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Litter-forager termite mounds enhance the ectomycorrhizal symbiosis between *Acacia holosericea* A. Cunn. Ex G. Don and *Scleroderma dictyosporum* isolates

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Abstract

The hypothesis of the present study was that the termite mounds of *Macrotermes subhyalinus* (MS) (a litter–forager termite) were inhabited by a specific microflora that could enhance with the ectomycorrhizal fungal development. We tested the effect of this feeding group mound material on (i) the ectomycorrhization symbiosis between *Acacia holosericea* (an Australian Acacia introduced in the sahelian areas) and two ectomycorrhizal fungal isolates of *Scleroderma dictyosporum* (IR408 and IR412) in greenhouse conditions, (ii) the functional diversity of soil microflora and (iii) the diversity of fluorescent pseudomonads. The results showed that the termite mound amendment significantly increased the ectomycorrhizal expansion. MS mound amendment and ectomycorrhizal inoculation induced strong modifications of the soil functional microbial diversity by promoting the multiplication of carboxylic acid catabolizing microorganisms. The phylogenetic analysis showed that fluorescent pseudomonads mostly belong to the *Pseudomonads monteillii* species. One of these, *P. monteillii* isolate KR9, increased the ectomycorrhizal development between *S. dictyosporum* IR412 and *A. holosericea*. The occurrence of MS termite mounds could be involved in the expansion of ectomycorrhizal symbiosis and could be implicated in nutrient flow and local diversity.

Introduction

In recent decades, there has been increasing evidence that soil microorganisms have an important effect on soil fertility and plant health (Gianinazzi & Schüepp, 1994). Amongst the microbial populations living in the rhizosphere, mycorrhizal fungi have been found to be essential components of sustainable soil–plant systems (Amato & Ladd, 1988; Bethlenfalvay & Linderman, 1992; Hooker & Black, 1995; Van der Hejden *et al*., 1998; Hart *et al*., 2003; Dickie & Reich, 2005). Over 80% of all land plants form some type of symbiotic association with mycorrhizal fungi. By increasing the absorptive surface area of their host plant, this fungal symbiosis influences plant growth and the uptake of nutrients, particularly phosphorus, a highly immobile element in the soil, which thus frequently limits plant growth in tropical areas. In addition to this positive effect on plant growth, the hyphae that grow outwards from the mycorrhizae into the surrounding soil interact with other soil microorganisms and constitute an important pathway for the translocation of energy-rich plant compounds to the soil. The expanding mycorrhizal mycelium exploits a larger volume of soil that would otherwise be inaccessible to plant roots. As mycorrhizal symbiosis modifies the microbial communities of its surrounding soil through changes in root exudation, this microbial compartment is usually named the 'mycorrhizosphere' (Linderman, 1988), rather than the rhizosphere. The mycorrhizosphere includes the more specific term 'hyphosphere', which refers only to the zone surrounding individual hyphae. Numerous studies have described the effect of the mycorrhizosphere on bacterial communities, such as fluorescent pseudomonads (Frey *et al*., 1997; Founoune *et al*., 2002a) or rhizobia (Duponnois & Plenchette, 2003). However, some bacteria belonging to the mycorrhizosphere compartment may

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promote the development of mycorrhizal symbiosis (Garbaye, 1994). These bacterial strains have been named mycorrhiza helper bacteria (MHB), and the MHB effect has been recorded in different plant–fungus combinations (Dunstan *et al.*, 1998; Founoune *et al.*, 2002b; Duponnois & Plenchette, 2003).

Mycorrhizal establishment usually depends on the plant species, soil type, soil phosphorus and mycorrhizal fungal species (Smith & Read, 1997). The mycorrhizosphere effect will therefore be influenced by soil disturbance (grazing or erosion) and by the impact of natural events in ecosystem functioning. For instance, the structures produced by the soil fauna strongly determine the diversity of the functional groups in their spheres of influence, at specific space and time scales (Lavelle, 1996). Termites, as ecosystem engineers, modulate the availability of resources for other species, such as microorganisms and plants (Lavelle, 1997). For example, fruit bodies of the ectomycorrhizal fungus *Scleroderma* spp. are regularly observed around the termite mounds of *Macrotermes subhyalinus* (a litter–forager termite) in the south of Burkina Faso (K. Sanon, pers. commun.) and Australia (Spain *et al.*, 2004). In order to explain this positive effect of the termite mound on fungal fructification, we hypothesized that the epigeal mound material was inhabited by a specific microflora that enhanced ectomycorrhizal fungal development.

In order to verify this hypothesis, we tested the effect of the mound material of this feeding group on the ectomycorrhizal symbiosis between *Acacia holosericea* (an Australian *Acacia* introduced in sahelian areas) and two ectomycorrhizal fungal isolates of *Scleroderma dictyosporum* (isolates IR408 and IR412), which are known to form ectomycorrhizae with *A. holosericea* seedlings in pot experiments. The influence of mound material amendment on the functional diversity of soil microflora was also assessed. As it has been demonstrated previously that most MHB belong to the fluorescent pseudomonad group (Frey-Klett *et al.*, 1997), and that termite mounds of *M. subhyalinus* are inhabited by this bacterial genus (Duponnois *et al.*, 2005), we investigated their diversity and their effect on IR412 ectomycorrhizal establishment.

Materials and methods

Chemical and microbiological analysis of the sampled epigeal mounds

Five termite mounds of *Macrotermes subhyalinus* were collected in a shrubby savanna, 50 km north of Ouagadougou, near the village of Yaktenga (Burkina Faso). The soil was shallow and rich in gravel above the hardpan level. Large hydromorphic spots intertwined with the deepest soils characterized the landscape. *Macrotermes* mounds were predominantly localized on deeper soils. Termite mounds (about 5 kg each) were crushed and passed through a 2 mm sieve before use.

