Microbial Ecology

Soil Bacterial Diversity Responses to Root Colonization by an Ectomycorrhizal Fungus are not Root-Growth-Dependent

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Abstract

The hypothesis tested in this present study was that the ectomycorrhizosphere effect on the bacterial community was not root-growth-dependent. The impacts of ectomycorrhizal infection (*Pisolithus albus* COI007) and a chemical fertilization to reproduce the fungal effect on root growth were examined on (1) the structure of bacterial community and (2) fluorescent pseudomonad and actinomycete populations in the mycorrhizosphere of *Acacia auriculiformis* using both culture-independent and culture-dependent methods. *A. auriculiformis* plants were grown in disinfested soil in pots with or without addition of the ectomycorrhizal fungus or N/P/K fertilization (to reproduce the fungal effect on root growth) for 4 months and then transferred to 20-L pots filled with nondisinfested sandy soil. The fungal and fertilizer applications significantly improved the plant growth after 4-month culture in the disinfested soil. In the nondisinfested cultural substrate, these positive effects on plant growth were maintained. The total soil microbiota was significantly different within the treatments as revealed from DNA analysis [denaturing gradient gel electrophoresis (DGGE)]. The structure of fluorescent pseudomonad populations was also affected by fungal and fertilizer applications. In contrast, no qualitative effect was observed for the actinomycete communities within each treatment, but fungal inoculation significantly decreased the number of actinomycetes compared to the fertilizer application treatment. These results show that the mycorrhizosphere effect is not root-growth-dependent but is mainly due to the presence of the ectomycorrhizal fungus and more particularly to the extramatrical mycelium.

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Introduction

Soil microorganisms associated with plant roots have an important influence on plant nutrition, growth promotion, and disease interactions. The structure and function of rhizosphere communities are mainly determined by plant species [26, 34, 37], plant genotype [5], and soil characteristics (pH, aeration, physicochemical characteristics) [7, 23]. It is usually believed that plant species effects come from differences in the composition of root cell components and root exudates [24, 36], plant age, N supply [31], and infection by mycorrhizal fungi. In this last case, the fungal symbiosis modifies root functions, and therefore, microbial communities associated with mycorrhizas differ from those of the nonmycorrhizal plants and of the surrounding soil [20–22]. This microbial compartment is commonly named "mycorrhizosphere" [32]. The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus. It includes the soil surrounding individual fungal hyphae that has been named "hyphosphere" [27].

It is known that ectomycorrhizal symbiosis increases the growth of fast-growing tropical tree species such as *Eucalyptus* [8], Australian *Acacia* [9], or *Allocasuarina* spp. [9]. In addition, it has been shown that ectomycorrhizal mycelium influences the surrounding bacterial community (such as fluorescent pseudomonads [14] or rhizobia [9]), and, in return, some bacteria can enhance the development of ectomycorrhizal infection associated with these tropical tree species (mycorrhiza helper bacteria, MHB) [9, 12, 15].

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Interactions within the microbial community are of special interest because some microorganisms associated with the mycorrhiza may complement mycorrhizal activities (i.e., N_2-fixing bacteria, phosphate-solubilizing bacteria) [47, 50], especially in tropical degraded soils that are the result of a deterioration of physicochemical and biological soil properties (soil structure, plant nutrient availability, organic matter content). In tropical areas, the response of fluorescent pseudomonad populations to the ectomycorrhizal symbiosis has mainly been assessed [14], but other bacterial groups are also of great importance in soil ecology. For instance, actinomycetes significantly contribute to the turnover of complex biopolymers such as lignocellulose, hemicellulose, pectin, keratin, and chitin [52]. However, most of the studies on the influence of ectomycorrhizal symbiosis on soil microorganisms have relied on culture-dependent techniques (i.e., fluorescent pseudomonad populations) [14]. Such methods are very selective as only a small proportion of soil microorganisms can be cultured [3]. Therefore, microbial community analysis should be assessed through culture-independent methods such as fatty acid extraction [6, 26] and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) [13, 25, 39, 40, 43]. Besides, the mycorrhizal symbiosis generally stimulates root growth of the host plant, which induces higher root soil colonization. This positive fungal effect has rarely been assessed in most of the studies focused on the mycorrhizosphere effect.

Using both culture-independent and culture-dependent methods, the aims of the present study were to examine the effects of ectomycorrhizal infection compared to chemical fertilization to reproduce the fungal effect on the root growth on (1) the total structure of the bacterial community and (2) fluorescent pseudomonad and actinomycete populations in the mycorrhizosphere of *Acacia auriculiformis* (Australian acacia introduced and widely planted in tropical Africa).

