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REGULAR ARTICLE

Responses of *Pinus halepensis* growth, soil microbial catabolic functions and phosphate-solubilizing bacteria after rock phosphate amendment and ectomycorrhizal inoculation

L. Ouahmane · J. C. Revel · M. Hafidi · J. Thioulouse · Y. Prin · A. Galiana · B. Dreyfus · R. Duponnois

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Abstract We examined the effects of an ectomycorrhizal (EM) fungus, *Pisolithus* sp., on of the growth of *Pinus halepensis* (Allepo pine) seedlings, soil microbial functions and rock phosphate solubilization in a un-disinfected soil amended or not with a Moroccan rock phosphate. Allepo pine seedlings were inoculated with an EM fungus (*Pisolithus* sp. strain PH4) isolated from a *P. halepensis* plantation and selected for its high ability to mobilize P from an inorganic form of phosphate. After 4 month's culture in a disinfected substrate, plants were transferred in 10 l-containers filled with a natural forest soil and amended or not with rock phosphate powder. After 12 month's culturing, the growth, needle nutrient concentrations of *P. halepensis* plants were measured. Soil microbial catabolic diversity was assessed by measuring CO_2 production of substrate induced respiration responses. Fluorescent pseudomonads were isolated from each soil treatment and tested in axenic conditions for their ability to solubilize a source of inorganic phosphate. The results clearly showed that (i) *P. halepensis* growth was greatly promoted by the presence of the ectomycorrhizal

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L. Ouahmane · M. Hafidi
Laboratoire Ecologie et Environnement,
Faculté des Sciences Semlalia, Université Cadi Ayyad,
Marrakech, Maroc

L. Ouahmane
Direction Régionale des Eaux & Forêts du Haut Atlas,
Marrakech, Maroc

J. C. Revel
Ecole Nationale Supérieure Agronomique de Toulouse,
Castanet-Tolosan, Eco. Lab., UMR 5245,
CNRS-UPS-INPT,
Toulouse, France

J. Thioulouse
Laboratoire de Biométrie et Biologie Evolutive
(UMR 5558); CNRS; Univ. Lyon 1,
43 bd 11 nov,
69622 Villeurbanne Cedex, France

Y. Prin · A. Galiana
CIRAD. UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2.
Laboratoire des Symbioses Tropicales et Méditerranéennes
(LSTM). TA10/J, Campus International de Baillarguet,
Montpellier, France

B. Dreyfus · R. Duponnois (✉)
IRD. UMR 113 CIRAD/INRA/IRD/SUP-AGRO/UM2.
Laboratoire des Symbioses Tropicales et Méditerranéennes
(LSTM). TA10/J, Campus International de Baillarguet,
Montpellier, France
e-mail: robin.duponnois@ird.sn

Present address:
R. Duponnois
IRD. Laboratoire Commun de Microbiologie
IRD/ISRA/UCAD. Centre de Recherche de Bel Air,
BP 1386 Dakar, Sénégal

fungus *Pisolithus* strain PH4 in a disinfected soil/vermiculite mixture and in a non disinfected soil, (ii) ectomycorrhizal inoculation induced significant changes in the functions of soil microbial communities and selected microorganisms potentially beneficial to the plant growth (i.e. phosphate-solubilizing fluorescent pseudomonad) and (iii) rock phosphate solubilisation was mainly dependent on EM inoculation and mycorrhizosphere microorganisms. These results were in accordance with previous studies where it was demonstrated that EM symbiosis has a beneficial effect on plant growth through a direct effect on the host plant but also an indirect effect *via* a selective pressure on soil microbiota that favours microorganisms potentially beneficial to plant growth.

Keywords Bacteria · Ectomycorrhizosphere effect · Fluorescent pseudomonads · *Pinus halepensis* · *Pisolithus* sp. · Rock phosphate · Soil functional abilities · Morocco

Introduction

In large areas of arid and semiarid ecosystems, degradation of natural plant cover has lead to an acceleration of soil degradation and desertification processes resulting in a loss or reduction of major physico-chemical and biological soil properties (Requena et al. 2001). Together with the severe climate (low and irregular precipitation and frequent drought periods), soil characteristics (nutrient availability, microbial activity, soil structure, etc) are usually constrictive for the establishment of tree species (Caravaca et al. 2005). Hence it is necessary to apply sustainable cultural methods which enhance soil quality and improve the growth of tree seedlings under unfavorable conditions.

