International Journal of Ecology and Ecosolution
Vol. 1(1), pp. 1-15, April 2014
Full Length Research Paper

# Ectomycorrhizal fungi on the early colonizing shrub *Sarcolaena oblongifolia* F. facilitate the establishment of an endemic tree *Uapaca bojeri* L. in Madagascarian highland forests

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Accepted 8 April, 2014

**ABSTRACT**

Ectomycorrhizal fungal community is largely integrated into the overall diversity and ecosystem processes. This study aims to determine the below-ground structure of ectomycorrhizal fungi community associated with *Uapaca bojeri* and *Sarcolaena oblongifolia* in highland sclerophyllous forest of Madagascar and to assess the impact of shared ectomycorrhizal symbiont on tree seedling recruitment and plant succession facilitation. Ectomycorrhizal fungi were identified based on morphotyping and RFLP analysis of rDNA internal transcribed spacer (ITS)-large subunit followed by sequence analysis of each RFLP pattern. Impacts of shared ectomycorrhizal symbionts on seedling development of *U. bojeri* were investigated in soil microcosm collected at different distances from naturally established of both host plant species. The ECM fungal communities associated with both plants showed high similarity and were dominated by the genus *Russula*. Expressed as RFLP types, 21 taxa of ECM fungi were collected from root tips. More than half of these ECM taxa were shared between the two plant species. The better development of *U. bojeri* seedling was observed in soil samples naturally influenced by *S. oblongifolia* and located between 5 and 10 m from established *U. bojeri* where seedlings were colonized particularly by shared ectomycorrhizal symbionts. This study demonstrates that natural establishment of secondary colonizing hosts may contribute to tree seedling recruitment and development by providing compatible ectomycorrhizal symbionts.

**Keywords:** Below-ground ectomycorrhizal community, secondary successional processes, facilitation, ITS-RFLP, Madagascar.

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## INTRODUCTION

Mycorrhizal fungi are ubiquitous components of most ecosystems throughout the world and are considered key ecological factors in governing the cycles of major plant nutrients and in sustaining the vegetation cover (van der

Hejden et al., 1998; Requena et al., 2001; Schreiner et al., 2003). Two major forms of mycorrhizas are usually recognised: the arbuscular mycorrhizas (AM) and the ectomycorrhizas (ECMs). Arbuscular mycoorrhizal symbiosis is the most widespread mycorrhizal association type with plants that have true roots, that is, pterydophytes, gymnospermes and angiosperms (Read et al., 2000). They affect about 80 to 90% land plants in natural, agricultural, and forest ecosystems (Brundrett, 2002). ECMs affect trees and shrubs and usually result from the association of Homobasidiomycetes with about 20 families of mainly woody plants (Smith and Read, 2008). These fungal symbionts improve host performance by enhancing nutrient and water uptake from the soil and protecting host roots from pathogens and toxic compound (Smith and Read, 2008).

High diversity of ectomycorrhizal fungi is frequently reported in natural forests and forest plantations (Bruns, 1995; Horton and Bruns, 2001; Taylor, 2002; Ramanankierana et al., 2007). Numerous studies have shown that ectomycorrhizal fungal species diversity and abundance may be affected by various soil properties such as organic matter content (Harvey et al., 1986), moisture content (O'Dell et al., 1999), fertility (Lilleskov et al., 2001) and plant cover composition (Dighton et al., 1986; Dickie et al., 2002; Wardle, 2002; Ishida et al., 2007; Morris et al., 2008). At a global scale, availability of ectomycorrhizal propagules are supported by ectomycorrhizal vegetation that determines the distribution in terms of presence, abundance, community composition of ectomycorrhizal fungi (Molina et al., 1992; Newton and Haigh, 1998; Wardle, 2002; Ishida et al., 2007; Morris et al., 2008). These relationships are of particular importance since a loss of ectotrophic plant cover leads to decrease in availability of ectomycorrhizal fungi and limits seedling establishment (Jones et al., 2003; Dickie and Reich, 2005). It is well known that ectotrophic vegetation is unlikely to survive in locations free of ectomycorrhizal fungi (Marx, 1991). In addition plant cover alterations are often associated with damages to the biological, physical and chemical status of the soil (Francis and Thornes, 1990; Vagen et al., 2006a, b).

In degraded areas, it could be critical to permit survival of ectomycorrhizal fungi because of the scarcity of ectomycorrhizal vegetation and of the effect of soil erosion that reduces ectomycorrhizal propagules (Marx, 1991; Jasper et al., 1991; Herrera et al., 1993; Dickie and Reich, 2005). It has been previously suggested that revegetation programmes using planting early-successional shrubs rather that tree species, would help prevent erosion and facilitate natural regeneration of late-successional tree species (Kennedy and Sousa, 2006; Sanchez-Gomez et al., 2006; Richard et al., 2009). This facilitation of forest woody species growth provided by early-successional shrubs has been called the "nurse plant effect" (Franco and Nobel, 1988; Scarano, 2002). The role of nurse plants in facilitating plant establishment in stressful environment is usually explained by the ability of these early colonists to colonize disturbed sites (Scarano, 2002), by creating buffer zone to moderate the effects of wind and solar exposure and to ameliorate chemical, physical and biological soil properties (Nara and Hogetsu, 2004; Cline et al., 2005). More recently, experimental investigations have shown that mycorrhizal fungi had key roles in this nurse plant effect (Baxter and Dighton, 2001; Dickie et al., 2002; Rillig and Mummey, 2006; Nara, 2006; Richard et al., 2009). For instance ECM communities mediate plant species coexistence through shared interspecific hyphal links (Dickie et al., 2004; Selosse et al., 2006; Nara, 2006) or by providing compatible fungal symbionts (Simard et al., 2002; Dickie et al., 2004; Bever and Schultz, 2005). Since infective ectomycorrhizal propagules are quiet limited in degraded soils or in the early stages of primary successions, ectotrophic nurse plant species accompanied tree species might facilitate the establishment and growth of late-successional tree species by improving the ectomycorrhizal soil potential (Nara and Hogetsu, 2004; Nara, 2006; Richard et al., 2009). Despite numerous investigations, structure of ECM communities and its impact on seedling recruitment and secondary succession were rarely documented in tropical ecosystems.

Madagascarian highland sclerophyllous forest is almost the only remnant of the forest that previously covered large areas of the southern half of the central plateau in Madagascar. Tree species composition is widely dominated by *Uapaca bojeri*, an endemic Euphorbiaceae of Madagascar that is associated with *Asteropeia micraster* (Asteropeiaceae) in the southern part of the forest. Understorey vegetations harbor some ectomycorrhizal host species belonging to Sarcolaenaceae and Asteropeiaceae, two botanical families entirely endemic of Madagascar (Ducousso et al., 2004, 2008). Populations of *U. bojeri* drastically decreased at the edge of the forest and contrastingly, these are mainly dominated by Sarcolaenaceae-type vegetation (e.g., *Sarcolaena oblongifolia*). In a previous study, we reported high diversity of above-ground ECM sporophores associated with *U. bojeri* and underlined that the development of this tree species is highly ectomycorrhizal dependant (Ramanankierana et al., 2007). Since *S. oblongifolia* frequently accompanied *U. bojeri* in sclerophyllous forests, we hypothesized that this early-successional shrub could act as a plant nurse to ensure the establishment and development of *U. bojeri* seedlings. To test this first hypothesis, direct field-based studies on ECM associations have been carried out in stands dominated either by *U. bojeri* or *S. oblongifolia* and in mixed stands point out patterns of ECM community recruited by the plant species. We implemented morphological and molecular-based methods to identify plant and fungal species of ectomycorrhizal structures collected in the field. From

these data we ascertained through the second experiment the relative importance of generated ECM community by each of the two host plant species on *U. bojeri* seedlings development testing the hypothesis that *S. oblongifolia* facilitate *U. bojeri* seedling establishment by providing compatible ectomycorrhizal fungal symbionts. Therefore, the objectives of our study were: (i) to describe structure and diversity of below-ground ECM communities associated with *U. bojeri* and *S. oblongifolia*; and (ii) to assess the importance of the nursing effect of *S. oblongifolia* on *U. bojeri* seedling development.

