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Ectomycorrhizal *Pisolithus albus* inoculation of *Acacia spirorbis* and *Eucalyptus globulus* grown in ultramafic topsoil enhances plant growth and mineral nutrition while limits metal uptake

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ABSTRACT

Ectomycorrhizal fungi (ECM) isolates of *Pisolithus albus* (Cooke and Massee) from nickel-rich ultramafic topsoils in New Caledonia were inoculated onto *Acacia spirorbis* Labill. (an endemic *Fabaceae*) and *Eucalyptus globulus* Labill. (used as a *Myrtaceae* plant host model). The aim of the study was to analyze the growth of symbiotic ECM plants growing on the ultramafic substrate that is characterized by high and toxic metal concentrations *i.e.* Co, Cr, Fe, Mn and Ni, deficient concentrations of plant essential nutrients such as N, P, K, and that presents an unbalanced Ca/Mg ratio (1/19). ECM inoculation was successful with a plant level of root mycorrhization up to 6.7%. ECM symbiosis enhanced plant growth as indicated by significant increases in shoot and root biomass. Presence of ECM enhanced uptake of major elements that are deficient in ultramafic substrates; in particular P, K and Ca. On the contrary, the ECM symbioses strongly reduced transfer to plants of element in excess in soils; in particular all metals. ECM-inoculated plants released metal complexing molecules as free thiols and oxalic acid mostly at lower concentrations than in controls. Data showed that ECM symbiosis helped plant growth by supplying uptake of deficient elements while acting as a protective barrier to toxic metals, in particular for plants growing on ultramafic substrate with extreme soil conditions. Isolation of indigenous and stress-adapted beneficial ECM fungi could serve as a potential tool for inoculation of ECM endemic plants for the successful restoration of ultramafic ecosystems degraded by mining activities.

Introduction

Ultramafic soils are produced by weathering and pedogenesis of ultramafic rocks that are characterized by high levels of Ni, Cr, and sometimes Co, but contain low levels of essential nutrients such as N, P, K and Ca (Rajkumar et al., 2009). The presence of heavy metals at high concentrations in these soils is toxic for many plants (Brooks, 1987; Harrison and Rajakaruna, 2011). Previous studies have shown that ultramafic soils are characterized by a high biological diversity of plants able to adapt to this extreme environment (Proctor, 2003; Brady et al., 2005; Kazakou et al., 2008). In addition, these soils contain a high diversity of microorganisms such as bacteria and fungi that use various mechanisms to cope with the extreme soil conditions, in particular, adaptation to toxic heavy metals (Rajkumar et al., 2009; Branco, 2010). Recently, major studies of the ecological traits of ultramafic soils have been reviewed to propose these soils as a model system in ecology and conservation, mostly because of their high plant diversity (Harrison and Rajakaruna, 2011).

In ultramafic ecosystems, most of the plants are known to be involved in mycorrhizal associations to face these extreme soil conditions (Alexander et al., 2007; Schechter and Bruns, 2008; Branco and Ree, 2010). The endo- and ectomycorrhizal associations are known to significantly enhance plant nutrition such as P assimilation, and to strongly reduce abiotic stresses on plants such as metal toxicity (Finlay, 2008; Smith and Read, 2008). Previous studies on ectomycorrhizal (ECM) fungal communities in ultramafic soils showed a high diversity of fungal species developing ECM symbioses with plants growing on these substrates (Moser et al., 2005; Urban et al., 2008). In addition, it was recently demonstrated that ultramafic soils do not limit and can even promote ectomycorrhizal fungal diversity (Moser et al., 2009; Branco and Ree, 2010). Comparisons of ECM fungal diversity between ultramafic and non-ultramafic soils showed differences within the fungal population

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structure (Brearley, 2006), sometimes with the presence of unique species (Moser et al., 2005). The study of physiological behaviours such as metal tolerance within the same fungal species present on both ultramafic and non-ultramafic soils have suggested adaptive evolution, raising questions about the adaptation and evolution of fungal species on these soils (Gonçalves et al., 2007, 2009; Jourand et al., 2010a,b). The abundance of ECM found on plant roots living on ultramafic soils has also raised questions about their specific roles in plant nutrition and adaptation to such extreme soil conditions (Kazakou et al., 2008), in particular ECM symbioses which are known to limit metal accessibility and uptake by the plant (Colpaert et al., 2011).

Here, we present the results of a set of experiments carried out on the ECM fungi *Pisolithus albus* (Cooke and Massee) collected from ultramafic soils in New Caledonia, a tropical archipelago located in the South Pacific Ocean (Fig. 1). In New Caledonia, the ultramafic soils cover one-third of the main island because of natural geological evolution (Fig. 1). As a result of the presence of such ultramafic outcrops, specific biological endemic ecosystems have developed (Jaffré, 1992) making the main island a biodiversity hot spot (Myers et al., 2000). In addition, in the neo-Caledonian ultramafic ecosystem, it has been reported that the ectotrophic mycorrhizal symbioses are dominant and might play an important role in plant adaptation to the extreme soil conditions (Prin et al., 2012). In this study, *P. albus* ultramafic ecotype isolates were inoculated onto (i) the endemic neo-Caledonian *Fabaceae*, *Acacia spirorbis* Labill. and (ii) the *Myrtaceae*, *Eucalyptus globulus* Labill., which used as a model plant to study the ECM symbiosis between *Pisolithus* and its host-plant at physiological and molecular levels (Duplessis et al., 2005; Bellion et al., 2006; Jourand et al., 2010b; Majorel et al., 2012). The aim of the study was to assess the importance of the ECM symbiosis on plant growth parameters, uptake of major mineral elements, control of heavy metal uptake and exudation of molecules involved in metal binding, when cultivated in ultramafic substrate. The final objective was to evaluate the potential of ECM symbioses in plant adaptation to ultramafic soils containing high concentrations of heavy metals, which is a prerequisite for their use in strategies for ecological restoration of mine sites (in particular chromium and nickel mining) as suggested by Reddell et al. (1999) in restoring forests following mining in Australia, or by Perrier et al. (2006a) in rehabilitation of ultramafic soils degraded after nickel mining in New Caledonia and, more recently as reported by Khosla and Reddy (2008) in post-mining of bauxite in Brazil.

Material and methods

Ultramafic site, topsoil and fungal ecotype isolate descriptions

A map location of the New Caledonia archipelago including sites used in the current study is presented in Fig. 1. The ultramafic sites were located in the south of the main island: Bois du Sud (site 1, Fig. 1) and Mont Dore (site 2, Fig. 1), and located in the north of New Caledonia: Pindaï Peninsula (site 3, Fig. 1) and Koniambo Massif (sites 4 and 5, Fig. 1). A full description of the ultramafic sites including climate, geology, geomorphology, vegetation structure, site geographical location, soil sampling and mineral analyses has been previously described in Jaffré (1992). The ultramafic topsoil geochemical analyses from the different sites were carried out as reported in Perrier et al. (2006b) and data are summarized in ESM Table 1. The description of *Pisolithus albus* isolates used in this study is presented in Table 1, which includes isolate codes, site location of collection, DNA ITS Genbank accession number, ergosterol content of mycelium cultivated *in vitro* (the method is described in more detail below) and references.

Mycelial culture and inoculum

Stock cultures from each sporocarp of *P. albus* isolates were obtained by aseptically transferring a piece of the pileus trama to solid modified Melin-Norkrans Medium (MNM) (Marx, 1969) containing: KH_2PO_4 (0.5 g L^{-1}), $(NH_4)_2HPO_4$ (0.25 g L^{-1}), $CaCl_2$ (0.05 g L^{-1}), NaCl (0.025 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.15 g L^{-1}), thiamine hydrochloride (100 μg L^{-1}), $FeCl_3{\cdot}6H_2O$ (0.03 g L^{-1}), glucose (10 g L^{-1}), malt extract (3 g L^{-1}) and agar (14 g L^{-1}). The pH was adjusted to 5.6 with 1 M HCl and the medium autoclaved for 20 min at 120 °C. All fungal strains were maintained as sub-cultures at 24 °C on the same medium. Vegetative inocula were prepared as described in Brundrett et al. (1996). Briefly: large scale inocula were produced by incubating each fungal mycelium from cultures on Petri dishes inoculated into a sterile solid substrate (peat-vermiculite mixture, 1:10, w:w), saturated with MNM liquid media in large plastic bags (1.5 L). The incubation was carried out at 30 °C in a dark room for 8 weeks.

Plant growth and treatments

Eucalyptus globulus Labill. subsp. *bicostata* (Maiden et al.) J.B. Kirkp. seed lot N°16731 was obtained from the Australian Tree Seed Centre (Kingston, ACT, Australia). *Acacia spirorbis* subsp. *spirorbis* Labill. seed lot N°036/11 was obtained from the New Caledonian Agronomic Institut (IAC, Païta, New Caledonia). A set of experiments was carried out in a glasshouse as described in Brundrett et al. (1996). Briefly, seeds were pre-treated for 2 min in 70% ethanol supplemented with 0.1% (v/v) Tween 20, then surface sterilized for 5 min in H_2O_2 (30%) and washed 3 times in sterile water. Germination occurred after 1 week at 28 °C in the dark. Seedlings were then placed for a week in a growth chamber with an 18/6 h day/night cycle at a light intensity of 200 μmol m^{-2} s^{-1} photosynthetic active radiation and at temperature 25/18 °C. Two-week-old seedlings were transplanted into plastic containers of 1 litre capacity and filled with (i) neutral growth substrate composed of vermiculite or (ii) ultramafic substrate from site 4 (Mont Dore). Plants were inoculated with 100 mL of 8-week-old mycelial inoculum prepared as described above. Plants inoculated with inoculum killed after sterilization by autoclaving (20 min at 120 °C) were used as non mycorrhizal controls. All plants received nutrient by watering the containers to field capacity with a nutrient solution (as suggested in Brundrett et al., 1996) in which the final concentrations of mineral elements expressed in mg kg^{-1} of substrate (w/w) were: NH_4NO_3, 36; K_2SO_4, 111.6; $Ca(H_2PO_4)_2{\cdot}H_2O$, 40; $CaSO_4{\cdot}2H_2O$, 51.5; $MgSO_4{\cdot}7H_2O$, 33.7; $FeCl_3$, 11; $MnSO_4{\cdot}4H_2O$, 16.9; $CuSO_4{\cdot}5H_2O$, 8.2; $ZnSO_4{\cdot}7H_2O$, 9.2; $Na_2B_4O_7{\cdot}10H_2O$ 1.1; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.46; $CoCl_2{\cdot}6H_2O$, 0.34. Plants were grown in the glasshouse under a natural light cycle for 12 months with average maximum and minimum temperatures of 25 ± 2 °C and 20 ± 2 °C, respectively. Water was applied by spraying 2 min per hour, 6 times per day. The watering system operated day and night. Once per month, a top dressing of NH_4NO_3 was applied at 30 mg kg^{-1} of substrate. All experiments were carried out on three independent assays, with each treatment containing 15 plant replicates.

Analyses

Plants were harvested 12 months after the mycelial inoculation. Root exudates were collected, filtered and lyophilized as described by Silva et al. (2004). Lyophilized root exudates were re-suspended with ultrapure water (20 mL). Organic acids were determined by HPLC (Laboratoire LIVE, Nouméa, Université de Nouvelle-Calédonie) with the following conditions: HPLC Waters Alliance system®, with PDA detector at 210 nm (Waters SA, Guyancourt, France); column ODS 100 V 4.6 mm diameter, 250 mm length,

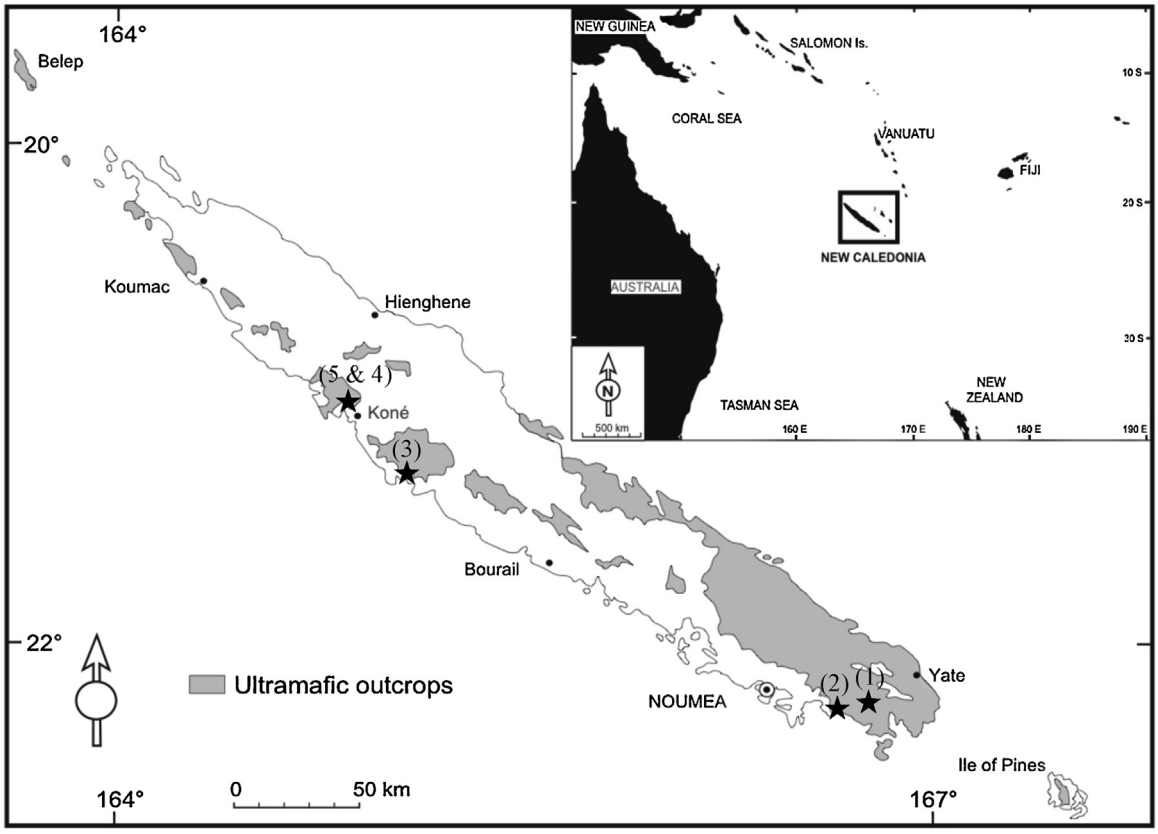

Fig. 1. General geographical map describing the New Caledonian archipelago, location of ultramafic massifs (in grey) and sites where *Pisolithus* sp. isolates were collected.

silica particle size of 5 μm (Tosoh Bioscience, Lyon, France); mobile phase was used at a flow rate of 0.7 mL min^{-1} and was constituted of an isocratic solvent composed of 98% H_3PO_3 HPLC grade at 0.1% in H_2O (v/v) and 2% of CH_3CN HPLC grade. Calibrations were made with standard organic acids from Sigma–Aldrich kit N°47264 (Sigma–Aldrich, L'Isle d'Abeau, France). Total free thiols were also assayed in lyophilized root exudates using Ellman's test and reagent (DTNB) as described in Bulaj et al. (1998). Shoot and root dry biomass were measured in mg after drying plants for 72 h at 60 °C. Relative gain or loss of biomass (expressed as %) was calculated compared with controls as reported in Duponnois et al. (2011). Aliquots of dried plant tissues (shoot or root) were collected for mineral analyses, which were performed by ICP-OES as reported in Perrier et al. (2006a,b). Ergosterol is the major sterol in most fungi and is either absent or a minor component of higher plants and so was used to estimate fungal biomass (Martin et al., 1990). Ergosterol analysis was carried out in dried roots and in pure mycelium after methanolic extraction as reported in Martin et al. (1990), followed by HPLC analysis with the following conditions: HPLC Waters Alliance system®, with PDA detector at 280 nm, column Agilent Extend C18 2.1 mm diameter, 150 mm length, silica particle size of 5 μm (Agilent, Massy, France), mobile phase constituted of Methanol HPLC grade 100% at a flow rate of 0.2 mL min^{-1}. A standard ergosterol was used for calibration (Sigma–Aldrich, L'Isle d'Abeau, France). On the basis of the ergosterol determination, a relative fungal biomass in plants roots was determined by using as conversion factor the mean of ergosterol per gram fresh weight of mycelium which was calculated after measuring ergosterol extracted from a pure culture of fresh mycelium as previously described in Martin et al. (1990).

Table 1
Pisolithus albus isolates used in this study: isolate codes, site location, DNA ITS Genbank accession number, ergosterol content of mycelium cultivated *in vitro*, nickel phenotype and references.

Pisolithus albus isolate code	Site on map	Location and GPS coordinates	DNA ITS Genbank or EMBL accession number	Ergosterol content in mycelium cultivated *in vitro* (in μg mg^{-1} of FW)[a]	Nickel phenotype	Reference
MD09-001	1	Bois du Sud (22°10′18″ S; 166°44′49″ E)	FR852891	1.1 ± 0.1	Tolerant	Majorel et al. (2012)
MD09-045	2	Mont-Dore, Plum Road (22°15′17″ S; 166°36′46″ E)	FR852892	1.9 ± 1.1	Tolerant	Majorel et al. (2012)
MD09-063	3	Pindaï peninsula (21°19′49″ S; 164°58′22″ E)	FR852893	1.8 ± 0.5	Sensitive	Majorel et al. (2012)
MD09-078	4	Pindjen Waterfall Road Koné (21°02′19″ S; 164°46′26″ E)	FR852890	5.2 ± 1.2	Sensitive	Majorel et al. (2012)
MD06-337	5	Trazy-Guerioum, Koniambo Massif (21°00′28″ S; 164°49′50″ E)	AM947121	1.9 ± 0.6	Tolerant	Jourand et al. (2010b)

Abbreviations: FW, fresh weight.
[a] Data are means ± standard deviation of 3 replicates.

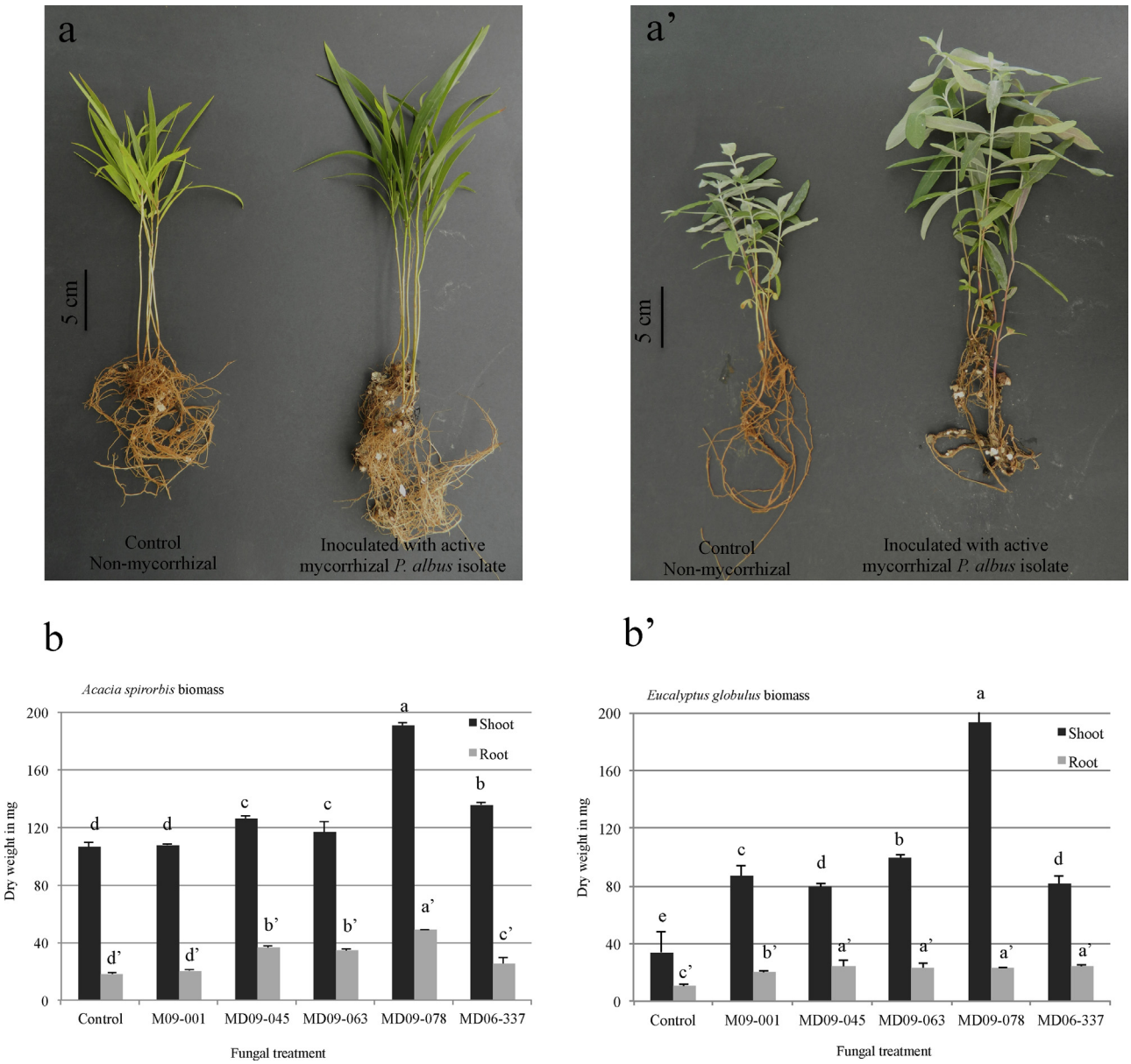

Fig. 2. *Acacia spirorbis* (a) and *Eucalyptus globulus* (a′) seedlings after 12 months of growth on ultramafic substrate. The biomass of their respective shoot and root (b: *Acacia* and b′: *Eucalyptus*) were measured as dry weight tissue expressed in mg. Bars represent means, and error bars represent standard deviation of means ($n = 15$) of three independent experiments. The different letters above columns indicate significant differences as determined by Tukey HSD test ($P \leq 0.05$).

Statistics

All data were subjected to a variance analysis (ANOVA) and means were compared using the honestly significant difference (HSD) of Tukey test at $P < 0.05$ using XLSTAT computer software (Addinsoft, 2011).

