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Effect of a genetically modified *Rhizobium meliloti* inoculant on the development of arbuscular mycorrhizas, root morphology, nutrient uptake and biomass accumulation in *Medicago sativa*

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SUMMARY

A soil microcosm system was used to evaluate the impact of a genetically modified (GM) *Rhizobium meliloti* strain on development and function of arbuscular mycorrhiza in alfalfa plants (*Medicago sativa* L.). There was no indication that this GM *Rhizobium* strain, which had an enhanced nodulation competitiveness ability, interfered with mycorrhiza formation by *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe. Indeed, inoculation with the GM *Rhizobium* strain greatly increased the number of mycorrhizal entry points in the alfalfa root system in comparison to the wild-type strain. Mycorrhizal development and quality of nodulation increased with time and coincided with increased biomass of and nutrient (N, P) uptake by the host plant. The establishment of the symbiotic interactions also induced changes in root morphology; in particular, the degree of branching increased and the number of lateral roots was greater in plants inoculated with the GM *Rhizobium* strain. These results demonstrate that the GM *Rhizobium* strain does affect adversely the performance of arbuscular mycorrhizal symbiosis, a biosafety model system based on a functional rhizosphere.

Key words: *Rhizobium*, arbuscular mycorrhizas, genetically modified micro-organisms, biosafety, root system morphology.

INTRODUCTION

Current interest in low-input agriculture is stimulating research on the rational manipulation of soil micro-organisms, particularly with regard to improving the use of efficient microbial inoculants (Elliott & Lynch, 1995). Recent developments in recombinant DNA technology are facilitating research into the natural diversity of soil micro-organisms for isolation of new strains, and development of superior strains by genetic modification (O'Gara, Dowling & Boesten, 1994). However, it is important not only to test the effectiveness of the genetically developed microbial inoculants in improving plant performance, but also to assess their impact on key rhizosphere processes in order to ensure biosafety of the inoculant release. Because of the importance of arbuscular mycorrhizal (AM) associations in rhizosphere ecology, it has been proposed that either the AM fungi or the AM symbiosis be included as target biosafety model system for the reliable application of the inoculants when released at the soil–plant interfaces, particularly for inocula of genetically modified micro-organisms (Barea *et al.*, 1993). In relation to these biosafety-related purposes a series of experiments are being carried out to compare the effects on AM formation and function of a wild type (WT) *Rhizobium meliloti* strain with those of its genetically modified (GM) derivative developed to improve the nodulation competitiveness of the WT strain (Sanjuán & Olivares, 1991).

In a preliminary study (Tobar *et al.*, 1996), it was found that the GM rhizobial strain did not interfere with spore germination and mycelial growth of the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe. Indeed the GM rhizobial strain increased the number of mycorrhizal entry points in a short-term pot experiment.