## MATERIALS AND METHODS

### Study area

The study was conducted within the central part of Madagascarian highland sclerophyllous forest (20° 39' S, 47° 06' E). This forest formation has been modified by the annual grassland fire regimes and its persistence is due to the resistance of the *U. bojeri* adult trees to these fires. The main annual rainfall varied from 1136 to 1554 mm with 6 months long of dry season. Study areas were selected among the surface areas where little anthropogenic disturbance occurred during at least the last five years. The small cluster of these forest structures occurs on sandstone to quartzite and forms mixed stands with *S. oblongifolia*. Forest cover is generally species-poor with a flora on the surrounding exposed rocks containing many endangered orchids and succulents such as miniature *Aloe* and *Pachypodium* species. *S. oblongifolia* is an early-successional shrub and often occurred in clumped distribution on degraded and exposed surface areas of *U. bojeri* formation or at forest edge. This shrub species has a long stem (1.5 to 2.5 m), stiff spreading hairs. Sclerophyllous forest ecosystem is the driest region from which *S. oblongifolia* is known, this shrub species exhibit morphological characteristic that reflect this, including small leaves and short petioles, as well as very reduced inflorescence. *U. bojeri* is a tree species long to 10 to 12 m, often smaller. Seedlings and adult trees of these two plant species have shallow root systems. To the east of the study site, *U. bojeri* trees were replaced by shrub formation where *S. oblongifolia* is associated with some shrub species such as *Aphloia theaformis* (Vahl.) Benn., *Helychrysum rusillonii* Hochr., *Leptolaena pauciflora* (Baker.), *Leptolaena bojeriana* (Baill.) Cavaco. and *Psiadia altissima* (D.C.) Drake.

### Morphotyping and molecular analysis of ectomycorrhizal communities (Experiment 1)

#### *Root and soil sampling*

Soil cores were collected in December 2006 from three microhabitats on the basis of ECM host plant composition: (i) a homogenous population of *U. bojeri* trees devoid of *S. oblongifolia* or other ECM host plant, (ii) a clumped population of *S. oblongifolia* saplings devoid of other ECM host plant and, (iii) a mixture formation of *U. bojeri* and *S. oblongifolia*. Five plots were randomly identified for each type of host plant assemblage.

We aimed to collect soil cores likely to contain ectomycorrhizas from exclusively *U. bojeri* or *S. oblongifolia* in one hand and ectomycorrhizas from both *S. oblongifolia* and *U. bojeri* saplings in the other hand. In order to avoid combining roots from different host plant species, we developed a sampling scheme according to which the nearest undesirable ECM host plant was located at least 20 to 25 m from the sampling point. Soil cores were sampled between two individuals of *U. bojeri* or two individuals of *S. oblongifolia,* depending on case, occurring within 3 to 4 m from each other. For the mixture samples, soil cores were sampled near seedling of *U. bojeri* (about one year old) occurring under *S. oblongifolia* sapling where at least three other seedlings of *U. bojeri* were observed within a circle of 1 m radius. Ectomycorrhizas were collected with a 10 cm diameter soil corer driven from the humus organic horizon to a depth of 30 cm. One soil core was taken in each plot for a total of 15 soil cores classified into three groups according to the host plants assemblage. Soil cores were kept at 4°C and processed for ECM root tips within two weeks.

Soil cores were soaked in water for 12 h at room temperature and roots were isolated and recovered thought a soil sieve (0.5 mm mesh size). Roots were carefully washed in running tap water before sorting by morphology under dissecting microscope. Mycorrhizal features criteria 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. To avoid combining different fungal species with similar morphotypes, ectomycorrhizas from each core were treated separately until RFLP type identification. All morphological types of ECM 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.

#### *PCR amplification of DNA*

DNA was extracted from ectomycorrhizas using Qiagen Dneasy Plant Mini Kits (Qiagen SA, Courtaboeuf, France) following the manufacturer's recommendations. This method extracted both host plant and fungi DNA. Fungal mitochondrial rDNA was amplified using the specific primers ML5 (5'-CTCGGCAAATTATCCTCATAAG-3') and ML6 (5'-CAGTAGAAGCTGCATAGGGTC-3') (White et al., 1990). PCR reaction was performed in a total volume of 25 µl, containing aliquots of 1 µl of genomic DNA, 1 µM of each primer, 1.5 units of *Taq* DNA Polymerase (Amersham Pharmacia Biotech), 10 mM of Tris-HCl, 50 mM of KCl, 1.5 mM of $MgCl_2$, 200 µl of each dNTP. Amplification was run in a Thermocycler (GenAmp PCR System 2400, Pekin Elmer, Foster City, California) as followed: 1 cycle for 5 min at 95°C followed by 40 cycles at 94°C for 45 s, 55°C for 30 s, 72°C for 1 min 30 s, and extension at 72°C for 7 min. Negative controls without DNA were used to detect DNA contaminations of reagents in each PCR. We confirmed amplification in a 2% agarose gel stained with ethidium bromide.

#### *RFLP analysis*

Accuracy of morphotype identity was performed by RFLP analysis using a part of amplified DNA. Fungal ITS-RFLP patterns were produced by digesting aliquots of 10 µl of each amplified DNA with 2 units of endonuclease *Hae*III or *Hinf*I in a 20 µl of supplied reaction buffer for 2 h at 37°C. The restriction bands were separated by electrophoresis in 2.5% high-resolution agarose gels [38% agarose + 62% NU-sieve agarose (FMC Bioproducts, Philadelphia, PA, USA)] stained in a 1x TAE with ethidium bromide at 10 mg/ml in the running buffer. The 100pb DNA ladder (GOBCO BRL) was used as a size standard. Gels were photographed under UV light and RFLP band sizes were calculated using gel compar software. Samples that displayed identical RFLP patterns within cores, including any sub-molar bands were considered to belong to the same RFLP types. Three groups of RFLP types were then created following the host plants species: *U. bojeri*, *S. oblongifolia*

**Table 1.** Identification by ITS sequence of the most RFLP types collected on *U. bojeri* and-/or *S. oblongifolia.*

| RFLP types | Closest GenBank species | GenBank accession number | BLAST expected value | Identified host plant |
|---|---|---|---|---|
| Ja *c* | *Russula earlei* | AF518722 | 2e-142 | *S. Oblongifolia* and *U. bojeri* |
| Bl *c* | *Uncultured ectomycorrhizal fungus clone c71* | KC424542 | 6e-168 | *S. oblongiofolia* and *U. bojeri* |
| Ja *a* | *Boletellus projectellus* | DQ534582 | 0.0 | *U. bojeri* |
| Br *a* | *Bondarcevomyces taxi* | DQ534583 | 2e-169 | *S. Oblongifolia* |
| Mr *d* | *Bondarcevomyces taxi* | DQ534583 | 0.0 | *S. oblongiofolia* and *U. bojeri* |
| Mr *e* | *Bondarcevomyces taxi* | DQ534583 | 0.0 | *S. Oblongifolia* |
| Nr *b* | *Bondarcevomyces taxi* | DQ534583 | 0.0 | *S. oblongiofolia* and *U. bojeri* |
| Nr *a* | *Bondarcevomyces taxi* | DQ534583 | 0.0 | *S. oblongiofolia* and *U. bojeri* |
| Br *b* | *Boletellus projectellus* | DQ534582 | 0.0 | *S. oblongiofolia* and *U. bojeri* |
| Bl *b* | *Scleroderma dictyosporum* | AJ509787 | 2e-175 | *S. Oblongifolia* and *U. bojeri* |
| Bl *a* | *Scleroderma citrinum* | AF393149 | 2e-175 | *S. oblongiofolia* and *U. bojeri* |
| | *Tapinella panuoides* | DQ534594 | 2e-98 | *S. Oblongifolia* |
| Ja *f* | *Russula exalbicans* | AY293269 | 9e-167 | *U. bojeri* |
| Mr *c* | *Russula fulvo-ochrascens* | AM117671 | 2e-169 | *S. Oblongifolia* |
| Ja *b* | *Russula earlei* | AF518722 | 9e-154 | *S. oblongifolia* and *U. bojeri* |
| Ja *d* | *Russula earlei* | AF518722 | 7e-143 | *U. bojeri* |
| Mr *a* | *Russula earlei* | AF518722 | 2e-144 | *U. bojeri* |
| Bl *d* | *Russula sp. C353gn* | AM117670 | 2e-110 | *S. oblongiofolia* and *U. bojeri* |
| Bl *e* | *Russula earlei* | AF518722 | 4e-158 | *S. oblongifolia* |
| Mr *b* | *Tomentella sp.* | U86859 | 1e-152 | *S. Oblongifolia* and *U. bojeri* |
| Ja *g* | *Thelephoroid mycorrhizal sp.* | AJ509798 | 3e-154 | *S. Oblongifolia* |

and mixture host plants.

