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Article

Arbuscular Mycorrhizal Fungi as Biofertilizers to Increase the Plant Quality of Sour-Orange Seedlings

Josefa María Navarro [1,*] and Asunción Morte [2]

[1] Equipo de Riego y Fisiología del Estrés, Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental, C/Mayor s/n, La Alberca, 30150 Murcia, Spain
[2] Departamento de Biología Vegetal (Botánica), Facultad de Biología, Campus de Espinardo, Universidad de Murcia, 30100 Murcia, Spain; amorte@um.es
* Correspondence: josefam.navarro2@carm.es

Abstract: In addressing the agricultural challenges posed by climate change, the use of biofertilizers, derived from living organisms, promotes environmentally friendly crop cultivation, and represents an adaptive strategy for sustainable agriculture in the face of climate uncertainty. Careful selection of the arbuscular mycorrhizal fungus (AMF) would represent a crucial step in mycorrhizal inoculation, considering the varying levels of compatibility between the AMF and the host plant. This study aimed to assess the impact of two AMF species that are prevalent in citrus soils of south-eastern Spain (*Rhizophagus irregularis* and *Funneliformis mosseae*) on the *Citrus aurantium* seedlings' behavior. Sour-orange plants showed a high mycorrhizal dependence regardless of the specific AMF species. Both *R. irregularis* and *F. mosseae* fungi exhibited high colonization percentages, with *R. irregularis* outperforming *F. mosseae* in root colonization. Inoculation with both AMF yielded notable growth improvements, but *R. irregularis* exhibited higher positive effects in the long term. The heightened P nutrition and increased chlorophyll concentration significantly enhanced the performance of AMF-inoculated plants. With *F. mosseae*, plants showed more pronounced improvements in P nutrition and a stronger correlation of their dry mass with P concentration; however, in general, inoculation with *R. irregularis* produced a higher sour-orange-plant performance. Both *R. irregularis* and *F. mosseae* fungi produced strong positive effects in sour-orange growth, which positioned them as viable biofertilizer options. These results can contribute to enhancing understanding for the development of an improved design of biofertilizers used in regions that are vulnerable to climate change, such as south-eastern Spain. This promotes a shift towards more sustainable and environmentally friendly agricultural practices by reducing dependence on chemical fertilizers.

Keywords: *Citrus aurantium*; *Rhizophagus irregularis*; *Funneliformis mosseae*; phosphorus; mycorrhizal dependency

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1. Introduction

In the coming decades of the 21st century, and due to factors such as climate change and the deterioration of biodiversity, agriculture will face significant obstacles in order to meet the growing demand for healthy foods [1]. By 2050, global food production must double, and to address the challenge of feeding the world's growing population and ensuring food security amid climate change, there is an urgent need to transition towards agriculture that is sustainable and climate smart, as well as able to withstand environmental challenges, with agricultural intensification being considered the primary solution despite its associated environmental risks [2]. Hence, it is imperative to promote increased agricultural production while minimizing the strain on available arable lands.

In recent years, agriculture has shifted toward high-input, chemical-dependent practices that, in spite of having increased global food production, have also damaged soil and long-term crop yields, causing pollution and environmental degradation [3]. Agricultural

intensification poses environmental threats, requiring a shift to environmentally friendly practices, as we reduce the use of inorganic fertilizers and increase the use of organic fertilizers and biofertilizers [4]. Thus, biofertilizers are explored as a sustainable alternative, using soil microorganisms to optimize production while preserving environmental health [5,6].

Biofertilizers are beneficial microbial inoculants derived from the rhizosphere that contain specific microorganisms. They are advocated as a secure partial alternative to replace chemical fertilizers and pesticides, offering cost effectiveness, eco friendliness, and easy farm-level production [7]. To maximize these advantages, it is recommended that we integrate them into the overall fertilization system alongside synthetic fertilizers, aiming to enhance soil properties and sustain horticultural crop productivity [8]. Biofertilizers represent a secure and eco-friendly alternative to chemical fertilizers and pesticides, reducing environmental pollution and offering direct benefits such as increased crop yields, fruit quality, nitrogen fixation, and phosphorus solubilization, improved plant growth, enhanced plant resistance to pests and diseases, reduced costs, and improved soil vitality and soil properties, as well as enhancing and conserving natural resources [7,9].

Arbuscular mycorrhizal fungi (AMF) have been known for establishing symbiotic relationships with approximately 80% of terrestrial plant species [10]. Due to their positive impact on soil organic-matter degradation and nutrient cycling, improving soil fertility, plant nutrition, water absorption, soil-aggregate stability, salinity, and drought-stress reduction, together with overall crop growth and productivity, can be employed as bioinoculants in sustainable agriculture [11–14]. Therefore, AMF can be used as an amendment to enhance long-term soil fertility, plant nutrition, crop productivity, and yield quality, contributing to protection in agriculture and revival of agro-ecosystems [15,16]. Moreover, mycorrhizal-based products are often more cost-effective than conventional fertilizers, particularly in regions where phosphorus depletion in soils is present [16].

Citrus is one of the most important horticultural crops in the world, being widely cultivated in south-eastern Spain, where climate conditions are those typical of a semi-arid area. On the other hand, in the northern hemisphere, fresh lemon (*Citrus limon* [L.] Osbeck) is mainly produced in the Mediterranean area. Specifically, in Spain, it is grown mainly in the south-eastern region, which concentrates almost 90% of its production [17]. In this area, the Intergovernmental Panel on Climate Change (IPCC) has proposed the use of desalinated seawater (DSW) as a potential option to adapt agriculture to the impacts of climate change [18]. In the design of new plantations of lemon, the choice of appropriate rootstock genotypes plays a fundamental role in plant health, especially when citrus trees are irrigated with DSW [19]. This is due to the fact that this water has high concentrations of Na^+, Cl^-, and boron [20], and citrus trees are sensitive to high concentrations of these elements [21,22]. Among commercial citrus rootstocks, sour orange (*Citrus aurantium* L.) is considered to be salt tolerant when compared with other rootstocks that are commonly used [23,24], which adds to a good tolerance to high B concentrations [25]. Moreover, sour orange is one of the rootstocks that are more frequently used in lemon-tree orchards [26], and, due to its low vigor compared with other more vigorous rootstocks, it confers a higher water-stress tolerance, and is recommended in regions where the availability of water is not assured [27]. In addition to its relevance as a rootstock in lemon plantations, sour orange is also important due to the bioactivity of its secondary metabolites, or phytochemicals, with properties that are of pivotal importance to human health, comprising, among others, anti-cancer, antiproliferative, hypolipidemic, and cardio-protective activities [28].

On the other hand, different studies indicate that citrus rootstocks show a broad variation in their mycorrhizal dependency [29,30], and the selection of AM fungi for a particular citrus rootstock under specific edaphic conditions may be necessary [31]. Although AM fungi are not host specific, previous studies have shown that mycorrhizal fungi vary widely in their effectiveness [31]. In the major citrus-growing regions of eastern Spain, *F. mosseae* and *R. irregularis* were the most prevalent AMF associated with citrus roots, with *R. irregularis* being the most efficient fungus in promoting growth due to its rapid colonization ability [32], which facilitates the formation of an extensive and

effective network of external hyphae around roots [31]. The effectiveness of an AMF in stimulating growth and its infectivity in colonizing roots seem to vary depending on the specific fungus–host interaction. In this sense, the influence of the rootstock on mycorrhizal dependency has been demonstrated, with sour orange exhibiting a high mycorrhizal dependency and *F. mosseae* being the least infective and least effective in this rootstock [32].

