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Response of nitrogen-transforming microorganisms to arbuscular mycorrhizal fungi

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Abstract We studied fluctuations in the numbers of autotrophic ammonium oxidizers, ammonifying microorganisms and denitrifying microorganisms in pot cultures of mycorrhizal and non-mycorrhizal maize. The populations were enumerated after 0, 15, 30, 45 and 60 days of plant growth. Two arbuscular mycorrhizal (AM) fungi belonging to different *Glomus* species were investigated. Pot cultures with AM-infected maize had significant quantitative and qualitative changes in the root-associated population of N-transforming bacteria compared with the non-mycorrhizal controls. The occurrence of autotrophic ammonium oxidizers in pot cultures of the AM fungi *Glomus mosseae* and *G. fasciculatum* was significantly higher than in non-mycorrhizal cultures throughout maize growth. The occurrence of these bacteria was delayed by 15 days in non-mycorrhizal as opposed to *Glomus*-colonized soil. Ammonifying and denitrifying bacterial populations were significantly decreased in the pot cultures of AM plants compared with the control. The distribution patterns of the physiological groups of bacteria tested were similar for both AM treatments but different from that of the non-mycorrhizal controls. Activity measurements expressed on a per cell basis showed changes with respect to the form of N in the mycorrhizal soil. *G. fasciculatum* was more active than *G. mosseae* during the earlier stages of plant growth.

Key words Arbuscular mycorrhiza · Nitrogen-transforming microorganisms · *Glomus mosseae* · *G. fasciculatum*

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Introduction

In general, soil productivity and nutrient cycling are influenced by soil microbial populations. In many circumstances N limits plant growth, but the significance of biological factors related to N cycling in the soil are not fully understood. In natural ecosystems the activity of microorganisms may be used to evaluate soil productivity (Bethlenfalvay and Schüepp 1994). Environmental problems associated with N in the soil are related to transformation processes which are largely microbial. Nitrification, as it occurs in soil, is a strictly biological process due, so far as it is known, to the activity of a few genera of chemoautotrophic bacteria. Few studies have been done on the ecology of N-transforming microbial groups, and data on their survival and growth are important in order to better understand natural processes.

Arbuscular mycorrhizal (AM) symbiosis is a widespread phenomenon and there is evidence of a direct effect of mycorrhizal fungi on inorganic N metabolism (Ho and Trappe 1975; Ames et al. 1983) and on N acquisition and assimilation by symbiotic systems (Azcón et al. 1982; Tobar et al. 1994a, b). Mycorrhizal status may alter the number and activity of N-transforming microorganisms, since changes in the soil environment are expected to affect microbial groups. Negative, as well as positive, AM effects on total viable counts of bacteria have been reported (Secilia and Bagyaraj 1987). In the present study we examined the influence of roots colonized by AM fungi compared with non mycorrhizal roots on bacterial populations which play a role in N transformations in soil.

Materials and methods

Experimental design

The experiment used a randomized complete-block design. The factors were: AM colonization (two AM isolates) or a control,

and growth periods (0, 15, 30, 45 or 60 days). Three replicates per treatment were used.

Growth conditions

Four maize seeds were sown into pots containing 1 kg of the experimental soil and were thinned after emergence to one seedling per pot. Plants were grown for 60 days in a greenhouse under controlled conditions at 25 °C during the day and 19 °C during the night, with a 16 h photoperiod (photosynthetic photon flux density of 400–700 μmol m^{-2} s^{-1}) and a relative humidity of 70–90%. Water was supplied daily after weighing each pot in order to mantain the soil water potential at a level close to 100% of the water-holding capacity of the test soil.

Test soil

Soil was collected from Güejar-Maitena, Granada Province, Spain. It was a cambisol type (14.91% clay, 26.37% loam and 58.71% sand) containing 0.8% organic matter and 14.8 mg P kg^{-1}, with a $pH_{(H_2O)}$ of 6.8 (Esteban et al. 1974). The N content was 2.6 g kg^{-1}, with 2.2 mg NO_3^- kg^{-1}. The soil was sieved (<2 mm), diluted with sand (5:2 v/v), steam-sterilized (100 °C for 1 h for 3 consecutive days) and then inoculated with a soil extract [20 ml pot^{-1} of a soil/water mixture (1:1 v/v) filtered through a Whatman no. 1 paper] to reintroduce the native microbial community, with the exception of propagules of AM fungi.

