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SHORT NOTE

Response of the grapevine rootstock Richter 110 to inoculation with native and selected arbuscular mycorrhizal fungi and growth performance in a replant vineyard

Amelia Camprubí · Victoria Estaún · Amaia Nogales · Francesc García-Figueres · Marta Pitet · Cinta Calvet

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Abstract Two indigenous arbuscular mycorrhizal (AM) fungi from the Mediterranean wine growing area in the Northeast of Spain were isolated and classified as *Glomus intraradices* Schenck & Smith. Both native fungi were found to increase the growth of the vine rootstock 110 Richter under greenhouse conditions compared with *G. intraradices* (BEG 72) and a phosphorus (P) fertilization treatment. The effectivity of field inoculation of Cabernet Sauvignon plants grafted on Richter 110 with the former native fungi and with *G. intraradices* BEG 72 in a replant vineyard severely infested by the root-rot fungus *Armillaria mellea* (Vahl ex Fr.) Kummer was assessed. The native fungi were not effective at enhancing plant development, and only *G. intraradices* BEG 72, resulted in a positive response. Field inoculation with this selected fungus increased plant shoot dry weight at the end of the first growing season.

Keywords *Armillaria* · *Glomus intraradices* · Isolation · Molecular characterization

A. Camprubí (✉) · V. Estaún · A. Nogales · M. Pitet · C. Calvet
Institute of Agro-Food Research and Technology (IRTA),
Ctra. de Cabrils s/n,
08348 Cabrils, Barcelona, Spain
e-mail: amelia.camprubi@irta.es

F. García-Figueres
Departament de Agricultura, Alimentació i Acció Rural,
Lab. Sanitat Vegetal, Generalitat de Catalunya,
08040 Barcelona, Spain

Introduction

Arbuscular mycorrhizal (AM) fungi are important partners in natural plant communities (St. John and Coleman 1983; Karagiannidis and Nikolaou 1999). They function as an extension of the root system of the plant increasing the absorptive area and improving the uptake of phosphorus and other nutrients with slow soil mobility. Other benefits include increased tolerance to plant root diseases (Barea et al. 1996; Pinochet et al. 1996; Azcón-Aguilar et al. 2002). These fungi form the symbiosis spontaneously with the roots of grapevine and are present in most of the commercial vineyards evaluated (Menge et al. 1983; Schubert and Cravero 1985; Linderman and Davis 2001; Cheng and Baumgartner 2004); however, intensive fertilization, tilling and pesticide use have decreased AM fungi variability and efficacy. The introduction of selected AM fungi into agroecosystems may benefit the crop growth by optimising the nutrition and by enhancing plant survival. This effect can be most significant in replanted vineyards, where grapevines grow slowly and show low vigour symptoms, including chlorosis and a poor root system development (Loubser 1997).

The objective of this study was to isolate native AM fungi from vineyard soils and to evaluate their effectiveness when compared with a collection isolate of *Glomus intraradices* Schenck & Smith (BEG 72) in both, a greenhouse experiment and in a field experiment conducted in a replant soil to determine whether mycorrhizal inoculation contributes to improve plant establishment and growth.

Materials and methods

Isolation of AM fungi from vineyard soils

Soil samples were taken from two vineyards both with replant problems, located in the Mediterranean area, Northeast of Spain. In one of the vineyards (location 1: pH 8.4, organic matter content 1.1% and 28 mg/kg of P), *Armillaria mellea* was detected in woody debris and in rotted plant root fragments, meanwhile in the other (location 2: pH 8.6, organic matter content 1.4% and 23 mg/kg of P) the root-rot fungus was not present and the stunted plants growth was attributed to abiotic factors. Four rhizosphere 5-l samples from the upper 20-cm layer were collected under the grapevines canopy in each location. Grapevine roots were extracted from the samples, washed free of soil and debris and, after clearing and staining (Koske and Gemma 1989; Phillips and Hayman 1970), they were observed under a binocular microscope to evaluate mycorrhizal colonization.

