



# Interactions between ectomycorrhizal symbiosis and fluorescent pseudomonads on *Acacia holosericea*: isolation of mycorrhiza helper bacteria (MHB) from a Soudano-Sahelian soil

H. Founoune <sup>a,b</sup>, R. Duponnois <sup>a,\*</sup>, J.M. Meyer <sup>c</sup>, J. Thioulouse <sup>d</sup>, D. Masse <sup>a</sup>,  
 J.L. Chotte <sup>a</sup>, M. Neyra <sup>e</sup>

<sup>a</sup> IRD, UR 'IBIS' (Interactions Biologiques dans les Sols des systèmes anthropisés tropicaux), BP 1386 Dakar, Senegal

<sup>b</sup> Université Moulay Ismail. Laboratoire de Biotechnologie et d'Amélioration des plantes, BP 4010 Meknes, Senegal

<sup>c</sup> Laboratoire de Microbiologie et de Génétique, Université Louis-Pasteur/CNRS FRE 2326, 28 rue Goethe, 67083 Strasbourg, France

<sup>d</sup> CNRS, UMR 5558, Université de Lyon 1, 69 622 Villeurbanne Cedex, France

<sup>e</sup> IRD, Laboratoire de Microbiologie, BP 1386 Dakar, Senegal

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## Abstract

*Acacia holosericea* seedlings were planted in 1-l pots filled with a soil collected from an Australian *Acacia* plantation in Southern Senegal. After 6 months of culture, mycorrhizosphere soil, roots, galls induced by root-knot nematodes and *Rhizobium* nodules were sampled from each pot. The diversity of this bacterial group was characterized by siderotyping (pyoverdine IsoElectric Focusing (IEF) analysis) and by restriction fragment length polymorphism (RFLP). The effect of these isolates on the establishment of the ectomycorrhizal symbiosis between an Australian *Acacia* (*A. holosericea*) and *Pisolithus* sp. strain IR100 was studied. In the mycorrhizosphere soil, the population of fluorescent pseudomonads was represented by strains of two different siderovars (groups of bacterial strains presenting an identical pyoverdine-IEF pattern): siderovar 1 (74%) and siderovar 2 (26%). The siderotyping of the isolates around galls of the root-knot nematodes revealed three siderovars (40% from siderovar 1, 40% from siderovar 2 and about 15% from siderovar 3). RFLP of 16S rDNA divided the isolates into four different groups with *MspI*, two with *HhaI* and two with *HaeIII* endonucleases. The establishment of the ectomycorrhizal symbiosis with *A. holosericea* was promoted by 14 bacterial strains isolated from the mycorrhizosphere soil, three isolates from the roots and four from the galls. Shoot biomass of *A. holosericea* seedlings was stimulated by eight bacterial isolates from soil, six isolates from galls and seven from roots. These mycorrhiza helper bacteria could have a great ecological importance in tropical areas through the reforestation programs. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

**Keywords:** Fluorescent pseudomonads; Restriction fragment length polymorphism; Siderotyping; *Pisolithus* sp.; Ectomycorrhizal symbiosis; *Acacia holosericea*

## 1. Introduction

As low-input agriculture has been promoted during the last decades, a great interest in soil microorganisms able to increase soil fertility or to stimulate plant nutrition and/or health has resulted. In tropical areas, several groups of microbial inoculants, such as mycorrhizal fungi (ectomycorrhizal or arbuscular mycorrhizal associations) or rhizob-

bia with the leguminous species, have been identified and tested, with respect to their ability to improve plant growth. For instance, the growth of some Australian *Acacia* species (i.e. *A. mangium*, *A. holosericea*, *A. auriculiformis*) was enhanced when they were inoculated with different strains of *Pisolithus* spp. or rhizobial isolates [1–5]. *Acacia* is the largest mimosoid genus and includes nearly 1200 species [6]. They are abundant in savanas and arid regions of Australia, Africa, India and the Americas. Some of them prevent wind and rain erosion, control sand dunes, are sources of wood and provide fodder for browsing livestock. In addition, much of the nitrogen fixed in leaves and roots by the rhizobial symbiosis is returned

\* Corresponding author. Present address: IRD, UR 'IBIS' (Interactions Biologiques dans les Sols des systèmes anthropisés tropicaux), 01 BP 182, Ouagadougou, Burkina Faso. Tel.: +226 30 67 37/39; Fax: +226 31 03 85. E-mail address: robin.duponnois@ird.bf (R. Duponnois).

to the soil with the natural loss of leaves and the resulting humus improves the fertility of the soil and its physical properties. One of the fast-growing leguminous trees, brought from Australia and introduced in Western Africa appears to be well adapted to these climatic conditions [7].

So far, however, inoculation experiments were performed under glasshouse conditions using sterilized soils which minimized the microbial interactions between the soil microbial microflora and the fungal symbionts. In fact, it has been demonstrated that establishment of ectomycorrhizas on tree roots could be affected by the bacterial communities of the rhizosphere [8,9]. This bacterial effect can have a positive or negative impact on mycorrhiza formation [8–10]. Some soil bacteria, especially fluorescent pseudomonads, stimulate the establishment of the ectomycorrhizal symbiosis. These bacteria were called MHB for mycorrhiza helper bacteria [11]. However these studies have generally been conducted using symbiotic tree/ectomycorrhizal fungus associations from temperate environments [12,13]. The influence of soil bacteria on the development of ectomycorrhizal symbiosis is still largely unknown in Sahelian and Soudano-Sahelian areas. Therefore, the objectives of this research were (i) to determine the heterogeneity of fluorescent pseudomonad strains isolated from the soil and along the root systems of *A. holosericea* seedlings growing in a soil collected from an Australian *Acacia* plantation where fruit bodies of *Pisolithus* spp. have been recorded and (ii) to measure the effect of these bacterial isolates on the establishment of the ectomycorrhizal symbiosis between *A. holosericea* and an isolate of *Pisolithus* sp.