***Host plant identification***

Ectomycorrhizas from mixture sample could be associated with the two host plants species either *U. bojeri* or *S. oblongifolia*. Host plant identification of ectomycorrhizas type was performed by sequencing a portion of the chloroplast genome encoded the ribulose-biphosphate carboxylase gene (*rbc*L). The plant specific primer *rbc*LR and *rbc*LN (Käss and Wink, 1997) were used to amplify plant rbcL DNA. Both strands DNA were sequenced with the same primers with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, California) 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). They were then compared with published sequences of the two plants in the Genbank NCBI.

***Sequencing and molecular identification of ECM symbionts***

To specify their taxonomic position, all RFLP types were sequenced by using the same primers as in fungal mitochondrial rDNA amplification. Sequencing was performed in the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, California) and released sequences were aligned and corrected as described above. All sequences from individual RFLP types were identified according to BLAST analysis at the NCBI page http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, using default settings (Table 1).

**Dynamic of *U. bojeri* seedling development on soil samples collected from different microhabitats (Experiment 2)**

***Soil sampling, chemical properties and enzymatic activity assessments of sampled soils***

Soil samples were collected from sampling points identified in a 20 m transect line drawn from individual *U. bojeri* tree

**Table 2.** Sampling design, host plant colonization by ectomycorrhizal fungal and fungal diversity.

| Parameter | Origins | | |
|---|---|---|---|
| | *U. bojeri* | *S. oblongifolia* | Mixture vegetation |
| Number of soil cores | 5 | 5 | 5 |
| Number of ectomycorrhizas root tips | 665 | 580 | 780 |
| Number of RFLP types (without soil core comparison) | 38 | 46 | 53 |
| Percentage of shared identified taxa* between the two host plant species (%) | 60 | 58,33 | 76,92 |
| Simpson's diversity index | 6.75 | 10.58 | 11.22 |
| Shannon-Wiener information index | 0.87 | 1.05 | 1.07 |

*Taxa are identified as RFLP types based on similarity of ITS region to sequences in GenBank.

to forest edge passing through clumped population of *S. oblongifolia*. Thus, soils were sampled at 0, 1, 5, 10 and 20 m from individual tree of *U. bojeri* of transect line. Five transect lines were drown and each soil sample consisted of five sub-samples mixture collected at 10 to 20 cm depth. Control samples were randomly collected from bare soil sites, away from plant influence. For each soil sample, pH of a water soil suspension (10 g dry soil/15 ml deionized water) was determined. The total organic carbon was measured according to the ANNE method (Aubert, 1978) and the total nitrogen by the Kjeldhal method. The available phosphorus was assessed calorimetrically (Olsen et al., 1954) and total phosphorus content by spectrophotometer readings.

All soil samples were characterized by measuring microbial global activity. Total microbial activity in soil was measured by using fluorescein diacetate (3'6'-diacetyl fluorescein [FDA]) hydrolysis assay according to the method of Alef (1998). This enzymatic conversion released a final product that can be determined colorimetrically at 490 nm, after 1 h of soil incubation. Total microbial activity was expressed as µg of hydrolysis product corrected for background fluorescence per hour and per gram of soil. Deshydrogenase activity of soil sample was measured by absorbance readings at 490 nm following the method of Prin et al. (1989) and Schinner et al. (1996) with iodo nitro-tetrazolium (INT) as an artificial electron acceptor to form IDT-Formazan (INTF).

### *Seedling development assessment*

Soil samples collected at identified sampling points (0, 1, 5, 10 and 20 m) through transect lines and soil controls were packed in 1 L pots. Seeds of *U. bojeri* were collected at random from wild plants growing in the study site. They were surface-sterilized in hydrogen peroxide for 10 min, rinsed and soaked in sterile distilled water during 12 h and germinated on 1% agar. After 10 days of incubation at 30°C in the dark, one-pregerminated seed was planted per pot. Pots were arranged in a randomized complete block design with 50 replicates per treatment. Plants were screened from the rain, grown under natural light (day length approximately 12 h, average daily temperature 25°C) and watered daily with tap water during 5 month of culture.

The overall mortality rate was assessed monthly for each treatment during 5 months of growth. Then, plants were uprooted and the oven dry weight (1 week at 65°C) of the shoot was measured. Root systems were gently washed, cut into 1 cm root pieces, and mixed. Numbers of ectomycorrhizal roots and non-ectomycorrhizal roots were determined under a stereomicroscope (magnification x 60) for each lateral root to determine the percentage of ectomycorrhizal colonization (number of ectomycorrhizal short roots/total number of short roots). Characteristics of each ECM morphotype were then described using mycorrhizal features criteria following Agerer (1987 - 1996) and their numbers were determined for each seedling. Three ectomycorrhizal root tips randomly selected from each morphotype groups were screened by RFLP analysis following the method described above.

### Statistical analysis

Plant growth measurements 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. Data from experiment 2 were analyzed with Principal Component Analysis (PCA). PCA computations were performed with the ade4 package for the R software (Thioulouse and Dray, 2007; R Development Core Team, 2010). Simpson index which take into account the number of taxon present, as well as the relative abundance of each taxon was used to assess ECM diversity on each community.

## RESULTS

### Description of ectomycorrhizal fungi community

A total of 2025 ECM root tips were collected from 15 soil cores and classified into 165 ECM morphotypes without mixing root samples from different soil cores. DNA extraction and RFLP analysis were carried out on 495 ectomycorrhizas and revealed 137 RFLP types (Table 2). By comparing RFLP types from soil cores collected from the same host plants species assemblage types, 10 RFLP types were found with *U. bojeri*, 12 with *S. oblongifolia* and 13 in mixed plant community (Figure 1). Among all RFLP types from each of the three groups of host plants composition, 21 individuals RFLP pattern were found and identified based on similarity of the ITS region to sequences in GenBank (Table 1). Except for one RFLP type that was identified as Thelephoroid mycorrhizal, RFLP types were identified to the level of genus (3 RFLP types) or species (17 RFLP types). At the genus level, *Russula* was the most abundant represented by 8 RFLP types followed by *Bondarcevomyces* (5 RFLP types), *Scleroderma* (2 RFLP types) and *Amanita*, *Tapinella* and *Tomentella* with one RFLP type respectively (Table 1).

