Restoring native forest ecosystems after exotic tree plantation in Madagascar: combination of the local ectotrophic species Leptolena bojeriana *and* Uapaca bojeri *mitigates the negative influence of the exotic species* Eucalyptus camaldulensis *and* Pinus patula

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ORIGINAL PAPER

Restoring native forest ecosystems after exotic tree plantation in Madagascar: combination of the local ectotrophic species *Leptolena bojeriana* and *Uapaca bojeri* mitigates the negative influence of the exotic species *Eucalyptus camaldulensis* and *Pinus patula*

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Abstract The objectives of this study were to determine the impact of two exotic tree species (pine and eucalypts) on the early growth of *Uapaca bojeri* (an endemic tree species from Madagascar) via their influence on soil chemical, microbial characteristics, on ectomycorrhizal fungal community structures in a Madagascarian highland forest and to test the ability of an early-successional ectomycorrhizal shrub, *Leptolena bojeriana*, to mitigate the impacts of these exotic species. Finally, we hypothesized that *L. bojeriana* could act as a natural provider for ectomycorrhizal propagules. Soil bioassays were conducted with *U. bojeri* seedlings grown in soils collected under the native tree species (*U. bojeri* and *L. bojeriana*) and two exotic tree species (*Eucalyptus camaldulensis* and *Pinus patula*) and in the same soils but previously cultured by *L. bojeriana* seedlings. This study clearly shows that (1) the introduction of exotic tree species induces significant changes in soil biotic and abiotic characteristics, (2) exotic-invaded soil significantly reduces the early growth and ectomycorrhization of *U. bojeri* seedlings and (3) *L. bojeriana* decreased these negative effects of the exotic tree species by facilitating ectomycorrhizal establishment and consequently improved the *U. bojeri* early growth. This study provides evidence that *L. bojeriana* can facilitate the ectomycorrhizal infection of *U. bojeri* and mitigates the negative effects of the introduction of exotic tree species on the early growth of the native tree

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species. From a practical point of view, the use of ectotrophic early-successional shrub species should be considered to improve forest resaturation after exotic invasion.

Keywords Ectomycorrhizas · *Uapaca bojeri* · Exotic tree species · Degraded forest ecosystems · Nurse plant · Restoration ecology · Revegetation strategies

Introduction

Numerous agricultural practices lead to soil degradation and losses of biodiversity in tropical areas. These anthropogenic impacts do not only degrade natural plant communities (population structure and species diversity) but also physico-chemical and biological soil properties such as nutrient availability, microbial activity, and soil structure (Styger et al. 2007). In order to reverse this loss of fertility and to limit soil erosion, some revegetation programmes have been undertaken in Madagascar using fast-growing exotic trees. Reforestation with eucalyptus (*E. robusta*, *E. rostrata*, *E. camaldulensis*) and later pine (*P. khesya*, *P. patula*) provided wood for the region (Gade 1996). By the 1930s, plantations have been set out by local communities, institutions, and individuals (Parrot 1925). However, exotic trees can threaten ecosystems or habitats by altering ecological interactions among native plants (Rejmanek 2000; Callaway and Ridenour 2004) that could compromise their role in sustainable development. Exotic plants can act directly on native plant communities by allelopathic effects or by higher performance in an introduction site that influence vegetation dynamics, community structure, and composition (del Moral and Muller 1970; Thébaud and Simberloff 2001). They also can alter biochemical cycling compared with native plants (Ashton et al. 2005). As exotic and native plants have different evolutionary histories and traits, it has been also suggested that plant introduction could affect belowground soil microbial communities (Hawkes et al. 2005; Batten et al. 2006; Kisa et al. 2007; Kivlin and Hawkes 2011). Among soil microbial communities, mycorrhizal fungi are considered as key components of the sustainable soil–plant system (Johansson et al. 2004; Dickie and Reich 2005). This symbiotic process influences soil development as well as plant growth (Schreiner et al. 2003; Duponnois et al. 2007).

Numerous studies have shown that ectomycorrhizal (ECM) vegetation is highly dependent on ECM fungi for their growth and survival (Smith and Read 2008). Limitation of the presence, abundance, and community composition of ectomycorrhizal fungi can result from natural (Terwilliger and Pastor 1999) or anthropogenic disturbance (Jones et al. 2003) and the lack of established ectomycorrhizal fungi in soils may limit the establishment or re-establishment of ECM tree species seedlings (Marx 1991). It has been well demonstrated that exotic plant species could disrupt mutualistic associations involved in native ecological associations (Callaway and Ridenour 2004; Kisa et al. 2007; Remigi et al. 2008; Faye et al. 2009) that could limit the natural regeneration of native tree species.

However, these negative impacts on soil microbiota may be counterbalanced by utilizing mycorrhizal native species that enhance the abundance, diversity, and function of mycorrhizal propagules in soil (Kisa et al. 2007; Faye et al. 2009). Recent studies have shown that some early-successional shrubs can preserve and/or increase the abundance and diversity of mycorrhizal propagules of AM fungi (Ouahmane et al. 2006) or ectomycorrhizal fungi (Dickie et al. 2004) and subsequently facilitate forest woody species growth. Improvement of seedling growth by pioneer shrubs, also called the "nurse plant effect", is a general facilitative process (Niering et al. 1963). Nurse plants facilitate vegetation growing beneath their canopies by ameliorating the physical environment and by increasing soil fertility (Franco and Nobel 1988; Callaway and Pennings 2000; Scarano 2002).

In Madagascar, the impacts of exotic tree species such as pine and eucalypts on diversity and abundance of mycorrhizal fungal communities as well as on the early growth of endemic tree species remain unknown. The aims of the present study were to determine in situ and under glasshouse conditions the impact of *Eucalyptus camaldulensis* and *Pinus patula* (two exotic tree species) on soil chemical characteristics, microbial activities and on ECM community structures. We hypothesized that soil microbial activities and mycorrhizal communities will differentiate under these exotic species leading to a decrease of the early growth of a native tree species, *Uapaca bojeri*. We further hypothesized that an enhancement of ectomycorrhizal diversity provided by an early-successional ectomycorrhizal

shrub, *Leptolena bojeriana*, would minimize the negative effects of these exotic species and consequently improve *U. bojeri* growth through a well-developed ectomycorrhizal root colonization. Finally, we tested the hypothesis that *L. bojeriana* could act as a natural provider for ectomycorrhizal propagules and could preserve the abundance and diversity of ectomycorrhizal fungi in stressful environments.