## 2. Materials and methods

### 2.1. Soil samples and microbial isolation

During a survey to determine the occurrence of the ectomycorrhizal symbiosis between *Pisolithus* sp. and Australian *Acacias*, fruit bodies of this fungal symbiont were recorded in a plantation of Australian *Acacia* localized at Cassankil (Casamance, South of Senegal). Sporocarps of *Pisolithus* spp. were sampled, brushed free of adhering soil and fractured carefully in a laminar flow hood. A small amount of tissue was then removed with a fine forceps and placed on MMN agar medium in a Petri dish (Melin and Norkrans modified by Marx) [14]. The fungal cultures were incubated at 25°C in the dark and subcultured until all contaminating microorganisms were eliminated. Among several fungal isolates, one strain called IR 100 was chosen because of its positive influence on the plant growth of Australian *Acacia* species.

Fluorescent pseudomonad strains were isolated from 6-month-old *A. holosericea* growing in 1-l pots filled with the soil collected around each sporocarp. Although this method risks losing some of potentially heterogeneity in field

soil, it was required to obtain more homogenous plant and soil material. Seeds of *A. holosericea*, provenance Bel Air (Dakar, Senegal), were surface-sterilized with concentrated 36 N sulfuric acid for 60 min. The acid solution was then decanted off and the seeds were rinsed and imbibed 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 medium. These plates were incubated for 2 days at 25°C in the dark. Then one germinated seed was transferred into each pot, placed in a glasshouse (35°C day, 30°C night, 12-h photoperiod) and watered twice a week without fertilizer. The measurements described below were performed on 10 plants of *A. holosericea*. After 6 months in culture, the pots were full of roots and mycelium. Then the plants were uprooted and, along the root systems, different structures have been distinguished. The *Rhizobium* nodules and the roots (mycorrhizal or not) were collected from each root system. The non-mycorrhizal roots and mycorrhizal roots were not distinguished. Although some fungal hyphae had been observed everywhere on the root systems, no well-developed mycorrhizas were found. *Pisolithus* mycorrhizas are generally clearly distinct from uninfected roots but, after a few months of culture in a non-disinfected soil, the yellow fungal mantle tends to disappear and, consequently, it is difficult to spot mycorrhizas among the root systems. Some galls induced by root-knot nematodes (*Meloidogyne* spp.) were also detected [15]. These plant cell modifications result from the invading nematode, which induces giant cell formation. In addition, the surrounding cells are stimulated to divide and the root swells to form the gall [16]. *Meloidogyne* species were identified using the method of Dalmasso and Bergé [17].

Soil subsamples (1 g fresh weight, fw), fresh ungallo roots and galls (1 g fw) with the adhering soil were suspended in 10 ml sterile magnesium sulfate solution (0.1 M) and blended in an Ultraturax blender. The *Rhizobium* nodules and their adhering soil were put in Eppendorf tubes (1 nodule per tube) filled with 1 ml sterile magnesium sulfate solution (0.1 M), further vigorously shaken and crushed with plastic pestle. Then serial dilutions of homogenized suspensions were plated on King's B medium [18] to isolate fluorescent pseudomonads and incubated for 48 h at 30°C. The King's B medium plates were examined under UV light and fluorescent colonies were counted and randomly selected. The isolates of fluorescent pseudomonads (76 bacterial strains) were purified, subcultured on King's B medium and cryopreserved at –80°C in glycerol 60%/TSB (tryptic soy broth, 3 g l<sup>-1</sup>) culture (1:1, v/v).

### 2.2. Characterization of fluorescent pseudomonad strains

#### 2.2.1. PCR amplification and restriction fragment length polymorphism (RFLP) analysis of 16S rDNA

The primers rD1 (5'-AAGCTTAAGGAGGTGATC-

CAGCC-3') and fD1 (5'-AGAGTTTGATCCTGGCT-CAG3-') [19] were used to amplify the 16S rDNA gene. This primer pair allows amplification of nearly the full-length of 16S rDNA gene for many bacterial genera.

The amplification was made in a total volume of 25  $\mu$ l and performed with lyophilized beads (Ready-to-go PCR beads, Pharmacia Biotech). The beads contain 1.5 U of *Taq* polymerase, 10 mM of Tris-HCl at pH 9 and ambient temperature, 50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 200  $\mu$ M of each deoxynucleoside triphosphate, 1  $\mu$ M of each primer and 2  $\mu$ l of bacterial cell suspension. Amplification reactions were performed in a GeneAmp PCR System 2400 automatic thermocycler (Perkin-Elmer, Applied Biosystems, CA, USA). The program was as follows: initial denaturation 5 min at 94°C, 35 cycles of denaturation (1 min at 94°C), annealing (45 s at 55°C) and extension (1.5 min at 72°C) and the last extension (5 min at 72°C).

An aliquot (3  $\mu$ l) of amplification product was visualized by horizontal electrophoresis in 1% (w/v) agarose gels (Sigma, La Verpillière, France). The gels were stained for 30 min with ethidium bromide (1 mg l<sup>-1</sup>) and integrated with the Image Analysis software BIOCAPT (Vilbert Lourmat, France) under UV light.

