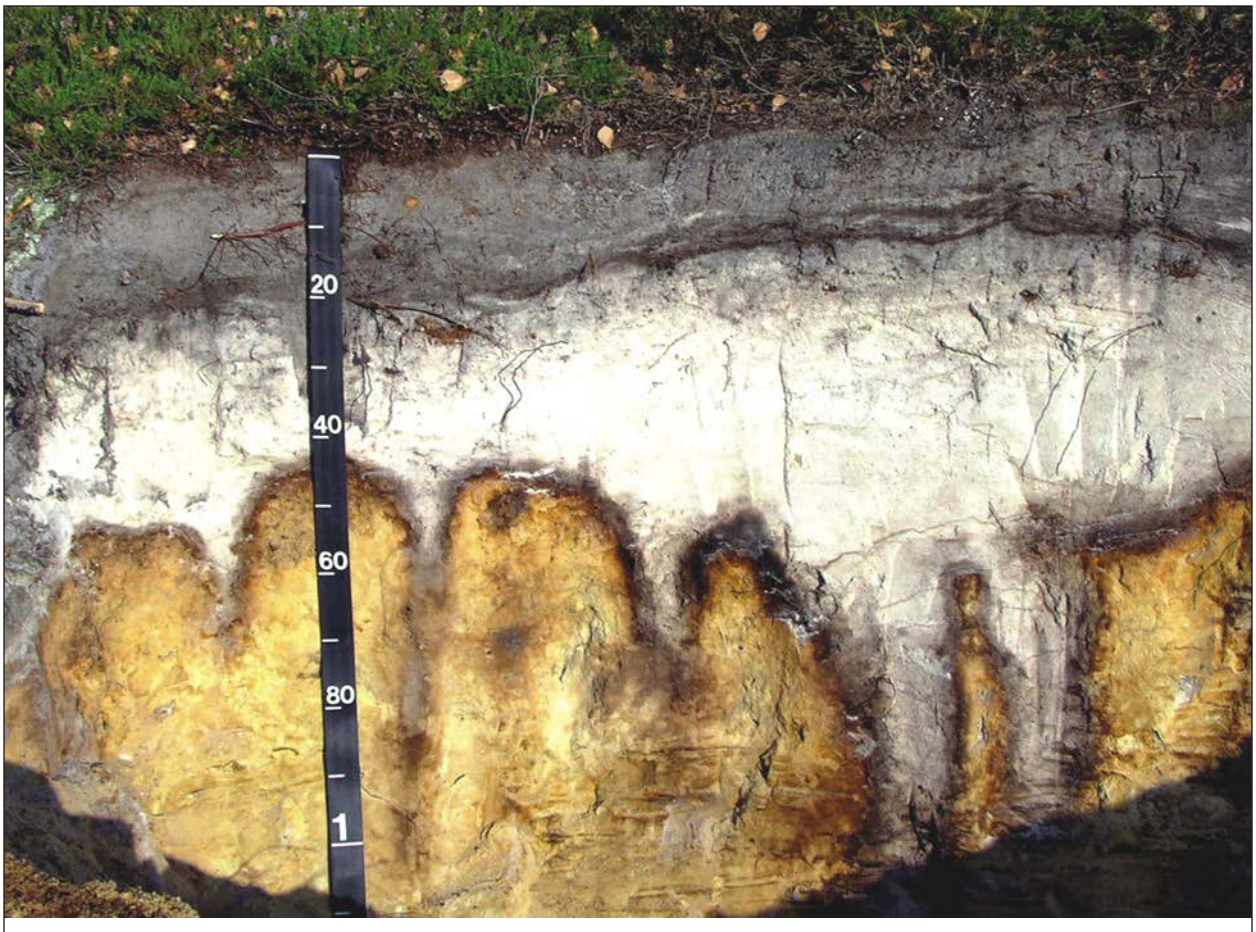

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The Ectomycorrhizosphere Effect Influences Functional Diversity of Soil Microflora