Therefore, selection of the AMF is a key factor before mycorrhizal inoculation, since there are different levels of compatibility between the AMF and the host plant. The objective of this work was to evaluate the effects of *R. irregularis* and *F. mosseae*, two of the AMF that are most commonly found in citrus soils of eastern Spain, on the growth of *C. aurantium* seedlings. This rootstock was selected considering that sour orange is one of the most common rootstocks used in lemon trees, being widely cultivated in the Spanish south-east, and its adoption has also been recommended in regions with water-scarcity problems; moreover, sour orange has shown a good relative response to irrigation with DSW, which is becoming increasingly prevalent each day in these areas. Understanding which mycorrhizal fungus produces a better plant response entails important advantages for its application as a biofertilizer. By identifying the most compatible fungus, we can enhance nutrient uptake, improve plant growth, and increase overall crop yields. This knowledge enables us to develop targeted and eco-friendly agricultural practices, fostering sustainable farming while reducing the need for chemical fertilizers.

2. Materials and Methods

2.1. Plant Culture and Experimental Design

The experiment was carried out in a walk-in controlled-environment room (3 m × 6.5 m) at the IMIDA under a 16 h photoperiod (07:00 a.m.–11:00 p.m.). Day–night variation caused fluctuations in temperature (20–24 °C) and relative humidity (65–85%). Seeds of *C. aurantium*, provided by the germplasm bank of the IMIDA, were surface sterilized for 10 min in 20% $NaClO_4$, rinsed four times with sterile distilled water, and sown in plastic trays containing moistened vermiculite. Forty-day-old seedlings were inoculated with *R. irregularis* or *F. mosseae* at the moment of transplantation from the germination tray to 1.1-litre pots. Pot substrate was a mixture of silica sand and clay-loam soil (soil:sand 1:3, *v*/*v*), previously sterilized in an autoclave for 1 h at 100 °C for three times on alternate days.

The inoculum per plant consisted of 25 g of bulk inoculum of *R. irregularis* or *F. mosseae* (a mix of spores, mycorrhized roots, and substrate, with an average of 1200 total fungal propagules per g of inoculum), propagated with the hybrid of *Sorghum bicolor* (L.) Moench and *Sorghum sudanense* (Piper) Hitch (*S. bicolor* × *sudanense*) as a trap plant. The inoculum was supplied by the Mycology-Mycorrhizas Laboratory, Department of Plant Biology, University of Murcia (Spain).

Experimental design consisted of two treatments, inoculated plants (+AM) and non-inoculated plants (−AM), and two fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), combined in four different treatments. Eight mycorrhizal and eight non-mycorrhizal seedlings of uniform size were selected for each fungus, providing a total of 32 pots, whose positions were changed every week to eliminate environmental variation. Plants were watered to maintain relative humidity, and 250 mL of modified Hoagland's solution [33] (6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.065 µM $(NH_4)_6Mo_7O_{24}$, and 20 µM Fe^{3+}-masquolate) were applied weekly to −AM plants. Inoculated plants were also irrigated weekly using the same solution without P.

2.2. Growth and Plant Analysis

The experiment was ended 4 months after plants were inoculated. At the end of the experiment, leaf water potential (Ψ_{leaf}) was measured in mature fully expanded leaves with a Schölander-type pressure chamber (model 3000; Soil Moisture Equipment Corp., Santa Barbara, CA, USA), following Turner's recommendations [34]. After that, plant roots were carefully separated from the substrate and washed with distilled water. Fresh

weights of the stems, leaves, and roots; length and diameter of the stem; and number of leaves from each plant were independently measured and processed. After roots and stems were oven-dried at 60 °C for 48 h (until a constant weight was reached) and leaves were freeze-dried, their dry weights were determined. To evaluate the degree to which plants depended on the mycorrhizal condition to produce their maximum growth, mycorrhizal dependency (MD, [35]) and mycorrhizal growth response (MGR, [36]) were calculated as follows:

$$MD\ (\%) = 100\ (X_i - X_n)/X_i$$

$$MGR = \log_e\ [X_i/X_n]$$

where X_i is the dry weight of the inoculated plant, and X_n is the dry weight of the non-inoculated plant.

Freeze-dried leaves were ground and analyzed for their mineral and chlorophyll contents. Dried and ground plant tissues were digested, ashes were dissolved in HNO_3, and Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe, Cu, Mn, Zn, B, and P were analyzed using an inductively coupled plasma optical emission spectrometer (Varian MPX-OEX Vista). Nitrogen (N) content was determined using a LECO FP-528 elemental analyzer. Chlorophyll contents were estimated by extracting 20 mg of ground material with N,N-dimethylformamide, and the absorbance was measured at 664.5 and 647 nm in a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) [37].

2.3. Determination of Mycorrhizal Colonization

For every plant, fragments of root were taken from the middle part of the root system to obtain an estimation of mycorrhizal colonization at the end of the experiment. One sample of each root system was cleaned and stained with trypan blue [38], but using lactic acid instead of lactophenol, and 100 root segments per plant were mounted on slides, squashed by pressing on the coverslips, and quantified for AM colonization [39].

2.4. Statistical Analysis

All data were analyzed using analysis of variance (ANOVA) procedures in Statgraphics Plus Version 5.1 (1994) software (Statistical Graphics Corporation, Warrenton, VA, USA). When there was a significant effect (value of $p < 0.05$), means were separated using Duncan's multiple range test. The Pearson correlation coefficients between the leaf P and the plant growth were calculated using the same statistical software.

3. Results and Discussion

In the current study, all of the inoculated sour-orange roots that were examined were colonized with arbuscular mycorrhizal fungi (AMF) and displayed typical AMF structures, such as hyphae that were present both inside and between cells, arbuscules, and vesicles (Figure 1). Occasionally, intraradical spores were also observed, either individually or grouped together within root tissues. The presence of AMF hyphae in citrus roots was consistently observed in all of the samples, and there was an abundance of arbuscules.

Sour-orange roots inoculated with AMF were colonized with *R. irregularis* besides *F. mosseae*, showing very good colonization 120 days after inoculation. No colonization was found on non-inoculated plants. Among two microbial inoculations, a significantly higher root fungal colonization was found with *R. irregularis* than with *F. mosseae*, with colonization percentages of 87% and 64%, respectively (Table 1). This lower colonization of Fm in sour orange plants had been previously described by other authors, who found that Fm was not as effective or infective in sour orange compared with other rootstocks such as Troyer citrange or Cleopatra mandarin [32]. However, the colonization capability of citrus roots with Fm is highly variable, and it depends on the host genotype [40]; additionally, studies with Poncirus trifoliata have shown higher colonization percentages of Fm with regard to Ri [41,42]. These different colonization responses of both AMF species in other

genotypes reveal the significant influence of host-plant species on the composition of the root-colonizing AMF community, as has been demonstrated in several studies [43,44].