Mycorrhizal inoculation

The mycorrhizal inoculum (5 g pot^{-1}) consisted of spores, mycelium and mycorrhizal root fragments of the AM fungi, *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *G. fasciculatum* (Thax. *sensu* Gerd.) Trappe and Gerd., obtained from the collection of the Estación Experimental del Zaidín. It was introduced into a hole in the appropriate potted soils. Mycorrhiza-free filtrates from the mycorrhizal inoculum were added to the controls in the same way as above.

Most probable number enumeration method: evaluation of autotrophic ammonium oxidizers, ammonifying bacteria and denitrifying bacteria

Activity measurements are commonly expressed on a per cell basis and thus require numerical estimation of the population. The usual approach to enumeration is the most probable number (MPN) technique which provides an indirect estimate of the population involved in a particular step.

Three subsamples of soil (20 g) were taken from each pot after 0, 15, 30, 45 and 60 days of maize growth. Samples were collected by driving a test tube (15 cm long × 1.3 cm inside diameter) into the soil, at a distance of 3 cm from the emerging shoot. The tube was then carefully withdrawn, with the core of soil remaining inside it.

Soil samples of 10 g (wet weight) were placed in an oven at 70 °C to obtain the soil moisture percentage. The other 10 g were used to prepare a ten-fold dilution series in sterile water to which was added a drop of Tween 80. To calculate the MPN of each group of bacteria, test tubes containing 10 ml of suitable liquid media were inoculated with 1 ml of each serial dilution as described by Alexander (1982).

Inoculated MPN tubes and controls containing ammonifying medium (Pochon and Tardieux 1962) were incubated at 26–28 °C. The tubes were examined every 3 days by removing approximately 0.5 ml under sterile conditions and inserting the sample into small tubes. NH_4^+ was detected by using the Nessler reagent. The MPN tubes were generally incubated for 12 days.

The nitrifier tubes and controls containing 10 ml of Soriano and Walker medium (Watson et al. 1981) were incubated at 26–28 °C. Initial observations were made after 2 weeks and showed a colour change, from rose to colourless, in the pH indicator, indicating active acid production. The presence of NO_2^- was detected by a spot test. An aliquot of 0.1 ml of a diazotizing reagent plus a coupling reagent was added (modified Griess-Ilosyay reagents). The colour production (pink to red) indicated that NO_2^- was present (Schmidt and Belser 1982). The tubes were monitored for 6 weeks.

Screw-top tubes were filled with sufficient denitrifying medium (Jeter and Ingraham 1981) to displace about two-thirds of the total volume. After inoculation of the test tubes with appropriate dilutions, the caps were tightened to exclude the diffusion of atmospheric O_2 during a 2-week incubation.

Durham tubes were used to record the evolution of gaseous products. To confirm denitrification, 0.5 ml of the medium was withdrawn and tested for NO_3^- by adding, drop-wise, up to six drops of diphenylamine reagent (Tiedje 1982). Blue indicated the presence of NO_3^-; a colourless response was evidence of denitrification.

Measurements of the-maize plants

Plants were harvested after 60 days. The dry weights, of shoots and roots were recorded after drying in an oven at 70 °C. At this time the percentage of root length infected by AM fungi was estimated by examining stained samples (Phillips and Hayman 1970). The grid line-intersect method of Giovannetti and Mosse (1980) was used.

Statistical analysis

To calculate the MPN of organisms in the original sample, Mac Grady's tables were used (Posgate 1969).

Data from replicates of each treatment were subjected to analysis of variance, and the means were differentiated by Duncan's multiple range test (least significant difference, $P<0.05$).

Results

A significant increase in growth was found in the AM treatments compared with the control. However, no differences in plant growth or in mycorrhizal colonization between mycorrhizal treatments was found (Table 1).