Materials and Methods

Plant and Fungal Inoculum and Soil Preparation

Plant. Seeds of *A. auriculiformis* Cunn ex. Benth (Provenance Bel Air, Dakar, Senegal) were surface-sterilized with 95% concentrated sulfuric acid for 60 min. Seeds were then transferred aseptically to petri dishes filled with 1% (w/v) agar/water medium. After 8 days of incubation at 25°C in the dark, the germinating seeds were used when rootlets were 1–2 cm long.

Fungal Inoculum. The ectomycorrhizal fungus *Pisolithus albus* COI007 was routinely maintained on modified Melin–Norkrans (MMN) agar medium at 25°C [35]. The ectomycorrhizal fungal inoculum was prepared according to Duponnois and Garbaye [10]. Glass jars (1 L) were filled with 600 mL of a mixture of vermiculite and peat moss (4/1, v/v) and autoclaved (120°C, 20 min). The substrate was then moistened to field capacity with 300 mL of liquid MMN medium. The jars were sealed with cotton floats and autoclaved at 120°C for 20 min. After cooling, the substrate was inoculated with fungal plugs taken from the margin of the fungal colonies and incubated for 6 weeks at 28°C in the dark.

Soil Preparation. *Acacia* seedlings were grown in 1-L pots filled with an autoclaved sandy soil (140°C, 40 min) collected in a stand of *Acacia holosericea* located east of Dakar (Senegal). After sampling, the soil was crushed, passed through a 2-mm sieve, and autoclaved for 40 min at 140°C to eliminate the native microbiota. After autoclaving, its physicochemical 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; total nitrogen (%) 0.02; Olsen phosphorus 4.8 mg kg^{-1}; total phosphorus 39 mg kg^{-1}.

Experimental Design. Three treatments were carried out: control, preplanting fertilizer application (FA), and ectomycorrhizal inoculation (EC). In ectomycorrhizal inoculation (EC), the soil was mixed with fungal inoculum (10/1, v/v). Treatments without fungus (control and preplanting fertilizer application) received an autoclaved mixture of moistened vermiculite/peat moss (MMN liquid medium) at the same rate. Preplanting fertilizer application (FA) was applied by adding 10 Osmocote™ granulates into each pot (N/P/K, 11/8/17). This fertilization rate has been calculated according to the results of previous experiments (data not published). All of the planted pots were kept in a greenhouse (daylight approximately 12 h, average daily temperature 25°C) and were watered regularly with tapwater without fertilizer. They were arranged in a randomized complete block design with 14 replicates per treatment.

After 4-month culturing, seven plants were randomly chosen from each treatment; they were uprooted and their root systems gently washed. The oven dry weight (1 week at 65°C) of the shoot was measured. Some nodules were observed along the root systems of the *Acacia* seedlings despite disinfestation of the soil and the seed surface. For each treatment, root nodules were counted, and their dry weight (1 week at 65°C) was determined. Then the root systems were cut into 1-cm root pieces and mixed. The percentage of ectomycorrhizal short roots (number of ectomycorrhizal short roots/total number of short roots) was determined on a random sample of at least 100 short roots per treatment under a stereomicroscope (magnification ×40). The dry

weight (1 week at 65°C) of roots was then measured for each treatment.

Remaining plants were transferred into 20-L pots filled with the same sandy soil but not autoclaved. The experimental design was the same as before. *Acacia* seedlings were grown under nursery conditions at ambient temperature, from 25 to 35°C, with watering every 2 days. After 8-month culturing, the height of the plants was measured. Plants were uprooted and their root systems were gently washed. In the EC treatment, the soil was highly colonized by the yellow fungal hyphae. The soil of each pot was carefully mixed, and 100-g subsamples were taken. Half of them were kept at –70°C for the assessment of the bacterial 16S ribosomal DNA (rDNA) community composition, whereas the others were placed at 4°C for other analysis. Twenty grams of moist soil was collected from each pot to determine the microbial biomass using the fumigation–extraction method [1]. The NH_4^+ and NO_3^- contents were determined according to a method described by Bremner [4].

Soil subsamples from each pot (2 g fresh weight) were suspended in 10-mL sterile magnesium sulfate solution (0.1 M) and blended in an Ultraturax blender. Then serial dilutions of homogenized suspensions were plated onto three replicate plates of two different culture media to assess the amount of fluorescent pseudomonads and actinomycetes. Fluorescent pseudomonads were isolated on King's B medium [28]. Actinomycetes were isolated on Waksman medium (glucose 10 g L^{-1}, casitone 5 g L^{-1}, sodium chloride 5 g L^{-1}, beef extract 3 g L^{-1}, agar 20 g L^{-1}) [46]. The King's B medium plates were incubated for 48 h at 30°C. Then they were examined under UV light, and fluorescent colonies were counted and randomly selected. The isolates of fluorescent pseudomonads were purified, subcultured on King's B medium, and cryopreserved at –80°C in glycerol 60%/tryptic soy broth (TSB, 3 g l^{-1}) culture (1/1, v/v). Actinomycetes were enumerated after 7 days of incubation at 30°C in the dark.