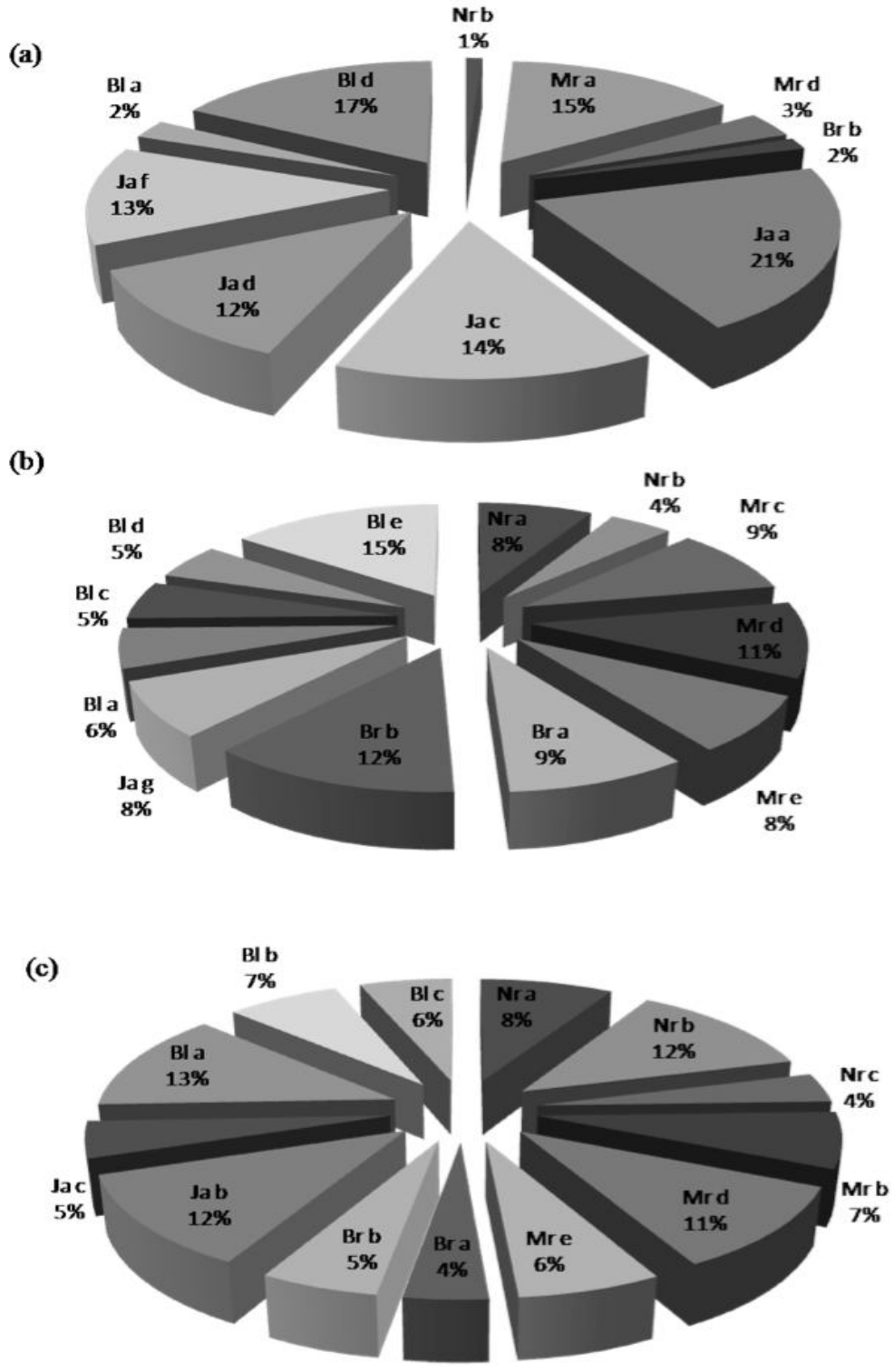


**Figure 1.** Structure of below-ground ectomycorrhizal community expressed by RFLP types percentage with respect to the three groups of host plant composition. (a): *Uapaca bojeri*; (b): *Sarcolaena oblongifolia*; (c): mixed vegetation of the two host plants. Ja *c*: *Russula earleri*; Bl *c*: *Amanita* sp.; Ja *a*: *Boletellus projectellus*; Br *a*: *Bondarcevomyces taxi*; Mr *d*: *Bondarcevomyces taxi*; Mr *e*: *Bondarcevomyces taxi*; Nr *b* : *Bondarcevomyces taxi*; Br *b*: *Boletellus projectellus*; Bl *b*: *Scleroderma dictyosporum*; Bl *a*: *Scleroderma citrinum*; Nr *c*: *Tapinella panuoïdes*; Ja *j*: *Russula exalbicans*; Mr *c*: *Russula fulvo-ochrascens*; Ja *b*: *Russula earlei*; Ja *d*: *Russula earlei*; Mr *a*: *Russula earlei*; Bl *d*: *Russula sp*; Bl *e*: *Russula earlei*; Mr *b*: *Tomentella*; Ja *g*: Thelephoroïd.

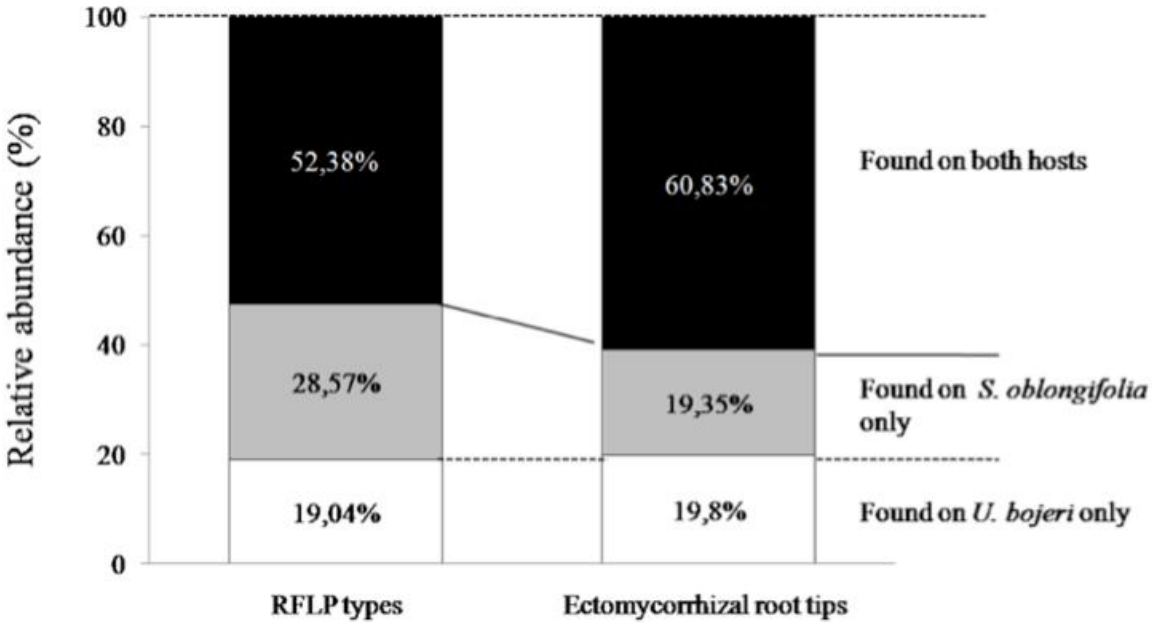


**Figure 2.** Distribution of ectomycorrhizal fungi between *Uapaca bojeri* and *Sarcolaena oblongifolia* expressed by percentage of RFLP types and percentage of total number of sampled ectomycorrhizal root tips.

**Ectomycorrhizal community distribution between both host plant species**

Overall, 52.38% of identified RFLP types were found in both *U. bojeri* and *S. oblongifolia* root system. The remaining RFLP types were found exclusively on either *U. bojeri* (19.04%) or *S. oblongifolia* (28.57%) (Figure 2). Simpson diversity index of below ground ectomycorrhizal community was similar between *S. oblongifolia* and mixed host plant vegetation. However, this diversity index is lower for *U. bojeri* suggesting that this tree species, in homogenous vegetation, harbored more rare species than it does with the multi-assemblage community. Similarity between the three origins of ectomycorrhizal fungi communities was revealed by the Shannon-Wiener information index (Table 2).

