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Development and resistance to *Verticillium dahliae* of olive plantlets inoculated with mycorrhizal fungi during the nursery period

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SUMMARY

The current study, performed in Castilla-La Mancha (Spain) in 2003–04, reports the growth, nutrition, tolerance to transplanting stress, and resistance to *Verticillium dahliae* of olive plantlets (*Olea europaea* L.) inoculated with different arbuscular mycorrhizal (AM) fungi (*Glomus mosseae*, *G. intraradices* and *G. claroideum*). Inoculated plants tolerated the stress of transplanting better than non-inoculated plants. Compared with controls, plantlets inoculated with any of these three *Glomus* species grew taller, had more and longer shoots, and showed higher plant N, P and K concentrations. However, colonization seemed to have no influence on resistance to *V. dahliae*.

INTRODUCTION

Olives are the most important oil crop of Castilla-La Mancha (central Spain), where some 278 443 ha are occupied by olive trees. The cultivar Cornicabra has a stable composition: the proportion of the fresh weight that is oil is 0·19 and oleic acid constitutes 0·77 of the oil (Barranco 1998). It occupies some 269 000 ha, making it the foremost cultivar of the region and the second most important in Spain.

In recent years, the Spanish olive-growing sector has experienced a phase of expansion, both in terms of production and new land brought under cultivation. This expansion is due to the increasing demand for olive oil by national and international markets, and has been accompanied by an upturn in nursery activity. According to data supplied by the National Institute of Nursery Seeds and Plants, 261 Spanish nurseries generated over 5·5 million olive plantlets in 1999/2000 (Anon. 2004).

Mist propagation of semi-woody olive tree cuttings has been widely adopted by nursery growers due to its improvement of rooting efficiency and the advantages it offers in terms of plant health (Porras *et al*. 1997; Caballero & del Rio 1999). However, two major problems arise in creating new olive plantations with mist-propagated plants: the slow growth of the plantlets in the sand-peat substrate (1:1 v:v) typically used by nurseries (Porras *et al*. 1997) (a minimum of 18 months is required before such plants can be marketed, although they are still rather small; Porras *et al*. 1991), and the appearance of *Verticillium* wilt.

Verticillium wilt is a disease of the vascular system of plants (Stapleton & Duncan 2000), caused by the soil-borne fungus *Verticillium dahliae* Kleb. (Jiménez Díaz *et al*. 1984; Trapero & Blanco 1999; Soriano *et al*. 2000). The species is characterized by being able to survive in the ground for many years (Adams & Thomas 1998). The rhizosphere of transplanted plants or the substrates in which they are raised may also be a source of infection. The disease can occur anywhere in the world and has recently appeared in the olive groves of Castilla-La Mancha (Trapero & Blanco 1999). It attacks new plantations especially, sometimes very severely. In adult plants the infection generally spreads slowly, but to such an extent that they can eventually die. Young plants can recover from mild attacks, but they can die in one season if infection is heavy (Trapero & Blanco 1999).

The control of this disease is difficult and expensive (Sinclair & Hudler 1998). Conventional fungicides

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are ineffective; therefore treatment usually involves cultivation practices (Tjamos *et al.* 1991, 2000).

Unlike *Verticillium*, arbuscular mycorrhizal (AM) fungi often enter into symbiotic relationships with plant roots. After colonizing them, the external mycelia appear to trigger extension of the root system, increasing its contact with the soil and thereby improving water and nutrient absorption (Hayman *et al.* 1976; Harper *et al.* 1991; Ruiz-Lozano *et al.* 1996*a*, *b*; Rinaldelli & Mancuso 1998; Barea *et al.* 1999) and plant growth (Vidal *et al.* 1992; Davies *et al.* 2000). Numerous papers have reported that many plants depend on mycorrhiza for achieving a higher growth rate. Colonized plants produce higher yields, are more tolerant to the stress of transplanting, show greater affinity for soil nutrients, and are more resistant to certain diseases (Barea *et al.* 1987; Requena *et al.* 1997; Azcón-Aguilar *et al.* 1999; Camprubi *et al.* 2000; Estaún *et al.* 2003; Ganz *et al.* 2002; Caravaca *et al.* 2002; Querejeta *et al.* 2003).

