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Impact of a genetically modified *Rhizobium* strain with improved nodulation competitiveness on the early stages of arbuscular mycorrhiza formation

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Abstract

Arbuscular mycorrhizal (AM) fungi and the AM symbiosis are key components in agroecosystems, thus, they have been used as biosensors to evaluate the impact of a *Rhizobium meliloti* strain, that was genetically modified to improve its nodulation competitiveness, when applied as inoculant. It was found that such a rhizobial strain did not interfere with any of a series of processes related to mycorrhiza formation by the representative AM fungus *Glomus mosseae*. The parameters tested include spore germination, mycelial growth from the mycorrhizal propagules and AM entry point formation on the developing root system of the common host plant *Medicago sativa* L. Indeed, the genetically modified *Rhizobium* increased the number of AM colonization units and the nutrient acquisition ability in the mycorrhizal plant, with respect to the wild type rhizobial strain.

Keywords: *Rhizobium* inoculants; Arbuscular mycorrhizas; Genetically modified microorganisms; Biosafety and biosensors; Rhizosphere ecology; Sustainable agriculture

1. Introduction

Current concerns in the general context of sustainable, environment-friendly, agricultural practices are renewing interest in microbial inoculants which are introduced into the agroecosystems either as biofertilizers or for the biocontrol of plant pathogens (Elliott and Lynch, 1995). New methodological approaches are enhancing the availability and success of microbial inoculants (O'Gara et al., 1994). For example, microbial molecular ecology techniques are presently being applied to improve our understanding of how the biodiversity in soil environments can be exploited as a source of new microbial strains, and ad hoc biotechnological approaches are currently generating genetically modified microorganisms whose superior traits make them useful as inoculants in agriculture/forestry.

When released in soil environments, however, microbial inoculants can produce deleterious effects on the indigenous microbial community. In general, there is a special interest in assessing the impact on key biological components of the rhizosphere as

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produced by genetically-modified (GM) organisms, in comparison to that of their corresponding wild-type (WT) strains, from which the GM organisms derive. Particularly important it would be an evaluation of the impact on beneficial resident soil microbiota. The arbuscular mycorrhizal (AM) fungi are among those beneficial microorganisms living in the soil-plant systems. These fungi form symbiotic associations with plant roots, i.e., arbuscular mycorrhizas, which are found in the roots of most plant species, including those of agronomical interest (Barea et al., 1993a). This symbiosis is not only ubiquitous concerning the host plant, but it also has a universal distribution regarding soil type, climate, geographical region, etc. The AM fungi are, therefore, present in most natural and agricultural ecosystems where they are involved in many key processes related to plant performance and ecosystem stability. The mycorrhizal effects include the enhancement of plant rooting and establishment, promotion of nutrient cycling, soil structure improvement, and plant health protection against biotic and abiotic stresses (Bethlenfalvay and Linderman, 1992; Gianinazzi and Schüepp, 1994; Barea and Jeffries, 1995).

Because of the role played by AM fungi, it seemed important to assess how they are affected by either GM or WT microbial inoculants, not only by considering AM/AM fungi as biosensors for a safe release of the inocula, but also in terms of whether these inoculants can even interact with the AM fungi to improve plant establishment, nutrition or health (Barea et al., 1993b).

Microbial interactions involving AM fungi are recognized as fundamental events which concern rhizosphere/mycorrhizosphere establishment, maintenance and function (Azcón-Aguilar and Barea, 1992, Linderman, 1992). These interactions are particularly interesting in the case of *Rhizobium*, since both the nitrogen-fixing rhizobia and the AM fungi co-colonize symbiotically the root system of legumes (Barea et al., 1992). The interactions between both types of endophytes at improving plant nutrition is well documented. However, it has been argued that these can also take place at the precolonization stages, when both microorganisms interact as rhizosphere inhabitants, or at the earlier stages of root colonization. The aim of this study was to compare the effects on AM formation of the inoculation of a WT *Rhizobium meliloti* strain with that of its GM derivative specifically developed to improve its nodulation competitiveness in alfalfa plants (Sanjuan and Olivares, 1991). According to the available information concerning the events/processes involved in AM formation (Williams, 1992, Koske and Gemma, 1992, Abbott and Gazey, 1994, Azcón-Aguilar and Barea, 1995, Bonfante and Bianciotto, 1995), the parameters used to evaluate the biosafety level of the release of such rhizobial inoculants include mycorrhizal fungus spore germination, plant-independent mycelial growth (both processes to be challenged in vitro), and fungal entry point formation on the developing root system of *Medicago sativa* L. (in soil microcosms). The growth and nutritional plant response at an incipient dual symbiotic colonization stage of alfalfa also need to be complementarily evaluated.