Phosphorus (P) is one of the most essential macronutrients required for the growth and development of plant (Illmer and Schinner 1992). To reduce P deficiencies and ensure plant productivity, large quantities of soluble forms of P fertilizers are applied worldwide every year. However up to 80% of P chemical fertilizer amendment are lost as it is easily precipitated into insoluble forms ($CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$) and becomes unavailable for plant uptake (Lopez-Bucio et al. 2000). The use of rock phosphate (RP) as an alternative source of fertilizer, has received significant interest in recent years since they are natural, inexpensive and available fertilizers. However their solubilization rarely occurs in non-acidic soils (Caravaca et al. 2004). Some soil microorganisms are known to be involved in the solubilization of insoluble phosphate by excreting organic acids, phenolic compounds, protons and siderophores (Illmer et al. 1995). Among phosphate-solubilizing microorganisms, it has been reported that ectomycorrhizal (EM) fungi have the ability to actively mobilize and translocate nutrients from minerals, directly to their host plant (Smith and Read 1997). EM fungi are associated with trees and shrubs, gymnosperms (Pinaceae) and angiosperms, and usually result from the association of homobasidiomycetes with about 20 families of mainly woody plants (Smith and Read 1997). EM fungi increase plant nutrient uptake and water absorption and decrease moisture and nutritional stresses, especially under unfavorable environmental conditions (Smith and Read 1997). In addition, it has been recently demonstrated that ectomycorrhizal symbiosis had a selective pressure on bacterial communities and favored bacteria potentially beneficial to the symbiosis and to the host plant (i.e. fluorescent pesudomonads) (Frey-Klett et al. 2005).

Aleppo pine (*Pinus halepensis* Mill.) is one of the most representative tree species of the Mediterranean basin and cover about 3.5 million hectares in the Mediterranean area (Quezel and Barbero 1992). Its origin in Morocco, Spain and France is likely natural and Moroccan populations seem to be relic (Quezel and Barbero 1992). However, environmental conditions encountered in Mediterranean semi-arid and arid areas (climatic conditions, soil properties) frequently limit the performance of reforestation tasks. Numerous studies have focused on the optimization of nursery practices to produce high-quality seedlings (Caravaca et al. 2005; Rincon et al. 2006). Among the tested cultural practices, inoculation with selected EM fungi has often been identified has a promising nursery cultural practice to improve the quality of the seedlings and their performance after out-planting (Duponnois et al. 2005).

The objectives of this study were to assess how inoculation with an EM fungus, *Pisolithus* sp., affects (i) the early growth of Aleppo pine seedlings, (ii) the functional diversity of soil microflora, (iii) the abundance of fluorescent pseudomonads able to solubilize inorganic forms of phosphorus in a soil amended with rock phosphate (RP).

Materials & methods

Fungal isolation and preparation of the EM fungal inoculum

Surface forest soil (0- to 20-cm depth) was collected from a native stand of *P. halepensis* plantation located at Tizgui (31° 35′ latitude north, 6° 56′ latitude west) in the Moroccan High Atlas. It was crushed, passed through a 2-mm sieve, carefully mixed and distributed in 1-l pots. Its physico-chemical characteristics were as follows: pH (H_2O) 6.8; clay (%) 2.6; silt (%) 40; sand (%) 57.4; carbon (%) 2.5; nitrogen (%) 0.12; C/N 10.5; P (Olsen) 14.5 mg kg^{-1}. The seeds of *P. halepensis* collected in the Tizghi site were surface sterilized in hydrogen peroxide for 30 min, rinsed and soaked in sterile distilled water for 12 h, and germinated on 1% agar. After 4 days of incubation at 25°C in the dark, one pre-germinated seed was planted per pot. The seedlings were screened from the rain and grown under natural light (daylight of approximately 12 h, average daily temperature of 22°C). They were watered regularly with tap water without fertilizer. After 5 month's culture, the plants were uprooted and their root systems gently washed with sterile distilled water. Healthy EM root tips were collected from roots and surface-sterilized in 30% hydrogen peroxide for 30 min, rinsed and soaked in sterile distilled water (120°C, 20 min) for 1 h. Then about 200 EM root tips were transferred aseptically to Petri dishes filled with modified Melin-Norkrans (MMN) medium at 28°C (Marx 1991). The EM isolates were purified, sub-cultured on MMN medium until all contaminating microorganisms were eliminated. Then they were maintained in Petri dishes on MMN agar medium at 25°C in the dark. Among ten fungal isolates, one strain called PH4, belonging to the genus *Pisolithus*, was chosen because of its high ability to solubilize tricalcium orthophosphate in axenic conditions according to the method of Lapeyrie et al. (1990) (Data not shown). The ectomycorrhizal fungal inoculum was prepared according to Duponnois and Garbaye (1991). 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.

Experimental design

Seeds of *P. halepensis* were treated as describe above. They were individually grown in 1-L pots filled with a mixture of Tizgui soil/vermiculite (1:1, v/v). Soil was prepared as described above and autoclaved at 120°C for 40 min before mixing with the autoclaved vermiculite. In ectomycorrhizal inoculation, the substrate was mixed with fungal inoculum (10/1, v/v). The treatment without fungus (control) received an autoclaved mixture of moistened vermiculite/peat moss (MMN liquid medium) at the same rate. All the planted pots were screened from rain and grown under natural light (daylight of approximately 12 h, average daily temperature of 22°C) and were watered regularly with tap water. They were arranged in a randomized complete block design with 14 replicates per treatment.