Because of the short-term nature (28 d) of this

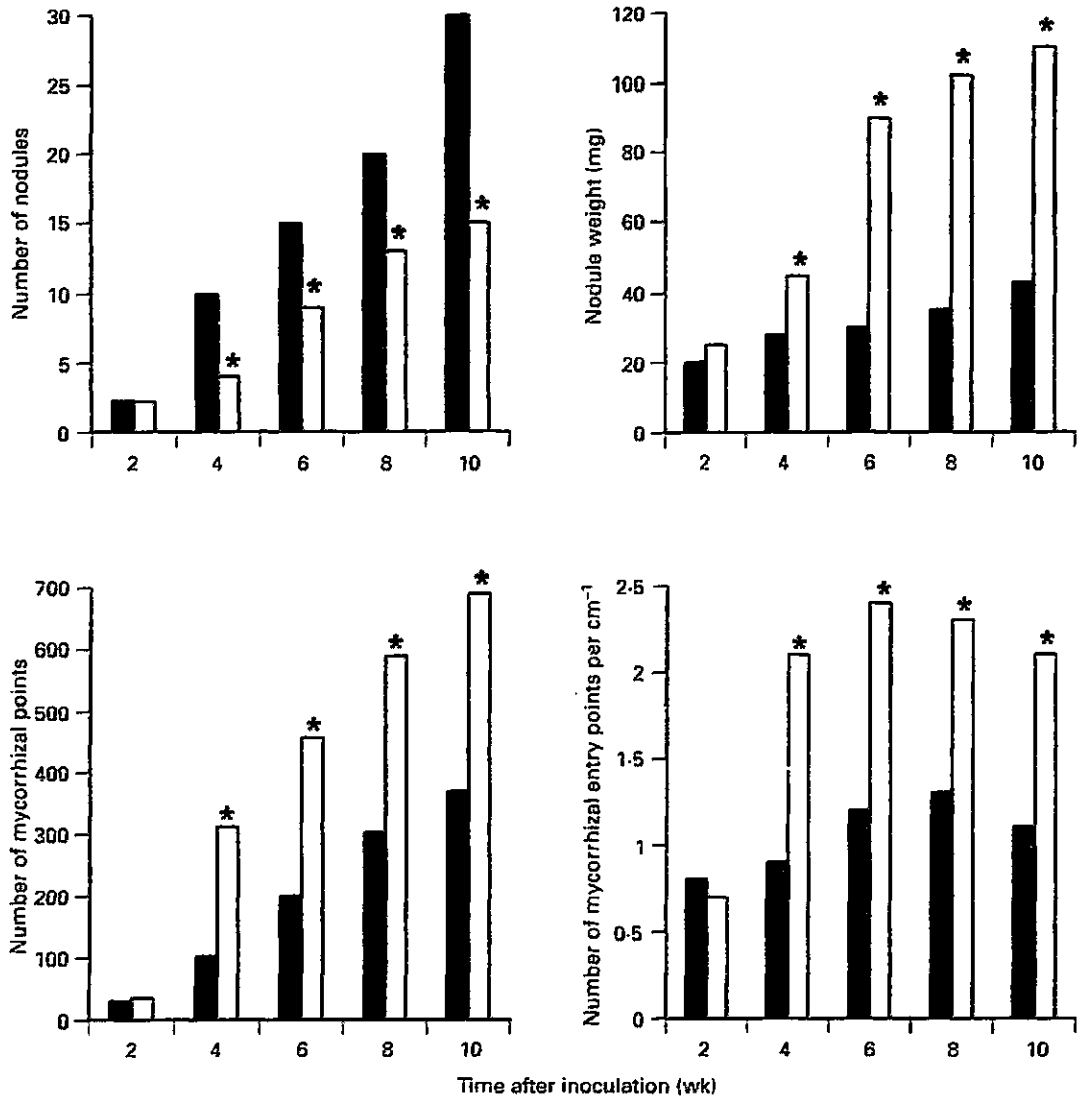

Figure 1. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on nodulation and 'entry point' formation, in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5 % level.

preliminary assay, it was considered that information on the impact of *Rhizobium* inoculants on AM formation should be extended to further stages of the development of the mycorrhizal plants. Furthermore, and although they warrant confirmation, some apparent effects on root morphology seem to add new insights to other well-documented endophyte interactions in legume roots (Barea, Azcón & Azcón-Aguilar, 1992). It has recently been recognized that AM colonization affects a range of morphological parameters in developing root systems. The most commonly described effect of AM colonization on root development is an increased root branching. However, other parameters, such as the length of lateral roots, specific root length, root diameter are also affected by AM colonization (Schellenbaum *et al.*, 1991; Hooker, Munro & Atkinson, 1992; Berta, Fusconi & Trotta, 1993; Atkinson, Berta & Hooker, 1994; Berta *et al.*, 1995). A time-course experiment was thus carried out in a soil microcosm system to compare the effect of the WT *Rhizobium meliloti* strain with that of its GM derivative, on the development and patterns of the AM colonization and nodulation processes, as well as to observe the subsequent consequences of the tripartite interactions on root system morphology, nutrient uptake by and biomass accumulation in *Medicago sativa* L.

MATERIALS AND METHODS

The AM fungus used was *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, a representative species from temperate agrosystems (Walker, 1992).

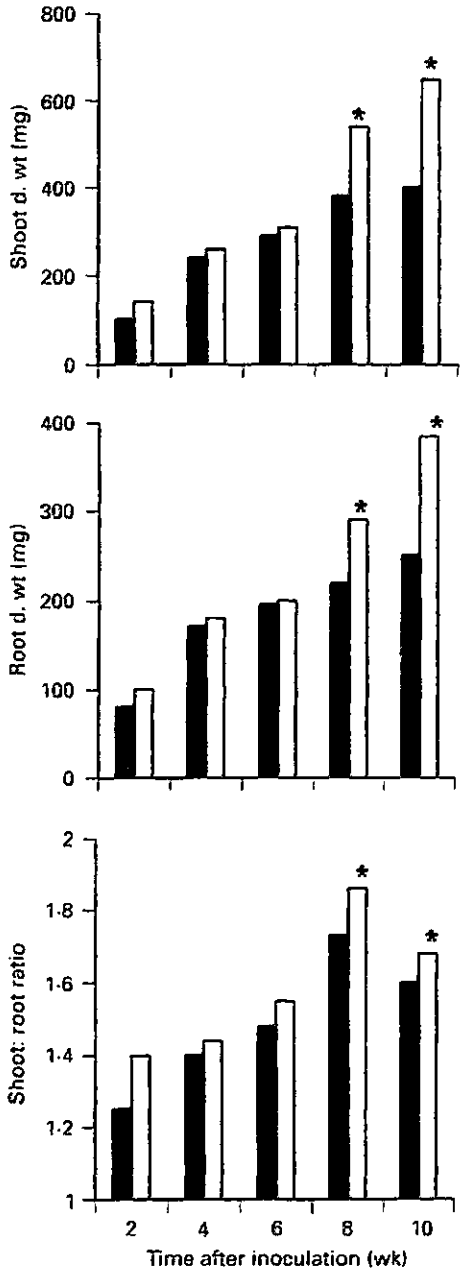

Figure 2. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on biomass accumulation and the shoot:root ratio, in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5 % level.

