Activity of the arbuscular mycorrhizal fungus, *Glomus iranicum* var. *tenuihypharum* var. *nova*, and its effect on citrus development in southeastern Spain

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Abstract

Arbuscular mycorrhizae (AM) are symbiotic associations between fungi of the Phylum *Glomeromycota* and most plants, and has direct benefits for plant nutrition, increased exploration of the soil and greater nutrient uptake through the hyphae and roots. The goal was to study the use of *Glomus iranicum* var. *tenuihypharum* (GIT) in different cultivars and species of citrus. The first experiment was aimed to ascertain the number of GIT applications necessary to obtain the best physiological response in lemon 'Fino'. Parameters measured included colonization percentage and mycelium production together with the gas exchange plants parameters net photosynthesis, stomatal conductance and water use efficiency. A second experiment to ascertain the effectiveness of two AM strains, GIT and *Rhizophagus irregularis* on the production of lemon 'Fino'. For this, several mycorrhizal parameters were measured, including colonization percentage and mycelium production, together with fruit caliber and production. The third experiment consisted of a battery of trials in tangerine plantations of 'Oronules', 'Clemenvilla' and 'Clemenpons' cultivars where GIT had been applied at a rate of 3 kg ha^{-1} through the irrigation system. Development variables, such as fruit production and caliber were measured. The use of GIT with double inoculation produced the best physiological and growth effects of lemon 'Fino' plants of one year, promoting in turn the highest mycorrhizal activity. Fruit production of the plants treated with GIT was higher than of the ones treated with *Rhizophagus irregularis* (35.67%). The production of treated plants increased from 7.20 to 26% in the tangerine cultivars studied in the third experiment. Fruit reached a commercial color earlier in the sub-plot treated with GIT making it possible to harvest more fruit in the first pass. The greatest production increase in response to GIT application was in 'Oronules' (26.3%) followed by 'Clemenvilla' (7.9%) and 'Clemenpons' (8.3%).

Keywords: lemon 'Fino', tangerines, net photosynthesis, mycorrizal activity, citrus caliber

INTRODUCTION

In their natural habitats, plant species are dependent on the mycorrhizal condition for maximum growth or greatest yield at a given level of soil fertility (Gerdemann, 1975). Mycorrhizal association is a symbiosis between plant roots and arbuscular mycorrhizal fungi (AMF), and is responsible for nutrient uptake, especially P, N, K, Mg, Mn, Fe, Zn and Cu. Early establishment and growth of mycorrhizae-dependent (MD) plants require mycorrhizal formation (Graham et al., 1982). Trees, especially citrus, are highly MD (Graham and Eissenstat, 1994; Krikun and Levy, 1980; Ortas et al., 2002).

Citrus plants have adaptive and/or regulatory processes that control the interaction between environmental factors and nutrition, and which interact with above-ground processes such as photosynthesis (Lukac et al., 2010; Rennenberg and Schmidt, 2010). One of the most important features of tree plants is how they make use of new resources and how the efficiency of nutrient acquisition can be improved by mycorrhizal fungi.

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There are several types of mycorrhizal associations, the arbuscular endomycorrhizae which interact with nearly 90% of terrestrial plant species, including citrus plant being the most common. (Gadkar et al., 2001). This has a broad host range and approximately over 150 species of AMF colonize 225,000 species of plant hosts (Wu and Zou, 2009). The main mycorrhizae spore in citrus rhizosphere is the *Glomus* species (Davies and Albrigo, 1994).

The efficacy of AMF, when used in inoculants, is influenced by numerous soil, crop and environmental factors, including crop species compatibility, size and effectiveness of indigenous microbial populations, soil fertility and management practices (Adholeya et al., 2005). Despite the successful laboratory results obtained with certain strains of AMF, many authors agree that scale-up of their use for agriculture has been slow, probably due to the relative ineffectiveness of the inoculation process (Ryan and Graham, 2002), or other factors, such as the technical difficulty involved in their application, the degree of soil compatibility (Herrera-Peraza et al., 2011) and field carrying capacity (Fernández et al., 2014).

Considering the above information, our objective was to select an AM species that could have a stable positive effect, regardless of external conditions, when used as a biological inoculant in intensive agriculture. In this context, the main criteria taken in account were the soil environment and the adaptation of the AMF to a specific range of soil pH levels (Herrera-Peraza et al., 2011). The species chosen would have to show constant results in the special conditions required by different intensive agriculture systems and crop families. Therefore, the fundamental objective of this work was to show the benefits of the application of *Glomus iranicum* var. *tenuihypharum* on plant physiology, nutrition and productivity of lemon and tangerine cultivars.

MATERIALS AND METHODS

Strain selected and inoculum

The strain *G. iranicum* var. *tenuihypharum* (hereinafter GIT) was previously isolated from a hydromorphic and highly compacted sodium saline soil classified as Solonetz Gley type. It is a very alkaline soil (pH_{H2O} 9.5) with high concentrations of Ca, Na and Mg, a low C:N ratio and low organic matter content. The inoculum (120 propagules g^{-1} (Porter, 1979)) was supplied through a drip irrigation system (injection pump regulated at 25 L h^{-1}) at the beginning of the vegetative growth phase at a dose of 3 kg ha^{-1} in each trial. In case of two applications, the same dose was applied (Juarez and Fernandez, 2015).

Series of experiments

1. Test 1. Effect of the number of applications of *Glomus iranicum* var. *tenuihypharum* on lemon 'Fino' seedlings for one year.