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Abstract: The aims of this study were to determine whether the microbial activities in soil compartments are influenced by ectomycorrhizal symbiosis and to determine the functional diversity of fluorescent pseudomonads associated with the symbiosis of *Uapaca* sp. and ectomycorrhizal fungi. *Uapaca* sp. seedlings were cultured in soils collected under ectomycorrhizal tree species. After 8 months culturing, the soil highly colonized by hyphal stands (hyphosphere soil, HS), as well as the non colonized soil (bulk soil, BS) was sampled from each pot. The non-mycorrhizal and mycorrhizal short roots with their adherent soil were collected and vigorously shacked, to recover the Rhizosphere Soil (RS) and the Mycorrhizosphere Soil (MS). The patterns of *in situ* catabolic potential (ISCP) of microbial communities have been measured and the results showed that functional activities of soil microbial communities are mainly dependent on fungal activities. In addition, this effect is different between the hyphosphere and mycorrhizosphere zones. The number of fluorescent pseudomonads was significantly more numerous in the HS, MS and RS compartments than in the bulk soil. The highest size of fluorescent pseudomonad population was in the Hyphosphere Soil (HS) compartment. The ectomycorrhizal symbiosis has also modified the functional activities of fluorescent pseudomonads. This fungal qualitative effect was mainly detected in the hyphosphere soil compartment on the ability of fluorescent pseudomonads to solubilize tricalcium orthophosphate and to produce lipases. Close interactions occur between the ectomycorrhizal symbiosis and the soil bacterial communities that could increase the efficiency of the fungal symbiosis for the host plant development.

Key words: Ectomycorrhizal symbiosis, fluorescent pseudomonads, microbial catabolic diversity

Introduction

Mycorrhizal fungi, as ubiquitous components of most terrestrial ecosystems, play an important role in soil processes (Smith and Read, 1997). One recognised activity of mycorrhizal fungi is to increase plant uptake of low mobility minerals, such as phosphorus (Bolan, 1991; Plenchette and Fardeau, 1988), micronutrients (Cooper, 1984; Bürkert and Robson, 1994) and nitrogen

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(Barea *et al*., 1991). Mycorrhizal symbiosis also improves water absorption (George *et al*., 1992) and plant health by providing protection against some pathogens (Dehne, 1982).

Ectomycorrhizal symbiosis is predominant among the members of the Pinaceae and Fagaceae from the temperate areas and with members of the Myrtaceae and Dipterocarpaceae from tropical regions (Smith and Read, 1997). Ectomycorrhizal fungi alter root exudation both quantitatively and qualitatively (Rambelli, 1973; Leyval and Berthelin, 1993), as they catabolize some of the root exudates and modify root metabolic functions. The microbial communities of the soil surrounding mycorrhizal roots and extramatrical mycelium are different from those of the rhizosphere of non mycorrhized plants and the bulk soil (Garbaye, 1991). Therefore the rhizosphere concept has been widened to include this fungal effect, resulting in the introduction of terms "mycorrhizosphere" and "hyphosphere" (Rambelli, 1973; Linderman, 1988). The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus whereas the hyphosphere is the zone surrounding individual fungal hyphae (Linderman, 1988).

Several papers have studied the effects of colonization by mycorrhizal fungi on different microbial groups, such as rhizobia (Duponnois and Plenchette, 2003), actinomycetes (Assigbetse *et al*., 2005), protozoa (Wamberg *et al*., 2003), microarthropods (Cromack *et al*., 1988) and microfungi (Neal *et al*., 1964). This mycorrhizosphere effect has been particularly investigated on fluorescent pseudomonads (Frey *et al*., 1997; Founoune *et al*., 2002). More recently, Frey-Klett *et al*. (2005) characterized the effect of the ectomycorrhizal symbiosis between Douglas fir and *Laccaria bicolor* on the genotypic and functional diversity of *Pseudomonas fluorescens* soil populations. They concluded that the ectomycorrhizosphere selected *P. fluorescens* populations were potentially beneficial to the symbiosis and to the plant.