Materials and methods

Study area

The field experiment was conducted within the central part of Madagascarian highland sclerophyllous forest in a forest located at 50 km to the west of Antananarivo (Arivonimamo site). The average annual rainfall was 1,398 mm with a average monthly temperature of 26 °C. The vegetation is a mosaic of *U. bojeri* islands and very scattered individuals of introduced tree species, *P. patula* and *E. camaldulensis*. These trees dominate an understorey mainly composed by early-successional plant species such as *Leptolaena bojeriana*, *Leptolaena pauciflora*, *Erica* sp., *Helychrisum rusillonii*, *Aphloia theaformis*, *Psiadia altissima*, *Rhus taratana*, *Vaccinium emirnensis*, *Rubus apelatus* and *Trema* sp. *L. bojeriana* was the most representative plant species in this site with a cover contribution of about 43 %.

Analysis of the mycorrhizal status of trees and early-successional plant species

Root samples were collected during the rainy season. Root identity was ascertained by tracing from the trunk to the fine root tips. Samples of 1–5 g (fresh weight) of fine roots were washed under running water and stored at 4 °C for further examination. Fine roots were examined for ECM infection under a dissecting microscope. Morphological parameters following Agerer (1987–1996) such as mantle color and structure, branching pattern and characteristics of rhizomorphs were used to categorize ectomycorrhizas into morphological type (morphotype) groups. For AM infection, fine roots were stained following the method of Phillips and Hayman (1970). The root pieces were placed on a slide for microscopic observation under 250 magnification (Brundrett 1991). About fifty 1-cm root pieces were randomly chosen from each root sample collected from each plant species.

Bioassays of soils collected under exotic tree species (*E. camaldulensis* and *P. patula*) and the native tree species (*U. bojeri*)

Seven adult trees of each exotic species and of *U. bojeri* were randomly chosen in an approximately 5 ha area in the Arivonimamo forest. In order to avoid disruption of soil and more particularly changes in mycorrhizal networks, seven intact blocks of soil were collected near each adult tree (about 50 cm from the trunk). Seven additional intact blocks were collected at 10-15 m from any targeted tree species (*E. camaldulensis*, *P. patula,* and *U. bojeri* trees) or other known ectomycorrhizal plants. Intact monoliths of soil were cut with shovel and immediately transferred into 15 cm diameter, 16 cm height plastic pots.

In addition, soil samples were taken near each soil block from the 0–10 cm layer and stored in sealed plastic bags at field moisture content at 4 °C for further measurements. For each soil sample, pH of a water soil suspension was determined. The total organic carbon (TOC) was measured according to the ANNE method (Aubert 1978) and the total nitrogen by the Kjeldahl method. The available and total phosphorus soil contents were analyzed by colorimetry (Olsen et al. 1954). Acid and alkaline phosphatase activities were measured using *p*-nitrophenol benzene as substrate (Schinner et al. 1996), and production of the *p*-nitrophenol product was determined colorimetrically at 650 nm. Fluorescein diacetate (FDA) hydrolysis was assayed to provide a measurement of the microbial global activity (Alef 1998).

Seeds of *U. bojeri* collected in the Arivonimamo forest were surface sterilized in hydrogen peroxide for 10 min, rinsed and soaked in sterile distilled water for 12 h, and germinated on 1 % agar. The germinating seeds were used when rootlets were 1–2 cm long. One pre-germinated seed was planted per pot filled with intact monolith of soil. The pots were randomized in the greenhouse and seedlings grown under natural light (daylight of approximately 12 h, average daily temperature of 25 °C). They were watered regularly with tap water without fertilizer.

After 5 months of culturing, *U. bojeri* seedlings were gently uprooted from the pots in order to keep the

root systems intact and to avoid root disruption. Then, they were gently washed with running water. The percentage of ectomycorrhizal short roots (number of ectomycorrhizal short roots/total number of short roots) was assessed under a dissecting microscope by counting all single root tips. Ectomycorrhizal or non-ectomycorrhizal 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, ECM root tips were classified by morphotypes based on characteristics of their mantle and extra-matrical mycelium (branching, surface color, texture, emanating hyphae, and rhizomorphs (Agerer 1995). All morphological types of ectomycorrhizas were stored at −20 °C in 700 µl CTAB lysis buffer (2 % cetylammoniumbromide; 100 mM Tris–HCl, 20 mM EDTA, 1.4 M NaCl) before molecular analysis. Three ectomycorrhizas randomly selected from each morphotype groups were screened by RFLP analysis and one sample of each unique RFLP patterns was sequenced.

DNA was extracted from root tips using Qiagen DNeasy Plant Mini Kits (Qiagen SA, Courtaboeuf, France) following the manufacturer's recommendations. Fungal mitochondrial rDNA extracts were amplified with ML5 and ML6 primers (White et al. 1990) and restriction digested *Hae*III or *Hin*fI enzymes. Then, one sample of each individual RFLP type was sequenced with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an applied Biosystems model 310 DNA sequencer (Perkin-Elmer). Sequences were aligned by using Clustal X 1.80 (Thompson et al. 1997) and alignment was subsequently manually corrected using Genedoc (Nicholas and Nicholas 1997). All sequences were identified according to BLAST analysis at the NCBI page http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, using default settings. Sequences were deposited in GenBank.

For each *U. bojeri* seedlings, the oven dry weight (1 week at 65 °C) of the aerial and root part was then 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 (Kjeldahl) determination, they were digested in 15 ml H_2SO_4 (36N) containing 50 g l^{-1} of salicylic acid.

Impact of early-successional ectomycorrhizal shrub, *Leptolena bojeriana* on the characteristics of soils collected under exotic tree species (*E. camaldulensis* and *P. patula*) and the native tree species (*U. bojeri*) and on *U. bojeri* early growth

Seeds of *L. bojeriana* were collected from the Arivonimamo forest. They were surface sterilized and were pre-germinated for 1 week in Petri dishes on humid filter paper. A germinated seed was then transplanted into each of plastic pots filled with soil monoliths sampled as described above under exotic and native tree species. One set of pots was unplanted. There were 3 replicates for the unplanted pots and 6 for the planted pots. The pots were randomized in a greenhouse under natural light (daylight of approximately 12 h, average daily temperature of 25 °C) and watered daily with deionized water. After 4 months of growth, half of the *L. bojeriana* seedlings were cut and their aerial parts discarded without any disruptions of the cultural soil and *L. bojeriana* root systems. Removal of aerial parts allowed to test the capacity of *L. bojeriana* seedlings to act as a provider of ectomycorrhizal propagules without any competitive processes between each plant species for C acquisition and consequently to reduce symbiosis costs. Then, one pre-germinated seed of *U. bojeri* (treated as previously described) was planted per pot randomized in the greenhouse and seedlings were cultivated under natural light (daylight of approximately 12 h, average daily temperature of 25 °C). They were watered regularly with tap water without fertilizer. There were 3 treatments: (1) control (without pre-cultivation with *L. bojeriana*), (2) pre-cultivation and dual cultivation with *L. bojeriana* (*L. bojeriana* treatment), and (3) pre-cultivation dual cultivation with *L. bojeriana* without aerial parts (*L. bojeriana* WA treatment). After 5 months of cultivation, measurements of chemical and enzymatic soil characteristics as well as *U. bojeri* ectomycorrhizal status, growth, and leaf mineral contents (N, P) were determined as described before.

