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REDUCTION OF THE JUVENILE PERIOD OF NEW OLIVE PLANTATIONS THROUGH THE EARLY APPLICATION OF MYCORRHIZAL FUNGI

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This study reports the responses of olive plantlets (cv. Cornicabra) to arbuscular mycorrhizal (AM) colonization by *Glomus intraradices*, *Glomus mosseae*, and *Glomus claroideum*, in terms of changes in the number and length of shoots and trunk diameter. In colonized plants, the number of shoots increased by 29% 3 months after transplanting, reaching as high as 121% at 12 months after transplanting. *G. mosseae* was the most influential species in this respect. Six months after transplanting, the colonized plants showed a greater number of shoots than did the control plants at 12 months after transplanting. Arbuscular mycorrhizal colonization may therefore provide a mechanism by which the juvenile growth period of these olive trees can be reduced. *G. claroideum*–colonized plants showed 118% more shoots than did control plants at 6 months after transplanting; at 12 months, they showed 100% more shoots. This fungus was the most effective at increasing trunk diameter and plant biomass at 12 and 24 months after transplant. Three months after transplanting, the trunk diameter in *G. claroideum*–colonized plants was similar to that observed 12 months after transplanting in noncontrol plants—a 10-month growth advantage. To maximize root and shoot growth and to reduce juvenile growth, Cornicabra olive plantlets apparently need to enter into a symbiotic relationship with *Glomus* species. The present results show the effectiveness of *Glomus* colonization in reducing the juvenile period of these trees; inoculation with *Glomus* species is therefore recommended as an olivicultural practice. (Soil Science 2006;171:52–58)

Key words: Juvenile period, mycorrhizal fungi, semi-hardwood cutting, inoculation, glomus spp.

OLIVES (*Olea europaea* L.) are a typical crop of the Mediterranean. In Spain, the area given over to olive trees is approximately 2,300,000 ha, traditionally distributed over 10 areas with different production characteristics. In the central area (covering the regions of Madrid and Castilla–La Mancha), the predominant cultivar is Cornicabra, with a planted area of approximately 300,000 ha (MAPA, 1999).

Olive trees have typically been grown on marginal soils and/or those with low fertility because the species shows great adaptability to adverse soil conditions as well as both tolerance and resistance to drought (Barranco et al., 1997). However, growth in trunk diameter and overall development are slow under stressed conditions.

In Spain, new plantations (usually with about 200-300 olive trees/ha) are established with plants obtained via the mist propagation of hardwood olive cuttings. This mode of plant production has been widely adopted by nursery growers owing to the advantages it offers in terms of rooting efficiency and plant health (Caballero and del Rio, 1994).

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Plants raised in nurseries require approximately 18 months to develop to a stage at which they are ready for sale. During this time, they are grown in polyethylene bags or pots containing an artificial substrate of sand and peat (1:1, v/v). After planting, young olive trees then require a period of 5 to 7 years during which time they have to be irrigated, followed by a further 10 years of rain-fed growth (Porras et al., 1999) before shaker-harvesters can safely be used to collect their fruit. From a physiological point of view, this period is unproductive: fruit production is at best very low and commonly nil. Shortening this period is one of the greatest challenges in oliviculture.

Owing to their design characteristics and the principle on which they work, shaker-harvesters require olive tree trunks have a minimum diameter of about 12 cm. If smaller, the shaker claw can cause a significant loss of bark which can lead to the entry of pests and disease organisms. The result is a loss of vigor and perhaps even the death of the tree.

Fontanazza (1984) in Italy and Pastor and Humanes (2000) in Spain established the most suitable pruning rules for shortening the juvenile period—but it remains too long. Establishing a symbiotic relationship between these trees and arbuscular mycorrhizal (AM) fungi might, however, provide a means of improving growth and thus minimizing this period. AM fungi can certainly increase the growth of plants (Harper et al., 1991; Vidal et al., 1992; Ruíz-Lozano et al., 1996a, 1996b, Rinaldelli and Mancuso, 1998; Barea et al., 1999; Porras et al., 2005), although not all species produce the same effects (Jaízme-Vega and Rodríguez-Romero, 1997; Citernesi et al., 1998; Barea et al., 1999). Such differences indicate a degree of functional compatibility between host variety and fungal species, as previously reported (Azcón and Ocampo 1981, Estaún et al., 1987; Monzón and Azcón 1996 Monzón and Azcón 1996).