The objective of the trial was to know the effect of the double application of *Glomus iranicum* var. *tenuihypharum* on the physiological and mycorrhizal activity of lemon 'Fino' plants for one year. Ten plants of lemon 'Fino' per treatment were studied using one and two applications of a commercial formulation (MycoUp) based on *Glomus iranicum* var. *tenuihypharum* mycorrhizal fungus at the commercial dose of 3 kg ha^{-1} through localised irrigation and an untreated control treatment. The applications were made in spring and 90 days after the first inoculation. The physiological activity of the trees was studied by measuring Spad index and gaseous exchange, as well as the mycorrhizal activity of the plants.

2. Test 2. Comparative study of the efficacy of the application of *Glomus iranicum* var. *tenuihypharum* and *Rhizophagus irregularis* on the production of lemon 'Fino'.

The aim of this trial was to compare the efficacy of the application of two species of mycorrhiza-forming fungi on the production of an eight-year-old lemon 'Fino' plantation during one harvest cycle. Two sectors with identical irrigation water management and fertilization programs were inoculated. Two treatments were carried out, one sector was treated with the commercial product MycoUp (based on *Glomus iranicum* var.

tenuihypharum) at a dose of 3 kg ha^{-1} and in the other sector a commercial product composed of *Rhizophagus irregularis*, at the same doses.

Both products were applied in early spring at the beginning of sprouting. The results were evaluated by comparing the yields of four trees from each of the sectors, with similar characteristics of bearing and vegetative development, as judged by the technicians involved. For the evaluation of the results, at the time of harvesting, the fruits of the trees were harvested separately before being weighed and calibrated in the field.

3. Test 3. Effect of the application of *Glomus iranicum* var. *tenuihypharum* on the productivity and calibre of 'Clemenpons', 'Oronules' and 'Clemenvilla' mandarin cultivars.

The aim of this work was to study the efficacy of the application of *Glomus iranicum* var. *tenuihypharum* on the productivity and calibres of the mandarin cultivars 'Clemenpons', 'Clemenvilla' and 'Oronules'. MycoUp was applied in two irrigation sectors with an area of 4 ha, leaving a similar area as a control. The applications were carried out in spring and September (to 'Clemenpons' and 'Oronules') or October ('Clemenvilla') at a dose of 3 kg ha^{-1}, in the plots described above and harvesting was carried out in three passes, calibrating and weighing 500 fruits from 5 treated and untreated trees chosen from the sectors described above. The calibers were grouped into 3-mm intervals ranging from 35 to >70 mm. The harvest was made in October for 'Clemenpons' and 'Oronules' and in December for 'Clemenvilla'.

General measurements

1. Mycorrhizal development.

Lemon 'Fino' young root samples from rhizosphere soil were collected at a depth of 20-30 cm to assess symbiotic development. Five root samples per treatment were used with four replicates each one. The percentage of mycorrhizal root colonization was estimated following the gridline intersect method under a microscope (100× magnification) after clearing and staining roots (Phillips and Hayman, 1970). Arbuscular endophyte (AE), extramatrical mycelium (EM), and the ratio EM:AE was measured by estimating the visual presence in an area (Herrera-Peraza et al., 2011).

2. Net photosynthesis and stomatal conductance.

Instantaneous measurements of net photosynthesis (*A*) and stomatal conductance (gs) were performed on four leaves $plot^{-1}$ (one leaf from each inner tree), using an open gas exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated leaf chamber fluorometer (Li-6400-40; Li-Cor Inc., Nebraska, USA). All measurements were performed on young, fully expanded leaves, at a photosynthetic photon flux density of 1500 µmol m^{-2} s^{-1} to ensure light saturation, with a CO_2 concentration in the cuvette of 400 µmol CO_2 mol^{-1} air (Pou et al., 2012). Measurements were carried out twice at 75 and 150 days after planting. Additionally, intrinsic water use efficiency (WUE) was determined and computed as the A/gs ratio (Pou et al., 2012).

3. Chlorophyll (in SPAD units).

Chlorophyll was measured in 10 plants on two occasions (days 75 and 150) during the assay, using a portable meter (chlorophyll meter SPAD-502, Konica Minolta), which makes instantaneous and non-destructive measurements of the relative chlorophyll index.

4. Leaf ion contents.

The ion content of leaves was obtained by the ICP-OES technique (Iris Intrepid II XDL, OribaSci.) 75 days after treating only in the trial 1. The ions measured were nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca), and the trace elements were manganese (Mn), iron (Fe) and zinc (Zn).

5. Yield and calibers of fruits.

Considering the natural stepped process of citrus maturation, three or two cuts were performed at commercial maturity as determined primarily by color. The first cut was of the greatest commercial value. Finally, the total yield was determined as the sum of the yield weighed at each cut. To determine the calibers of fruits grapes, 500 fruits from each treatment were measured by an electronic calibrator.

Data analysis

Data were analyzed using a one-way analysis of variance, using SPSS V21 for Windows to detect any significant differences between the parameters measured. In addition, when differences were significant, Tukey's range test at the 95% confidence level was carried out for a comparison between treatments. Percentage values of root colonization were arcsine (square root (X)) transformed before statistical analysis.