One gram of fresh root was randomly collected along the root system of each plant to evaluate the intensity of mycorrhizal symbiosis and was kept in ethanol (70%). The percentages of ectomycorrhizal root pieces (ectomycorrhizal rates) were calculated as described above. Then the root systems were oven-dried (1 week, 65°C) and weighed.

Molecular Characterization of Fluorescent Pseudomonad Isolates. Fluorescent pseudomonads were grown overnight on TSB agar plates at 28°C. For each strain, a single colony was picked and resuspended in 50 mL of lysis buffer (0.05 M NaOH, 0.25% SDS), vortexed for 60 s, heated to 95°C for 15 min, and centrifuged at 13,000 rpm for 10 min. The lysate cell suspensions were diluted (1/10, v/v) with sterile distilled water. The primers rD1 (5′ AAGCTTAAGGAGGT GATCCAGCC 3′) and fD1 (5′ AGAGTTTGATCCTGG CTCAG 3′) were used to amplify the 16S rDNA gene [17]. Amplification reactions were performed in a GeneAmp PCR System 2400 automatic thermal cycler (PerkinElmer, California, USA) using Pure Taq Ready-To-Go PCR beads (Amersham Biosciences), 1 μM of each primer, and 2 μL of bacterial cell suspension in 25-μL reaction mixtures. The mixture was submitted to 5-min initial denaturation followed by 35 cycles at 94°C for 1 min, 55°C for 45 s, and 72°C for 1.5 min. A final elongation step was performed at 72°C for 5 min. PCR products (7 μL) were digested in total volume of 20 μL at 37°C for 2 h using the endonucleases *Hae*III and *Msp*I (Promega, France) as described by the manufacturer. Restriction fragments were separated by horizontal electrophoresis in a 2.5% (w/v) Metaphor gel (FMC, Rockland, MA, USA). After 2 h of running at 80 V, the gel was stained for 30 min with ethidium bromide (1 mg L^{-1}) and integrated with Image Analysis software BIOCAPT (Vilbert Lourmat, France) under UV light.

PCR-DGGE Analysis of Total Soil Bacterial and Actinomycete Communities

DNA Extraction. Total DNA was extracted from 0.5-g aliquots of soil, collected from three pots randomly chosen in each treatment, by adding 0.2-g glass beads (Sigma, 0.1 mm) and 1-mL lysis buffer (0.25 M NaCl, 0.1 M EDTA; pH 8) and treating the suspension by bead beating (BeadBeater, Biospec Products) two times for 2 min with an intermittent 2-min heat (65°C) treatment. The subsequent steps were carried out as described by Porteous *et al.* [44]. Briefly, crude extract was concentrated with potassium acetate 5 M and PEG 8000 and centrifuged for 15 min at 13,000 × *g*. The pellet was dissolved in cetyltrimethylammonium bromide 2% and then extracted with equal volume of chloroform. DNA was precipitated at –20°C for 15 min with 0.7 vol isopropanol. Pellets were obtained by centrifugation at 4°C for 15 min at 13,000 × *g*. Pellets were then again dissolved and precipitated with ammonium acetate (2.5 M) and ethanol, washed with 70% ethanol, and air-dried. The DNA was dissolved in 100 μL 1 × TE [TE buffer (10 mM Tris/HCl, 1mM Na2EDTA; pH8)].

PCR Amplifications of 16S rDNA. For the specific amplification of 16S rDNA fragments of actinomycetes, a nested PCR protocol was applied using a combination of actinomycete-specific primer F243 [26] with 16S rDNA bacteria target primers 984GC and R1378. The PCR fragment 226–1401 was first amplified with primer pair F243–R1378. The resulting fragment was excised from the agarose gel, extracted, and recovered by spin columns (QIA-quick gel extraction kit; Qiagen, Ger-

Table 1. Response of *A. auriculiformis* seedling growth, ectomycorrhizal colonization, and rhizobial infection to the *P. albus* COI007 inoculation and the preplanting fertilizer application after 4-month culture in a disinfected soil

	Treatments		
	Control	*Preplanting fertilizer application*	*P. albus COI007 inoculation*
Shoot biomass (g dry weight)	1.47 a[a]	2.49 b	2.61 b
Root biomass (g dry weight)	0.52 a	1.83 b	1.85 b
Number of nodules per plant	0.0 a	0.0 a	16.8 b
Total nodule weight per plant (mg dry weight)	0.0 a	0.0 a	42.3 b
Ectomycorrhizal colonization (%)	0.0 a	0.0 a	42.6 b

[a] Data in the same line followed by the same letter are not significantly different according to the one-way analysis of variance ($p < 0.05$).

many). It was used as a template for a second PCR with primer pair F984GC–R1378 [41] to obtain a fragment of 430 bp suitable for DGGE analysis. PCR mixture of 25 μL contained 2.5 μL of 10× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.1 μM of each primer, 20 ng of DNA template, and 1.25 U of *Taq* DNA polymerase (Roche Molecular Biochemicals). Amplification was performed using 35 cycles of 1-min denaturation at 94°C, 1 min at 60°C for primer annealing, and 2 min at 72°C for primer extension, followed by a final step at 72°C for 10 min.