After 4 months of 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. After drying plant tissues were ground, ashed (500°C), digested in 2 ml HCL 6N and 10 ml HNO_3 N for nitrogen and then analyzed by colorimetry for P (John 1970). For nitrogen (Kjeldhal) determination, they were digested in 15 ml H_2SO_4 (36N) containing 50 g l^{-1} of salicylic acid. 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 x 40). The dry weight (1 week at 65°C) of roots was then measured for each plant. Mycorrhizal dependency of *P. halepensis* was determined by expressing the difference between the total dry weight of the mycorrhizal plant and the total dry weight of the nonmycorrhizal plant, as a percentage of the total dry weight of the mycorrhizal plant (Plenchette et al. 1983).

For both treatments, the remaining seven plants were transferred with their cultural substrate into 10-L capacity containers filled with the same soil as before (Tizgui soil) but not disinfected. The Khouribga Rock Phosphate (Morocco) was ground with pestle and mortar and passed through a 90 μm sieve. Its chemical

characteristics were as follows (%): SiO_2 3.1; Al_2O_3 0.5; Fe_2O_3 0.27; P_2O_5 33.4; MgO 0.5; CaO 54; K_2O 0.06; Na_2O 0.76; CaP 2.64; CO_2 2.1 (Hafidi 1996). In each pot, the surface substrate (0 cm to 10 cm depth) was mixed with the insoluble rock phosphate powder (0.1%, w/v) or not for the un-amended rock phosphate treatments. The containers were arranged in a complete randomized block design and placed outside in the Campus International de Recherche IRD (Institut de Recherche pour le Développement)/ISRA (Institut Sénégalais de Recherches Agricoles) of Dakar (Senegal) in a clean area. The plants were grown without any solar protection and at ambient temperature from 18–30°C with daily watering.

After 12 month's culture, *P. halepensis* seedlings were carefully uprooted from the pots in order to keep the root system intact and avoid root disruption and their oven-dried weights were determined (2 weeks at 65°C). Shoot nitrogen and phosphorus concentrations were determined using the methods previously described. Then their root systems were gently washed and cut into 1-cm segments to evaluate the intensity of mycorrhizal symbiosis (see below). Then the root systems were oven-dried (1 week at 65°C). In each container, the soil collected along the root systems was carefully mixed and kept at 4°C for further measurements.

Ectomycorrhizal assessment

The percentage of ectomycorrhizal short roots was assessed under the stereomicroscope by counting all single root tips. Mycorrhizal or non-mycorrhizal short roots were detected according to the presence or absence of fungal mantle and mycelium and to the presence or lack of root hairs. In each treatment, ectomycorrhizal root tips were classified by morphotypes based on characteristics of their mantle and extra-matrical mycelium (branching, surface colour, texture, emanating hyphae and rhizomorphs (Agerer 1995).

Measurement of microbial catabolic diversity

Microbial catabolic diversity was assessed by adding a range of 31 simple organic compounds to the soil and determining the short-term respiration responses (Degens and Harris 1997). Measurements were made on three soil samples randomly collected from each treatment. One gram of dry soil was moistened with each of the 31 substrates suspended in 2 ml sterile distilled (West and Sparling 1986). CO_2 production from basal respiratory activity in the soil samples was measured by adding 2 ml sterile distilled water to 1 g equivalent dry weight of soil. Bottles were immediately sealed with a Vacutainer stopper after the addition of the substrate solutions to soil samples and incubated for 4 h in darkness at 28°C. 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 subtracted from the CO_2 basal production expressed as μg CO_2 g^{-1} soil h^{-1}. Ten amino acids (L-phenylalanine, L-glutamine, L-serine, L-arginine, L-histidine, L-lysine, L-glutamic acid, L-tyrosine, L-cystein), three carbohydrates (D-glucose, D-mannose, sucrose), two amides (D-glucosamine, succinamide) and 16 carboxylic acids (ascorbic acid, citric acid, fumaric acid, gluconic acid, quinic acid, malonic acid, formic acid, α-ketoglutaric acid, α-ketobutyric acid, succinic acid, tartric acid, uric acid, oxalic acid, gallic acid, malic acid, hydroxybutyric acid) were tested. The amines and amino acids were added at 10 mM, whereas the carbohydrates were added at 75 mM and the carboxylic acids at 100 mM (Degens and Vojvodic-Vukovic 1999). Catabolic evenness (a measure of relative variability in the catabolic functions) was determined using the Simpson-Yule index, $E = 1/p_i^2$ with p_i = [respiration response to individual substrates]/[total respiration activity induced by all substrates for a soil treatment] (Magurran 1988). Microbial biomass C was calculated with the substrate-induced respiration method as described by Sparling (1995). One gram oven-dry weight of soil was suspended in 2 ml of 75 mM glucose solution in 10 ml bottles, sealed with a vacutainer stopper and incubated at 25°C for 4 h. After correction for CO_2 produced in bottles with only deionised water added, Microbial Biomass C (MBC) was calculated as MBC (μg C g^{-1} soil) = 50.4 x respiration rate (μl CO_2 g^{-1} soil h^{-1}).