The numbers of autotrophic ammonium oxidizers (Fig. 1A) in the soil of mycorrhizal pot cultures of *G.*

Table 1 Effect of arbuscular mycorrhizal fungi on shoot and root growth and mycorrhizal colonization of maize plants. Each value is the mean of five pots

Treatments	Shoot dry weight (g)	Root dry weight (g)	Mycorrhizal infection (%)
Control	1.34b	1.45b	0
Glomus mosseae	1.63a	1.99a	51
G. fasciculatum	1.66a	1.95a	46

[a,b] Column values followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

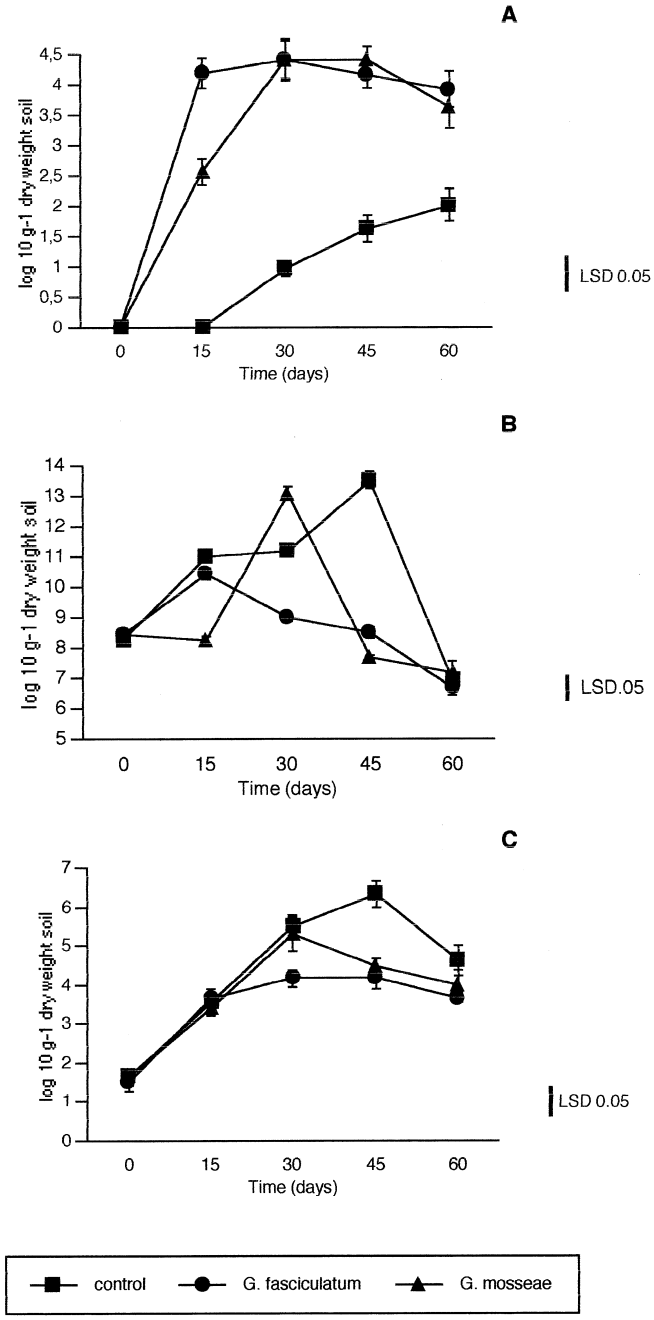

Fig. 1A–C Effects of arbuscular mycorrhizal fungi (*Glomus mosseae* or *G. fasciculatum*) on the numbers of autotrophic ammonium oxidizers (**A**), numbers of ammonifying microorganisms (**B**) and numbers of denitrifying bacteria (**C**) during the growth of maize. *Bars* indicate the standard deviation ($n=3$)

mosseae and *G. fasciculatum* were significantly greater than in the controls. Nevertheless, this effect was more pronounced in *G. fasciculatum*-colonized plants than in *G. mosseae* ones. Initially (day 0) no ammonium oxidizers were detected in the pot cultures, but after 15 days they were found in the mycorrhizal pots; few were detected in the soil from control plants after 30 days.

Ammonifying microorganisms were the most abundant of all the physiological groups of bacteria examined (Fig. 2). In contrast to those before, the incidence of ammonifying microorganisms (Fig. 1B) was less only after 15 and 45 days in all the mycorrhizal soil. The highest abundance of this group was recorded after 45 days for controls, 30 days for *G. mosseae*-colonized roots and 15 days for *G. fasciculatum*-colonized roots. AM pots harboured less denitrifying bacteria than non-mycorrhizal pot cultures after 30 or 45 days of plant growth (Fig. 1C). As with ammonifying bacteria, the incidence of denitrifying bacteria increased after 45 days in control soils, but in the case of *G. mosseae*-colonized soils the highest number was recorded after 30 days, while the soil cultures of *G. fasciculatum*-colonized plants maintained the greatest numbers from day 15 onwards.