RESULTS

Test 1. Effect of the number of applications of *Glomus iranicum* var. *tenuihypharum* on one-year-old lemon 'Fino' seedlings

The parameters gas exchange and water use efficiency (WUE) were analysed 90 and 180 days after inoculation in one-year-old potted plants with one and two applications of MycoUp during the growing cycle (Table 1), and the following differences were noted.

Table 1. Gaseous exchange parameters and WUE (A/Gs) taken in lemon 'Fino' plants 75 and 150 days after planting and inoculated with *Glomus iranicum* var. *tenuihypharum* at 30 and 120 days and their respective untreated control plants.

Date	Treatments	A (μmol CO_2 m^{-2} s^{-1})	Gs (mmol H_2O m^{-2} s^{-1})	WUE (μmol CO_2 mol^{-1} H_2O)
75 days	Untreated	15.25 a	140 a	108.9 a
	First inoculation	17.12 b	145 a	118.06 b
	First inoculation	17.22 b	148 a	116.35 b
	Significance level	1.12***	3.5 ns	2.1 **
150 days	Untreated	11.03 a	110 a	100.27 a
	First inoculation	15.32 b	121 b	126.6 b
	Two inoculation	15.90 b	123 b	129.3. b
	Significance level	0.88***	2.1**	2.6***

Different letters in the same column correspond to significantly different values according Tukey's range test.

As regards the colorimetric content of SPAD (Figure 1), significant differences were observed between treated and untreated plants, both at 75 and 150 days, with the highest values always found in the presence of MycoUp. Among treated plants, the greatest differences were observed in plants with receiving a double inoculation at 175 days, in which values of up to 52 were recorded, compared with 48 for single inoculation and 40 in the untreated controls.

In terms of photosynthesis, there was a significant difference between untreated plants (15.25) and those to which MycoUp was applied, both at 30 and 120 days (Table 1). As for the stomatal conductivity values, in the first 75 days, there were no significant differences between the treatments studied, a situation that changed after 150 days in favour of greater activity in trees treated with MycoUp, with values significantly higher than the control although there were no significant differences between treated variants.

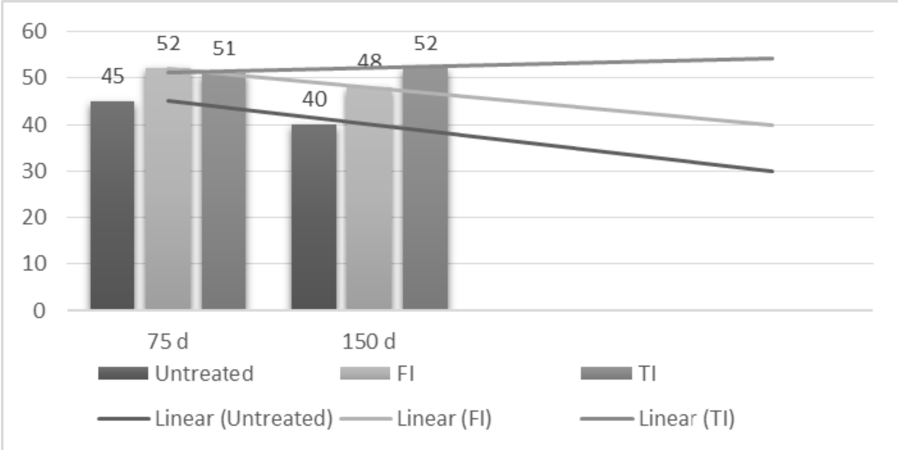

Figure 1. SPAD measurements taken in lemon ‘Fino’ plants 75 and 150 days after planting in trees inoculated with *Glomus iranicum* var. *tenuihypharum* at 30 and 120 days and their respective untreated controls.

This behaviour was generalized in terms of efficient water use, which always showed the same pattern: lower efficiency in untreated plants (108.9 and 100.27 at 75 and 150 days respectively) and higher efficiency/activity in *Glomus iranicum* var. *tenuihypharum* inoculated plants (118.06 and 116.35, respectively), in plants inoculated with a single application and 126.6 and 129.3, respectively, in plants inoculated twice, with no significant differences between them (Table 1).

The analysis of the arbuscular endophyte contents showed that the presence of *G. iranicum* var. *tenuihypharum* strain in lemon ‘Fino’ plants produced significantly higher values than those observed in untreated plants, an effect that was increased with double inoculation (Table 2). There were already significant differences in the height of the plants at 75 days, at least in one of the repetitions after the first application (42.5 cm). After 150 days, marked differences were found between the control (46.5 cm) and the plants receiving applications of the fungal inoculant: 72.2 cm in the case of double inoculation and 62.3 cm after single inoculation, values statistically different from each other and from the control.

The same pattern was found in the case extramatrical mycelium, as a consequence of the mycorrhizal colony extending beyond the root, with higher values reported in plants treated with MycoUp. In untreated plants this value at 75 and 150 days moved further from equilibrium, promoting a ratio of 3.76 and 2.62, respectively, as opposed to plants treated with MycoUp, where the values were much lower, and, in the case of double inoculation, values were much closer to symbiotic equilibrium at 150 days.

Test 2. Comparative study of the effect of applying *G. iranicum* var. *tenuihypharum* and *Rhizophagus irregularis* on the production of lemon ‘Fino’

As regards the number of fruits harvested, there was a statistically significant difference between the two treated areas can be seen, 991 more fruits being collected for the trees treated with MycoUp (Table 3). The difference in harvested fruits also has an impact on the total weight of the sample the difference being greater than that related to the number of fruits, and in this case, there was a 35.67% increase in the total weight of the sample treated with *G. iranicum* var. *tenuihypharum* (539.9 kg) compared with *Rhizophagus irregularis* (398.0 kg). As far as the weight of the fruits is concerned, the same trend can be observed as in the case of the fruit average.