2. Materials and methods

2.1. Microbial strains

The AM fungus used was *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe, a representative species from temperate agrosystems (Walker, 1992). The *Rhizobium meliloti* strains tested were the wild type GR4 isolate, and its genetically modified derivative GR4(pCK3), containing plasmid pCK3, which carries the *Klebsiella pneumoniae nifA* gene, constitutively expressed from a Kanamycin gene promoter (Sanjuan and Olivares, 1991). This genetic modification has been shown to enhance *R. meliloti* nodulation competitiveness (Sanjuan and Olivares, 1991).

2.2. Microcosm experiment

Alfalfa (*Medicago sativa* L., cultivar Aragón) was the test plant. 5-day old seedlings obtained from surface sterilized seeds were transplanted to the soil microcosms. An agricultural soil collected from Granada province (Spain) was used. The characteristics of this test soil, a Cambisol, were as follows: pH (H_2O), 6.8; available ($NaHCO_3$-extractable) P, 15 mg l^{-1}; total N, 2600 mg l^{-1}; organic C, 0.8%; a texture with sand, 58.7%; silt, 26.4% and clay, 14.9%. Alfalfa had never been cultivated in this soil.

The experimental soil was sieved (4 mm), steam-

sterilized (100°C for 1 h on 3 consecutive days) and then reinoculated with 2 ml per pot of a soil filtrate containing its natural microbial population except propagules of AM fungi. The soil filtrate was obtained by suspending 100 g of the experimental soil in 500 ml sterile water. After shaking and decanting, the suspension was filtered twice (Whatman No. 1). This allows to evaluate the effects of specific mycorrhizal inoculation treatments. The soil was distributed into pots (500 ml).

At transplanting, seedlings (one per pot) received the microbial treatments. The mycorrhizal inoculum was obtained from a stock-pot culture (Azcón et al., 1991), using *Allium cepa* L. as a host plant, to produce a rhizospheric soil containing five sporocarps g^{-1} (with six mature spores per sporocarp, on average), together with some single spores, mycelium and mycorrhizal root fragments. 50 g of such mycorrhizal inoculum was added per pot and was thoroughly mixed with the soil in the pot. The rhizobial cultures were prepared following standard procedures (Azcón et al., 1991) and contained 10^8 cells ml^{-1}. Each pot received 1 ml of the appropriate *Rhizobium* strain.

There were four microbial treatments: C = control uninoculated plants, having the natural soil microbiota, except mycorrhizal propagules; Myc = mycorrhizal inoculation; Myc + WT = mycorrhiza and the WT *Rhizobium* inoculation; Myc + GM = mycorrhiza and the GM *Rhizobium* inoculation. These four treatments were replicated five times for a total of 20 microcosm units. A completely-random arrangement was followed.

The plants were grown in a greenhouse under a day/night cycle of 16/8 h, 21/15°C, 50% relative humidity and a photosynthetic photon flux density of 600–700 μmol m^{-2} s^{-1}. During the assay plants were fertilized (10ml/week/pot) with Long Ashton nutrient solution (Hewitt, 1952) lacking N and P. Throughout the experiment, the pots were weighed every day and the water loss was compensated for by top watering to reach field capacity.

After a growth period of 30 days the plants were harvested. Shoot dry weight was recorded after drying at 70°C. Shoot P and N concentrations were measured after Kjeldahl digestion (for N) or by the molybdenum blue (for P) procedures (Lachica et al., 1983).

Roots were carefully washed and the number of nodules was assessed visually. Roots were separated into branching orders and the root length of each order laterals was measured using video images with a Digital Image Analysis, version 1.10 A. Delta T Devices. Then, the roots of each branching order were divided into two batches: a part to be stained for mycorrhizal entry points (Phillips and Hayman, 1970), and the other for dry weight determination.