The samples were combined to yield one sample per location; the resulting rhizosphere soil was used to determine the number of infective propagules and to recover the AM fungi present. The number of infective AM fungal propagules was estimated using the Most Probable Number technique (MPN), with tenfold series of soil dilutions with autoclaved sandy soil as a diluent (Porter 1979; Powell 1980) and leek (*Allium porrum* L.) as host plant. The AM fungi were recovered from soil and roots using leek plantlets as a trap plant. Leek seedlings were transplanted into 1-l containers filled with soil from each location and kept in a greenhouse. Once mycorrhizal colonization was checked, leek plants were transplanted into sterilized sandy soil to allow fungal development and the formation of chlamydospores. After 9 months of growth in pot cultures, AM spores were extracted by wet sieving and decanting (Gerdemann and Nicolson 1963). Spores were mounted in water, in polyvinyl-lactoglycerol (PVLG) and in PVLG with a drop of Mezler's reagent for microscopic examination. Spores mounted in Mezler's reagent were crushed to observe the staining of the different spore wall layers. At least 20 spores of each of the different morphotypes found were mounted in PVLG and ten spores mounted in PVLG + Melzer's reagent to observe their morphology. The spores were morphologically identified after the original descriptions (Schenck and Perez 1990) and also with internet published reference culture data bases (http://invam.caf.wvu.edu).

Molecular identification of isolated AM spores

Ten spores of each of the morphotypes isolated were used for DNA extraction, as well as spores from *G. intraradices* BEG 72, also used in the greenhouse and field assays. The DNA extraction was done using the Power Soil DNA isolation kit (MoBio Laboratories Inc, Carlsbad, CA, USA). Spores were crushed with a micro-pestle in the extraction buffer, and the following steps were done according to the manufacturer's instructions. Primary polymerase chain reaction (PCR) was performed with the eukaryote specific primers LSU0061(LR1)/LSU0599 (NDL22) (Van Tuinen et al 1998), with 2 μl of the DNA spore extract as template, 2 μl of a 10-μM solution of each primer and 10 μl of Eppendorf Master Mix 2.5× (Eppendorf AG, Hamburg Germany) in a total volume of 25 μl. PCR conditions were: initial denaturation at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, the last cycle was followed by a final extension at 72°C for 7 min. Amplicons were then used as templates in two separate PCRs with specific primers for the Glomerales: LSURK4f/LSURK7mr (Kjoller and Rosendahl 2000) and FLR3/FLR4 (Gollote et al. 2004). Nested PCRs were carried out in a 50-μl volume, and the reaction conditions were identical for the two primer combinations and differed from the primary PCR conditions on the annealing phase that was done at 60°C instead of 54°C, and on the length of the final extension phase at 72°C changed to 10 min instead of 7 min. PCR products were visualised and separated by electrophoresis in 2% agarose gels stained with ethidium bromide. Bands were subsequently cut, and amplified DNA was purified with the High Pure PCR Product purification kit (Roche Diagnostic GmbH, Mannheim, Germany). Sequencing was carried out in both directions by Secogen SL (Madrid, Spain) using the corresponding primers.

Results were manually aligned using the program BIOEDIT Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared to existing NCBI data.

Inoculum production

Spores recovered from the isolation process were used to establish pure fungal cultures. Two native AM fungi isolates and *G. intraradices* BEG 72 were used for inoculum production. Ten spores of each morphotype, isolate 1, isolate 2 and *G. intraradices* BEG 72, were used to inoculate leek plantlets. Plants were germinated in axenic conditions, spores were placed directly on the leek roots and each leek was then planted in a 60-ml pot filled with autoclaved sand. After 8 weeks of growth, plants were transplanted into a 1-l container filled with autoclaved sandy soil (pH, 7.8 and P content, 8 mg/kg). Plants were harvested after 8 months of growth under greenhouse conditions. Before the harvest, plants were left with no irrigation for 1 month and wilted. The dried shoots and the

top soil layer were discarded and the rest of the rhizosphere soil and roots were mixed and stored at 4°C. Pots inoculated with the same strain were grouped to achieve a homogeneous batch of an approximate volume of 10 l, sufficient for the experimental purposes of the work.