Fluorescent pseudomonad efficacy for phosphorus mobilization

Soil subsamples (1 g fresh weight) collected along the root systems from each pot in each treatment were suspended in 10 ml sterile magnesium sulfate solution (0.1 M) and shaken manually (ten times up and

down). Then serial dilutions of homogenized suspensions were plated on King's B medium (King et al. 1954) and incubated at 30° for 2 days. The King's B medium plates were examined under UV light and fluorescent colonies were counted and randomly selected. The isolates of fluorescent pseudomonads (30 bacterial strains per treatment) were purified, sub-cultured on King's B medium and cryopreserved at -80°C in glycerol 60%/TSB (Tryptic Soy Broth, 3 g l^{-1}) culture (1:1; v/v). The TCP (tricalcium orthophosphate) medium was used to test the ability of fluorescent pseudomonads to solubilize tricalcium orthophosphate (Frey-Klett et al. 2005). Its composition was as follows: 4 g $Ca_3(PO_4)_2$, 10 g glucose, 5 g NH_4Cl, 1 g NaCl, 1 g $MgSO_4$, 7 H_2O and 20 g agar per liter at pH=7.2. Petri dishes (5.5 cm diameter) were filled with 10 ml of TCP agar medium per dish (Frey-Klett et al. 2005). Bacterial isolates were then picked up from their mother cultures and placed in the center of Petri dishes on TCP agar medium. The plates were incubated at 25°C for 5 days. All the bacterial isolates grew on TCP agar medium. Clear zones around the bacterial colonies indicated phosphate solubilization. Phosphate solubilizing ability was classified as "0" or "+" depending on the presence of well defined clear zone produced by bacterial colony.

Statistical analysis

Data were treated with one-way ANOVA. Means were compared using the Newman-Keuls test ($p<0.05$). The percentages of the mycorrhizal colonization were transformed by arcsin(sqrt) before the statistical analysis. The proportion of fluorescent pseudomonads able to solubilize the inorganic phosphate was compared between each treatment using a χ^2 test ($p<0.05$). The patterns of *in situ* catabolic potential of microbial communities from soil samples submitted to the four treatments (Control, Rock phosphate amendment, *Pisolithus* sp. inoculation, *Pisolithus* sp. inoculation + Rock phosphate amendment) were analysed using the between-group analysis (BGA, Culhane et al. 2002). BGA is an ordination method considered as a robust alternative to the discriminant analysis (Huberty 1994). A permutation test (Monte-Carlo method) was used to check the statistical significance of the between-group differences. BGA computations were performed with the free ADE 4 software (Thioulouse et al. 1997).

Results

Plant growth and ectomycorrhizal colonization

After 4 months of culturing in the disinfected soil/vermiculite mixture, shoot and root biomass, total biomass, phosphorus and nitrogen needle concentrations of the *P. halepensis* seedlings inoculated with *Pisolithus* sp. strain PH4 were significantly higher than in the control (Table 1). Compared to the control treatment, the shoot and root growth of ectomycorrhizal plants were stimulated 1.6 and 1.2 times, respectively (Table 1). No ectomycorrhizas were detected in the uninoculated treatment whereas the extent of EM colonization was 46.8% for *Pisolithus* strain PH4-inoculated seedlings (Table 1).

Table 1 Growth response of *P. halepensis* seedlings, needle nitrogen and phosphorus concentration and mycorrhizal colonization in soils inoculated *Pisolithus* sp., strain PH4 after 4-month culture in a disinfected soil

	Treatments	
	Control	*Pisolithus* sp. strain PH4
Shoot biomass (mg dry weight)	159.3 [(1)] (2.6) [(2)] a [(3)]	248.6 (3.9) b
Root biomass (mg dry weight)	68.6 (8.5) a	82.3 (6.9) b
Total biomass (mg dry weight)	227.9 (11.1) a	330.9 (10.8) b
Root/shoot ratio	0.43 (0.067) a	0.33 (0.08) b
Needle N concentration (%)	1.3 (0.2) a	1.6 (0.3) b
Needle P concentration (g kg^{-1})	7.7 (0.11) a	9.4 (0.13) b
Mycorrhizal colonization (%)	–	46.8 (4.9)
Mycorrhizal dependency (%)		31.1 (2.9)

[(1)] Mean of seven replicates. [(2)] Standard error of the mean. [(3)] Data in the same line followed by the same letter are not significantly different according to the Newman & Keuls test ($p<0.05$).

Twelve months after the transplantation in the non-disinfected soil, no significant effect on plant growth was recorded in the KRP treatment (Table 2). The beneficial effects of *Pisolithus* strain PH4 on plant growth were recorded for all the measured parameters (shoot and root biomasses, phosphorus and nitrogen needle concentrations; Table 2). However the highest *P. halepensis* seedling growth and nutrient uptake was found in the KRP + *Pisolithus* strain PH4 treatment (Table 4). For example and compared to the control, EM inoculation stimulated 1.43 times the plant total biomass whereas the soil amendment with KRP combined with ectomycorrhizal inoculation improved the total plant growth by 1.75x (Table 2). Ectomycorrhizas were detected on the root systems of *P. halepensis* seedlings in all the treatments and EM colonization indexes ranged from 23.2% (control) to 66.5% (KRP + *Pisolithus* strain PH4 treatment) (Table 2). No significant KRP effect was recorded on the ectomycorrhizal colonization rate but the highest ectomycorrhization percentages were calculated in the KRP + *Pisolithus* strain PH4 treatment (Table 2).