The distribution patterns, given as percentages of the three different physiological groups of bacteria occurring in the soil from the mycorrhizal and non-mycorrhizal treatments are presented in Fig. 2. The non-mycorrhizal control harboured a greater proportion of ammonifying and denitrifying bacteria, while in mycorrhizal treatments the percentages of autotrophic ammonium oxidizers were highest.

Discussion

Populations of N-transforming microorganisms in the soil varied throughout plant growth, in both the mycorrhizal and non-mycorrhizal treatments. The metabolic activity of roots over time seems to be involved in this, since the populations of each physiological group of bacteria varied during the experiment in all the treatments. Increases in microbial populations have often been attributed to soluble organic substances (sugars, organic acids and amino acids) exuded by roots (Foster 1988; Lynch 1989). Ammonifying microorganisms were the most abundant group with high numbers during the early period of plant growth. In contrast, autotrophic ammonium oxidizers were not present when the first count was made. Mycorrhizal fungi enhanced the number of cells in each particular growth stage. Root exudation varies according to the age of a plant (Haller and Stolp 1985). In fact, microbial populations related to N-transformation differed qualitatively as well as quantitatively with time, and the presence or absence of mycorrhiza had a pronunced influence on specific bacterial groups.

The bacterial populations could have reacted differently to plants infected by AM fungi by day 15, depending on the fungus involved, but the abundance of nitrifying bacteria over time was quite similar in both AM fungal treatments. Meyer and Linderman (1986) reported that VA mycorrhizae affected specific groups of bacteria. Different AM fungi may specifically stimulate certain groups of bacteria in the soil. In the study reported here, the different *Glomus* species caused