The use of *Glomus* species in olive plant production has been studied in micropropagated commercial olive plants (cv. Arbequina and Leccino) (Calvente et al., 2004). The results suggest that these plants should be inoculated during nursery production. Based on these results, the aim of the present study was to determine the influence of *Glomus mosseae*, *Glomus intraradices*, and *Glomus claroideum* (three drought- and infertile soil–adapted AM fungi isolated from the olive rhizosphere) on the growth of cv. Cornicabra olive plants obtained by mist propagation and the length of their juvenile period. This cultivar was selected because it is the most common in Castilla–La Mancha and because information on its responses to AM colonization is lacking.

MATERIAL AND METHODS

Semihardwood cuttings of cv. Cornicabra olive trees were cut to lengths of about 15 cm, leaving three pairs of leaves at the top. The basal end of each cutting was treated with INABAR-PLANT IV, a commercial powder product containing 0.4% indolebutyric acid, 0.4% naphthalene acetic acid, and 15% Ziram. These cuttings were planted in perlite at a density of 1500 plants/m^2 and placed inside a propagation tunnel equipped with a high precision environmental control system (Porras et al., 2000). The substrate was heated to 22 °C, and the air temperature maintained at 20 °C. The cuttings were automatically moistened using mirror-type nozzles (diameter, 0.8 mm; pressure, 0.3 MPa) so that the leaves remained moist at all times.

The rooted cuttings were inoculated during transplanting (or not, thus providing uninoculated control plants) with fragments of colonized root spores and mycelia of either *G. intraradices* (BEG 125) Schenck and Smith, *G. mosseae* (BEG 124) (Nicol and Gerard) Gerdeman and Trappe, or *G. claroideum* (BEG 125) Schenck and Smith. All three fungi were isolated from dry infertile soil (Spain) from the rhizosphere of long established olive trees and cultivated in a mixture of sand and sepiolite (1:1 an average v/v) using alfalfa (*Medicago sativa*) as a host. Three grams of inoculum of each fungus was located directly under the roots when placing the plants in pyramidal (truncated), pressed peat containers (120 cm^3) filled with a sand-peat (1:1, v/v) substrate. This substrate was previously steam-sterilized at 98 °C for 1 h on 3 consecutive days. All treatments (including the noninoculated controls) were replicated 18 times, giving a total of 72 pots. These were arranged in a randomized complete block design. The controls were treated with a corresponding sterilized *Glomus* inoculum, plus a 2-mL aliquot of a fungus-free filtrate (<20 nm) produced from a corresponding fresh inoculum, to provide a microbial population free of AM propagules.

During the acclimatization period, the plantlets were placed in the same propagation tunnel used for their rooting. The substrate was heated to 22 °C, and the air maintained at

20 °C. Six weeks later, the 72 plantlets with their corresponding pressed peat containers were placed in 2-L black polyethylene pots filled with sterilized substrate and randomly positioned in a shaded area covered with a polyethylene net. This area was equipped with microsprinklers controlled by an electronic sensor (Porras et al., 2000).

After eight weeks in this area, the plants were transferred to a climate-controlled greenhouse (18-24 °C) to protect them from temperatures lethal to young olive trees (which can occur in both summer and winter in the greenhouses of this region). The plants were distributed on randomized blocks and watered to field capacity twice a week when there was strong sunshine, and once a week when there was less.

The first stage of the experiment involved 48 plants (12 inoculated with *G. intraradices*, 12 with *G. mosseae*, 12 with *G. claroideum*, and 12 noninoculated controls). These were kept in the same 2-L pots from December 1 until the same date on the following year. The development of these plants was recorded during this time, measuring the maximum height and the number and length of the shoots attained by each.

After the first year, these 12 plants from each group were carefully removed from their pots, and the dry weights of the aerial and underground parts were determined.

The second stage of the experiment involved 24 plants (6 inoculated with *G. intraradices*, 6 with *G. mosseae*, 6 with *G. claroideum*, and 6 noninoculated controls). These were all pruned to leave them with a single trunk. After removing them from their pots, they were placed in 100-L black polyethylene containers filled with the same substrate—this time, not sterilized to simulate conditions closer to those experienced by field crops. They were then tied to a bamboo stake using an HT-B Tapener binding machine. These plants were kept in these 100-L containers for 12 months, during which time all the shoots issuing from the trunk were removed. In addition, they were marked with indelible paint 10 and 60 cm from the base, and the trunk diameter at these positions was measured monthly with an MIT digital slide caliper.