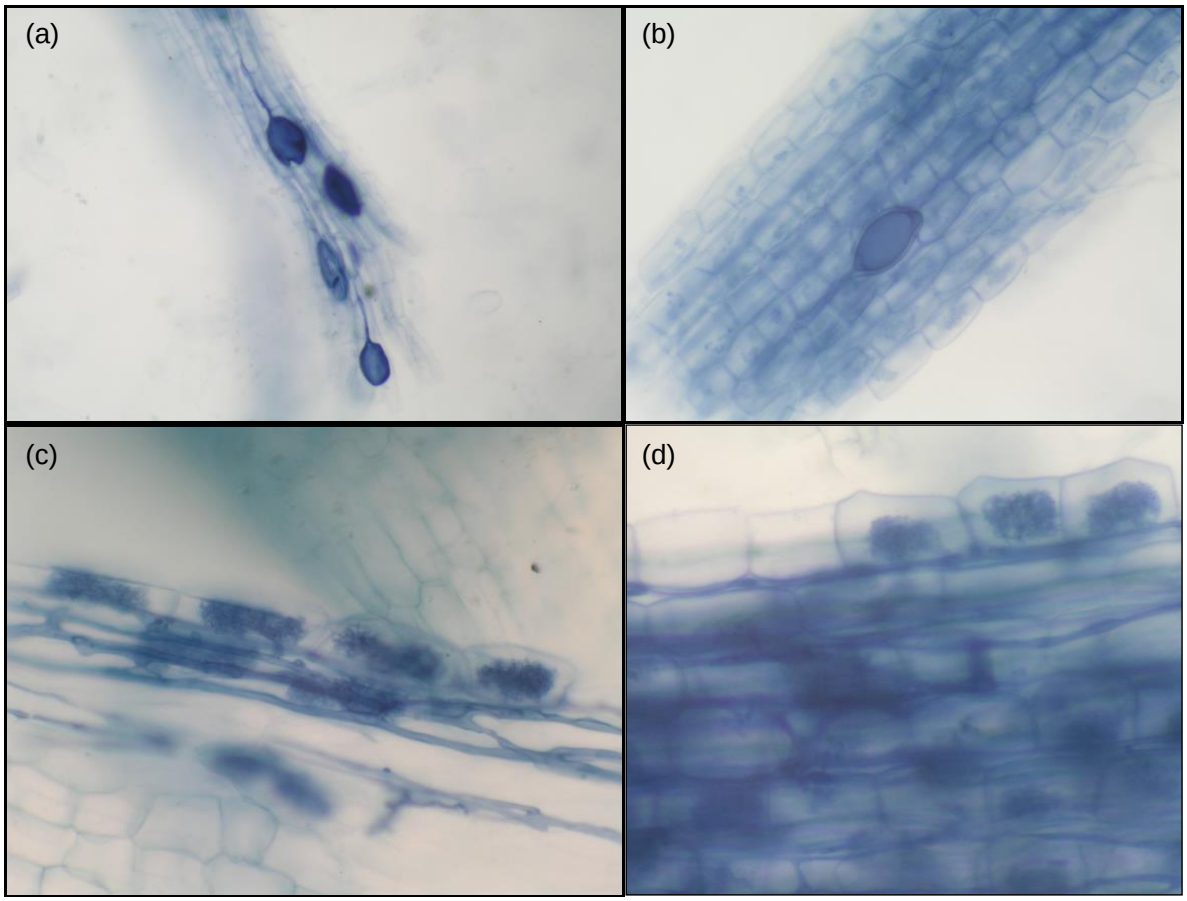

Figure 1. Microscopic examinations of *R. irregularis* (Ri) and *F. mosseae* (Fm) colonization of *C. aurantium* roots 60 days after fungal inoculation. (**a**,**b**) Hyphae and vesicles formed by Fm and Ri in the roots of the host sour orange. (**c**,**d**) Arbuscules and hyphae formed by Fm and Ri in the root cortex cells of the host sour orange.

Table 1. Root AM colonization of *C. aurantium* seedlings 120 days after inoculation with *R. irregularis* (Ri) and *F. mosseae* (Fm). −AM: non-inoculated plants; +AM: inoculated plants.

		Colonization Percentage (%)
Ri	−AM	22.3 a
	+AM	86.6 c
Fm	−AM	22.6 a
	+AM	64.1 b
ANOVA		***

*** indicates significant differences at the 0.001 level of probability. Different letters indicate significant differences according to Duncan's multiple range test at the 95% confidence level.

In this study, a high root infection with AMF significantly increased sour-orange growth from the early stages of the experiment. However, it is not clear whether *R. irregularis* or *F. mosseae* colonized the root of sour orange first, since 60 days after fungal inoculation, a large number of mycorrhizal intraradical hyphae, vesicles, and arbuscules were found in both *R. irregularis* and *F. mosseae*-colonized plants (Figure 1). Eighty days after inoculation, the size of sour orange plants inoculated with Fm was higher than that of Ri-inoculated plants (Figure 2a,b). However, at the end of the experiment, Ri-inoculated plants showed a higher height than Fm-inoculated plants (Figures 2c and 3). Seemingly,

over a brief period, Fm demonstrated a greater proneness to fostering positive outcomes in plant growth. Nonetheless, Ri exhibited superior positive effects compared with Fm over an extended period of time.

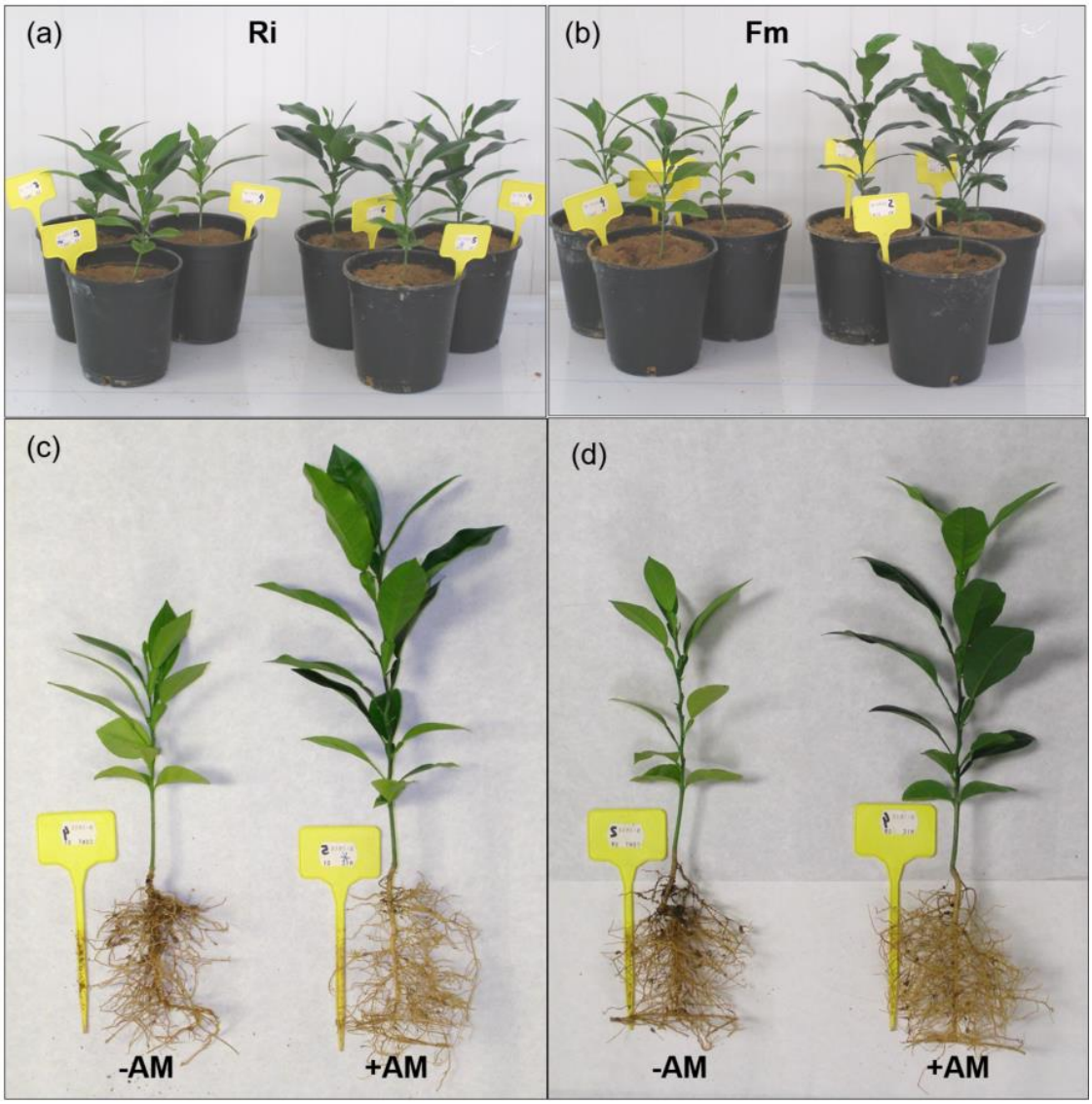

Figure 2. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on Citrus aurantium seedling growth 80 days after fungal inoculation (**a**,**b**) and at the end of the experiment, 120 days after fungal inoculation (**c**,**d**). −AM: non-inoculated plants; +AM: inoculated plants.