PCR products (7  $\mu$ l) were digested in total volume of 20  $\mu$ l at 37°C for 2 h using the endonucleases *Hha*I, *Hae*III and *Msp*I (Gibco-BRL, Cergy Pontoise, France) as described by the manufacturer. Restriction fragments were separated by horizontal electrophoresis in a 2.5% (w/v) Metaphor gel (FMC, Rockland, Marine, USA). After 2 h of running at 80 V, the gel was stained with ethidium bromide (1 mg l<sup>-1</sup>) and photographed under UV light.

### 2.2.2. Siderotyping

The method used for distinguishing different fluorescent *Pseudomonas* strains was based on the pyoverdine (PVD) IsoElectric Focusing (IEF) analysis of the PVDs produced during growth in CAA medium, the composition of which was (g l<sup>-1</sup>): low-iron casein hydrolysate (Difco, Bacto Casamino Acid), 5; K<sub>2</sub>HPO<sub>4</sub>•3H<sub>2</sub>O, 1.54; MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.25 [20].

The analysis was performed according to a method described by Koedam et al. [21] using a Bio-Rad model 111 Mini IEF Cell. Polyacrylamide (5%) gels containing ampholines (Byolite 3/10, Bio-Rad, France) were prepared according to the manufacturer's recommendations. The pH gradient which developed during electrophoresis was from 3.5 to 9.3. 1  $\mu$ l of each of the 20-fold-concentrated (through lyophilization) CAA-culture supernatants was loaded on the gel. PVD bands corresponding to the different PVD isoforms accumulated in the medium during growth [22] were visualized under UV light at 365 nm. Their corresponding isoelectric pH values (pHi) were determined according to a standard curve which was obtained by slicing the IEF gels into 0.5-cm bands immediately after electrophoresis. These bands were incubated

separately in 2 ml KCl (10 mM) solution for 30 min and the pH was measured.

### 2.3. Effect of fluorescent pseudomonads on the establishment of the ectomycorrhizal symbiosis between *A. holosericea* and *Pisolithus* sp.

These experiments described below have been performed with bacterial strains isolated from soil (26 isolates, HR45, HR46, HR48–HR55, HR57–HR65, HR67–HR72, HR74), root (20 isolates, HR1–HR11, HR13–HR20, HR22), and galls (18 isolates, HR23, HR24, HR28, HR29, HR31–HR44). All of them belong to the most representative siderovars (sv., 1 and 2).

#### 2.3.1. Effect of fluorescent pseudomonads on in-vitro fungal growth

The fungal strain of *Pisolithus* sp. (IR 100) isolated from the Australian *Acacia* plantation (see below) was maintained in Petri dishes on MMN agar medium at 25°C in the dark [14]. Agar plugs (6 mm diameter; 4 mm thick) were taken from the margin of the fungal colonies. The confrontation between bacterial strains and the fungus *Pisolithus* IR 100 was done using two-compartment dishes [11]. One compartment contained the fungal plugs, surface side up on the dry bottom of the dish, while the other was filled with TSB agar medium inoculated with each bacterial isolate. There were no bacteria in the control treatment. Then the dishes were sealed with tape to prevent drying during incubation and placed at 25°C in the dark. Three dishes, each one with three mycelial plugs, were prepared for each bacterial treatment. After 8 days of incubation, the mean radial growth in two perpendicular directions was calculated for each fungal plug with a stereomicroscope fitted with an ocular scale. The data were analyzed with a one-way analysis of variance. Mean values were compared using Student's *t*-test ( $P < 0.05$ ).

#### 2.3.2. Effect of fluorescent pseudomonads on mycorrhization and plant growth

The ectomycorrhizal fungus *Pisolithus* sp., strain IR 100, was routinely maintained on MMN agar medium [14]. 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 the fungal colonies. The jars were then sealed and incubated for 6 weeks at 28°C in the dark.

The bacterial strains, tested for their effect on the fungal growth in axenic conditions, were grown in 100 ml TSB medium (Difco) for 72 h at 25°C in the dark on a rotary shaker. Each bacterial suspension was then centrifuged (2400  $\times$  g, 30 min) and the supernatants discarded. The pellets were resuspended three times in 0.1 M MgSO<sub>4</sub>