The inoculated and noninoculated plants were fertilized monthly with 100 mL of Hewitt's (1952) nutrient solution, modified as follows: 0.368 g/L $MgSO_4 \cdot 7H_2O$, 0.0245 g/L EDTA-Fe, 0.00223 g/L $MnSO_4 \cdot 7H_2O$, 0.00024 g/L $CuSO_4 \cdot 5H_2O$, 0.00029 g/L $ZnSO_4 \cdot 2H_2O$, 0.00186 g/L H_3BO_3, 3.5×10^{-5} g/L $Na_2MoO_4 \cdot 2H_2O$, 0.303 g/L KNO_3, 1.416 g/L Ca $(NO_3)_2$, and 0.0208 g/L $NaPO_4H_2 \cdot 2H_2O$.

To verify the colonization of the roots by the fungi, more than 50 root segments of about 1 cm were selected at the end of each phase of the assay (1 g/plant) and stained using the Phillips and Hayman (1970) modified technique. The roots were carefully washed in tap water, cut into segments of 1 to 2 cm, and submerged in a 10% KOH solution for 20 min at 100 °C. After washing again with tap water, roots with excess pigmentation were submerged in a solution of H_2O_2 (10% vol.) to counterstain them. All roots were then immersed in 0.1 *N* HCl for 15 min to neutralize the KOH. The HCl was eliminated, and the roots were submerged in 0.05% trypan blue (5 trypan blue: 95 lactic acid, v/v) for 15 min at 100 °C. Excess stain was removed with water. The stained roots were kept in a 50% solution of lactic acid and water until analysis by light microscopy (NIKON BH 40x). Mycorrhizal colonization was quantified using the grid-line intersect method described by Giovannetti and Mosse (1980).

After 1 year, the plants from the 100-L containers were carefully removed from their containers (100 L); the percentage of root colonization was confirmed, and the dry weight of the shoots and roots was recorded.

Roots from uninoculated control plants were examined, but no AM colonization was detected. Thus, these plants remained free from mycorrhiza throughout the experiment.

The efficiency of the mycorrhizal inoculum was calculated as the percentage increase in shoot weight produced by each *Glomus* species with respect to the uninoculated control plants.

Data were examined using the Pearson chi-square test and analysis of variance (repeated-measures design). All calculations were made using Statgraphics Plus 2.1 software.

RESULTS

Mycorrhizal colonization was found to be critical in increasing the growth (in terms of shoot number, length, and maximum height) of the olive plantlets. All three *Glomus* species used were effective in increasing all the growth variables measured (Tables 1, 2, and 3).

The results show the effectiveness of all three *Glomus* species to be similar in the early growth period (both at 12 months, i.e., just before transplanting to the 100-L containers,

TABLE 1

Effect of mycorrhizal *Glomus* species on the number of shoots per plant [A], the length of shoots (cm) [B], the length of shoot (cm)/number of shoot ratio [C], and the maximum height (cm) of shoots [D] over four growth periods

	Months after inoculation			
	3	6	9	12
[A]				
Control	3.4 a	4.5 a	7.0 a	8.4 a
G. intraradices	4.3 a	11.1 b	17.0 b	18.2 b
G. mosseae	4.4 a	10.0 b	22.2 b	27.0 b
G. claroideum	4.1 a	10.4 b	19.9 b	23.2 b
[B]				
Control	4.8 a	16.3 a	39.7 a	54.6 a
G. intraradices	7.4 b	35.7 b	80.1 b	100.3 b
G. mosseae	5.5 a	32.0 b	63.6 b	71.9 b
G. claroideum	5.1 a	34.5 b	86.7 b	109.1 b
[C]				
Control	1.4 a	3.6 a	5.7 c	6.4 c
G. intraradices	1.7 a	3.2 a	4.7 b	5.5 b
G. mosseae	1.2 a	3.2 b	2.9 a	2.7 a
G. claroideum	1.2 a	3.3 a	4.3 b	4.7 b
[D]				
Control	6.6 a	16.3 a	40.6 a	45.8 a
G. intraradices	8.1 b	35.7 b	80.1 b	85.1 b
G. mosseae	8.1 b	32.8 b	64.3 b	69.3 b
G. claroideum	7.4 a	35.8 b	90.1 b	96.3 b

