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Ectomycorrhizal symbiosis enhanced the efficiency of inoculation with two *Bradyrhizobium* strains and *Acacia holosericea* growth

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Abstract Two strains of *Bradyrhizobium* sp., Aust 13C and Aust 11C, were dually or singly inoculated with an ectomycorrhizal fungus, *Pisolithus albus* to assess the interactions between ectomycorrhizal symbiosis and the nodulation process in glasshouse conditions. Sequencing of strains Aust 13C and Aust 11C confirmed their previous placement in the genus *Bradyrhizobium*. After 4 months' culture, the ectomycorrhizal symbiosis promoted plant growth and the nodulation process of both *Bradyrhizobium* strains, singly or dually inoculated. PCR/RFLP analysis of the nodules randomly collected in each treatment with Aust 13C and/or Aust 11C: (1) showed that all the nodules exhibited the same patterns as those of the *Bradyrhizobium* strains, and (2) did not detect contaminant rhizobia. When both *Bradyrhizobium* isolates were inoculated together, but without *P. albus* IR100, Aust 11C was recorded in 13% of the treated nodules compared to 87% for Aust 13C, whereas Aust 11C and Aust 13C were represented in 20 and 80% of the treated nodules, respectively, in the ectomycorrhizal treatment. Therefore Aust 13C had a high competitive ability and a great persistence in soil. The presence of the fungus did not significantly influence the frequencies of each *Bradyrhizobium* sp. root nodules. Although the mechanisms remain unknown, these results showed that the ectomycorrhizal and biological nitrogen-fixing symbioses were very dependent on each other. From a practical point of view, the role of ectomycorrhizal symbiosis is of great importance to N_2 fixation and, consequently, these kinds of symbiosis must be associated in any controlled inoculation.

Keywords *Acacia* · Ectomycorrhizas · Rhizobia · Microbial interaction

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Introduction

Acacia are abundant in savannas and arid regions of Australia, Africa, India and Americas. *Acacia* is the largest mimosoid genus including nearly 1,200 species (Pedley 1986). They can be used to prevent wind and rain erosion, to control sand dunes or as a source of wood and fodder for browsing livestock. As with many N_2-fixing trees and shrubs, *Acacia* species are very dependent on mycorrhizas to absorb nutrients necessary for plant growth and for efficient N_2 fixation (Cornet and Diem 1982). Two morphological types of mycorrhizas can be associated with *Acacia* species, arbuscular mycorrhizas (AM) and ectomycorrhizas (EM) (Le Tacon et al. 1989). As with other tree genera native to Australia (i.e. *Casuarina* and *Eucalyptus*), it has been established that some Australian *Acacia* can be associated with either ectomycorrhizal and/or endomycorrhizal fungi (Le Tacon et al. 1989; Ducousso 1990).

Each type of mycorrhiza can dramatically increase root nodulation. The main explanation for this is that the improvement of P uptake by the host plant resulting from mycorrhizal symbiosis enhances nodulation and N_2 fixation (Cornet and Diem 1982). Positive effects of AM fungi on nodulation have been demonstrated in *Acacia holosericea* with *Glomus intraradices* (Duponnois and Plenchette 2003), *G. fasciculatum* (Senghor 1998) and *G. mosseae* (Cornet and Diem 1982). In the same way, it has been shown that ectomycorrhizal fungi can enhance the number of nodules per plant and increase nodule weights in Australian *Acacia* species such as *A. holosericea* and *A. mangium* (Founoune et al. 2002a,b,c; Duponnois et al. 2002; Duponnois and Plenchette 2003). However, a controlled inoculation of rhizobial isolates together with EM

fungi, has been rarely achieved in these research works. Usually, this positive fungal effect has been assessed on contaminant rhizobia.

In order to verify these positive interactions between ectomycorrhizal symbiosis and the nodulation process, the purpose of this study was to evaluate the effect of an EM fungus, *Pisolithus albus*, on the root nodulation of *A. holosericea* with two strains of *Bradyrhizobium* sp., Aust 13C and Aust 11C, dually or singly inoculated.