Table 1. Biological and chemical characteristics of *Macrotermes subhyalinus* mound powder

Biological and chemical characteristics	*M. subhyalinus*
NH_4^+ (μg N g^{-1} of dry mound powder)	9.4
NO_3^- (μg N g^{-1} of dry mound powder)	3408.9
Available P (μg g^{-1} of dry mound powder)	3.5
Microbial biomass (μg C g^{-1} of dry mound powder)	22.5
Fluorescent pseudomonads ($\times 10^2$ CFU g^{-1} of dry mound powder)	79.3
Actinomycetes ($\times 10^2$ CFU g^{-1} of dry mound powder)	39.5
Ergosterol (μg g^{-1} of dry mound powder)	0.316

The chemical and microbiological analyses have been described in a previous study (Table 1) (Duponnois *et al.*, 2005). The NH_4^+ and NO_3^- contents were measured according to the method of Bremner, 1965, whereas available phosphorus was determined according to Olsen *et al.* (1954). The content of ergosterol was determined using the method of Grant & West (1986). The fumigation–extraction method was used to estimate the microbial biomass (Amato & Ladd, 1988). The enumeration of colony-forming units was carried out on King's B agar medium for the fluorescent pseudomonads (King *et al.*, 1954) and on actinomycete isolation agar medium (Difco Laboratories, Detroit, MI) for the actinomycetes. The isolates of fluorescent pseudomonads were randomly selected (18 bacterial strains), purified, subcultured on King's B medium and cryopreserved at $-80\,^{\circ}C$ in glycerol 60%-TSB (tryptic soy broth, 3 g L^{-1}) culture [1/1, volume in volume (v/v)].

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 up and suspended in 50 mL of lysis buffer [0.05 M NaOH, 0.25% sodium dodecylsulphate (SDS)], vortexed for 60 s, heated to 95 °C for 15 min and centrifuged at 2400 ***g*** for 10 min. The lysate cell suspensions were diluted (1/10, v/v) with sterile distilled water. The primers rD1 (5′-AAGCTTAAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) were used to amplify the 16S rDNA gene (Frey-Klett *et al.*, 1997). PCR was performed in a GeneAmp PCR System 2400 thermal cycler (Perking-Elmer, Foster City, CA) using PureTaq Ready-To-Go PCR beads (Amersham Biosciences, Orsay, France), 1 μM of each primer and 3 μL of bacterial cell suspension in 25 μL reaction mixtures. The mixture was submitted to 5 min of 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

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was performed at 72 °C for 5 min. PCR products (7 μL) were digested in a total volume of 20 μL at 37 °C for 2 h using 10 U of the endonucleases *Hae*III and *Msp*I (Promega, Charbonnières, France), as described by the manufacturer. Restriction fragments were separated by horizontal electrophoresis in a 2.5% (weight in volume, w/v) Metaphor gel (FMC, Rockland, ME). 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 the Image Analysis software BIOCAPT (Vilbert Lourmat, Paris, France) under UV light.

The amplified DNA fragments were purified using a Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France), and then ligated into the pGEM-T vector and transformed into cells (*Escherichia coli* DH5α) according to the instructions of the manufacturer (pGEM-T easy vector system, Promega). PCR amplification with the primers T7 and Sp6 was performed directly from the selected white colonies (presumed transformants) to confirm the presence of the insert of the correct size. The plasmid insert from a clone representing each isolate was sent for sequencing (Genome Express, Montreuil, France). The sequence data were compared with gene libraries [GenBank and European Molecular Biology Laboratory (EMBL)] using BLAST (Heinemeyer *et al.*, 1989) and FASTA (Pearson & Lipman, 1988) programs.

Twenty-eight different *Pseudomonas* species were retrieved from the Ribosome Database Project (RDP) (http://www.cme.m-su.edu/RDP) for phylogenetic comparison with our *Pseudomonas* isolates. The phylogenetic analysis was performed using the MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 package (Kumar *et al.*, 2001). Multiple sequence alignments were carried out using the ClustalW program (Thompson *et al.*, 1994). Phylogenetic analysis was performed by the neighbour-joining method, and the relative support for groups was determined on the basis of 1000 bootstrap trees.

The nucleotide sequence obtained in this study has been deposited in the GenBank database and assigned Accession number AY327816.

Glasshouse experiment

Fungal and bacterial inoculum

The ectomycorrhizal fungi, strains IR408 and IR412, have been identified as *Scleroderma dictyosporum* on the basis of rDNA internal transcribed spacer phylogeny (Sanon, 1999). They were isolated from sporocarps under *Uapaca guineensis* in the province of Houet (Burkina Faso). The fungal isolates were maintained on modified Melin–Norkrans (MMN) agar (Marx, 1969) at 25 °C. The ectomycorrhizal fungal inoculum was prepared according to Duponnois & Garbaye (1991). Glass jars were filled with 600 mL of a vermiculite–peat moss mixture (4/1, v/v) and autoclaved (120 °C, 20 min). This substrate was moistened to field capacity with 300 mL of liquid MMN medium. The jars were sealed with a cotton float and autoclaved (120 °C, 20 min). Finally, 10 fungal plugs were placed aseptically into each glass jar and incubated for 6 weeks at 28 °C in the dark.

One strain of fluorescent pseudomonad (*Pseudomonas* sp. KR9) was randomly chosen from the selected bacterial isolates. It was grown in 0.3% TSB (Difco Laboratories) liquid medium for 3 days at 28 °C on a rotary shaker, centrifuged (2400 ***g***, 20 min) and suspended in 0.1 M $MgSO_4$. The final concentration of the bacterial suspension was about 10^8 CFU mL^{-1}, estimated by enumeration on a plate count agar medium (King's B medium) (King *et al.*, 1954). This suspension was used as inoculum.

Effect of the mound powder on IR408 and IR412 ectomycorrhizal development

Seeds of *Acacia holosericea*, originating in Ndioum/Podor (Senegal), were surface sterilized with concentrated 18 M sulphuric acid for 60 min. The acid solution was decanted off and the seeds were washed for 12 h in four rinses of sterile distilled water. The seeds were then transferred aseptically to Petri dishes filled with 1% (w/v) agar–water medium. These plates were incubated at 25 °C in the dark. The germinating seeds were used when the rootlets were 1–2 cm in length.