Results

Geochemical characterization of the ultramafic rhizospheric soils

The five sites are located in the ultramafic ligno-herbaceous maquis (Fig. 1). The results of soil characterizations indicated that sites 1, 2 (Koniambo) and 3 (Pindaï) topsoil horizons presented ultramafic Ferralsol geochemical characteristics, while that of sites 4 (Mont Dore) and 5 (Bois du Sud) presented Cambisol geomorphological characteristics. Full data of chemical soils analyses are presented in ESM Table 1. All sites shared geochemical characteristics of ultramafic soils and showed (i) a low content of major plant nutrients (N, P, and K) with mean concentrations of 1.18 ± 0.68; 0.09 ± 0.04 and $0.07 \pm 0.04\ g\ kg^{-1}$, respectively; (ii) an unbalanced Ca/Mg ratio with mean concentrations of 0.61 ± 0.36 and $7.0 \pm 3.6\ g\ kg^{-1}$, respectively (molar ratio of Ca/Mg was 1/19); and (iii) high levels of putatively toxic metals with DTPA exchangeable cation mean concentrations of 51 ± 7; 0.56 ± 0.2, 103 ± 18; 703 ± 480 and $65 \pm 29\ mg\ kg^{-1}$, for Co, Cr, Fe, Mn and Ni, respectively.

Plant and mycelial growth

Summarized plant growth data are presented in Fig. 2. Full data are accessible in ESM Table 2. On ultramafic substrate, ectomycorrhizal treatment with *P. albus* induced enhancement of plant growth when compared with controls (Fig. 2): in *A. spirorbis* the biomass was significantly enhanced with an increase of shoot dry weight from +8.7% to +44.3% (except for plants inoculated with

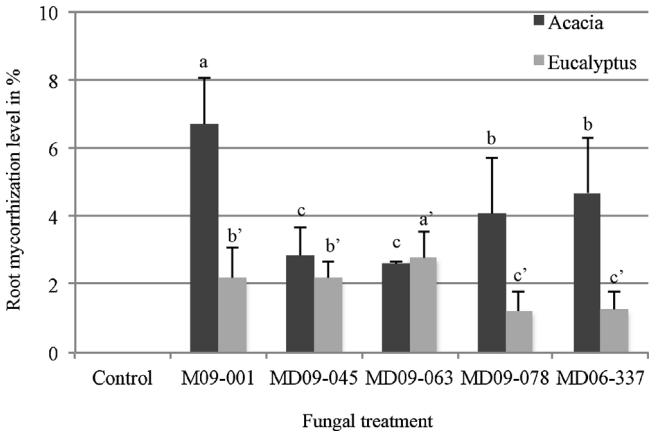

Fig. 3. Mycorrhizal plant root colonization expressed as % of mycelium content in root systems (mg of FW mycelium/100 mg of DW total root biomass) in *Acacia spirorbis* and in *Eucalyptus globulus* seedlings after 12 months of growth on ultramafic substrate. Bars represent means and error bars represent standard deviation of means ($n = 15$) of three independent experiments. Different letters above columns indicate significant differences as determined by Tukey test ($P < 0.05$). Abbreviations: FW: fresh weight; DW: dry weight.

P. albus isolate MD09-001) and an increase of root dry weight from +10.8% to 62.3%; in *E. globulus* the plant shoot dry weight was significantly enhanced from +57.1% to +82.4% and the root dry weight from +47.4% to +56.1%, all when compared with controls. Ergosterol was also measured in roots as indicator of the quantity of mycelium at the root level. In pure cultures of fungal isolates, the ergosterol concentration varied from 1.1 ± 0.1 to 5.2 ± 1.2 μg mg^{-1} of mycelium fresh weight as reported in Table 1. The conversion factor of ergosterol into mycelium fresh weight was calculated using the mean and was 2.38 ± 0.7 μg of ergosterol mg^{-1} of mycelium fresh weight. Full data for ergosterol content in plant roots and conversion into relative fresh mycelium biomass using the conversion factor are presented in ESM Table 3. Summarized values of fresh mycelium biomass in the root system are presented in Fig. 3: in *A. spirorbis* the mycelial biomass attributable to ECM was from 2.61 ± 0.06% to 6.71 ± 1.35% and in *E. globulus* the mycelial biomass attributable to ECM was from 1.22 ± 0.57% to 2.77 ± 0.76%.

Plant mineral nutrition analyses

The major elements needed for plant nutrition (*i.e.* N, P, K, Ca and Mg contents) were analyzed in shoots of ECM and control plants exposed to the ultramafic substrate. Results are summarized in Fig. 4 while full data are reported in ESM Table 4a. The presence of ECM symbiosis significantly affected major element contents in shoot tissues when compared with controls. In shoots of *A. spirorbis*, significant enhancement of N content in tissues (from 33.7% to 46.5%) was observed; P assimilation was significantly enhanced from 20.3% to 57.5% (except for plants inoculated with isolates MD09-045 and MD09-063); K assimilation was significantly enhanced from 21.4% to 35.8%; Ca assimilation was significantly enhanced from 6.1% to 18.1% (except in shoots of plants inoculated with the isolate MD09-63) and Mg was significantly reduced by 16.6% on average (except in plants inoculated with MD09-078 and MD06-337). In shoots of *E. globulus* significant enhancement of N content in tissues (from 8.3% to 20.5%) was observed (except for plants inoculated with isolates MD09-045 and MD09-063); P assimilation was significantly enhanced from 14.2% to 45.1%; K assimilation was significantly enhanced from 27.8% to 38.0%; Ca assimilation was significantly enhanced from 29.4% to 48.0%; and Mg was significantly reduced by 13.5% on average. Similarly, the metals that were present at high concentrations in the ultramafic substrate (Co, Cr, Fe, Mn and Ni) were analyzed in shoot tissues of ECM and control plants. Results are summarized in Fig. 5 while full data are reported in ESM Table 4b. Presence of ECM symbiosis significantly reduced metal contents in shoot tissues when compared with controls. Considering metal shoot tissue contents of both *A. spirorbis* and *E. globulus*: on average Co was reduced by 1.8×; Cr by 3.2×; Fe by 2.5×; Mn by 0.9× and Ni by 2.5×.

Free thiols and oxalic acid contents in plant root exudates

To determine whether adaptation to the ultramafic substrate could be related to the release of metal binding compounds, root exudates were analyzed. The two major chemical components of the exudate solution were non-protein free thiols (-SH) and oxalate. Data are presented in Fig. 6: the amounts of these compounds are expressed per gram dry weight of root (full data are reported in ESM Table 5). For *E. globulus*, mycorrhizal plants released from 1.64–43.7× less thiol and from 1.3–7.8× less oxalate than the plant controls. For *A. spirorbis*, mycorrhizal plants released from 1.65–5.92× less thiol than plant controls, but we observed a higher release of oxalate from ECM plants than controls (from 1.36–3.15× more), except for plants inoculated with MD09-078, which showed a lower release of oxalate (1.44× less) than controls.

Discussion

This study is the first to report data about ectomycorrhizal inoculation of plants growing on ultramafic substrate and showing that ECM symbioses (i) improve plant growth when cultivated on ultramafic soil, (ii) limit metal transfer to plants and (iii) vary in its symbiotic and physiological responses to the plant.

Ectomycorrhizal symbiosis improves plant development on ultramafic soil

The promotion of plant growth by ECM symbiosis has been widely reviewed: ECM associations are known to affect plant root growth, improve the general plant mineral nutrition and so improve plant fitness (Marschner, 2012). In ultramafic soil and topsoil deriving from weathering of ultramafic rocks, essential plant nutrients such as N, P and K are at low levels or deficient (Brooks, 1987). In addition, the Ca/Mg ratio in the ultramafic substrate is strongly unbalanced in favour of Mg (1/19 in the present study). It is not surprising that, in our study, most of the ECM symbioses contributed to improving plant mineral nutrition such as N, P, K and Ca assimilation and consequently improved plant biomass production. Regarding N and P assimilation improvement, our results are in agreement with common knowledge about the role of ECM in the assimilation and the recycling of the two elements (Read and Perez-Moreno, 2003). Concerning the other major elements (Ca, Mg and K), our data indicate that K and Ca assimilations were improved in most of the ECM symbioses tested, while Mg was reduced. Recent data about Ca, Mg and K assimilation in ECM roots suggest that the fungal sheath might act as an apoplastic barrier for the entry of these elements in the root cortex (Bücking et al., 2012). Our study suggests that in ultramafic soils, ECM helped the plant in finding deficient elements such as K and Ca and regulated the excess supply of Mg.

Role of ECM in metal tolerance

In this study, we demonstrated that an ultramafic ecotype of *P. albus* isolates could improve plant metal tolerance on ultramafic substrate when metals were present at high concentration in the soil. The mechanisms that are involved in metal homeostasis and

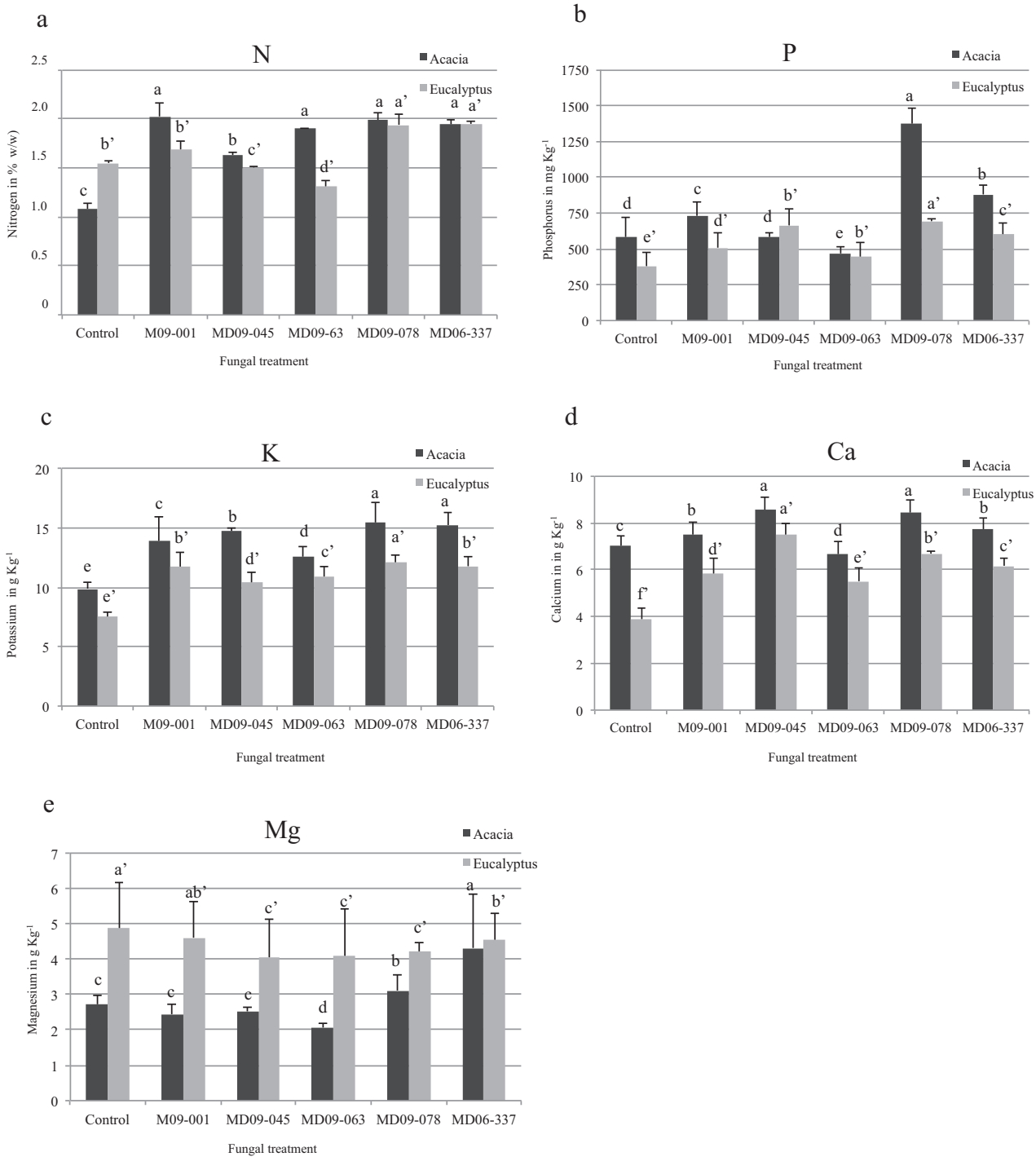

Fig. 4. Major element content in plant shoots of *Acacia spirorbis* and *Eucalyptus globulus* expressed as % (w/w), or mg kg^{-1}, or g kg^{-1} of dry weight tissue. (a) Nitrogen (N); (b), Phosphorus (P); (c): Potassium (K); (d), Calcium (Ca) and (e), Magnesium (Mg). Bars represent means and error bars represent standard deviation of means ($n = 15$) of three independent experiments. Different letters above columns indicate significant differences as determined by Tukey test ($P < 0.05$).

detoxification of essential and non-essential metals in ECM fungi are the same as those that are present in other eukaryotes (Colpaert et al., 2011). Heavy metal tolerance can be due to several processes: (i) extracellular binding mechanisms on external mycelium or the fungal mantle by excreted ligands, (ii) surface sequestration by binding to the fungal cell wall, (iii) enhanced metal efflux from the fungal cell, (iv) binding to peptides or proteins in the fungal cytoplasm or released in the rhizosphere, or (v) sequestration in the fungal vacuole (Bellion et al., 2006) and (vi) reduction of metal accumulation by activation of magnesium transport (Nies, 1999). These processes decrease the metal concentration in the soil solution in the mycorhizosphere, in the roots and so in the shoot of the ECM host plant (Marschner, 2012). In our study two major results argue in favour of the hypothesis that ECM regulates and detoxifies metals in the rhizosphere: (i) metal levels in ECM plant shoot analysis revealed a strong and significant decrease when compared with non ECM plants; (ii) ECM plants released both free thiols and oxalic acid, molecules known to be involved in

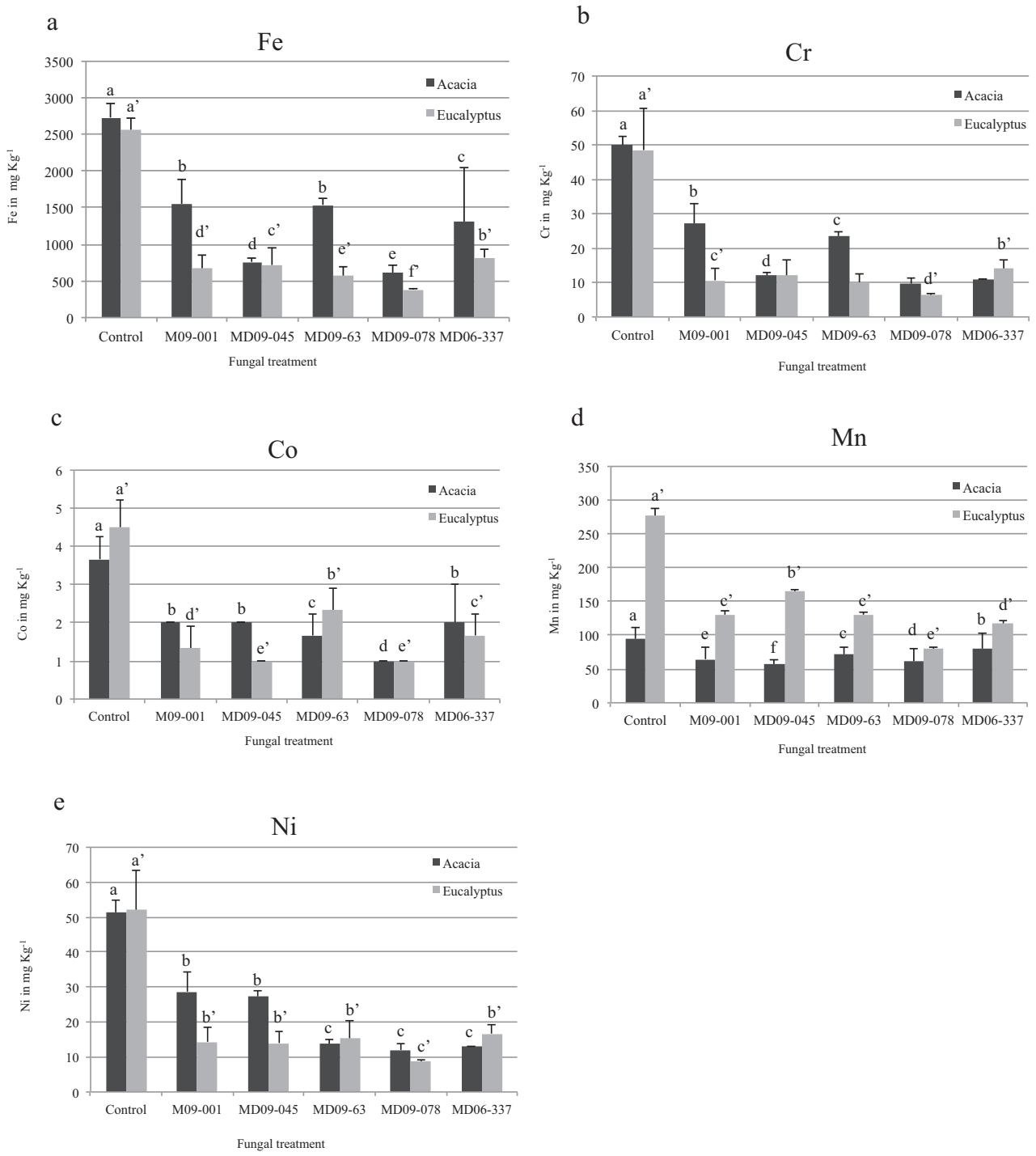

Fig. 5. Metal contents in plant shoot of *Acacia spirorbis* and *Eucalyptus globulus* expressed as mg kg^{-1} of dry weight tissue. (a) Iron (Fe); (b), Chromium (Cr); (c): Cobalt (Co); (d), Manganese (Mn) and (e), Nickel (Ni). Bars represent means and error bars represent standard deviation of means ($n = 15$) of three independent experiments. Different letters above columns indicate significant differences as determined by Tukey test ($P < 0.05$).

metal binding (Machuca, 2011). However, in the presence of most ECM *P. albus* isolates tested, levels of free thiols and oxalic acid released were lower in inoculated plants than in non-ECM plants (controls). Such results are in agreement with previous observations reported by Jourand et al. (2010b) testing nickel toxicity on ECM plants: excretions of oxalate and non-protein thiols were relatively unresponsive to Ni addition when the plant was mycorrhizal. All these data suggest that the fungal sheath on roots might act as a strong barrier to metal toxicity and significantly contributes to the plant physiological fitness. Moreover, when regarding nickel as one of the major ultramafic markers because of its toxicity (Majorel et al., 2012), and as we tested both Nickel-tolerant and Ni-sensitive *P. albus* isolates (see Table 1), our results showed that best performing isolate on plant growth and response to metals is the Ni-sensitive isolate (MD09-078). This suggests that (i) nickel is not a sufficient indicator to select high-performing fungal isolates to be used as inocula in rehabilitation strategies, and (ii) plant adaptation to ultramafic metallic constraints might result from a complex

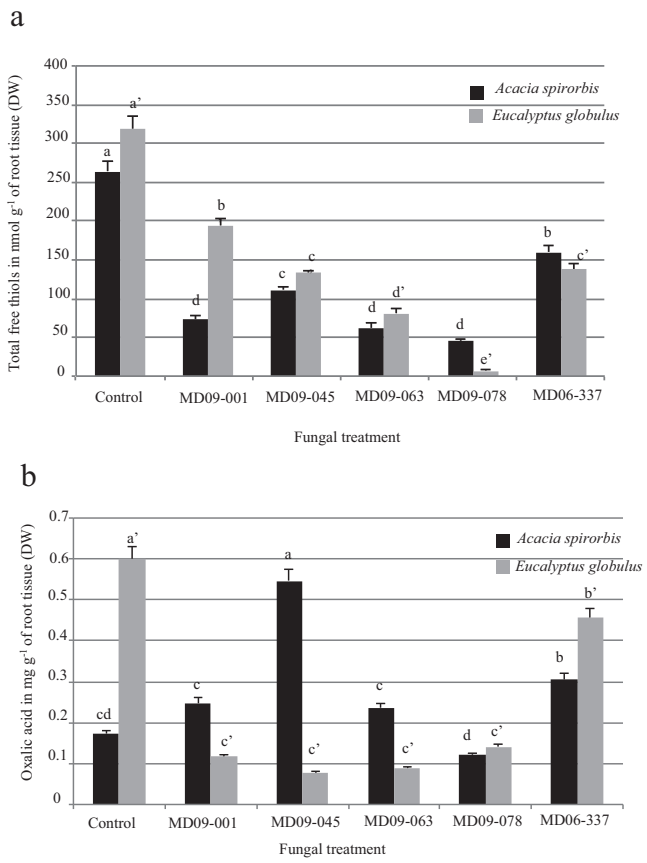

Fig. 6. Comparison of total free thiols (a) and oxalic acid (b) contents in root exudates of *Acacia spirorbis* and *Eucalyptus globulus* seedlings after 12 months of growth on ultramafic substrate. Bars represent means and error bars represent standard deviation of means ($n = 15$) of three independent experiments. Different letters above columns indicate significant differences as determined by Tukey test ($P < 0.05$). *Abbreviation*: DW: dry weight.

strategy linking ECM symbiosis and plant responses to the toxic substrate.

Mycorrhizal responsiveness

The analysis of the full dataset from our study shows that some *Pisolthus albus* isolates are more efficient than others: in particular, inoculation of isolates MD09-078 and MD06-337 contributed to higher yields in plant biomass production, efficient plant mineral nutrition and metal detoxification, than inoculation with isolates MD09-001 and MD09-063. In addition to the drastic extreme soil conditions of the ultramafic soils, many other factors may influence plant host ectomycorrhizal symbiosis specificity and efficiency: (i) the capacity of plants to allocate carbon to ECM (Nehls and Hampp, 2000) and (ii) the influence of root exudates to select and promote specific ECM populations as well as mycorrhizal helper bacteria (Hartmann et al., 2009). Moreover, comparison of results obtained with the two plants tested showed differences in particular in Mg and Mn accumulation as well as oxalic acid excretion: in the endemic plant *A. spirorbis* the uptake of Mg and Mn are controlled by the plant regardless of the presence or absence of ECM, while in *E. globulus* only the presence of ECM strongly reduced metal uptake (Figs. 3 and 4). The endemic plant seems to respond better to the ultramafic edaphic constraints than the model plant host suggesting that the endemic plant is probably adapted and specialized to such an extreme habitat as already described for other endemic plants growing from ultramafic soils (Harrison and Rajakaruna, 2011).