PCR amplification targeting total soil 16S rDNA bacterial community was performed with the eubacterial primer pair 338f–GC [42] and 518r [40]. PCR was performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, USA) using Pure Taq Ready-To-Go PCR beads (Amersham Biosciences), 10 ng of DNA, and 1 μM of each primer in 25-μL PCR mixtures. The following thermocycling regime was used: 5 min at 94°C (denaturation), 1 min at 65°C (annealing), and 1 min at 72°C (elongation) with a 1°C touchdown every second cycle during annealing for 20 cycles, followed by 10 cycles with an annealing temperature of 55°C and a final cycle consisting of 10 min at 72°C. All PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gels and stained for 30 min with ethidium bromide (1 mg L^{-1}).

DGGE analysis. 16S rDNA-DGGE was performed using 6% acrylamide gels (37.5/1 and 60/1 acrylamide/bis-acrylamide for total bacterial and actinomycete amplicons, respectively), containing a denaturant gradient of 30–60% (100% denaturant contained 7 M urea and 40% formamide). The gels, loaded with an amount of 300 ng of amplicons, were electrophoresed in 0.5× Tris–acetate–EDTA buffer at 60°C at constant voltage of 20 V for 10 min and then 150 V for 6 h by using the Dcode system (Bio-Rad Laboratories, Hercules, CA). The gels were stained for 30 min with ethidium bromide (1 mg L^{-1}) and washed for 10 min with MilliQ H_2O prior to UV transillumination. Banding patterns were digitized using a charge-coupled device camera and the Biocapt software program (Vilber Lourmat). 16S rDNA-DGGE patterns were analyzed with the Bio-Profil Biogene program (Vilber Lourmat). Then, DGGE fingerprints were scored by the presence or absence of comigrating bands, independent of intensity. Profile similarity was calculated by determining Dice's coefficient for the total number of bands. Dendrograms were constructed by using the unweighted pair group method with arithmetic averages (UPGMA).

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 arc sin ($\sqrt{x}$) and by $\log(x + 1)$ before statistical analysis. The composition of fluorescent pseudomonad population was compared between each treatment with 2 × 2 contingency tables and chi-square test and with Yates correction for small numbers.

Table 2. Effects of *P. albus* COI007 inoculation and preplanting fertilizer application on *A. auriculiformis* seedling growth, ectomycorrhizal colonization, and rhizobial infection after 8-month culture in a nondisinfected soil

	Treatments		
	Control	*Preplanting fertilizer application*	*P. albus COI007 inoculation*
Height (cm)	32.2 a[a]	59.5 b	58.3 b
Shoot biomass (g dry weight)	2.79 a	22.85 b	24.29 b
Root biomass (g dry weight)	1.35 a	7.41 b	7.06 b
Number of nodules per plant	0.5 a	31.0 b	94.9 c
Total nodule weight per plant (mg dry weight)	2 a	314 b	324 b
Ectomycorrhizal colonization (%)	0 a	0 a	23.4 b

[a] Data in the same line followed by the same letter are not significantly different according to the one-way analysis of variance ($p < 0.05$).

Table 3. Effects of *P. albus* COI007 inoculation and preplanting fertilizer application on soil nitrogen content, microbial biomass, and abundance of actinomycetes and fluorescent pseudomonads after 8-month culture in a nondisinfected soil

	Treatments		
	Control	*Pre-planting fertilizer application*	*P. albus COI007 inoculation*
Microbial biomass (μg C g^{-1} of soil)	23.3 a[a]	39.7 b	22.2 a
NH_4^+ (μg N g^{-1} of soil)	1.3 a	2.1 a	1.4 a
NO_3^- (μg N g^{-1} of soil)	10.9 a	4.4 b	3.5 b
Fluorescent pseudomonads (CFU[b] g^{-1} of soil × 10^3)	487.5 a	458.3 a	416.7 a
Actinomycetes (CFU g^{-1} of soil × 10^3)	112.5 ab	200.0 b	66.7 a

[a]Data in the same line followed by the same letter are not significantly different according to the one-way analysis of variance ($p < 0.05$).
[b]CFU: colony forming unit.

Results

After 4-month culturing, the shoot and the root growth of both mycorrhizal and fertilized *A. auriculiformis* seedlings was significantly higher than in the control treatment (Table 1). Rhizobial nodules were only recorded in the EC treatment, whereas the noninoculated plants remained uninfected by ectomycorrhizal inoculant throughout the experiment and had no nodules (Table 1).