These results suggest a close relationship exists between the plant, the fungal symbiont and the mycorrhizosphere micro-organisms.

This functional selective pressure of ectomycorrhizal symbiosis has usually been determined on specific groups of bacteria (i.e., fluorescent pseudomonads) or on soil microflora in temperate areas (Timonen *et al*., 1998; Heinonsalo *et al*., 2000; Heinonsalo *et al*., 2001). Few studies have been carried out to compare the diversity of microbial functionalities in different compartments (rhizosphere, mycorrhizosphere, hyphosphere and bulk soil) in a tropical environment.

The aims of this study were (i) to characterize the microbial activities in each of the soil compartments whether they are influenced or not by the ectomycorrhizal symbiosis and (ii) to determine the functional diversity of fluorescent pseudomonads associated with the symbiosis of *Uapaca* sp. and ectomycorrhizal fungi and isolated from each of the soil compartment.

Materials and Methods

Soil Samples

Soil samples were collected in may 2004 from forests located in southwestern Burkina Faso (9°45' N - 12°15' N and 3°10' W - 5°25' W) where the mean annual rainfall varies from 1000 to 1200 mm, with a long dry season from October to May. They were sampled under *Afzelia africana*, *Isoberlinia doka* and *Uapaca somon* that are known to be ectomycorrhizal dependent (Sanon *et al*., 1997). Each of the soil samples was carefully mixed, crushed and passed through a 2 mm sieve. Chemical characteristics of this soil mixture were as follows: pH (H_2O) 6.4; carbon (%) 0.9; organic matter (%) 1.56; nitrogen (%) 0.06, C/N 15; total P (ppm) 114.9 and Bray P (ppm) 3.7. Then the soil was packed in 1 dm^3 polythene pots.

The seeds of *Uapaca* sp. were collected from forests located in southwestern Burkina Faso and conserved at 4°C in a damp atmosphere. Seeds were scarified in hydrogen peroxide for 10 min, rinsed and soaked in sterile distilled water during 12 h and germinated on 1% agar. After one week of incubation at 30°C in the dark, one pre-germinated seed was planted per pot.

Plants were watered daily with tap water (pH 6.0) without fertilizer. In May 2004, fifteen pots were placed in a glasshouse in the IRD Experimental station of Burkina Faso under natural light (daylight approximately 12 h, mean temperature 25°C).

After 8 months culturing, the plants were uprooted. As *Uapaca* root system was not highly ramified and mainly constituted by strong roots, the designation of mycorrhizal from non-mycorrhizal roots was easy. In addition, ectomycorrhizal infection was patchy which allowed fungal colonized soils or free root and fungus soils to be sampled separately. These characteristics avoided to use selective mesh barrier techniques usually recommended to obtain compartmentalisation in most other studies of this nature (Mansfeld-Giese *et al*., 2002). From each pot, the soil highly colonized by hyphal stands (hyphosphere soil, HS), as well as the non-colonized soil (bulk soil, BS) was sampled. The non mycorrhizal short roots with their adherent soil were collected and vigorously shacked, to recover the rhizosphere soil (RS). The same method was used with the ectomycorrhizal short roots and their adherent soil (mycorrhizosphere soil, MS). Fifteen HS and fifteen MS soil samples (20 g fresh weight per pot) were collected. Only 3 soil samples (20 g fresh weight per pot) of the rhizosphere and bulk soil were taken, because most of the plants were highly mycorrhized and their cultural substrate was highly colonized by ectomycorrhizal fungi.