Our data suggest that selecting fungal ECM isolates based on (i) their capacity to adapt to the ultramafic abiotic stresses such as presence of metals and unbalanced Ca/Mg ratio, and (ii) their efficacy in improving plant growth and mineral nutrition, might be a prerequisite to use ECM as a biotechnological tool for inoculum in rehabilitation strategies of ultramafic ecosystems degraded by mining activities as suggested by O'Dell and Claassen (2011). In addition, our results confirm that the use of endemic plants might also a prerequisite to succeed in such strategies as previously reported by Jaffré et al. (1994).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.10.011.

References

Addinsoft. XLSTAT software version 7.5.2; 2011, http://www.xlstat.com
Alexander E, Coleman R, Keeler-Wolfe T, Harrison S. Serpentine geoecology of Northern North America. New York: Oxford University Press; 2007.
Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol Lett 2006;254:173–81.
Brady K, Kruckberg A, Bradshaw H. Evolutionary ecology of plant adaptation to serpentine soils. Ann Rev Ecol Evol Syst 2005;36:243–66.
Branco S. Serpentine soils promote ectomycorrhizal fungal diversity. Mol Ecol 2010;19:5566–76.
Branco S, Ree RH. Serpentine soils do not limit mycorrhizal fungal diversity. PLoS ONE 2010;5:e11757.
Brearley FQ. Differences in the growth and ectomycorrhizal community of *Dryobalanops lanceolata* (*Dipterocarpaceae*) seedlings grown in ultramafic and non-ultramafic soils. Soil Biol Biochem 2006;38:3407–10.
Brooks R. Serpentine and its vegetation. Portland: Dioscorides Press; 1987.
Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N. Working with mycorrhizas in forestry and agriculture. Canberra, Australia: Australian Centre for International Agricultural Research; 1996.
Bücking H, Liepold E, Ambilwade P. The role of the mycorrhizal symbiosis in nutrient uptake of plants and the regulatory mechanisms underlying these transport processes. In: Dhal NK, Sahu SC, editors. Plant Science; 2012. p. 108–32, Intech ISBN 980-953-307-033-4.
Bulaj G, Kortemme T, Goldenberg DP. Ionization-reactivity relationships for cysteine thiols in polypeptides. Biochemistry 1998;37:8965–72.
Colpaert JV, Wevers J, Krznaric E, Adriaensen K. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Ann Forest Sci 2011;68:17–24.
Duplessis S, Courty PE, Tagu D, Martin F. Transcripts patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. New Phytol 2005;165:599–611.
Duponnois R, Ba A, Mousain D, Galiana A, Baudoin E, Dreyfus B, et al. Biotechnological processes used in controlled ectomycorrhiza practices. In: Rai M, Varma A, editors. Diversity and biotechnology of ectomycorrhizae. Soil biology. Berlin/Heidelberg, Germany: Springer-Verlag; 2011 p. p. 143–55.
Finlay RD. Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. J Exp Bot 2008;59:1115–26.
Gonçalves SC, Portugal A, Goncalves MT, Vieira R, Martins-Loucao MA, Freitas H. Genetic diversity and differential *in vitro* responses to Ni in *Cenococcum geophilum* isolates from serpentine soils in Portugal. Mycorrhiza 2007;17:677–86.
Gonçalves SC, Martins-Louçao MA, Freitas H. Evidence of adaptative tolerance to nickel in isolates of *Cenococcum geophilum* from serpentine soils. Mycorrhiza 2009;19:221–30.
Harrison S, Rajakaruna N. Serpentine: the evolution and ecology of a model system. Berkeley and Los Angeles, CA: The University of California Press; 2011.
Hartmann A, Schmid M, Van Tuinen D, Berg G. Plant-driven selection of microbes. Plant Soil 2009;321:235–57.
Jaffré T. Floristic and ecological diversity of the vegetation on ultramafic rocks in New Caledonia. In: Baker AJM, Proctor J, Reeves RD, editors. The vegetation of ultramafic soils. Andover, UK: Intercept Ltd; 1992. p. 101–7.

Jaffré T, Rigault F, Sarrailh JM. La végétalisation des anciens sites miniers de Nouvelle-Calédonie. Bois et Forêts des Tropiques 1994;242:1–13.
Jourand P, Ducousso M, Loulergue-Majorel C, Hannibal L, Santoni S, Prin Y, et al. Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype. FEMS Microbiol Ecol 2010a;72:238–49.
Jourand P, Ducousso M, Reid R, Majorel C, Richert C, Riss J, et al. Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant *Eucalyptus globulus* at toxic nickel concentrations. Tree Physiol 2010b;30:1311–9.
Kazakou E, Dimitrakopoulos PG, Reeves RD, Baker AJM, Troumbis AY. Hypotheses, mechanisms, and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. Biol Rev 2008;83:495–508.
Khosla B, Reddy MS. Response of ectomycorrhizal fungi on the growth and mineral nutrition of eucalyptus seedlings in bauxite mined soil. Amer-Eurasian J Agri Environ Sci 2008;3:123–6.
Machuca A. Metal-chelating agents from ectomycorrhizal fungi and their biotechnological potential. In: Rai M, Varma A, editors. Diversity and biotechnology of ectomycorrhizae. Soil biology series. Berlin, Germany: Springer-Verlag; 2011. p. 347–70.
Majorel C, Hannibal L, Soupe ME, Carriconde F, Ducousso M, Lebrun M, et al. Tracking nickel adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach. Mol Ecol 2012;21:2208–23.
Marschner P. Mineral nutrition of higher plants. London, UK: Academic Press Limited; 2012.
Martin F, Delaruelle C, Hilbert JL. An improved ergso- terol assay to estimate fungal biomass in ectomycorrhizas. Mycol Res 1990;94:1059–64.
Marx DH. The influence of ectotropic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. Phytopathology 1969;59:153–63.
Moser AM, Petersen CA, D'Allura JA, Southworth D. Comparison of ectomycorrhizas of *Quercus garryana* (*Fagaceae*) on serpentine and non serpentine soils in southwestern Oregon. Am J Bot 2005;92:224–30.
Moser AM, Frank J, D'Allura JA, Southworth D. Ectomycorrhizal communities of *Quercus garryana* are similar on serpentine and non serpentine soils. Plant Soil 2009;315:185–94.
Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. Biodiversity hotspots for conservation priorities. Nature 2000;403:853–8.
Nehls U, Hampp R. Carbon allocation in ectomycorrhizas. Physiological and Molecular Plant Pathology 2000;57:95–100.
Nies DH. Microbial heavy-metal resistance. Appl Microbiol Biotechnol 1999;51:730–50.
O' Dell RE, Claassen VP. Restoration and revegetation of harsh soils. In: Harrison S, Rajakaruna N, editors. Serpentine: the evolution and ecology of a model system. Berkeley and Los Angeles, CA: The University of California Press; 2011. p. 383–413.
Perrier N, Amir H, Colin F. Occurrence of mycorrhizal symbioses in the metal-rich lateritic soils of the Koniambo Massif, New Caledonia. Mycorrhiza 2006a;16:449–58.
Perrier N, Ambrosi JP, Colin F, Gilkes RJ. Biogeochemistry of a regolith: the New Caledonian Koniambo ultramafic massif. J Geochem Explor 2006b;88:54–8.
Prin Y, Ducousso M, Tassin J, Béna G, Jourand P, Dumontet V, et al. Ectotrophic mycorrhizal symbioses are dominant in natural ultramafic forest ecosystems of New Caledonia. In: Hafidi M, Duponnois R, editors. The mycorrhizal symbiosis in mediterranean environment: importance in ecosystem stability and in soil rehabilitation strategies. New York, USA: Environmental Science, Engineering and Technology, Nova Science Publishers; 2012. p. 25–48.
Proctor J. Vegetation and soil and plant chemistry on ultramafic rocks in the tropical Far East. Perspect Plant Ecol 2003;6:105–24.
Rajkumar M, Prasad MNV, Freitas H, Ae N. Biotechnological applications of serpentine soil bacteria for phytoremediation of trace metals. Crit Rev Biotechnol 2009;2:120–30.
Read DJ, Perez-Moreno J. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? New Phytologist 2003;157:475–92.
Reddell P, Gordon V, Hopkins MS. Ectomycorrhizas in *Eucalyptus tetrodonta* and *E. miniata* forest communities in tropical Northern Australia and their role in the rehabilitation of these forests following mining. Aust J Bot 1999;47:881–907.
Silva IR, Novais RF, Jham GN, Barros NF, Gebrim FO, Nunes FN, Neves JCL, Leite FP. Responses of eucalypt species to aluminum: the possible involvement of low molecular weight organic acids in the Al tolerance mechanism. Tree Physiol 2004;24:267–1277.
Smith S, Read D. Mycorrhizal symbiosis. 2nd edn London, UK: Academic Press; 2008.
Schechter S, Bruns T. Serpentine and non-serpentine ecotypes of *Collinsia sparsiflora* associate with distinct arbuscular mychorrizal fungal assemblages. Mol Ecol 2008;17:3198–210.
Urban A, Puschenreiter M, Strauss J, Gorfer M. Diversity and structure of ectomycorrhizal and co-associated fungal communities in a serpentine soil. Mycorrhiza 2008;18:339–54.

CHAPTER 6

Abundance, Distribution, and Function of *Pisolithus albus* and other Ectomycorrhizal Fungi of Ultramafic Soils in New Caledonia

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1. Introduction

Ultramafic soils, also known as "serpentine soils" in literature, are a weathered product from ultramafic bedrock that covers less than 1% of the earth's surface (Coleman and Jove 1992). These soils are characterized by high concentrations of iron oxides (up to 85% w/w), unbalanced calcium-

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to-magnesium ratio (up to 1/30 that consequently may influence both Mg and Ca plant nutrition), and the presence of various heavy metals at high concentrations, such as chromium, cobalt, manganese and nickel, all of which are mostly toxic for many plants (Brooks 1987). They are also extremely deficient in elements that are essential for plant nutrition, including nitrogen, phosphorus and potassium (Brooks 1987, Chiarucci and Baker 2007). Previous studies have shown that ultramafic soils are characterized by a high biological diversity of plants as described in Proctor (2003) and micro organisms that use various mechanisms to cope with the extreme edaphic conditions, in particular adaptation to toxic heavy metals (Brady et al. 2005, Kazakou et al. 2008, Rajkumar et al. 2009). Recently, major data about the ecological traits of ultramafic soils have been reviewed to propose these soils as a model system in ecology and conservation,mostly because of their high plant diversity (Harrisson and Rajakaruna 2011).

In ultramafic ecosystems, it is well known that most plants tolerant to these extreme soils are involved in mycorrhizal associations, which may greatly enhance plant nutrition (such as P assimilation) and reduce metal toxicity on plants (Alexander et al. 2007, Smith and Read 2008). Studies carried out on ectomycorrhizal (ECM) fungal communities in ultramafic soils showed a high diversity of fungal species developing ECM symbioses with plants growing on these substrates (Moser et al. 2005, Urban et al. 2008). In addition, it was recently demonstrated that ultramafic soils do not limit, and can even promote, the ectomycorrhizal fungal diversity (Moser et al. 2009, Branco and Ree 2010, Branco 2010). However, the comparison of ECM fungal diversity between serpentine and non-serpentine soils showed differences within the fungal population structure (Brealey et al. 2006), sometimes with the presence of unique species (Moser et al. 2005). Moreover, studies about physiological behaviour such as metal tolerance within a same fungal species present on both serpentine and non-serpentine soils have suggested adaptive evolution, raising questions about the adaptation and evolution of fungal species on these soils (Gonçalves et al. 2007, 2009).

Here, we have presented a review carried out on ECM fungi collected from ultramafic soils in New Caledonia, which is a tropical archipelago located in the South Pacific Ocean (Fig. 1). In New Caledonia, these soils cover one-third of the main island due to geological evolution (Fig. 1). As a result of the presence of such ultramafic outcrops, numerous endemic ecosystems have developed (Jaffré, 1992), making the main island a biodiversity hot spot (Myers et al. 2000). In the first section, we have summarized results from existing studies and new results of ECM fungal diversity found on these extreme soils. In the second section, we have gathered data about the ECM *Pisolithus albus* (Cooke and Massee) isolated from ultramafic soils in New Caledonia: diversity, metal-tolerance and symbiotic interactions with its host plant are presented.

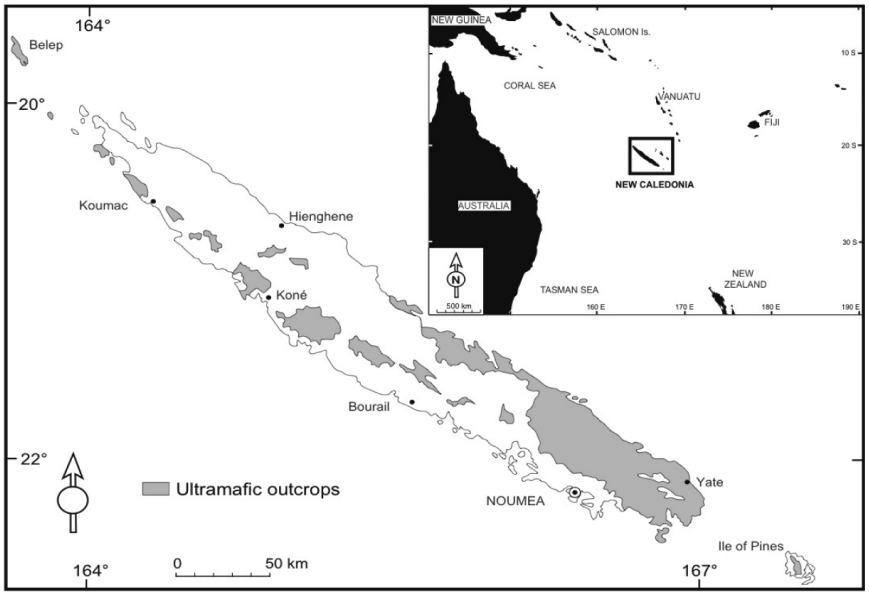


Fig. 1. Geographical map of the New Caledonian archipelago in the South Pacific Ocean with location of ultramafic massifs (in grey). Data from Perrier et al. (2006a).

2. Plant ECM Status and Fungal Diversity in Ultramafic Soils of New Caledonia

Considering the vascular plants:fungi ratio of 1:6 as reported by Hawksworth (1991, 2001) and the number of vascular plants of 3,371 species identified in New Caledonia (T. Jaffré, personal communication), we could hypothesize that at least 20,000 fungal species inhabit the archipelago. Referring to the available literature and herbarium data, the mycologists Horak and Mouchacca listed about 420 Ascomycota and Basidiomycota taxa in New Caledonia (Horak and Mouchacca 1998, Mouchacca 1998, Mouchacca and Horak 1998), which would indicate that approximately 2% of the species have been inventoried.

The studies undertaken by Perrier, though preliminary, are to date the only ones that have characterized the ECM status of some New Caledonian plant species and the related ECM fungal diversity (Perrier 2005, Perrier et al. 2006a,b). In New Caledonia, two main plant formations are basically distinguished on ultramafic rocks: sclerophyllous scrubland formations, called "maquis" or "maquis minier", and rain forest formations (Jaffré and L'Huillier 2010). According to the type of soil, the altitude, and the floristic composition, many groups are further identified. The plant formations

studied by Perrier were located on the ultramafic Koniambo Massif and correspond to four distinct vegetation groups (Fig. 2): a maquis with emerging *Araucaria* trees, a lingo-herbaceous maquis, a *Tristaniopsis* spp. maquis and a rain forest dominated by *Nothofagus balansae* with patches of *N. codonandra*. Investigation of the root systems of 19 species revealed that two *Tristaniopsis* species, *T. calobuxus* and *T. guillainii*, are involved in ECM symbioses. These species belong to the Myrtaceae (Leptospermoideae group), a well-known plant family frequently found to be associated with ECM fungi (Smith and Read 2008, Wang and Qiu 2006). *Nothofagus balansae* and *N. codonandra* roots were also characterized by the presence of a fungal mantle and a Hartig net. Another New Caledonian species, *N. aequilateralis*, has also been previously shown to be able to develop ECM associations (McCoy 1991). *Nothofagus* are indirectly, by the presence of putative ECM fungal fruit bodies, and/or directly, by investigation of the root system, defined in other regions of the world (i.e., Australia, New Zealand, Papua New Guinea and South America) as ECM trees (Horak and Wood 1990, Garnica et al. 2003, Tedersoo et al. 2008, Dickie et al. 2010), and subsequently recognized as an important ECM genus in the Southern Hemisphere (Smith and Read 2008).

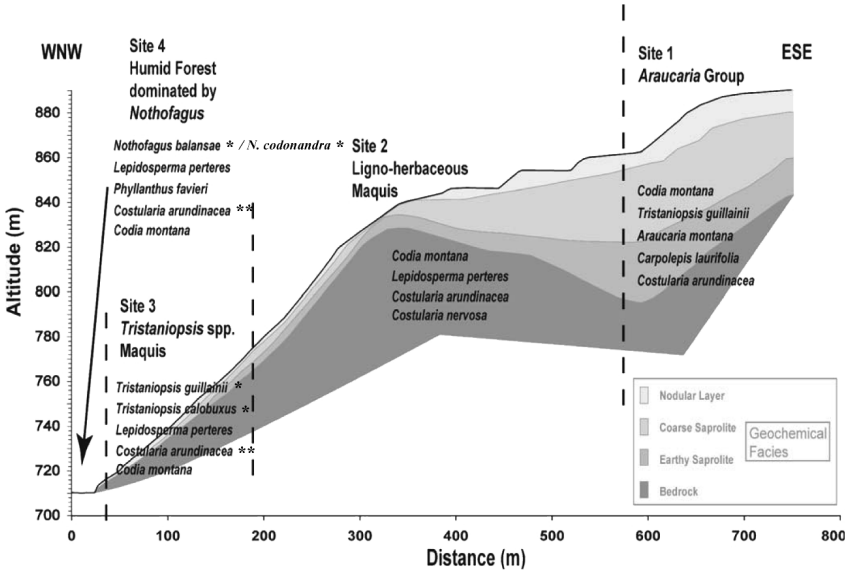


Fig. 2. Repartition of the four distinct plant formations (sites 1 to 4) on the topographic sequence studied by Perrier et al. (2006) at the Koniambo Massif. Most abundant and potential species for restoration purpose within each vegetation type are shown. Plants with ECM structures or ECM-like-structures on their root systems are indicated by an asterisk or two asterisks, respectively. The distance in meters (m) from the valley to the plateau and the altitude are given in abscissa and ordinate, respectively. Modified data from Perrier et al. (2006a).

Surprisingly, Perrier et al. (2006a) observed ECM-like structures on Cyperaceae roots as *Costularia arundinacea*, with the presence of a fungal mantle but the absence of a Hartig net. Such observation has already been done on two other Cyperaceae species belonging to the genus *Carex* (Harrington and Mitchell 2002). However, the colonization of *C. arundinacea* roots was only observed on sites 3 and 4, dominated by *T. guillainii* and *N. balansae* respectively (Fig. 2). The vicinity of both ECM plants may thus explain the colonization of *C. arundinacea* root systems. Further investigations led to the identification of other plant species as ECM (Table 1) (Amir and Ducousso 2010, F. Carriconde personal communication). Regarding the Myrtaceae, four additional *Tristaniopsis* species, two *Melaleuca* species and the monospecific and endemic genus *Arillastrum* are involved in such associations (Table 1). The Fabaceae *Acacia spirorbis* has also been identified as ECM (Ducousso et al. 2012). Finally, in New Caledonia, the ECM status has been characterized for only 13 plant species among Fabaceae, Myrtaceae and Nothofagaceae (Table 1). Giving the large representation of the Myrtaceae family, especially the Leptospermoidae group, and the Fabaceae family in New Caledonia (Morat et al. 2012), we

Table 1. Plant families and species in New Caledonia characterized as ECM. The biogeographical native status is given according to Jaffré et al. (2001) (N: native, i.e., species for which their natural distribution area extend beyond the boundaries of New Caledonia; E: endemic species; EE: endemic genus). The types of soils on which species are encountered are also indicated (C: calcareous; UM: ultramafic soils; VS: volcano-sedimentary). For species known to be present on more than one type of soil, the predominant types are highlighted in bold. Modified data from Amir and Ducousso (2010).

Family	Species	Biogeographical status	Type soil
Fabaceae	*Acacia spirorbis*	N	C, **VS**, **UM**
Myrtaceae	*Arillastrum gummiferum*	EE	UM
	Melaleuca pancheri	E	UM
	Melaleuca quinquenervia	N	C, **VS**, UM
	Tristaniopsis calobuxus	E	**UM**, VS
	Tristaniopsis glauca	E	UM
	Tristaniopsis guillainii	E	UM
	Tristaniopsis macphersonii	E	UM
	Tristaniopsis ninndoensis	E	VS
	Tristaniopsis vieillardii	E	UM
Nothofagaceae	*Nothofagus aequilateralis*	E	UM
	Nothofagus balansae	E	UM
	Nothofagus codonandra	E	UM

could expect a large number of species to be involved in such mutualistic interaction. This clear lack of knowledge is furthermore well-illustrated by the fact that the ECM status remains unknown for two *Nothofagus* species in New Caledonia: *N. baumanniae* and *N. discoidea*.

The aboveground and belowground fungal diversity has been, to some extent, investigated on the topographic sequence at the Koniambo Massif (Fig. 2) by collecting sporocarps, ectomycorrhizal root tips and hyphal mats in the soil (Table 2, Perrier 2005). Molecular identification has been carried out by sequencing the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS), a widely used marker in mycology and recently defined as the reference region for fungal DNA barcoding (Schoch et al. 2012). Twenty-nine sporocarps, 11 ECM root tips and 7 hyphae collected from soil cores were successfully sequenced (Table 2, Perrier 2005). Comparison of the generated ITS sequences to the international available database GenBank using the BLAST algorithm (Altschul et al. 1990) showed the presence of several genera (Table 2). Interestingly, out of the total of 47 samples, 45 presented a percentage of similarity less than 97% (Table 2), a value commonly used to differentiate ECM species (e.g., Tedersoo et al. 2003, Izzo et al. 2005, Smith et al. 2007). Two sporocarps, K66C and KC03C, had a percentage of similarity >97% with samples from Australia and New Zealand, respectively. Overall, these results suggest there is a diverse and unique ECM fungal assemblage at these study sites and possibly across New Caledonia at a regional scale.