Regardless of the species of fungus, the effects of colonization with the two AMF on seedling growth in this experiment were very clear 120 days after inoculation, with large visible differences between non-inoculated and inoculated plants, mainly in the shoot growth (Figures 2 and 3). On the whole, plant height, number of leaves, and stem diameter were significantly greater in +AM plants (63%, 29%, and 16%, respectively) than in −AM plants, showing the positive effect of AMF on the growth of sour-orange plants. Growth enhancement due to AMF has been described in different culture conditions for C. aurantium [45–47], but also for other citrus species [48–51]. On the other hand, as it was found with plant height, sour-orange plants inoculated with Ri were the plants with the greatest number of leaves at the end of the experiment (Figure 3).

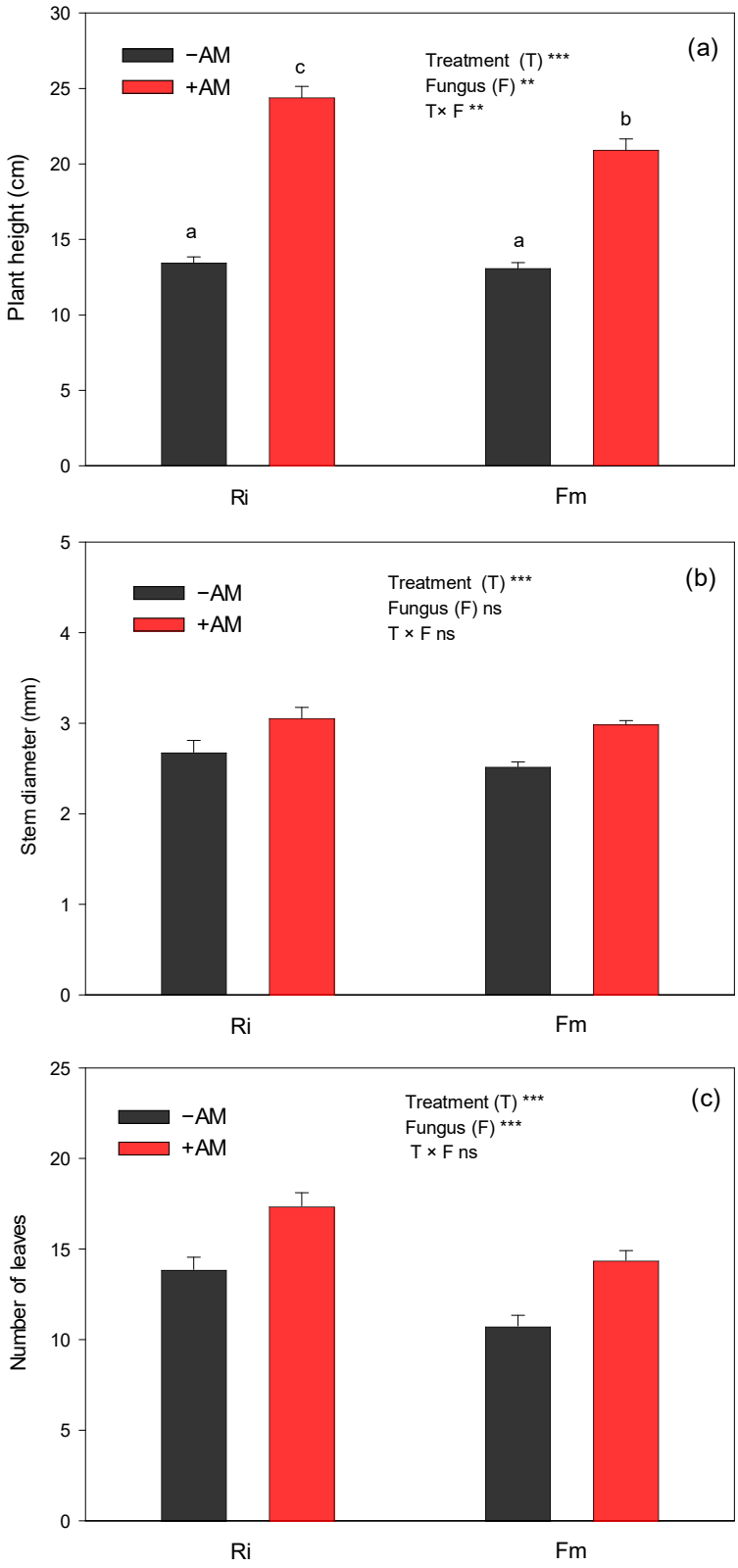

Figure 3. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on plant height (**a**), stem diameter (**b**), and number of leaves (**c**) of *C. aurantium* seedlings 120 days after fungal inoculation. −AM: non-inoculated plants; +AM: inoculated plants. Data are means ± SE (n = 8). ** and *** indicate significant differences at the 0.01 and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences according to Duncan's multiple range test at the 95% confidence level.

With regard to the rest of the growth parameters that were analyzed, after 4 months of culture, sour-orange plants inoculated with AMF had significantly higher fresh and dry weights compared with non-inoculated plants (Table 2). The highest increase in fresh weight due to AMF inoculation was observed in the leaves, where this amounted to 113% compared with that registered by −AM plants. Moreover, with AMF inoculation, the increase of weight was higher in the shoots than in the roots, given the lower root/shoot ratios, both in fresh and dry weights, with regard to non-inoculated plants. The whole plant had a significantly better growth response (higher number of leaves and fresh weight of leaves and shoots) when it was inoculated with Ri (Table 2). However, although Ri had the best positive effect on plant-growth performance, no differences were found between the two studied AMF regarding the root and plant dry weight.

Table 2. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on the fresh and dry weights of *C. aurantium* seedlings 120 days after fungal inoculation. −AM: non-inoculated plants; +AM: inoculated plants.

		Fresh Weight (g)					Dry Weight (g)				
AM		**Root**	**Leaf**	**Shoot**	**Plant**	**Root/Shoot**	**Root**	**Leaf**	**Shoot**	**Plant**	**Root/Shoot**
−AM		3.14	2.26	3.01	6.3	1.11	0.68	1.21	0.96	1.60	0.73
+AM		5.19	4.82	6.17	11.4	0.86	1.36	1.54	2.31	3.47	0.56
Fungus											
Ri		4.31	4.14	5.13	9.6	0.92	0.99	1.57	1.90	2.65	0.59
Fm		4.02	2.94	4.05	8.1	1.04	1.05	1.19	1.38	2.41	0.70
Ri	−AM	3.41	2.76	3.59	7.3	1.04	0.68	1.47	1.08	1.68	0.66
	+AM	5.21	5.52	6.67	11.9	0.81	1.30	1.66	2.71	3.63	0.53
Fm	−AM	2.87	1.76	2.43	5.3	1.17	0.68	0.96	0.83	1.51	0.80
	+AM	5.17	4.12	5.67	10.8	0.92	1.42	1.42	1.92	3.31	0.59
ANOVA											
AM		***	***	***	***	***	***	***	***	***	***
Fungus (F)		ns	***	**	*	ns	ns	***	**	ns	**
AM × F		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*, ** and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of probability, respectively. ns indicates non-significant differences at the 0.05 level of probability.