Measurement of Catabolic Diversity of Microbial Communities in Soil Compartments

Microbial functional diversity in soil treatments was assessed by measuring the patterns of *in situ* catabolic potential (ISCP) of microbial communities (Degens and Harris, 1997). Thirty one substrates, comprising a range of amino acids, carbohydrates, organic acids and amides, were screened for differences in SIR (Substrate Induced Respiration) responsiveness between soil treatments (Table 1). The substrate concentrations providing optimum SIR responses are indicated in Table 1

Table 1: Organic compounds and their appropriate concentrations used to assess patterns of *in situ* catabolic potential (ISCP) of soil treatments

Organic substrates	Conc. (mM)	Organic substrates	Conc. (mM)
Amino acids		Carboxylic acids	
L-Phenylalanine	15	Ascorbique acid	100
L-Glutamine	15	Fumaric acid	100
L-Serine	15	Gluconic acid	100
L-Arginine	15	Quinic acid	100
L-Asparagine	15	Malonic acid	100
L-Histidine	15	Formic acid	100
L-Lysine	15	α-ketoglutaric acid	100
L-Glutamic acid	15	Succinic acid	100
L-Tyrosine	15	Tartaric acid	100
L-Cysteine	15	Uric acid	100
		Oxalic acid	100
Carbohydrates		Gallic acid	100
		Malic acid	100
D-Glucose	75	Tri-citrate	100
D-Mannose	75	DL-α-Hydroxybutyric acid	100
Sucrose	75		
Amides			
D-Glucosamine	15		
Succinamide	15		

(Degens and Harris, 1997). Each substrate (0.5 g equivalent dry weight of soil) was suspended in 1 mL sterile distilled water (West and Sparling, 1986) in 10 mL bottles. CO_2 production from basal respiratory activity in the soil samples was also determined, by adding 1 mL sterile distilled water to 0.5 g equivalent dry weight of soil. After the addition of the substrate solutions to soil samples, bottles were immediately closed and kept at 28°C for 4 h. CO_2 fluxes from the soils were measured using an infrared gas analyser (IRGA) (Polytron IR CO_2, Dräger™) in combination with a thermal flow meter (Heinemeyer *et al*., 1989). Results were expressed as µg CO_2 g^{-1} soil h^{-1}.

Catabolic diversity was measured by catabolic richness and catabolic evenness. Catabolic richness, R, is the number of substrates used by microorganisms in each soil treatment. Catabolic evenness, E, (variability of substrate used among the range of substrates tested) was calculated using the Simpson-Yule index, $E = 1/\sum p_i^2$ with p_i = respiration response to individual substrates/total respiration activity induced by all substrates for a soil treatment (Magurran, 1988).

Microbial Isolation and Functional Activities Assessment of Fluorescent Pseudomonads

Soil subsamples (1 g fresh weight) were suspended in 10 ml sterile magnesium sulphate solution (0.1 M) and blended in an Ultraturax blender. Then serial dilutions of homogenized suspensions were plated on King's B medium (King *et al*., 1954) and incubated for 48 h at 30°C in order to isolate fluorescent pseudomonads. The King's B medium plates were examined under UV light to detect fluorescence. Fluorescent bacterial colonies were counted and randomly selected. Fluorescent pseudomonad isolates (120 bacterial strains per soil compartment) were isolated, purified and cryopreserved at -80°C in King's B liquid medium supplemented with 20% glycerol.

Functional activities of fluorescent pseudomonad isolates were assessed by *in vitro* assays carried out to detect their phosphate solubilization, lypolitic activity potentialities and their capacity to grow on trehalose, the most abundant carbohydrate accumulated in the ectomycorrhizal mycelium (Frey *et al*., 1997).

The ability of fluorescent pseudomonads to solubilize tricalcium orthophosphate (TCP) was assessed by using TCP medium. Its composition was as follows: 4 g $Ca_3(PO_4)_2$, 10 g glucose, 5 g NH_4Cl, 1 g NaCl, 1 g $MgSO_4$ and 20 g agar per litre at pH = 7.2. Petri dishes (9 cm diameter) were filled with 25 mL of medium per dish. Bacterial isolates were then picked up from their mother cultures and placed on TCP medium (20 bacterial colonies per dish, 1 cm apart). The plates were incubated at 25°C for 5 days. Phosphate solubilization was indicated by clear zones around the bacterial colonies. Phosphate solubilizing ability was classified as "0" or "+" depending on the presence of well defined clear zone produced by bacterial colony.