Indeed, the description in the last few years of new putative ECM species, such as the impressive *Podoserpula miranda* (Fig. 3), thought to be associated with *Arillastrum gummiferum* in the South of New Caledonia (Ducousso et al. 2009), or the chanterelle, *Cantharellus garnieri* (Fig. 3) collected under distinct potential host trees in different localities and type of soils (Ducousso et al. 2004), strengthened the idea of the high fungal diversity in the archipelago. Regarding the abundance of the different fungal genera at Koniambo's sites, samples belonging to the *Cortinarius* genus were largely represented. Indeed, out of the 29 sporocarps, 11 ECM root tips and 7 hyphal mats collected, 11 (~38%), 6 (~55%), and 5 (~71%) were assigned to this genus. The large belowground representation of *Cortinarius* has already been highlighted in *Nothofagus* forests in Australia and New Zealand (Tedersoo et al. 2008, Dickie et al. 2010). Co-evolution between Cortinariaceae and *Nothofagus* in Australia has been suggested (Bougher et al. 1994), and could thus be one of the main driving forces that may have led to the diversification of this fungal group in the Pacific region.However, the limited sampling size of Perrier's study (in total only 47 samples), and particularly the very restricted number of studies undergone to date on fungal diversity, do not allow us to draw any conclusions on the diversity level and the structure of this diversity on the archipelago.

Table 2. Sporocarps, ECM root tips and hyphae samples collected in the *Tristaniopsis* spp. maquis (site 3) and the rain forest dominated by *Nothofagus balansae* (site 4), located on the topographic sequence at the Koniambo Massif and genotyped by sequencing of the ITS region. The host plant (putative), the morphospecies when available, the ITS sequence length, the closest BLAST match and the related information are presented. ITS sequence data generated by Perrier (2005) were recently analyzed.

Sample reference	Sample type	Plant formation	Host plant (putative) †	Morphospecies	GenBank accesion number	Sequence lenght (bp)	Closest species BLAST match ‡	Bases matched	% Similarity	Best match GenBank accesion number
K66C	Sporophore	3	*Tristaniopsis guillainii*	*Pisolithus sp*	FJ656011	527	*Pisolithus sp*	520/526	99%	AF270787
K02C	Sporophore	4	*Nothofagus balansae*	*Boletus sp*	FJ656001	609	*Boletus sp*	384/449	86%	EU569234
K05C	Sporophore	4	*Nothofagus balansae*	nd	FJ656002	551	*Cortinarius subgemmeus**	498/561	89%	JX000354
K06C	Sporophore	4	*Nothofagus balansae*	-	FJ656003	605	*Phellodon sp*	544/597	91%	GU222318
K09C	Sporophore	4	*Nothofagus balansae*	nd	FJ656004	547	*Austrogautieria macrospora**	438/504	87%	GQ981492
K10C	Sporophore	4	*Nothofagus balansae*	nd	FJ656005	670	*Tricholoma imbricatum*	628/668	94%	AY573537
K10C	Sporophore	4	*Nothofagus balansae*	nd	FJ656005	670	*Tricholoma imbricatum*	626/668	94%	AY573537
K12C	Sporophore	4	*Nothofagus balansae*	nd	FJ656006	572	*Cortinarius austrovenetus*	534/573	93%	GQ890318
K14C	Sporophore	4	*Nothofagus balansae*	*Inocybe sp*	FJ656007	506	*Dermocybe largofulgens**	483/504	96%	GU233324
K16C	Sporophore	4	*Nothofagus balansae*	nd	FJ656008	706	*Lactarius scrobiculatus*	660/719	92%	EU597079
K18C	Sporophore	4	*Nothofagus balansae*	Lactaroides	FJ656009	598	*Russula zonaria * *	526/569	92%	DQ421990
KC02C	Sporophore	4	*Nothofagus balansae*	nd	FJ656012	436	*Cortinarius lividus*	390/433	90%	AF539734
KC05C	Sporophore	4	*Nothofagus balansae*	nd	FJ656014	580	*Inocybe aeruginascens**	491/569	86%	GU949591
KC08C	Sporophore	4	*Nothofagus balansae*	nd	FJ656016	670	*Lactarius olympianus*	624/684	91%	EF685079
KC11C	Sporophore	4	*Nothofagus balansae*	nd	FJ656018	609	*Russula sp*	577/608	95%	GU222292
KC12C	Sporophore	4	*Nothofagus balansae*	nd	FJ656019	518	*Cortinarius flammuloides*	460/540	85%	AF539716
KC16C	Sporophore	4	*Nothofagus balansae*	nd	FJ656020	574	*Cortinarius multiformis*	518/589	88%	AF389135
KC17C	Sporophore	4	*Nothofagus balansae*	nd	FJ656021	574	*Leratiomyces ceres*	394/411	96%	HQ604750
KC19C	Sporophore	4	*Nothofagus balansae*	nd	FJ656022	583	*Cortinarius singularis**	508/584	87%	JQ287672
KC22C	Sporophore	4	*Nothofagus balansae*	nd	FJ656023	528	*Austrogautieria macrospora**	442/503	88%	GQ981492
KD37C	Sporophore	4	*Nothofagus codonandra*	nd	FJ656038	582	*Cortinarius eutactus**	559/582	96%	JX000366
KC23C	Sporophore	4	*Tristaniopsis guillainii*	nd	FJ656024	414	*Cortinarius elaiops**	241/262	92%	JX000369
K01C	Sporophore	4	*Nothofagus balansae*	nd	FJ656000	701	*Tricholoma ustale*	632/713	89%	AF458435
K22C	Sporophore	4	*Nothofagus balansae*	nd	FJ656010	582	*Inocybe aeruginascens**	491/569	86%	GU949591
KC03C	Sporophore	4	*Nothofagus balansae*	nd	FJ656013	612	*Russula sp*	593/613	97%	GU222292
KC06C	Sporophore	4	*Nothofagus balansae*	nd	FJ656015	436	*Phaeocollybia redheadii*	394/411	96%	JN102541
KC10C	Sporophore	4	*Nothofagus balansae*	nd	FJ656017	447	*Cortinarius aff. austrosanguineus*	427/454	94%	GQ890317
KD36C	Sporophore	4	*Nothofagus codonandra*	nd	FJ656037	585	*Cortinarius elaiops**	554/586	95%	JX000369
KD42C	Sporophore	4	*Nothofagus codonandra*	nd	FJ656039	485	*Tricholoma ustale*	402/455	88%	AF458435
KE01-2M	ECM	3	*Tristaniopsis guillainii*	-	FJ656040	457	*Piloderma sp*	400/450	89%	JQ711951
KE02M	ECM	3	*Tristaniopsis guillainii*	-	FJ656041	511	*Cortinarius vernicifer**	387/449	86%	JX000370
KE04M	ECM	3	*Tristaniopsis guillainii*	-	FJ656042	438	*Piloderma sp*	383/430	89%	JQ711951
KD10M	ECM	4	*Nothofagus balansae*	-	FJ656025	552	*Oidiodendron chlamydosporicum*	477/519	92%	AF062789
KE06M	ECM	4	*Nothofagus balansae*	-	FJ656043	584	*Cortinarius amoenus*	544/590	92%	AF389160
KE12-1M	ECM	4	*Nothofagus balansae*	-	FJ656045	620	*Tricholoma ustale*	557/640	87%	AF458435
KD18M	ECM	4	*Nothofagus codonandra*	nd	FJ656026	474	*Cortinarius calyptratus**	425/476	89%	EU525980

KD29-2M	ECM	4	*Nothofagus codonandra*	-	FJ656033	608	*Cortinarius elaiops**	558/606	92%	JX000369
KD31"-2M	ECM	4	*Nothofagus codonandra*	-	FJ656034	570	*Cortinarius elaiops**	518/600	86%	JX000369
KD31'M	ECM	4	*Nothofagus codonandra*	-	FJ656035	682	*Tomentellopsis submollis*	642/684	94%	JQ711898
KD36-2M	ECM	4	*Nothofagus codonandra*	-	FJ656036	584	*Cortinarius singularis**	509/584	87%	JQ287672
KD19_1S	Hyphae	3	*Tristaniopsis guillainii*	-	FJ656027	698	*Lycoperdon sp*	682/726	94%	JX029934
KD19_2S	Hyphae	3	*Tristaniopsis guillainii*	nd	FJ656028	592	*Cortinarius eutactus**	560/597	94%	HQ533023
KD19_9S	Hyphae	3	*Tristaniopsis guillainii*	-	FJ656029	410	*Cortinarius sp*	350/402	87%	JQ287690
KD20_5S	Hyphae	3	*Tristaniopsis guillainii*	-	FJ656031	585	*Cortinarius sp*	543/594	91%	JN942302
KD20_6S	Hyphae	3	*Tristaniopsis guillainii*	nd	FJ656032	615	*Cortinarius sp*	552/621	89%	JN942302
KE12_2S	Hyphae	4	*Nothofagus balansae*	-	FJ656046	608	*Tricholoma ustale*	554/629	88%	AF458435
KE18_2S	Hyphae	4	*Nothofagus codonandra*	-	FJ656047	540	*Cortinarius subgemmeus**	477/560	85%	JX000354

† ECM root tips were sampled by tracing the roots from the tree trunks.
‡ Voucher specimens are indicated by an asterisk.

Fig. 3. Two new fungal species recently identified in New Caledonia: (A) *Podoserpula miranda* (*Atheliaceae*) and (B) *Cantharellus garnierii* (*Cantharellaceae*). Photos provided: courtesy Ducousso Marc, CIRAD.

Color image of this figure appears in the color plate section at the end of the book.

Although preliminary analysis of the ECM diversity has been achieved, a thorough description of ECM and fungal communities and the interaction with host-plants in New Caledonia should be carried out. In order to really investigate such fungal diversity and better understand the mechanisms involved, molecular ecology studies on ECM communities by sequencing sporocarps and ectomycorrhizas using the classical Sanger approach, complemented by the use of next generation sequencing on soil cores, should be undertaken.

3. *Pisolithus albus* from Ultramafic Soils in New Caledonia: Diversity and Physiological Response to Nickel

Pisolithus albus (Cooke and Massee) is a fungal species belonging to *Pisolithus* Alb. and Schwein known to be one of the major ectomycorrhizal *Boletale* distributed on a worldwide scale that forms ectomycorrhizal symbioses with a broad range of angiosperm and gymnosperm tree species (Marx 1977, Martin et al. 2002). *Pisolithus* is also regarded as an early colonizer that persists on sites subject to edaphic stresses (Anderson et al. 1998). In New Caledonia, *P. Albus* fruit bodies are very abundant. The species also develops ectomycorrhizal associations with many endemic plants belonging to various genera of the *Myrtaceae* such as *Melaleuca*, *Tristaniopsis* and *Sannantha*, and one *Mimosaceae*, i.e., *Acacia spirorbis* (Perrier 2005). In New Caledonia most of the plants able to form ECM with *P. albus* dominate specific zones in their respective ecosystem: for example, *Tristaniopsis* genus colonizes specific zones of the ultramafic ecosystem at an altitude from 400 to 900 meters (L'Huillier et al. 2010). Altogether, the abundance of *P. albus* and its ability to develop ECM symbioses with endemic plants that colonize specific ecosystems in New Caledonia has led to the study of the genetic diversity of *P. albus* in New Caledonia.

3.1 Diversity of Pisolithus albus and their symbioses in New Caledonia

Isolates of ectomycorrhizal *P. albus* were sampled from both ultramafic and non-ultramafic soils in New Caledonia in order to investigate the relationships between (i) genetic diversity and (ii) the edaphic constraintssuch as the deficiency of major nutrient elements (N, K and P), the unbalanced Ca/Mg ratio and the presence of heavy metals at high concentrations (Jourand et al. 2010a). Fruiting body description, spore morphology (Fig. 4) and phylogenetic analysis based on internal transcribed spacer (ITS) rDNA (as previously reported by Martin et al. 2002) sequences confirmed that all isolates belong to *P. albus* and are closely related to other Australasian specimens (Fig. 5). In addition, the ecology of *P. albus* isolated from New Caledonia confirmed the dominant association with endemic plants belonging to genera of the Myrtaceae family (e.g., *Melaleuca*, *Sannantha*, *Tristaniopsis*) or the Fabaceae family (e.g., *Acacia*).

Altogether, the ecological and molecular data of *P. albus* isolated in New Caledonia were in agreement with the phylogeography of the ectomycorrhizal *Pisolithus* genus inferred from rDNA-ITS sequences, suggesting that (1) evolutionary lineages within *Pisolithus* are related to the biogeographical origin of their plant hosts (Martin et al. 2002) and (2) a long-distance dispersal event of ectomycorrhizal fungi from Australia

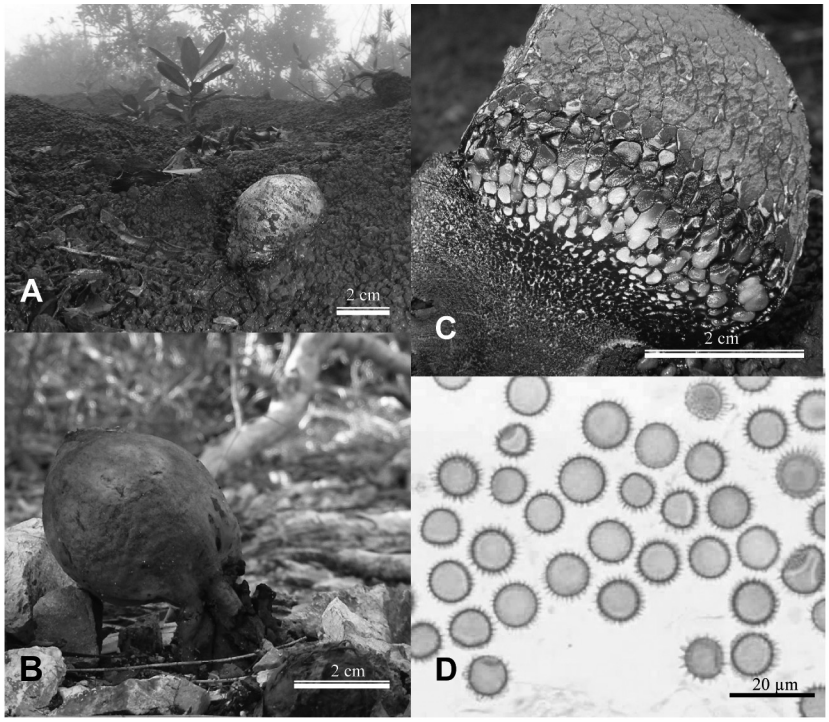


Fig. 4. *Pisolithus albus* from New Caledonia. A: *Pisolithus albus* MD07-117 from the Koniambo massif; B: *Pisolithus albus* MD07-228 from the Ouen-Toro, Noumea; C: cross section of *Pisolithus albus* MD07-166 from Pindjen water-fall and D: globose spores (8.77 to 9.62 µm) of *Pisolithus albus* MD06-379 from Poum, erected spines (1.2 µm) are clearly visible. From Jourand et al. (2010a).

Color image of this figure appears in the color plate section at the end of the book.

might explain the introduction of *Pisolithus* species in the South Pacific zone (Moyersoen et al. 2003). Interestingly, the use of other molecular tools such as ITS-restriction fragment length polymorphism (Fig. 6A) and amplified fragment length polymorphism markers (AFLP) (Fig. 6B), showed the existence of one genotype within *P. albus* grouping isolates from ultramafic soils (Jourand et al. 2010a). Such results raised the question of the presence a fungal ecotype on ultramafic soils, as described for plants found on these soils (Harrison and Rajakaruna 2011). They also contribute to the hypothesis of a link between the phylogenetic population structure and the ecological adaptation due to the particular mineral constraints,

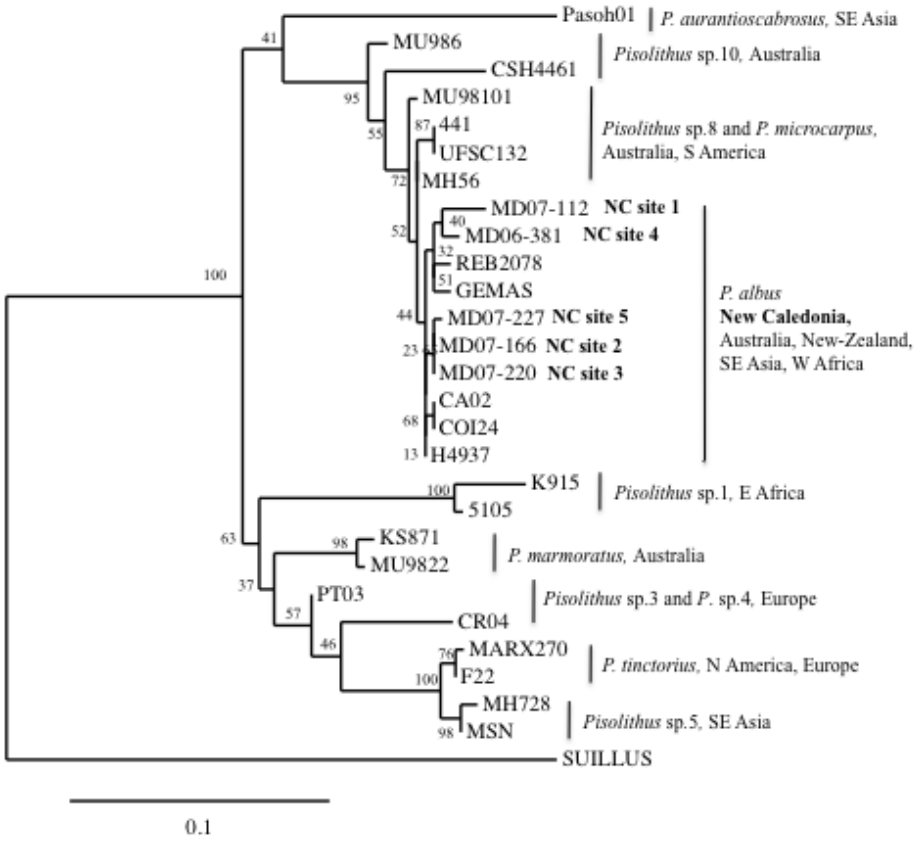
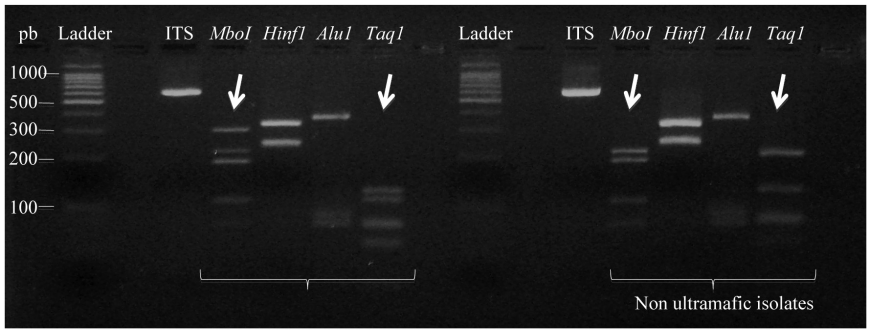


Fig. 5. Phylogenetic synthetic relationships among representative *Pisolithus* sp. from New Caledonia collection sites and worldwide reference isolates. The phylogeny is based on the analysis of the rDNA ITS1, 5.8S and ITS2 sequences. The tree was rooted with *Suillus luteus* ITS sequences. Significant bootstrap frequencies are indicated. Abbreviations: S America: South America; SE Asia: South East Asia; W Africa: West Africa, E Africa: East Africa. From Jourand et al. (2010a).

in particular ultramafism, as observed in ectomycorrhizal communities from other ultramafic soils (Urban et al. 2008). To further investigate this hypothesis, considering that nickel is one of the most toxic and bioavailable metal found at high concentrations in these soils (Echevarria et al. 2006), *P. albus* molecular and physiological responses to nickel were assessed in a further study.

A) *P. albus* ITS RFLP profiles



B) *P. albus* AFLP profile relationship

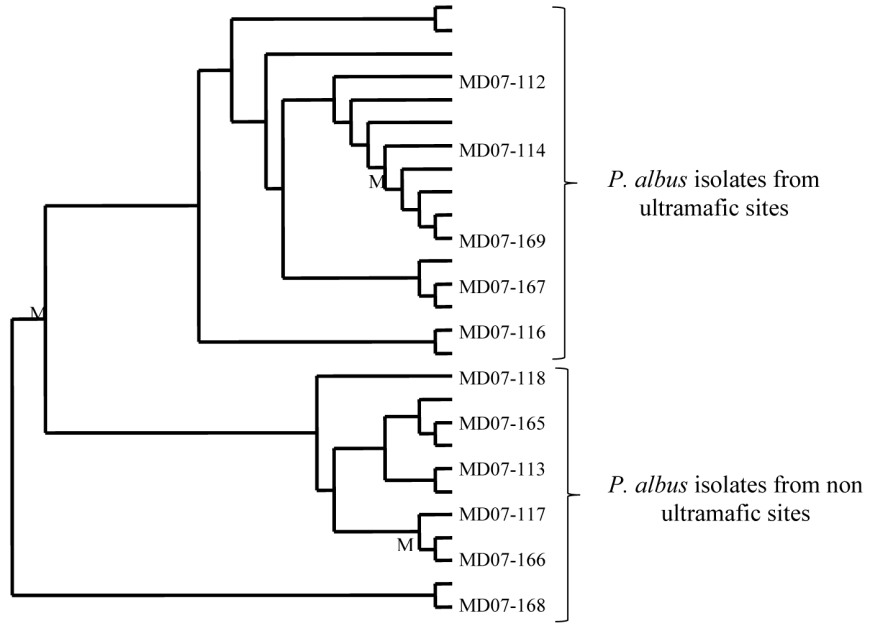


Fig. 6. A) Representative patterns of ITS restriction fragment length polymorphism (RFLP) profiles of *Pisolithus albus* isolates from both ultramafic and volcano sedimentary soils compared to both undigested amplified ITS and 100 pb DNA Ladder (Promega). Arrows highlight major differences between profiles. B) Genetic relationship within *P. albus* isolates from New Caledonia according to AFLP analysis. Bootstrap consensus UPGMA tree obtained for 882 AFLP scored fragments obtained with the 9 selective primers pairs on the 27 *P. albus* isolates (100 replicates). Data from Jourand et al. (2010a).