Acacia holosericea seedlings were grown in 1 L pots filled with soil collected from a millet field near Ouagadougou (Burkina Faso). Before use, the soil was crushed, passed through a 2 mm sieve and autoclaved for 40 min at 140 °C. One week after autoclaving, its chemical and physical characteristics were as follows: pH (H_2O) 5.6; clay, 4.6%; fine silt, 0.0%; coarse silt, 0.8%; fine sand, 25.5%; coarse sand, 69.1%; carbon, 0.204%; nitrogen, 0.04%; carbon/nitrogen, 5.2; soluble phosphorus, 0.0043 mg g^{-1}; total phosphorus, 0.116 mg g^{-1}. The soil was mixed with 10% (v/v) of mound powder and/or 10% (v/v) IR408 or IR412 fungal inoculum. The control treatment was not mixed with either mound powder or fungal inoculum. There were six treatments: control (C), fungal isolate inoculation (IR408 and IR412), termite mound amendment (MS) and fungal inoculum and termite mound added together to the soil (IR408+MS and IR412 + MS). The plants were placed in a glasshouse (25 °C by day, 15 °C by night, 10 h photoperiod) and watered regularly with tap water without the addition of fertilizer. They were arranged in a randomized complete block design with eight replicates per treatment.

After 4 months of culture, the plants were collected and their root systems were gently washed under running tap water. The oven dry weight (1 week at 65 °C) of the shoot was measured. Some nodules were detected along the root systems despite disinfection of the soil and the seed surface.

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The root nodules were counted. The root systems were cut into 1 cm root pieces and mixed. The percentage of ectomycorrhizal colonization [(number of ectomycorrhizal short roots/total number of short roots) × 100] was determined under a stereomicroscope at ×40 magnification on a random sample of at least 100 short roots per root system. The arbuscular mycorrhizal fungal colonization was assessed after clearing and staining the root pieces according to the method of Phillips & Hayman (1970). The root pieces were placed on a slide for microscopic observations at ×250 magnification (Brundrett *et al.*, 1985). About 50 1 cm root pieces were observed per plant. Arbuscular mycorrhizal colonization was expressed in terms of the fraction of the root length with mycorrhizal internal structures (vesicles or hyphae): [(length of colonized root fragments/total length of root fragments) × 100]. The dry weight (65 °C, 1 week) of the roots was then determined.

The soil from each pot was mixed and kept at 4 °C for the assessment of the catabolic diversity of microbial communities.

Assessment of the catabolic diversity of microbial communities

The microbial functional diversity in soil treatments was assessed by the determination of the *in situ* catabolic potential patterns of microbial communities (Degens & Harris, 1997). A range of amino acids, carbohydrates, organic acids, amides and a polymer were screened for differences in substrate-induced respiration (SIR) responsiveness between soil treatments (Table 2). The substrate concentrations providing optimum SIR responses are indicated in Table 2 (Degens & Harris, 1997). Four replicates (soil samples) were randomly chosen from each treatment. One gram equivalent of dry weight soil for each sample was suspended in 2 mL of sterile distilled water in 10 mL bottles (West & Sparling, 1986). CO_2 production from basal respiratory activity in the soil samples was also determined by adding 2 mL of sterile distilled water to 1 g equivalent of dry weight soil. The bottles were immediately closed and kept at 28 °C for 4 h after the addition of the substrate solutions to the soil samples. CO_2 fluxes from the soils were assessed using an infrared gas analyser (Polytron IR CO_2, Dräger, Germany) in combination with a thermal flow meter (Heinemeyer *et al.*, 1989). Results were expressed as micrograms of CO_2 per gram of soil per hour.

Effect of the fluorescent pseudomonad isolate KR9 on IR412 ectomycorrhizal development

Seeds of *A. holosericea* were prepared as described above; *A. holosericea* seedlings were individually grown in 1 L pots filled with the same autoclaved sandy soil (140 °C, 40 min)

Table 2. Organic compounds and their concentrations used to assess patterns of ISCP of soil treatments

Organic substrates	Organic substrates
Amino acids (15 mM)	Carboxylic acids (100 mM)
L-Glutamine	2-Keto-glutaric acid
L-Arginine	3-Hydroxybutyric acid
L-Serine	Ascorbic acid
L-Histidine	D-quinic acid
Phenylalanine	DL-malic acid
L-Asparagine	Formic acid
L-Tyrosine	Fumaric acid
L-Glutamic acid	Gallic acid
L-Lysine	Gluconic acid
L-Cystein	Ketobutyric acid
	Malonic acid
Carbohydrates (75 mM)	Oxalic acid
D-Glucose	Succinic acid
D-Mannose	Tartaric acid
Sucrose	Tri-sodium citrate
	Uric acid
Amides (15 mM)	
D-Glucosamine	Polymer (100 mM)
N-methyl-D-Glucamine	Cyclohexane
Succinamide	

ICSP, *in situ* catabolic potential.

as used in the previous glasshouse experiment. The substrate was mixed with 10% (v/v) IR412 fungal inoculum or with a 10% vermiculite–peat mixture (4/1, v/v) for treatments without fungus. Immediately after planting, the young seedlings from the experimental group were inoculated with 5 mL of fluorescent pseudomonad KR9 suspension (10^8 bacterial cells), whereas those from the control group were inoculated with 5 mL of 0.1 M $MgSO_4$ solution. The plants were placed in a glasshouse (25 °C by day, 15 °C by night, 10-h photoperiod) and watered regularly with tap water without the addition of fertilizer. The pots were arranged in a randomized complete block design with eight replicates per treatment.

After 4 months of culture, the shoot and root biomass, the number of nodules and the percentage of ectomycorrhizal colonization were determined for each plant in each treatment, as described above.