It is well known that the improvement in crop performance brought about by colonization depends not only on the combination of AM fungus and host species (Barea *et al.* 1999) but also on the AM fungus-cultivar combination (Citernesi *et al.* 1998; Linderman & Davis 2004; Alguacil *et al.* 2003). In *Olea europaea*, *Glomus intraradices* and *G. claroideum* have been shown to be the most influential AM fungi (Caravaca *et al.* 2003*a*, *b*); *G. mosseae* has been successfully used in the mycorrhization of other woody species (Pirazzi *et al.* 1999; Monticelli *et al.* 2000). The aim of the present study was to examine the influence of these three AM fungi on the growth, nutrition, tolerance to transplantation and resistance to *Verticillium dahliae* of cv. Cornicabra olive plantlets obtained by mist propagation of semi-woody cuttings.

MATERIALS AND METHODS

Plant material

Semi-woody cuttings of cv. Cornicabra trees were obtained from an olive grove and cut to lengths of 15 cm. All cuttings were left with three pairs of leaves at the top (Porras *et al.* 1997). The bottom of each cutting was treated with 4 mg/g indole butyric acid, 4 mg/g naphthaleneacetic acid and 150 mg/g Captan (Inabarplant 4, San Adrián 42-08030 Barcelona, Spain). All 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 leaves were kept moist by spraying water at 0·3 MPa from 0·8 mm diameter nozzles.

AM fungi

The three species of fungi used in the assays all belonged to the genus *Glomus*, which accounts for 0·99 of the AM fungi in Spanish soils (Azcón, personal communication). Rooted cuttings were inoculated with spores, mycelia and mycorrhized root fragments produced by stock cultures of *G. intraradices* Schenck & Smith, *G. claroideum* Schenck & Smith and *G. mosseae* Nicol. & Gerd. These fungi originally came from non-agricultural soils in Murcia (southeastern Spain) and were multiplied by Dr J. M. Barea (Agricultural Experimentation Centre CSIC, Zaidín, Granada, Spain) in a mixture of sand and sepiolite using alfalfa (*Medicago sativa* L.) as the host. Four blocks of 1000 plantlets per block were transplanted to 120 ml pyramid-trunk pressed peat pots containing sand and blonde peat (1:1 v:v; one cutting per pot; this large number of plantlets was used for the present and other trials). The plants of one control block for each of the three AM fungi received 3 g of inoculum directly under their roots. The rooting substrate was previously pasteurized at 98 °C for 1 h on 3 consecutive days.

The plantlets were then returned to the propagation tunnel. After 6 weeks the roots began to emerge through the pressed-peat containers. Without removing them from these containers, 69 plantlets from each of the four blocks, selected for the uniformity of their size, were then retransplanted to 2 litre black polyethylene pots filled with sterile substrate. These were placed randomly in a tunnel covered with a black polyethylene mesh and equipped with a microsprinkler system controlled by an electronic humidity sensor. The plants were kept in the tunnel for 8 weeks before being transferred to a greenhouse with an air conditioning system, where they were kept from the beginning of January until the end of December. All plants were watered twice weekly during the hottest period, and once a week when the temperature was lower.

Mycorrhizal association achieved

Four blocks of 18 plants per block were established – one control block plus one block for each of the three AM species assayed. Every 2 months, the proportion of roots with mycorrhizae was determined in three plants from each block using the Phillips & Hayman (1970) staining method. The plants were taken out of the pots and a sample of their roots cut into pieces 1·5 cm long. These were placed in a plastic test tube, at the base of which six 1-mm diameter holes had been bored. The perforated base was then positioned in a precipitation vessel containing 100 g/kg KOH for 2 days, then in another containing HCL 0·1 N for 30 min, and finally in one containing trypan blue diluted with lactic acid (0·5 mg/g)

for 5 h. Excess stain was eliminated by placing the same test tubes in precipitation vessels containing distilled water. The root fragments were then removed from the perforated tubes, mounted on glass slides and soaked in lactic acid. After covering with a coverslip they were observed using an optical microscope (Nikon Alphaphot-2 YS2). Using contiguous sections (2 mm apart), the number of fragments of each root showing typical fungal structures, such as mycorrhiza, hyphae, arbuscules, vesicles or spores, was counted. Proportional colonization was determined using the following formula:

$$\text{MIC} = \text{number of mycorrhizal intersections} / \text{total number of observed intersections}$$