3.2 Tolerance and adaptation to nickel of Pisolithus albus from New Caledonia

In ultramafic soils, nickel (Ni) is one the most bioavailable and phytotoxic element: nickel content may reach up to 10 g/kg in ultramafic soils when compared with the average 50 mg/kg in cultivated soils (Wenzel and Jockwer 1999, Echevarria et al. 2006). This mineral element is a crucial selecting factor for plant survival on ultramafic soils: to grow on such high concentrations of nickel as found in serpentine environments (often coinciding with high concentrations of other heavy metals), plants had to develop major adaptations that include exclusion of the absorption of the toxic metal by the roots and/or metal hyperaccumulation with internal complexation and compartmentation (Kazakou et al. 2008). In addition, ECM symbioses might contribute to limit the metal accessibility and uptake by the plant (Colpaert et al. 2011).

3.2.1 Pisolithus albus nickel content and in vitro tolerance

In the previous study, the nickel concentration in fruiting body tissues of *Pisolithus albus* isolates from New Caledonia was assessed, as well as the *in vitro* nickel tolerance of cultivated mycelia from isolates collected from soil type (ultramafic vs non-ultramafic) where *P. albus* were collected (Jourand et al. 2010a). In fruiting bodies of *P. albus* from ultramafic soils, the nickel concentration reached an average of 5.7µg/g of dried tissue. In contrast, tissue of carpophores of isolates collected from non-ultramafic soils contained 2.5 times less nickel. In addition, *P. albus* mycelia from ultramafic soils included isolates with high variations of *in vitro* nickel-tolerance, with both nickel-tolerant isolates (with an average that half the maximal effective concentration of Ni that reduced fungal growth by 50% was 575 mM) and nickel-sensitive isolates (average Ni EC_{50} 37 mM). In contrast, all isolates from non-ultramafic soils were found to be nickel-sensitive (average Ni EC_{50} at 32 mM).

Within *Pisolithus* spp., previous studies have showed that some isolates were able to tolerate high concentrations of nickel. For example isolates of *Pisolithus tinctorius* were found to tolerate nickel with a Ni EC_{50} ranging from 126 to 170 mM (Tam 1995). Aggangan et al. (1998) also described one isolate of *P. tinctorius* from ultramafic soils in New Caledonia able to grow on nickel from 20 to 200 µM. More recently, Blaudez et al. (2000) and Ray et al. (2005) reported isolates of *P. tinctorius* that are able to grow on medium with nickel concentrations ranging from 17 to 350 µM. The mycelia from *P. albus* isolates from New Caledonian ultramafic soils displayed both *in vitro* nickel-sensitive and nickel-tolerant phenotypes. In addition, the nickel-tolerant isolates presented a noteworthy tolerance to Ni with an

average Ni EC_{50} two to three times higher than the Ni EC_{50} already reported for other *Pisolithus* spp. mentioned above. To explain the high variability in nickel-tolerance observations, it was first hypothesized that such variations could be correlated to high real fluctuations of bioavailable nickel content in ultramafic soils, which is assessed as the DTPA-Ni fraction according to Echevarria et al. (2006). Perrier et al. (2006a) reported that the nickel-DTPA concentrations in ultramafic soils varied in a range from 17 to 980 µmol/kg. Assuming that the average nickel-DTPA concentration does not reflect real fluctuations of bioavailable nickel in ultramafic soils, and considering the range of nickel-DTPA concentrations in ultramafic soils reported by Perrier et al. (2006a,b), it is not surprising to find isolates of *P. albus* with high variations in nickel tolerance from the same ultramafic site. Similar variations in metal-tolerant fungal populations in correlation to metal-soil content have already been reported. For instance, in Suilloid fungi, populations displayed zinc tolerance relative to zinc concentrations in polluted soils, suggesting an evolutionary adaptation of fungi to the soil environment (Colpaert et al. 2004). More recently, evidence of adaptation to nickel was provided in isolates of *Cenococcumgeophilum* from ultramafic soils in Portugal and the USA (Gonçalves et al. 2009). No clear relationship between the phenotypic physiological response to nickel and the population genetic differentiation observed within *P. albus* from soils could be established as the nickel-tolerant isolates from ultramafic soils did not cluster in a homogeneous group. It was thus tempting to speculate that the capacity of some *P. albus* to tolerate high nickel concentrations reflects the expression of an adaptive response to high concentrations of bioavailable nickel in soils as suggested for other fungi in response to high heavy metal levels (Hartley et al. 1997, Colpaert et al. 2004, Gonçalves et al. 2009). However, if New Caledonian population of *P. albus* seems to be structured into one ecotype, nickel tolerance alone might not be a sufficient feature to explain such results. Thus, the ultramafic constraint should be considered as a whole, even if each factor (N, P, K contents, Ca/Mg imbalance, heavy metal presence) is studied separately, as suggested by Kazakou et al. (2008).

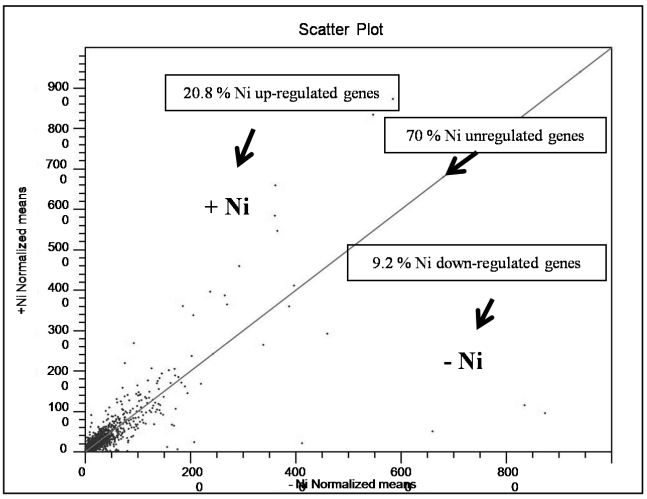
3.2.2 Pisolithus albus transcriptomic response to nickel

In another study on nickel-tolerant *Pisolithus albus* isolated from ultramafic soils in New Caledonia, the comparison of the transcriptomes of a nickel-tolerant isolate in the presence and absence of nickel was monitored by using pyrosequencing and quantitative polymerase chain reaction (qPCR) approaches in order to identify genes involved in the specific molecular response to nickel and to quantify their expression (Majorel et al. 2012). As a result of the experiment, two non-normalized cDNA libraries were obtained from one nickel-tolerant *P. albus* isolate grown in the presence and

absence of nickel. A total of 19,518 genes could be obtained through the de novo assembly of the sequence reads from the two non-normalized cDNA libraries. The expression of 30% of these genes was regulated by nickel. Further analysis identified 4,211 genes (21%) that were up-regulated by nickel and 1763 genes (9%) that were down-regulated by nickel. The global statistical distribution of these 19,518 genes is presented on a scatter plot in Fig. 7A. The genes, for which expression was induced most markedly by nickel, encoded products that were putatively involved in a variety of biological functions, such as the modification of cellular components (53%) and the regulation of biological processes (27%) and molecular functions (20%) (Fig. 7B). Compared to most previous studies conducted on ECM samples isolated from soils polluted with heavy metals as a result of human activities (Jacob et al. 2004, Muller et al. 2007, Ruytinx et al. 2011), this study was the first repository of its kind. These results clearly suggested a positive transcriptomic response of the fungus to nickel-rich environments, which may contribute to the tolerance of the fungus to the extreme conditions as found in New Caledonia. The analysis of the results based on gene ontology (GO) analysis and functional genetic tools also suggests the role of these genes as putative adaptive mechanisms of nickel tolerance in *P. albus*. The majority of genes up-regulated by nickel belonged to the GO category 'cellular component'. Information on the annotations of these genes is valuable for the further investigation of gene functions, cellular structures and biological processes that might be involved in the tolerance of fungi to nickel via extracellular and intracellular mechanisms, as suggested by Bellion et al. (2006).

In the second step of the experiment, ten genes that were analyzed as the most nickel-induced in pyrosequencing were characterized by qPCR analysis in both nickel-tolerant and nickel-sensitive *P. albus* isolates from ultramafic soils. Among them, six genes were expressed exclusively in nickel-tolerant isolates as well as in ECM samples *in situ*. In addition, in the nickel-tolerant isolates, the presence of nickel increased their level of expression by between one- and nine-fold (Fig. 8). Their functional classification showed that these genes encoded for putative proteins involved either in chitin cell wall rearrangements as GPI-anchor-like protein and class III chitinase, or biological regulations as vacuolar protein sorting and APC amino acid permease, suggesting a possible role of fungi in metal immobilization and consequently in reducing metal toxicity when in symbiosis with plants. In previous studies involving fungi, many genes involved in the response to stress induced by heavy metals were found to encode proteins that function as metal transporters or metal-binding proteins (Jacob et al. 2004, Bellion et al. 2006, Bolchi et al. 2011). However, in Majorel et al. (2012), most of the genes overexpressed in the presence of nickel did not encode proteins that are generally involved in metal-stress responses. This suggested that the

A) Gene expression level repartition in Ni-tolerant *Pisolithus albus*



B) Gene functional GO terms assignment and distribution in Ni-tolerant *P. albus*

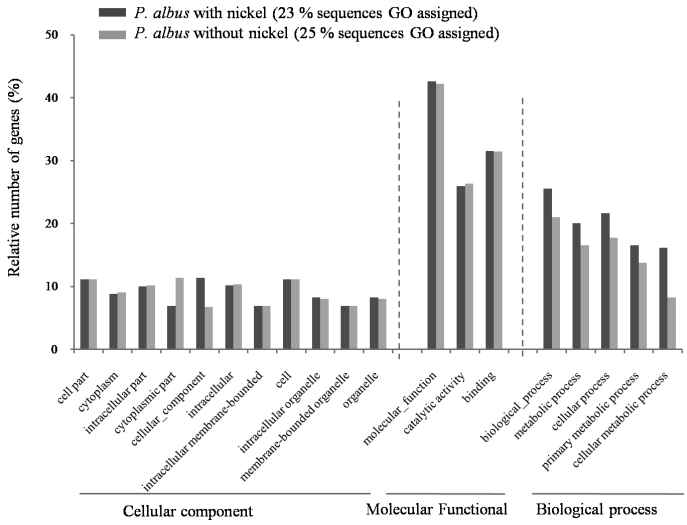


Fig. 7. A) Scatter plot presenting gene expression levels in *Pisolithus albus* Ni-tolerant ecotypefree-living mycelium grown without or with Ni at 250 µM. The expression levels of genes were normalized using a scale of 0 to 10,000. Each circle in the plot represents expression of one gene. B) Functional GO terms assignment and distribution of total sequences of two transcriptomes of Ni-tolerant *P. albus* with (+250 µM) and without nickel, among Gene Ontology (GO) biological process, molecular function and cellular component. From Majorel et al. (2012).

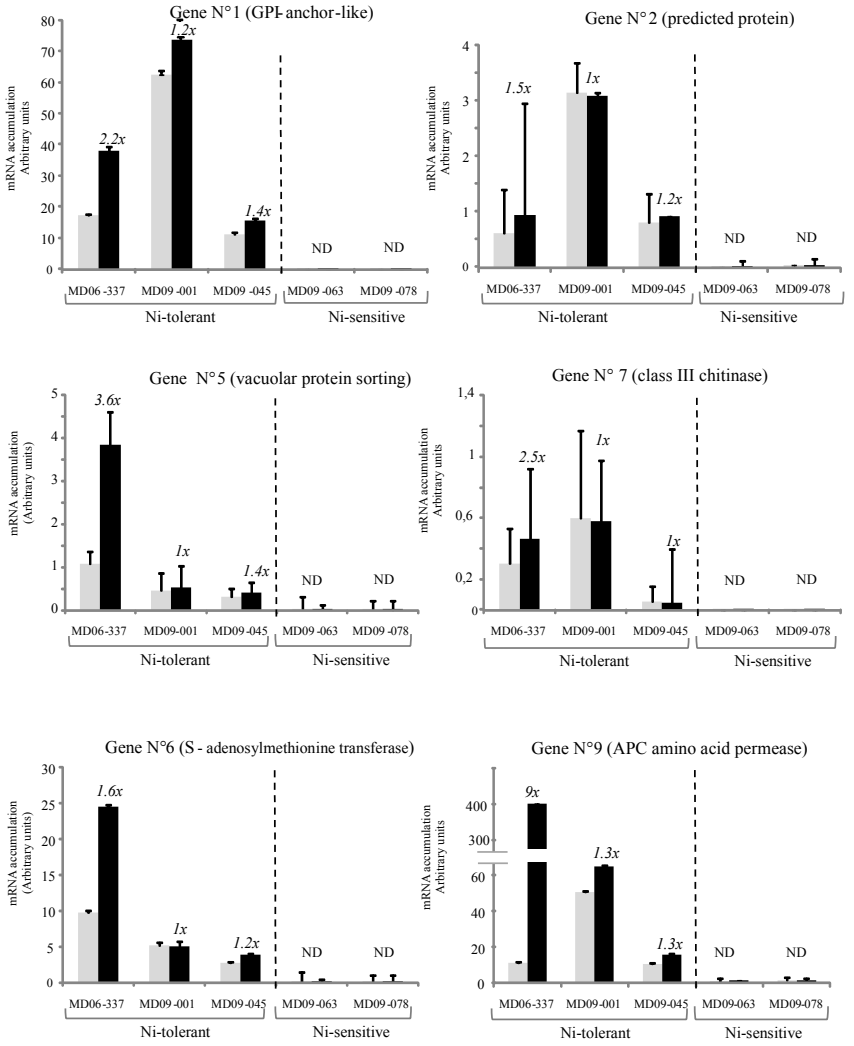


Fig. 8. Comparison of mRNA accumulation profiles for six selected Ni up-regulated genes in five *P. albus* isolates from ultramafic soil in presence of nickel 50 µM (black columns) and in absence of nickel (grey columns). Three nickel-tolerant isolates (MD06-337, MD09-045, and MD07-001) and two nickel-sensitive isolates (MD09-078 and MD09-063) were compared. Transcript accumulation was quantified by qPCR using $2^{-\Delta\Delta CT}$ method with normalization to two reference genes, GAPDH and EF4α, and is expressed as arbitrary units. The data indicate mean values ± S.D. values, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the black columns in italics. ND: mRNA non-detected (Ct values >37). From Majorel et al. (2012).

mechanisms that underlie the nickel tolerance in *P. albus* from ultramafic soils might differ from those of other fungi. In particular, that might reflect a long-term adaption to nickel in natural environment, in contrast to short-term adaptation on metal contaminated soils. Among the genes in which expression was remarkably induced in presence of nickel, and exclusively expressed in nickel-tolerant, it was interesting to identify genes that encode chitinase-like and glycosylphosphatidylinositol (GPI) cell-wall structural proteins that are involved in extracellular processes and encode putative cell-wall proteins. Recently, it was suggested that modifications of structural elements of the cell wall, such as the rearrangement of chitin and biosynthesis of glucan- or galactosamine-containing polymers, might play a key role in modulating the integrity of the cell wall and its capacity to immobilize heavy metals. In this way, such modifications could confer tolerance to metals and affect the ability of fungi to survive in stressful environments (Meharg 2003, Bellion et al. 2006, Fuchs and Mylonakis 2009).

Altogether, these results evidenced a strong and specific transcriptomic response to nickel of ultramafic-adapted *P. albus* both *in vitro* and *in situ*. This led the authors to hypothesize that the presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats (Majorel et al. 2012).

3.3 Role of ECM symbiose between nickel-tolerant Pisolithus albus and its host plant Eucalyptus globulus exposed at toxic nickel concentrations

As ECM symbioses are known to play a major role in the fitness of plants in the presence of heavy metals (Jentschke and Godbold 2000), experiments were carried out to analyse the symbiotic interactions between *P. albus* and one of its host plants in the presence of nickel. Ectomycorrhizal *Pisolithus albus* isolated in nickel-rich ultramafic soils from New Caledonia and showing *in vitro* adaptive nickel tolerance were inoculated to *Eucalyptus globulus* Labill used as a *Myrtaceae* plant-host model to study ectomycorrhizal symbiosis. Plants were then exposed to a nickel dose-response experiment with increased nickel treatments up to 60 mg/kg soil as maximum extractable nickel content found in ultramafic soils (Perrier et al. 2006a). Results showed that plants inoculated with ultramafic ECM *P. albus* were able to tolerate high and toxic concentrations of Ni (up to 60 mg/kg) while uninoculated controls were not (Fig. 9). At the highest nickel concentration tested, root growths were more than 20-fold higher and shoot growths more than 30-fold higher in ECM plants compared with

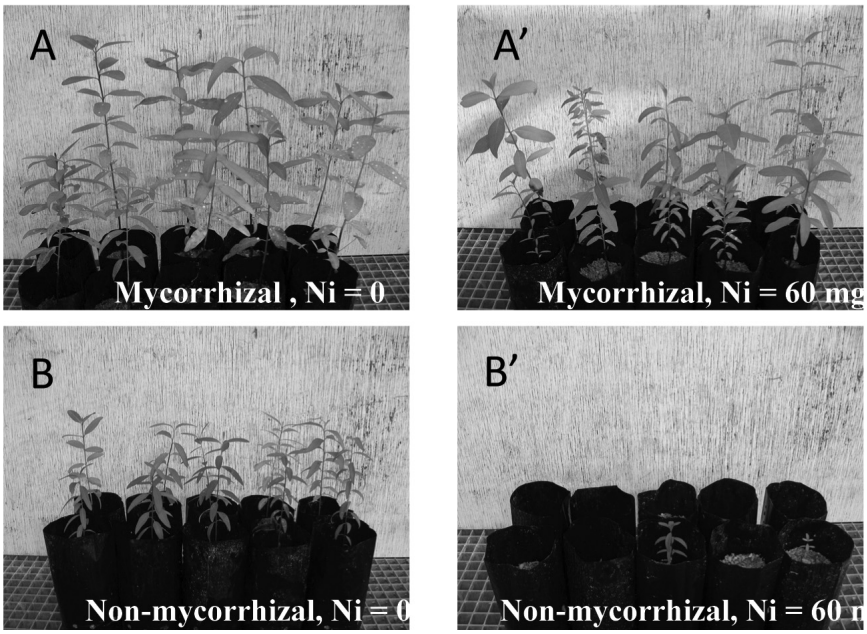


Fig. 9. *Eucalyptus globulus* seedlings after 12-weeks growth. A and A′ mycorrhizal; B and B′: non-mycorrhizal (controls). A and B: no nickel added; A′ and B′ seedlings treated with Ni. From Jourand et al. (2010b).

Color image of this figure appears in the color plate section at the end of the book.

control plants. Ergosterol was also measured in roots as it is a major sterol in fungi and is a good indicator of the level of mycorrhizal colonization of roots (Martin et al. 1990). Without nickel, roots had a mean level of 19.7% ectomycorrhization. At low nickel concentrations (0.6 and 6 mg/kg), the level of root ECM colonization varied from 15.6 to 27.8%. At high and toxic nickel concentrations (30 and 60 mg/kg), the level of root colonization was significantly reduced to around 9%, but confirmed the presence of viable ECM. At the highest nickel concentration tested, the improved growth in ECM plants was also associated witha 2.4-fold reduction in root nickel concentration but a massive 60-fold reduction in transfer of nickel from root to shoots, while for all other major plant nutrient elements analyzed, i.e., N, P, K, Ca and Mg, no significant differences in concentration were noted in either shoots or roots in response to nickel treatments or fungal treatment. To determine whether nickel tolerance was related to the release of metal binding compounds, exudates from roots were analyzed. The two principal chemical components of the exudate solution were non-protein thiols and oxalate. Control plants excreted significantly more thiols and oxalate than plants developing ECM symbiose with *P. albus*, with the increase being more

evident at higher nickel concentrations: control plants released 5-fold more thiols at 30 and 60 mg/kg of nickel, and 12- and 8-fold more oxalate at 30 and 60 mg/kg nickel, respectively. All these results confirmed that the nickel tolerance of the ECM has a substantial beneficial effect on the plant host. Ultramafic ECM isolates produced significant increases in growth in both the absence and low concentrations of nickel (from 0.6 to 6 mg/kg). Plant root surface was greatly increased, and the high level of mycorrhizal colonization is consistent with previous data on the interaction between *Pisolithus* and *Eucalyptus* (Martin et al. 1990, Brundrett et al. 1996). At low nickel concentrations, the increase in both shoot and root biomass observed in ECM plants compared with non-inoculated plants is probably a consequence of better mineral nutrition (Marschner 1995, Finlay 2004). However, at toxic levels of nickel the contribution of the ECM symbiosis with ultramafic *P. albus* to host nickel tolerance was more substantial. Interestingly, *P. albus* isolates could withstand *in vitro* high nickel concentrations but accumulated very little nickel in its tissue (Jourand et al. 2010b). The lower nickel uptake by mycorrhizal plants could not be explained by increased release of metal-complexing chelates since these were 5- to 12-fold lower in mycorrhizal plants at high nickel concentrations. It was proposed that the fungal sheath covering the plant roots acts as an effective barrier to limit transfer of nickel from soil into the root tissue.

4. Conclusions

Overall, the observations about ECM diversity found on ultramafic soils in New Caledonia raise very compelling questions about the evolutionary processes involved in fungal diversification in New Caledonia and at a regional scale. The focus on ECM *Pisolithus albus* isolated from soils in New Caledonia highlighted the identification of an ultramafic nickel-tolerant ecotype as reported in Jourand et al. (2010a), showing specific and adaptive molecular response to this metal (Majorel et al. 2012), and having a key role in plant host adaptation to toxic nickel concentrations as found in these soils (Jourand et al. 2010b). Together, these results constitute an important step in evaluating the potential of ECM symbioses for plant adaptation to ultramafic soils containing high concentrations of heavy metals, which is a prerequisite for their use in strategies for ecological restoration of mine sites as suggested by Reddell et al. (1999), Perrier et al. (2006a) and, more recently, Khosla and Reddy (2008). Further characterization of ECM fungal communities in New Caledonia would increase knowledge about fungal diversity and identify fungal species that might be relevant for plant inoculation purpose and their direct implications in restoration strategies.