Statistical analysis

The data were treated with one-way analysis of variance. Means were compared using Fisher's protected least significant difference test ($P < 0.05$). The percentages of mycorrhizal colonization were transformed by arcsin (sqrt) before statistical analysis. Co-inertia analysis was performed for plant growth, mycorrhizal colonization indices and SIR responses. Co-inertia analysis (Chessel & Mercier, 1993; Dolédec & Chessel, 1994) is a multivariate analysis

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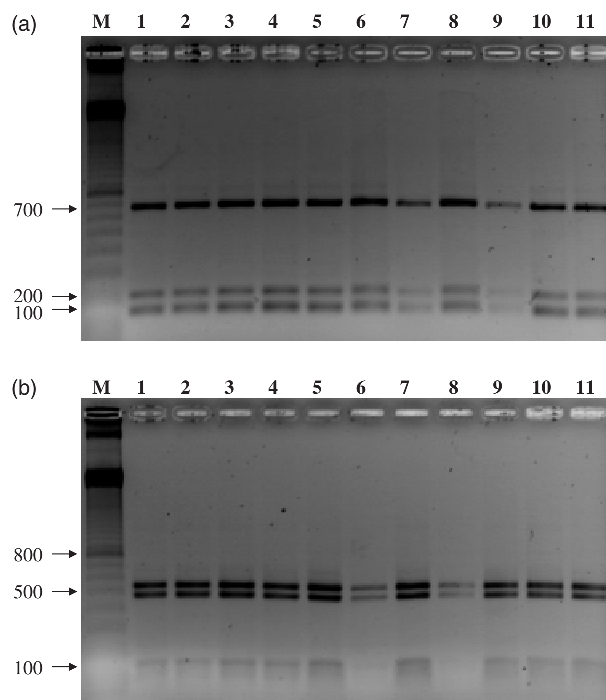

Fig. 1. Gel electrophoresis of PCR-amplified 16S rDNA fragments of fluorescent pseudomonad isolates digested with *Hae*III (a) and *Msp*I (b). Lanes 1–11: fluorescent pseudomonads isolated from termite mounds of *Macrotermes subhyalinus*. Lane M, 100 bp molecular size ladder.

technique that describes the relationships between two data tables. Numerous methods have been suggested for this [e.g. canonical analysis (Gittins, 1985), canonical correspondence analysis (Ter Braak, 1986) and partial least squares regression Höskuldsson, 1988], but one of the simplest, from a theoretical point of view, is co-inertia analysis. Computations and graphical displays were prepared with free ADE-4 software (Thioulouse *et al.*, 1997), available at http://pbil.univ-lyon1.fr/ADE-4/.

Results

Genotypic fingerprinting of fluorescent pseudomonad strains isolated from termite mounds of *Macrotermes subhyalinus*

Eighteen randomly chosen fluorescent pseudomonad strains were subjected to PCR/restriction fragment length polymorphism (RFLP) analysis. PCR conditions allowed the amplification of a single DNA fragment of the 16S rDNA gene with the same size of 1000 bp for all 18 *Pseudomonas* isolates studied. Digestion of the PCR products with two restriction enzymes (*Hae*III and *Msp*I) did not show any polymorphism in the patterns of the 16S rDNA fragments (Fig. 1).

Sequence analysis of the 16S rDNA of all the *Pseudomonas* sp. isolates studied showed 100% identity, signifying that they were all identical. Only one isolate (*Pseudomonas* sp. KR9) was chosen for phylogenetic analysis. The rDNA sequence demonstrated high identity (99.7%) with 16S rDNA sequences of *Pseudomonas monteillii* HR13 (Accession no. AY032725), *P. mosselii* (Accession no. AF072688), *P. putida* (Accession no. AB029257), *P. plecoglossicida* (Accession no. AB09457) and *P. monteillii* (Accession no. AF064458).

Phylogenetic analysis with other selected *Pseudomonas* species from RDP was performed with the neighbour-joining method using *Escherichia coli* as outgroup. The sequence of the *Pseudomonas* isolate KR9 clustered highly with the sequences of *P. monteillii* HR13, *P. mosselii*, *P. putida*, *P. plecoglossicida*, *P. monteillii* and *P. mevalonii* (Fig. 2).

Effect of termite mound amendment on IR408 and IR412 ectomycorrhiza formation

After 4 months of culture, the shoot growth of *Acacia holosericea* seedlings was significantly stimulated by both ectomycorrhizal fungal strains in comparison with the noninoculated treatment (control) (Table 3). The termite mound amendment also significantly improved the shoot biomass (Table 3). No significant differences were recorded between the *M. subhyalinus* treatment and the termite mound amendment/ectomycorrhizal fungal inoculation (Table 3). Compared with the control, *Scleroderma dictyosporum* IR408 significantly enhanced the root growth of *A. holosericea* seedlings, whereas no significant effects were recorded with *S. dictyosporum* IR412 (Table 3). In the soil amended with the termite mound, the root growth was significantly higher than that recorded in the control (Table 3). This termite mound effect was significantly enhanced when ectomycorrhizal fungi were inoculated (Table 3). No significant differences were recorded between the treatments with regard to the total number of nodules per plant. The arbuscular mycorrhizal colonization indices were very low and not significantly different between the soil treatments (Table 3). No ectomycorrhizal short roots were detected in the soil amended with the termite mound (Fig. 3). The ectomycorrhizal colonization indices of *A. holosericea* seedlings inoculated with *S. dictyosporum* IR408 and IR412 were not significantly different (13.5%). The termite mound amendment significantly increased ectomycorrhizal formation, which reached around 25% (Fig. 3).

Catabolic diversity of microbial communities in soil treatments

Co-inertia analysis of the relationship between plant growth, mycorrhizal formation and SIR responses is shown in Fig. 4. The four figures (Fig. 4a–d) can be superimposed to allow