Eight months after the transplantation, the beneficial effects of the EC and FA treatments on plant growth (height, shoot, and root biomasses) maintained (Table 2). The highest number of nodules per plant was recorded in the EC treatment. Nodule formation was also observed on the roots of *A. auriculiformis* seedlings supplied with N/P/K (FA treatment), and their development was significantly higher than in the control (Table 2). No ectomycorrhizas were detected on *A. auriculiformis* root systems in the FA and control treatments (Table 2).

Microbial biomass was significantly higher in the FA treatment soil than in the other treatment soils (Table 3). No significant difference occurred in NH_4^+ soil content between treatments, whereas NO_3^- soil content was significantly higher in the control treatment than in the other ones (Table 3). The number of fluorescent pseudomonads per gram of soil was not significantly different between treatments. On the contrary, the amount of actinomycetes was significantly higher in the FA treatment than in the soil colonized by *P. albus* COI007 isolate (EC treatment) (Table 3).

Eighty-nine isolates of fluorescent pseudomonads, randomly chosen from the treatments, were subjected to PCR/RFLP analysis of their 16S–23S rDNAs. All the amplified DNAs were digested with the endonucleases *Msp*I and *Hae*III. Amplification of the 16S rDNA for each isolate yielded a single band of approximately 1600 bp. Six RFLP fingerprints were observed with *Hae*III (groups I–VI; Fig. 1A), whereas *Msp*I gave the same clustering, but subdivided the second group (groups II and VII; Fig. 1B). The PCR/RFLP pattern of group II was most frequently obtained, and it included most of the isolates (53/89), more than 59% of the analyzed profiles.

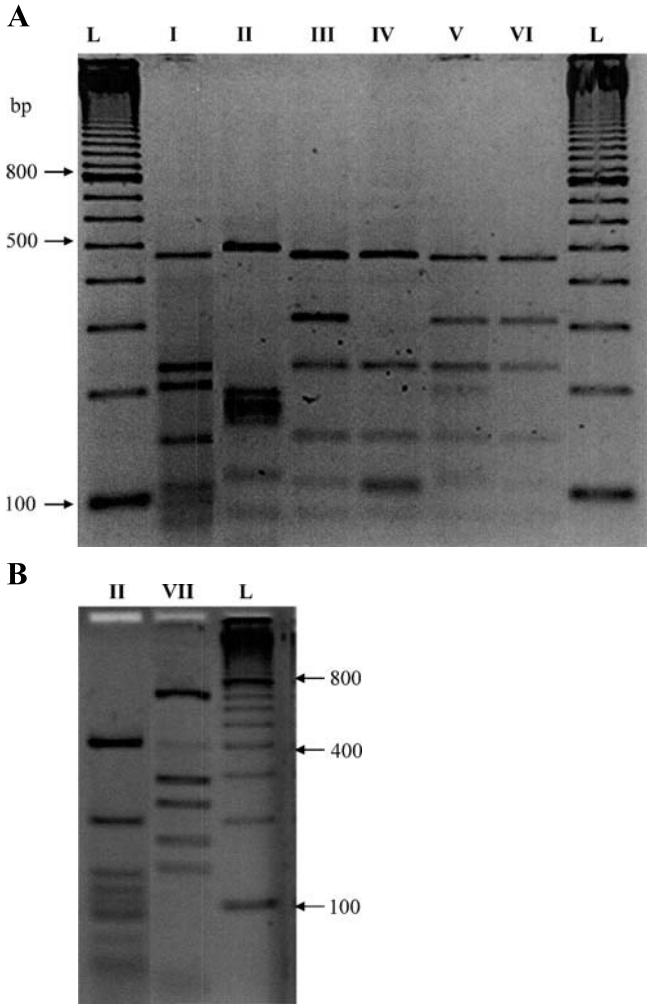

Figure 1. (A) *Hae*III restriction patterns of PCR-amplified 16S rDNA fragments of fluorescent pseudomonad isolates; *lane 1*: PCR/RFLP group I; *lane 2*: PCR/RFLP group II; *lane 3*: PCR/RFLP group III; *lane 4*: PCR/RFLP group IV; *lane 5*: PCR/RFLP group V; *lane 6*: PCR/RFLP group VI. The size marker L is a 100-bp ladder (Amersham Biosciences). (B) *Msp*I restriction patterns of PCR-amplified 16S rDNA fragments of fluorescent pseudomonad isolates belonging to the *Hae*III-digested RFLP group II that gathered most of the pseudomonad isolated. *Msp*I group II profile was found among fluorescent pseudomonad strains sampled from all the treatments. The RFLP group VII was only present in the FA treatment. The size marker L is a 100-bp ladder (Amersham Biosciences).