The data point to an increase of 6.12% in the average weight of fruit treated with the strain present in MycoUp (122.5 g) (Table 3). This difference is especially important when it is related to the above-mentioned information since, in addition to achieving a significantly higher number of fruits, a greater average weight has been achieved. There was also a shift in the classification of the fruits harvested towards larger sizes, which usually translates into greater commercial value.

Table 2. Mycorrhizal activity found in lemon 'Fino' plants 75 and 150 days after planting and inoculated with *Glomus iranicum* var. *tenuihypharum* at 30 and 120 days and their respective untreated control plants.

Date	Treatment	Arbuscular endophyte (mg g^{-1} soil)	Extramatrical mycelium (mg mycelium g^{-1} soil)	EM:AE (mg:mg)	Plant height (cm)
75 d	Untreated	1.8/0.25 a	5/0.69 a	3.76 a	31.2 a
	First inoculation	20/1.30 b	18/1.25 b	1.96 b	42.5 b
	Second inoculation	22/1.34 b	19/1.27 b	1.94 b	39.8 ab
	Transformed	Log x	Log X	Log X+1	
	Significance level	0.08**	0.09***	0.019**	2.01*
150 d	Untreated	2.1/0.32 a	7/0.84 a	2.62 a	46.5 a
	First inoculation	29/1.46 b	31/1.49 b	2.02 b	62.3 b
	Second inoculation	70/1.84 c	39/1.59 c	1.86 c	72.2 c
	Transformed	Log x	Log X	Log X+1	
	Significance level	0.05***	0.012***	0.015***	1.60***

Different letters in the same column correspond to significantly different values according Tukey's range test.

Table 3. Production and fruit calibres from lemon 'Fino' plants treated with *Glomus iranicum* var. *tenuihypharum* (G. i.v.t.) and *Rhizophagus irregularis* (R. i.).

Treatments	Fruits (*n*)	Calibre (mm)					Total weight (kg)	Fruits average (g)[1]
		45-50	50-55	55-60	60-70	>70		
G. i.v.t.	4407 a	43	552	1853 a	1794 a	165 a	539.9 a	122.5 a
R. i.	3447 b	43	532	1466 b	743 b	16 b	398.0 b	115.5 b
Significance level	12.25**	Ns	Ns	25.4**	10.2**	0.9***	9.56***	0.91**

[1]Total weight/numbers of fruits.
Different letters in the same column correspond to significantly different values according Tukey's range test.

The analysis of foliar nutrient concentrations at 75 d of cultivation reflected the results obtained for the other variables studied (Table 4). Significant differences were found for most of the elements between the studied treatments, except the K and Zn contents. Once again, the highest values were recorded in the treatments that showed the greatest production and largest caliber.

Table 4. Concentrations of some nutritional elements (ppm) in lemons leaves plants treated with *G. iranicum* var. *tenuihypharum* (G. i.v.t.) and *R. irregularis* (R. i.) after 75 days of treatment.

Treatments	N (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mn (ppm)	Fe (ppm)	Zn (%)
G. i.v.t	3.0 a	0.15 a	6.6	3.3 a	161.4 a	650 a	42.5
R. i.	2.5 b	0.12 b	6.2	2.6 b	132.2 b	342 b	35.2
Significance levels	0.012***	0.002***	ns	0.09**	0.25***	1.25***	ns

Different letters in the same column correspond to significantly different values according Tukey's range test.

It is also important to highlight the high values of the trace elements Fe and Mn (essential for photosynthesis and growth activity in general) measured in plants treated with *G. iranicum* var. *tenuihypharum,* which differed significantly from that measured in the plants treated with *Rhizophagus irregularis*, which reflects the lower absorption of these elements by this species in this treatment.

Test 3. Effect of the application of *Glomus iranicum* var. *tenuihypharum* on the productivity and caliber of the cultivars of mandarin, 'Clemenpons', 'Oronules' and 'Clemenvilla'

1. 'Clemenpons'.

This study has demonstrated the effectiveness of MycoUp in increasing in production of this cultivar, with an increase in yield of 7.94% with respect to untreated plants (Table 5), and for increasing the size of the fruits harvested. It could be seen that the distribution curve of calibres of the treated plants is displaced to the right with respect to the untreated plants, which translates into an overall increase in calibre of the evaluated fruits (Figure 2).

Table 5. Effect of the application of *Glomus iranicum* var. *tenuihypharum* on the production of mandarin, 'Clemenpons', 'Oronules' and 'Clemenvilla' cultivars.

Tangerin	Treatments	Yield (kg tree^{-1})	Yield (kg ha^{-1})	Increment (%)
Clemenpons	Untreated	53.77	24,442,42	7.94
	MycoUp	58.05	26,384.55	
Oronules	Untreated	57	48,032	26.3
	MycoUp	72.2	60,686	
Clemenvilla	Untreated	214.39	107,196	8.83
	MycoUp	233.33	116,666	

2. 'Oronules'.

The application of MycoUp in this cultivar had the expected effect, an increase in yield ha^{-1} and an increase in fruit size. In the case of production, the application of MycoUp generated a total increase with respect to the control of 26% (Table 5). As far as the sizes are concerned, the percentage of fruit smaller than size 5 (46-56 mm) decreased with respect to the control values, while the percentage of higher sizes increased (Figure 2).