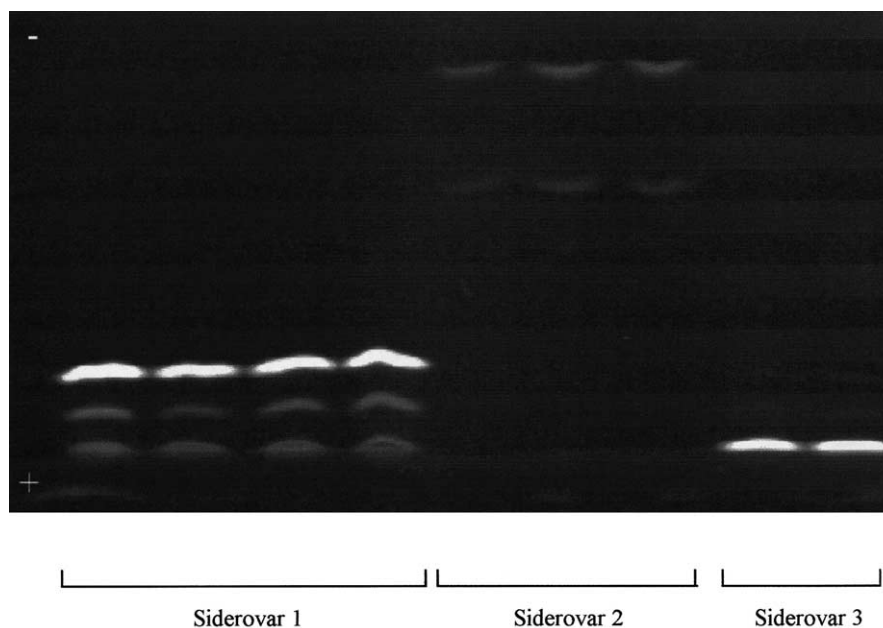


Fig. 1. PVD-IEF patterns allowing the classification of strains in three siderovars.

and centrifuged ( $2400 \times g$ , 30 min) to eliminate the culture medium; each bacterial pellet was finally suspended in 0.1 M  $MgSO_4$  at about  $10^9$  colony forming units (CFU)  $ml^{-1}$ .

*A. holosericea* seedlings were grown in 60 ml containers filled with a disinfected sandy soil collected in a stand of *A. holosericea* at the east of Dakar (Sangalkam). Before use, the soil was crushed, passed through a 2-mm sieve and autoclaved for 40 min at  $140^\circ C$  to eliminate native microflora. One week after autoclaving, its chemical and physical 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 Olsen P (ppm) 4.8. Each container was inoculated with the *Pisolithus* IR 100 strain and one fluorescent pseudomonad isolate by mixing the autoclaved soil with 10% (v/v) IR 100 fungal inoculum and 5 ml of the bacterial suspension, both prepared as described above. The control received 5 ml of 0.1 M magnesium sulfate instead of the bacterial suspension. The number of bacterial cells inoculated per container was about  $5 \times 10^9$  CFU  $ml^{-1}$ . The plants were arranged in a randomized, complete block design with 10 replicates per treatment. They were placed in a glasshouse under natural light (daylength approximately 12 h, mean temperature  $30^\circ C$  day) and watered daily without fertilizer.

The plants were harvested after 3 months of culturing and their root systems were washed under running tap water, cut into short pieces and mixed. The percentage of ectomycorrhizal colonization of all lateral roots was determined for each treatment (number of mycorrhizal roots/total number of roots  $\times 100$ ) under a stereomicroscope at  $\times 160$  magnification. The dry weights of shoots and roots (1 week at  $60^\circ C$ ) were measured.

#### 2.4. Statistical analysis

Data were subjected to a one-way analysis of variance and means were compared with the Newman-Keuls multiple range test ( $P=0.05$ ). Fungal percentage colonization and bacterial counts were transformed, respectively, by arcsin (vx) and by  $\log(X+1)$  before statistical analysis.

The origins and siderovars of bacterial isolates and their effect on the plant growth, mycorrhizal rate and fungal growth were compared with a principal component analysis [23] with a one-way analysis of variance.

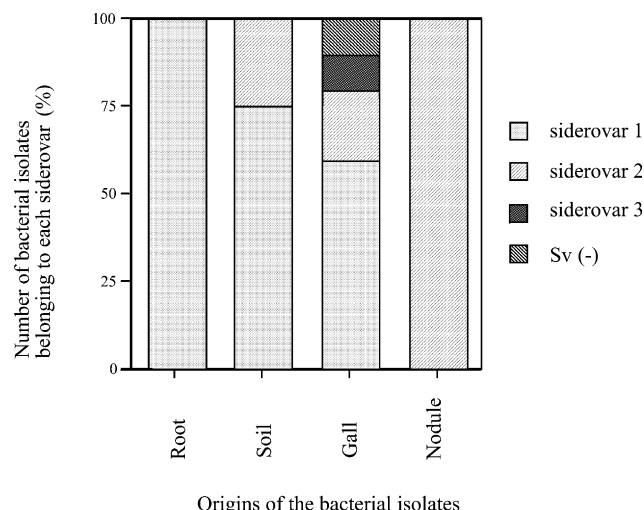


Fig. 2. Total percentage of bacterial isolates of each siderovars from each compartment.