The most common morphotypes detected in all the treatments were *Suillus* sp., *Cenococcum geophilum* and *Pisolithus* sp. Their abundances were not significantly different among the treatments except for *Pisolithus* that was significantly more represented in the *Pisolithus* strain PH4 inoculation treatment with or without KRP amendment.

Soil microbial analysis

After 12 month's culture, the catabolic evenness of the soil collected under *Pisolithus* strain PH4 inoculated seedlings with or without KRP amendment was significantly lower than those recorded in the other treatments (Table 3). The highest average respiration SIRs (Substrate Induces Respirations) to carboxylic acids, amino-acids and amides were recorded with the soil inoculated with the strain PH4 and amended with KRP. No significant difference was found in the average SIRs to carbohydrates between the *Pisolithus* PH4 treatments with or without KRP amendment (Table 3).

The four treatments gave very different SIR profiles (Figs. 1 and 2). The highest SIRs have been recorded with α-ketoglutaric acid and D-Glucosamine (Fig. 1). The permutation test of BGA showed that microbial catabolic functions were very different according to the soil treatment ($p<0.001$) (Fig. 2). The four treatments were very well separated on the BGA second axes with the un-inoculated and un-amended soils on the left and the inoculated and KRP amended soils on the right of the figure (Fig. 2). The substrates preferentially used in samples collected from the control treatment were lysine and formic acid whereas it was succinic acid, glutamic acid and malic acid in the uninoculated soil but amended with KRP (Fig. 2). In contrast, the substrates preferentially used in the *Pisolithus* inoculated soil without KRP were OH-butyric acid, tyrosine, gallic acid and succinamide whereas it was asparagine, oxalic acid, serine and glucosamine in the *Pisolithus* inoculated soil with KRP (Fig. 2).

Soil microbial biomass ranged among the treatments as follow: *Pisolithus* inoculated soil with KRP > *Pisolithus* inoculated soil without KRP > KRP amendment = control treatments (Table 4). The abundance of fluorescent pseudomonads was

Table 2 Growth response of *P. halepensis* seedlings, needle nitrogen and phosphorus concentrations and mycorrhizal colonization in soils inoculated with *Pisolithus* sp. strain PH4 and/or rock phosphate amendment after 12-months culture in a non-disinfected soil

	Treatments			
	Control	KRP [1]	*Pisolithus* sp. PH4	PH4 + KRP
Shoot biomass (mg dry weight)	321.2 [2] (24.7) [3] a [4]	318.6 (14.9) a	432.7 (21.5) b	542.9 (14.6) c
Root biomass (mg dry weight)	159.7 (21.6) a	149.7 (22.4) a	254.6 (21.8) b	297.5 (14.5) c
Total biomass (mg dry weight)	480.9 (22.5) a	468.3 (18.3) a	687.3 (21.6) b	840.4 (14.8) c
Needle N content (%)	1.2 (0.1) a	1.1 (0.2) a	1.5 (0.2) b	1.5 (0.1) b
Needle P content (g kg^{-1})	4.1 (0.11) a	4.3 (0.14) a	7.3 (0.13) b	9.5 (0.14) c
Mycorrhizal colonization (%)	23.2 (1.5) a	25.4 (4.3) a	52.5 (1.2) b	66.5 (1.6) c

[1] KRP: Khouribga Rock Phosphate (Morocco). [2] Mean of seven replicates. [3] Standard error of the mean. [4] Data in the same line followed by the same letter are not significantly different according to the Newman & Keuls test ($p<0.05$).

Table 3 Catabolic evenness and average substrate-induced respiration (SIR) responses (μg CO_2 g^{-1} soil h^{-1}) with each substrate group (carboxylic acids, amino-acids, amides and carbohydrates) in the soil treatments

	Treatments			
	Control	KRP [1]	*Pisolithus* sp. PH4	PH4 + KRP
Catabolic evenness	9.37 [2] (0.29) [3] a [4]	9.51 (0.22) a	11.63 (0.38) b	10.58 (0.33) b
Carboxylic acids	23.3 (0.64) a	25.4 (0.72) a	24.9 (0.86) a	28.2 (0.69) b
Amino-acids	3.1 (0.39) a	2.1 (0.15) a	5.3 (0.22) b	6.3 (0.33) c
Amides	15.1 (0.43) a	18.7 (0.32) b	19.4 (0.11) b	22.9 (1.03) c
Carbohydrates	2.4 (0.19) a	3.3 (0.51) a	5.9 (0.41) b	6.5 (0.49) b

[1] KRP: Khouribga Rock Phosphate (Morocco). [2] Mean of five replicates. [2] Standard error of the mean. [3] Data in the same line followed by the same letter are not significantly different according to the Newman & Keuls test ($p<0.05$).

significantly higher in the soil collected from the *Pisolithus* inoculated treatment with or without KRP amendment (Table 4). The percentages of fluorescent pseudomonad isolates able to solubilize the inorganic phosphate were significantly higher in the soils collected from the *Pisolithus* inoculated treatments (with or without KRP amendment) than in the other soil treatments (Table 4).