Statistical analysis

Plant growth measurements and soil characteristics were treated with one-way analysis of variance and means were compared with the Newman–Keul multiple range test ($p < 0.05$). The fungal colonization

indexes were transformed by arcsin ($\sqrt{x}$) before statistical analysis. A principal component analysis (PCA) was applied to the soil, plant, and microbial parameters. The software used was the ade4 package (Dray and Dufour 2007) for the R software for statistical computing (R Development Core Team 2010).

Results

Mycorrhizal status of trees and early-successional plant species in the Arivonimamo forest

All tree and shrub species recorded in the Arivonimamo forest formed mycorrhizas. Among these, 8 presented AM infections and 5 were found with both AM and ECM (Table 1).

Impact of targeted tree species on soil chemical characteristics, ectomycorrhizal colonization, and growth of *U. bojeri* seedlings

The highest soil acidity was recorded with the *E. camaldulensis* origin followed by *P. patula*, *U. bojeri,* and the bulk soil (Table 2). For N and P soil contents, the opposite ranking was found with the highest values recorded with *E. camaldulensis* soil (Table 2). The total organic matter in soil was significantly higher in *U. bojeri* and the lowest value was found in the bare soil whereas *P. patula* and *E. camaldulensis* soils had intermediate TOC contents (Table 2).

The acid phosphatase and FDA activities were significantly higher in the soils collected under the targeted tree species compared to the bulk soil but these activities were higher in the soils sampled under exotic tree species than in the *U. bojeri* origins (Table 2). With the alkaline phosphatase activity, an opposite pattern was found with a higher activity in the *U. bojeri* soil followed by the *P. patula* soil and finally by the bulk and *E. camaldulensis* soils (Table 2).

After 5 months of culturing, shoot and root biomass, total biomass of *U. bojeri* seedlings were significantly lower in the soil collected under *E. camaldulensis* than in the other soil origins, whereas the highest root and total growth were found in the *U. bojeri* soil (Table 3). Compared with the control (bulk soil), no significant effect of *P. patula* origin was recorded for the root and total biomass except for the shoot biomass (Table 3). According to the soil origins, root/shoot ratios ranged as follows: *U. bojeri* > *P. patula* > bulk soil (control) > *E. camaldulensis* (Table 3). Nitrogen leaf contents were not significantly different among soil origins, whereas phosphorus foliar content of *U. bojeri* seedlings was significantly higher in the soil originating from around *U. bojeri* compared with *P. patula* soil (Table 3).

Compared with the bulk soil, the extent of ectomycorrhizal colonization was significantly higher in the soil collected under *U. bojeri* (73.7 %) and significantly lower in the *E. camaldulensis* soil (16.3 %) (Table 3). Structures of ectomycorrhizal

Table 1 Mycorrhizal status of trees and early-successional plant species in the Arivonimamo forest

Shrub and tree species	Family	Mycorrhizal status
Leptolaena pauciflora Baker.	Sarcolaenaceae	ECM & AM
Leptolaena bojeriana (Baill.) Cavaco.	Sarcolaenaceae	ECM & AM
Trema sp.	Cannabaceae	AM
Vaccinium emirnense Hook.	Ericaceae	AM
Aphloia theaeformis (Vahl.) Benn.	Aphloiaceae	AM
Rhus taratana (Baker.) H. Perrier	Anacardiaceae	AM
Helychrysum rusillonii Hochr.	Asteraceae	AM
Psiadia altissima (*D.C.*) Drake	Asteraceae	AM
Rubus apetalus Poir.	Rosaceae	AM
Erica sp.	Ericaceae	AM
Eucalyptus camaldulensis Dehn.	Myrtacea	ECM & AM
Pinus patula Schiede ex Schtdl. & Cham.	Pinaceae	ECM & AM
Uapaca bojeri L.	Euphorbiaceae	ECM & AM

ECM ectomycorrhizas, *AM* arbuscular mycorrhizas, *ECM & AM* co-existence of arbuscular mycorrhizas and ectomycorrhizas

Table 2 Chemical and biochemical characteristics of rhizosphere soils collected under a native tree species (*Uapaca bojeri*), two exotic tree species (*Pinus patula* and *Eucalyptus camaldulensis*) and from the bare soil (control) in the Arivonimamo forest

	Soil origins			
	Control	*U. bojeri*	*P. patula*	*E. camaldulensis*
pH (H_2O)	5.26 (0.03)[1] d[2]	4.94 (0.01) c	4.78 (0.01) b	4.52 (0.01) a
Total nitrogen (%)	0.09 (0.006) a	0.19 (0.003) c	0.15 (0.006) b	0.22 (0.006) d
Soluble P (mg kg^{-1})	1.45 (0.02) a	2.85 (0.02) c	2.14 (0.07) b	3.09 (0.02) d
Total organic matter (%)	1.76 (0.009) a	4.26 (0.038) d	3.23 (0.041) b	3.53 (0.026) c
Total microbial activity (μg of hydrolyzed FDA h^{-1} g^{-1} of soil)	5.61 (0.05) a	6.69 (0.25) b	11.54 (0.65) c	15.33 (2.05) c
Acid phosphatase activity (μg *p*-nitrophenol g^{-1} of soil h^{-1})	130.56 (31.8) a	314.01 (11.7) b	867.06 (50.7) c	586.51 (104.9) c
Alkaline phosphatase activity (μg *p*-nitrophenol g^{-1} of soil h^{-1})	166.51 (6.91) a	302.54 (7.44) c	170.95 (8.47) b	82.54 (5.59) a

[1] Standard error of the mean. [2] 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 Response of *U. bojeri* seedling growth and ectomycorrhizal colonization in soils from different tree species (*Uapaca bojeri*, *Pinus patula* and *Eucalyptus camaldulensis*) and from the bare soil (control) after 5 months culturing in glasshouse conditions

	Soil origins			
	Control	*U. bojeri*	*P. patula*	*E. camaldulensis*
Shoot biomass (mg dry weight)	131 (11)[1] b[2]	125 (15) b	85 (12) a	83 (9) a
Root biomass (mg dry weight)	113 (12) b	295 (35) c	119 (10) b	27 (4) a
Total biomass (mg dry weight)	244 (12) b	419 (48) c	205 (22) b	110 (8) a
Root:shoot ratio	0.88 (0.15) b	2.37 (0.16) d	1.42 (0.12) c	0.34 (0.08) a
N leaf mineral content (mg per plant)	0.89 (0.06) a	0.85 (0.1) a	0.65 (0.09) a	0.65 (0.07) a
P leaf mineral content (mg per plant)	71.1 (7.3) ab	94.1 (9.9) b	58.9 (8.7) a	62.3 (7.3) ab
Ectomycorrhizal colonization (%)	36.1 (2.08) b	73.7 (3.18) c	29.3 (5.55) ab	16.3 (2.40) a