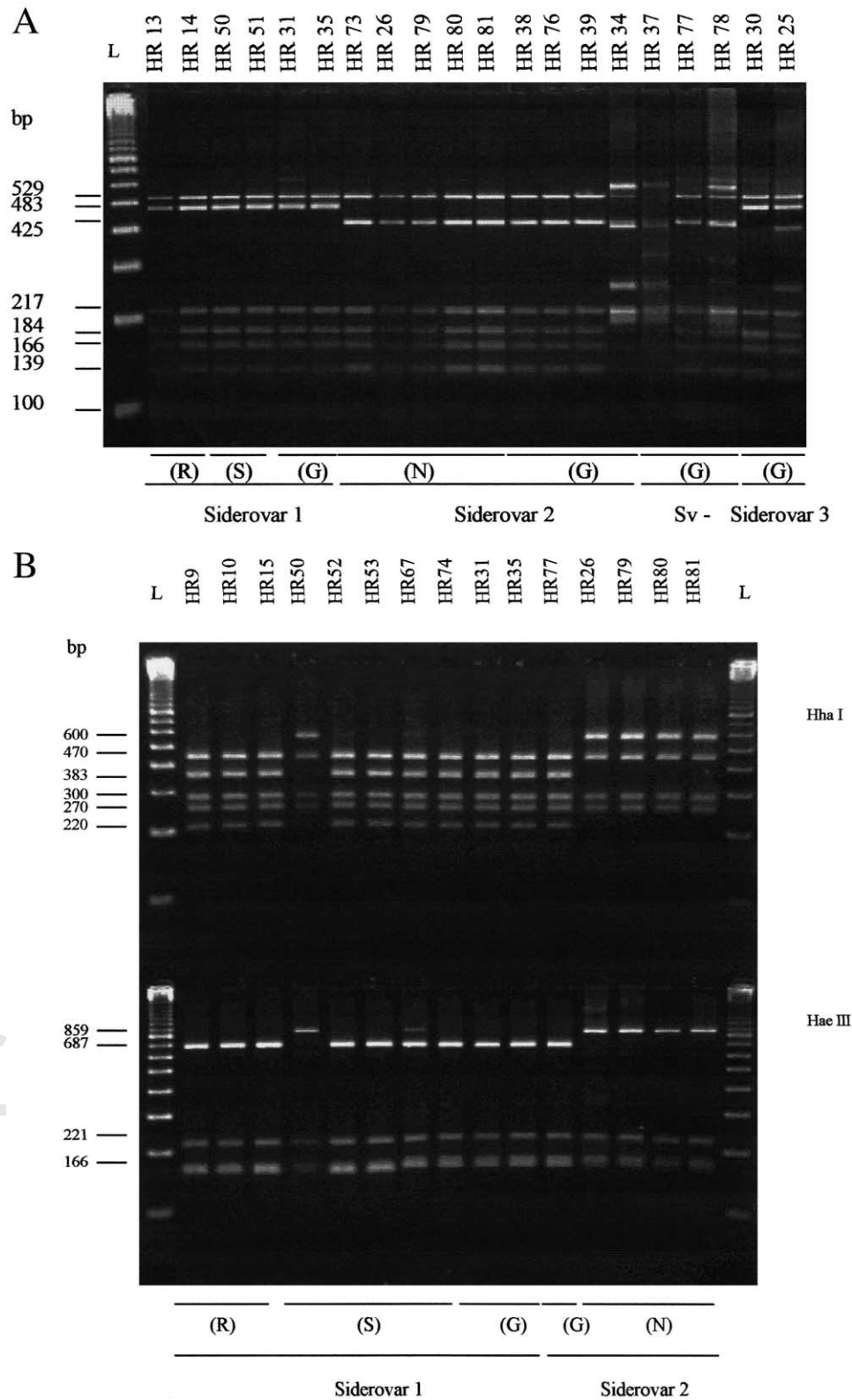


Fig. 3. A: Gel electrophoresis of PCR-amplified 16S rDNA fragments digested with *MspI* restriction enzyme. L: molecular mass markers 100 bp; R: root; S: soil; G: gall; N: nodule. B: Gel electrophoresis of PCR-amplified 16S rDNA fragments digested with *HhaI* and *HaeIII* restriction enzymes.

### 3. Results

#### 3.1. Microbial isolation and characterization of fluorescent pseudomonad strains

The fungal strain IR 100 isolated from the Australian *Acacia* plantation in Cassankil (Senegal) was identified as

*Pisolithus alba* according to its ITS sequence (F. Martin, personal communication).

After 6 months of *A. holosericea* culture in the test soil, the plants were highly infected by the root-knot nematode *Meloidogyne incognita*, identified according to the method of Dalmasso and Bergé [17]. The populations of fluorescent pseudomonads were significantly larger in the soil