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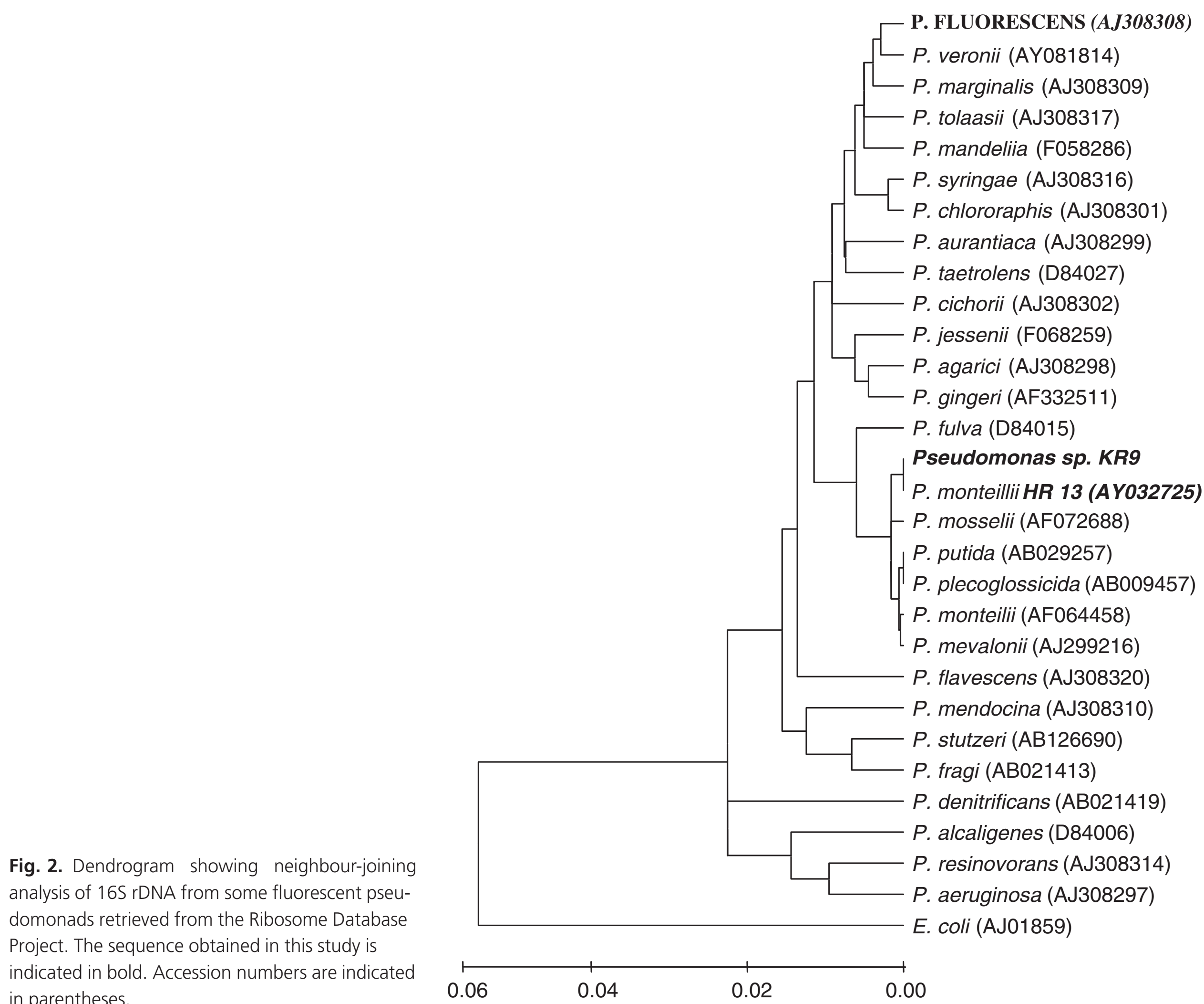

Fig. 2. Dendrogram showing neighbour-joining analysis of 16S rDNA from some fluorescent pseudomonads retrieved from the Ribosome Database Project. The sequence obtained in this study is indicated in bold. Accession numbers are indicated in parentheses.

Table 3. Effects of fungal inoculation and *Macrotermes subhyalinus* mound powder amendment on the *Acacia holosericea* growth, on the total number of nodules per plant and on the arbuscular mycorrhizal colonization after 4 months of culturing in greenhouse conditions

Treatments	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Arbuscular mycorrhizal colonization index (%)	Total number of nodules per plant
Control	261 a*	33 a	0 a	0.5 a
Scleroderma sp. IR408	1458 c	318 bc	0 a	2.3 a
S. dictyosporum IR 412	964 b	190 ab	0 a	2.5 a
M. subhyalinus (MS)	1288 bc	238 b	0.5 a	1.2 a
IR 408+MS	1051 b	432 cd	0.5 a	0.5 a
IR 412+MS	1140 bc	606 d	1.8 a	1.0 a

*Data in the same column followed by the same letter are not significantly different according to the one-way analysis of variance ($P < 0.05$).

the analysis of the relationships between these variables. The Monte-Carlo test showed that there was a statistically significant, although not extremely strong, relationship ($P = 0.025$). Figure 4a and 4c shows the positive effect of fungal inoculation on plant growth: the points corresponding to the inoculated treatments (IR408 and IR412) are shifted towards the right of the figures, which correspond to higher root and shoot biomass. The positive effect of *Macrotermes subhyalinus* mound powder amendment on plant growth is also clearly visible: treatments MS, IR408 +

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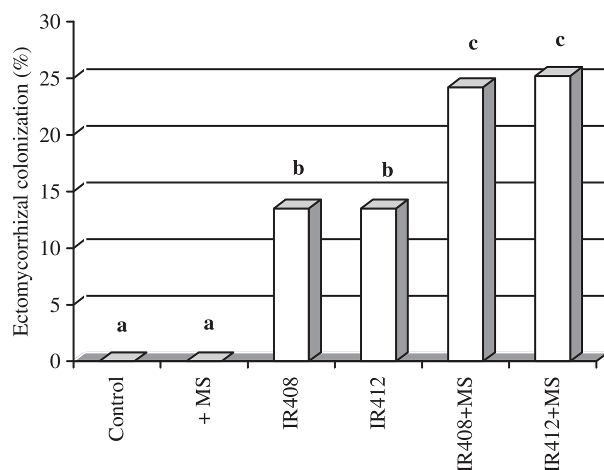

Fig. 3. Ectomycorrhizal formation of *Scleroderma* sp. IR408 and *Scleroderma dictyosporum* IR412 on *Acacia holosericea* root systems in soil amended and not amended with *Macrotermes subhyalinus* mound powder after 4 months of culture in glasshouse conditions. Columns indicated by the same letter are not significantly different according to one-way analysis of variance ($P < 0.05$). MS, *M. subhyalinus* mound powder amendment.

MS and IR412 + MS are also shifted towards the right of the figure. This positive effect seems to be stronger for the IR412 strain than for the IR408 strain. With regard to SIR responses, Figs 4b and 4d clearly show that the positive effect of *M. subhyalinus* mound powder amendment corresponds to a strong modification of the functional microbial diversity for the three treatments.