Verticillium dahliae

The defoliating pathotype isolate Vd 117 (provided by the Plant Pathology Department of the E.T.S.I.A.M., Córdoba) was employed in the present study. The fungus was maintained in Petri dishes containing potato-dextrose-agar (PDA) medium. Hyphal disks (1 cm diameter) were extracted from the edge of the colony of single-spore cultures, incubated for 10 days in the dark at 22 °C, transferred to Petri dishes containing PDA, and then incubated under the same conditions for 10 days followed by 4 days under black light at room temperature. After the incubation period, 3 ml of distilled sterile water was added to each Petri dish. After leaving for a few minutes, the plate was scraped and the fungal material obtained filtered through four layers of surgical gauze. The conidial suspension this provided was adjusted to a concentration of 10^7 conidia/ml.

Plant fertilization

All plants were provided monthly with 100 ml of Hewitt's (1952) modified nutrient solution (20 ml $MgSO_4 \cdot 7H_2O$/l [18·4 g/l], 10 ml EDTA-Fe/l [2·45 g/l], 1 ml $MnSO_4 \cdot 7H_2O$/l [2·23 g/l], 0·1 ml $CuSO_4 \cdot 5H_2O$/l [2·4 g/l], 0·1 ml $ZnSO_4 \cdot 2H_2O$/l [2·9 g/l], 0·1 ml H_3BO_3/l [18·6 g/l], 0·1 ml $Na_2MoO_4 \cdot 2H_2O$/l [0·35 g/l], 10 ml KNO_3/l [30·3 g/l], 20 ml Ca $[NO_3]_2$/l [70·8 g/l] and 1 ml Na $H_2PO_4 \cdot 2H_2O$/l [20·8 g/l]).

Evaluation of plant growth

To evaluate the growth of the plantlets, 4 blocks of 15 plants/block were used (one block used as a control, plus three blocks of 15 plants each inoculated with either *G. mosseae*, *G. intraradices* or *G. claroideum*). Plant growth was evaluated by counting the number of shoots per plant, calculating the mean shoot length, and measuring plant height. These variables were assessed every 2 months. At the end of the assay, both shoot and root dry weight was determined after drying the plant material at 70 °C for about 20 h until reaching a constant weight.

Evaluation of N, P and K absorption

At the end of the evaluation of plant growth, the leaf N, P and K concentrations were determined using the method of Lachica *et al.* (1973). Leaves were taken from the olive plantlets and ground in a mill with a 0·25 mm sieve. Nitrogen levels were determined using an injection flow autoanalyser, P was measured colorimetrically, and K by atomic absorption (all performed at the *Centro de Investigación y Formación Agraria de la Venta del Llano*, Mengíbar, Jaén, Spain).

Resistance to Verticillium dahliae

Resistance to *V. dahliae* was determined in plants whose roots were left intact in their pots, in plants removed from their pots, and in those whose roots were intentionally damaged.

For plants with intact roots, 4 blocks of 36 plants per block (one block for each of the AM species plus one control block) were used. Every 2 months, six plantlets in each block were inoculated with *V. dahliae* by adding 50 ml of an aqueous conidial solution (10^7 conidia/ml) to the soil. The plants thus inoculated were transferred to a growth chamber where they were incubated at 22 °C under a 16 h photoperiod (450 mM $E/m^2 \cdot s$). These were watered by hand to pot capacity once per week. Each group of plantlets was kept in the growth chamber for 3 months, during which time they were periodically inspected for disease symptoms. At the end of this time, the number of dead plants in each group due to *V. dahliae* attack was recorded.

To determine the resistance of injured plants, 5 blocks of 36 plants/block (a positive control [P-control] block, a negative control [N-control] block, and one block for each of the AM species) were employed. Plants were extracted from six pots in each block every 2 months and their roots washed in water to remove all soil. Cuts were then made in five root ends of each plant. The roots of N-control plants were immersed in water, while those of the P-control and mycorrhized plants were soaked for 10 min in a precipitation vessel containing the *V. dahliae* inoculum (10^7 conidia /ml). These plants were then replanted in their own pots and watered with 50 ml of an aqueous conidial solution of *V. dahliae* at a concentration of 10^7 conidia/ml (except the N-control plants, which received 50 ml water). The inoculated plants were then transferred to a growth chamber where they were incubated at 22 °C under a 16-h photoperiod (450 mM $E/m^2 \cdot s$). All were watered manually to field capacity once a week. Plantlets were kept in the growth chamber for 3 months, during