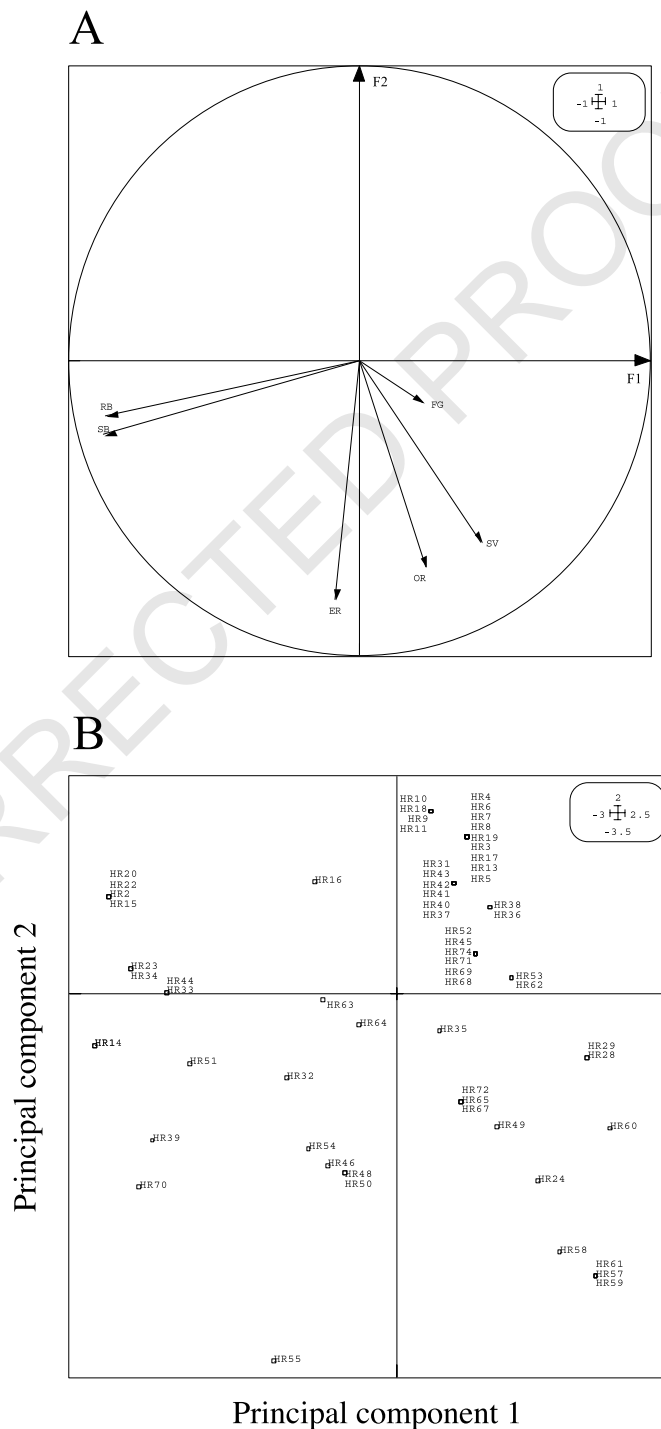


Fig. 4. Principal component analysis. A: Correlation circle of the first two principal components. The six variables are: SB: shoot biomass; RB: root biomass; OR: origin; SV: siderovar; ER: ectomycorrhizal rate; FG: fungal growth. B: Scores of bacterial isolates on the first two principal components. Many isolates have the same profile and are superimposed; their labels have been arranged so that they are readable (for example, isolates HR2, HR15, HR20 and HR22 in the top left part of the graph).

( $11.7 \times 10^3$  CFU  $g^{-1}$  fw) and in the galls ( $11.0 \times 10^3$  CFU  $g^{-1}$  fw) than in the roots (non-ectomycorrhizal or ectomycorrhizal roots) ( $3.0 \times 10^3$  CFU  $g^{-1}$  fw) and in the nodules ( $3.2 \times 10^3$  CFU  $g^{-1}$  fw) according to the one way analysis of variance ( $P < 0.05$ ) calculated on log-transformed data.

The studies on bacterial populations were performed on 27 pseudomonad strains isolated from the soil, 20 from the roots, 24 from the galls, and five from the nodules. According to the PVD they produced, the bacterial isolates were classified into three siderovars. The 20 pseudomonad strains isolated from the root presented an identical PVD-IEF pattern characterized by three bands at pHi 4.9, 4.4 and 4.0, respectively (Fig. 1). Thus, the 20 strains were grouped into the same siderovar (sv. 1) (Fig. 2). Strains isolated from the nodules showed an original PVD-IEF pattern with two bands at pHi 9.1 and 7.7 (Fig. 1). Therefore, these strains were grouped into sv. 2 (Fig. 2). According to the PVD-IEF patterns developed by the 27 strains isolated from soil, 74% of the fluorescent pseudomonad population could be classified into sv. 1 and 26% into sv. 2 (Fig. 2). The diversity for strains isolated from the galls was higher. Fifteen strains (63%) developed the PVD-IEF pattern typical of sv. 1 strains, four strains (17%) the PVD-IEF pattern of sv. 2, while a third pattern (sv. 3) was identified with a major band at pHi 3.9 and two minor bands at pHi 4.9 and 4.4 (Fig. 1). Three strains belonging to the gall isolates group, did not produce PVD and, therefore, could not be classified (sv (-) group) (Fig. 2).

This diversity among the bacterial strains tested was also observed with the genotypic fingerprinting of fluorescent pseudomonads. The isolates were divided into four different 16S rDNA groups with *MspI*, two with *HhaI* and two with *HaeIII*. Amplification of the 16S rDNA for each isolate yielded a 1600-bp PCR product. All the amplified 16S rDNAs were tested with each of the three restriction endonucleases (*HhaI*, *HaeIII* and *MspI*). Depending on the bacterial strain and the restriction enzyme, four to 11 restriction fragments were recorded (Fig. 3A,B). For example, the restriction pattern of 'sv. 1' bacteria was defined with six fragments (139, 166, 184, 217, 483 and 529 bp) when the strain was digested with *MspI* enzyme (Fig. 3A).

### 3.2. Effect of bacteria on fungal growth, mycorrhizal establishment and plant growth

The correlation circle (Fig. 4A) of the first two principal components of the analysis performed on the origin and siderovar of bacterial isolates, shoot and root biomass, ectomycorrhizal rate and fungal growth, showed that shoot and root biomass are highly correlated (grouped in the left part of the graph), and opposed to the siderovar, which is on the right. Fig. 4B shows the scores of bacterial isolates on the first two principal components. This figure is easier to interpret if the bacterial isolates

are labelled with their siderovar (sv. 1 or sv. 2, Fig. 5A). Bacterial isolates belonging to sv. 2 are all grouped in the right part of the graph (with only one exception), which means that they correspond to plants with small shoot and root biomass. Conversely, bacterial isolates belonging to sv. 1 are mostly in the left part of the graph, hence corresponding to high shoot and root biomass. This means that bacteria belonging to sv. 1 have a positive influence on plant growth, while those belonging to sv. 2 have a negative influence.

