after AM fungi inoculation, both mixed-AMF and FM treatments showed significant improvement in the internal and external quality of fruits in Ponkan mandarin (Table 1; Figure 4). Compared with the non-AMF treatment, single fruit weight, equatorial diameter, polar diameter, fruit peel weight, sarcocarp weight, total soluble solids content, and coloration value were significantly increased by 38.8, 18.0, 14.0, 70.0, 43.1, 14.6, and 4.7%, respectively under mixed-AMF inoculation conditions, and by 32.2, 13.0, 14.2, 56.1, 36.7, 9.1, and 2.8% under FM inoculation conditions, respectively. Moreover, mixed-AMF-inoculated fruits had significantly higher equatorial diameter and fruit peel weight compared to FM inoculation alone. Root AM colonization was significantly and positively correlated with these internal and external quality parameters of fruits (Table 1).

Table 1. Effects of AMF inoculation on internal and external quality of fruits and Pearson's correlations between root AM colonization and fruit quality.

Treatments	Polar Diameter (mm)	Equatorial Diameter (mm)	Single Fruit Weight (g)	Coloration Value	Fruit Peel Weight (g)	Sarcocarp Weight (g)	Soluble Solids Content (%)
Non-AMF	32.0 ± 0.8 b	41.6 ± 1.2 c	33.2 ± 2.5 b	72.4 ± 1.3 b	7.3 ± 0.4 c	24.7 ± 2.1 b	11.5 ± 0.8 b
FM	36.5 ± 0.6 a	47.1 ± 1.1 b	43. 9 ± 2.7 a	74.4 ± 1.7 a	11.4 ± 0.9 b	33.8 ± 1.6 a	12.6 ± 0.8 a
Mixed-AMF	36.5 ± 1.2 a	49.2 ± 1.2 a	46.1 ± 2.0 a	75.8 ± 0.9 a	12.4 ± 0.5 a	35.3 ± 2.5 a	13.2 ± 0.7 a
Correlation Root AM colonization	0.65 **	0.66 **	0.62 **	0.67 **	0.59 *	0.71 **	0.54 *

Means ± SD (n = 5) followed by different letters indicate significant differences ($p < 0.05$) among treatments. * $p < 0.05$; ** $p < 0.01$. The correlation data were collected in 2019, the second year after AMF inoculation.

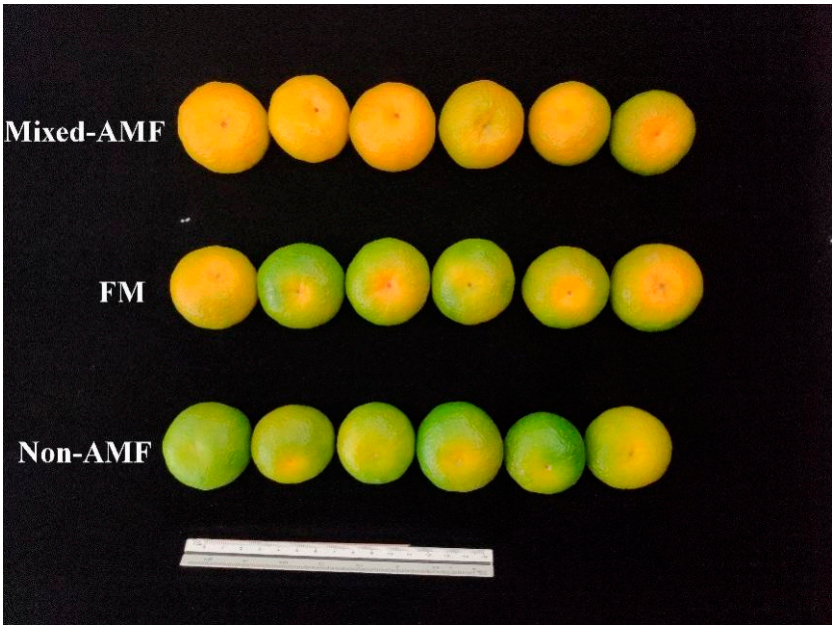

Figure 4. Changes in the appearance of fruits of Ponkan mandarin inoculated with *Funneliformis mosseae* (FM) and mixed-AMF, compared to uninoculated variety (non-AMF). The data were collected in 2019, the second year after AMF inoculation.