The *Rhizobium meliloti* strains tested were the wild-type GR4 isolate, and its genetically modified derivative GR4(pCK3), developed by Sanjuán & Olivares (1991) to improve the nodulation competitiveness of the wild-type strain.

Alfalfa (*Medicago sativa* L., cv. Aragón) was the test plant. Five-day-old seedlings obtained from surface-sterilized seeds were transplanted to a soil microcosm system. An agricultural soil collected from Granada province (Spain) was used. The characteristics of this test soil, a Cambisol, were: pH (H_2O), 6·8; available ($NaHCO_3^-$-extractable) P, 15 mg l^{-1}; total N, 2600 mg l^{-1}; organic C, 0·8 %; and texture: sand, 58·7 %; silt, 26·4 % and clay, 14·9 %. Alfalfa had never been grown in this soil.

The experimental soil was sieved (4 mm), steam-sterilized (100 C for 1 h on three consecutive days) and then re-inoculated with a soil filtrate, obtained by shaking the soil with water, assumed to contain the natural soil microbial population minus propagules of AM fungi, which were retained on the filter paper (Whatman no. 1). The soil was distributed into pots (1 l).

At transplanting, seedlings (one per pot) were inoculated. The mycorrhizal inoculum, obtained from a stock-pot culture (Azcón, Rubio & Barea, 1991) where *Allium cepa* L. was the host plant, contained five sporocarps g^{-1} with on average six mature spores per sporocarp, together with some single spores, mycelium and mycorrhizal root fragments. Fifty grams per pot of this mycorrhizal inoculum was thoroughly mixed with the soil in the pot. The rhizobial inoculum consisted of 1 ml per seedling of the corresponding *Rhizobium* strain culture. The rhizobial cultures were prepared following standard procedures (Azcón *et al.*, 1991) and contained 10^8 cells ml^{-1}. All the plants were inoculated with the AM inoculum but there were two rhizobial treatments: the wild type *Rhizobium* strain and its genetically modified derivative. These two treatments were replicated 25 times giving a total of 50 microcosm units. The plants were arranged randomly in a glasshouse and grown under a day/night cycle of 16/8 h, 21/15 C, 50 % r.h. A photosynthetic photon flux density of 600–700 μmol m^{-2} s^{-1} was applied as supplementary light. Plants were fertilized (10 ml wk^{-1} per pot) with Long Ashton nutrient solution (Hewitt, 1952) lacking N and P. The pots were weighed and watered to field capacity daily.

Five plants per treatment were harvested after 2, 4, 6, 8 and 10 wk of growth. Shoot and root d. wt were recorded after drying at 70 °C to constant weight. Shoot N and P concentrations were measured after Kjeldahl digestion or molybdenum blue procedures respectively (Lachica, Aguilar & Yañez, 1973).

The roots were washed carefully and the number of nodules was assessed visually and then weighed (fresh). Roots were separated into branching order and the root length of each order measured using video images with a Digital Image Analysis, version 1.10 A, Delta T Devices Ltd. The roots were then divided into two batches: one half was stained to aid identification of mycorrhizal 'entry points' (Phillips