The ability of fluorescent pseudomonads to produce extracellular lipases was measured with a solid medium method (Thompson *et al*., 1999). Solid medium was prepared by adding 1% Tween 20 (v/v) to medium containing 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$ $2H_2O$ and 20 g agar per litre at pH = 6. Petri dishes (9 cm diameter) were filled with 25 mL of the medium per dish. Bacterial strains were cultured as described above. Petri dishes were incubated at 25°C for 5 days and lipase production was detected by the presence of Ca laurate extracellular crystals around the bacterial colonies. Lipase production ability was classified as "0" or "+" depending on the presence of Ca laurate crystals produced by bacterial colonies.

The ability of fluorescent pseudomonads to grow on trehalose was measured on M9 medium (minimal medium) amended with 0.1% (w/v) Trehalose. Its chemical composition was as follows: 100 mL Salt mixture; 100 mL Glucose 4% (m/v); 10 mL $CaCl_2$ 0.01 M; 10 mL $MgSO_4$, $7H_2O$ 0.1 M; 0.2 mL Fe - citrate 0.3% (w/v) and 780 mL distilled water. Petri dishes were filled with 25 mL of medium per dish. Bacterial isolates were cultured in plates as described above. Petri dishes were incubated at 25°C for 5 days. Bacterial growth was classified according to the size of the bacterial colonies ("0": diameter of the bacterial colony < 5 mm and "+": diameter > 5 mm).

Statistical Analysis

Data were treated with one-way analysis of variance. Means were compared using PLSD Fisher test ($p<0.05$).

Between-group analysis (BGA, Dolédec and Chessel 1987; Dolédec and Chessel, 1989; Culhane, 2002) is an ordination method that can be used in Ecology as a robust alternative to Discriminant Analysis (DA). Specifically, BGA can be used even when the number of cases is lower than the number of variables. A permutation test (Monte-Carlo method) allows to check the statistical significance of the between-groups differences. The free ADE4 software (Thioulouse *et al*., 1997) was used to perform BGA computations.

Fluorescent pseudomonad populations were expressed as Log transformed CFU (Colony Forming Unit) per gram of soil. For each *in vitro* assay, the percentage of fluorescent pseudomonads from each soil compartment, whether showing or not showing the tested functional activity, was compared with 2×2 contingency tables and chi-square test (χ^2 test) with Yates correction for small numbers.

Results

After 8 months culturing, mainly white ectomycorrhizas with a thick mantle and a dense extramatrical mycelium were observed. In addition, large soil zones were highly colonized by a dense white mycelium coming from ectomycorrhizas.

The permutation test of BGA indicated that the four soil compartments were very different ($p<1/1000$). The percentages of total inertia of the first two axes are equal to 60 and 24%, respectively and the between-group inertia is equal to 13% of the total inertia. The Fig. 1A shows that the use of glucose and ketoglutaric, tartaric and quinic acids is characteristic for the mycorrhizosphere, which is not the case with cystein, glucosamine and succinamide.

Figure 1B gives the graphical outputs of BGA. The lower graph shows the 36 samples, labeled by stars according to the soil compartment to which they belong. The four compartments are ordered from left to right: bulk soil, rhizosphere, hyphosphere and mycorrhizosphere, the last one being more distinctly separated from the other three.

The catabolic richness is higher in the MS and HS compartments than in the BS compartment (Table 2). As for the catabolic evenness, it is higher in the MS compartment than in the RS compartment (Table 2).

The number of fluorescent pseudomonads is significantly higher in the hyphosphere soil than in the rhizosphere and mycorrhizosphere soil ($p<0.05$) (Table 2). The lowest fluorescent pseudomonad population size is recorded in the bulk soil (Table 2).