The number of spores was assessed for each batch before being used as inoculum in the greenhouse and field experiments. Spore numbers for each batch were counted in a 50-g sample after wet sieving and decanting (Daniels and Skipper 1982) and shredding the roots in a blender to recover intraradical spores. The resulting concentration of mycorrhizal propagules was approximately 1,120 spores/10 g of soil inoculum, 960 spores/10 g of soil inoculum and 1,500 spores/10 g of soil inoculum for isolate 1, isolate 2 and *G. intraradices* BEG 72, respectively.

Greenhouse response of Richter 110 rootstock plants to AM fungi

Three fungi were used as inoculants in the greenhouse experiments: isolate 1 from location 1, isolate 2 from location 2 and *G. intraradices* BEG ID 72 (Camprubí and Calvet 1996), an isolate recommended for a wide range of experimental agricultural situations (Camprubí et al. 1995; Estaún et al. 2003; Calvet et al. 2004).

Hardwood cuttings, 20–30 cm long, were collected from 110 Richter vines (*Vitis berlandieri* Planch. × *Vitis rupestris* Scheele), immersed during 15 min in a 0.1% oxyquinoleine sulphate solution and dipped in the rooting mix Inabarplant IV® (AIB 0.4%, ANA 0.4% and Captan 15%). Cuttings were planted in perlite rooting beds in a heated greenhouse and watered twice a day until root emergency and sprouting.

One month after rooting was initiated homogenous cuttings were transplanted to 2-l containers filled with a pasteurized mixture of sandy soil, quartz sand and sphagnum peat substrate (3:2:1 *v*/*v*) and low P content (8 mg/kg). There were five treatments, with 15 plants each: 1) non-inoculated control, 2) phosphorus fertilisation with 0.035 g KH_2PO_4/kg substrate, 3) inoculation with *G. intraradices* BEG 72, 4) inoculation with isolate 1 and 5) inoculation with isolate 2. A mixed leached (5 ml per plant) of the inocula was added to the plants.

To ensure a sufficient number of AM propagules, 20 g of inocula from the corresponding isolates were placed under the root cuttings when transferred from the rooting beds to the pots. Plants were maintained in a greenhouse with controlled temperature (20°C±5°C and 16 h daylight) during 5 months. Plant growth and AM root colonization were assessed at the end of the experiment. Root samples were stained with 0.05% trypan blue in lactic acid (Phillips and Hayman 1970; Koske and Gemma 1989). The percentage of root colonization was determined using the grid-line intersect method (Giovannetti and Mosse 1980).

Field response of grapevine

A field experiment was carried out in a vineyard located in the wine production area of Appellation d'Origine "Conca de Barberà" in northeastern Spain (pH 8.1, organic matter content 2.26% and 15 mg/kg of P), in the same area where the AM fungi isolation process was carried out (41°23′ N and 1°10′ E geographical coordinates). Old oak trees had been removed 10 years ago to establish a vineyard and grape plants had shown replant decline symptoms caused by *A. mellea* root-rot ever since.

At the beginning of the experiment, the number of infective AM fungal propagules present in the soil was estimated using the MPN technique (Porter 1979; Powell 1980).

In March 2004, plants of Cabernet Sauvignon grafted onto Richter 110 rootstock were replanted in the empty spaces left by previously removed dead grapevines. There were four treatments: (1) non-inoculated control, (2) inoculation with *G. intraradices* BEG 72, (3) inoculation with isolate 1 and (4) inoculation with isolate 2. To ensure a sufficient number of AM propagules, 100 g of inocula from each AM fungus were placed under the plant roots at transplant. Seventy-five plants for each treatment were established at random in the empty loci in the vineyard.