2.3. Pure culture experiments

These experiments were conducted as described by Azcón-Aguilar et al. (1986), using 9 cm diameter plastic Petri dishes containing water-agar (1% Difco Bacto) adjusted to pH 6.8. Sporocarps of *G. mosseae* were obtained from rhizospheres of *Allium cepa* L. plants grown in stock-pot cultures, as previously indicated (Section 2.2). The rhizosphere samples were kept for some weeks in polyethylene bags at 4°C and after collection the sporocarps were stored on damp filter paper at 4°C. Resting spores freshly excised from the sporocarps were surface sterilized by using a solution containing Chloramine T, streptomycin and Tween 80 (Mosse, 1962) for 20 min and were then washed five times in sterile water. The inocula of the rhizobial strains (WT and GM) were prepared as previously indicated (Section 2.2). Cultures of both *Rhizobium* strains were adjusted to an optical density of 0.4 (λ = 650nm) corresponding to 10^8 colony forming units per ml (cfu ml^{-1}). Serial dilutions (10^{-1} to 10^{-6}) were made to test the 10^{-4}, 10^{-5} and 10^{-6} dilutions. The bacterial cells were spread (50μl of the corresponding dilution per plate) on the agar surface.

Six surface-sterilized spores of *G. mosseae* were transferred individually to each Petri dish, located on the vertices of an imaginary hexagon of about 3.5 cm side. The plates being sealed with parafilm to reduce dehydration and contamination risks and incubated at 25°C in the dark. Each treatment had five replicate plates, and five were kept as controls with the *G. mosseae* spores growing axenically.

Spore germination rate was evaluated every 4 days. A spore was considered germinated if a germtube was clearly visible. Hyphal growth from germinated resting spores, and the number of secondary spores formed on the mycelium were assessed under

a light microscope at the end of the experiment, after 26 days incubation.

2.4. Statistical analysis

Data from the percentage of spore germination in each treatment were subjected to a hypothesis test against those of the corresponding controls. The Z statistic was calculated from the experimental data and compared with the tabulated value at the 5% significance level. Data of mycelial growth and from the microcosm experiment were processed by ANOVA and Duncan's new multirange test ($P \leq 0.05$).

3. Results

There was no indication that the genetic modification induced in the wild type *Rhizobium meliloti* GR4 interfered negatively with mycorrhizal entry point formation between *Glomus mosseae* and *Medicago sativa* under the microcosm conditions tested (Figs. 1 and 2). The GM strain improved nutrient (N and P) acquisition in mycorrhizal plants, with respect to the WT strain, in spite of the fact that both *Rhizobium* (WT and GM) produced plants of similar size at the harvest time tested (Fig. 1). The GM derivative increased the length of individual primary, secondary and tertiary mycorrhizal roots and mycorrhizal entry point formation, in comparison to the WT strain (Fig. 2). The GM effect on the number of entry points, according to the root order laterals, was particularly noteworthy for secondary roots, this affecting thereby the total number of infection units in the alfalfa plant root system (Fig. 2).

The number of nodules formed by the GM strain

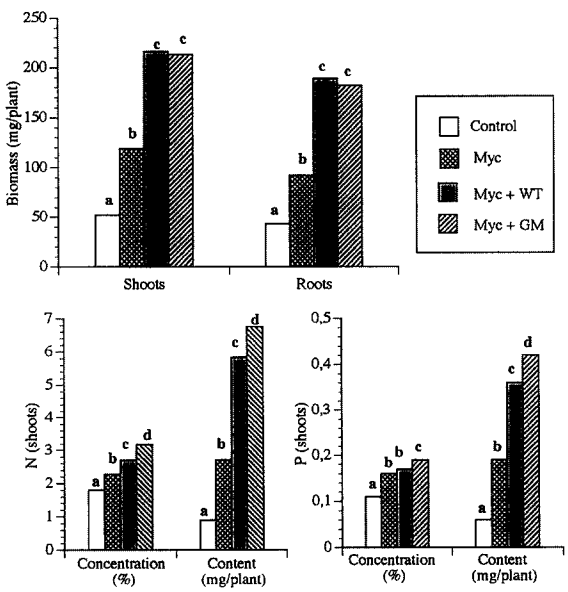

Fig. 1. Comparative effects of the GM and WT *Rhizobium* strains on biomass (dry weight), and nutrient (N and P) accumulation by mycorrhizal alfalfa plants growing for 30 days in microcosms under greenhouse conditions. For each parameter, the means (five replicate pots) sharing a common letter do not differ significantly at the 5% level.