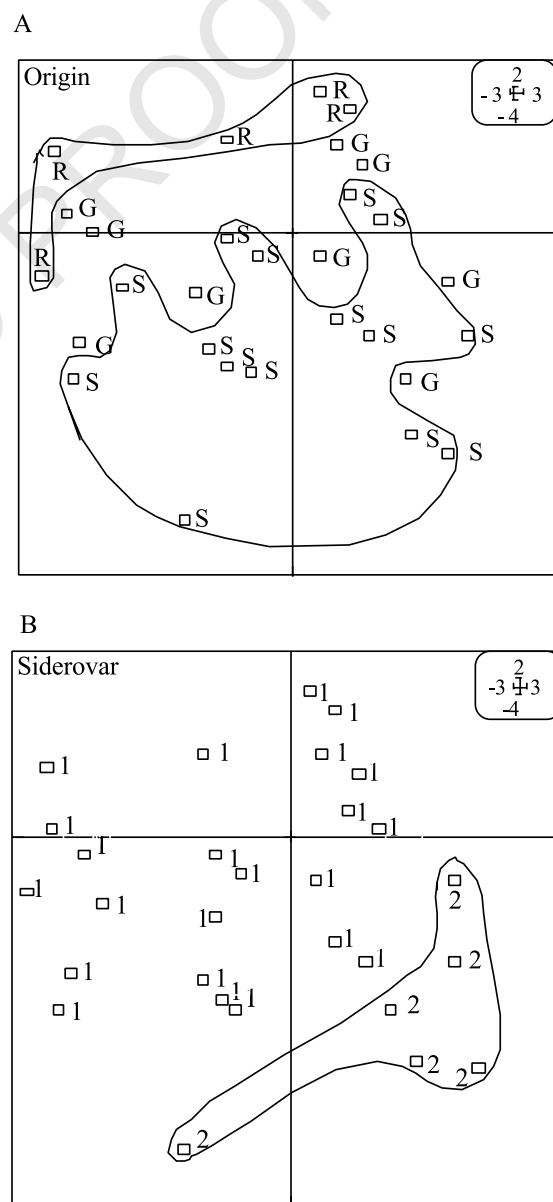


Fig. 5. Scores of bacterial isolates on the first two principal components (same as Fig. 4B). The bacterial isolates are labeled according to their siderovars (B; 1: sv. 1; 2: sv. 2) and their origins (A; R: root; S: soil; G: gall) instead of their experiment code. On both graphs, isolates having the same profiles are superimposed and are represented by only one point (Fig. 4B gives the experiment codes of superimposed isolates at each point).

On the second principal component (vertical axis), the ectomycorrhizal rate, origin and siderovar of bacterial isolates are well grouped in the lower part of the graph, while fungal growth is near the center (Fig. 4A). Fig. 5B shows the scores of bacterial isolates (same as Fig. 4B and 5A), labelled with the origin of the isolates: root (R), soil (S) or gall (G). Bacterial isolates from the soil are grouped in the lower part of the graph, corresponding to samples with high ectomycorrhizal rate. Bacterial isolates from the roots are in the upper part of the graph, corresponding to samples with low ectomycorrhizal rate. This means that bacteria isolated from the soil have a positive influence on ectomycorrhizal rate, as opposed to bacteria isolated from the roots.

After 3 months culture, 14 bacterial strains isolated from the soil (HR46, HR48, HR49, HR50, HR54, HR55, HR57, HR58, HR59, HR61, HR65, HR67, HR70 and HR72) stimulated mycorrhiza formation (Table 1). A few bacterial strains isolated from the other compartments (roots and galls) had also enhanced the establishment of the ectomycorrhizal symbiosis. Compared to the control (fungus alone), the shoot biomass of the *Acacia*

seedlings was significantly stimulated by seven bacterial strains isolated from the roots (HR 1, HR 2, HR 14, HR 15, HR 16, HR 20 and HR 22), eight from the soil (HR 48, HR 50, HR 51, HR 54, HR 55, HR 63, HR 64 and HR 70) and six from the galls (HR 23, HR 32, HR 33, HR 34, HR 39 and HR 44)(Table 1). Among all the bacteria tested, the root growth was significantly increased by HR 2, HR 14, HR 15, HR 20 and HR 22 (root origin), HR 51, HR 55 and HR 70 (soil origin), HR 23, HR 33, HR 34, HR 39 and HR 44 (gall origin) (Table 1).

#### 4. Discussion

The fluorescent pseudomonad populations were about four times greater in the soil and in the galls for *Acacia* plants than in the roots and in the *Rhizobium* nodules. These results reveal discrepancy with other results obtained with several mycorrhizal plant species such as Douglas fir [24], hybrid larch, Sitka spruce [25] and hazel trees mycorrhized with truffles [26]. However, in the present study, the soil was sampled just under some *Piso-*

Table 1

Effect of some bacterial strains on the mycorrhizal establishment (expressed by the percentage of ectomycorrhizal formation) and the plant growth after 3 months culture.