Materials and methods

Fungal inocula and rhizobia

The ectomycorrhizal fungus in this study has been identified as *Pisolithus albus* on the basis of rDNA ITS phylogeny (Martin et al. 2002). It was isolated from a sporocarp sampled from a monospecific forest plantation of *A. mangium* in southern Senegal (Duponnois and Plenchette 2003). It is routinely maintained on MMN agar (Marx 1969) at 25°C in the dark. The inoculum was prepared in 1.6-l glass jars containing 1.3 l vermiculite-peat mixture (4:1, v:v) moistened with liquid MMN medium and autoclaved for 20 min at 120°C. This substrate was then inoculated aseptically with fungal plugs taken from the margin of fungal colonies.°C in the dark (Duponnois and Garbaye 1991).

Bacterial strains Aust 13C and Aust 11C were isolated from nodules collected in natural stands of *A. mangium* in Australia. Both strains were shown to be very efficient and identified as *Bradyrhizobium* sp. (Galiana et al. 1990, 1994). They are cultured in glass flasks containing liquid yeast extract-mannitol medium (Vincent 1970).

Molecular characterization of bacterial strains

One representative strain (Aust 1C and Aust 13C) of each group of similarity of Aust 11C and Aust 13C was selected for rDNA sequencing. Strains Aust 11C and Aust 13C had been preliminarily analysed by PCR amplification of the rRNA operon, comprising the 16S and the 16S/23S intergenic spacer (IGS), followed by RFLP analysis of the PCR products with four different restriction enzymes (Frémont et al. 1999). The dendrogram of genotype variations allowed us to differentiate them but to place them together in the same Australian cluster. Using the same four enzymes (data not shown), Aust 11C could not be differentiated by all of them from Aust 1C, another Australian strain.

DNA was extracted from 1 ml of a 1-week-old bacterial culture as described by Koponen et al. (2003). DNA amplification was conducted in a Genamp 2400 thermocycler (PE Applied Biosystems, Courtaboeuf, France), using the following primers (Oligos Etc., USA), targeting the rDNA operon: FGPS6:16S (beginning), 5′-GGAGAGTT AGATCTTGGCTCAG-3′, sense (Simonet et al. 1991); and FGPL132′:23S (beginning), 5′-CCGGGTTTCCCC ATTCGG-3′, antisense (Ponsonnet and Nesme 1994). The 25-µl reaction mixture contained 200 µM of each deoxynucleoside triphosphate, 0.8 µM of each primer, 15 mM of $MgCl_2$, 1.25 U of Taq DNA polymerase (Promega, Charbonnières, France) and the buffer supplied with the enzyme. After the final elongation step at 72°C for 3 min, the PCR products were run on a 1% agarose gel (Sigma, L'Isle d'Abeau, France).The amplified fragments were purified with a QIAquick gel extraction kit (Qiagen, Courtaboeuf) following the manufacturer's instructions.

Double-strand sequencing was carried out by Genome Express (Meylan, France) for Aust 13C, whereas, for Aust 1C, a 630-pb portion of 16S rRNA was sequenced on both DNA strands, using the following internal sequencing primers: 213, sense, 5′ CCTGGGGAGTACGGTCGCAAG 3′, *Escherichia coli* numbering 882–902, and FGPS 1509′, antisense, 5′ AAGGAGGGGATCCAGCCGCA 3′, at the end of 16S rDNA (Normand et al. 1996). Sequencing reactions were analysed on an Applied Biosystems model 310 DNA automated sequencer with BigDye Terminator chemistry (PE Applied Biosystems).

DNA sequences were deposited to the GenBank database of the National Center for Biotechnology Information at the National Institute of Health, Bethesda, Maryland (http://www.ncbi.nlm.nih.gov/). ClustalX software (Thompson et al. 1997) was used to align our sequences with previously published *Bradyrhizobium* and related genera reference sequences. Phylogenetic analyses were made using the neighbour-joining method (Saitou and Nei 1987). A bootstrap confidence analysis was performed with 1,000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein 1993). The resulting tree was drawn by using the NJplot software of M. Gouy (Laboratoire de Biométrie, UMR CNRS 5558, Université Lyon I). The Aust 1C 16S rDNA sequence and the total Aust 13C 16S rDNA-IGS 16S-23S sequence have been deposited in the GenBank database under accession nos. AY603955 and AY603956, respectively.