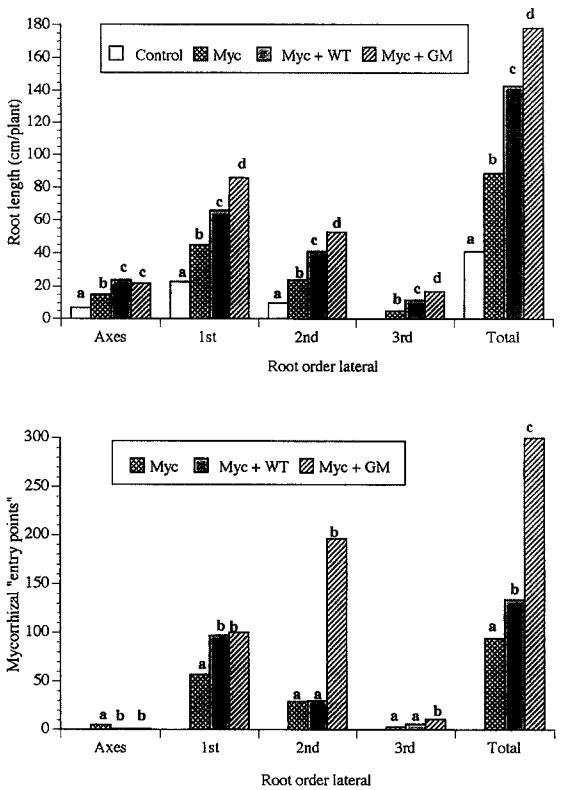

Fig. 2. Comparative effects of the GM and WT *Rhizobium* strains on root morphology and mycorrhizal entry point formation by *Glomus mosseae* in alfalfa plants growing for 30 days in microcosms under greenhouse conditions. For each parameter, and root order laterals, the means (five replicate pots) sharing a common letter do not differ significantly at the 5% level.

was nine per plant, significantly lower than the 16 formed by the WT strain.

None of the rhizobial strains (GM and WT) affected the percentage of spore germination but both of them enhanced the development of the mycelium arising from resting spores, and the production of secondary spores per germinating resting spore (Fig. 3). Nevertheless, the time-course evaluation of spore germination, showed that both *Rhizobium* strains induced an increase in the rate of germination which was statistically significant between days 8–22 (Fig. 4).

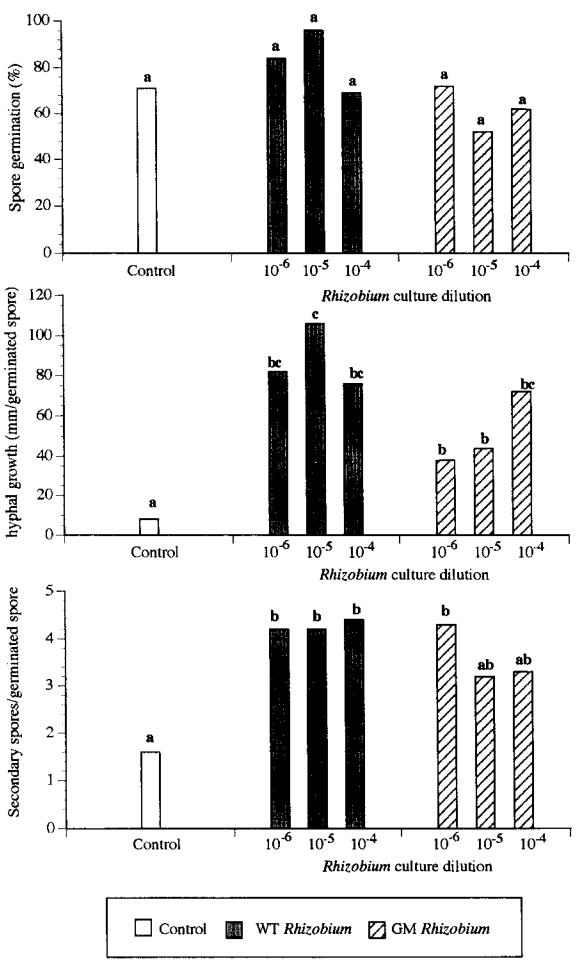

Fig. 3. Comparative effects of the GM and WT *Rhizobium* strains, applied at different concentrations, on spore germination and mycelial growth of the mycorrhizal fungus *Glomus mosseae* after 26 days of co-culture under monoxenic conditions. For spore germination there were no significant differences between *Rhizobium* treatments and the control. For the other parameters, means sharing a common letter do not differ significantly at the 5% level.