Origin of the bacterial isolates	Bacterial treatments	Mycorrhizal establishment (%)	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)
Root	Control	14.2	127.0	53.4
	HR1	32.0*	315.4*	107.8
	HR2	13.0	336.1*	155.2*
	HR14	25.4*	294.2*	169.4*
	HR15	14.4	238.4*	115.6*
	HR16	9.6	272.8*	102.2
	HR20	20.8	259.6*	123.1*
Soil	HR22	27.6*	312.2*	136.1*
	HR46	32.6*	213.4	132.8*
	HR48	33.2*	263.8*	105.2
	HR49	24.6*	98.2	71.1
	HR50	28.0*	330.7*	103.8
	HR51	21.6	332.1*	126.2*
	HR54	27.6*	237.8*	109.4
	HR55	26.5*	319.1*	126.5*
	HR57	27.0*	76.7	37.7
	HR58	24.4*	149.8	52.4
	HR59	25.2*	193.8	62.8
	HR61	28.8*	145.2	48.2
	HR63	23.0	256.3*	42.3
	HR64	6.6	238.6*	68.0
HR65	25.0*	135.7	67.3	
HR67	31.8*	159.8	70.8	
HR70	25.4*	262.1*	129.2*	
HR72	25.2*	141.2	49.8	
Gall	HR23	17.4	297.4*	123.1*
	HR24	26.8*	232.8	55.6
	HR32	29.2*	334.0*	109.8
	HR33	18.0	331.4*	155.2*
	HR34	11.4	284.8*	153.6*
	HR35	32.6*	154.4	101.6
	HR39	25.8*	306.2*	136.4*
	HR44	20.8	289.8*	114.4*

Values followed by an asterisk are significantly different from the control according to the one-way analysis of variance ( $P < 0.05$ ).



*lithus* fructifications where the yellow mycelial stands of *Pisolithus* sp. were visible in the soil. It suggests that this soil has been highly colonized by the fungal hyphae and, furthermore, could be considered as a soil compartment such as the mycorrhizosphere and mycosphere soil. These results are in accordance with the mycorrhizosphere activity which exerts a significant stimulating effect on the populations of fluorescent pseudomonads in the soil. The high level of fluorescent pseudomonad populations isolated from the galls has been already recorded in an experiment performed with tomato (a plant susceptible to *Meloidogyne* spp.) in a soil highly colonized with fluorescent pseudomonads (R. Duponnois, unpublished data). The low numbers of fluorescent pseudomonads recorded from the roots could be attributed to the sandy soil used in this study. In this type of soil, the amount of adhering soil is usually low [27]. The bacterial strains isolated from the roots were probably localized inside the roots (endophytic isolates) or at the root vicinity (rhizoplane isolates). In these compartments, the numbers of bacteria are usually lower than in the rhizosphere [27].

Besides the differences on the size of the fluorescent pseudomonad population, the phenotypic and genotypic analyses show a different distribution of the bacterial isolates among the compartments. A higher diversity was recorded in the mycorrhizosphere soil and in the galls. The siderotyping of the fluorescent pseudomonads populations gave three main different PVD-related siderotypes. This diversity is generally higher [28] under natural conditions. With the method used to isolate the bacterial strains (6 month culture in 1-l pots), the substrate was highly colonized by the fungal hyphae and the roots which could have exerted a strong selection for the bacterial populations. In contrast and according the method used to analyze the genotypic diversity of fluorescent pseudomonad population, the structure of bacterial populations from the roots and the nodules is less diverse but closely different (sv. 1 in the root and sv. 2 in the nodule). These results suggest that the metabolic activities (secretion, excretion of different organic substrates, etc.) of compartments are different tending selection of the bacterial strains.

Multivariate analyses of the bacterial effects on the fungal growth, plant growth and ectomycorrhizal symbiosis showed two main results: (i) plant growth stimulation was linked with a specific PVD-IEF pattern and (ii) bacteria isolated from the mycorrhizosphere soil significantly increased the ectomycorrhizal establishment.

Among fluorescent pseudomonad isolates collected from the different compartments, some of them significantly enhanced the establishment of the ectomycorrhizal symbiosis between *P. alba* and *Acacia holosericea*. The presence of MHB associated with an Australian *Acacia* and the ectomycorrhizal fungus *Pisolithus* was demonstrated. Moreover, this stimulatory effect is significantly linked with the origin of the bacterial isolates. Bacteria from the my-

corrhizosphere compartment act positively on the mycorrhizal symbiosis. This result suggests that the MHB are not strictly rhizobacteria. This hypothesis is in accordance with that of Frey-Klett et al. [24] who demonstrated that helper bacteria (BBc6) of the mycorrhizal symbiosis between *Laccaria bicolor* and Douglas fir is associated with the mycelium of the fungus in the soil. Moreover, Sen et al. [29] have demonstrated that BBc6 was attached to the hyphal wall of *L. bicolor* in in vitro experiments.

As ectomycorrhizal rate and plant growth was significantly correlated, it suggests that a metabolic interaction between mycorrhizae and bacteria may occur and that a consequence of this interaction could stimulate plant growth. Frey-Klett et al. [24,30] suggest different mechanisms which could explain the beneficial effect of these MHBs but emphasize a positive bacterial effect on the presymbiotic growth of the fungus. This hypothesis is supported by the results of other studies in which the stimulation of the mycorrhizal infections by MHB is linked with the bacterial effect on the fungal growth in axenic conditions [8,9]. However there is no significant correlation between the bacterial effect on the ectomycorrhizal infection and the effect on the fungal growth in the present study. Although the confrontation between the bacteria and the fungal strain IR 100 has been only tested with an experimental system where the only connection between the bacterial and fungal isolates was atmospheric [31], other mechanisms might be involved, such as the improvement of the root receptivity to the fungus or the bacterial implication in the recognition mechanisms between the two symbionts.

From a practical point of view, this study shows that MHBs could have a great ecological importance in tropical areas through reforestation programs. If we consider the main properties of these MHBs demonstrated in temperate areas (reduction of the mycorrhizal inoculum, specificity to the fungal symbiont) [13], the use of such bacterial inoculum could optimize the effect of the fungal inoculation on the plant growth.

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