One-way analysis of variance confirmed these conclusions (Table 4). Ectomycorrhizal establishment was mainly characterized by higher SIR responses with L-arginine, whereas termite mound amendment was indicated by higher SIR responses with sucrose, D-glucosamine, keto-glutaric, hydroxy-butyric, ascorbic, quinic, gluconic, keto-butyric, malonic, oxalic, succinic, tartaric and uric acids, trisodium citrate and cyclohexane (Table 4). The SIR response with gallic acid was significantly higher when termite mound and ectomycorrhizal inoculum were both added to the soil (Table 4). The highest catabolic richness was recorded in the IR412 treatment, whereas the highest catabolic evenness was recorded in the IR408+MS treatment (Table 4).

Effect of a fluorescent pseudomonad strain (isolate KR9) on IR412 ectomycorrhizal development

After 4 months of culture, *S. dictyosporum* IR412 had colonized *A. holosericea* seedlings and had significantly increased shoot and root growth (Table 5). By contrast, no significant effect of the bacterial inoculant KR9 was recorded on plant growth. When KR9 was co-inoculated with IR412, plant growth was significantly higher than that measured when IR412 was inoculated alone; ectomycorrhizal colonization was also significantly increased (from 28.3% to 48.5%) (Table 5). The total biomass of the plants correlated significantly with the mycorrhizal rates ($r^2 = 0.78$). Nodules were observed in all treatments. Ectomycorrhizal inoculation significantly enhanced the number and total weight of nodules per plant. This fungal positive effect was significantly increased when *S. dictyosporum* was co-inoculated with KR9 (Table 5). The number and total biomass of nodules per plant were significantly linked with the mycorrhizal rates ($r^2 = 0.76$ and $r^2 = 0.79$, respectively).

Discussion

The main objectives of this study were to test the effect of a *Macrotermes subhyalinus* mound structure amendment on the formation of ectomycorrhizae between *Acacia holosericea* and two isolates of *Scleroderma dictyosporum* and to evaluate the role of fluorescent pseudomonads inhabiting the mound in these interactions.

In a previous study, spores of ectomycorrhizal fungi were detected in the mounds of wood-, litter- and grass-feeding termites (Spain *et al.*, 2004). The authors showed that there was a greater diversity and more concentrated populations of ectomycorrhizal fungal spores in the mounds than in the surrounding soil. They also detected basidiocarps of the common genera *Pisolithus* and *Scleroderma* species on the mound surfaces. This localization of fruit bodies indicated that the hyphae in the mounds originated from the nearest putative host plants. In our study, no ectomycorrhizal short roots were detected in the *M. subhyalinus* treatment without ectomycorrhizal fungal inoculation. This result seems to contradict the conclusions of Spain *et al.* (Spain *et al.*, 2004). However, the termite mounds of *M. subhyalinus* were collected in a shrubby savanna where all the plant species were associated with arbuscular mycorrhizal fungi (Duponnois *et al.*, 2001). As no potential ectomycorrhizal host tree species was present in these areas, termite mounds could be overspread by ectomycorrhizal short roots. In addition, in a previous study (Duponnois & Lesueur, 2005), the formation of ectomycorrhizae was not observed after 4 months of culture when spores of ectomycorrhizal fungi were inoculated in the soil.

In the present study, termite mound amendment significantly enhanced the ectomycorrhizal expansion of both fungal isolates. This promoting effect could be attributed to: (1) the enhancement of plant growth (particularly root growth) induced by termite mound amendment; (2) inoculation (via the termite mound) by a bacterial group (i.e. fluorescent pseudomonads) that could act as MHB (Duponnois & Plenchette, 2003); and (3) the development of

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Table 4. Effect of ectomycorrhizal inoculation and *Macrotermes subhyalinus* mound powder amendment on *in situ* catabolic potential (ISCP) of microbial communities and catabolic richness, catabolic evenness in soil treatments

	Treatments					
Organic substrates	Control	IR 408	IR 412	*M. subhyalinus* (MS)	IR 408+MS	IR 412+MS
L-Glutamine	5.44 ab*	4.16 ab	4.13 ab	3.89 a	8.05 b	4.20 ab
L-Arginine	8.48 ab	14.14 c	15.45 c	6.09 a	16.53 c	11.96 bc
L-Serine	1.96 ab	3.24 bc	1.96 ab	1.96 ab	3.48 c	1.52 a
L-Histidine	0.0 a	0.0 a	0.87 b	0.22 ab	0.0 a	0.87 b
Phenylalanine	0.26 ab	0.70 ab	0.47 ab	0.02 a	1.79 b	0.89 ab
L-Asparagine	4.64 a	7.41 a	7.84 a	6.09 a	4.79 a	6.53 a
L-Tyrosine	3.79 c	3.58 bc	2.93 bc	0.94 a	2.93 bc	1.84 ab
L-Glutamic acid	4.76 a	4.15 a	3.72 a	3.89 a	5.11 a	4.19 a
L-Lysine	3.26 ab	2.61 ab	2.61 ab	3.92 b	1.96 a	3.05 ab
D-Glucose	5.44 a	6.96 ab	11.5 b	7.6 ab	9.13 ab	11.31 b
D-Mannose	2.61 a	3.48 a	3.26 a	2.61 a	3.26 a	2.83 a
Sucrose	2.39 a	3.26 a	3.26 a	6.09 b	7.18 b	6.75 b
D-Glucosamine	5.66 a	6.31 a	8.49 a	18.5 b	11.3 a	5.87 a
N-methyl-D-Glucamine	3.51 ab	3.72 b	3.50 ab	3.50 ab	2.89 a	3.94 b
Succinamide	3.26 abc	4.57 c	2.17 ab	2.83 abc	4.14 bc	1.52 a
2-Keto-glutaric acid	66.61 a	70.71 ab	75.74 ab	90.77 c	73.14 ab	84.05 bc
3-Hydroxybutyric acid	1.23 ab	0.87 a	1.09 a	4.57 c	3.92 bc	3.92 bc
Ascorbic acid	1.96 a	3.05 a	2.61 a	6.09 b	5.44 b	6.21 b
D-Quinic acid	1.52 a	1.74 a	4.13 a	13.49 b	14.81 b	15.01 b
DL-Malic acid	1.52 ab	3.05 b	2.39 b	0.0 a	2.87 b	2.39 b
Formic acid	7.35 b	9.74 c	6.69 b	2.34 a	10.46 c	4.09 b
Fumaric acid	0.65 a	1.31 a	0.43 a	4.13 b	2.61 ab	1.96 ab
Gallic acid	5.66 a	6.53 ab	5.88 a	5.22 a	10.01 c	9.36 bc
Gluconic acid	3.92 a	4.13 a	7.18 ab	10.88 b	10.01 b	9.58 b
Ketobutyric acid	59.86 a	65.3 a	62.47 a	87.72 b	65.08 a	82.28 b
Malonic acid	3.23 a	4.57 ab	4.78 ab	20.68 c	11.07 ab	12.61 bc
Oxalic acid	19.22 ab	18.94 a	26.34 ab	38.53 c	25.69 ab	28.08 b
Succinic acid	1.96 a	4.35 a	2.61 a	8.05 b	4.57 a	4.12 a
Tartaric acid	2.39 a	3.70 a	3.51 a	13.49 c	11.75 c	7.57 b
Tri-sodium citrate	3.71 a	3.27 a	3.71 a	9.79 c	6.96 b	9.36 c
Uric acid	5.88 a	8.05 ab	8.71 abc	11.10 bc	14.98 d	11.97 cd
Cyclohexane	4.35 a	4.79 a	3.71 a	6.96 b	6.96 b	7.18 b
Catabolic richness	30.7 ab	31.2 b	32.0 b	29.8 a	31.0 ab	30.8 ab
Catabolic eveness	2.55 a	2.65 a	2.62 a	2.62 a	2.84 b	2.66 a