Plant biomass production was measured as shoot length, after 2 and 5 months growth in 20 plants per treatment, chosen at random. At the end of the growing season, after 8 months of growth, plants were pruned, leaving two leaves in the main stem per plant, the dry weight of the pruned stems and leaves was measured and the plant mortality recorded. At the beginning of the experiment and after the first growing season the mycorrhizal propagule density was evaluated taking samples from under each of the replaced trees and processed taking into account the inoculation treatment. Biomass data were analysed by analysis of variance (ANOVA) and means were compared using Tukey's multiple range test ($P \leq 0.05$).

Results

Three AM fungal species were recovered from the two field sites and were classified according to Schenck and Perez (1990), and are now kept in the IRTA culture collection. From location 1 two isolates were obtained. The spores of the first isolate (isolate 1, ref. MV1 IRTA culture collection) were produced in clusters outside the roots and morphologically identified as *Glomus aggregatum* Schenck & Smith emend. Koske, and the spores of the second isolate (isolate 1B, ref. MV2 IRTA culture collection) were morphologically identified as *Glomus microaggregatum* Koske, Gemma & Olexia. Isolate 2 (ref. PV1 IRTA culture

collection), from location 2, with spores produced mainly within the roots, was identified as *G. intraradices*. The sequence data were aligned with NCBI sequences and the results grouped isolate 1, isolate 2 and BEG 72 in the *G. intraradices* group. Isolate 1B is homogeneous with sequences belonging to *G. microaggregatum*. The sequence data obtained were entered in the NCBI GenBank with accession numbers: EU234488, EU234491, EU234490 and EU234492 belonging to the sequences of *G. intraradices* BEG 72, isolate1, isolate 1B and isolate 2, respectively.

In the greenhouse experiment, there was a significant growth increase in the 110 Richter rootstock plants inoculated with AM fungi (Table 1). The increase in dry weight in mycorrhizal plants over non-inoculated plants was significant for all fungi used; the effect of P fertilization was comparable to AM inoculation. The colonisation obtained by isolate 2 was 33%, lower than the colonisation achieved by isolate 1 (54%) and *G. intraradices* BEG 72 (60%).

In the field experiment, *A. mellea* growth was apparent and identified on woody plant material left on the replant soil after dead vines had been removed. The most probable number of native AM propagules present in the field soil before the establishment of the plants was estimated as 114 infective propagules in 100 ml of soil. Under these conditions, the "in situ" inoculation with *G. intraradices* BEG 72 significantly increased the growth of grapevine plants during the first growing season, measured as shoot length 2 and 5 months after planting (Fig. 1). Inoculation with the native isolates did not significantly increase the growth of plants when compared with the non-inoculated control treatment. Plant mortality at the end of the growing season was 14% for the non-inoculated control treatment and 12%, 7% and 2% for inoculation treatments with *G. intraradices* isolate 2, *G. intraradices* isolate 1 and *G. intraradices* BEG 72, respectively.

Table 1 Plant growth response (fresh weight, dry weight, number of leaves per plant) and percentage of AM colonization in Richter 110 rootstock plants inoculated with two native AM fungal isolates (1 and 2) from grapevines and with *Glomus intraradices* BEG 72 after six months growth under greenhouse conditions

Treatment	Dry weight (g)	No. leaves/ plant	AM colonization (%)
Control	6.08 a	63 a	0
P	8.50 b	80 ab	0
Isolate 1	9.10 b	87 b	54±7
Isolate 2	9.46 b	88 b	33±7
G. intraradices	9.59 b	96 b	60±4

Different letters in the same column indicate significant differences between treatments (Tukey $P \leq 0.05$).

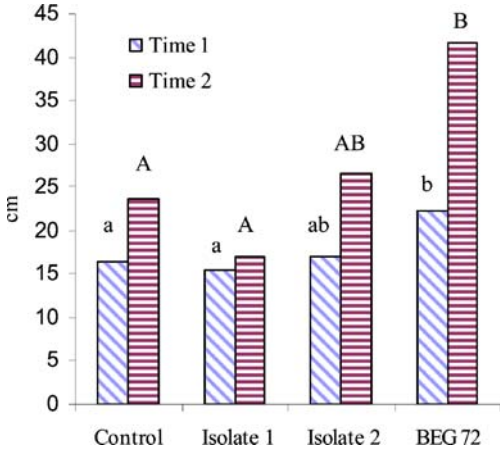

Fig. 1 Plant growth response measured as shoot lengths (cm) of grapevine plants (Cabernet Sauvignon grafted onto Richter 110 rootstock) inoculated with two native AM fungal isolates from vineyards and with *Glomus intraradices* BEG 72 after two (time 1) and five (time 2) months growth under field conditions. Different letters behind treatment values within the same time indicate significant differences between treatments (Tukey $P \leq 0.05$)