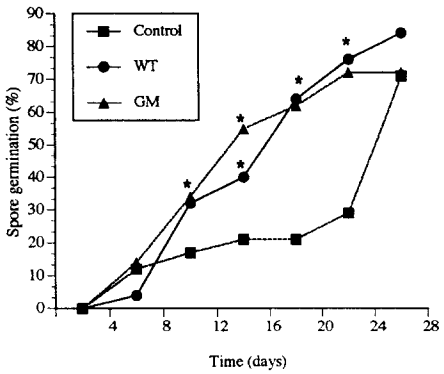

Fig. 4. Time-course effects of a representative dilution (10^{-6}) of the *Rhizobium* cultures (WT and GM) on the germination of the mycorrhizal fungus *Glomus mosseae* spores after 26 days of co-culture under monoxenic conditions. (*) Denotes significant effects over control at the given measurement, at the 5% level.

4. Discussion

Mycorrhizal fungus populations are considered as an important ecological factor whose activity can affect the performance of microbial inoculants (Azcón-Aguilar and Barea, 1992). In addition, these fungi could be used as biosensors for the evaluation of a safe release of such inoculants in agricultural soils (Barea et al., 1993b). For this purpose the process of AM formation must be considered as a whole (Sanders and Sheikh, 1983, Abbott and Gazey, 1994). Thus, for the examination of the effect of a microbial inoculant the system must include mycorrhizal propagules, a soil-based living substrate and a host plant.

The effects found in this microcosm study on entry point formation implicitly indicated that the *Rhizobium* inoculants did not inhibit any of the events/processes involved in AM formation i.e., spore germination, plant-independent mycelial growth and the saprophytic rhizosphere colonization (Azcón-Aguilar and Barea, 1995). Indeed, the experiments developed in vitro under axenic/monoxenic conditions, corroborated these findings concerning

spore germination and hyphal growth. The final germination percentage of *G. mosseae* spores, which readily germinated axenically, did not change when any of the two *Rhizobium* was co-inoculated. Nevertheless, the germination rate was hastened by the presence of *Rhizobium*. Both such an effect on the germination and the stimulation of the subsequent mycelial growth by *Rhizobium* co-inoculation, have been described for other microorganisms (Azcón-Aguilar et al., 1986). The mechanisms accounting for these microbial stimulation have not yet been clearly demonstrated. However, there is overwhelming evidence, that microorganisms develop many functions in the rhizosphere which affect both the plant and other microbial members of the soil community (Lynch, 1990, Kloepper et al., 1991). The beneficial activities, based on the production of vitamins, amino acids, hormones, etc., must be operating in the microbe–microbe relationships involving AM fungi (Azcón-Aguilar and Barea, 1992), and may possibly justify the effects found in this study.

Those interactions between AM fungi and *Rhizobium* strains, which improve legume performance are well documented (Barea et al., 1992). The reported findings corroborate selective relationships concerning AM fungi and *Rhizobium* strain interactions (Azcón et al., 1991), also applied for genetically modified bacteria. It should be emphasized that the GM strain increased N concentration and content in mycorrhizal plants, with respect to the WT. However, this contrasts with the fact that the GM strain produces less nodules than the WT strain, but those of the GM are more effective, corroborating previous results (Sanjuan and Olivares, 1991).

The effects on plant root morphology are also of particular interest. In fact it has been recently demonstrated that mycorrhizal inoculation induced changes in root morphogenesis (Schellenbaum et al., 1991; Hooker et al., 1992), and apparently the *Rhizobium* × arbuscular mycorrhiza interactions, as reported here, also induce such changes in root morphology. In conclusion, we can say that a *Rhizobium meliloti* strain genetically modified for an improved nodulation competitiveness did not negatively interfere with mycorrhiza formation by *Glomus mosseae* in alfalfa. Moreover, the modified strain appears to enhance the mycorrhizal mycelium development and the formation of mycorrhizal colonization units on a developing root system, in comparison with the wild type strain from which it derived. Therefore, the *Rhizobium* inoculants derived from the genetically improved bacteria tested in the reported experiments can be used in a safe way in that concerning to their impact on arbuscular mycorrhiza performance.

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