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

communities associated with *U. bojeri* root systems in the different soil origins were significantly different (Table 4; Fig. 1). The RFLP types UA1 (*Russula earlei*), UA2 (*Amanita* sp.), UA3 (Thelephoroid symbiont), and UA4 (uncultured ECM fungus) were only recorded on *U. bojeri* seedlings grown in *U. bojeri* soil, whereas in the soils collected under exotic tree species, UD1 (*Bondarcevomyces*), UC3 (*Russula exalbicans*), and UB6 (*Boletellus projectellus*) were found. In the bare soil, the RFLP type UC3 was mainly detected and two other types, UC2 (*Boletus rubropunctus*) and UB5 (*Coltricia perennis*) at lower abundances (Fig. 1). The RFLP type UB4 (*Xerocomus chrysenteron*) was only recorded in the *E. camaldulensis* soil treatment (Fig. 1).

Responses of soil characteristics and *U. bojeri* growth to the *L. bojeriana* cultivation

A data table with 36 rows and 12 columns was constructed with the soil, plant, and microbial activity parameters. The 12 variables were: pH, soluble phosphorus, total nitrogen and total organic matter, total microbial activity, acid and alkaline phosphatase activities, shoot and root biomass of *U. bojeri* seedlings, ectomycorrhizal rate, leaf nitrogen and phosphorus contents, and the Shannon diversity index of the ectomycorrhizal fungal morphotypes. The 36 rows corresponded to three samples of the four soil origins: soil collected under *E. camaldulensis*, *P. patula*, *U. bojeri*, or bare soil. For each soil origin, three treatments were considered: *U. bojeri* seedling

Table 4 Identification by ITS sequence of RFLP types for ectomycorrhizas collected on *U. bojeri* seedling after 5 month culturing in glasshouse conditions on soils collected under a native tree species (*Uapaca bojeri*), two exotic tree species (*Pinus patula* and *Eucalyptus camaldulensis*) and from the bare soil (control) in the Arivonimamo forest

RFLP types	GenBank accession number	Closest GenBank species	BLAST expected value
UA1	AF518722	*Russula earlei*	2e−144
UD1	DQ534583	*Bondarcevomyces taxi*	3e−138
UA2	AM117659	*Amanita* sp.	0.0
UA3	AJ509798	Telephoroid mycorrhizal sp.	1e−154
UC3	AY293269	*Russula exalbicans*	2e−170
UA4	AY157720	Uncultured ECM homobasidiomycete Clone E2	0.0
UB6	DQ534582	*Boletellus projectellus*	0.0
UC2	FJ480421	*Boletus rubropunctus*	2e−171
UB5	None	*Coltricia perennis*	2e−141
UB4	AD001659	*Xerocomus chrysenteron*	4e−173

was planted alone, with a *L. bojeriana* seedling, or with a *L. bojeriana* seedling that aerial part was cut after 4 months of cultivation, but keeping intact its root system. The resulting data table was submitted to a principal component analysis (PCA) to describe the main structures of this data set.

The Fig. 2 showed the results of this PCA. The upper part (Fig. 2a) graphic was the correlation circle of all the parameters, and the lower part graphic (Fig. 2b) was the map of sample scores on the first two principal components. The correlation circle (Fig. 2a) showed that the first principal component (PC1) was well correlated to plant growth, with better growth toward the right of the graphic (shoot biomass, leaf phosphorus and leaf nitrogen contents) and also to the microbial activities (total microbial activity, acid and alkaline phosphatase activity), to the ectomycorrhizal rate, and to the Shannon diversity index of ectomycorrhizal fungi. The second principal component (PC2) was negatively correlated to root biomass increase and soil total nitrogen (downward arrows) and positively to organic matter and pH (upward arrows).

The map of sample scores (Fig. 2b) showed on the PC1 the very strong effect of the *L. bojeriana* plant (solid arrows pointing right). This effect was positive, as it corresponded to an increase of *U. bojeri* seedling growth, of microbial activities, and of ectomycorrhizal fungal diversity. This effect was highest when the

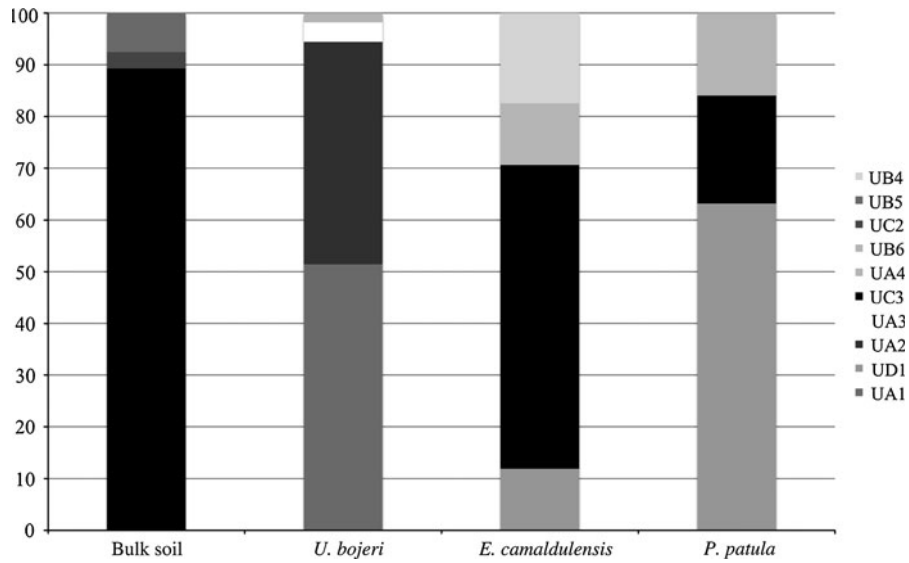

Fig. 1 Similarities in ectomycorrhizal communities between *U. bojeri* seedlings growing in soils collected under *Uapaca bojeri*, *Eucalyptus camaldulensis*, *Pinus patula* and from a bulk soil (d). Values are expressed by RFLP type percentages with regards to the soil treatments. UA1: *Russula earlei*, UD1: *Bondarcevomyces taxi*, UA2: *Amanita* sp., UA3: Telephoroid mycorrhizal sp., UC3: *Russula exalbicans*, UA4: Uncultured ECM homobasidiomycete Clone E2, UB6: *Boletellus projectellus*, UC2: *Boletus rubropunctus*, UB5: *Coltricia perennis*, UB4: *Xerocomus chrysenteron*