Discussion

Glomus aggregatum is a fungus often found in field samples, frequent in arid land areas (Uhlmann et al. 2005). When this fungus is isolated and subcultured the newly formed spores have, in many instances, additional outer layers in the spore wall, which would include the fungus in the *G. intraradices* taxon. Authors (Morton et al., INVAM web page http://invam.caf.wvu.edu) have suggested that these two species might be synonymous. Our molecular results confirm that the fungus isolated from the vineyard defined as isolate 1, and morphologically identified as *G. aggregatum* was in fact a *G. intraradices* isolate.

The ability of mycorrhizal fungi to enhance grapevine growth has been described for many rootstocks, although differences among rootstocks and fungi have been reported by several authors (Schubert et al. 1988; Aguín et al. 2004). In our greenhouse experiment, under controlled conditions using a sterile sandy soil mixture with low levels of P all fungi studied, the native endophytes and *G. intraradices* BEG 72, increased the growth of rootstock Richter 110 plants. The native fungi were isolated with the trap plant method, a system that selects fast colonising fungi, and the results obtained in the greenhouse experiment showed that both fungi were good growth enhancers as well; therefore they were considered to be good candidates to be used as inoculants in field experimental trials (Estaún et al. 2002).

Menge et al. (1983) suggested that AM fungal inoculation would be of interest in those vineyards previously fumigated, with low fertility. The replanted vineyard in our experiment, had a considerable number of AM propagules (natural mycorrhizal potential of 114 propagules/100 ml), and the results show that in the field, only *G. intraradices*

BEG 72 was able to increase significantly transplant survival and early plant growth while the native isolates did not. These native isolates proved to be effective at increasing plant growth under controlled greenhouse conditions, with no naturally occurring endophytes and low fertility. Factors linked to the soil biological and chemical characteristics must have a large influence on mycorrhizal performance (Schreiner 2003), and repeated intensive cropping can result in a negative selection of mycorrhizal fungi, inefficient at increasing plant survival and growth in specific environments. In this field trial *G. intraradices* BEG 72, a fungus isolated from similar soil and climatic conditions but originally associated to an entirely different host plant (*Citrus* sp.) (Camprubí and Calvet 1996), was better at increasing grapevine growth than fungi isolated from vineyards in the same grapevine growing area. The field trial was set in a soil heavily infested by a damaging root rot fungus *A. mellea*. Mycorrhizal inoculation directly ameliorates plant nutrition (Gerdemann 1968) through the fungal mycelium, but additionally affects root architecture (Linderman 1992), by increasing lateral finer roots, non-woody, that are not a substrate for *A. mellea* growth but the site of plant absorption and mycorrhizal colonisation. The rootstock used, Richter 110, has been shown to respond to the AM inoculation with a significant growth increase in the first and second order lateral roots, compared to other rootstocks, where changes in root architecture were not as important (Aguín et al. 2004). Rootstock Richter 110 is a *V. berlandieri* × *V. rupestris* cross, bred for resistance to drought stress and tolerance to calcareous soils. It is therefore widely used in many Mediterranean vine growing areas, although it poorly assimilates potassium and magnesium.

In our experiment, early field inoculation with a selected AM fungus was beneficial for the establishment and growth of the Richter 110 rootstock. The first year results show an increased survival of mycorrhizal vines that can also be attributed to a greater plant fitness (Baumgartner and Rizzo 2000) and they point out to an AM fungus isolate-specific effect and indicate the need to inoculate vines with selected inocula because not all AM fungi, even within the same species, will lead to successful results in the field.

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