4. Discussion

The results of this study showed that inoculation of field-grown Ponkan mandarin with *F. mosseae* and mixed-AMF could significantly increase the root mycorrhizal colonization, soil hyphal length, and root vitality, indicating that the current inoculation method of AM fungi was successful in improving the status of citrus mycorrhizas under field conditions. The effects of mixed-AMF on fruit quality, root mycorrhizal colonization, and soil hyphal length were better than that of single FM inoculation, which may be due to a higher number of spores and AM-colonized root segments in mixed-AMF inocula versus FM inocula. Regvar et al. [16] inoculated pepper and tomato with AM fungi in the field and found a promoted effect on root mycorrhizal formation. It meant that although indigenous AM fungi existed in the field, the number of AM fungal spores was rather low and could not meet the requirements of plant roots to establish significant mycorrhizal symbiosis. Field inoculation with exotic AM fungi effectively promoted mycorrhizal formation and development in roots and soils in citrus. However, AM fungi are aerobic and determine the spatial distribution of AM fungal development for plants growing in field conditions [25]. The incompatibility of exogenous AMF inoculations with citrus roots in the field may be related to the selectivity between AMF and host species. Therefore, further study needs to screen an effective strain or a combination of AM fungi before being used in the field. We also need to analyze the temporal and spatial effects of field AM fungi inoculation on the development of root mycorrhiza of citrus trees and to evaluate the changes induced by exotic and indigenous AM fungal populations in the rhizosphere of field citrus.

Glomalin-related soil proteins (GRSP) are released into the soil by mycelia and spores of AM fungi [26]. It has been shown that EE-GRSP is readily produced by AM fungi as a stable and effective substance that helps to improve soil aggregate stability, soil moisture-holding capacity, and plant growth, while DE-GRSP is the turnover product of EE-GRSP and might contain toxic substances [27]. The colonization of AM fungi was negatively correlated with DE-GRSP content [28]. Our results showed that both FM and mixed-AMF inoculation significantly increased the content of EE-GRSP but decreased the content of DE-GRSP. This finding indicates that exotic AM fungi inoculation had the ability to improve soil structure by the increase in EE-GRSP effectively, but not DE-GRSP and to reduce growth inhibition of plants by DE-GRSP. In addition, GRSP also might contribute to

soil nutrient cycling [6,7], but whether the nutrient contribution of GRSP is related to the improved fruit quality of citrus remains to be further studied.

Studies have shown that the degree of mycorrhizal colonization was positively correlated with plant growth responses [29]. In our study, FM and mixed-AMF inoculation significantly improved fruit size, fruit weight, coloration value, and soluble solid content, whilst soluble solid content was positively correlated with root mycorrhizal colonization. Arbuscular mycorrhizas could enhance the absorption of mineral nutrients and water by citrus roots [1] and promote the synthesis of chlorophyll and photosynthetic intensity [30], thus, accelerating fruit sugar accumulation.

In our study, the field inoculation of AM fungi into the rhizosphere of citrus had certainly promoted fruit quality and mycorrhizal formation, especially the mixed-AMF showed better advantage than single FM inoculation. Crossay et al. [31] found that co-inoculation of AM fungi from different families improved plant biomass, mineral nutrition, and stress tolerance better than single AM fungal inoculation. Also, Husna et al. [32] reported that mixed-AMF inoculation was more effective on plant growth and nutrient acquisition of *Kalappia celebica* than a single inoculation. In addition, Singh et al. [33] found that inoculation of mixed AMF increased total phenols and total chlorophyll contents of *Punica granatum* more effectively than an inoculation of a single AMF. Mycorrhizal colonization and soil hyphal length were improved to a higher extent under mixed-AMF treatment compared to a single FM treatment, indicating that mixed-AMF species had a synergistic effect promoting root colonization by each other. However, the relevant interaction mechanism needs to be studied in further detail.

5. Conclusions

The results of this study strongly confirmed that field inoculation with exotic AM fungi, especially mixed-AMF, promoted fruit quality and the root physiological activity of citrus. Therefore, our study provides strong support for applying exotic AM fungi in field cultures of citrus and confers a new path for quality-increasing in citrus fruits. However, this study analyzed the effects of AM fungi on fruit quality and root physiological activity of citrus only in the second year after inoculation, and the tested trees were just four years old at the time of analysis. A long-term evaluation of field inoculations with AM fungi on citrus fruit quality and tree growth is needed, for supporting success of field inoculations and to evaluate the benefits of mycorrhizae applications in field citrus cultures.

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

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