Greenhouse experiment

Seeds of *A. holosericea* (provenance Bel Air, Dakar) were surface sterilized with 95% concentrated sulphuric acid for 60 min. Then, the acid solution was decanted and the seeds were rinsed for 12 h in four rinses of sterile distilled water. Seeds were then transferred aseptically to Petri dishes filled with 1% (w/v) water agar. The plates were incubated for 4 days at 25°C. The germinating seeds were used when rootlets were 1–2 cm long.

The germinated seeds were individually grown in 0.5-l pots filled with a sandy soil collected from a 17-year-old plantation of *A. holosericea* in an experimental station at Sangalkam (50 km east of Dakar). Before use, the soil was crushed, passed through a 2-mm sieve, autoclaved for 40 min at 140°C and stored for 1 week in a dry room to avoid any soil toxicity. One week after autoclaving, its physico-chemical characteristics were as follows: pH (H_2O) 5.3; clay (%) 3.6; fine silt (%) 0.0; coarse silt (%) 0.8; fine sand

(%) 55.5; coarse sand (%) 39.4; carbon (%) 0.17; nitrogen (%) 0.02; C/N 8.5; total P (ppm) 39 and P-Bray 1 (ppm) 2.1.

The disinfected soil was mixed with 10% (v/v) fungal inoculum or 10% vermiculite-peat mixture (4:1; v:v) for the treatments without fungus. The plants were placed in a glasshouse (25°C day, 15°C night, 10-h photoperiod) and watered regularly with non-sterilized water but without fertiliser addition. The pots were arranged in a randomised complete block design with eight replicates per treatment. After 1 week's culture, the young seedlings were inoculated with 5 ml of the Aust 13C or Aust 11C suspensions (10^9 bacterial cells) or 5 ml of the culture medium without bacteria for the control treatments. When *Bradyrhizobium* strains were inoculated together, the same final volume of inoculum and the same densities of each bacterial strain (5 ml, 10^9 bacterial cells) were added to the soil.

Harvesting and plant growth assessment

After 4 month's culture, the height of the plants was measured. Then they were uprooted and the root systems gently washed. Shoot dry matter was determined after drying at 80°C for 1 week. Root nodules were counted, surface-disinfected with calcium hypochlorite (33 g l^{-1}) for 5 min, then with 96° ethanol for 5 min, and rinsed with sterile distilled water. They were cryopreserved at –80°C in glycerol 20%. The root systems were cut into pieces 1 cm long, mixed and the percentage of ectomycorrhizal root pieces [colonization index=(number of ectomycorrhizal root pieces/total number of root pieces)×100] was determined under a stereomicroscope (magnification×40) on a random sample of at least 100 root pieces per plant. Then root dry weight (60°C, 1 week) was determined for each plant.

Molecular tracing of bacterial strains in planta

Nodules were randomly collected in each treatment with Aust 13C and/or Aust 11C and crushed in 150 µl sterile distilled water with a plastic pestle. Then crushed nodules were suspended in 150 µl of CTAB/PVPP buffer (0.2 M TRIS-HCl, pH 8; 0.04 M EDTA pH 8; 2.8 M NaCl; 4% w:v CTAB; 2% w:v PVPP). The mixture was incubated at 65°C for 60 min and centrifuged for 10 min at 11,000 *g* to remove cell fragments. Phenol-chloroform-isoamyl alcohol (150 µl) (25:24:1; v:v:v) was mixed with the supernatant and centrifuged at 13,000 *g* for 15 min. DNA was purified from phenol by adding 150 µl of chloroform-isoamyl alcohol (24:1; v:v) followed by centrifugation at 13,000 *g* for 15 min. DNA from the aqueous phase was precipitated overnight at −20°C by adding sodium acetate–absolute ethanol (1:25; v/v). The solution was centrifuged at 13,000 *g*. The DNA pellet was washed with 70% ethanol and the suspension was centrifuged at 13,000 *g* for 15 min, vacuum dried and desorbed into 25 µl of ultrapure water. DNA samples were stored at −20°C for further analysis.