Except for ten individual taxa that differed between the two host plant species (6 with *S. oblongifolia* and 4 with *U. bojeri*), pattern of overall community structure were similar and many of the common species were detected on both *U. bojeri* and *S. oblongifolia* (Figure 3). For example, *Bondarcevomyces* (Mr *d*) was the most abundant species on *S. oblongifolia* and was also found on *U. bojeri*. In all, 11 taxa were shared by the two host plant species. Especially for ectomycorrhizas sampled from mixture of the two host plant species, 76.92% of the identified taxa were shared by the two plant species (Table 2). On the other hand, 4 taxa (3 *Russula* and 1 *Boletellus*) were only found in *U. bojeri* including the most frequent species on this host plant, *Boletellus prejectellus* (Ja *a*). Taxa detected exclusively in *S. oblongifolia* included 2 *Bondarcevomyces*, 2 *Russula*, one *Tapinella* and the unidentified Thelephoroïd.

**Soil chemical conditions within soil sample origins**

Chemical characteristic of soil samples varied slightly along the transect line. Among of total carbon and total organic matter allowed classifying soil samples in three groups. Soil collected from 0 and 1 m recorded the highest quantities of total carbon and total organic matter followed by soil samples from 5 and 10 m (Table 3). The lowest contents were recorded in control and in soil samples at 20 m from the *U. bojeri* adult tree. Total phosphorus contents in soil samples from 0 and 1 m were significantly high in comparison with quantities of this nutrient recorded in the others soil samples (5, 10, 20 m). There was no significant difference between soil samples for pH, total nitrogen and soluble phosphorus (Table 3). Total microbial activity recorded was significantly different within soil samples collected at different distances from tree of *U. bojeri*. Thus, soil samples from 5 and 10 m displayed the highest activities. The same significant distinction was found for deshydrogenase activity which values decreased as follows 10 m > 5 m > 1 m > 0 m > 20 m > Control (Table 3).

**Seedlings development assessment**

Mortality rate of *U. bojeri* seedlings was higher in control and in soil samples collected at 20 m of adult tree than in the others soil samples after three months of culture. At

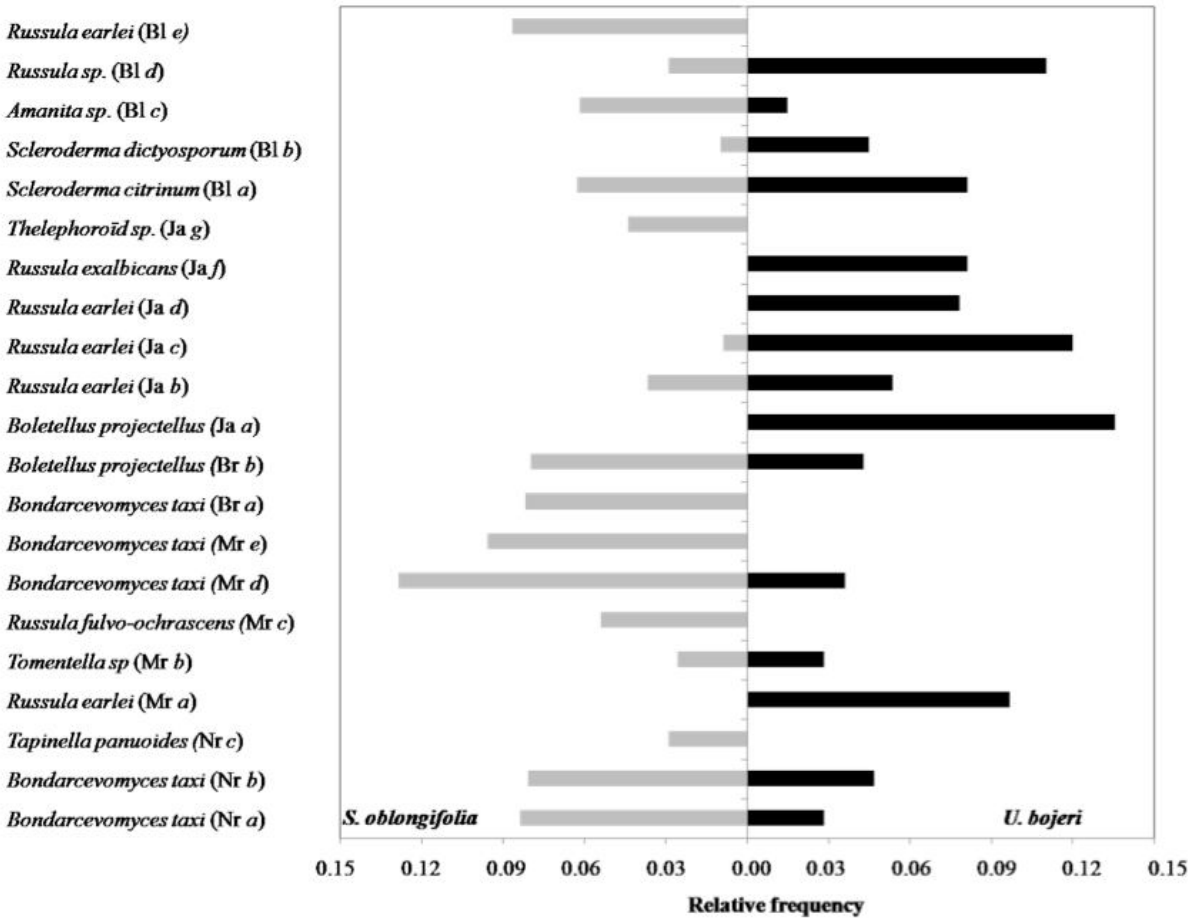


**Figure 3.** Relative frequency of identified RFLP types based on ITS region sequences on roots of *Uapaca bojeri* and *Sarcolaena oblongifolia*. Relative frequency was calculated as the number of occurrences of each RFLP type divided by the total number of occurrences of all RFLP types.

**Table 3.** Chemical characteristics and enzymatic activities of soil samples collected at different distances from *U. bojeri* individual tree and from the area away from plant influence (control).

| Parameter | Control | Distance from *U. bojeri* adult tree | | | | |
|---|---|---|---|---|---|---|
| | | 20 m | 10 m | 5 m | 1 m | 0 m |
| pH | 6.08a[(1)] | 5.27[a] | 5.32[a] | 5.24[a] | 5.41[a] | 5.37[a] |
| Total nitrogen (%) | 0.07[a] | 0.1[a] | 0.11[a] | 0.09[a] | 0.12[a] | 0.13[a] |
| Total carbon (%) | 0.86[a] | 0.98[a] | 1.71[b] | 1.78[b] | 2.77[c] | 2.92[c] |
| Total organic matter (%) | 0.92[a] | 1.19[a] | 2.47[b] | 2.26[b] | 5.61[c] | 5.13[c] |
| Total phosphorus (mg $kg^{-1}$) | 9.92[a] | 10.22[a] | 12[ab] | 12.49[ab] | 13.67[b] | 14.63[b] |
| Available phosphorus (mg $g^{-1}$) | 4.92[a] | 5.03[a] | 5.31[a] | 5.6[a] | 5.95[a] | 6.27[a] |
| Total microbial activity (µg of hydrolyzed fluorescein diacetate $h^{-1}$ $g^{-1}$ of soil) | 7.48[a] | 8.79[a] | 68.71[c] | 65.49[c] | 17.89[b] | 12.37[a] |
| Deshydrogenase activity (µg INTF $d^{-1}$ $g^{-1}$ of soil) | 27.05[a] | 32.48[a] | 141.17[d] | 112.86[c] | 65.49[b] | 56.34[b] |

[(1)] Data in the same line followed by the same letter are not significantly different according to the Newman-Keul test ($p< 0.05$).

the end of the experiment (5 months), up to 66 and 46% of seedlings were dead in control treatment and in 20 m soil samples respectively (Figure 4). In the others soil samples, mortality rate of seedlings were stabilized after the third month of culture. The lowest mortality was recorded with seedlings grown on soil sampled at 5 m of