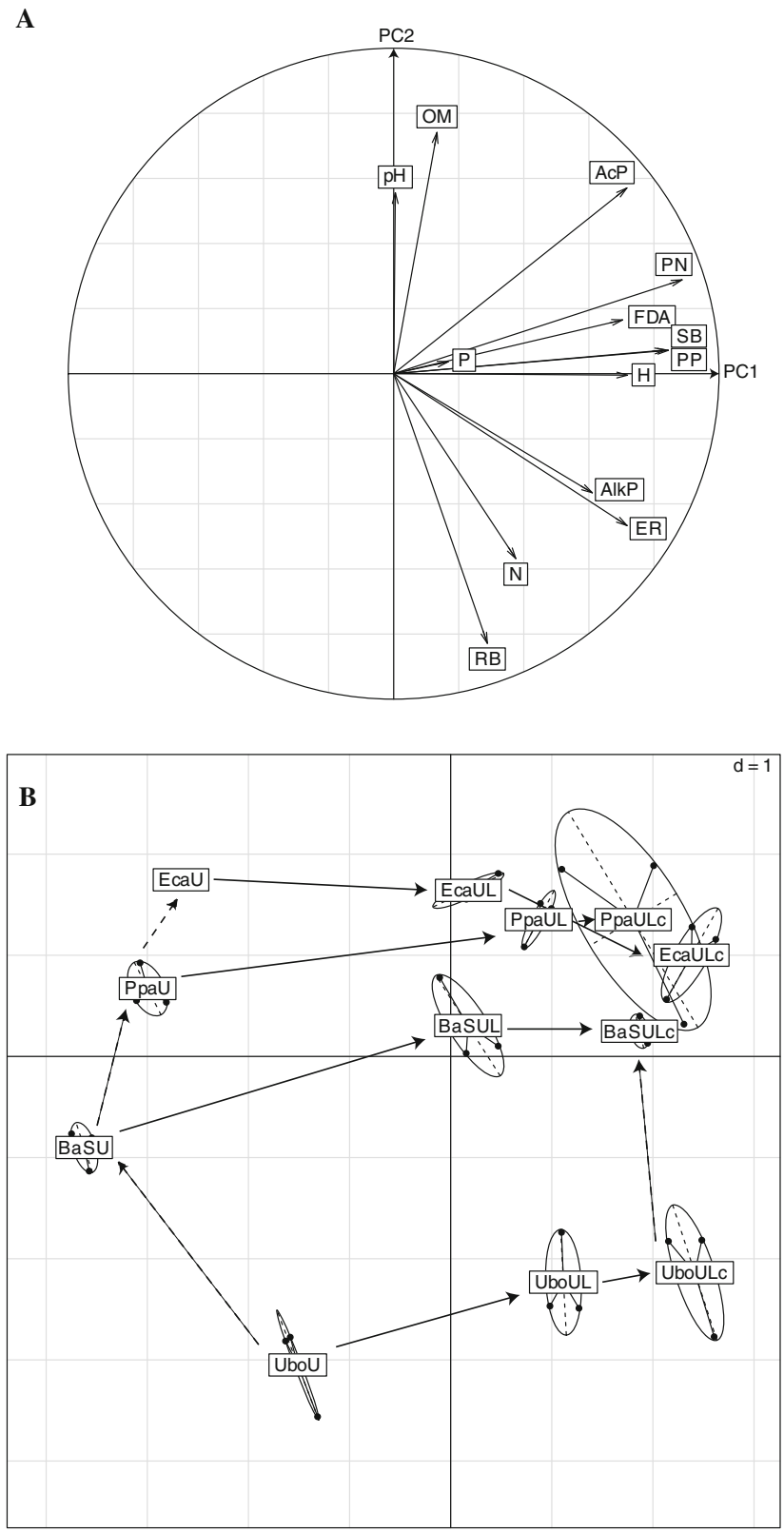

Fig. 2 Results of the PCA on the data table of soil, plant, and microbial activity parameters. **a** Correlation *circle* of all the parameters. The 12 variables are: pH = pH, P = total phosphorus (mg kg^{-1}), N = total nitrogen (%), OM = total organic matter (%), FDA = total enzymatic activity, AcP = acid phosphatase, AlkP = alkaline phosphatase, SB = shoot biomass (g), RB = root biomass (g), ER = ectomycorrhizal rate (%), PN = leaf nitrogen (%), PP = leaf phosphorus ($mg.kg^{-1}$), H = Shannon diversity index of ectomycorrhizal fungi. **b** Map of sample scores on the first two principal components. Samples are coded as follows. The first three characters correspond to the soil origin: Eca = soil collected under *E. camaldulensis*, Ppa = soil collected under *P. palida*, Ubo = soil collected under *U. bojeri*, BaS = bare soil. The treatment applied to the *U. bojeri* seedlings is coded as folows. U = *Uapaca* plant alone, UL = *Uapaca* plant + *L. bojeriana*, Ulc = *Uapaca* plant + *L. bojeriana* cut after 4 months cultivation. For example, sample coded "EcaULc" is a *U. bojeri* seedling grown in soil collected under *E. camaldulensis* in which a plant of *L. bojeriana* was grown and cut after 4 month cultivation

Leptolena plant was cut and only the root system was left before planting *U. bojeri* seedlings. It was also interesting to notice that this effect was the same for bare soil, for soils collected under exotic tree species or for soil collected under a *Uapaca* adult tree. On the same graphic (Fig. 2b), the PC2 showed the soil origin effect (dotted arrows pointing upward), corresponding to the negative influence of exotic tree species (*E. camaldulensis*, *P. patula*) on root biomass. Root biomass was higher in soils collected under *U. bojeri* adult tree and lower in soils collected under exotic tree species. Bare soils have an intermediate position. Conversely, pH and total organic matter are higher in soils collected under exotic tree species.

For each soil origins, the impact of *L. bojeriana* (with or without aerial parts) on soil characteristics, *U. bojeri* growth, and ectomycorrhizal communities was indicated in Tables 5, 6, and 7. For the bulk soil origin and compared with the control, the treatment with *L. bojeriana* without aerial parts provided the highest positive effects on pH, soluble P, soil N content, organic matter content and on microbial enzymatic activities (Table 5). The dual cultivation of *L. bojeriana* with or without aerial parts significantly improved shoot and root biomass and mineral nutrition of *U. bojeri* seedlings (N, P) (Table 6). Ectomycorrhizal colonization was significantly improved when the dual cultivation was performed with *L. bojeriana* without aerial parts (Table 6). Strong modifications in the composition of ectomycorrhizal communities occurred in the treatments with *L. bojeriana* (Table 7). RFLP types, UC3 and UC2 recorded in the control treatment, were not found in the dual cultivation treatments and replaced by the RFLP types UA1, UA2, and UB4. The RFLP type UB6 was only recorded in the treatment with entire *L. bojeriana* seedlings (Table 7).