The primers MBAS3 (5′-TGCGGCTGGATCACCTCCTT-3′) and MBAL2 (5′-GTGGGTTCCCCATTCGG-3′) were used to amplify the 16S-23S rDNA spacer region. The amplification was done in a total volume of 25 µl and carried out with lyophilised beads (Ready to Go PCR beads; Pharmacia Biotech) containing 1.5 U of Taq polymerase, 10 mM TRIS-HCl at pH 9 and ambient temperature, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 1 µM of each primer and 4 µl of pure total DNA extract (10–50 ng μl^{-1}). Amplification reactions were carried out in a Gene-Amp PCR System 2400 automatic thermocycler (PE Applied Biosystems). The program was as follow: initial denaturation 5 min at 95°C, 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C) and extension (1 min at 72°C) and a final extension step (7 min at 72°C). PCR-amplified DNAs in 3-µl aliquots were visualized by horizontal electrophoresis on 1% (w/v) agarose gel (type II; Sigma, La Verpilliere, France). The gels were stained for 30 min with ethidium bromide (1 mg l^{-1}) and integrated with BIOCAPT image analysis software (Vilbert Lourmat, France) under a 260-nm UV source.

PCR products (7 µl) were digested in a total volume of 20 µl at 37°C for 2 h using the endonucleases HaeIII and MspI (Gibco, Cergy Pontoise, France) as described by the manufacturer. Restricted DNA was analysed by horizontal electrophoresis in a 2.5% (w/v) Metaphor gel (FMC, Rockland, Ma.). After 3 h at 80 V, the gels were stained with ethidium bromide (1 mg l^{-1}) and photographed under UV light.

Statistical analysis

Plant growth data were treated with one-way ANOVA and means were compared with the Newman-Keuls multiple range test ($P<0.05$). For the fungal colonization index, data were transformed by arcsin ($\sqrt{x}$) before statistical analysis. The numbers of both rhizobial strain nodules along the root systems of ectomycorrhized or non-mycorrhized *A. holosericea* plants were compared with 2×2 contingency tables and chi-square test (χ^2-test) and Yates correction for small numbers.

Results

Molecular characterization of the inoculant bacterial strains

The 16S rDNA BLAST analysis showed that Aust 1C and Aust 13C sequences were closely related to *Bradyrhizobium elkanii* type strain USDA76^T (accession no. U35000) both with 99% identity. 16S rDNA partial sequences of Aust 1C and Aust 13C were identical. Figure 1 shows the 16S rDNA partial sequence-based phylogenetic relationships between the strains Aust 1C and Aust 13C and re-