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References

Alexander, E., R. Coleman, T. Keeler-Wolfe and S. Harrison. 2007. Serpentine Geoecology of Northern North America. Geology, Soils, and Vegetation. Oxford University Press, New York.

Aggangan, N., B. Dell and N. Malajczuk. 1998. Effects of chromium and nickel on growth of the ectomycorrhizal fungus *Pisolithus* and formation of ectomycorrhizas on *Eucalyptus urophylla* S.T. Blake. Geoderma 84: 15–27.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–10.

Amir, H. and M. Ducousso. 2010. Les bactéries et les champignons du sol sur roches ultramafiques. *In*: L. L'huillier, T. Jaffré and A. Wulff (eds.). Mines et environnement en Nouvelle-Calédonie: les milieux sur substrats ultramafiques et leur restauration. IAC, Nouméa, Nouvelle-Calédonie, pp. 129–145.

Anderson, I.C., S.M. Chambers and J.W.G. Cairney. 1998. Use of molecular methods to estimate the size and distribution of mycelial individuals of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. Mycol. Res. 102: 295–300.

Bellion, M., M. Courbot, C. Jacob, D. Blaudez and M. Chalot. 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol. Let. 254: 173–181.

Blaudez, D., C. Jacob, K. Turnau, J.V. Colpaert, U. Ahonen-Jonnarth, R. Finlay, B. Botton and M. Chalot. 2000. Differential responses of ectomycorrhizal fungi to heavy metals *in vitro*. Mycol. Res. 104: 1366–1371.

Bolchi, A., R. Ruotolo, G. Marchini, E. Vurro, L. Sanità di Toppi, A. Kohler, E. Tisserant, F. Martin and S. Ottonello. 2011. Genome-wide inventory of metal homeostasis-related gene product including a functional phytochelatin synthase in th hypogeous mycorrhizal fungus *Tuber melanosporum*. Fungal Genet. Biol. 48: 573–584.

Bougher, N.L., B.A. Fuhrer and E. Korak. 1994. Taxonomy and biogeography of Australian *Rozites* species mycorrhizal with *Nothofagus* and Myrtaceae. Aust. J. Bot. 7: 353–375.

Brady, K., A. Kruckberg and H. Bradshaw. 2005. Evolutionary ecology of plant adaptation to serpentine soils. Ann. Rev. Ecol. Evol. Syst. 36: 243–266.

Branco, S. 2010. Serpentine soils promote ectomycorrhizal fungal diversity. Mol. Ecol. 19: 5566–5576.

Branco, S. and R.H. Ree. 2010. Serpentine soils do not limit mycorrhizal fungal diversity. PLoS One 5:e11757.

Brearley, FQ. 2006. Differences in the growth and ectomycorrhizal community of Dryobalanops lanceolata (Dipterocarpaceae) seedlings grown inultramafic and non-ultramafic soils. Soil Biol. Biochem. 38:3407–3410.

Brooks, R. 1987. Serpentine and its vegetation. Dioscorides Press, Portland.
Brundrett, M., N. Bougher, B. Dell, T. Grove and N. Malajczuk. 1996. Working with mycorrhizas in forestry and agriculture. ACIAR Monogr. 32. Australian Centre for International Agricultural Research, Canberra, Australia, p. 374.
Chiarucci, A. and A.J.M. Baker. 2007. Advances in the ecology ofserpentine soils. Plant Soil 293: 1–217.
Coleman R.G. and C. Jove. 1992. Geological origin of serpentinites. *In*: A.J.M. Baker, J. Proctor and R.D. Reeves (eds.). The Vegetation of Ultramafic (Serpentine) Soils. Proceedings of the First International Conference on Serpentine Ecology. Intercept, Hampshire, UK, pp. 1–17.
Colpaert, J.V., L.A.H. Muller, M. Lambaerts, K. Adriaensen and J. Vangronsveld. 2004. Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi. New Phytol. 162: 549–560.
Colpaert, J.V., J. Wevers, E. Krznaric and K. Adriaensen. 2011. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Annals of Forest Science 68: 17–24.
Dickie, I.A., N. Bolstridge, J.A. Cooper and D.A. Peltzer. 2010. Co-invasion by *Pinus* and its mycorrhizal fungi. New Phytol. 187: 475–484.
Ducousso, M., C. Contesto, M. Cossegal and Y. Prin. 2004. *Cantharellus garnierii* sp. nov., une nouvelle chanterelle des maquis nickélifères de Nouvelle-Calédonie. Cryptogamie Mycol. 25: 135–145.
Ducousso, M., S. Proust, V. Denis and G. Eyssartier. 2009. *Podoserpula miranda* nom prov., une nouvelle espèce de champignon très spectaculaire découverte en Nouvelle-Calédonie. Bois et Forêts des Tropiques 302: 73–75.
Ducousso, M., F. Carriconde, E. Frischt, F. Juillot, L. Hannibal, C. Majorel and Jourand P. 2012. Diversité des champignons ectomycorhiziens d'*Acaciaspirorbis* dans des habitats très contrastés en Nouvelle-Calédonie. Journées Francophones Mycorhizes, troisième édition, 5-7 septembre 2012, Nancy, France.
Echevarria, G., S. Massoura, T. Sterckeman, T. Becquer, C. Schwartz and J.L. Morel. 2006. Assessment and control of the bioavailability of Ni in soils. Environ. Toxicol. Chem. 25: 643–651.
Finlay, R.D. 2004. Mycorrhizal fungi and their multifunctional roles. Mycol. 18: 91–96.
Fuchs, B.B. and E. Mylonakis. 2009. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot. Cell 8: 1616–1625.
Garnica, S., M. Weib and F. Oberwinkler. 2003. Morphological and molecular phylogenetic studies inSouth American *Cortinarius* species. Mycol. Res. 107: 1143–1156.
Gonçalves, S.C., A. Portugal, M.T. Goncalves, R. Vieira, M.A. Martins-Loucao and H. Freitas. 2007. Genetic diversity and differential *in vitro* responses to Ni in *Cenococcum geophilum*isolates from serpentine soils in Portugal. Mycorrhiza 17: 677–686.
Gonçalves, S.C., M.A. Martins-Louçao and H. Freitas. 2009. Evidence of adaptative tolerance to nickel in isolates of *Cenococcum geophilum* from serpentine soils. Mycorrhiza 19: 221–230.
Harrington, T.J. and D.T. Mitchell. 2002. Colonisation of root systems of *Carex flacca* and *C. pilulifera* by *Cortinarius* (*Dermocybe*) *cinnamomeus*. Mycol. Res. 106: 452–459.

Harrison, S. and N. Rajakaruna. 2011. Serpentine: the Evolution and Ecology of a Model System. The University of California Press, Berkeley and Los Angeles, CA.

Hartley, J., J.W.G. Cairney and A.A. Meharg. 1997. Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? Plant Soil 189: 303–319.

Hawksworth, D.L. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol. Res. 95: 641–655.

Hawksworth, D.L. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol. Res. 105: 1422–1432.

Horak, E. and J. Mouchacca. 1998. Annoted checklist of New Caledonian Basidiomycota. I. Holobasidiomycetes. Mycotaxon 68: 75–129.

Horak, E. and A.E. Wood. 1990. *Cortinarius* Fr. (Agaricales) in Australasia. 1. Subgen. *Myxacium* and subgen. *Paramyxacium*. Sydowia 42: 88–168.

Izzo, A., J. Agbowo and T.D. Bruns. 2005. Detection of plot-level changes in ectomycorrhizal communities across years in a old-growth mixed-conifer forest. New Phytol. 166: 619–630.

Jacob, C., M. Courbot, F. Martin, A. Brun and M. Chalot. 2004. Transcriptomic responses to cadmium in the ectomycorrhizal fungus *Paxillus involutus*. FEBS Letters 576: 423–427.

Jaffré, T. 1992. Floristic and ecological diversity of the vegetation on ultramafic rocks in New Caledonia. *In*: A.J.M. Baker, J. Proctor and R.D. Reeves (eds.). The Vegetation of Ultramafic Soils. Intercept Ltd, Andover, UK, pp. 101–107.

Jaffré, T., Morat P., Veillon J.M., Rigault F., Dagostini G. 2001.Composition and characterisation of the native flora of New Caledonia. Documents Scientifiques et Techniques - IRD : II, 121 p.

Jaffré, T. and L. L'Huillier. 2010. La végétation des roches ultramafiques ou terrains miniers. *In*: L. L'huillier, T. Jaffré and A. Wulff (eds.). Mines et environnement en Nouvelle-Calédonie: les milieux sur substrats ultramafiques et leur restauration. IAC, Nouméa, Nouvelle-Calédonie, pp. 45–103.

Jentschke, G. and D.L. Godbold. 2000. Metal toxicity and ectomycorrhizas. Physiol. Plant 109: 107–116.

Jourand, P., M. Ducousso, C. Loulergue-Majorel, L. Hannibal, S. Santoni, Y. Prin and M. Lebrun. 2010a. Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype. FEMS Microbiol. Ecol. 72: 238–249.

Jourand, P., M. Ducousso, R. Reid, C. Majorel, C. Richert, J. Riss and M. Lebrun. 2010b. Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant *Eucalyptus globulus* at toxic nickel concentrations. Tree Physiol. 30: 1311–1319.

Kazakou, E., P.G. Dimitrakopoulos, R.D. Reeves, A.J.M. Baker and A.Y. Troumbis. 2008. Hypotheses, mechanisms, and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. Biol. Rev. 83: 495–508.

Khosla, B. and M.S. Reddy. 2008. Response of ectomycorrhizal fungi on the growth and mineral nutrition of eucalyptus seedlings in bauxite mined soil. Amer-Eurasian J. Agri. Environ. Sci. 3: 123–126.

L'Huillier, L., T. Jaffré and A. Wulf. 2010. Mines et environnement en Nouvelle-Calédonie : les milieux sur substrats ultramafiques et leur restauration. IAC Ed, Noumea, New Caledonia.

Majorel, C., L. Hannibal, M.E. Soupe, F. Carriconde, M. Ducousso, M. Lebrun and P. Jourand. 2012. Tracking nickel adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach. Mol. Ecol. 21: 2208–2223.

Martin, F., C. Delaruelle and J.L. Hilbert. 1990. An improved ergsoterol assay to estimate fungal biomass in ectomycorrhizas. Mycol. Res. 94: 1059–1064.

Martin, F., J. Diez, B. Dell and C. Delaruelle. 2002. Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from ribosomal DNA ITS sequence. New Phytol. 153: 345–357.

Marschner, H. 1995. Mineral nutrition of higher plants 2nd edn. Academic Press, London, UK, p. 889.

Marx, D.H. 1977. Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. Can. J. Microbiol. 23: 217–223.

McCoy, S.G. 1991. Edaphic controls influencing the distribution of *Nothofagus aequilateralis* on ultrabasic soils at the Col the Mouirange, New Caledonia, Australian National University.

Meharg, A.A. 2003. The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. Mycol. Res. 107: 1253–1265.

Morat, P., T. Jaffré, F. Tronchet, J. Munzinger, Y. Pillon, J.-M. Veillon and M. Chalopin. 2012. The taxonomic database "FLORICAL" and characteristics of the indigenous flora of New Caledonia. Adansonia 34, in press.

Moser, A.M., C.A Petersen, J.A. D'Allura and D. Southworth. 2005. Comparison of ectomycorrhizas of *Quercus garryana* (*Fagaceae*) on serpentine and non serpentine soils in southwestern Oregon. Am. J. Bot. 92: 224–230.

Moser, A.M., J. Frank, J.A. D'Allura and D. Southwood. 2009. Ectomycorrhizal communities of *Quercus garryana* are similar on serpentine and non serpentine soils. Plant Soil 315: 185–194.

Mouchacca, J. 1998. Ascomycetes described from New Caledonia, South Pacific region. Mycotaxon 67: 99–121.

Mouchacca, J. and E. Horak. 1998. Annotated cheklist of New Caledonia Basidiomycota. II. Rusts and Smuts. Mycotaxon 69 : 13–30.

Moyersoen, B., Beever, R.E. and F. Martin 2003. Genetic diversity of *Pisolithus* in New-Zealand indicates multiple long-distance dispersal from Australia. New Phytol. 160: 569–579.

Muller, L.A., J. Vangronsveld and J.V. Colpaert. 2007. Genetic structure of *Suillus luteus* populations in heavy metal polluted and non-polluted habitats. Mol. Ecol. 16: 4728–4737.

Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. da Fonseca and J. Kent. 2000. Biodiversity hotspots for conservation priorities. Nature 403: 853–858.

Perrier, N. 2005. Bio-géodiversité fonctionelle des sols latéritiques miniers: application à la restauration écologique (Massif du Koniambo, Nouvelle-Calédonie). PhD thesis. University of New Caledonia, New Caledonia.

Perrier, N., H. Amir and F. Colin. 2006a. Occurrence of mycorrhizal symbioses in the metal-rich lateritic soils of the Koniambo Massif, New Caledonia. Mycorrhiza 16: 449–458.

Perrier, N., J.P. Ambrosi, F. Colin and R.J. Gilkes. 2006b. Biogeochemistry of a regolith: the New Caledonian Koniambo ultramafic massif. J. Geochem. Explor. 88: 54–58.

Proctor, J. 2003. Vegetation and soil and plant chemistry on ultramafic rocks in the tropical Far East. Perspect. Plant Ecol. 6: 105–124.

Rajkumar, M., M.N.V. Prasad, H. Freitas and N. Ae. 2009. Biotechnological applications of serpentine soil bacteria for phytoremediation of trace metals. Crit. Rev. Biotechnol. 2: 120–130.

Ray, P., R. Tiwari, G.U. Reddy and A. Adholeya. 2005. Detecting the heavy metal tolerance level in ectomycorrhizal fungi *in vitro*. World J. Microb. Biot. 21: 309–315.

Reddell, P., V. Gordon and M.S. Hopkins. 1999. Ectomycorrhizas in *Eucalyptus tetrodonta* and *E. miniata* forest communities in tropical Northern Australia and their role in the rehabilitation of these forests following mining. Aust. J. Bot. 47: 881–907.

Ruytinx, J., A.R. Craciun, K. Verstraelen, J. Vangronsveld, J.V. Colpaert and N. Verbruggen. 2011. Transcriptome analysis by cDNA-AFLP of *Suillus luteus* Cd-tolerant and Cd-sensitive isolates. Mycorrhiza 21: 145–154.

Schoch, C.L., K.A. Seifert, S. Huhndorf, V. Robert, J.L. Spouge, C.A. Levesque, W. Chen and F.B. Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. P. Natl. Acad. SCI USA 109: 6241–6246.

Smith, S. and D. Read. 2008. Mycorrhizal Symbiosis, 2nd edn. Academic Press, London.

Smith, M.E., G.W. Douhan and D.M. Rizzo. 2007. Ectomycorrhizal community structure in a xeric *Quercus* woodland based on rDNA sequence analysis of sporocarps and pooled roots. New Phytol. 174: 847–863.

Tam, P.C.F. 1995. Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by Pisolithus tinctorius. Mycorrhiza 5: 181–187.

Tedersoo, L., U. Kõljalg, N. Hallenberg and K.-H. Larsson. 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytol. 159: 153–165.

Tedersoo, L., T. Jairus, B.M. Horton, K. Abarenkov, T. Suvi, I. Saar and U. Koljalg. 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon specific primers. New Phytol. 180: 479–490.

Urban, A., M. Puschenreiter, J. Strauss and M. Gorfer. 2008. Diversity and structure of ectomycorrhizal and co-associated fungal communities in a serpentine soil. Mycorrhiza 18: 339–354.

Wang, B. and Y.-L. Qiu. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16: 299–363.

Wenzel, W.W. and F. Jockwer. 1999. Accumulation of heavy metals in plants grown on mineralised soils of the Austrian Alps. Environ. Pollut. 104: 145–15.

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Tracking nickel-adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach

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Abstract

The fungus *Pisolithus albus* forms ectomycorrhizal (ECM) associations with plants growing on extreme ultramafic soils, which are naturally rich in heavy metals such as nickel. Both nickel-tolerant and nickel-sensitive isolates of *P. albus* are found in ultramafic soils in New Caledonia, a biodiversity hotspot in the Southwest Pacific. The aim of this work was to monitor the expression of genes involved in the specific molecular response to nickel in a nickel-tolerant *P. albus* isolate. We used pyrosequencing and quantitative polymerase chain reaction (qPCR) approaches to investigate and compare the transcriptomes of the nickel-tolerant isolate MD06-337 in the presence and absence of nickel. A total of 1 071 375 sequencing reads were assembled to infer expression patterns of 19 518 putative genes. Comparison of expression levels revealed that 30% of the identified genes were modulated by nickel treatment. The genes, for which expression was induced most markedly by nickel, encoded products that were putatively involved in a variety of biological functions, such as the modification of cellular components (53%), regulation of biological processes (27%) and molecular functions (20%). The 10 genes that pyrosequencing analysis indicated were induced the most by nickel were characterized further by qPCR analysis of both nickel-tolerant and nickel-sensitive *P. albus* isolates. Five of these genes were expressed exclusively in nickel-tolerant isolates as well as in ECM samples *in situ*, which identified them as potential biomarkers for nickel tolerance in this species. These results clearly suggest a positive transcriptomic response of the fungus to nickel-rich environments. The presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats.

Keywords: ectomycorrhiza, New Caledonia, nickel, *Pisolithus albus*, pyrosequencing

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Introduction

The presence of high levels of heavy metals in terrestrial environments can result from natural or human-related factors, such as pollution. It may influence the ecology and evolution of plants, fungi, animals and other organisms (Branco & Ree 2010; Colpaert *et al.* 2011). In the presence of high levels of heavy metals, ectomycorrhizal (ECM) fungal populations are differentiated into those that are highly tolerant of metal toxicity and those that are sensitive to such metals (Hartley *et al.* 1997).

In recent years, several studies have characterized and reviewed the various molecular mechanisms and cellular responses to stress that enable ECM fungi to

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tolerate heavy metals (Meharg 2003; Bellion *et al.* 2006). These mechanisms involve the uptake and subcellular sequestration of the metal (Blaudez *et al.* 2000a), intracellular binding of the metal (Jacob *et al.* 2004), responses to oxidative stress by the production of molecules that are involved in detoxification processes (Lanfranco 2007; Ramesh *et al.* 2009) and the synthesis of proteins involved in the transport and accumulation of metals. Additional mechanisms include antioxidative responses (Muller *et al.* 2007a; Ruytinx *et al.* 2011), extracellular chelation and binding of the metal to the cell wall (Bellion *et al.* 2006). The involvement of all of the above-mentioned mechanisms was confirmed recently using the complete genome and transcriptome data of the ECM fungal species *Tuber melanosporum* (Bolchi *et al.* 2011), which enabled the detection of genes related to metal homeostasis in this plant-symbiotic ascomycete. The genes that were identified were subdivided into three major functional classes, namely those involved in: (i) transport of metals, (ii) defence against the effects of oxidative stress and (iii) detoxification of metals (Bolchi *et al.* 2011). Such molecular processes could play a key role in enabling plants, fungi or microorganisms to persist in extreme habitats, such as serpentine soils, which are naturally rich in heavy metals (Branco 2009; Gonçalves *et al.* 2009; Rajkumar *et al.* 2009; Wright & Stanton 2011).

Serpentine soils are derived from ultramafic bedrock and are characterized by high concentrations of magnesium, iron and various heavy metals, such as chromium, cobalt, nickel and manganese. They are also extremely deficient in elements that are essential for plant nutrition, including nitrogen, phosphorus and potassium (Brooks 1987). Previous studies have shown that serpentine soils are characterized by a high biological diversity of plants and microorganisms that use various mechanisms to cope with the edaphic conditions (in particular, adaptation to toxic heavy metals, such as nickel) (Kazakou *et al.* 2008; Branco & Ree 2010). For example, it was shown recently that samples of the ECM fungus *Cenococcum geophilum* that were isolated from serpentine soils were highly tolerant of nickel, which suggests that the organism had adapted to its presence (Gonçalves *et al.* 2009). Recently, similar findings of fungal tolerance to nickel were observed in *Pisolithus albus* (Cooke & Massee) (Jourand *et al.* 2010a).

Pisolithus albus belongs to the genus *Pisolithus* Alb. & Schwein., a major ECM genus in the *Boletales* order, members of which are distributed globally and form ECM associations with a broad range of angiosperm and gymnosperm trees and shrubs (Marx 1977; Martin *et al.* 2002). *Pisolithus albus* grows on serpentine soils in New Caledonia, a tropical archipelago in the Southwest Pacific that is considered to be a hotspot of biodiversity (Myers *et al.* 2000; Jourand *et al.* 2010a). Serpentine soils cover a third of the surface area of the main island (Perrier *et al.* 2006). In New Caledonia, isolates of *P. albus* from these soils cluster genetically into a single group that comprises both nickel-tolerant and nickel-sensitive isolates (Jourand *et al.* 2010a). It was suggested that the observed phenotypic variations in nickel tolerance might arise from variations in the concentrations of bioavailable nickel in these soils. In addition, the nickel-tolerant isolates of *P. albus* were found to improve the growth and tolerance of nickel in plant hosts that were exposed to toxic concentrations of the metal (Jourand *et al.* 2010b).

Given these findings, it is important to clarify the molecular mechanisms of ECM adaptation to serpentine soils and tolerance of nickel in particular. In the study reported here, we examined changes in the transcriptome upon exposure of a single nickel-tolerant *P. albus* isolate from the ultramafic soils of New Caledonia to nickel to identify biomarkers that are involved in the molecular responses to the metal. Data on the complete transcriptome were obtained using pyrosequencing. We identified genes for which expression was changed by the presence of nickel and verified their differential expression in nickel-tolerant and nickel-sensitive isolates by using real-time quantitative PCR (RT-qPCR). The use of these procedures enabled us to generate the first database of expressed sequence tags (ESTs) from a nickel-tolerant fungus and to identify five genes expressed exclusively in nickel-tolerant isolates of *P. albus*. The latter suggests that this species shows a molecular adaptive response to nickel-rich environments.