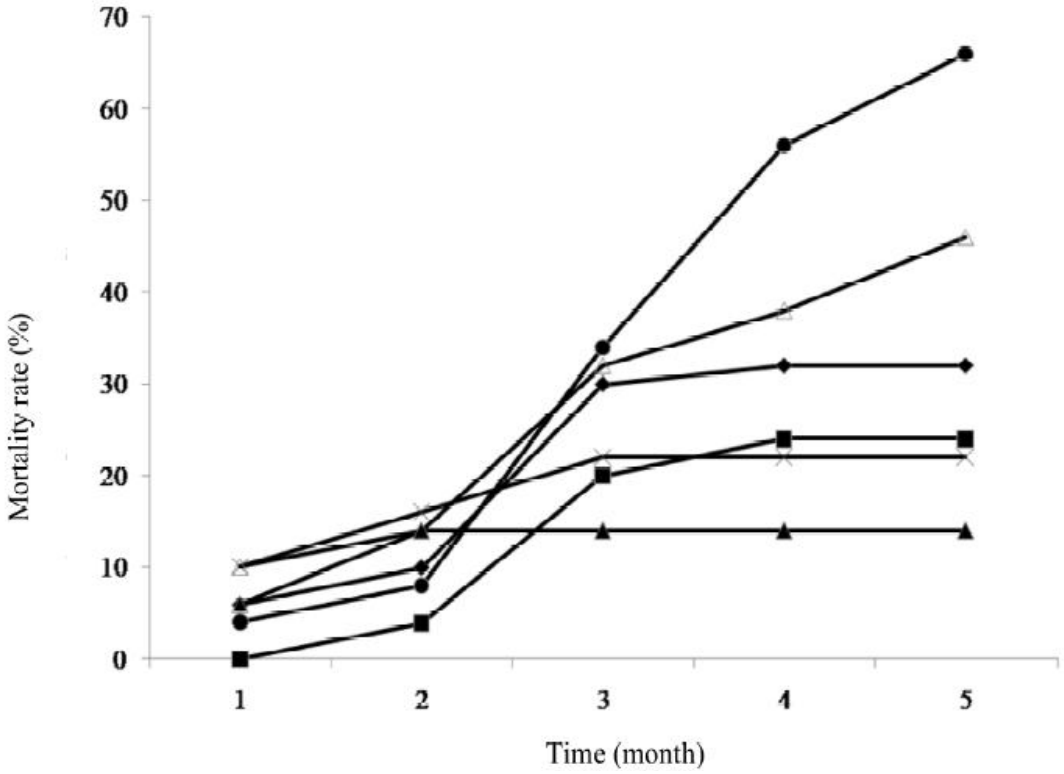


**Figure 4.** Mortality rate of *Uapaca bojeri* seedlings grown in soils collected at different distances from established *U. bojeri* adult tree during the first 5 months of development (● : control; Δ : soil samples collected at 20 m; × : soil samples collected at 10 m; ▲ : soil samples collected at 5 m; ■ : soil samples collected at 1 m; ♦ : soil samples collected at 0 m.

trees of *U. bojeri*. Mortality rate of seedlings changed as follows: 5 m < 10 m < 1 m < 0 m < 20 m < Control (Figure 4).

The growth of *U. bojeri* seedling was significantly higher in soil sampled from areas with established adult tree of *U. bojeri* than in the control treatment and in the soil sampled at 20 m. However the highest development of seedling were recorded in 5 and 10 m soil samples where shoot growth of seedling was stimulated on average 3.09 times compared to those measured in the control and the 20 m soil sample and 1.7 times compared to those recorded in the 0 and 1 m soil samples (Figure 5, Table 4). The same significant difference was observed for the root growth with highest values recorded in 5 and 10 m soil samples as well as for the rates of ectomycorrhizal colonization. Shoot: Root mass ratios of *U. boyeri* seedlings were significantly different between the 0 and 10 m soil origins (Table 4). Growth stimulation of *U. bojeri* seedling in 5 and 10 m soil samples was linked with significant higher ectomycorrhizal colonization rates (Figure 5, Table 4). No significant differences were found between ectomycorrhizal colonization of seedlings grown in 0 and 1 m soil samples. However, seedlings cultured in these soil samples displayed significantly higher ectomycorrhizal colonization rates than those grown in control treatment and in 20 m soil sample (Figure 5, Table 4).

**Pattern of early ectomycorrhizal fungi community associated with *U. bojeri* seedling**

At the end of the experiment, a total of 309 ectomycorrhizal tips were harvested from 30 seedling of *U. bojeri* (5 seedlings per soil sample). A total of 6 individuals of RFLP pattern were revealed by RFLP analysis. Seedlings were poorly colonized by ectomycorrhizal fungi except in 5 and 10 m soil samples where ectomycorrhizal colonization per seedling reached 22.05 and 21.06%, respectively (Figure 5, Table 4). Similarly, the mean ectomycorrhizal taxa richness, expressed as RFLP types, was higher with seedlings grown in these two soil samples compared to those grown in others soil samples. However, taxa richness remains low in all soil samples ranged from 0.5 to 3.5 taxa per seedling (Table 4). Ectomycorrhizal diversity estimated by Simpson's index was significantly higher in seedlings grown in 5 and 10 m soil samples compared to the other treatments (Figure 5).

All of the 6 individuals RFLP types were already detected within described ectomycorrhizal communities in experiment 1. Expressed as RFLP types, 4 of the 6 corresponding taxa (Bl *a*, Bl *d*, Br *b*, Nr *b*) in experiment 1 were associated with both of the two host plant species and the two others were exclusively found in either *S.*