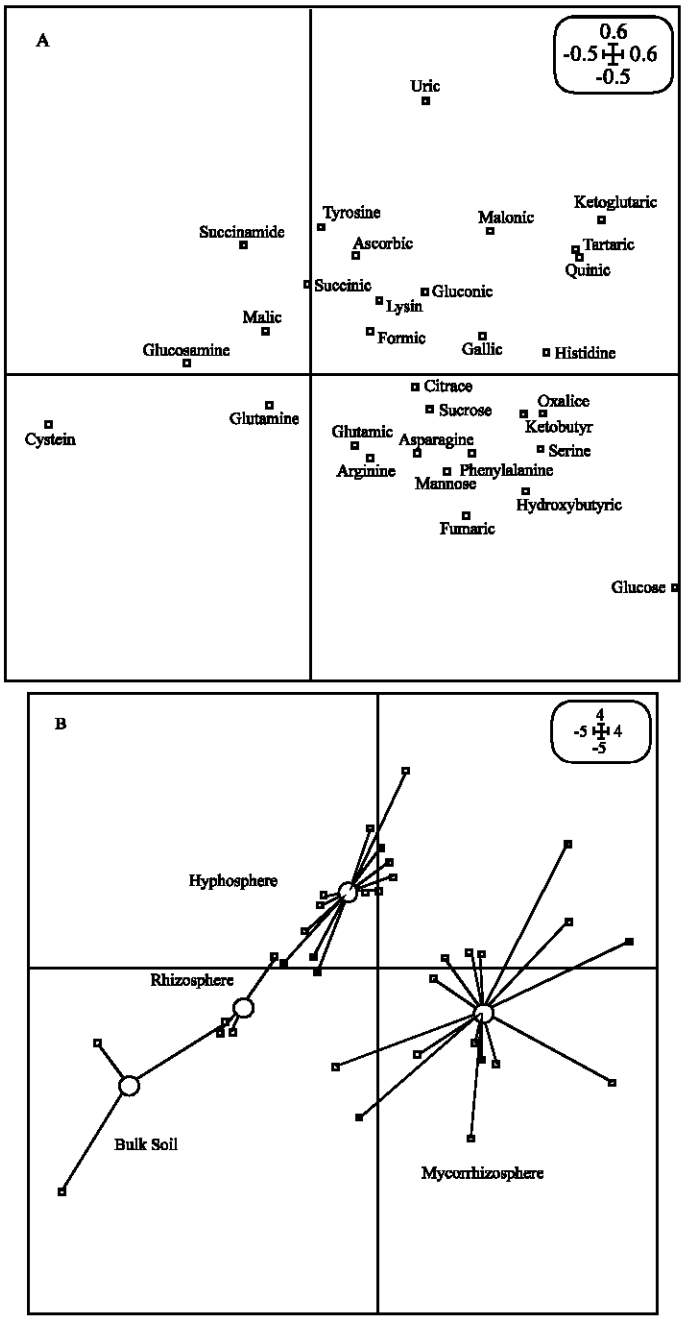

Fig. 1: Between-group analysis of the SIR responses of the bulk soil, rhizosphere, hyphosphere and mycorrhizosphere soil compartments
A: Factor map of the SIR responses
B: Factor map of SIR responses of soil compartments

Table 2: Catabolic diversity (A) catabolic eveness (B), number of fluorescent pseudomonads (C), distribution of the bacterial isolates according to their functional abilities (D) (solubilization of tricalcium orthophosphate and production of extracellular lipases) in each soil compartments. Data indexes by the same letter are not significantly different according to the one way analysis of variance for A, B and C and according to the chi-square test (Ψ^2 test) ($p < 0.05$) for D. BS: Bulk Soil; RS: Rhizosphere Soil; MS: Mycorrhizosphere Soil; HS: Hyphosphere Soil