3. 'Clemenvilla'.

This study demonstrated the effectiveness of MycoUp, with an 8.83% increase in yield (Table 5) accompanied by a higher calibre of harvested mandarins. As can be seen, the distribution curve of calibres of the treated part is displaced to the right with respect to the control plants reached which translates into a general increase in calibre of the mandarins treated with this bio fertilizer (Figure 2).

DISCUSSION

Effect of the number of applications of *Glomus iranicum* var. *tenuihypharum* on one-year-old lemon 'Fino' seedlings

Plants treated with *Glomus iranicum* var. *tenuihypharum* showed increases of 33 and 55% compared to the control at 150 days, with a single and double application, respectively. Reyes-Santamaría et al. (2000) reported that photosynthesis induces increases in dry matter accumulation as well as increases in the specific weight of citrus leaves. Alarcón et al. (1998), Aguilera-Gómez et al. (1999), Manjarrez et al. (1999) and Davies et al. (2000) mentioned that AMF are able to modify the gaseous exchange and induce higher photosynthetic rate in their hosts, making water use and the activity of the rubisco enzyme to capture CO_2 more efficient.

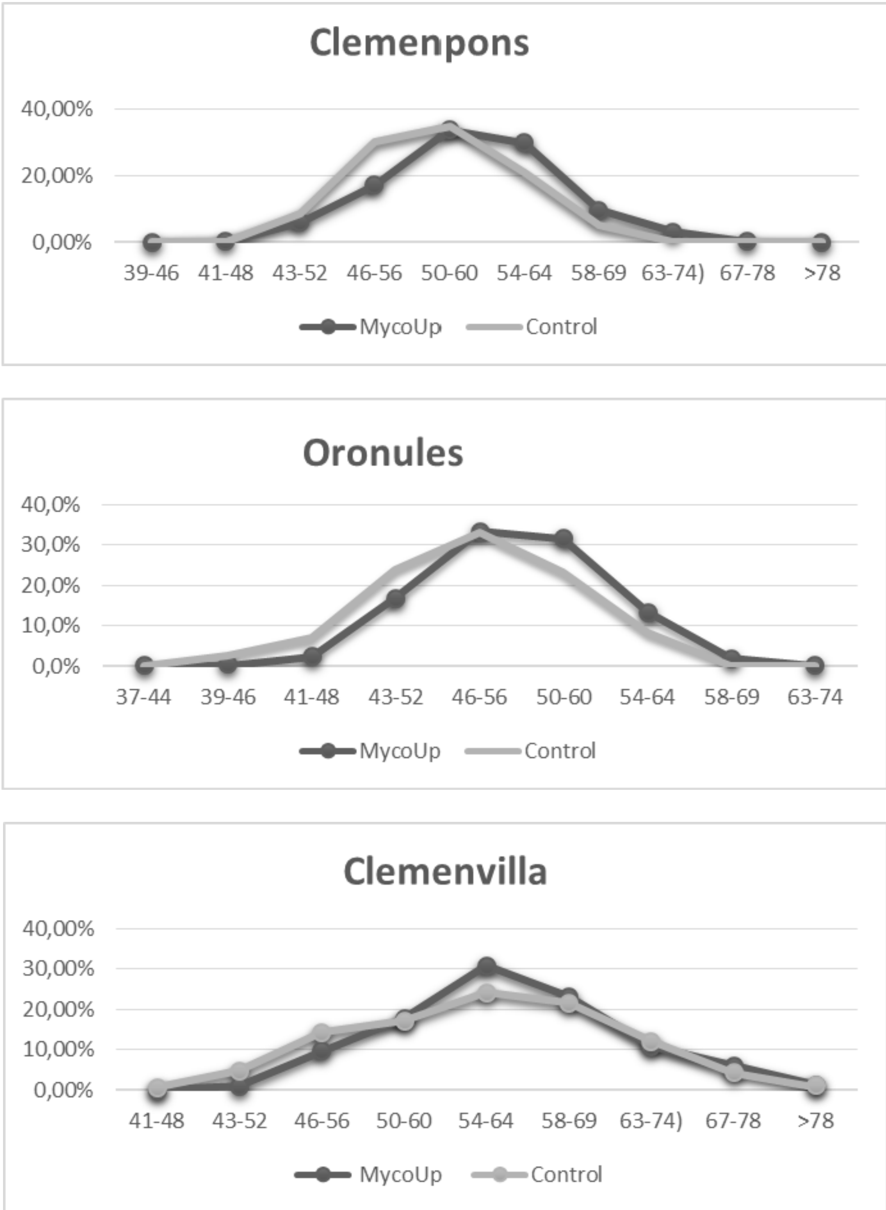

Figure 2. Effect of the application of *Glomus iranicum* var. *tenuihypharum* on the calibre of the cultivars of mandarin, 'Clemenpons', 'Oronules' and 'Clemenvilla'.

This effect allows the plant to synthesize highly energetic carbon compounds that not only influence plant growth, but also meet fungal requirements. To a large extent, the benefit of AMF in photosynthesis is related to the increase of chlorophyll content of leaves (Davies et al., 2000), which is greater in citrus plants in the middle of the growing season, since at this stage there is greater demand for photo assimilates (Reyes-Santamaría et al., 2000). Although the effect of AMF on height was not significant in one of the cases at 75 days, the presence of AMF structures in roots possible favoured the stimulation photosynthetic activity since 75 to 150 days, expressing greater mycorrhizal activity.