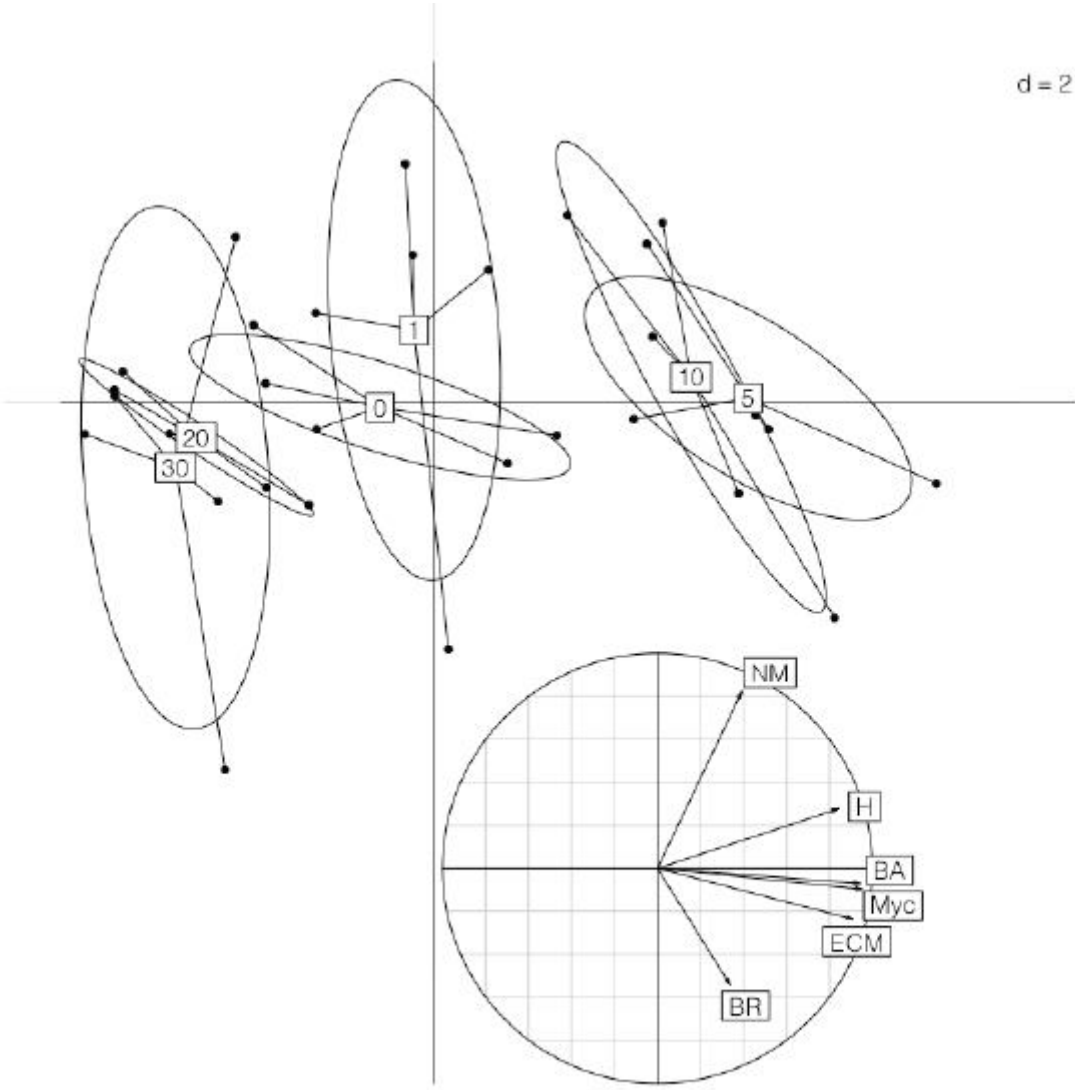


**Figure 5.** Responses of plant growth and ectomycorrhizal colonization according to the distance from *U. bojeri* tree adult analyzed with Principal Component Analysis (PCA). NM: Non mycorrhizal root types; Myc: ectomycorrhizal root types; ECM: ectomycorrhizal colonization rate; BA: shoot biomass; BR: root biomass; H: Simpson's index.

*oblongifolia* or *U. bojeri* (Table 5). RFLP types were differently distributed along the transects and 3 groups have been distinguished: Group 1 (Ja *c* and Nr *b*) highly represented at the lowest distances from *U. bojeri* adult trees, Group 2 (Bl *a*, Ja *b* and Bl *b*) highly represented at the 5 to 10 m distances and Group 3 (Bl *d*) recorded from all the seedlings (Figure 6).

## DISCUSSION

Ectomycorrhizal communities were increasingly recognized as among the key players in tropical ecosystem processes and functions (Henkel et al., 2002; Alexander, 2006; McGuire, 2007; Tedersso et al., 2008). On the other hand, host specificity of ectomycorrhizal fungi may be one of the origin of community differentiation (Tedersoo et al., 2008). Here, we investigated the structure and the ecological importance of below-ground ectomycorrhizal fungal communities associated with two tropical plants, *U. bojeri* and *S. oblongifolia* in order to verify the hypothesis that both host plants share some ectomycorrhizal symbionts which are involved in plant succession facilitation by enhancing development of *U. bojeri* seedling and improve the natural regeneration process of this tree species.

The main results of this study show that: (i) a large diversity of ectomycorrhizal symbionts was recorded under *U. bojeri* and that ectomycorrhizal fungal community varied with distance from adult tree, (ii) *S.*

**Table 4.** Growth and ectomycorrhizal development of *U. bojeri* seedlings after 5 months of culture in soil samples collected at different distances from established *U. bojeri* adult tree.

| Parameter | Control | Distances from *U. bojeri* adult tree | | | | |
|---|---|---|---|---|---|---|
| | | 20 m | 10 m | 5 m | 1 m | 0 m |
| Seedling development | | | | | | |
| Shoot biomass (mg per plant) | 92$^{a\ (1)}$ | 120$^{a}$ | 313$^{c}$ | 335$^{c}$ | 199$^{b}$ | 183$^{b}$ |
| Root biomass (mg per plant) | 61$^{a}$ | 75$^{a}$ | 168$^{bc}$ | 211$^{c}$ | 128$^{b}$ | 137$^{b}$ |
| Total biomass (mg per plant) | 154$^{a}$ | 195$^{a}$ | 481$^{c}$ | 546$^{c}$ | 328$^{b}$ | 320$^{b}$ |
| Shoot / Root mass ratio | 1.53$^{ab}$ | 1.65$^{ab}$ | 1.86$^{b}$ | 1.59$^{ab}$ | 1.64$^{ab}$ | 1.42$^{a}$ |
| Number of root tips per mg of root biomass | 0.83$^{b}$ | 0.73$^{b}$ | 0.51$^{ab}$ | 0.39$^{a}$ | 0.63$^{ab}$ | 0.49$^{a}$ |
| Ectomycorrhizal colonization (%) | 3.19$^{a}$ | 5.88$^{ab}$ | 21.06$^{c}$ | 22.05$^{c}$ | 11.70$^{bc}$ | 10.49$^{bc}$ |
| Ectomycorrhizal diversity | | | | | | |
| Taxa richness (expressed as RFLP type) | 0.5$^{a}$ | 0.8$^{a}$ | 3$^{c}$ | 3.5$^{c}$ | 1.6$^{ab}$ | 2$^{b}$ |

(1) Data in the same line followed by the same letter are not significantly different according to the Newman-Keul test ($p< 0.05$).

**Table 5.** Relative frequency of RFLP types harvested in *U. bojeri* seedlings and host plant species in experiment 2.

| RFLP types | Relative frequency | Host plant species of correspondant RFLP type in experiment1 | Closest GenBank species |
|---|---|---|---|
| Bl *a* | 0.25 | *S. oblongiofolia* and *U. bojeri* | *Scleroderma citrinum* |
| Bl *d* | 0.08 | *S. oblongiofolia* and *U. bojeri* | *Russula* sp. C353gn |
| Ja *b* | 0.2 | *U. bojeri* | *Russula earlei* |
| Ja *c* | 0.15 | *S. Oblongifolia* | *Russula earlei* |
| Bl *b* | 0.18 | *S. oblongiofolia* and *U. bojeri* | *Scleroderma dictyosporum* |
| Nr b | 0.12 | *S. oblongiofolia* and *U. bojeri* | *Bondarcevomyces taxi* |

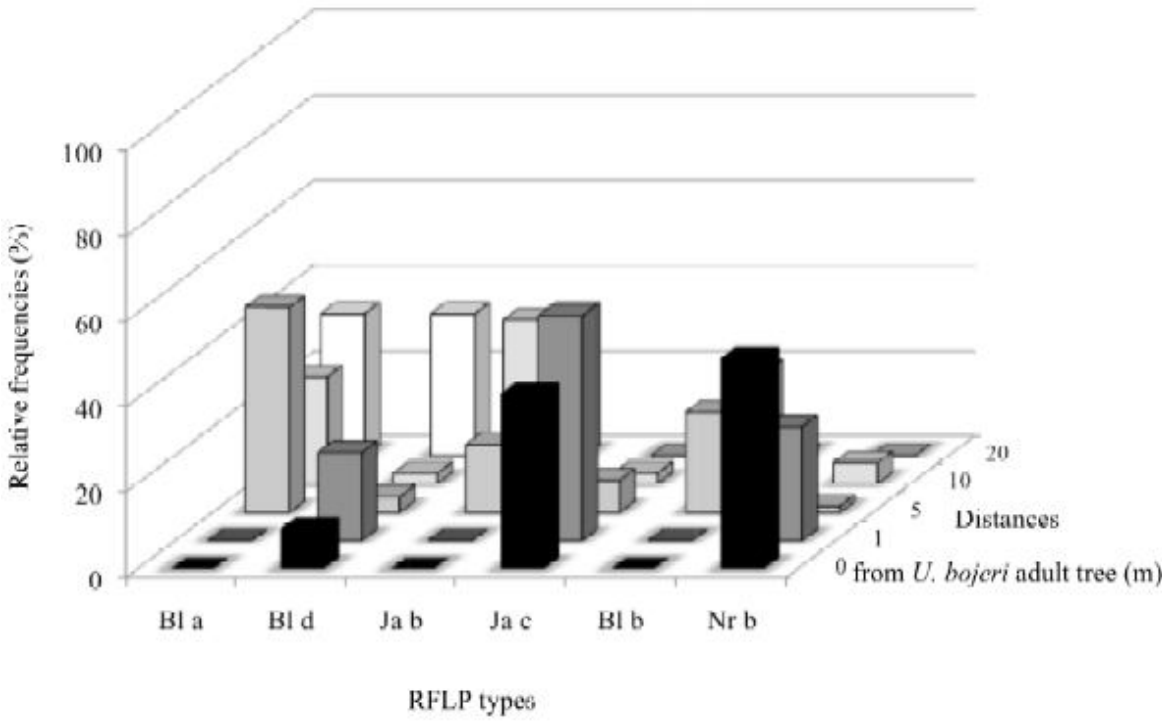


**Figure 6.** Relative frequencies of RFLP types according to the distance from *U. bojeri* adult tree.

*oblongifolia* facilitates ectomycorrhizal formation of *U. bojeri* seedlings and (iii) the nurse plant effect providing by *S. oblongifolia* improves *U. boyjeri* growth and soil functions.