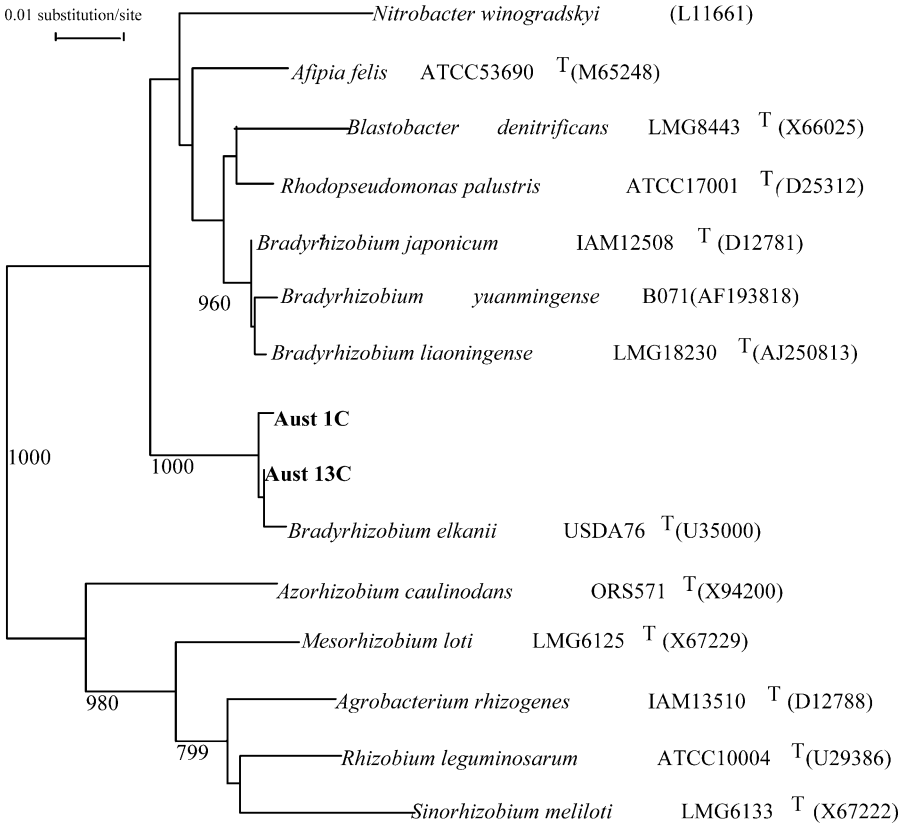

Fig. 1 Neighbour-joining dendrogram showing phylogenetic relationships of partial 16S rDNA sequences of *Bradyrhizobium* sp. strains Aust 13C and Aust 1C isolated from *Acacia mangium*, bradyrhizobia reference strains, and related organisms. Sequence accession numbers are given *in parentheses*. Bootstrap values, expressed as a percentage of 1,000 replications are given at the nodes, when ≥75%. The *bar* represents 1% sequence difference

lated organisms in the α-subclass of the Proteobacteria. Grouping of strains Aust 1C and Aust 13C with *B. elkanii* reference strain USDA 76^T, isolated from soybean, in a separate cluster, was supported by high confidence values from the bootstrap analysis.

Glasshouse experiment

After 4 months' culture, no effect on plant growth was recorded with the *Bradyrhizobium* strains in either treatment (single or dual bacterial inoculation) (Table 1). When each rhizobial inoculation, data in the same column followed by the *same letter* are not significantly different according to one-way ANOVA. *DW* Dry weight

Table 1 Effect of *Pisolithus albus* IR100 and/or *Bradyrhizobium* sp. Aust 13C and/or Aust 11C, singly or dually inoculated, on the growth of *Acacia holosericea* and its nodulation and mycorrhiza formation after 4 months' culture under glasshouse conditions. For

Treatments	Height (cm)	Shoot biomass (mg DW)	Root biomass (mg DW)	Ectomycorrhizal colonization (%)	Nodules per plant	Nodule biomass (mg DW)
Control	16.1 a[a]	159.2 a	76.4 a	0	0.2 a	0.18 a
Aust 11C	12.3 a	180.2 a	105.4 a	0	0.2 a	0.18 a
IR100	33.6 b	733.8 b	273.8 b	45.2 a	30.0 b	41.9 b
Aust 11C+IR 100	31.1 b	725.0 b	261.6 b	48.3 a	23.6 b	40.4 b
Control	16.0 a	159.2 a	76.4 a	0	0.2 a	0.18 a
Aust 13C	13.7 a	221.6 a	112.1 a	0	0.4 a	0.12 a
IR100	33.6 b	733.8 b	309.6 b	45.2 a	22.0 b	41.9 b
Aust 13C+IR 100	34.3 b	915.1 c	373.8 b	39.6 a	30.0 b	30.8 b
Control	16.0 a	159.2 a	76.4 a	0	0.2 a	0.18 a
Aust 13C/Aust 11C	16.4 a	244.8 a	105.2 a	0	3.2 a	0.24 a
IR100	33.6 b	733.8 b	373.8 b	45.2 a	24.8 b	41.9 c
Aust 13C/Aust 11C+IR 100	37.4 b	1006.6 c	464.4 b	41.6 a	30.0 b	43.7 b