Data are expressed as $\mu g\ CO_2\ g^{-1}$ soil h^{-1}.

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

multitrophic interactions between the ectomycorrhizal symbiosis and the soil microflora.

Termite mounds (*Isoptera*) are a ubiquitous feature of tropical ecosystems, especially in savanna environments. Through termite activities, large amounts of soil are translocated from various depths of the soil profile (Holt & Lepage, 2000). In some areas, such termitaria represent a soil volume of more than 300 m^3 above the ground. These structures strongly influence their environment. In their review, Lobry de Bruyn & Conacher (1990) reported a soil quantity of up to 4.7 tonnes ha^{-1} $year^{-1}$. This termite activity has a considerable influence on soil physical and chemical properties (Lee & Wood, 1971; Lobry de Bruyn & Conacher, 1990; Black & Okwakol, 1997; Holt & Lepage, 2000), and largely explains the termite role as ecosystem engineers. In the present study, termite mound amendment stimulated root growth, probably through an enhanced supply of nitrogen, which, in turn, increased the number of fungal infection sites.

Recent studies have suggested that termite mounds could be sites of great bacterial and fungal diversity. Termite nests generally contain a diversity of fungi (Sannasi & Sundara-Rajulu, 1967; Mohindra & Mukerji, 1982). In *Macrotermes bellicosus* mound soil in Nigeria, Thomas (Thomas, 1987a) found 21 species of fungi. Other authors have found large populations of active bacteria in termite mounds, different from those of the parent soil: eight functional bacterial groups were found in a *Macrotermes* mound in Rhodesia (Meiklejohn, 1965). The higher microbial diversity in termite mounds was attributed to higher organic matter levels

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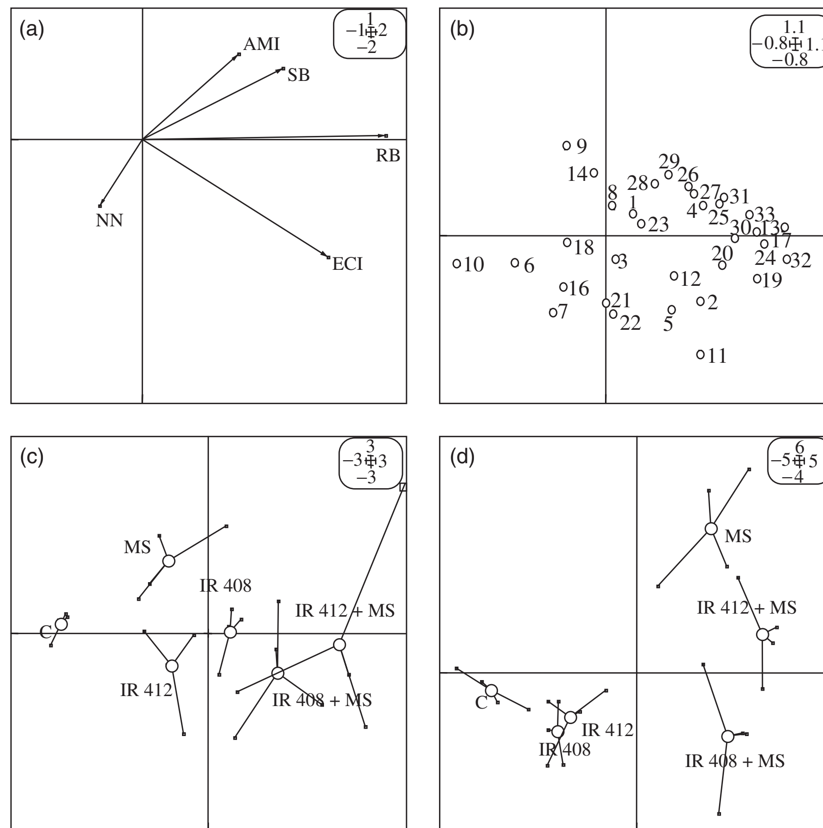