As can be seen, there was a notable difference in the production of extramatrical mycelium between the untreated plants at 75 and 150 days in relation to their treated equivalents (5 and 7 mg g^{-1} soil, respectively). In this case the double inoculation (39 mg g^{-1} of soil) further reinforced the activity of the network of extramatrical mycelium compared with a single application in spring (31 mg g^{-1} soil).

According to some authors, the maximum symbiotic relationship is reached when the mycorrhizal components (EM:AE) achieves a balance in which the contents are matched in both the external and internal phases, approaching the ideal value of 1 or below it. As can be observed in the results, in the case of untreated plants, a ratio of 3.76 and 2.62 was observed at 75 and 150 days, respectively, while plants treated with selected AMF showed a value that was much closer to symbiotic equilibrium and a lower ratio with the double inoculation (1.86).

Comparative study of the efficacy of the application of *G. iranicum* var. *tenuihypharum* and Rhizophagus irregularis on the production of lemon 'Fino'

The increased production observed in plants inoculated with *G. iranicum* var. *tenuihypharum* reflected the high degree of mycorrhization (Baslam et al., 2011a). Mycorrhizal symbiosis in lemon plants might have favored the absorption of water and nutrients (Navarro García et al., 2011), increasing production and calibers. The higher fruit production detected in plants treated with *G. iranicum* var. *tenuihypharum* can be mainly attributed to an increase in the nutrient absorption rate, which would improve the nutritional status of the lemon plants because of a more developed root system as a result of the mycorrhizal symbiosis (Marjanović and Nehls, 2008).

Hirrel and Gerdemann (1980) indicated that the increased uptake of P by plants inoculated with AMF seems to be one of the key factors responsible for increased vegetable production. In the present experiment, it was found that P uptake and growth only clearly increased in the case of *G. iranicum* var. *tenuihypharum* but not with *Rhizophagus irregularis* treated plants. Potassium plays an important role in stomatal movements, protein synthesis and in the response to changes in leaf water status (Evelin et al., 2013), but no significant differences in these respects were observed in the present study, while the increase in Ca detected in plants treated with *G. iranicum* var. *tenuihypharum* agreed with the observations of Cantrell and Linderman (2001).

Although whole mycorrhizal plants enhance the uptake of relatively immobile metal micronutrients, such as Fe and Zn (Baslam et al., 2011b), significant differences were observed between the strains used. Ca, Mg, Mn and Fe were higher, and P was within the optimum range calculated by the diagnosis and recommendation integrated system (DRIS) and proposed by Hartz et al. (2007). Different authors suggest great variability in these optimum ranges. For instance, in the case of Ca, Mg and Mn, Jones et al. (1991), reported different optimum ranges (Ca 1.5-3%; Mg 0.36-0.50% and Mn 25-250 ppm). In the case of phosphate, Hochmuth et al. (1991) indicated that optimum values range between 0.25 and 0.50%. Bearing in mind all these assumptions, all macro- and micronutrients were deemed to be within the optimal nutritional ranges.

Effect of the application of *G. iranicum* var. *tenuihypharum* on the productivity and calibre of the mandarin cultivars, 'Clemenpons', 'Oronules' and 'Clemenvilla'

As observed in the other trials, the application of *G. iranicum* var. *tenuihypharum* to tangerines/mandarins, not only generated significant increases in production, but also produced larger calibres, regardless of the cultivar under study. These results are supported by the above mentioned comments that the efficacy of this type of mycorrhizal fungus is closely related to the high dependence of citrus, which are unavoidably mycorrhizal-dependent (Graham, 1986; Graham and Eissenstat, 1994; Graham and Syvertsen, 1985; Krikun and Levy, 1980; Menge et al., 1982; Ortas et al., 2002) and the increase in production generated by a high physiological activity that, together with the symbiotic association with the species present in MycoUp, generates higher photosynthetic rates and at the same time greater biostimulation of treated plants.

Application of *G. iranicum* var. *tenuihypharum* to maximize the productive potential of citrus cultivation

As we have seen throughout this work, double mycorrhizal inoculation plays an important role in the development of the mycorrhizal colony, and the photosynthetic activity of the plant, favouring the development of the cultivation of citrus, which, like most fruit trees and olive trees, are highly dependent on the arbuscular-type mycorrhizal associations. The action of *G. iranicum* var. *tenuihypharum*, both in the spring and in the growth and fattening stage of the fruit, plays an essential role, because it forms an effective mycorrhizal colony that carries nutrients to the plant, and accumulates reserve substances in the roots such as starch and arginine. The double inoculation reinforces and helps the AMF colony, to inoculate the new flash roots emitted during the stage of fattening of the fruit with a lot of propagules active and ready to start new colonization.

The general conclusions in this respect are: in early cultivars, the application should be made in spring and 40 days before the proposed harvest date; in medium season cultivars, application is recommended in the spring and between 30 to 40 days before the harvest date; in late cultivars, application is recommended in spring and up to 60 days before harvest.