Discussion

This study clearly shows that (i) *P. halepensis* growth is greatly promoted by the presence of the ectomycorrhizal fungus *Pisolithus* strain PH4 in a disinfected soil/vermiculite mixture and in a non disinfected soil, (ii) ectomycorrhizal inoculation induces significant changes in the functions of soil microbial communities and selects microorganisms potentially beneficial to the plant growth (i.e. phosphate-solubilizing fluorescent pseudomonad) and (iii) rock phosphate solubilization is mainly dependent on EM inoculation and mycorrhizosphere microorganisms.

Numerous studies have reported the high susceptibility for *P. halepensis* to be colonized by ectomycorrhizal fungi (Gonzalez-Ochoa et al. 2003; Rincon et al. 2007). Inoculations with different fungal species such as *Suillus collinitus*, *Pisolithus tinctorius* and *Rhizopogon roseolus* have been demonstrated to improve the performance of *P. halepensis* plantations (Querejeta et al. 1998). The present study confirmed the high mycorrhizal dependency of this *Pinus* species. The inoculation with *Pisolithus* strain PH4 improved the growth of *P. halepensis* seedlings and stimulated its nitrogen and phosphorus assimilation in shoot tissues in a disinfected cultural substrate. It is

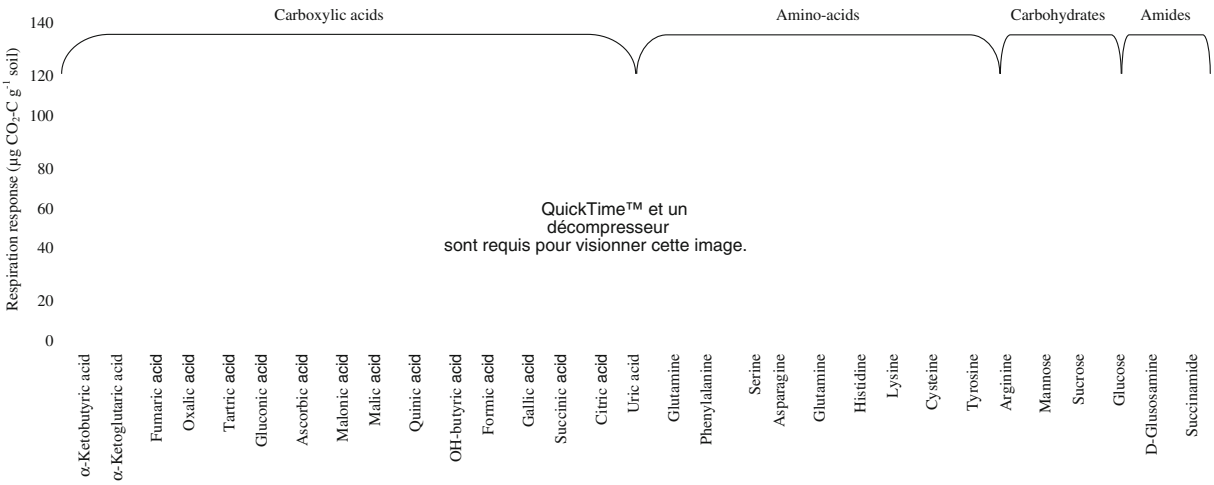

Fig. 1 Catabolic responses for each soil treatments. Errors bars represent standard errors ($n=5$). White bars, control; Clear grey bars, KRP amendment; Dark grey bars, PH4 inoculation; Black bars, KRP amendment + PH4 inoculation