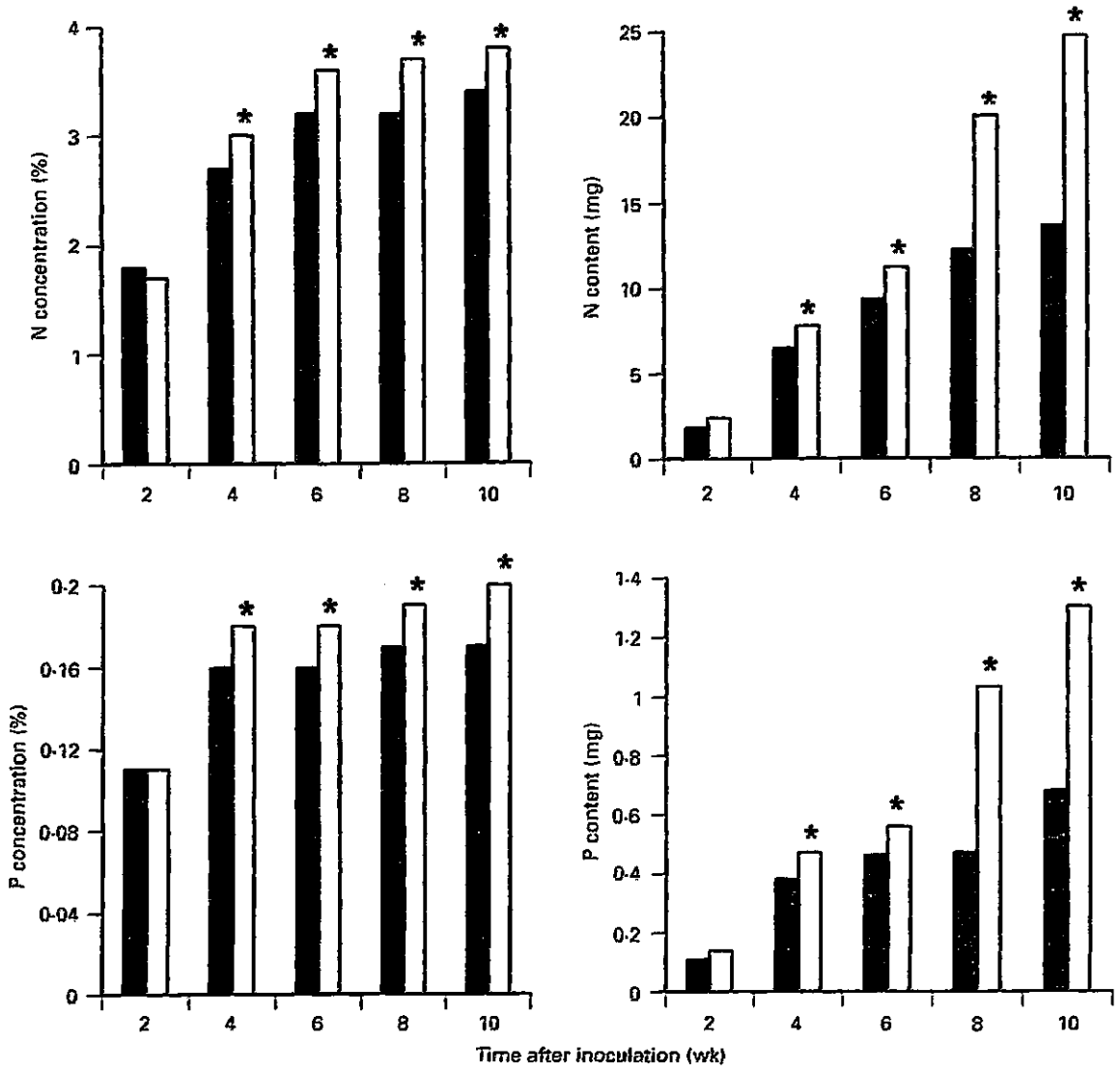

Figure 3. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on nitrogen and phosphorus accumulation in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5% level.

& Hayman, 1970), and the other for d. wt determination.

At each harvest, the following morphometric parameters were determined: number of roots of each order, number of axes, length of the roots present in each order, total root length, the degree of branching of the root system, lateral root frequency and the specific root length (Berta *et al.*, 1995). The following formulae were applied:

$$\text{degree of branching} = \frac{\text{total root number}}{\text{total root length}}$$

$$\text{lateral root frequency} = \frac{\text{number of roots of order } n}{\text{length of roots of order } n-1}$$

$$\text{specific root length} = \frac{\text{total root length}}{\text{total d. wt}}$$

Data from all measurements were processed by ANOVA ($P \leqslant 0{\cdot}05$).

RESULTS

Development of symbioses

Figure 1 shows the time-courses for the development of nodular and mycorrhizal symbioses. The GM *Rhizobium* strain formed fewer nodules although they weighed more, than those produced by the WT strain. The GM strain did not interfere with AM development. Indeed, it increased, with respect to the WT strain, the number of mycorrhizal 'colonization units' in alfalfa roots, evaluated as either the total number of entry points per plant or as their number per unit of root length (cm).

Biomass accumulation and nutrient acquisition in alfalfa plants

Figure 2 shows the time-course for the effect of *Rhizobium* strain x mycorrhiza interaction on plant biomass accumulation. At 6 wk no differences were evident in the shoot and root d. wt. By contrast, at 8 wk plants in the GM treatment were significantly heavier than those in the WT treatment.