Previous data resulting from fruit-body surveys in *Uapaca* forests have reported that ectomycorhizal fungi were identified as belonging to ten genera *Afroboletus*, *Amanita*, *Boletus*, *Cantharellus*, *Lactarius*, *Leccinum*, *Rubinoboletus*, *Scleroderma*, *Tricholoma* and *Xerocomus* (Ramanankierana et al., 2007). *Russula* was the most frequent ectomycorrhizal genus recorded under *U. bojeri*. Compared with these data, the present study reports a lowest diversity with only 6 genera (*Amanita*, *Boletellus*, *Bondarcevomyces*, *Scleroderma*, *Tapinella* and *Russula*). However, our investigation underlined the abundance of the genus *Russula*, well known to be often dominant in tropical rainforest in Africa, Asia and Madagascar (Buyck et al., 1996; Lee et al., 1997; Walting and Lee, 1998; Riviere et al., 2006). Furthermore, our result detected three new taxa such as *Bondarcevomyces*, *Tapinella* and *Tomentella* that have not been spotted during fruitbody surveys. This confirms that the absence of sporocarps does not indicate a lack of colonization but also earlier observations on the complementary of above and below ground analyses to determine an exhaustive view of community composition (Gardes and Bruns, 1996; Peter et al., 2001; Richard et al., 2005). These results also show that ectomycorrhizal fungal community declined with distance from ectomycorrhizal vegetation. Previous studies have reported that ectomycorrhizal fungi are not uniformly distributed in forest ecosystems with variability in terms of presence, abundance and community composition (Baylis, 1980; Perry et al., 1989; Dickie et al., 2004; Dickie and Reich, 2005). Environmental factors can influence patterns of fungal distribution such as soil characteristics, soil microbiota (Smith and Read, 2008) but it is largely admitted that ectomycorrhizal vegetation is the main factor influencing the availability of ectomycorrhizal fungal propagules in soil (Dickie and Reich, 2005). These biological processes provide large implications for the establishment of ectomycorrhizal plant cover and for fungal community ecology.

In the present study, two ectomycorrhizal plant species (*U. bojeri* and *S. oblongifolia*) can support patches of ectomycorrhizal fungi and consequently facilitate the establishment of *U. bojeri* seedlings. Numerous studies have reported the importance of living roots of adult trees as source of fungal inoculum in tropical forests through a common mycorrhizal network that connects different host plants (Onguene and Kuyper, 2002; Simard and Durall, 2004). Hence *U. bojeri* mature trees can facilitate ectomycorrhizal colonization of young seedling regenerations as it has been reported from this present study with a higher growth and ectomycorrhizal colonization near mature trees. The role of external fungal mycelium radiated from mature trees on young seedling ectomycorrhizal formation was evidenced in a Guinean tropical forest (Diédhiou et al., 2010), in Mediterranean holm oak (Richard et al., 2005) and in temperate coniferous forests (Jonsson et al., 1999; Kranabetter and Friesen, 2002). However, in our experimental site, the ectomycorrhizal shrub, S. *oblongifolia* displayed a higher effect on ectomycorrhizal fungal infection of young seedlings.

Assuming that each RFLP type corresponds more or less to one species, our result shown similar structure between both ectomycorrhizal communities associated with *S. oblongifolia* and *U. bojeri* respectively, despite the contrasted distribution of the two host plants at the study site. In this part of the Madagascarian sclerophyllous forest, *S. oblongifolia* was naturally established especially at forest edge or in degraded surface area free of *U. bojeri* adult trees. Our results demonstrate that more than half of collected ectomycorrhizal taxa were shared between *S. oblongifolia* and *U. bojeri* grown separately or in mixed formation. It suggests that *S. oblongifolia*, as pioneering species, may persist on disturbed sites and facilitate the survival of ectomycorrhizal fungi that could potentially infect *U. bojeri* seedling root. Our results show that this nursing effect was particularly efficient by enhancing the ectomycorrhizal fungal diversity associated with *U. bojeri* young seedlings and the rate of ectomycorrhizal infection. These results are similar to reports on facilitation of ectomycorrhizal infection of *Pseudotsuga menziesii* seedlings by pioneering hardwood (Borchers and Perry, 1987) or the positive effect of *Helianthemum bicknellii* on ectomycorrhizal infection of *Quercus* spp. (Dickie et al., 2004). This promoting effect displayed by *S. oblongifolia* was particularly marked on the ectomycorrhizal infection with 3 fungal symbionts, *S. citrinum*, *S. dictyosporum* and *R. earlei*. These fungal species can be found either on adult tree and/or seedlings in tropical forests (Diedhiou et al., 2010). *Scleroderma* species are considered “early-stage” symbionts (Deacon et al., 1983; Ba et al., 1991) and can form mycorrhizas with a wide range of tropical tree species such as *Afzelia africana* (Ba and Thoen, 1990), *A. quanzensis*, *Isoberlinia doka*, *I. dalziellii* and *Brachystegia speciformis* (Sanon et al., 1997). For instance, it has been previously reported that *Scleroderma* isolates could significantly improve *U. bojeri* growth in disinfected and in non-disinfected soil (Ramanankierana et al., 2007). The differences between plant growths at the distances from *U. bojeri* adult tree show that biological and chemical soil characteristics differ in their effects on plant physiological attributes. For example, the shoot:root ratio of *U. bojeri* seedlings as well as root tip densities varied along the transect showing that nutrient acquisition and nutrient foraging ability are largely influenced by above-ground and below-ground characteristics. In particular, plant growth is significantly dependant to the extent and diversity of ectomycorrhizal infection. It is admitted that mycorrhizal diversity improves plant growth in nutrient-poor soils as

different fungal species may access different nutrient sources (Bruns, 1995; Jonsson et al., 2001; Dickie et al., 2002; Tedersoo et al., 2003).

The results of the present study also show that the extent and species richness of ectomycorrhizal infection was positively linked with the total microbial and deshydrogenase activities. These enzymatic activities are directly involved in the transformation of soil organic matter to mobilize and transfer nutrients to plants (Caravaca et al., 2005; Courty et al., 2010). It is well known that ectomycorrhizal fungi are able to produce extracellular and cell wall-bound enzymes which hydrolyse N- and P-compounds contained in soil organic matter: amino acids, proteins and chitin (Leake and Read, 1990; Hodge et al., 1996; Tibbett et al., 1999; Leake et al., 2002). In addition to these fungal properties, ectomycorrhizal symbiosis induces strong modifications in the genetic and functional diversity of soil microbiota leading to the so-called "mycorrhizosphere effect" defined by Linderman (1988). The mycorrhizosphere effect on soil microbial functions has been previously described in numerous studies (Caravaca et al., 2002; Duponnois et al., 2005; Frey-Klett et al., 2005) showing that mycorrhizal establishment increased soil microbial activities.

## Conclusion

The results of the present study show that some ectomycorrhizal established shrubs as *S. oblongifolia* are essential in facilitating the sustainable establishment of *U. bojeri* seedlings in forest sites following natural or anthropogenic disturbances. It also suggests that these nurse plant species have to be used in sylvicultural practices to enhance the performances of reforestation programs in Madagascar by improving ectomycorrhizal soil potential.

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