Moreover, AMF inoculation increased plant dry weight to a greater extent than fresh weight (180% more dry weight on the leaves of +AM plants than in −AM plants, versus the 115% of fresh weight, Table 2). In fact, the H_2O percentage of +AM plants was significantly lower than that of −AM plants, mainly in the roots and leaves (Figure 4); therefore, AMF promoted the dry weight increase in plants, primarily in the leaves, but also, to a lower extent, in the roots. On the other hand, Fm emerged as the fungus that facilitated the greatest accumulation of dry weight in both leaves and roots. This was evident because plants inoculated with Fm exhibited the lowest water percentages in these tissues (Figure 4).

One of the important implications of mycorrhiza inoculation on plant growth and nutrient uptake is mycorrhizal dependency (MD) [52], which represents the degree to which a plant species is dependent on the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility [53]. MD and mycorrhizal growth response (MGR) were calculated, and the results confirmed the high dependency of citrus species on AMF. They revealed that sour-orange seedlings are highly responsive to MD and MGR, since they increased the mycorrhizal dependency of sour orange over 50% for both Ri and Fm (Figure 5). Due to shallow root systems and underdeveloped root hairs, citrus is heavily dependent on arbuscular mycorrhizal fungi [54,55]. Thus, AM symbiosis partly replaces root hairs for the absorption of water and nutrients from the soil [56,57]. Moreover, citrus plants showed a higher root-hair density when they were inoculated, especially with *F. mosseae* [56].

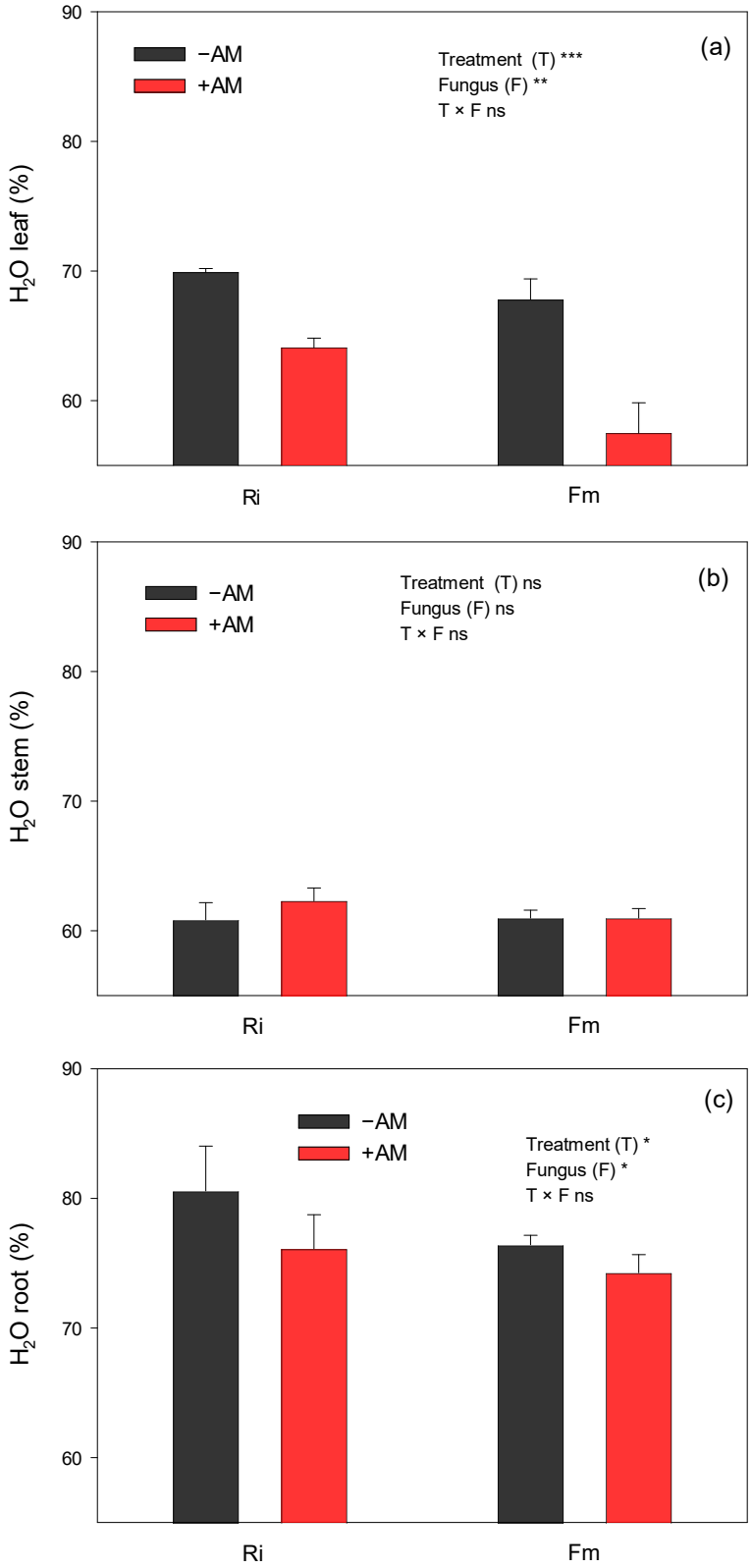

Figure 4. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on water percentage in leaves (**a**), stems (**b**), and roots (**c**) of *C. aurantium* seedlings 120 days after fungal inoculation. −AM: non-inoculated plants; +AM: inoculated plants. Data are means ± SE (n = 8). *, **, and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns.