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Table 1. *Changes in the proportion of roots associated with mycorrhizae*

	Sep 2003	Feb 2004	Apr 2004	Jun 2004	Aug 2004	Oct 2004	Dec 2004
Control	0	0	0	0	0	0	0
G. mosseae	0	0·88	0·96	0·97	0·99	0·99	0·10
G. intraradices	0	0·65	0·84	0·92	0·93	0·98	0·99
G. claroideum	0	0·85	0·90	0·95	0·93	0·94	0·99
S.E.D.	0·000	0·134	0·107	0·107	0·106	0·103	0·089
D.F.	3	3	3	3	3	3	3

Table 2. *Number of plants that died from* V. dahliae *attack through infection by soil application*

Non-injured plants	Feb	Apr	Jun	Aug	Oct	Dec
Control	2	0	2	0	1	1
G. mosseae	1	0	0	0	0	0
G. intraradices	1	1	1	0	0	1
G. claroideum	5	2	2	1	1	0

There were no significant differences within columns.

Table 3. *Number of plants that died from* V. dahliae *attack through infection by root immersion/soil application*

Injured plants	Feb	Apr	Jun	Aug	Oct	Dec
N-control	0	0	0	0	0	0
P-control	3	2	3	2	2	1
G. mosseae	2	3	3	1	2	3
G. intraradices	3	2	3	2	3	2
G. claroideum	1	3	2	3	2	3
S.E.D.	1·2	1·2	1·3	1·1	1·1	1·3
D.F.	4	4	4	4	4	4

which time they were periodically examined for disease symptoms. At the end of this period the number of plants that had died from *V. dahliae* attack was recorded.

Statistical analysis

The Pearson χ^2 test was used to determine whether the number of plantlets lost during transplanting and after inoculation with *V. dahliae* coincided with the expected results. Fisher's multiple range test was used to discriminate between means.

RESULTS

During transplanting in preparation for these assays, the proportion of control plants that died was 0·07 whereas 0·04 of the inoculated plants died (Pearson χ^2 test = 14·21; D.F. = 3).

The change in the proportion of mycorrhizal association of the roots over time (Table 1) shows that all plants inoculated with the AM fungi developed mycorrhiza. This demonstrates the great adaptability of olive plants to these *Glomus* species. Indeed, colonization levels were very high, even shortly after inoculation. Control plants developed no mycorrhizal association in any of the cases. There were no significant differences between plants inoculated with different *Glomus* species.

No significant differences were seen between the number of control and colonized plants that died through the soil application of *V. dahliae* (Table 2). Nor were any significant differences seen between the same groups after *V. dahliae* attack was initiated by immersing their injured roots in the conidial suspension followed by soil application (Table 3).

Colonization by the three *Glomus* species used in this investigation increased all three growth traits measured (number of shoots, length of shoots and plant height) (Table 4). This was apparent within 2 months post-inoculation. During the winter months, little difference was seen in growth between the inoculated and control plants. The dry weight of the roots and shoots of olive plantlets associated with mycorrhizae, and the ratio between the shoot and root dry weights, was always higher in the inoculated than in the control plants (Table 5). All three *Glomus* species were highly effective in increasing the root and shoot biomass. The number of shoots was increased most by *G. mosseae*, shoot length by *G. claroideum* and maximum height by *G. claroideum*. The weight (g) of N, P and K per plant were significantly higher in the inoculated plants than in the control plants (Table 6).

DISCUSSION

Inoculation with AM fungi appeared to help the olive plantlets withstand the stress produced by their transplantation to the pressed peat containers. This agrees with results obtained for other species such as avocado (Vidal *et al.* 1992) and *Hedysarum coronarium* (Barea *et al.* 1987). According to Porras-Piedra *et al.* (2005), this might be due to the achievement of a high proportion of plants with

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Table 4. *Development of inoculated and non-inoculated olive plantlets*