Means of 18 replicate values in columns followed by different letters indicate significant differences ($P \leq 0.05$).

and 12 months later). Although no significant differences between the effects of these fungi were found, some specific trends were noted. *Glomus mosseae* was the most effective at increasing the number of shoots per plant (48% more efficient than *G. intraradices* in the last evaluation, that is, 12 months after inoculation; Table 1), although it was the least efficient at increasing the length of olive shoots. The ratio between the length and number of shoots differed depending on the colonizing fungus (Table 1).

Six months after inoculation, the colonized trees had produced more shoots per plant than the controls had produced at 12 months after inoculation (Table 1). In fact, irrespective of the fungus involved, colonization advanced shoot production by about 6 months.

Twelve months after transplanting, olive plant biomass (shoot and root) was greatly increased by fungal colonization (Table 3). The stimulation of growth ranged from 122% (root) to 150% (shoot) when the colonizing fungus was *G. mosseae* and from 181% (root) to 200% (shoot) when the colonizing species was *G. claroideum* (Table 3).

The shoot/root dry weight ratio was increased in the colonized plants. *G. claroideum* was the most effective in increasing trunk diameter (whether measured at 10 or 60 cm from the base; Table 2). The effect of mycorrhizal colonization on trunk diameter 10 cm from the base was greater than on any of the other variables. Three months after inoculation, plants colonized with *G. claroideum* showed similar trunk radii (10 cm from the base) to those of control plants at 12 months—a 10-month growth advantage (Table 2).

After 24 months of growth, colonization with *G. intraradices* increased shoot dry biomass by 57% over that of controls, whereas *G. claroideum* increased the same by 150%. Root developments followed similar trends (Table 3). These results provide evidence of the great effectiveness of *Glomus* species, particularly *G. claroideum*, in improving Cornicabra growth. Nevertheless, the efficiency of these fungi was greater 12 months after transplanting than at 24 months.

These results support the idea that olive trees need to be colonized by mycorrhiza to achieve maximum development. The growth variables measured were similarly improved by each *Glomus* species used because no significant differences were found. No differences were seen in the ability of the different *Glomus* species to colonize the roots. All roots showed colonization.

DISCUSSION

Compared with the control plants, inoculation with any of the three *Glomus* isolates

TABLE 2

Effect of mycorrhizal *Glomus* species on trunk diameter (mm) measured at 10 cm [A] and at 60 cm [B] from the base over four growth periods

	Months after inoculation			
	3	6	9	12
[A]				
Control	6.36 a	7.06 a	7.81 a	8.52 a
G. intraradices	7.69 b	8.99 b	10.38 bc	11.52 c
G. mosseae	7.49 b	8.29 b	9.12 b	9.89 b
G. claroideum	8.40 b	9.40 b	10.59 c	11.64 c
[B]				
Control	3.86 a	4.31 a	5.02 a	5.68 a
G. intraradices	4.25 ab	5.04 b	6.06 bc	7.08 bc
G. mosseae	4.25 ab	4.78 a	5.58 ab	6.29 ab
G. claroideum	4.71 b	5.4 b	6.5 c	7.45 c

Means of 18 replicate values in columns followed by different letters indicate significant differences ($P \leq 0.05$).

TABLE 3

Effect of mycorrhizal *Glomus* species on shoot and root dry weight shoot/root ratio and percentage of AM colonized plants after 12 [A] and 24 months of growth [B]

	Plants AM colonized (%)	Dry weight (g)		S/R	Inoculum efficiency
		Root	Shoot		
[A]					
Control	0	8.0 b*	12.7 b	1.58	—
G. intraradices	100	18.4 a	32.3 a	1.75	254
G. mosseae	100	17.8 a	31.7 a	1.78	249
G. claroideum	100	22.5 a	38.1 a	1.69	300
[B]					
Control	0	81.8 a*	75.8 a	0.92	—
G. intraradices	100	111.4 b	119.4 b	1.07	157
G. mosseae	100	116.9 b	132.5 b	1.13	174
G. claroideum	100	153.9 c	189.8 c	1.23	250