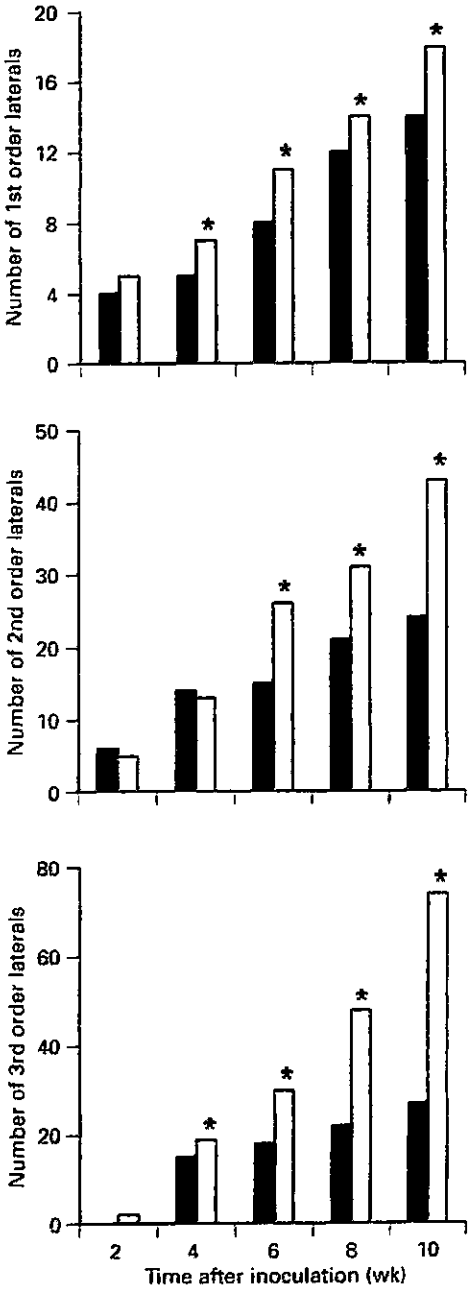

Figure 4. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on the number of roots present in each order (each plant produced only one axis), in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5 % level.

The shoot:root d. wt ratio (S:R) was similar for GM and WT mycorrhizal plants until 4 wk. However from 6 wk onwards, it was higher in GM-inoculated mycorrhizal plants.

Figure 3 records the time-course of accumulation of N and P in plant shoots. Both the nutrient concentration (given as percentage of d. wt) and the nutrient content (as mg per plant) were significantly higher in the GM-inoculated plants (Fig. 3), from the timed establishment of significant differences in the number of mycorrhizal colonization units (Fig. 1). The differences in nutrient uptake between GM and WT rhizobial treatments increased with time (Fig. 3). A positive relationship between development of symbioses, nutrient uptake and biomass accumulation was clearly evident (Figs 1, 2, 3).

Root system morphology

The root system of alfalfa plants consisted of only one axis produced directly from the base of the shoot. Figure 4 records the number of first-order, second-order and third-order lateral roots. It is evident that regardless of their order, the number of laterals was greater in plants inoculated with the GM *Rhizobium* than in those inoculated with the WT strain. The differences increased with time and also root order. At the final harvest (10 wk), the percentage contribution of the different orders to the total root number were: 21 (WT)/13 (GM), for primary; 36 (WT)/32 (GM), for secondary; 27 (WT)/74 (GM), for tertiary. Thus, the most obvious effect of GM vs. WT in this respect is that the largest proportion of the root system was found in the third-order laterals of the GM-treated plant.

In general, the length of the roots present in any order increased linearly with time (Fig. 5). There were no significant differences in length of root axes at any harvest. At 6 wk first-order laterals were shorter in GM-plants than in the WT-inoculated ones, whereas the second- and third-order laterals were longer in the GM treatment (Fig. 5). No significant differences in 'total root length' were observed between treatments at the final harvests (Fig. 7).

Figure 6 records the time-courses of the relationships between the number of roots present in each order and the length of these roots In general, the GM *Rhizobium* increased root branching frequency. Such an effect, which increased markedly with time, was particularly evident in the first-order laterals (Fig. 6).

It was also evident that the GM strain increased the total number of roots formed through increasing branching but not the total root length. The specific root length in alfalfa was similar for both rhizobial treatments with the exception of the final harvest (10 wk.) at which it was higher in WT-inoculated plants (Fig. 7).