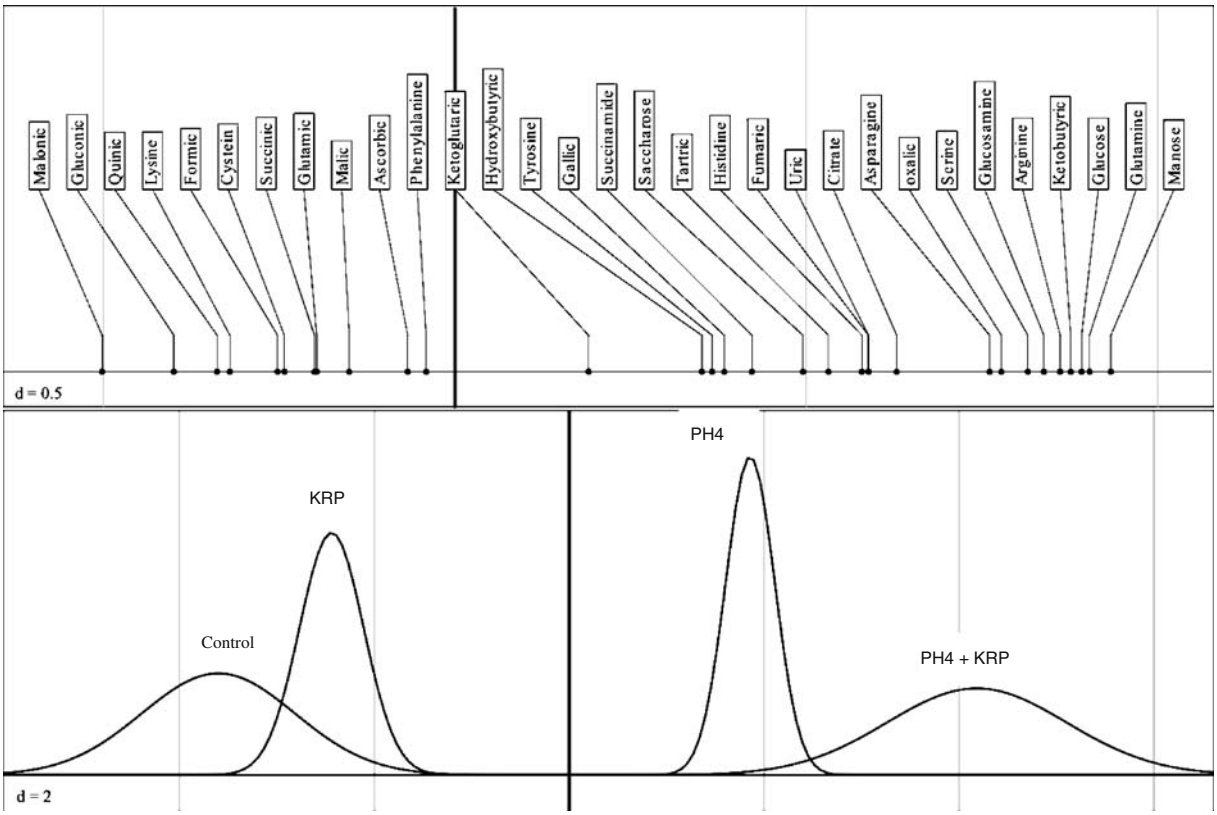

Fig. 2 Graphical display (biplot) of BGA axes showing the Substrate Induced Respirations with respect to the soil treatments. The upper and medium parts of the figure show the scores of the 31 substrates on the first and second BGA axes, respectively. The four Gauss curves in the medium and lower parts of the figure represent the mean and the variance of the scores of the soil samples on the first and second BGA axes. Control: un-inoculated and un-amended soil), KRP: soil amended with Khouribga Rock Phosphate, PH4: soil inoculated with *Pisolithus* sp. strain PH4, PH4 + KRP: soil amended with Khouribga Rock Phosphate and inoculated with *Pisolithus* sp. strain PH4. Substrates represented by lines curved in the same direction as corresponding Gauss curves tend to be used more in the corresponding soil samples

well known that EM fungi improve the phosphorus and nitrogen uptake of their associated host plant (Read and Perez-Moreno 2003). This ectomycorrhizal effect on plant growth has been mainly ascribed to the fact that the extramatrical mycelium increased the abilities of the host plant to explore a larger volume of soil than roots alone and to take up phosphate from a greater surface area (Smith and Read 1997). Our results

Table 4 Effect of *Pisolithus* sp. strain PH4 inoculation and/or Khouribga Rock Phosphate amendment on the soil microbial biomass, the number of fluorescent pseudomonads per gram of soil and the distribution of fluorescent pseudomonads according to their ability to solubilize tricalcium orthophosphate after 12-months culture in a non-disinfected soil

	Treatments			
	Control	KRP [1]	*Pisolithus* sp. PH4	PH4 + KRP
Soil microbial biomass (μg C g^{-1} soil)	62.9 (17.9) [2] a [3]	87.1 (23.9) a	174.3 (15.2) b	241.3 (11.7) c
Fluorescent pseudomonads (CFU g^{-1} of soil)	177 (11) a	186 (15) a	459 (26) b	546 (68) b
Phosphate solubilizing fluorescent pseudomonads (%)	40 a [4]	38 a	85 b	89 b

[1] KRP: Khouribga Rock Phosphate (Morocco). [2] Mean of seven replicates. [3] Standard error of the mean. [4] Data in the same line followed by the same letter are not significantly different according to the Newman & Keuls test ($p<0.05$). [5] Data in the same line followed by the same letter are not significantly different according to the χ^2 test ($p<0.05$).

showed that the positive effect of EM inoculation on the plant growth was maintained in a non-disinfected soil suggesting that the persistence and the beneficial influence of the ectomycorrhizal fungus could be maintained 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 (Slankis 1974). It has been also established that apart from host specificity, the environmental conditions play an important role in the occurrence of *Pisolithus* (Duponnois et al. 2007). It highlights the importance of criteria such as host-fungus compatibility, fungal ecological adaptability to the transplantation site, to select EM fungi for nursery mycorrhization programmes (Smith and Read 1997). Since the fungal symbiont tested in the present study has been isolated from the same soil as that used in the experiment, its high efficiency on the plant growth and its persistence in the soil could result from its adaptability to the biotic and abiotic soil characteristics.