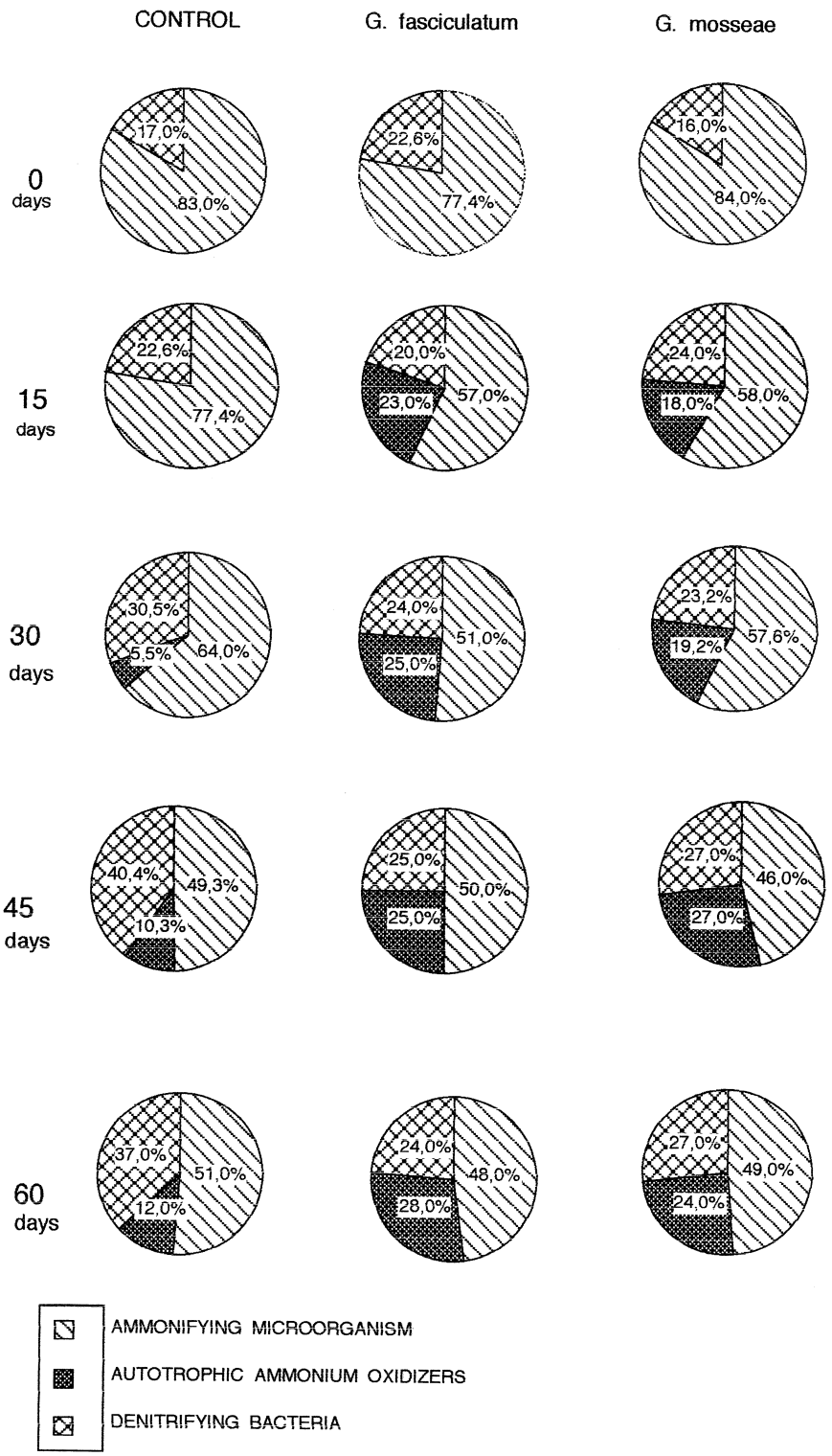

Fig. 2 *Areas of the circles* indicate the proportions of ammonifying microorganisms, autotrophic ammonium oxidizers and denitrifying bacteria in soil during the growth of mycorrhizal and non-mycorrhizal maize plants

quantitative changes in the bacterial populations, mainly during early growth. The presence of *G. fasciculatum* had stimulated, after 15 days, populations of the ammonifying bacteria (63%) and ammonium oxidizers (26%) compared to *G. mosseae* which was stimulated after 30 days. This effect suggests that specific AM fungi affects the physiology of the host in a different way with time.

It is difficult to predict the outcome of interactions between bacteria and AM fungi in relation to the total bacterial population as well as to specific groups of bacteria. Interactions between some of these bacterial populations are a consequence of changes in nutrient availability in the rhizosphere of mycorrhizal roots (Kloepper et al. 1985) and the presence of a new microorganism in the medium. AM fungi do not take up C from

the medium, but they can potentially affect the chemistry of the rhizosphere in several ways depending on their effect on the host's physiological processes (Secilia and Bagyaraj 1987; Paulitz and Linderman 1989). The different physiology of roots colonized by AM fungi compared with non-mycorrhizal roots affects root exudation (Schwab et al. 1983) and thereby leads to changes in the growth conditions for the general microbiota (Christensen and Jakobsen 1993). In the present study, plant growth parameters and AM colonization rates were examined as similar in both mycorrhizal treatments, but the influence of endophytes on counts of ammonium oxidizers as well as viable denitrifying bacteria was different at intermediate stages of plant growth (15 and 30 days).

Most denitrifying bacteria of significance in soil are heterotrophic. It is now recognized that denitrification is a specific metabolic process characterized for a relatively limited, yet large, number of bacterial genera. The most prevalent denitrifiers are species of *Pseudomonas,* especially *P. fluorescens* and *P. alcaligenes* (Gamble et al. 1977). Results concerning denitrifying bacteria in this study agree with those reported by Meyer and Linderman (1986) where the counts of fluorescent pseudomonas were lower in treatments with *G. fasciculatum* in association with *Zea mays* compared to non-AM controls. The AM may decrease the amount of plant-root-derived organic matter available for bacterial growth by altering the permeability of root cells, thus affecting exudation (Schwab et al. 1983). As Rambelli (1973) observed in the rhizosphere of mycorrhizal plants, the amounts of soluble compounds were modified and reduced. These findings agree with viable counts of total bacteria reported by Ames et al. (1984) and Secilia and Bagyaraj (1987). The number of microorganisms found in this study is similar to that reported by Gamble et al. (1977) and Martikainen (1985) for other soils.

The maximum microbial biomass was reached in the controls after 45 days of sowing, and in the mycorrhizal treatments the time taken to achieve this level was shortened to 30 days (*G. mosseae*) and 15 days (*G. fasciculatum*). However, the mycorrhizal fungi affected each bacterial group in a different way, leading to an increase in the number of ammonium oxidizers and a decrease in the numbers of ammonifying and denitrifying bacteria. These findings show that both mycorrhizal colonization and the specific stage of plant growth affect, certain microbial groups and, presumably, available nutrients in the soil.

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