For the *U. bojeri* soil origin, dual cultivation with entire *L. bojeriana* seedlings increased all the measured soil parameters except for pH (Table 5). Eliminating the aerial parts of *L. bojeriana* seedlings led to higher increases of N, organic matter soil contents and FDA activity but to a lower enhancement of soil soluble P content (Table 5). Dual cultivation had significantly improved plant nutrient (N and P) uptake with highest data for the treatment without aerial parts (Table 6). No significant effect has been found on root growth and root/shoot ratio but shoot growth of *U. bojeri* seedlings was significantly improved with *L. bojeriana* without aerial parts. Ectomycorrhizal colonization was significantly increased when *U. bojeri* seedlings were cultivated with *L. bojeriana* without aerial parts (Table 6). This positive impact was also recorded on the composition of ectomycorrhizal communities with the same RFLP types (except for UA3) as those found in the control treatment (UA1, UA2 and UA4) and two others only detected with the presence of *L. bojeriana* seedlings (Table 7).

With *E. camaldulensis* soil, dual cultivation treatments significantly improved soil pH, nitrogen content, and enzymatic activities with highest effects found in *L. bojeriana* seedlings without aerial parts for soil nitrogen content and FDA activity (Table 5). Opposite effects have been found for soil P content and soil organic matter (depressive effect provided by *L. bojeriana* seedlings without aerial parts). Dual cultivation treatments have enhanced the growth of *U. bojeri* seedlings and ectomycorrhizal colonization but no significant differences have been found between both *L. bojeriana* treatments (with or without aerial parts) and no effects have been recorded on the root/shoot values (Table 6). The presence of *L. bojeriana* seedlings allowed the development of some RFLP types not detected in the control treatment (UA1, UA2, UA3, UA4), increased the establishment of UB6 but limited UB4 multiplication (Table 7).

For the *P. patula* soil origin, dual cultivation treatments significantly improved soil P content and enzymatic activities, whereas the presence of entire *L. bojeriana* seedlings significantly decreased soil nitrogen and organic matter contents (Table 5). *U. bojeri* shoot growth and leaf foliar contents (N, P) have been significantly promoted by *L. bojeriana* seedlings (entire or not) (Table 6), and ectomycorrhizal colonization was higher in the dual cultivation treatment involving *L. bojeriana* seedlings without aerial parts (Table 6). Only UB6 RFLP type was detected in all the treatments, whereas UC3 recorded in the control treatment was absent in the dual cultivation treatments (Table 7). An opposite pattern was found with UA1 and UA4 RFLP types (Table 7).

Discussion

This study clearly shows that (1) the introduction of exotic tree species induces significant changes in the soil chemical characteristics, microbial activities and

Table 5 Effect of *L. bojeriana*/*U. bojeri* succession (pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings with aerial parts or without aerial parts) on soil chemical characteristics and enzymatic activities

Treatments	pH H_2O	Sol P[4]	Total N[5]	Total OM[6]	FDA[7]	Ac P[8]	Alk P[9]
Bulk soil							
Control[1]	5.7[10] (0.01) a[11]	2.00 (0.06) a	0.022 (0.001) a	4.20 (0.06) a	32.0 (6.4) a	498.8 (31.9) a	274.6 (6.2) a
L. bojeriana[2]	5.9 (0.01) b	4.47 (0.09) b	0.024 (0.001) a	6.33 (0.04) b	46.9 (1.4) ab	1,046.4 (52.1) b	359.5 (113.7) ab
L. bojeriana WA[3]	6.2 (0.02) c	5.50 (0.11) c	0.103 (0.001) b	9.65 (0.03) c	57.5 (3.6) b	1,334.5 (82.6) c	383.7 (22.1) b
U. bojeri soil							
Control	5.4 (0.01) b	5.35 (0.03) a	0.301 (0.001) a	7.32 (0.01) a	5.2 (0.36) a	715.6 (19.5) a	404.1 (11.6) a
L. bojeriana	5.4 (0.01) b	6.80 (0.06) c	0.412 (0.001) b	8.32 (0.04) b	49.8 (3.4) b	980.7 (23.4) b	512.2 (22.3) b
L. bojeriana WA	5.3 (0.02) a	6.32 (0.06) b	0.423 (0.001) c	8.72 (0.07) c	63.5 (2.2) c	1,044.3 (24.9) b	582.5 (17.7) b
E. camaldulensis soil							
Control[1]	5.3 (0.007) a	9.23 (0.03) c	0.054 (0.001) a	15.76 (0.09) b	6.1 (1.6) a	1,213.5 (19.9) a	214.3 (5.6) a
L. bojeriana[2]	6.3 (0.009) c	4.43 (0.09) b	0.064 (0.002) b	15.80 (0.06) b	21.4 (3.1) b	1,447.2 (48.1) b	417.1 (26.3) b
L. bojeriana WA[3]	5.4 (0.006) b	3.70 (0.06) a	0.071 (0.001) c	14.25 (0.03) a	68.3 (5.3) c	1,597.6 (8.3) c	394.4 (43.6) b
P. patula soil							
Control	6.2 (0.01) a	3.36 (0.09) a	0.087 (0.001) b	14.47 (0.09) b	22.2 (3.8) a	558.3 (55.2) a	288.5 (4.5) a
L. bojeriana	6.3 (0.01) a	7.60 (0.11) c	0.073 (0.001) a	14.05 (0.03) a	100.4 (8.6) c	1,257.1 (37.5) b	567.9 (18.3) c
L. bojeriana WA	6.2 (0.01) a	4.40 (0.06) b	0.090 (0.001) b	14.68 (0.06) b	66.4 (4.4) b	1,594.9 (49.3) c	331.4 (14.5) b

[1] *U. bojeri* without pre- and dual cultivation with *L. bojeriana*. [2] Pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings with aerial parts. [3] Pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings without aerial parts. [4] Soluble phosphorus (mg kg^{-1}). [5] Total nitrogen (%). [6] Total organic matter (%). [7] Total microbial activity (μg of hydrolyzed FDA h^{-1} g^{-1} of soil). [8] Acid phosphatase activity (μg *p*-nitrophenol g^{-1} of soil h^{-1}). [9] Alkaline phosphatase activity (μg *p*-nitrophenol g^{-1} of soil h^{-1}). [10] Standard error of the mean. [11] Data in the same column and for each soil origin followed by the same letter are not significantly different according to the Newman–Keuls test ($p < 0.05$)

Table 6 Effect of *L. bojeriana*/*U. bojeri* succession (pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings with aerial parts or without aerial parts) on the growth and ectomycorrhizal colonization of *U. bojeri* seedlings in soils collected under *Uapaca bojeri*, *Eucalyptus camaldulensis*, *Pinus patula* and from a bulk soil after 5 month culture in glasshouse conditions