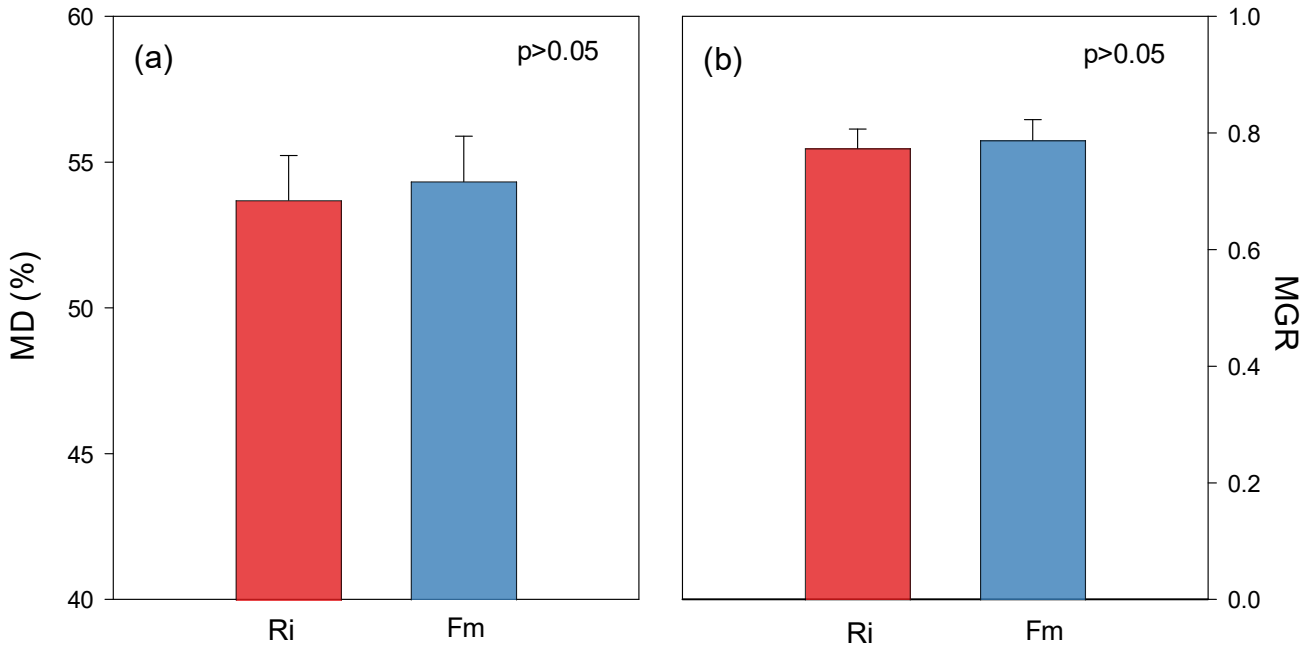

Figure 5. Mycorrhizal dependency (MD, (**a**) and mycorrhizal growth response (MGR, (**b**) of *C. aurantium* seedlings to the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), 120 days after fungal inoculation.

As AMF are the completely symbiotic fungi, and their growth and development depend on the photosynthetic products of the host plant [57], higher chlorophyll concentrations in +AM plants would help the host to enhance photosynthesis. The results of this study showed that, at the end of the experiment, chlorophyll a, chlorophyll b, and total chlorophyll concentrations were significantly higher in +AM than in non-AM seedlings, irrespective of AM species (Figure 6), which is consistent with previous results found for citrus plants [41,58]. However, although plant growth of the seedlings was greater with Ri (Table 2), no differences between Fm and Ri were found, and both fungi presented similar chlorophyll a, chlorophyll b, and total chlorophyll concentrations.

Several studies have shown that mycorrhizal citrus seedlings present higher root hydraulic conductivity, stomatal conductance, and transpiration rates than non-mycorrhizal seedlings [45,48,58]. Due to their greater stomatal openness, and, therefore, due to their higher transpiration rates, mycorrhizal plants show higher levels of water uptake through their roots and are able to lower the water potential of the soil in pots more than non-mycorrhizal plants [48]. Moreover, since root systems are constrained to a relatively low soil volume, the leaf-water potential declines more quickly in +AM than in −AM plants [48], since +AM plants are larger (Table 2) and deplete soil moisture reserves more quickly. Hence, both the greater plant size and the higher transpiration rates could be the reasons behind the lower Ψ_x of +AM when compared with −AM plants (Figure 7). In spite of the lower values of Ψ_x found in +AM plants, these did not show any visual symptoms of water stress, since watering was sufficient to satisfy the water requirements of the largest plants. For this reason, the greater size of plants inoculated with Ri with regard to those inoculated with Fm did not produce any differences to plant water status (Figure 7).

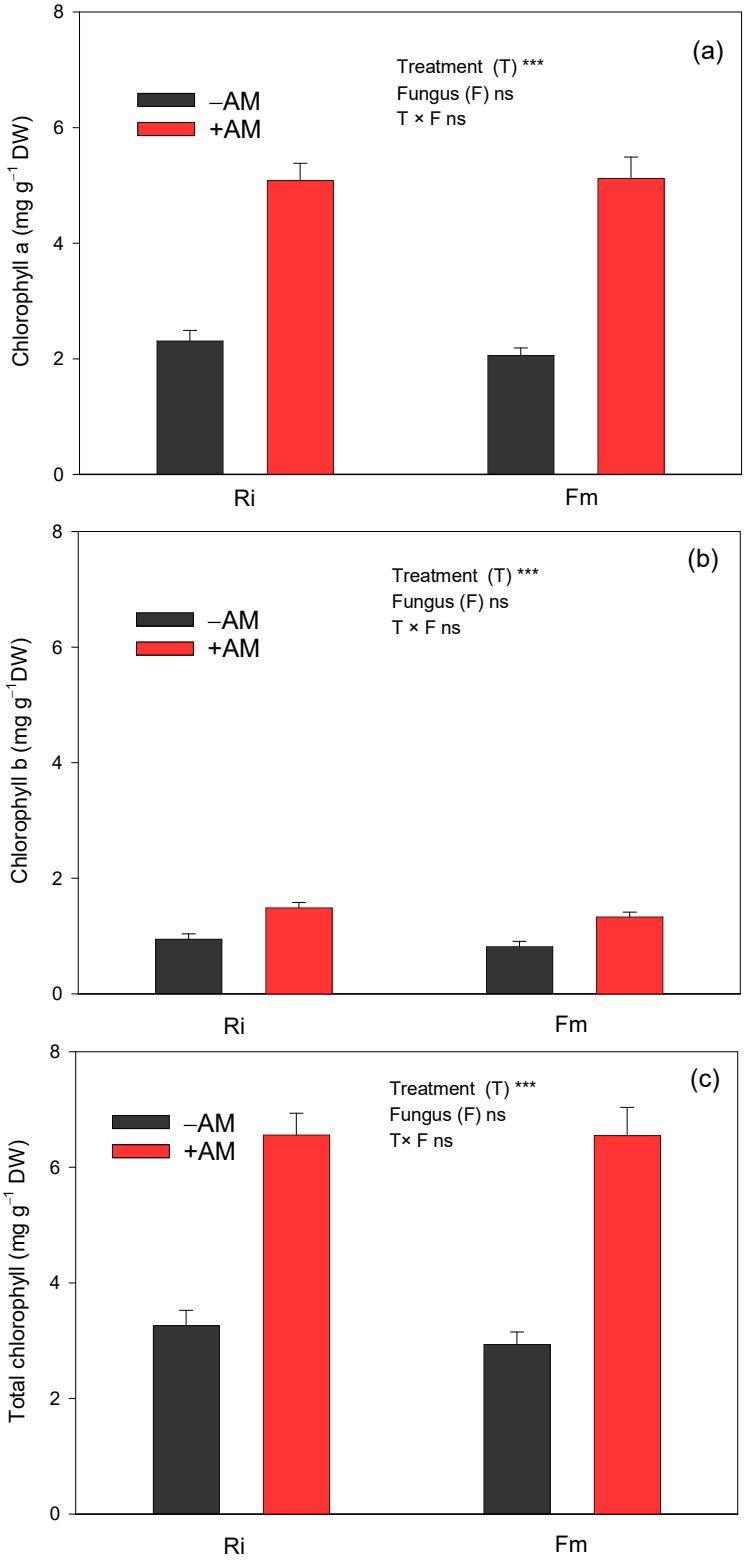

Figure 6. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on chlorophyll a (**a**), chlorophyll b (**b**), and total chlorophyll (**c**) in leaves of *C. aurantium* seedlings 120 days after fungal inoculation. −AM: non-inoculated plants; +AM: inoculated plants. Data are means ± SE (n = 8). *** indicates significant differences at the 0.001 level of probability. Non-significant differences at the 0.05 level of probability are indicated as ns.