Table 4. Number of fluorescent pseudomonads per PCR/RFLP groups for each treatment (control, preplanting fertilizer application, and *P. albus* COI007 inoculation)

	Treatments		
PCR-RFLP groups	*Control [36][a]*	*Preplanting fertilizer application [24]*	*P. albus COI007 inoculation [29]*
I	5 (13.8)[b]	1 (4.2)	6 (20.7)
II	27 (75.0)	14 (58.3)	12 (41.3)
III	0 (0)	0 (0)	7 (24.1)
IV	2 (5.6)	0 (0)	4 (13.9)
V	2 (5.6)	0 (0)	0 (0)
VI	0 (0)	2 (8.3)	0 (0)
VII	0 (0)	7 (29.2)	0 (0)

[a] Total number of fluorescent pseudomonad isolates analyzed by PCR/RFLP in each treatment.
[b] Total number and percentage of isolates of each PCR/RFLP group from each treatment.

Four PCR/RFLP groups were found in each treatment: groups I, II, IV, and V in the control treatment, groups I, II, VI, and VII in the FA treatment, and groups I, II, III, and IV in the EC treatment (Table 4). The PCR/RFLP group V has only been recorded in the control treatment, groups VI and VII were only detected in the FA treatment, and group III was only found in the EC treatment (Table 4). The spread of fluorescent pseudomonad groups was significantly different between the control and the FA treatment ($\chi^2 = 18.1$, $p = 0.0028$), control and EC treatment ($\chi^2 = 15.6$, $p = 0.0057$), and FA and EC treatments ($\chi^2 = 23.4$, $p = 0.0003$).

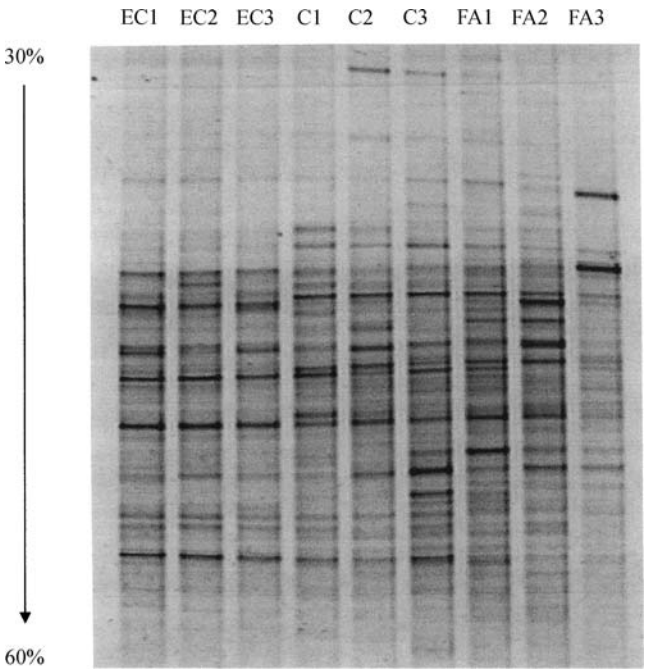

Figure 2. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA of total soil bacterial communities in the control (C), preplanting fertilizer application (FA), and ectomycorrhizal inoculation (EC) treatments. Migration was performed with a 6% acrylamide gel. Percent values indicate the percentage of denaturants at each position.

The 16S rDNA-DGGE patterns of the bacterial communities from the control, FA, and EC treatments are presented in Fig. 2.

Numerous DGGE bands of various intensities that resulted from differences between the 16S rDNA gene sequences of different bacterial species were detected. They ranged in mobility approximately from 30 to 60% with different DGGE patterns between each treatment (Fig. 2). Nevertheless, the banding patterns from different soil treatments shared some of the intensely stained DGGE bands, indicating that a stable bacterial population colonized the soil regardless of the applied treatment. The soils collected from the FA and control treatments displayed more complex banding patterns in comparison to the soil from the EC treatment (Fig. 2).

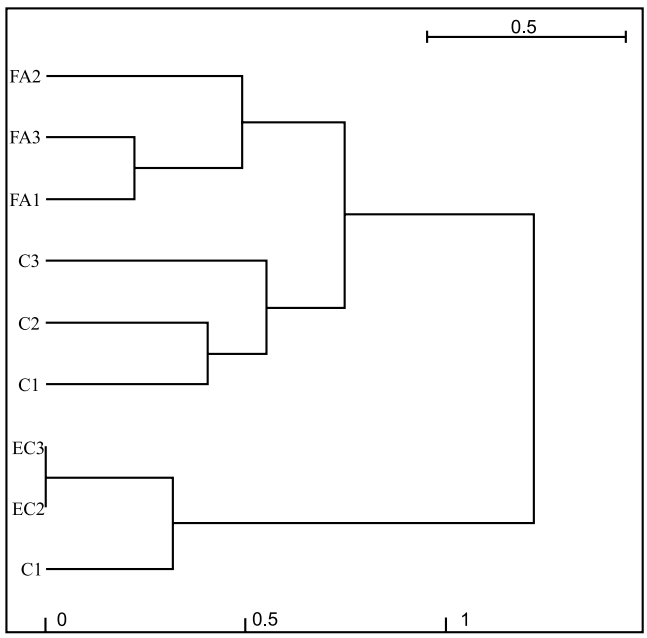

Figure 3. Similarities between PCR-DGGE profiles obtained from bacterial communities in each soil treatments: control (C), preplanting fertilizer application (FA), and *P. albus* COI007 inoculation (EC).