Means of 18 replicate (A) or 6 (B) values in columns followed by different letters indicate significant differences ($P \leq 0.05$).

increased the number and length of the shoots and the trunk diameter. Although the differences between these improvements were nonsignificant, some specific trends were seen. Inoculation with *G. mosseae* led to the production of the greatest number of shoots, with improvements of 29% over controls 3 months after inoculation and of 221% at 12 months after inoculation. Nevertheless, improvements in shoot length and trunk diameter were greater with *G. claroideum* and especially *G. intraradices*. At 12 months after inoculation, the shoots of the inoculated plants were more than 100% longer than those of the control plants; in addition, the trunk diameter of 10 cm from the base was 36% greater in the inoculated plants. In earlier work, Calvente et al. (2004) reported *G. intraradices* to be the most effective fungus for improving the development of cv. Arbequina and Leccino olive trees growing outside their normal area and therefore in soils with different characteristics.

In most studies, mycorrhizal colonization is reported to improve the macro and/or micronutrient status of plants. Colonization may influence the affinity of the uptake system (Km) and threshold concentration (C_{min}), values related to root physiological and morphological characteristics. Other mechanisms by which *Glomus* species might affect plant growth have also been described (Ruíz-Lozano and Azcón, 2000). For example, they can increase the photosynthetic output, transpiration, stomatal conductance, and the efficiency of water use. Some mechanisms could be more advantageous than others in terms of long-term plant survival under particular ecological conditions. In fact, under drought conditions, an improvement in the efficiency of water use is one of the most important benefits possible (Marulanda et al., 2000).

All three *Glomus* species had a positive influence on plant growth. Thus, regardless of the fungus used, mycorrhizal colonization seems to be critical to Cornicabra olive tree development, as reported by Azcón and Barea (1997) and Barea et al. (1999) for other plant species.

The inoculation of semihardwood cuttings after the emission of their first roots should allow the beneficial influence of these *Glomus* species to be gained from the earliest stage possible. This is in agreement with the results of Jaízme-Vega and Rodríguez-Romero (1997) for banana plants. In agreement with results reported by Barea et al. (1987) and Vidal et al. (1992), only 4% of the inoculated plants were lost after transplant compared to 7% of the uninoculated controls. The Pearson chi-square test showed that inoculation significantly increased ($P < 0.05$) resistance to transplant stress.

High input techniques (for achieving high yields) are often used when soil nutrients need to be replenished, but this practice can lead to environmental problems. Interest has therefore been growing in sustainable agricultural practices such as the use of biofertilizers. In this respect, microorganisms such as *Glomus* species have an important role to play. By providing balanced nutrition and promoting nutrient acquisition, they reduce the need for chemical fertilizer. In addition, *Glomus* colonization increases tolerance to transplantation.

The present results indicate the positive influence of *Glomus* species on Cornicabra olive trees growing in poor soils and provide

information on the role of AM associations in the maintenance of plant growth under limiting environmental conditions. During the winter months, there were very few differences between the growth of the AM inoculated and control plants; this was observed in both the early and later growth stages. This might be due to the typical vegetative winter pause experienced by olive trees in Castilla–La Mancha. This changed with the arrival of spring, when notable differences in the growth of inoculated plants were seen. These results agree with those of Porras et al. (2003, 2005).

Colonization with *Glomus* species has previously been shown to affect the growth of olive trees (Calvente et al., 2004, Porras et al., 2003, 2005). In fact, olive species are typically endomycorrhizal plants and are reliant upon such symbiosis. More information is needed, and comprehensive investigations of the way of which different AM endophytes influence the juvenile development of this important tree are required. Such knowledge should provide insight into the functional differences between different combinations of plant and *Glomus* species.

In the present study, the dry weights of the aerial and underground parts of the inoculated olive tree were approximately double those of the noninoculated plants. Thus, the colonization of Cornicabra olive plantlets by *Glomus* species can be used to reduce the juvenile period of new olive tree plantations, as shown in avocado by Vidal et al. (1992). The study provides the first description of the effects of *Glomus* colonization in shortening this period and on post transplant plant survival.

In summary, nursery growers should systematically inoculate olive plants (raised under mist propagation from hardwood cuttings) with *Glomus* species. The stimulus of trunk diameter growth could help reduce the period before shaker-harvesters can be used to collect fruit from these trees.

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