	BS	RS	MS	HS
Catabolic richness	17.7 a	20.3 ab	21.9 b	20.4 b
Catabolic eveness	5.2 ab	4.3 a	5.5 b	5.4 ab
No. of fluorescent pseudomonads ($\times 10^4$ CFU g^{-1} of soil)	0.3 a	1.5 b	1.8 b	3.3 c
Phosphate solubilizing bacteria ($\times 10^4$ CFU g^{-1} of soil)	5.7 a	8.8 b	8.9 b	14.2 c
Lipase producer bacteria ($\times 10^4$ CFU g^{-1} of soil)	0.6 a	0.9 a	2.8 b	5.7 c

The percentages of phosphate solubilizing and lipase producing fluorescent pseudomonads are significantly higher in the hyphosphere soil than in the other soil compartments ($p<0.05$) (Table 2). They are ordered as follows: BS < RS < MS < HS (Table 2). No significant differences were recorded between each soil compartment for the percentages of bacteria that have the ability to grow on trehalose amended medium.

Discussion

In the present work, we showed that functional activities of soil microbial communities are mainly dependent on fungal activities. This mycorrhizal effect is different between the hyphosphere and mycorrhizosphere zones.

In the literature, there are indications of both negative and positive effects of mycorrhizal symbiosis on the activity of soil bacterial community. It has been previously demonstrated that the decomposition rate of litter increased after exclusion of mycorrhizal fungi (Gadgil and Gadgil, 1971). Olsson *et al.* (1996a) showed that ectomycorrhizal mycelium decreased bacterial activities by using the thymidine incorporation technique. This result differed from the one obtained with mycelium of arbuscular mycorrhiza where, by using the same technique, no fungal effect was recorded (Olsson *et al.*, 1996b). The explanation for this negative effect remains unknown though it is well known that ectomycorrhizal fungi produce antibacterial substances as it has been demonstrated for *Paxillus involutus* and *Hebeloma crustuliniforme* in pure culture (Marx, 1973) and for *Cenococcum graniforme* in mycorrhizal symbiosis (Krywolap *et al.*, 1964). It has been suggested that the extramatrical mycelium allocated carbon amounts in the root free soil and thus increased bacterial growth in the bulk soil (Söderström, 1992). In the present study, the patterns of *in situ* catabolic potential (ISCP) of microbial communities from each of the four compartments were very different. More specifically, the hyphosphere and mycorrhizosphere soils were characterised by a high induced respiration with organic acids. The positive effects of ectomycorrhizal fungi on plant nutrition have usually been attributed to the extramatrical mycelium that take essential dissolved nutrients from the soil solution and, then, translocate them to the host plant *via* the hyphae. Ectomycorrhizal fungi also have access to organic N (Chalot and Brun, 1998) and inorganic or organic P sources (Landeweert *et al.*, 2001). In current researches, the capability of ectomycorrhizal fungi to solubilize surrounding weatherable minerals is getting more attention. Weathering of soil minerals by ectomycorrhizal fungi is mainly performed through fungal excretion of organic acids (Landeweert *et al.*, 2001). Using *in vitro* assays, it has been demonstrated that ectomycorrhizal fungal species produce oxalic acid and solubilize calcium phosphate (Lapeyrie *et al.*, 1991; Leyval and Berthelin, 1986). With long-term pot experiments, it has been established that phosphorus was mobilized from apatite by

ectomycorrhizal pine seedlings and that the P release was positively correlated to the oxalic acid concentration in the soil solution (Wallander and Wickman, 1999; Wallander, 2000). In natural conditions, the relationships between organic acid concentrations and ectomycorrhizal effects on weathering and nutrient uptake are usually concealed due to high microspatial variability of organic acid concentrations and rapid microbial consumption of these fungal exudates in the soil. Present results confirm these conclusions as ectomycorrhizal fungi through its exudates and particularly through their organic acids production, induce a selective pressure on soil microbial communities. In fact, the number of micro-organisms that can catabolise organic acids is higher or the microbial activity is higher in the zone influenced by ectomycorrhizal fungi, which can explain a higher organic acid induced respiration.