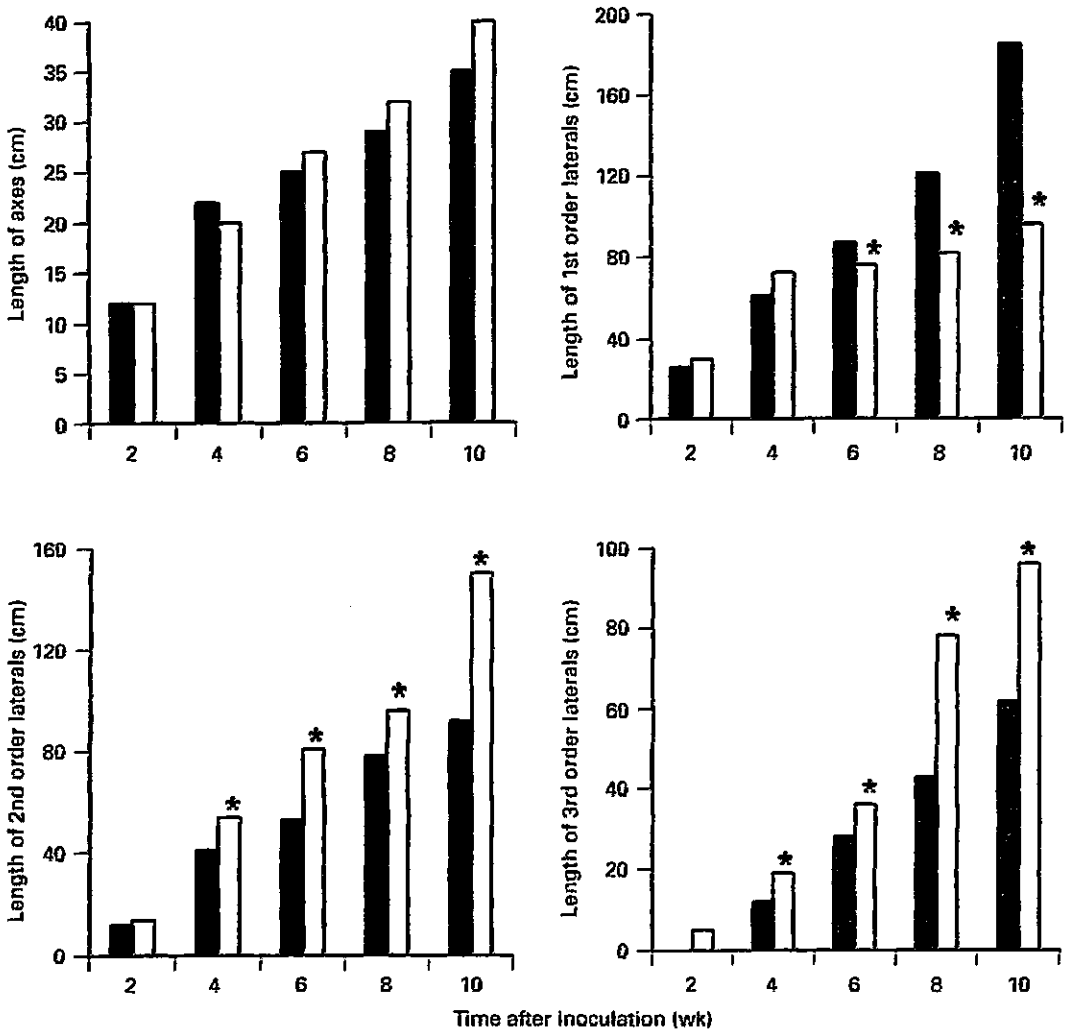

Figure 5. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on the length of the roots present in each order in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5% level.

DISCUSSION

The main objective of this study was to use the system *Medicago sativa–Glomus mosseae* as a biosafety model, in order to assess the impact of a GM *Rhizobium* strain, when released, on a key rhizosphere process, AM formation and function. A microcosm system was used to mimic interactions which occur in a living soil–plant situation. It was concluded that there was no evidence that the *Rhizobium* inoculant interfered with mycorrhiza development and function. On the contrary, the improved GM *Rhizobium* greatly benefited mycorrhiza performance.

Apparently, the primary effect of the GM *Rhizobium* on AM was to increase the number of mycorrhizal entry points in the alfalfa root system. This was probably as a result of GM *Rhizobium–G. mosseae* interactions at pre-colonization stages, as previously described. Tobar *et al.* (1996) found that GM *Rhizobium* increased the development of the mycelium arising from axenically germinated spores of *G. mosseae in vitro*, and also increased the formation of the 'first' entry points on alfalfa roots. Azcón-Aguilar & Barea (1992) discussed the mechanisms of the improvement of pre-colonization and early mycorrhizal colonization of legume roots by their specific *Rhizobium* strain. They concluded that an increased rate of root exudation induced by the extracellular *Rhizobium* polysaccharides, and/or the supply of plant hormones known to be produced by the bacteria, could be involved.