Treatments	SB[4]	RB[5]	RB:SB[6]	N[7]	P[8]	ECM[9]
Bulk soil						
Control[1]	131 (11)[10] a[11]	113 (12) a	0.88 (0.13) b	0.89 (0.06) a	71.1 (7.3) a	36 (2.1) a
L. bojeriana[2]	277 (11) b	140 (10) ab	0.51 (0.04) a	3.02 (0.12) b	253.4 (10.9) b	42 (6) a
L. bojeriana WA[3]	309 (26) b	166 (3) b	0.55 (0.04) ab	3.08 (0.27) b	332.1 (29.1) b	90.3 (3.2) b
U. bojeri soil						
Control	125 (15) a	295 (35) a	2.37 (0.16) b	0.85 (0.1) a	94.1 (9.9) a	73.7 (3.2) a
L. bojeriana	222 (38) ab	242 (38) a	1.21 (0.33) a	2.14 (0.32) b	197.7 (34.1) b	78 (2.1) a
L. bojeriana WA	332 (19) b	219 (39) a	0.67 (0.14) a	3.58 (0.19) c	303.9 (14.1) c	90.7 (2.4) b
E. camaldulensis soil						
Control[1]	83 (0.9) a	27 (4) a	0.34 (0.08) a	0.65 (0.07) a	62.3 (7.3) a	16.3 (2.4) a
L. bojeriana[2]	233 (41) b	99 (6) b	0.45 (0.09) a	2.30 (0.41) b	194.6 (35.5) b	65.3 (3.3) b
L. bojeriana WA[3]	250 (42) b	129 (12) b	0.57 (0.17) a	3.17 (0.57) b	268.6 (44.9) b	79.3 (4.1) b
P. patula soil						
Control	85 (12) a	119 (10) a	1.42 (0.12) b	0.65 (0.09) a	58.9 (8.7) a	29.3 (5.5) a
L. bojeriana	233 (9) b	146 (27) a	0.62 (0.11) a	2.28 (0.10) b	181.3 (5.7) b	30.3 (2.4) a
L. bojeriana WA	333 (66) b	127 (7) a	0.41 (0.08) a	3.90 (0.78) b	278.1 (53.9) b	65.3 (1.5) b

[1] *U. bojeri* without pre- and dual cultivation with *L. bojeriana*. [2] Pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings with aerial parts. [3] Pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings without aerial parts. [4] Shoot biomass (mg dry weight). [5] Root biomass (mg dry weight). [6] Root:shoot ratio. [7] N leaf mineral content (mg per plant). [8] P leaf mineral content (mg per plant). [9] Ectomycorrhizal colonization (%). [10] Standard error of the mean. [11] Data in the same column and for each soil origin followed by the same letter are not significantly different according to the Newman–Keuls test ($p < 0.05$)

on ectomycorrhizal communities, (2) exotic-invaded soil significantly reduces the early growth and ectomycorrhization of *U. bojeri* seedlings, and (3) ectotrophic early-successional shrub species such as *L. bojeriana* could lower these negative effects provided by *E. camaldulensis* and *P. patula* by facilitating ectomycorrhizal establishment and consequently improved the *U. bojeri* early growth.

Numerous studies have reported that the introduction of exotic tree species has an environmental impact on soil characteristics (i.e., soil nutrient contents, water dynamics, etc.) (Smith et al. 2000; Sicardi et al. 2004) but with opposite results on soil biofunctioning indicators. For instance, Sicardi et al. (2004) reported that the conversion of pasture land to planted *Eucalyptus grandis* forest decreased FDA hydrolysis, acid and alkaline phosphatase activities that are directly involved in the transformation of soil organic matter. On the opposite, other studies have shown higher availability of nitrogen in exotic-invaded soils (Kourtev et al. 1999; Ehrenfeld et al. 2001). Our results are in accordance with these previous studies for soil N contents. However, we report higher rates of acid phosphatase activity under exotic plant species (*P. patula* and *E. camaldulensis*) that probably result from the more acid conditions encountered under these two exotic species and in contrast suppress alkaline phosphatase activities (Acosta-Martinez and Tabatai 2000; Krämer and Green 2000). These results are in accordance with those of Kourtev et al. (2002) as the higher rates of acid phosphatase reflected the organic-rich horizons with large amounts of recalcitrant compounds which accumulate under *E. camaldulensis* and *P. patula*.

All these biological changes have resulted to a lowest early growth of *U. bojeri* seedlings and in particular to a decrease of ectomycorrhiza formation. A previous study suggested that *Pinus* spp. was enable to associate with native fungi in exotic habitats leading to unsuccessful establishment when ECM fungi are lacking (Mikola 1970). It agrees with our data where this tree species selected a few ectomycorrhizal

Table 7 Relative abundance of RFLP types harvested in *U. bojeri* seedlings in the cultural patterns with *L. bojeriana* (pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings with aerial parts or without aerial parts) in soils collected under *Uapaca bojeri*, *Eucalyptus camaldulensis*, *Pinus patula* and from a bulk soil after 5 month culture in glasshouse conditions

Treatments	Relative abundance of RFLP types (%)									
	UA1	UD1	UA2	UA3	UC3	UA4	UB6	UC2	UB5	UB4
Bulk soil										
Control[1]	0.0	0.0	0.0	0.0	89.4	0.0	0.0	3.1	7.5	0.0
L. bojeriana[2]	26.5	0.0	27.9	0.0	0.0	0.0	7.4	0.0	0.0	38.2
L. bojeriana WA[3]	19.3	0.0	49.5	0.0	0.0	0.0	0.0	0.0	0.0	31.2
U. bojeri soil										
Control	51.5	0.0	43.0	3.7	0.0	1.8	0.0	0.0	0.0	0.0
L. bojeriana	13.0	0.0	18.5	0.0	19.6	19.6	29.3	0.0	0.0	0.0
L. bojeriana WA	14.7	0.0	16.7	0.0	11.8	22.5	34.3	0.0	0.0	0.0
E. camaldulensis soil										
Control[1]	0.0	11.9	0.0	0.0	58.7	0.0	11.9	0.0	0.0	17.5
L. bojeriana[2]	23.8	0.0	0.0	20.6	0.0	12.8	42.8	0.0	0.0	0.0
L. bojeriana WA[3]	20.2	0.0	12.1	19.2	0.0	25.3	23.2	0.0	0.0	0.0
P. patula soil										
Control	0.0	63.2	0.0	0.0	20.8	0.0	16.0	0.0	0.0	0.0
L. bojeriana	22.6	0.0	0.0	0.0	0.0	28.3	49.1	0.0	0.0	0.0
L. bojeriana WA	17.8	0.0	0.0	0.0	0.0	35.6	46.6	0.0	0.0	0.0