Fig. 4. Co-inertia analysis of substrate-induced respiration (SIR) responses of soils inoculated or not with *Scleroderma dictyosporum* isolates IR408 and IR412 and amended or not with mound powder. In the four panels (a–d), the top-right inset gives the minimum and maximum of the horizontal and vertical coordinates. (a) Factor map of plant growth. Mycorrhizal and rhizobial variables: SB, shoot biomass; RB, root biomass; AMI, arbuscular mycorrhizal colonization index; ECI, ectomycorrhizal colonization index; NN, number of nodules per plant. (b) Factor map of SIR responses. 1, L-glutamine; 2, L-arginine; 3, L-serine; 4, L-histidine; 5, phenylalanine; 6, L-asparagine; 7, L-tyrosine; 8, L-glutamic acid; 9, L-lysine; 10, L-cysteine; 11, D-glucose; 12, D-mannose; 13, sucrose; 14, D-glucosamine; 15, *N*-methyl-D-glucamine; 16, succinamide; 17, 2-keto-glutaric acid; 18, 3-hydroxy-butyric acid; 19, ascorbic acid; 20, D-quinic acid; 21, D,L-malic acid; 22, formic acid; 23, fumaric acid; 24, gallic acid; 25, gluconic acid; 26, keto-butyric acid; 27, malonic acid; 28, oxalic acid; 29, succinic acid; 30, tartaric acid; 31, trisodium citrate; 32, uric acid; 33, cyclohexane. (c) Factor map of plant growth. Microbial and soil sample variables: C, control; MS, soil amended with *Macrotermes subhyalinus* mound powder; IR408, soil inoculated with *S. dictyosporum* strain IR408; IR412, soil inoculated with *S. dictyosporum* strain IR412; IR408+MS, soil inoculated with *S. dictyosporum* strain IR408 and amended with *M. subhyalinus* mound powder; IR412+MS, soil inoculated with *S. dictyosporum* strain IR412 and amended with *M. subhyalinus* mound powder. The star-like diagrams represent the four replicates of each treatment, and the dot inside each star is the mean of these replicates. (d) Factor map of SIR responses of soil samples (for details, see c).

Table 5. Effect of *Scleroderma dictyosporum* IR412 and/or the fluorescent pseudomonad strain, isolate KR9, on mycorrhiza formation, rhizobial development growth of *Acacia holosericea* after 4 months culture under glasshouse conditions

Treatments	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Number of nodules per plant	Total nodule weight per plant (mg)	Ectomycorrhizal colonization (%)
Control	532 a*	184 a	4.2 a	6.8 a	0 a
Isolate KR9	553 a	198 a	4.6 a	7.1 a	0 a
S. dictyosporum IR 412	1236 b	536 b	8.3 b	15.9 b	28.3 b
S. dictyosporum IR 412+Isolate KR9	1786 c	868 c	12.4 c	25.3 c	48.5 c

*Data in the same column followed by the same letter are not significantly different according to the one-way analysis of variance ($P < 0.05$).

and a better supply of nitrogen (Meiklejohn, 1965; Mohindra & Mukerji, 1982), and to higher moisture levels and higher substrate availability (Holt, 1987; Abbadie & Lepage, 1989).

The population and composition of microbial groups appear to vary according to the mound compartment considered (Brauman, 2000). Increasing evidence demonstrates that termites are able to control the number of

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microorganisms, and probably their diversity, in selected parts of their mounds (Sannasi & Sundara-Rajulu, 1967; Holt & Lepage, 2000). Previous microbiological studies of termite mounds have been carried out to compare the cultures of microbial communities in grass-, litter- and soil-feeding termite mounds (Duponnois *et al*., 2005). Fluorescent pseudomonads have been detected only in *M. subhyalinus* mound powder. The phylogenetic analysis performed in this study showed that these fluorescent pseudomonads mostly belonged to *Pseudomonas monteillii* species. It has been demonstrated that one isolate of *P. monteillii* (isolate HR13) can stimulate the establishment of ectomycorrhizal symbiosis in tropical conditions (Founoune *et al*., 2002b) and is considered as an MHB. This MHB effect has been recorded with different fungal isolates, such as *S. dictyosporum*, *S. verrucosum*, *Pisolithus albus* and *P. tinctorius*, on *A. holosericea* and other Australian *Acacia* species (Duponnois & Plenchette, 2003). As *P. monteillii* isolate KR9 stimulated ectomycorrhizal formation between *S. dictyosporum* IR412 and *A. holosericea*, these bacterial strains present in *M. subhyalinus* mounds could also be involved in the enhancement of ectomycorrhizal formation recorded in the present study.

Macrotermes subhyalinus mound amendment and ectomycorrhizal inoculation induced strong modifications of functional microbial diversity. In particular, important soil microflora, able to use carboxylic acids, were detected through high SIR responsiveness with these compounds. Biological and biochemical weathering is mediated by microorganisms that excrete organic acids, phenolic compounds, protons and siderophores (Drever & Vance, 1994). For instance, it is well known that many different fungal species produce these organic acids as the strongest chelators of trivalent metals (oxalate, malate and citrate) (Dutton & Evans, 1996; Gadd, 1999). In addition, amongst termites, the *Macrotermitinae* subfamily (also called 'fungus-growing termites') plays a major role in African ecosystem functioning, mainly in arid and semi-arid areas. The effect of these termites on soil microbiology is not only due to their influence on nonmutualistic microorganisms, but also to their specific exosymbiotic relationship with the fungus *Termitomyces*, which is only found in special structures within the mound, called 'fungus combs' (Sands, 1969; Thomas, 1987b; Wood & Thomas, 1989; Rouland-Lefèvre, 2000). It is suggested that these fungal communities (saprophytic and ectomycorrhizal fungi) could exert a selective influence on the soil microflora by promoting the multiplication of carboxylic acid catabolizing microorganisms.

Macrotermitinae-built structures constitute patches in the landscape in which the availability of soil nutrients for plants is improved (Jouquet, 2002). Associations between fungus-growing nests and grasses have recently been found in West African savanna (Jouquet *et al*., 2004). Mounds of grass- and litter-feeding termites form fertile 'islands' in the savanna, maintaining fertility in these, mostly highly weathered, soils (Okello-Oloya *et al*., 1985, 1986; Lobry de Bruyn & Conacher, 1990). This positive effect is generally attributed to the activity of termites, which translocate nutrient elements in food into their mounds. However, another translocation could be proposed, from the termite mound to the host plant, mediated by ectomycorrhizal roots.

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