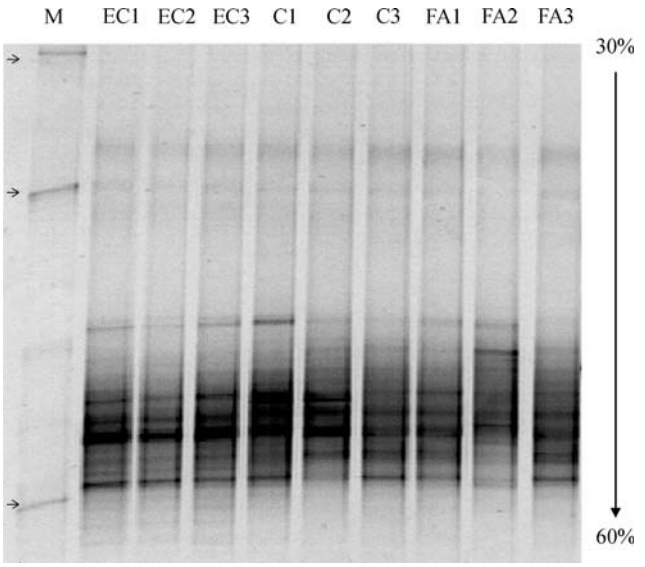

Figure 4. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA of total actinomycete communities in the control (C), the preplanting fertilizer application (FA), and the ectomycorrhizal inoculation (EC) treatments. Migration was performed with a 6% acrylamide gel. *M*, marker, composed of PCR products generated from the following bacteria strains (from *top* to *bottom*): *Enterococcus cecorum*, *Bacteroidaceae* sp., and *Pseudomonocardia zijingensis*. Percent values indicate the percentage of denaturants at each position.

Their DGGE patterns revealed a high number of intense and discrete bands, whereas a lower number of bands were detected in the soil of the EC treatment. The ectomycorrhizal formation influenced significantly the structure of the soil bacterial community by reducing its diversity. Hierarchical cluster analysis (Ward method) of the DGGE results for the three repetitions (1, 2, 3) and the three treatments (C, EC, FA) is presented in Fig. 3. The scale gives the distances, computed by the Ward method, between repetitions and tree nodes. The three treatments appear clearly different, but the three repetitions of the EC treatment are markedly set apart (Fig. 3).

The DGGE of 16S rDNA genes amplified by PCR with the actinomycete-specific primers displayed similar banding patterns (Fig. 4). The PCR-DGGE bands comigrated at the part of the gel (50–60%). No significant differences were revealed between the actinomycete communities from the soils of ECI, FA, and control treatments.

Discussion

The ectomycorrhizal fungus, *P. albus* strain COI007, dramatically stimulated plant growth in the disinfested soil compared to the nonfertilized control but not to FA treatment. Shoot and root biomasses of *A. auriculiformis* seedlings were increased nearly 1.7- and 3.5-fold, respectively, on a sandy soil commonly found in West Africa. This positive effect of ectomycorrhizal symbiosis on *A. auriculiformis* has already been observed with other ectomycorrhizal symbionts [9, 11]. In addition, ectomycorrhizal infection was linked with the presence of rhizobial nodules. The most plausible explanation of this bacterial contamination was that the irrigation tap water contained indigenous rhizobia, but air contamination was of course also possible. This observation has already happened in the same experimental conditions with other Australian acacias inoculated with different ectomycorrhizal fungi [9]. It is well known that mycorrhizal infection helps nodule formation and functioning under stress conditions (drought, salinity, low-nutrient soil, etc.) [2]. Mycorrhizas, through their positive effect on the root growth, favor rhizobia colonization and infection. However, other mechanisms could be considered, such as the fungal effect on the quality and the quantity of root exudates and other relationships between symbionts at physiological and molecular levels [29, 51]. Although the preplanting fertilizer application increased root growth of *A. auriculiformis* seedlings, no nodules were detected.