The time-course of the pattern of AM establishment indicated that the GM strain induced up to twice as many mycorrhizal entry points as did the WT strain at any harvest. Thus, from the second harvest (4 wk) onwards, the experimental system had two variables: (i) the *Rhizobium* inoculant, and (ii) the degree of AM colonization, depending on the rhizobial treatment. An improvement of nodule

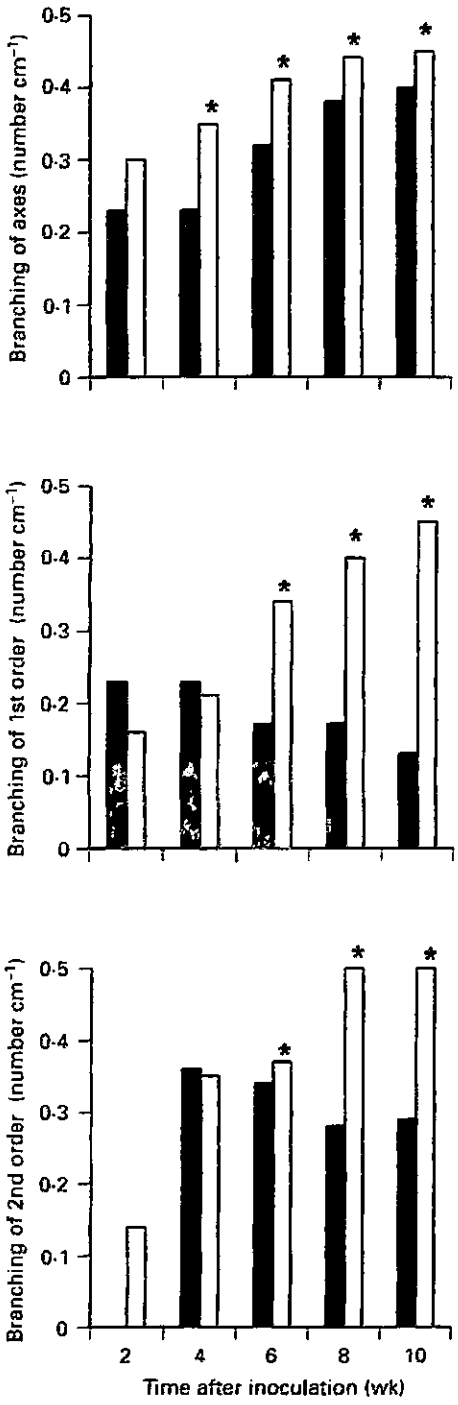

Figure 6. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on the number of branch roots, emerging from the different order laterals in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5% level.

quality in GM-inoculated plants was expected (Sanjuán & Olivares, 1991). The improvement in both the mycorrhizal development and the quality of the nodulation coincided with an increased plant biomass and nutrient (N and P) uptake. As is well-accepted (Jarrel & Beverley, 1981) the parameter 'nutrient content' can be used as a basis for discussion of the plant response to any nutrient-supplying treatment, since it takes into account well-balanced effects on nutrient concentration and biomass production. There was a close relationship between the time-course of mycorrhiza development and the time-course of nutrient content, as affected by the *Rhizobium* inoculant.

The efficiency of the symbiosis has been found to depend on the particular combination of *Rhizobium* strain and *Glomus* species involved, indicating selective compatibilities between strains and isolates of the legume microsymbionts (Azcón *et al.*, 1991). The results from the present study support the existence of such a selective compatibility also for GM bacterial derivatives, co-symbionts with the same AM fungus (*Glomus mosseae*).

Modifications to AM colonization induced by the *Rhizobium* inoculant merit some comments. Morphological, modelling and topological methods have been used to investigate effects of AM on root morphology and/or architecture (Atkinson *et al.*, 1994). We agree with Fitter & Stickland (1991), Schellenbaum *et al.* (1991), Berta *et al.* (1993) and Atkinson *et al.* (1994), that the topological method allows architectural approaches which, in turn, make it possible to evaluate the costs and benefits of root architecture related to the potential of the root for soil resource exploitation. This type of assessment cannot be achieved by applying the morphological, developmental, analysis. However, the latter is inherently dynamic and it is therefore most useful in describing changes which reflect developmental processes (Atkinson *et al.*, 1994). Because this fits into the aims of the present study, a simple developmental method was used. Such an approach allowed us to detect changes in root morphology induced by the symbiotic interactions. The main effects derived from an enhanced mycorrhizal status (GM-inoculated plants) were that the root number in any order lateral was higher than in WT-inoculated plants. This agrees basically with the findings by Schellenbaum *et al.* (1991), Hooker *et al.* (1992) and Berta *et al.* (1995), who compared AM with non-mycorrhizal control plants. Discussions of whether or not a set of morphogenetic modifications can be expressed by any single model have not reached general conclusions because of the complexity of the factors involved, such as AM fungal species, host plant, soil fertility and environment (Schellenbaum *et al.*, 1991; Atkinson *et al.*, 1994). The results from this study, particularly those showing changes in the length of the different orders of lateral roots, further reflect difficulties for any generalization.