CONCLUSIONS

- The use of *Glomus iranicum* var. *tenuihypharum* with double inoculation produced the best physiological and growth effects of lemon 'Fino' plants of one year, promoting in turn the highest mycorrhizal activity;
- Fruit production of the plants treated with *Glomus iranicum* var. *tenuihypharum* was higher than that obtained with plants treated with *Rhizophagus irregularis* (35.67%) and the root system was more developed;
- The production of treated plants increased by 7.20 to 26% in the tangerine cultivars studied. In 'Clemenpons', the percentage of 46-56 mm caliber fruit was lower in the treated plants than in the control, while the percentage of 56-70 mm caliber fruits was favored by *Glomus iranicum* var. *tenuihypharum* (9.18% increase);
- Fruit reached a commercial color earlier in the sub-plot treated with *Glomus iranicum* var. *tenuihypharum* making it possible to harvest more fruit in the first pass. The greatest increase in production in response to mycorrhizal application was observed in 'Oronules' (26.3%) followed by 'Clemenvilla' and 'Clemenpons' (7.9 and 8.3%, respectively).

Literature cited

Adholeya, A., Tiwari, P., and Singh, R. (2005). Large scale inoculum production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. In Soil Biology In Vitro Culture of Mycorrhizae, S. Declerck, D.G. Strullu, and A. Fortin, eds. (Berlin; Heidelberg: Springer-Verlag), p.315–38.

Aguilera-Gómez, L., Davies, F.T., Jr., Olalde-Portugal, V., Duray, S.L., and Phavaphutanon, L. (1999). Influence of phosphorus and endomycorrhiza (*Glomus intraradices*) on gas exchange and plant growth of chile ancho pepper (*Capsicum annuum* L. cv. San Luis). Photosynthetica *36* (*3*), 441–449 https://doi.org/ 10.1023/A:1007032320951.

Alarcón, A., Ferrera-Cerrato, R., Villegas-Monter, A., and Almaraz, A.A. (1998). Efecto de la simbiosis micorrízica en la fotosíntesis de *Citrus volkameriana* Tan & Pasq. In Avances de la Investigación Micorrízica en México, R.R Zulueta, M.A. Escalona A., and D. Trejo A., eds. (Xalapa, Veracruz, Mexico: Universidad Veracruzana), p.119–125

Baslam, M., Garmendia, I., and Goicoechea, N. (2011a). Arbuscular mycorrhizal fungi (AMF) improved growth and nutritional quality of greenhouse-grown lettuce. J. Agric. Food Chem. *59* (*10*), 5504–5515 https://doi.org/10.1021/jf200501c. PubMed

Baslam, M., Pascual, I., Sánchez-Díaz, M., Erro, J., García-Mina, J.M., and Goicoechea, N. (2011b). Improvement of nutritional quality of greenhouse-grown lettuce by arbuscular mycorrhizal fungi is conditioned by the source of phosphorus nutrition. J. Agric. Food Chem. *59* (*20*), 11129–11140 https://doi.org/10.1021/jf202445y. PubMed

Cantrell, I.C., and Linderman, R.G. (2001). Preinoculation of lettuce and onion with VA mycorrhizal fungi reduces deleterious effects of soil salinity. Plant Soil *233* (*2*), 269–281 https://doi.org/10.1023/ A:1010564013601.

Davies, F.S., and Albrigo, L.G., eds. (1994). Citrus. Crop Production Science in Horticulture, 2 (Wallingford, UK: CAB International)

Davies, F.T., Jr., Estrada-Luna, A.A., Finnerty, T.L., Egilla, J.N., and Olalde-Portugal, V. (2000). Applications of mycorrhizal fungi in plant propagation systems. In Ecología, Fisiología y Biotecnología de la Micorriza Arbuscular,

A. Alarcón, and R. Ferrera-Cerrato, eds. (Mexico: Mundi Prensa), p.120–138.

Evelin, H., Giri, B., and Kapoor, R. (2013). Ultrastructural evidence for AMF mediated salt stress mitigation in *Trigonella foenum-graecum*. Mycorrhiza *23* (*1*), 71–86 https://doi.org/10.1007/s00572-012-0449-8. PubMed

Fernández, F., Vicente-Sánchez, J., Maestre-Valero, J.F., Bernabé, A.J., Nicolas, E., Pedrero, F., and Alarcón, J.J. (2014). Physiological and growth responses of young tomato seedlings to drip-irrigation containing two low doses of the arbuscular mycorrhizal fungus *Glomus iranicum* var. t*enuihypharum* sp. *nova*. J. Hortic. Sci. Biotechnol. *89* (*6*), 679–685 https://doi.org/10.1080/14620316.2014.11513137.

Gadkar, V., David-Schwartz, R., Kunik, T., and Kapulnik, Y. (2001). Arbuscular mycorrhizal fungal colonization. Factors involved in host recognition. Plant Physiol. *127* (*4*), 1493–1499 https://doi.org/ 10.1104/pp.010783. PubMed

Gerdemann, J.W. (1975). VA mycorrhizae. In Development and Function of Roots, J.G. Torrey, and D.T. Clarkson, eds. (London, UK: Academic Press), p.575–591.

Graham, J.H. (1986). Citrus mycorrhizae - potential benefits and interactions with pathogens. HortScience *21* (*6*), 1302–1306.

Graham, J.H., and Eissenstat, D.M. (1994). Host genotype and the formation and function of VA mycorrhizae. Plant Soil *159* (*1*), 179–185 https://doi.org/10.1007/BF00000107.

Graham, J.H., and Syvertsen, J.P. (1985). Host determinants of mycorrhizal dependency of citrus rootstock seedlings. New Phytol. *101* (*4*), 667–676 https://doi.org/10.1111/j.1469-8137.1985.tb02872.x.