Materials and methods

*Fungal material (*in vitro *and* in situ*)*

The ECM fungal isolates used in the study were isolated from sporocarps collected from ultramafic soils in New Caledonia at collection sites 1, 2, 3, 4 and 5 described in Table 1 and Fig. S1 (Supporting information). Anatomical and morphological characterizations led to their identification as isolates of the fungal species *Pisolithus albus*. The Ni-tolerant isolate MD06-337, which was described previously (Jourand *et al.* 2010b), was also used in the study. The other isolates of *P. albus* that were included in the study were new samples and are described in Table 1. Whereas MD09-045 and MD09-001 are nickel-tolerant isolates, MD09-063 and MD09-078 are nickel-sensitive isolates. To confirm their identities at the genus and species levels, these new isolates were analysed phylogenetically on the basis of their internal transcribed spacer (ITS) rDNA sequences as described previously (Jourand *et al.* 2010a). The analysed ITS sequences were deposited in the EMBL Nucleotide

Table 1 Isolates of *Pisolithus albus* used in this study. In addition to description of the sites where isolates were sampled (map location and GPS coordinates), details regarding the accession number of ITS sequences and *in vitro* tolerance of nickel are provided for each isolate

Site description: ultramafic soils		*Pisolithus albus* isolates					
Site on map	Map location, GPS coordinates	Isolate code	DNA-ITS EMBL accession code	Nickel phenotype	*Ni EC_{50} µM MMN medium	*Ni EC_{50} µM UF-like medium	References
1	Trazy-Guerioum, Koniambo Massif (21°00′28″ S; 164°49′50″ E)	MD06-337	AM947121	Tolerant	569 ± 3	1800 ± 5	Jourand *et al.* 2010a
2	Pindjen Waterfall Road Koné (21°02′19″ S; 164°46′26″ E)	MD09-078	FR852890	Sensitive	42 ± 4	65 ± 1	This study
3	Pindai (21°19′49″ S; 164°58′22″ E)	MD09-063	FR852893	Sensitive	76 ± 2	29 ± 2	This study
4	Mont-Dore, Plum Road (22°15′17″ S; 166°36′46″ E)	MD09-045	FR852892	Tolerant	>1000	2600 ± 5	This study
5	Bois du Sud (22°10′18″ S; 166°44′49″ E)	MD09-001	FR852891	Tolerant	1194 ± 3	1500 ± 5	This study

EC_{50}, the effective concentration that inhibits growth by 50% (Blaudez *et al.* 2000b); ITS, internal transcribed spacer.
*Ni EC_{50} was determined in two media: Melin-Norkrans medium as reference medium (Blaudez *et al.* 2000b) and UF-like medium.

Sequence Database. To evaluate the expression of the nickel biomarkers in fungal material *in situ* further, samples from ECM root tips were collected at the study site where the nickel-tolerant isolate MD06-337 and the nickel-sensitive isolate MD09-078 were found. These fungal materials were stored in liquid nitrogen.

Growth conditions

To generate stock cultures from each sporocarp, a fresh piece of pileus trama was transferred aseptically to solid modified Melin-Norkrans (MMN) medium (Blaudez *et al.* 2000b). The MMN medium had the following components: KH_2PO_4 (0.5 g/L; K and P = 3.7 mM), $(NH_4)_2HPO_4$ (0.25 g/L; N = 3.78 mM and P = 3.67 mM), $CaCl_2$ (0.05 g/L; Ca = 0.34 mM), NaCl (0.025 g/L; NaCl = 0.43 mM), $MgSO_4.7H_2O$ (0.15 g/L; Mg = 0.61 mM), thiamine hydrochloride (100 µg/L), $FeCl_3.6H_2O$ (0.03 g/L), glucose (10 g/L), malt extract (3 g/L) and agar (14 g/L). The pH was adjusted to 5.6 using 1 M HCl to match the pH measured in ultramafic soils as reported by Perrier *et al.* (2006). The medium was autoclaved for 20 min at 120 °C. All fungal isolates were maintained as subcultures at 30 °C in this medium.

For further experiments, MMN medium was altered to mimic the extreme conditions of ultramafic soils. In the Koniambo Massif, where the *P. albus* isolate MD06-337 was sampled, the concentrations of chemical elements in the soil according to Jourand *et al.* (2010a) were as follows: N (3.6 g/kg, N = 257 mM), P (0.07 g/kg, P = 2.25 mM), K (0.09 g/kg, K = 2.3 mM), Ca (0.26 g/kg, Ca = 6.5 mM) and Mg (4.1 g/kg, K = 170 mM), with an unbalanced molar ratio of Ca/Mg (1/26) and an excess of extractable Ni (Ni-diethylene triamine pentaacetic acid) (19 mg/kg, Ni = 327 µM). To mimic these conditions, the MMN medium was altered to generate what is henceforth referred to as UF-like medium, which contained the following components: KH_2PO_4 (0.25 g/L; P = 1.83 mM and K = 1.83 mM), $(NH_4)_2HPO_4$ (0.125 g/L); N = 1.8 mM and P = 0.9 mM, NH_4NO_3 (10 g/L); N = 250 mM, $CaCl_2$ (0.05 g/L); Ca = 0.34 mM, KCl (0.033 g/L); K = 0.44 mM, NaCl (0.025 g/L), $MgSO_4{\cdot}7H_2O$ (1.95 g/L); Mg = 7.9 mM, thiamine hydrochloride (100 µg/L), $FeCl_3{\cdot}6H_2O$ (0.03 g/L), glucose (1 g/L) and malt extract (0.3 g/L). The pH was adjusted to 5.6 using 1 M HCl to match the pH measured in ultramafic soils as reported by Perrier *et al.* (2006). The medium was autoclaved for 20 min at 120 °C. When needed, nickel was added after autoclaving under sterile conditions from a stock solution of $NiCl_2$ (1 M) to the required final concentrations. To check the nickel tolerance of all the isolates used in the study, the EC_{50} of Ni was measured in both the reference MMN medium and the UF-like medium as described previously (Jourand *et al.* 2010a).

Extraction of total RNA

Total RNA was extracted from mycelium that had been cultivated *in vitro* as well as from frozen fungal

material that was collected *in situ*. A cetyltrimethyl ammonium bromide (CTAB)-based protocol was used as described by Salzman *et al.* (1999). Briefly, 1–5 g of frozen mycelium or ECM tissue was ground in liquid nitrogen using a mortar and pestle. Then, 0.6–1 mL of extraction buffer (2% CTAB, 2% polyvinylpyrrolidone PVP-K-40, 100 mM Tris-HCl, 25 mM EDTA and 2 M NaCl) was added to each tube, and the samples were incubated at 60 °C for 15 min. An equal volume of a mixture of chloroform:isoamyl alcohol (24:1) was added and mixed immediately for 2 min using a vortex mixer. The samples were then centrifuged at 10 000 *g* for 10 min at 4 °C. Supernatants solutions were transferred to a new tube, and a 1/3 volume of 8 M LiCl was added to each tube. The samples were mixed by inversion and stored at 4 °C overnight. Total RNA was purified using the silica membrane from an RNeasy Plant Mini kit (Qiagen, Courtaboeuf, France), in accordance with the manufacturer's instructions. The purified RNA was quantified using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at wavelengths of 230, 260 and 280 nm. The integrity of the RNA was verified by separating sample components in 1.2% agarose gels, as described by Salzman *et al.* (1999).

Processing of 454 pyrosequencing data and assembly of cDNA sequences from the nickel-tolerant Pisolithus albus *isolate* (MD06-337)

To compare the gene expression in the nickel-tolerant *P. albus* isolate (MD06-337) in the presence and absence of nickel, two sets of total RNA were isolated using the CTAB-based protocol from 15-day-old liquid cultures of the isolate cultivated in UF-like medium with or without nickel (250 μM $NiCl_2$). The two sets of extracted RNA were sent to GATC Biotech (Constanz, Germany), where the cDNA was pyrosequenced in accordance with the established procedures used by the company. The primer sequences were removed from the data. Sequence reads were then assembled into contigs using the Genomics Workbench software (CLC Bio, Aarhus, Denmark). Expression analysis was performed using the RPKM (reads per kilobase of exon model per million mapped reads) values (Mortazavi *et al.* 2008). The RPKM values, which are used to standardize sequence counts as a function of contig length and absolute size of the data set (i.e. mapped reads: assembled reads), were calculated as follows: (number of reads per contig) × (1000/length of contig) × (1×10^6/total number of mapped reads in the assembly) (Mortazavi *et al.* 2008). On the basis of the ratio of the RPKM values obtained in the presence and absence of nickel (which represents the fold induction of gene expression in response to nickel), we selected the genes for which expression was increased more than twofold above the background level for the pyrosequencing, as commonly performed in previous studies using comparative transcriptomic approaches (Chelaifa *et al.* 2010). This cut-off level for the induction of gene expression was used previously in another analysis of the transcriptomic response to a heavy metal in another ECM fungus (Jacob *et al.* 2004). The genes that presented both the highest RPKM values in the presence of nickel (RKPM ≥ 10) and the lowest RPKM values in the absence of nickel were selected as candidate biomarkers of nickel tolerance in *P. albus*. In addition, to identify genes, the contig sequences that were obtained by pyrosequencing were compared with sequences stored in the GenBank nonredundant database (nr), *Laccaria bicolor* database (http://mycor.nancy.inra.fr/imgc/laccariagenome/) and *Pisolithus microcarpus* database (free access was provided generously by the F. Martin (INRA laboratory, Nancy, France) using BlastX with an *E*-value cut-off of 10^{-5}, as described previously for other pyrosequencing transcriptome analyses (Schmid *et al.* 2010). All assembled contigs and their respective RPKM values sequences of the genes selected from the pyrosequencing data analysis were deposited in the EMBL Nucleotide Sequence Database and referenced under the accession numbers shown in Table 2.

Gene annotation using Gene Ontology (GO) terms

To identify the global functional categories of the unique contigs (with respect to the standard Gene Ontology (GO) categories of biological process, molecular function and cellular component), the two transcriptomic sequence banks and the pyrosequencing contig data obtained from *P. albus* MD06-337 in the presence or absence of nickel were analysed using the GO database. This analysis was based on the similarities of the sequences to those of known proteins in the GenBank nr protein database (*E*-value cut-off of 10^{-5}), which had been annotated with GO terms using the software Blast2GO V2.4.8 (Götz *et al.* 2008).

Quantitative PCR analysis

To confirm the nickel-responsive differential expression revealed by pyrosequencing, we used RT-qPCR to verify the fold induction of the genes that had been selected as biomarkers of nickel tolerance. This technique is the most sensitive and reproducible method to quantify gene expression, as described recently in a study using the pyrosequencing approach (Chelaifa *et al.* 2010). For the RT-qPCR experiments, *P. albus* isolates were grown in UF-like medium that contained

Table 2 Candidate biomarker genes, shown by pyrosequencing analysis to be up-regulated by nickel, which show differential expression in nickel-tolerant *Pisolithus albus* isolate MD06-337. EMBL accession codes are provided for all DNA sequences, as are the closest homologs identified by BlastN analysis

Pyrosequencing analysis					Gene sequence and BlastN identification			
No. Contig	No. gene	Size (bp)	RPKM 0 μM Ni	RPKM 250 μM Ni	EMBL accession number	Species	*E*-value	% Identity
C10864	1	517	0	64	FR852894	*Pisolithus microcarpus*	$2E^{-33}$	100%
C6406	2	411	0	65	FR852895	*P. microcarpus*	$1E^{-105}$	96%
C9200	3	443	0	10	FR852896	*P. microcarpus*	$3E^{-94}$	95%
C7318	4	411	0	90	FR852897	*P. microcarpus*	$2E^{-79}$	95%
C10795	5	504	0	90	FR852898	*P. microcarpus*	$1E\text{-}^{130}$	99%
C15327	6	235	0	17	FR852899	*P. microcarpus*	$1E^{-112}$	99%
C17653	7	1199	1	27	FR852900	*P. microcarpus*	$9E^{-44}$	98%
C18133	8	1125	1	35	FR852901	*P. microcarpus*	$1E^{-151}$	99%
C10491	9	665	6	30	FR852902	*P. microcarpus*	$9E^{-92}$	97%
C4552	10	1303	3	42	FR852903	*P. microcarpus*	$1E^{-155}$	99%

bp, base pair; RPKM, reads per kilobase of exon model per million mapped reads (Mortazavi *et al*. 2008).

either 50 or 250 μM nickel provided as $NiCl_2$. Flasks that contained 250 mL of UF-like medium were each inoculated with five plugs of mycelium from the MMN stock culture. The cultures were allowed to grow in the dark for 15 days, with continuous shaking at 130 rpm at 30 °C. The RNA was extracted from three biological replicates of the fungal isolate MD06-337, which had been cultivated *in vitro* as described above, and analysed by RT-qPCR. At the same time, we tested RNA samples extracted from ECM of the MD06-337 and MD09-078 isolates that had been isolated *in situ*. Furthermore, we analysed RNA samples extracted from other *P. albus* isolates (already described in Table 1). Aliquots of total RNA (1 μg) were treated with TurboDNase I (Applied Biosystems, Carlsbad, CA, USA) and used for cDNA synthesis, which was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's protocol. Real-time quantitative PCR was performed in duplicate in a volume of 20 μL, which contained 5 μL of diluted cDNA (1 ng of total RNA equivalent), 1× SYBR Green PCR Master Mix (Applied Biosystems) and 0.3 μM of each primer. Primers were designed from the *P. albus* contig data, which were obtained from the results of the pyrosequencing using the Primer Express software (Applied Biosystems). The primers amplified a gene fragment of 150 bp in length at an annealing temperature of 60 °C, as shown in Table S1 (Supporting information). PCR was applied to test the specificity of the primers and the efficiency of amplification (Table S1, Supporting information). The amplification profile consisted of an initial denaturation step at 95 °C for 15 min, then 40 cycles that each comprised a 95 °C step for 10 s, 59 °C for 30 s and 72 °C for 32 s, followed by a dissociation stage, in accordance with the manufacturer's instructions. Real-time PCR was performed using an ABI 7300 Sequence Detection System (Applied Biosystems) in the presence of SYBR Green. A melting curve analysis was performed at the end of each RT-qPCR by gradually increasing the temperature from 60 to 95 °C, while recording the change in fluorescence. A single peak at the melting temperature of the PCR product confirmed primer specificity, indicating that RT-qPCR had been performed with no contamination or formation of primer dimers.

Data and statistical analysis of qPCR

Data were analysed using two methods. The REST 2009 method, which is available at http://www.gene-quantification.de/rest-2009.html (Pfaffl *et al.* 2002), was used to determine the ratios of transcripts, whereas the $2^{-\Delta\Delta Ct}$ method was used to analyse mRNA accumulation (Livak & Schmittgen 2001). Each analysis was normalized with two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 4α (EF4α). For each treatment, we performed three technical replicates for each of the three biological replicates per isolate and per nickel treatment, to evaluate the relative expression. In the REST method, the RT-qPCR results were analysed using the hypothesis test [$P(H1)$], which represents the probability that the alternate hypothesis differentiates between the sample and control groups only owing to chance. In the $2^{-\Delta\Delta Ct}$ method, one-way analysis of variance (ANOVA) was performed and the results were compared by Student's *t*-test ($P < 0.05$) using the XLSTAT software (Addinsoft 2005).

Results

Phylogenetic analysis of the fungal ITS sequence

To confirm that the fungal samples used in the study belong to the species *Pisolithus albus*, we analysed the ITS sequences (ITS1/5.8S rRNA gene/ITS2) of each isolate. The ITS sequence is a commonly used phylogenetic marker for Dikarya (Ascomycetes and Basidiomycetes) and some lower fungi. The sequences were deposited in the EMBL Database and referenced under the accession numbers reported in Table 1. The phylogenetic analysis was performed as described previously (Jourand *et al.* 2010a). The results, which are presented as supplementary data in Fig. S2 (Supporting information) confirmed that the isolates belong to *P. albus*.

DNA pyrosequencing, assembly and sequence analysis of the nickel-tolerant Pisolithus albus *isolate MD06-337*

We obtained two non-normalized cDNA libraries from the nickel-tolerant *P. albus* isolate MD06-337 grown in the presence and absence of nickel. Pyrosequencing of these libraries yielded 1 071 375 single reads, which comprised 542 680 trimmed reads from *P. albus* grown in medium that contained nickel and 528 695 trimmed reads from *P. albus* grown in the absence of nickel. The average read lengths were 238 and 236 bp, respectively (Table S2, Supporting information). We used a two-step assembly procedure to process these reads and generated two groups of contigs by excluding low-quality sequences (sequences shorter than the length threshold, i.e. <200 bp, were discarded). The two groups corresponded to 18 800 contigs from *P. albus* exposed to nickel and 16 636 contigs from *P. albus* that had not been exposed to nickel. These two groups of contigs were pooled and reassembled to highlight the genes that were expressed differently in response to nickel. A final group of 19 518 contiguous sequences (from 813 651 total read counts) from *P. albus* MD06-337 was obtained, with an average length of 698 bp. There were also 254 424 singleton reads (24%; Table S2, Supporting information). Hereafter, the contiguous sequences are referred to as genes. Of the 19 518 genes that were obtained through the *de novo* assembly of the sequence reads, the expression of 30% of the genes was modulated by nickel. Further analysis identified 4211 genes (21%) that were up-regulated by nickel and 1763 genes (9%) that were down-regulated by nickel. The global statistical distribution of these 19 518 genes is presented on a scatter plot in Fig. 1. To identify the greatest possible number of descriptive annotations for each sequence, global gene prediction was carried out on the basis of a set of BLASTX searches (Table S2, Supporting information). Among the 19 518 unique contigs, the global BLASTX analysis indicated that 11 489 contigs (59%) had significant matches in the GenBank database, whereas 10 926 contigs (56%) had matches in the *Laccaria bicolor* database and 15 150 contigs (77.6%) in the *Pisolithus microcarpus* database (Table S2b, Supporting information).

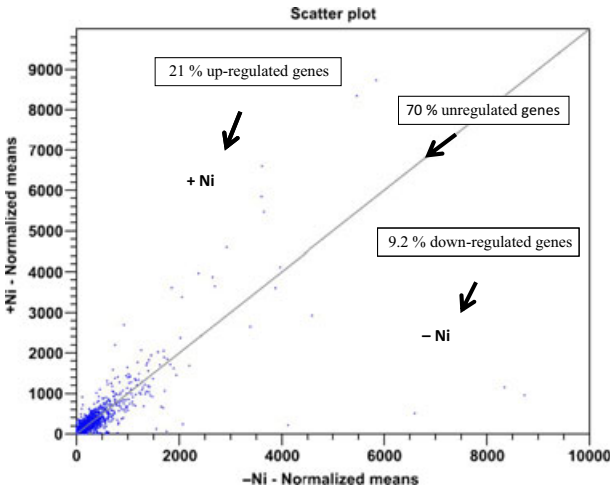

Fig. 1 Analysis of 454 pyrosequencing data. The scatter plot represents gene expression levels in a free-living mycelium of the nickel-tolerant *P. albus* isolate (MD06-337) grown without nickel or with nickel at 250 μM. The expression levels of genes were normalized using a scale of 0–10 000. Each circle in the plot represents the level of expression of one gene.

GO annotation of genes expressed differentially in Pisolithus albus

To determine the global variation in gene expression in the MD06-337 isolate of *P. albus* in the presence and absence of nickel, we assigned GO terms to the sequences identified in the two pyrosequencing transcriptomic databanks. The result showed that 23% and 25% of the sequences from the databanks for *P. albus* grown in the presence and absence of nickel, respectively, could be assigned a GO term (Fig. 2). The distribution of sequences among the following primary GO categories was analysed: cellular component, molecular function and biological process. This showed differences in distribution between the two conditions within the categories of biological process and cellular component (Fig. 2). To focus on the factors that underlie nickel tolerance in *P. albus*, we also performed a GO analysis of the 4211 contigs (21%) that corresponded to genes that were up-regulated by nickel (Fig. S3, Supporting information). The analysis indicated that only 12.6% of the up-regulated genes could be assigned to the three GO categories, among which 53% were assigned to cellular components, 27% were assigned to

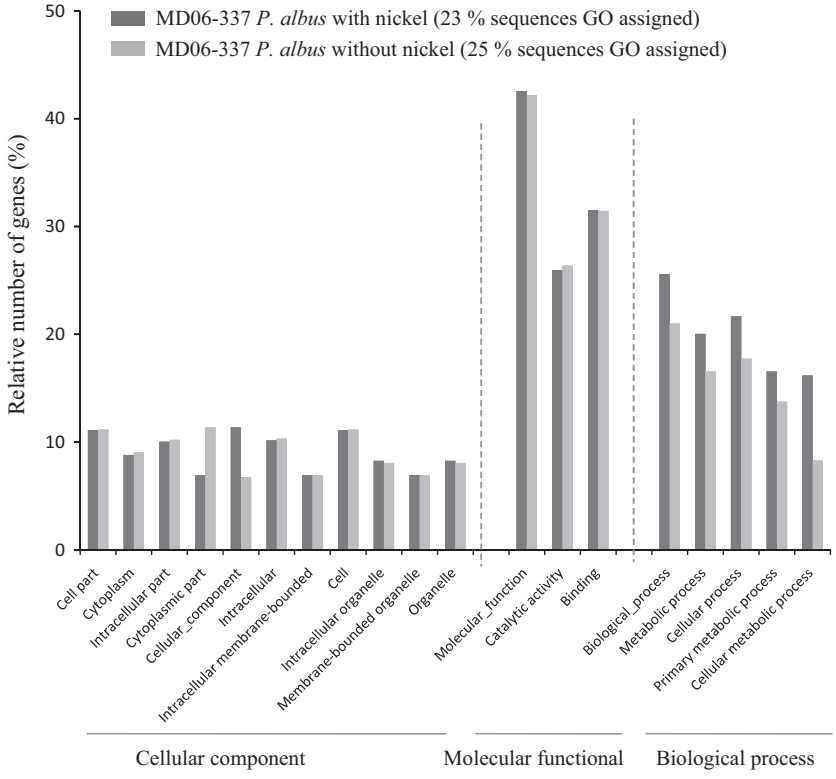

Fig. 2 Assignment of functional Gene Ontology terms and distribution of total sequences for two transcriptomes of the nickel-tolerant isolate MD06-337 of *P. albus* with (in the presence of 250 μM $NiCl_2$) and without nickel, in terms of the categories of biological process, molecular function and cellular component.

biological processes and 20% were assigned to molecular function (Fig. S3, Supporting information).