In addition to the effect on root morphology of enhanced mycorrhizal status, a direct effect of the GM *Rhizobium*, resulting from increased N supply

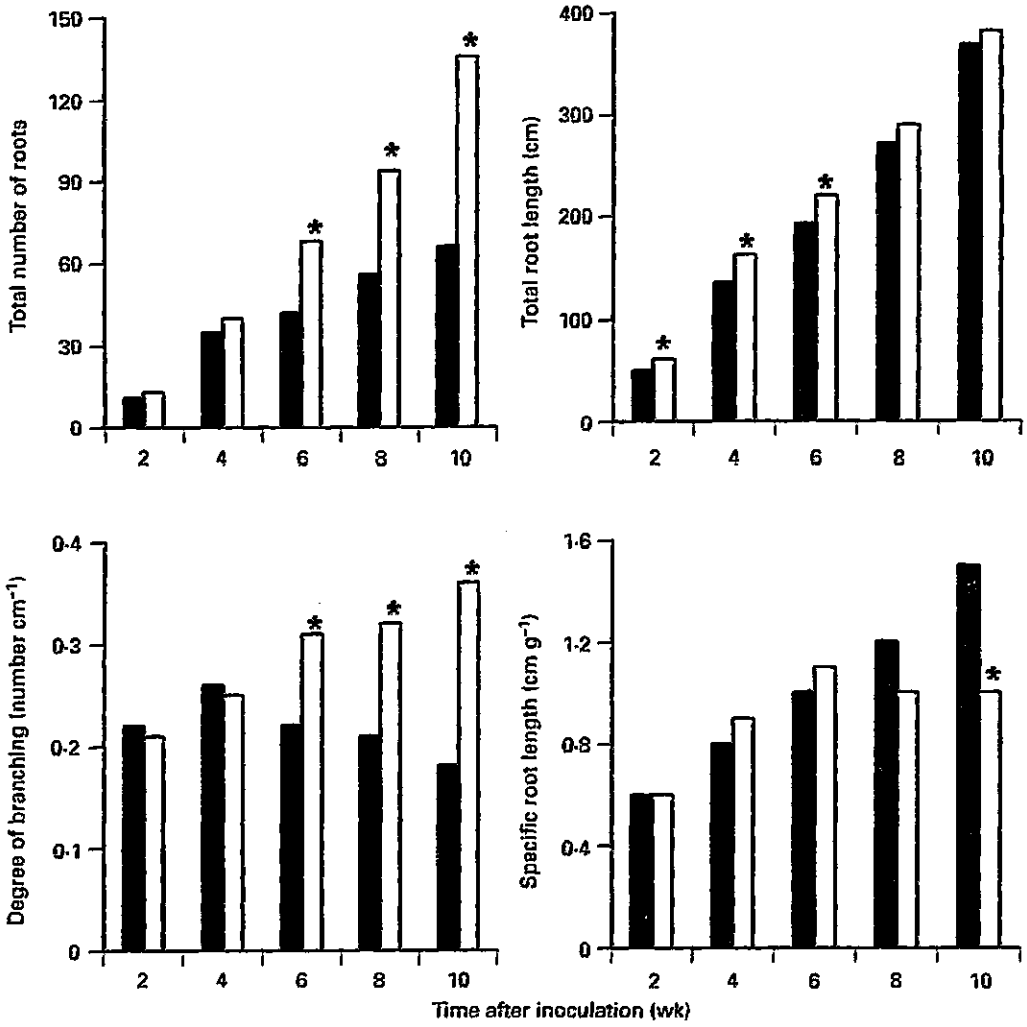

Figure 7. Effects of the two *Rhizobium meliloti* strains, the wild type (■) and its genetically modified derivative (□), on several parameters of root system development in mycorrhizal (*G. mosseae*) alfalfa plants. Data are given on a per plant (i.e. on a per microcosm unit) basis. The symbol (*) denotes that, for each parameter and harvest time, the differences between the effects of GM and WT are significant at the 5% level.

through improved N_2 fixation, could be also involved. It should be noted that an increased rate of branching is a general response of plant root to increased supply of major nutrients (Fitter, 1982).

The AM-induced decrease in the specific root length over time was similar to that found by Berta *et al.* (1995), but the data available from the present study do not permit any further discussion of the underlying mechanisms.

The close relationship between the time-course for nutrient uptake and the rate of lateral root formation found in the present study could support a P-mediated mechanism for the AM-induced changes in root system morphology. In leek the addition of P affected root morphology by increasing the initiation of first-order laterals (Amijee, Tinker & Stribley, 1989). However, as in previous discussions on this topic (Atkinson *et al.*, 1994; Berta *et al.*, 1995), other factor such as, for example, a mycorrhizal effect mediated by growth substances, cannot be ruled out. Indeed, the *G. mosseae* strain used in this study is known to be able to produce auxins, giberellins and cytokinins (Barea & Azcón-Aguilar, 1982).

In conclusion, it appears that an improved AM formation and the quality of nodulation first increase nutrient uptake, which can induce changes in root morphology. This, in turn, could also affect nutrient uptake patterns, finally to improve plant development.

ACKNOWLEDGEMENTS

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