Fungus	Feb	Apr	Jun	Aug	Oct	Dec
			Mean number of shoots			
Control	3·5	3·9	4·3	5·9	7·4	7·7
G. intraradices	4·3	7·2	12·8	17·0	17·9	18·5
G. mosseae	4·0	6·6	13·5	22·2	26·4	27·2
G. claroideum	4·1	6·3	13·0	19·9	22·9	23·4
S.E.D.	0·34	1·45	4·41	7·22	8·27	8·45
D.F.	3	3	3	3	3	3
			Mean length of shoots			
Control	5·0	11·1	31·0	48·4	50·3	50·8
G. intraradices	7·4	20·5	50·2	80·1	96·7	104·4
G. mosseae	5·5	18·9	43·7	63·6	71·3	75·0
G. claroideum	5·1	17·3	51·7	86·7	105·4	109·9
S.E.D.	1·12	4·11	9·43	17·21	25·02	27·48
D.F.	3	3	3	3	3	3
			Mean maximum height			
Control	6·5	9·9	22·1	41·7	45·8	46·9
G. intraradices	8·1	22·2	50·2	80·1	83·6	86·2
G. mosseae	8·1	18·6	44·7	64·3	67·6	69·8
G. claroideum	7·4	19·0	53·6	90·1	94·4	96·7
S.E.D.	0·76	5·27	14·18	21·10	21·13	21·70
D.F.	3	3	3	3	3	3

Table 5. *Dry weight of roots and shoots of inoculated and control olive plantlets*

	Dry matter (g)		
	Shoot	Root	Shoot/root
Control	12·7	8·0	1·6
G. intraradices	32·3	18·4	1·7
G. mosseae	31·7	17·8	1·8
G. claroideum	38·1	22·5	1·7
S.E.D.	11·05	6·15	0·08
D.F.	3	3	3

Table 6. *Total macronutrient (g) per plant in plants inoculated with* G. intraradices, G. mosseae *or* G. claroideum, *and of controls*

	Nutrient content (g per plant)		
	N	P	K
Control	218	29	88
G. intraradices	494	184	323
G. mosseae	371	124	289
G. claroideum	427	141	354
S.E.D.	117·5	63·3	120·2
D.F.	3	3	3

mycorrhizae soon after inoculation. The agricultural implications of this result are evident for nurseries – the losses they experience in the transplant of plants could be reduced.

Mycorrhization clearly induces growth-promoting effects. The results of the current experiments, which concur with those of others working on olive trees and other species (Vidal *et al*. 1992; Barea *et al*. 1999; Davies *et al*. 2000; Porras-Piedra *et al*. 2005; Sorianо-Martín *et al*. 2005), are of great economic importance: the length of time for plants to reach sale size is reduced, and their juvenile period is likely reduced when they are planted in orchards.

Little difference was seen in growth between the inoculated and control plants during the winter months (as is typical for olive trees in winter in Castilla-La Mancha). However, at the beginning of spring, significant differences began to appear ($P < 0{\cdot}05$ at 6 months post-inoculation). These results agree with those of studies performed on other fruit trees (Vidal *et al*. 1992).

After 18 months, the aerial and underground parts of the inoculated plants were significantly larger than those of the controls, coinciding with findings reported for other fruit trees treated in the same way (Vidal *et al*. 1992).

The well-known role of AM fungi in increasing the uptake of nutrients by their host plants (Vidal *et al*. 1992; Ruiz-Lozano *et al*. 1996*a*, *b*; Azcón-Aguilar *et al*. 1999) is well reflected in the present results (Table 6). The effect of mycorrhizal association differed for each macronutrient: N and K contents were increased less than the P content by all three fungi. In agreement with Caravaca *et al*. (2002, 2003*a*, *b*), the greater capacity of the inoculated plants to absorb nutrients might allow olives to be grown on poor soils, or reduce the need for fertilizers.

The olive plantlets were highly susceptible to infection by the defoliating *V. dahliae* pathotype used, as found by Trapero & Blanco (1999). None of the AM fungi provided any benefits in this respect, which agrees with the findings of Calvente (2003). In conclusion, the inoculation of olive plants with AM fungi during the nursery growth stage may help to improve transplant survival rates and to produce more vigorous and better-nourished plants for sale to growers. This is of great agricultural importance. No significant differences between the positive effects produced by the three fungi assayed were seen. Unfortunately, colonization by these fungi does not appear to improve the resistance of cv. Cornicabra olive plantlets to attack by *V. dahliae*.

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