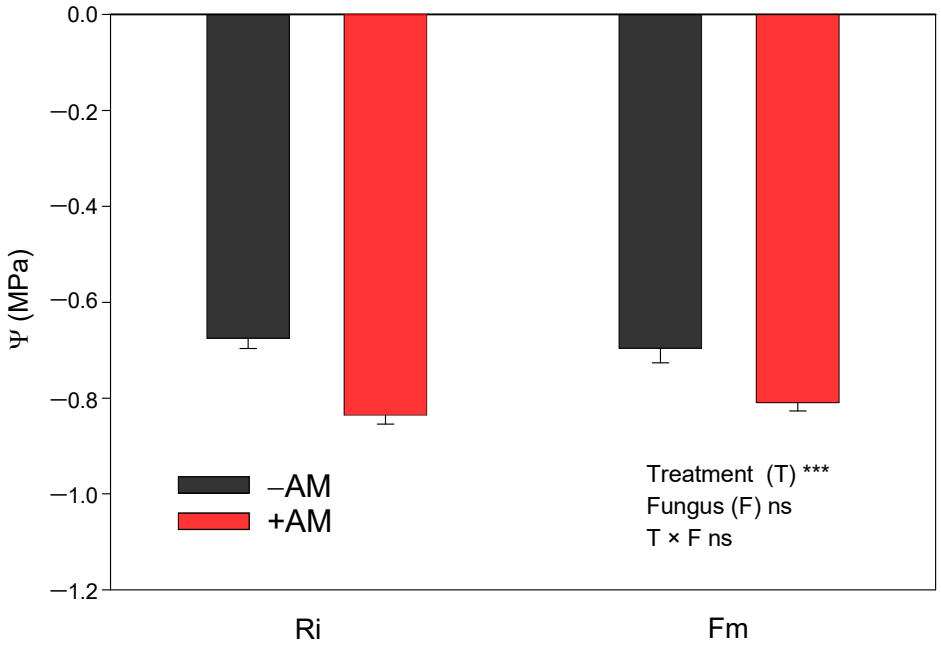

Figure 7. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on leaf water potential in leaves of *Citrus aurantium* seedlings 120 days after fungal inoculation. −AM: non-inoculated plants; +AM: inoculated plants. Data are means ± SE (n = 8). *** indicates significant differences at the 0.001 level of probability. Non-significant differences at the 0.05 level of probability are indicated as ns.

In our experiment, mycorrhizal inoculation significantly improved plant phosphorus acquisition (it increased by approximately 100%) compared with non-inoculated plants. *F. mosseae* showed a higher acquisition efficiency than *R. irregularis* (92% and 122%, respectively; Figure 8). Inoculation with *F. mosseae* also improved leaf phosphorus acquisition in licorice plants with regard to non-inoculated plants [59]. It is widely established that one of the primary benefits of AMF is the improved P uptake conferred on symbiotic plants, and the higher uptake of phosphorus by host plants is primarily due to extraradical hyphae and elevated acid phosphatase activity [60]. Moreover, the distribution of hyphae in soil zones from which roots are absent and the greater contact of the hyphae with the soil have a large contribution to the increased nutrient uptake [61]. In any case, foliar P concentrations found in the experiment in both +AM and −AM plants were below the optimum range of P for adult citrus trees [62]. Nonetheless, previous results showed that these values were sufficient to sustain the proper growth of young citrus seedlings without deficiency symptoms [48,63].

As a consequence of the higher phosphorus uptake in mycorrhizal plants, their growth is increased, and the great effect of mycorrhizal infection on plant growth has been related to the higher uptake of soil P by the extraradical mycorrhizal mycelium [60,64,65]. In this regard, leaf P has been positively correlated with root dry weight or root length in Citrus volkameriana inoculated plants [66]. In our experiment, positive and linear correlations were found between leaf P and root and leaf dry weights at the end of the experiment for both Ri and Fm fungi species (Figure 9). Moreover, although sour-orange growth was positively correlated with the leaf P status regardless of fungi species, this correlation was stronger when Fm fungus was used.

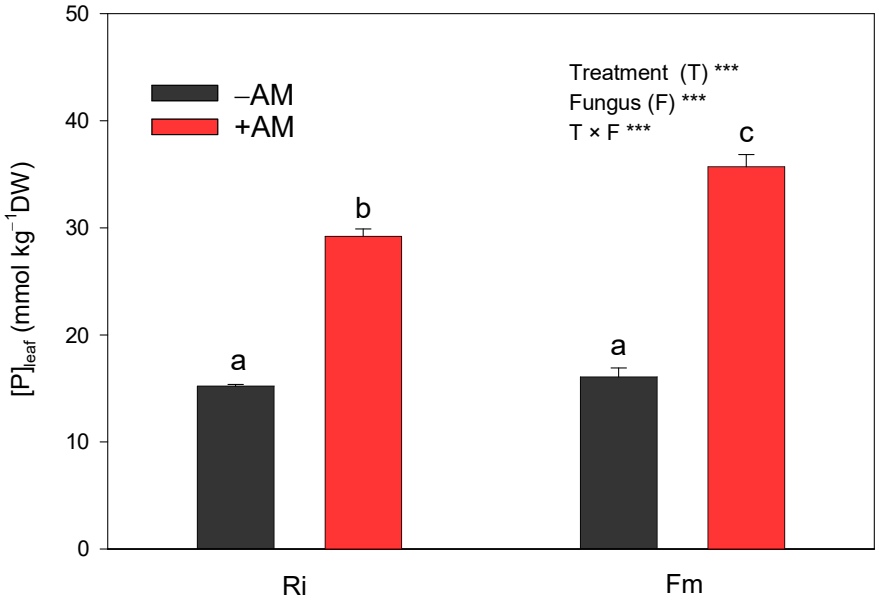

Figure 8. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on phosphorus concentration in leaves of *C. aurantium* seedlings 120 days after fungal inoculation. −AM: non-inoculated plants; +AM: inoculated plants. Data are means ± SE (n = 8). *** indicates significant differences at the 0.001 level of probability. Different letters indicate significant differences according to Duncan's multiple range test at the 95% confidence level.