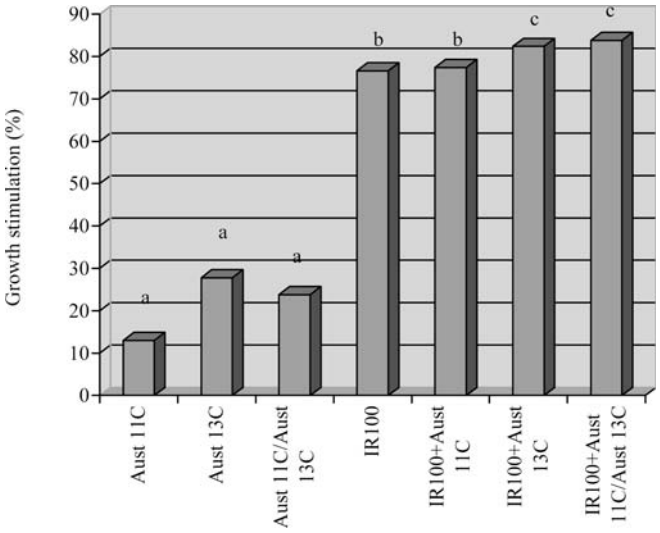

Fig. 2 Stimulation of shoot biomass by microbial treatments after 4 months' culture. The results are expressed as % shoot biomass measured in the control treatment. Treatments with the *same letter* are not significantly different according to one-way ANOVA

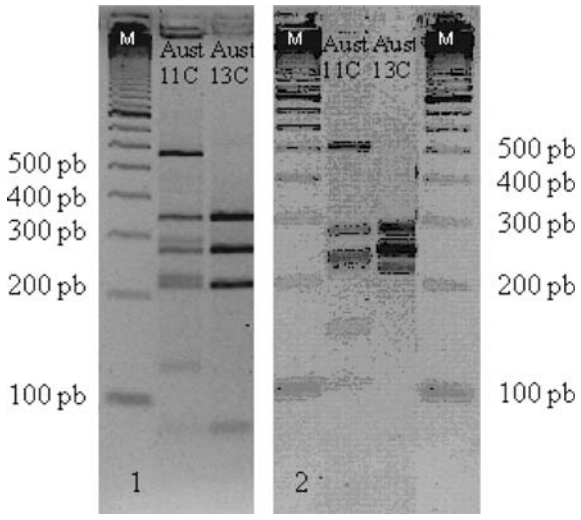

Fig. 3 Gel electrophoresis of PCR-amplified 16S-23S rDNA fragments of the bradyrhizobial strains Aust 13C and Aust 11C, digested with two restrictions enzymes [MspI. (*1*), HaeIII (*2*)]. *M* Molecular weight markers

bacterial strains were singly or dually inoculated, the number of nodules per plant and their dry weight were not significantly different from those of the non-inoculated treatment (control) (Table 1). No effect of Bradyrhizobial inoculations was recorded on ectomycorrhizal colonization (Table 1). Shoot and root biomasses were significantly increased in *P. albus* IR100 treatments and a significant difference in shoot biomass was found between the treatments *P. albus* IR100+Aust 13C with or without Aust 11C and *P. albus* IR100 singly inoculated (Table 1). In contrast, no effect of the dual inoculation of *P. albus* IR100 with Aust 11C was recorded for shoot growth compared to the *P. albus* IR100 treatment. Root biomass was mainly increased by fungal inoculation, whereas bacterial inoculation did not exert any significant effect. For all treatments combined, the inoculation with Aust 13C and *P. albus* IR100 stimulated shoot growth most (Fig. 2).

Although the soil was autoclaved and the seeds surface disinfected, some nodules were recorded on control plant roots, suggesting that plants were contaminated with indigenous rhizobia. This rhizobial contamination had been already observed and the main explanation for this was that the irrigation water could have contained N_2-fixing bacteria. The fungal strain, *P. albus* IR100 singly inoculated, significantly increased the number of nodules induced by rhizobial contaminants and the root infection by both *Bradyrhizobium* strains, singly or dually inoculated (Table 1).