Pisolithus inoculation has induced strong modifications in soil microbial catabolic functions. In the present study, the catabolic evenness of the un-inoculated and KRP un-amended soil was 9.37, which was rather low compared with previous studies in which values of catabolic evenness for soils under Pine forest conditions ranged from 15.1–22.3 (Degens et al. 2000). However, Degens et al. (2000) reported that there was no characteristic level of catabolic evenness for soils under pine forest and evenness was highly dependent to the soil type. It has been hypothesized that decreases in microbial catabolic diversity will cause declines in the resistance of soils to stress or disturbances (Giller et al. 1997). The inoculation of *Pisolithus* sp. enhanced the soil catabolic evenness, leading to a more resistant soil to disturbances. This result highlights the importance of EM symbiosis in the soil functioning. It is also well known that mycorrhizas modify root functions (in particular, root exudation) and, therefore, could modify qualitatively and quantitatively microbial communities (commonly termed the "mycorrhizosphere effect") (Linderman 1988). Moreover the extramatrical mycelium of ectomycorrhizal fungi could also influence soil microbiota by modifying the chemical composition and pH of the surrounding soil and excreting substances into it (Caravaca et al. 2002).

Whereas rock phosphate amendment had little effect on soil microbial functionalities compared to the control treatment, it induced strong modifications when it is added to the soil of inoculated *P. halepensis* seedlings. Rock phosphates can be solubilized under the influence of water, acids, complexing agents and oxygen. Biological weathering or biochemical weathering is realized by microorganisms which produce organic acids, phenolic compounds, protons and siderophores (Drever and Vance 1994). Soluble organic acids affecting rock phosphate weathering in soils could be of high molecular weight (i.e. humic substances) or low molecular weight. They are produced by plant roots and soil microorganisms (Ochs 1996). Many EM fungi excrete organic acids and more particularly oxalic acid (Lapeyrie et al. 1990; Wallander 2000). However the importance of organic acids in dissolving minerals remains uncertain since they could be easily degraded by microorganisms in the soil (Wallander 2000). In the present study, rock phosphate amendment combined with EM inoculation lead to a higher average SIR response with carboxylic acids and higher SIR responses with oxalic and α-ketobutyric acids. It suggests that large amounts of carboxylic acids excreted by the EM fungus could exert a selective influence on soil microbial communities through a multiplication of carboxylic acids-, oxalic acid- and α-ketobutyric acid-catabolizing microorganisms inducing a higher SIR. In addition, the ability of the EM fungus to enhance the uptake of P from rock phosphate has favoured a better shoot and root growth and a higher development of the EM symbiont along the root systems (increase of the EM colonization index) and probably a larger development of the extrametrical mycelium. In fact, most of fungi including members of the Ascomycotina, Basidiomycotina, Deuteromycotina and Mastigomycotina have walls which contain chitin and glucans or mannans whereas those of the Zygomycotina contain both chitin and chitosan (Bartnicki-Garcia 1968). Glucosamine being the building block of chitin originating from fungal cell walls, its measurement is frequently used to estimate the fungal biomass in the soil (Ekblad and Näsholm 1996; Appuhn et al. 2004). Hence the presence of this compound in soil is highly linked to the extent of extramatrical mycelium and could be also a source of C for soil microbiota. In the KRP + *Pisolithus* PH4 treatment, a higher SIR response with glucosamine suggested that glucosamine-catabolizing microorganisms were more abundant in this soil treatment

than in the others, resulting from a higher establishment of the EM symbiosis, along the roots and in the soil.

Our results showed that the number of fluorescent pseudomonads was significantly higher in the *Pisolithus* inoculated treatments. Since it could be assumed that, in the present study, soil samples represented mycorrhizosphere and hyphosphere compartments, these results are in accordance with those of Frey et al. (1997) and Grayston et al. (1994) from which it has been reported that the mycorrhizosphere activity exerts a significant stimulating effect on the populations of fluorescent pseudomonads in the soil.

This study clearly showed that most of the fluorescent pseudomonads strains isolated from the *Pisolithus* treatments were able to solubilise inorganic phosphate by contrast to the majority of those isolated from uninoculated treatments. It has been already reported that phosphate-solubilizing bacteria could positively interact with mycorrhizal fungi and enhance P supply to plants (Muthukumar et al. 2001). Our results are in accordance with those of Frey-Klett et al. (2005). These authors suggested that the enrichment of the mycosphere with phosphate-solubilizing fluorescent pseudomonads could be involved in an improvement of Douglas fir seedlings in a nursery soil as it has also been recorded in the present study.

In conclusion, this study shows that ectomycorrhizal fungus inoculation had a positive effect on the *P. halepensis* growth and had a significant impact on the solubilisation of rock phosphate. This fungal effect is also combined with an indirect effect of the ectomycorrhizal fungus on the functionalities of fluorescent pseudomonads by selecting bacteria potentially beneficial to the plant growth. It confirms that ectomycorrhizal symbiosis functioning results from microbial complex activities where multitrophic interactions take place. However field-based experimental research must be undertaken to determine the impacts of ectomycorrhizal symbiosis on plant growth and soil microbiota functionalities using rock phosphate amendment.

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