The number of fluorescent pseudomonads was significantly more numerous in the HS, MS and RS compartments than in the bulk soil. The highest size of fluorescent pseudomonad population was recorded in the HS compartment. Present results are in accordance with the results of Grayston *et al*. (1994) in the mycorrhizosphere of hybrid larch, Sitka spruce and sycamore, Frey *et al*. (1997) in the Douglas fir - *Laccaria bicolor* mycorrhizosphere and Founoune *et al*. (2002) in the *Acacia holosericea* - *Pisolithus albus* mycorrhizosphere. In these studies, the ectomycorrhizal effect on fluorescent pseudomonad populations has been determined on soil compartment that comprised both the HS and MS soil compartments. But, most of these works have not been designed to distinguish the effects of mycorrhizal roots from the effects of the mycelium alone. Present results show that the ectomycorrhizal mycelium increased the fluorescent pseudomonads growth and that this effect was different from the ectomycorrhizosphere effect. It is well known that 10 to 20% of photosynthetic assimilates are allocated by the host plant to their ectomycorrhizal fungus partner (Smith and Read, 1997). The hyphae of ectomycorrhizal fungi could be the sources of carbon to the soil microbial communities from fungal exudates (Sun *et al*., 1999) and/or from following senescence of hyphae (Bending and Read, 1995). This carbon allocation could be used by fluorescent pseudomonads and, consequently, could improve the growth of this bacterial group.

In addition to this quantitative fungal effect on fluorescent pseudomonad populations, the ectomycorrhizal symbiosis has also modified the functional activities of fluorescent pseudomonads. This fungal qualitative effect was mainly detected in the hyphosphere soil compartment. It has been previously demonstrated that extramatrical mycelium could absorb and then translocate to the host plant, soluble phosphorus from mineral and organic matter, through the excretion of organic acids and phosphatases, respectively (Landeweert *et al*., 2001). Present results showed that most of the fluorescent pseudomonad strains of the hyphosphere soil are able to solubilize tricalcium orthophosphate, compared to those isolated from the bulk soil. Frey-Klett *et al*. (2005) also showed that phosphate-solubilizing fluorescent pseudomonads were significantly more abundant in the hyphosphere than in the bulk soil. Previous studies have demonstrated that some phosphate-solubilizing bacteria can interact synergistically with mycorrhizal fungi for translocation of the soluble phosphorus to the host plant (Kim *et al*., 1997; Muthukumar *et al*., 2001). These results suggest that the selective effect of the extramatrical mycelium can improve the phosphorus soil content around the hyphae and, consequently enhance the phosphorus uptake by the host plant through these synergistic interactions.

The selective effect of the extramatrical mycelium has also been recorded with the lipase producing micro-organisms. Lipases are a group of enzymes that catalyse the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol (Thompson *et al*., 1999). This enzymatic activity could be involved in organic matter degradation and soil lipase activity could be an excellent indicator for monitoring oil decontamination (Margesin *et al*., 2000). Lipase

activity is also involved in the humification of litter processes (Lähdesmäki and Piispanen, 1988). In fact, these bacteria could degrade these complex compounds and facilitate their transfer to the extramatrical mycelium.

In conclusion, all these results showed that close interactions occur between the ectomycorrhizal symbiosis (more particularly with the extramatrical mycelium) and the soil bacterial communities, such as fluorescent pseudomonad populations, which increase the efficiency of the fungal symbiosis for the host plant development (i.e., resistance to the soil pollutants, enhancement of the phosphorus nutrition, etc). Consequently, the mycorrhizal symbiosis cannot be considered as an independent partner inside the symbiotic association but as a component of a multitrophic association between the soil microflora (hyphosphere microflora), the ectomycorrhizal fungal communities and the host plant. Ectomycorrhizal fungal diversity usually detected *in situ* conditions has not been considered in these studies. As it has been demonstrated that mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity (van der Hejden *et al*., 1998), this parameter has to be taken in account in further studies.

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