Restriction analysis was done with 68 nodules randomly collected from all the treatments (Table 2). The nodules analysed from the plants not inoculated with Aust 13C or Aus 11C showed PCR/RFLP patterns different from those of Aust 13C or Aust 11C (data not shown). In contrast, nodules collected from Aust 13C or/and Aust 11C exhibited the same patterns as those of the Bradyrhizobial strains (Fig. 3). When both Bradyrhizobia isolates were inoculated together but without *P. albus* IR100, Aust 11C was recorded in 13% of the treated nodules compared to 87% for Aust 13C, whereas no contaminant rhizobia pattern was recorded (Table 2). When the ectomycorrhizal fungus was inoculated with both Bradyrhizobia, Aust 11C and Aust 13C were represented in 20 and 80% of the treated nodules, respectively (Table 2). As before, no nodules formed by native rhizobia were detected. This mycorrhizal effect was not significant according to the χ-test (χ^2=0.3, P>0.05).

Table 2 Frequencies of Aust 11C and Aust 13C nodules in each treatment assessed by restriction analysis of PCR-amplified 16S-23S rDNA spacer region

Treatments	Number of isolates[a]	
	Aust 11C	Aust 13C
Aust 11C	0	0
Aust 13C	0	0
Aust 11C/Aust 13C	13 (2)	87 (13)
Aust 11C+IR 100	100 (14)	0
Aust 13C+IR 100	0	100 (14)
Aust 11C/Aust 13C+IR 100	20 (5)	80 (20)

[a]Percentage (number of analysed nodules) of each Bradyrhizobial strain from each treatment

Discussion

Sequencing of strains Aust 13C and Aust 1C/Aust 11C confirmed their previous placement (Frémont et al. 1999) in the genus *Bradyrhizobium*. As stated by Willems et al. (2001a), the genus *Bradyrhizobium* is very diverse. From the 16S sequencing, we would place the two strains within *B. elkanii*, with 99% homologies for both strains. However, this phylogenetic placement was not confirmed through ITS sequencing, the homology of strain Aust 13C showing <95.5% homology with the *B. elkanii* type strain, as shown by Willems et al. (2003). In *Bradyrhizobium*, strains with at

least 96% spacer sequence similarity did belong to the same genospecies, but strains with lower levels of spacer sequence similarity do not necessarily belong to different genospecies (Willems et al. 2001b). Actually, genospecies' numbers were assigned by Willems et al. (2001c) on the basis of DNA–DNA hybridizations and ITS sequence grouping. Willems et al. (2001c) found 11 genospecies among the *Bradyrhizobium* genus. Aust 13C had closest affinities with genospecies VI or XI. A formal species assignment of both Aust 11C and Aust 13C strains could probably only be obtained through DNA–DNA hybridization as described by Willems et al. (2001c). Without these hybridizations, uncertainty about ITS sequence homologies leads to their assignment as *Bradyrhizobium* sp. strains.

P. albus strain IR100 dramatically increased plant growth: shoot biomass was enhanced by 460%. This positive effect of the EM symbiosis has been previously described with other *P. albus* isolates such as *P. albus* COI007 (+448%) (Founoune et al. 2002b), *P. albus* COI024 (+142%) (Duponnois et al. 2000) but also with other ectomycorrhizal fungal genera such as *Scleroderma dictyosporum* IR109 (+212%) or *S. verrucosum* IR500 (+251%) (Duponnois and Plenchette 2003). It is well known that ectomycorrhizal fungi improve plant productivity in low fertility soils by enhancing mineral nutrient concentrations (Bolan 1991). From the present study, this fungal effect cannot only be attributed to *P. albus* inoculation as a lot of nodules were recorded on the *A. holosericea* root systems. The effect of these native rhizobial strains on plant growth has to be investigated in further experiments.