[1] *U. bojeri* without pre- and dual cultivation with *L. bojeriana*. [2] Pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings with aerial parts. [3] Pre-cultivation with *L. bojeriana* and dual cultivation with *L. bojeriana* seedlings without aerial parts. UA1: *Russula earlei*, UD1: *Bondarcevomyces taxi*, UA2: *Amanita* sp., UA3: Telephoroid mycorrhizal sp., UC3: *Russula exalbicans*, UA4: Uncultured ECM homobasidiomycete Clone E2, UB6: *Boletellus projectellus*, UC2: *Boletus rubropunctus*, UB5: *Coltricia perennis*, UB4: *Xerocomus chrysenteron*

symbionts such as *Russula exalbicans*. This ectomycorrhizal genus was largely distributed in tropical areas (Ducousso et al. 2004; Rivière et al. 2006; Diédhiou et al. 2010) and frequently recorded under tropical tree species (Rivière et al. 2005, 2006). In contrast to pine, it has been suggested that *Eucalyptus* spp. (i.e., *E. robusta*) was able to contract ectomycorrhizal associations in their introduction area with most of the native ectomycorrhizal symbionts (Tedersoo et al. 2007). Our results partially corroborated these data since ectomycorrhizal community associated with *U. bojeri* seedlings grown in soil collected under *E. camaldulensis* was more diverse than that found in soil sampled under *P. patula*. However, *E. camaldulensis* has negatively influenced the ectomycorrhizal establishment and consequently *U. bojeri* seedling growth largely than that which has been measured with *P. patula* soil. It is well known that *Eucalyptus* drastically alters the vegetation development where *Eucalyptus* litter accumulates through the release of allelochemicals (del Moral and Muller 1970). Hence, this allelopathic effect could limit the *U. bojeri* growth seedling and in particular root system development leading to a lower ectomycorrhiza establishment.

Uapaca bojeri seedlings growing in soil collected under *U. bojeri* adult tree showed much higher ectomycorrhizal infection and growth than those growing in the soil collected at a distance from established ectomycorrhizal vegetation. These data are consistent with results of previous works where it has been demonstrated that a lack of ectomycorrhizal infection distant from ectomycorrhizal vegetation or from adult tree that provides ectomycorrhizal propagules to the young seedlings could influence nutrient uptake and growth of seedling (Baxter and Dighton 2001; Lilleskov et al. 2002; Dickie and Reich 2005; Kisa et al. 2007). Moreover, it has been suggested that a rapid and early integration of seedlings into ectomycorrhizal mycelium radiating from mother plants could significantly improve survival and growth

of seedlings (Janos 1980, 1996; Onguene and Kuyper 2002). Our data support these observations with *U. bojeri* in a Madagascarian highland forest and showed that this tree species acts as a mother tree or nurse tree by promoting ectomycorrhizal formation and seedling growth. High root/shoot ratio has been identified as an important factor allowing plants to exploit reduced resource availability due to patchiness in distribution, both for water and nutrients (Reader et al. 1992). These high ratios would be of great importance in the regeneration process of native tree species especially during periods of drought or where nutrient resources are heterogeneously distributed. Hence, the exotic tree species (*P. patula* and *E. camaldulensis*) could limit the growth of *U. bojeri* young regeneration, whereas the presence of *U. bojeri* mother tree facilitated the early development of *U. bojeri* seedlings.

In tropical forests, one of the main biological processes that ensure recovery rates of tree species depends on the amount and activity of mycorrhizal inoculum. Ectomycorrhizal mycelia radiating from mother tree roots function as a source of ectomycorrhizal infection for neighboring host plants and more particularly for young tree regeneration (Jonsson et al. 1999; Matsuda and Hijii 2004; Nara 2005). In addition plants could become connected to a common mycorrhizal network that could be highly beneficial for growth and fitness of seedlings (Nara 2005). When ectomycorrhizal potential (abundance and diversity of ectomycorrhizal propagules) is lowered following natural or anthropogenic disturbance (Allen 1987; Jones et al. 2003), seedling establishment is limited and it is necessary to reinforce ectomycorrhizal infection potential. It has been previously demonstrated that a herbaceous ectomycorrhizal perennial of prairies, *Helianthemum bicknellii*, could permit the survival of ectomycorrhizal propagules and create patches of high ectomycorrhizal infection potential that facilitate the establishment of *Quercus*, an ectomycorrhizal tree species (Dickie et al. 2004). From the present study, similar effects have been provided by the ectomycorrhizal shrub species, *L. bojeriana*. Among ectotrophic early-successional plants recorded in the studied area, *Leptolaena* genus was highly represented and facilitated ectomycorrhizal infection and growth of *U. bojeri* seedlings but also enhanced soil chemical characteristics and enzymatic activities. Since *L. bojeriana* shared ectomycorrhizal fungi with *U. bojeri* (i.e. *Russula earlei*, *Amanita* sp., etc.), this shrub species has significantly enhanced ectomycorrhizal colonization of *U. bojeri* seedlings. This nursing effect was more particularly recorded in the treatments with exotic-invaded soils. In the *P. patula* and *E. camaldulensis* soils, *L. bojeriana* stimulated *U. bojeri* total growth by 2.3× and 3.4×, respectively, whereas this positive effect was 1.3× with the *U. bojeri* soil. This result supports the hypothesis that facilitation generally increasing in importance with increasing abiotic stress (Liancourt et al. 2005). In addition N, P nutrient uptake of *U. bojeri* seedlings was significantly enhanced in the dual cultivation treatments. Foliar N and P contents were significantly correlated with ectomycorrhizal colonization. Hence, by facilitating ectomycorrhizal propagule multiplication, *L. bojeriana* enhanced ectomycorrhizal infection of *U. bojeri* that is known to improve plant nutrient uptake (Dickie et al. 2002). In addition, *U. bojeri* nutrition may benefit from the ectomycorrhizal network radiating from *L. bojeriana* root systems that explores a larger volume of soil than *U. bojeri* alone. These connections could lead to N and P or carbon transfers between *U. bojeri* and *L. bojeriana* seedlings via mycorrhizal linkages (Simard et al. 1997). No significant effect has been recorded between treatments with entire *L. bojeriana* seedlings and *L. bojeriana* seedlings without aerial parts. It suggests that no competitive interactions occur between each plant species. The association in a common mycelial network of each plant species has probably lowered the cost of establishing mycorrhizal infection (Newman 1988).

This study provides evidence that *L. bojeriana* can facilitate the ectomycorrhizal infection of *U. bojeri* and mitigates the negative effects of the introduction of exotic tree species on the early growth and ectomycorrhizal formation of the native tree species. However, the mechanisms involved in this nursing effect have to be elucidated since multiple abiotic and biotic factors are involved. From a practical point of view, the use of ectotrophic early-successional shrub species has to be considered in tropical areas to improve the performances of reafforestation programs with native tree species.

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