Graham, J.H., Leonard, R.T., and Menge, J.A. (1982). Interaction of light-intensity and soil-temperature with phosphorus inhibition of vesicular arbuscular mycorrhiza formation. New Phytol. *91* (*4*), 683–690 https://doi.org/10.1111/j.1469-8137.1982.tb03347.x.

Hartz, T., Johnstone, P.R., Williams, E., and Smith, R.F. (2007). Establishing lettuce leaf nutrient optimum ranges through DRIS analysis. HortScience *42* (*1*), 143–146.

Herrera-Peraza, R.A., Hamel, C., Fernández, F., Ferrer, R.L., and Furrazola, E. (2011). Soil-strain compatibility: the key to effective use of arbuscular mycorrhizal inoculants? Mycorrhiza *21* (*3*), 183–193 https://doi.org/10.1007/s00572-010-0322-6. PubMed

Hirrel, M.C., and Gerdemann, J.W. (1980). Improved growth of onion and bell pepper in saline soils by two vesicular-arbuscular mycorrhizal fungi. Soil Sci. Soc. Am. J. *44* (*3*), 654–655 https://doi.org/10.2136/sssaj1980.03615995004400030046x.

Hochmuth, G., Maynard, D., Vavrina, C., and Hanlon, E. (1991). Plant Tissue Analysis and Interpretation for Vegetable Crops in Florida. Univ. Florida Special Publication SS-VEC-42.

Jones, J.B., Wolf, B., and Mills, H.A. (1991). Plant Analysis Handbook (Athens, Greece: Micro-Macro) pp.213.

Juarez, J., and Fernandez, F. (2015). *Glomus iranicum* var. *tenuihypharum* var. nov. Strain and Use Thereof as Bio-stimulant. Patent WIPO No: WO/2015/000612.

Krikun, J., and Levy, Y. (1980). Effect of vesicular arbuscular mycorrhiza on citrus growth and mineral-composition. Phytoparasitica *8* (*3*), 195–200 https://doi.org/10.1007/BF03158316.

Lukac, M., Calfapietra, C., Lagomarsino, A., and Loreto, F. (2010). Global climate change and tree nutrition: effects of elevated CO_2 and temperature. Tree Physiol. *30* (*9*), 1209–1220 https://doi.org/10.1093/ treephys/tpq040. PubMed

Manjarrez, M.M.J., Ferrera-Cerrato, R., and González-Chávez, M.C. (1999). Efecto de la vermicomposta y la micorriza arbuscular en el desarrollo y tasa fotosintética de chile serrano. Terra *17*, 9–15.

Marjanović, Z., and Nehls, U. (2008). Ectomycorrhiza and water transport. In Mycorrhiza (Berlin, Heidelberg: Springer), p.149–59.

Menge, J.A. et al. (1982). Predicting mycorrhizal dependency of troyer citrange on *Glomus fasciculatum* in California citrus soils and nursery mixes. Soil Science Society of American Journal *46* (*4*), 762–768.

Navarro García, A., Del Pilar Bañón Árias, S., Morte, A., and Sánchez-Blanco, M.J. (2011). Effects of nursery preconditioning through mycorrhizal inoculation and drought in *Arbutus unedo* L. plants. Mycorrhiza *21* (*1*), 53–64 https://doi.org/10.1007/s00572-010-0310-x. PubMed

Ortas, I., Ortakci, D., Kaya, Z., Cinar, A., and Onelge, N. (2002). Mycorrhizal dependency of sour orange in relation to phosphorus and zinc nutrition. J. Plant Nutr. *25* (*6*), 1263–1279 https://doi.org/10.1081/PLN-120004387.

Phillips, D.M., and Hayman, D.S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. *55* (*1*), 158–161 https://doi.org/10.1016/S0007-1536(70)80110-3.

Porter, N.W. (1979). The most probable number method for enumerating infective propagules of vesicular arbuscular mycorrhizal fungi in soil. Aust. J. Soil Res. *17* (*3*), 515–519 https://doi.org/10.1071/ SR9790515.

Pou, A., Medrano, H., Tomás, M., Martorell, S., Ribas-Carbó, M., and Flexas, J. (2012). Anisohydric behaviour in grapevines results in better performance under moderate water stress and recovery than isohydric behaviour. Plant Soil *359* (*1-2*), 335–349 https://doi.org/10.1007/s11104-012-1206-7.

Rennenberg, H., and Schmidt, S. (2010). Perennial lifestyle–an adaptation to nutrient limitation? Tree Physiol. *30* (*9*), 1047–1049 https://doi.org/10.1093/treephys/tpq076. PubMed

Reyes-Santamaría, M.I., Villegas-Monter, A.M., Colinas-León, T., and Calderón-Zavala, G. (2000). Peso específico, contenido de proteína y de clorofila, en hojas de naranjo y tangerino. Agrociencia *34*, 49–55.

Ryan, M.H., and Graham, J.H. (2002). Is there a role for arbuscular mycorrhizal fungi in production agriculture? Plant Soil *244* (*1-2*), 263–271 https://doi.org/10.1023/A:1020207631893.

Wu, Q.S., and Zou, Y.N. (2009). Arbuscular mycorrhizal symbiosis improves growth and root nutrient status of citrus subjected to salt stress. Sci. Asia *35* (*4*), 388–391 https://doi.org/10.2306/scienceasia1513-1874.2009.35.388.