After 8-month culturing in a nondisinfested soil, the ectomycorrhizal effect on the plant growth previously measured after 4 months of cultivation was significantly magnified, as shoot and root biomasses of *A. auriculiformis* seedlings increased nearly 8.7- and 5.3-fold, respectively. In contrast, in the disinfested soil, ectomycorrhizas were detected at a lower rate (23.4% versus 42.6%). These positive effects of the fungal isolate were similar to those recorded in the FA treatment, except for the nodule formation. These results clearly show that the persistence and the beneficial influence of the ectomycorrhizal fungus on plant growth are sustainable in these environmental conditions. The persistence of the introduced fungal strain generally depends on abiotic factors such as soil pH, soil fertility, moisture, and temperature [49]. It has been established that apart from host specificity, the environmental conditions play an important role in the occurrence of *Pisolithus* [45, 48]. Biotic factors, such as soil microbial communities, can also influence the ectomycorrhizal development. For instance, Garbaye and Bowen [21] showed that mycorrhizal infection of *Pinus* seedlings by three ectomycorrhizal fungi could be stimulated or inhibited by soil microbiota.

In the present study, fertilizer amendment and ectomycorrhizal inoculation affected the composition of the rhizosphere bacterial community, as revealed from DNA analysis (DGGE). As the preplanting fertilizer application significantly increased the root biomass, it is plausible to think that root exudation was more important in these treatments than in the control, and consequently, seedlings provided soil microbes with extra input of substrate that stimulated their growth. This was

also shown by the significantly higher microbial biomass in the fertilized soil than in the nonamended soil (control). Compared to the other treatments, ectomycorrhizal formation decreased the number of bands on the DGGE profiles. This result shows that ectomycorrhiza inhibits some specific bacteria in the rhizosphere. It has been previously demonstrated that ectomycorrhizal mycelia reduced bacterial activity in a sandy soil [42]. The cause of this decrease remains unknown, but it has been demonstrated that ectomycorrhizal fungi may produce antibacterial substances. This property has been recorded with other ectomycorrhizal fungi such as *Paxillus involutus* and *Hebeloma crustuliniforme in vitro* conditions [35] or *Cenococcum graniforme* in mycorrhizal symbiosis [30].

No significant influences of ectomycorrhizal inoculation and fertilizer amendments occurred on the amount of fluorescent pseudomonads. This result is contrary to other studies in which the mycorrhizosphere exerts a significant stimulating effect on the populations of fluorescent pseudomonads [14, 16]. However, the genotypic analysis shows a different distribution of the bacterial isolates between treatments. This result is in accordance with other studies obtained with the *A. holosericea–P. albus* symbiosis [14]. It suggests that the metabolic activities of the mycorrhizosphere (secretion, excretion of different organic substrates) are different from the activities in other treatments (control and FA), tending toward selection of bacterial strains. Moreover, Frey-Klett *et al.* [18] demonstrated that the ectomycorrhizosphere significantly structured the *Pseudomonas fluorescens* populations and selected strains potentially beneficial to the symbiosis and to the plant. The fertilization also induced differences in the structure of the fluorescent pseudomonad population. As for the total bacterial microbiota, better growth of the root system probably excreted a higher amount of root exudates that selectively favored some groups of the fluorescent pseudomonad populations.

Culturable actinomycetes isolated from the soil were estimated to be 0.7×10^5 cfu g^{-1} dry weight (EC treatment) and 2×10^5 cfu g^{-1} dry weight (FA treatment). Similar results were obtained from previous isolation experiments involving *Tuber melanosporum* mycorrhizal roots [33]. The fertilizer amendment significantly increased the number of actinomycetes in the soil. The actinomycetes (order Actinomycetales) are primarily saprophytic and are best known to contribute to the turnover of complex biopolymers such as lignocellulose, hemicellulose, pectin, keratin, and chitin [38]. As the *A. auriculiformis* seedlings supplied with fertilizer had larger root systems, the amount of viable and dead root materials is more important in the soil. That could explain this better actinomycete development. In the same way, as the abundance of actinomycetes associated with the ectomycorrhizal seedlings is lower than that with the fertilized seedlings, it can be concluded that ectomycorrhizas exert a negative effect on the development of this microbial group. The actinomycete group is mainly implicated in the biological processes involved in the litter degradation. Negative effect of ectomycorrhiza on this bacterial group could explain the fact that the decomposition rate of litter increased when ectomycorrhizal tree roots were excluded from a forest soil [19].

In conclusion, it appears that ectomycorrhizas have a strong effect on the structure of the total soil bacterial community, actinomycete community, and, as it has been already described, on fluorescent pseudomonad population. This effect is not root-growth-dependent but is mainly due to the presence and to soil colonization of the ectomycorrhizal inoculant. As the soil samples collected from the ECI treatment were highly colonized by the fungal hyphae, they could be considered as representative of a soil compartment such as the mycosphere soil that is a part of the overall mycorrhizosphere. Consequently, these results underline the importance of the extramatrical mycelium in such microbial interactions. Other studies have to be undertaken to describe the implications of these changes on the bacterial activities and on the soil biofunctioning.

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