Surprisingly, a low number of nodules were detected along the root systems of *A. holosericea* when their cultural substrate was inoculated with Aust 13C or Aust 11C. The PCR/RFLP analysis confirmed that these nodules were induced by contaminant rhizobia when each strain was singly inoculated. In contrast, Aust 13C and Aust 11C nodules were detected when both Bradyrhizobial strains were inoculated together. Duponnois et al. (2002) also observed that Aust 13C inoculation did not involve the formation of well-developed nodules on *A. mangium* seedlings after 4 months' culture in disinfected sandy soil. These authors observed some very small nodules, but did not verify if these nodules contained the Aust 13C isolate. Galiana et al. (2002) have also estimated the impact of Aust 13C on growth and nitrogen fixation in *A. mangium*. Unfortunately, none of the data indicated that this inoculated bacteria formed nodules in glasshouse conditions before transplantation to the field. The Aust 13C isolate has been used in field trials to test the effect of selected *Bradyrhizobium* strains on the growth of *A. mangium*. Nineteen months after tree transplantation, Aust 13C had a positive effect on tree growth and was predominant in nodules collected from *A. mangium* root systems. The Aust 13C survival and persistence in the field were assessed using a FITC labelled antibody technique (Somasegaran and Hoben 1985). These data confirmed the long-term stimulating effect of Aust 13C on plant growth, its high competitive ability against indigenous N_2-fixing bacteria and its persistence in soil. In conclusion, this bacterial strain was present in the disinfected soil during the glasshouse period but only nodulated after tree transplantation in a natural soil. It is well established that mycorrhizal fungi are an ubiquitous component of most ecosystems, play an important role in soil processes (Smith and Read 1997) and, therefore, their presence could facilitate nodule formation. A lot of studies have demonstrated that arbuscular mycorrhizal infection generally helps nodule formation and function under stress conditions (Azcon et al. 1988; André et al. 2003). The same positive effects have been recorded with ectomycorrhizal symbiosis (Duponnois et al. 2000, 2002; Duponnois and Plenchette 2003). This mycorrhizal-promoting effect could be due to the better root growth that favoured rhizobia colonization and infection. But, it is also well known that mycorrhizas modify root functions (in particular, root exudation) and, therefore, could modify microbial communities (commonly termed the "mycorhizosphere effect") (Katznelson et al. 1962; Linderman 1988). Specific relationships occur between mycorrhizal fungi and mycorhizosphere microflora (Garbaye 1991; Garbaye and Bowen 1989; Marshner et al. 2001). Moreover, the extraradical mycelium of ectomycorrhizal fungi could also influence the chemical composition and pH of the surrounding soil, and excrete substances into it (Frey et al. 1997; Caravaca et al. 2002). All these physical and chemical modifications could facilitate the development of the inoculated Bradyrhizobial strains and, consequently, improve the nodulation process. However, these hypotheses are not limited to the environment of the host plant/ectomycorrhizal fungus symbiosis. More specific relationships could occur during the development of the tripartite symbiosis, at the physiological and molecular level (Van Rhijn et al. 1997; Blilou 1999; Parniske 2000).

The high competitiveness level of Aust 13C in the treatment without IR 100 was not altered on ectomycorrhized plants. The co-inoculation improved the plant growth compared to that measured when *P. albus* was singly inoculated. In a previous study, André et al. (2003) have observed that an arbuscular mycorrhizal fungus, *Glomus intraradices*, could modify the development of bacterial inoculants along the root systems of *A. raddiana*. One of their hypotheses was that these bacteria metabolised trehalose, produced by many fungi (van Laere 1989) whereas most vascular plants are unable to excrete this carbohydrate (Muller et al. 1995). In our study, Aust 13C and Aust 11C did not assimilate this compound (data not shown). Consequently, other mechanisms must be involved to explain these microbial interactions.

In conclusion, these results showed that both the ectomycorrhizal and biological nitrogen-fixing symbioses were very dependent on each other. From a practical point of view, the role of ectomycorrhizal symbiosis is of great importance to N_2 fixation and, consequently, these kinds of symbiosis must be associated in any controlled inoculation. Moreover, the selection of Bradyrhizobia for their efficacy with respect to plant growth in field trials or glasshouse experiments has to be investigated by taking into account the interactions between bacterial and fungal symbionts.

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