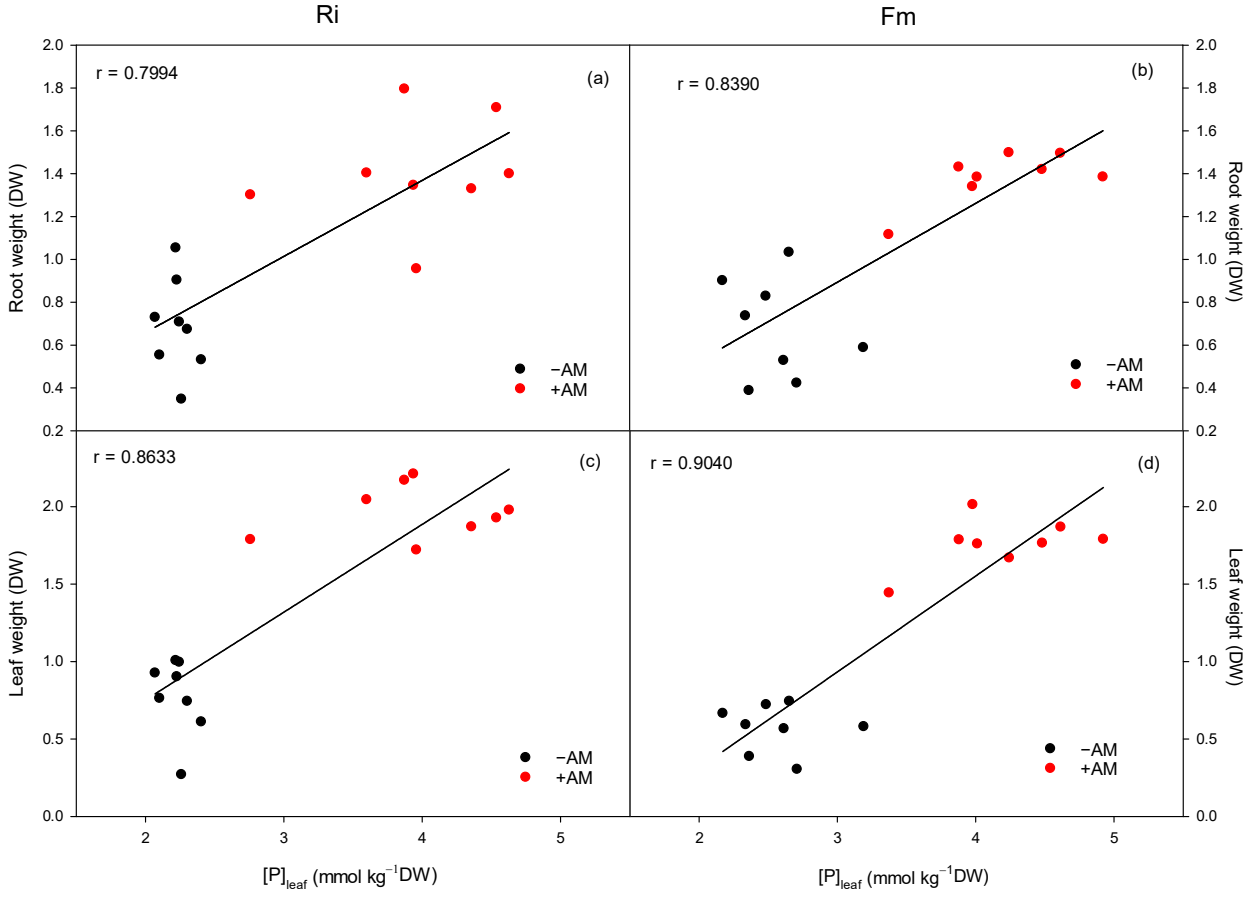

Figure 9. Effect of leaf phosphorus concentration on the growth of the root (**a**,**b**) and leaves (**c**,**d**) of *C. aurantium* seedlings for the two AM fungi, *R. irregularis* (Ri) and *F. mosseae*. −AM: non-inoculated plants; +AM: inoculated plants.

In general, AMF contributes to an accelerated acquisition of nutrients in the citrus plants [47,55,67,68]. However, in our experiment, mycorrhizal inoculation decreased foliar nitrogen levels (Table 3). Some results obtained with other citrus species show no effect of inoculation on nitrogen content [69]. Moreover, similarly to the results that we attained, it has been found that growth stimulation in inoculated plants resulting from the increased P acquisition can produce a reduction in N concentrations due to growth dilution [45]. This dilution effect caused by growth stimulation, a consequence of the better phosphorus nutrition, could also be the reason for the lower concentrations of foliar Ca^{2+}, K^{+}, Na^{+}, and Mg^{2+} found in mycorrhizal seedlings (Table 3). A similar effect on foliar K^{+} levels was found in other citrus studies [45], and even higher levels of K^{+} and Ca^{2+} were observed in non-inoculated plants with regard to *R. irregularis*-inoculated plants in trifoliate orange [70].

Table 3. Effect of the two AM fungi, *R. irregularis* (Ri) and *F. mosseae* (Fm), on the N, K, Ca, Mg, Na, B, Fe, Cu, Mn, and Zn concentrations of *C. aurantium* leaves 120 days after fungal inoculation. Concentrations are expressed as mmol kg^{-1} DW for all of the nutrients, except for N, which is expressed as % of DW. −AM: non-inoculated plants; +AM: inoculated plants.

AM		N	K	Ca	Mg	Na	B	Fe	Cu	Mn	Zn
−AM		3.66	589	695	156.7	136	4.09	1.53	0.477	0.672	0.313
+AM		2.75	341	522	91.5	111	2.40	1.49	0.314	0.548	0.251
Fungus											
Ri		3.29	470	593	122.8	122	3.09	1.40	0.492	0.590	0.345
Fm		3.11	460	623	125.4	125	3.40	1.63	0.299	0.629	0.220
Ri	−AM	3.76	567	685	160.2	146	3.96	1.33	0.650 b	0.666	0.421 c
	+AM	2.82	374	502	85.3	99	2.23	1.46	0.334 a	0.515	0.268 b
Fm	−AM	3.55	611	704	153.2	127	4.23	1.74	0.305 a	0.677	0.205 a
	+AM	2.67	309	542	97.7	123	2.57	1.52	0.294 a	0.581	0.234 ab
ANOVA											
AM		***	***	***	***	*	***	*	***	***	**
Fungus (F)		*	ns	ns	ns	ns	ns	ns	***	ns	***
AM × F		ns	ns	ns	ns	ns	ns	ns	***	ns	***

*, ** and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences according to Duncan's multiple range test at the 95% confidence level.

On the other hand, the effect of AM fungi in the micronutrients B, Fe, Cu, Mn, and Zn was similar, showing a lower concentration in +AM plants compared with −AM plants (Table 3). Again, a dilution effect due to the greater growth of inoculated plants could have taken place. Other authors found that AM symbiosis does not affect plant Cu concentrations or even reduces Mn concentration [48,71]. This decrease in Mn in mycorrhizal plants has been explained by changes in the physiology of the host, with reflections on the biological processes of Mn oxidation and alterations of Mn^{4+} reducing potential in the rhizosphere of mycorrhizal plants, probably due to a lower population of Mn-reducing organisms [72]. In general, AMF increase the Fe uptake and translocation in mycorrhizal plants, since the higher amount of hyphae and the larger surface area of roots could help the host plant obtain more Fe from the soil [73]. However, a lower Fe concentration was observed in the leaves of +AM than in −AM plants (Table 3); although, all of the plants had optimal leaf Fe concentrations regardless of the treatments. The literature describes the different impacts of AM fungi on Fe nutrition, from an increase in Fe uptake and translocation in mycorrhizal plants [73] to no response to mycorrhizal inoculation in citrus seedlings [74].

Anyhow, regardless of the differences found in the nutrition of mycorrhizal and non-mycorrhizal plants, the type of fungus had little influence on the nutritional status of the plants. In addition to the differences described in the nutrition of P, only N, Cu, and Zn concentrations were significantly higher in plants inoculated with Ri compared with Fm-inoculated plants (Table 3).

4. Conclusions

Sour-orange plants exhibit a strong positive response to arbuscular mycorrhizal fungi, showing a high mycorrhizal dependency regardless of the AMF species. Both *R. irregularis* and *F. mosseae* fungi displayed high colonization percentages, and yet *R. irregularis* exhibited a higher root colonization compared with *F. mosseae*.

Inoculation resulted in a significant growth improvement, with *F. mosseae* leading to higher plant-growth stimulation in the short term. However, *R. irregularis* demonstrated superior positive effects compared with *F. mosseae* in the long term. The improvement in P nutrition was more pronounced with *F. mosseae*, and the stimulation of plant growth was also higher in plants inoculated with *F. mosseae*. In spite of this, *R. irregularis* showed a better overall plant-growth performance, indicating that factors beyond P nutrition, such as overall plant nutrition, could contribute to an improved plant performance with this fungus.

In summary, both *R. irregularis* and *F. mosseae* fungi caused a positive response in sour-orange plants. Therefore, the combination of both fungal species could establish a more comprehensive and balanced symbiosis, mutually reinforcing each other and resulting in a more robust and healthier growth of citrus trees. All these findings position both fungi as viable options for use as biofertilizers in the cultivation of citrus trees grafted with sour-orange rootstock. This knowledge can help to promote the use of biofertilizers for citrus-species cultivation, particularly in areas that are especially vulnerable to climate change, promoting the reduction of chemical fertilizers for the sake of a more sustainable and environmentally friendly agriculture.

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