Tracking of nickel biomarkers in nickel-tolerant MD06-337 Pisolithus albus

Among the genes that were expressed differentially in response to nickel (4211 genes), we selected the 10 genes that showed the greatest variation on the basis of the RPKM values obtained in the pyrosequencing analysis, as shown in Table 2. The names of the sequences correspond to the contig numbers (No. contigs in Table 2). All nucleotide sequences have been deposited in the EMBL Database, and their accession number and lengths (in base pairs) are summarized in Table 2. These DNA sequences were analysed using BLASTN, and the sequences were found to be identical in approximately 95–100% of cases to sequences in the *Pisolithus microcarpus* database (currently not publicly available, personal communication from Martin F., INRA Nancy, France) (Table 2). In addition, the function of the genes was determined using BLASTX sequence alignments (with a threshold of $<1E^{-03}$). The results showed homologies to putative proteins from the following species: *Laccaria bicolor* (seven genes, No. 2, 3, 4, 6, 8, 9 and 10), *Coprinopsis cinerea* (two genes, No. 5 and 7) and *Saccharomyces cerevisiae* (gene No. 1). Full data from the BLASTX search are presented in Table 3. The analysis of gene function showed that genes No. 1, 3, 5, 6, 7, 8, 9 and 10 encoded proteins with predicted functions, whereas genes No. 2 and 4 encoded proteins with no known function (Table 3). The genes with a predicted function encoded for (i) cell-wall components (genes No. 1 and 7), (ii) components involved in metabolic processes and amino acid transport (genes No. 3, 6, 8 and 9), (iii) components involved in RNA processing (gene No. 10) and (iv) components involved in metallocarboxypeptidase processes (gene No. 5).

Validation of pyrosequencing results by RT-qPCR in nickel-tolerant MD06-337 Pisolithus albus

The 10 selected genes were analysed by RT-qPCR to confirm the differential expression that was indicated by the pyrosequencing data. For this purpose, specific primer sequences were designed from the DNA sequences obtained by pyrosequencing (Table S1, Supporting information). These primers were used in RT-qPCR to quantify gene expression in *P. albus* MD06-337 using two sets of cDNA. The first set corresponded to the cDNAs that were used for the pyrosequencing, whereas the second set corresponded to newly synthesized independent cDNAs from three independent mycelia. Both sets contained cDNAs that were obtained from *P. albus* grown in the presence of nickel and those of *P. albus* grown in the absence of nickel. The

Table 3 Functional description and assignment of GO terms for 10 candidate biomarker genes (No. 1–10), up-regulated following exposure to nickel, as selected from pyrosequencing analyses that revealed differential expression in nickel-tolerant *Pisolithus albus* isolate MD06-337

No. contig	No. gene	BLASTX functional identification					
		Best predicted function	Species	*E*-value	Blast hit	Functional classification	GO terms
C10864	1	GPI-anchored cell surface glycoprotein-like (AGA1)	*Saccharomyces cerevisiae*	$5E^{-03}$	YNR044W	Cell-wall anchorage	No hits found
C6406	2	Unknown function	*Laccaria bicolor*	$3E^{-08}$	XM_001881526.1	Unknown function	No hits found
C9200	3	Alpha-aminoadipate reductase Lys1p (LbLYS1)	*L. bicolor*	$8E^{-64}$	XM_001879583.1	Secondary metabolite biosynthesis	GO:0003824
C7318	4	Unknown function	*L. bicolor*	$3E^{-07}$	XP_001878436.1	Unknown function	No hits found
C10795	5	Vacuolar protein sorting-associated protein 70	*Coprinopsis cinerea*	$4E^{-20}$	XP_001837057.2	Metallocarboxypeptidase activity	GO:0004180
C15327	6	S-adenosylmethionine-dependent methyltransferase	*L. bicolor*	$2E^{-21}$	XP_001874602.1	Methyl transferase	GO:0016740
C17653	7	Class III chitinase	*C. cinerea*	$5E^{-08}$	XM_001828384.2	Chitin catabolic process	No hits found
C18133	8	Cytochrome P450 (CYP)	*L. bicolor*	$4E^{-40}$	XP_001878510.1	Monooxygenase activity	GO:0009055
C10491	9	APC amino acid permease	*L. bicolor*	$4E^{-39}$	XP_001875612.1	Amino acid transport	GO:0006560
C4552	10	bZip transcription factor	*L. bicolor*	$9E^{-10}$	XP_569310.1	RNA processing	GO:0009267

GO, Gene Ontology.

RT-qPCR results for the selected genes are presented in Table 4 and are given as the ratio of mRNA expression in the presence of nickel to mRNA expression in the absence of nickel (ratio +Ni/−Ni). The relative expression levels that were obtained from the cDNAs used in the pyrosequencing analysis and from the new cDNA samples (means ± SD of the three replicates) are compared in Table 4. The data show that there were no significant differences between gene expression profiles in relation to the cDNA template used (pyrosequenced

Table 4 Validation of the pyrosequencing data by RT-qPCR. Relative induction of expression by nickel of the 10 selected genes (up-regulated by nickel: No. 1–10) was measured in the MD06-337 nickel-tolerant isolate, using GAPDH and EF4α as reference genes

No. contigs/genes	C10864	C6406	C9200	C7318	C10795	C15327	C17653	C18133	C10491	C4552
Relative expression (cDNA 454)*	10***	10.4***	6.3	6.5	4.2***	8***	3.5	39***	17.3***	7.7***
(1) P(H1)	0.0001	0.0001	0.49	0.49	0.0001	0.0001	0.49	0.0001	0.0001	0.0001
†Relative expression (3n)	5.1**	4.1*	2.3	8***	5*	6.5**	6***	8.5	39.5**	5.2***
(2) P(H1)	0.004	0.05	0.175	0.0001	0.022	0.007	0.0001	0.066	0.005	0.0001

Relative expression values were determined by normalizing expression levels against those of the housekeeping genes that encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 4 α (EF4α).
*Values in line (1) represent the relative expression levels of the 10 genes as determined using the cDNA used for the pyrosequencing analysis of MD06-337 isolate.
†Values in line (2) represent the means of three biological independents relative expressions qPCR on the MD06-337 isolate.
All statistical computation and analyses of qPCR data were carried out using the REST 2009 software (Pfaffl *et al.* 2002).
We used the hypothesis tests P(H1).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
Values indicate the mean ± SD ($n = 3$).

DNA or new cDNA; $P > 0.05$). The ratios of gene expression that were obtained using RT-qPCR confirmed the transcriptomic profile of nickel-tolerant *P. albus* MD06-337 that was obtained by pyrosequencing. We also used the REST method to compare the relative expression patterns of the 10 selected genes in the nickel-tolerant isolate MD06-337 at 50 μM, with expression patterns in the presence of 250 μM of nickel. The results are presented in Fig. S4 (Supporting information) and show similar profiles for the two concentrations of nickel tested. In addition, RT-qPCR was performed to verify the levels of mRNA expression of the 10 genes selected in nickel tolerant MD06-337 ECM root tips sampled in situ. The results (Table 5) showed that the selected genes were also expressed in symbiotic ECM root tips sampled in situ, except for gene No.7, which was not detected in ECM root tips.

Comparison of expression patterns in nickel-tolerant (MD06-337) and nickel-sensitive (MD09-078) isolates of Pisolithus albus

We compared the expression profiles of the 10 selected genes that were up-regulated by nickel between the nickel-tolerant isolate (MD06-337) and a nickel-sensitive isolate (MD09-078) from the ultramafic soils. As shown in Fig. 3, the levels of expression of the genes analysed differed significantly between the nickel-sensitive isolate MD09-078 and the nickel-tolerant isolate MD09-337 ($P < 0.05$). Genes No. 1, 2, 5, 6 and 7 were expressed

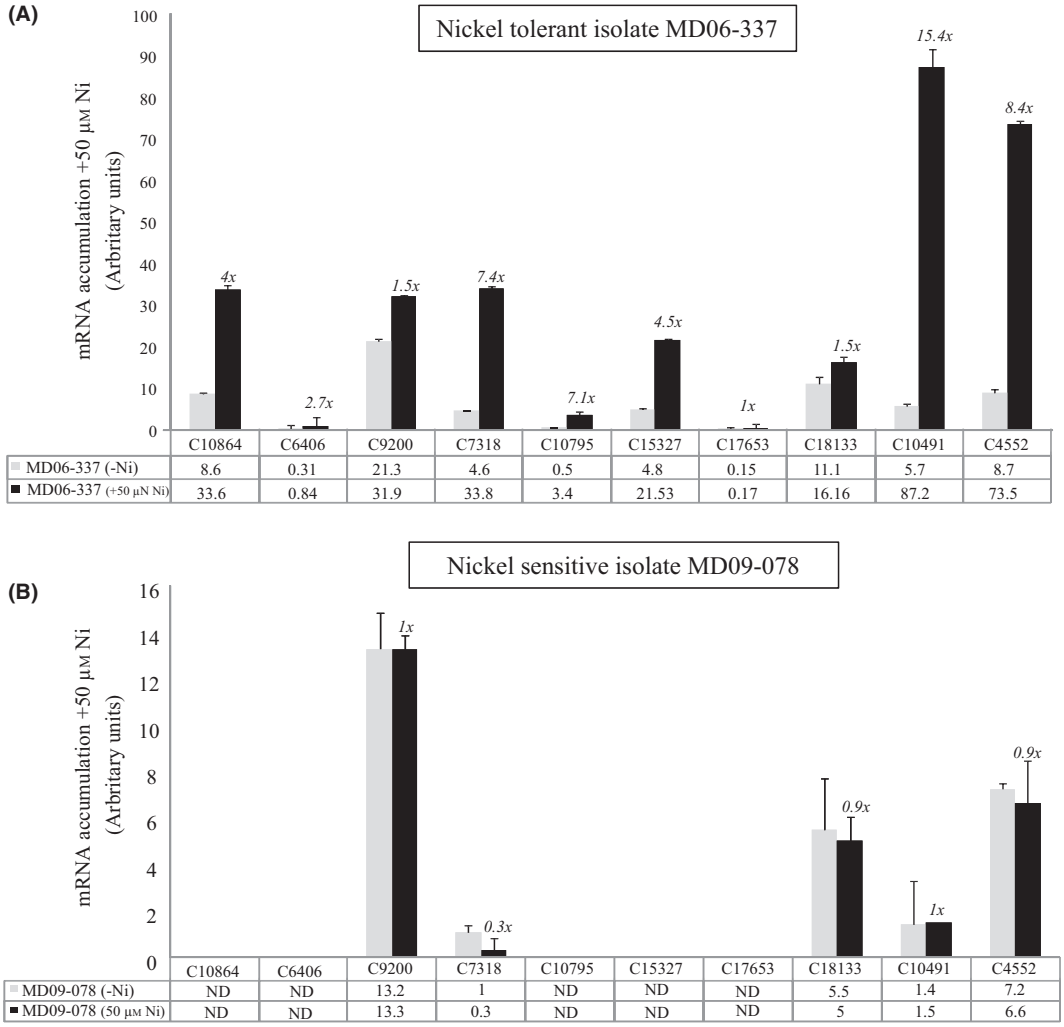

Fig. 3 Comparison of patterns of mRNA accumulation in *P. albus* isolates grown without nickel or with nickel (provided as 50 μM $NiCl_2$). Expression of the 10 genes up-regulated most by nickel, as determined by pyrosequencing, in nickel-tolerant (MD06-337) and nickel-sensitive (MD09-078) isolates. Transcript accumulation was quantified by RT-qPCR, using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase and EF4α as reference genes for normalization, and expressed as arbitrary units. The data indicate mean values ± SD, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the columns in italics. ND: mRNA nondetected (Ct values >37).

Table 5 *In situ* validation of the pyrosequencing data by RT-qPCR. Relative induction of expression by nickel of the 10 selected genes (up-regulated by nickel: No. 1–10) in ectomycorrhizal *Pisolithus albus* root tips (nickel-tolerant MD06-337 isolate and nickel-sensitive MD09-078 isolate) *in situ* was measured by comparison with the nickel-tolerant isolate MD06-337 and nickel-sensitive isolate MD09-078, *in vitro*. Transcript accumulation was quantified by RT-qPCR using the $2^{-\Delta\Delta ct}$ method with normalization using two reference genes, GAPDH and EF4α

No. gene	No. 1 C10864	No. 2 C6406	No. 3 C9200	No. 4 C7318	No. 5 C10795	No. 6 C15327	No. 7 C17653	No. 8 C18133	No. 9 C10491	No. 10 C4552
mRNA accumulation										
ECM (MD06-337) *in situ* Nickel-tolerant isolate	11	0.4	8.4	5.1	1.2	1.3	nd	0.8	3	7
Control (*in vitro*) MD06-337 (UF-like + 250 μM nickel)	10	10.4	6.3	6.5	4.2	8	3.5	39	17.3	7.7
ECM (MD09-078) *in situ* Nickel-sensitive isolate	0.25	0.74	48.2	35.7	0.24	0.41	0.24	18.5	0.34	26.6
Control (*in vitro*) MD09-078 (UF-like + 50 μM nickel)	nd	nd	22	3	0.30	0.24	0.21	8	1.5	3

nd, not detected (CT > 37); ECM, ectomycorrhizal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

very weakly or were not detectable (ND) in the MD09-078 isolate, whereas they were expressed strongly in MD06-337 isolate. As shown in Fig. 3, the normalized expression levels varied from 0.15 to 87 arbitrary units. The remaining genes, No. 3, 4, 8, 9 and 10, were expressed at substantial levels in both isolates. However, whereas in the nickel-sensitive isolate, the expression of these genes (No. 3, 4, 8, 9 and 10) was not affected by nickel (there was no significant induction), in the nickel-tolerant isolate, nickel induced an increase in expression of these genes of between 1.5- and 15-fold in the nickel-tolerant isolate. From this comparison of gene expression levels between nickel-sensitive and nickel-tolerant isolates, we identified genes No. 1, 2, 5, 6 and 7 as showing the most significant differences in terms of levels of expression. We concluded that they show the greatest promise as molecular markers (biomarkers) of tolerance to nickel in *P. albus* from the ultramafic soils of New Caledonia. We also included gene No. 9 in this set of promising biomarkers because it was induced strongly by nickel in the nickel-tolerant isolate. These six selected genes were used as biomarkers for the final analysis of nickel tolerance. In addition, RT-qPCR was performed to verify the levels of mRNA expression for the 10 selected genes in ECM samples from nickel-sensitive *P. albus* isolate MD09-078 sampled *in situ* (Table 5). The RT-qPCR results showed that the six genes that were selected as biomarkers were either not expressed, or only weakly expressed, in nickel-sensitive isolate from ECM root tips sampled *in situ*. These results are in agreement with those obtained by *in vitro* experiments conducted using the nickel-sensitive *P. albus* isolate MD09-078.

Comparison of biomarker expression between nickel-tolerant and nickel-sensitive Pisolithus albus *isolates*

We tested the expression levels of the six biomarkers in five isolates of *P. albus* from the ultramafic soils of New Caledonia. These included three nickel-tolerant isolates (MD06-337, MD09-045 and MD09-001) and two nickel-sensitive isolates (MD09-078 and MD09-063). The six biomarkers were expressed in all three nickel-tolerant isolates (MD06-337, MD09-045 and MD09-001), but they were not expressed in any of the nickel-sensitive isolates, as shown in Fig. 4. In the nickel-tolerant isolates, the presence of 50 μM nickel increased the expression of the six genes by between one- and ninefold (Fig. 4).

Discussion

We have used a transcriptomic approach to characterize the expression of potential biomarkers of nickel tolerance in the ECM fungus *Pisolithus albus* from the ultramafic soils of New Caledonia. The demonstrations that 21% of all genes in *P. albus* are up-regulated in the presence of nickel and that six genes are expressed exclusively in nickel-tolerant isolates of *P. albus* together suggest the presence of a molecular transcriptomic adaptive response to nickel-rich environments in this species.

Transcriptomic database of nickel-tolerant Pisolithus albus

In previous studies, genes that are regulated by heavy metals in ECM fungi have been identified by

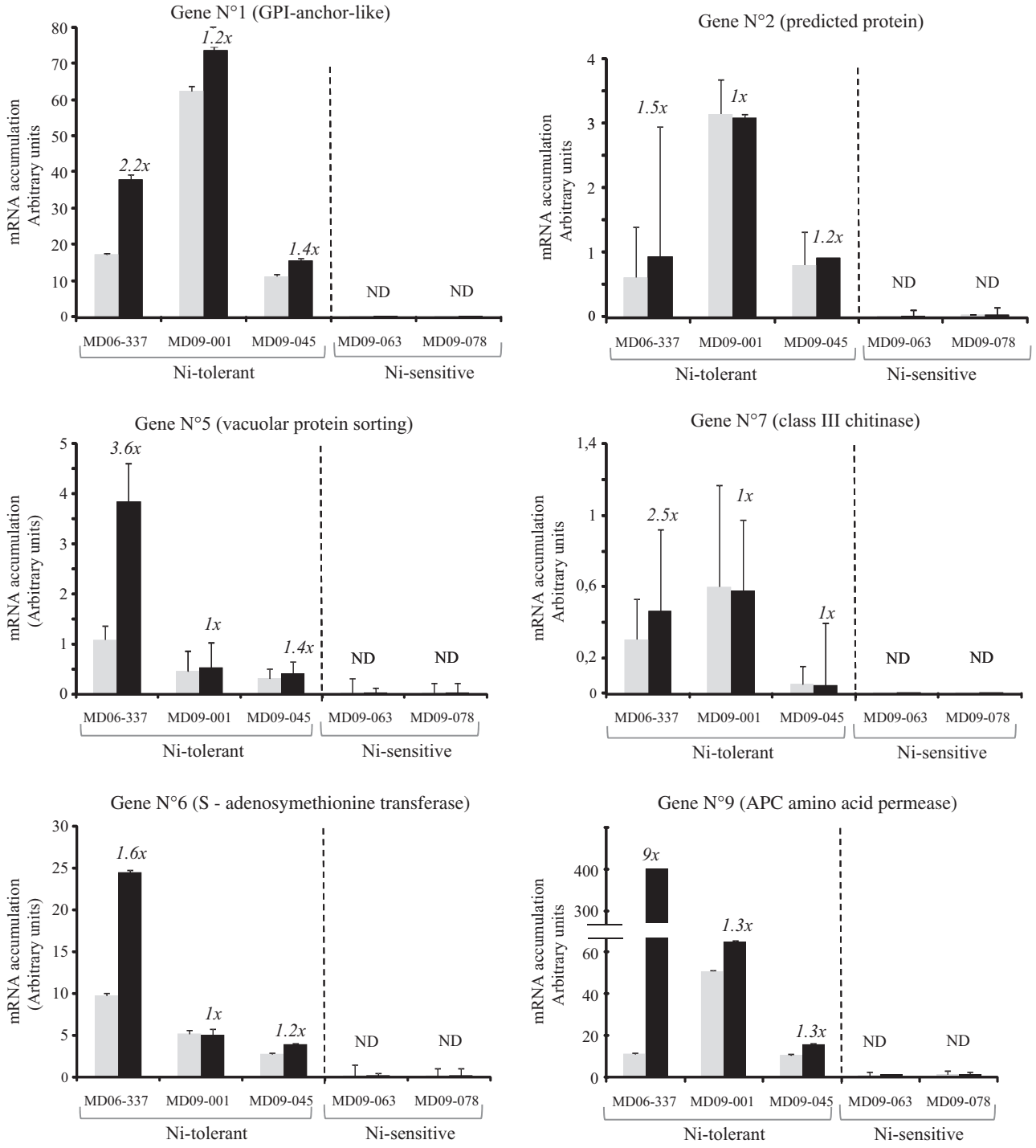

Fig. 4 Comparison of mRNA accumulation profiles for the six selected up-regulated genes in five *P. albus* isolates from ultramafic soil in the presence of nickel 50 μM (black columns) and in the absence of nickel (grey columns). Three nickel-tolerant isolates (MD06-337, MD09-045 and MD07-001) and two nickel-sensitive isolates (MD09-078 and MD09-063) were compared. Transcript accumulation was quantified by qPCR using $2^{-\Delta\Delta CT}$ method with normalization to two reference genes, glyceraldehyde-3-phosphate dehydrogenase and EF4α, and is expressed as arbitrary units. The data indicate mean values ± SD values, calculated from three technical replicates with triplicate biological samples. The fold induction by nickel is presented above the black columns in italics. ND: mRNA nondetected (Ct values >37).

transcriptomic approaches, such as DNA microarray or cDNA-AFLP (amplified fragment length polymorphism) analysis (Jacob *et al.* 2004; Muller *et al.* 2007a). These studies were conducted mostly on ECM samples isolated from soils polluted with heavy metals as a result of human activities (Jacob *et al.* 2004; Muller *et al.* 2007b; Ruytinx *et al.* 2011). Here, we report a transcriptome database, generated using pyrosequencing, for an isolate (MD06-337) of the ECM fungus *P. albus*, which was isolated from extreme soils that naturally contain high concentrations of heavy metals, particularly nickel, and is tolerant of nickel. To the best of our knowledge, this is the first repository of its kind.

We also characterized molecular biomarkers that were associated with tolerance to nickel in *P. albus*. The reference database that we generated for *P. albus* comprised 19 518 contigs, of which 56% were also found in the *Laccaria bicolor* database (Martin *et al.* 2008) and 77.6% in the *Pisolithus microcarpus* database (F. Martin, *P. microcarpus* database version 1.0; INRA laboratory, Nancy, France). Moreover, the differential transcriptomic analyses that we conducted showed that, after growth for 15 days in the presence of nickel, 30% of all genes in the *P. albus* genome varied in their levels of expression relative to the expression levels in the absence of nickel. Analysis of the changes in the transcriptome of *P. albus* between these two conditions showed that of the differentially expressed genes, 21% were up-regulated following exposure to nickel and 9% were down-regulated following exposure to nickel. In a previous study, it was demonstrated that environmental changes could markedly affect gene expression profiles (Gibson 2008). We also identified the genes for which expression was affected the most by exposure to nickel. Our results suggest that, at a minimum, all of the 5855 genes that showed differential expression in the nickel-tolerant *P. albus* isolate in response to nickel contribute to tolerance of the fungus to the extreme ultramafic environment in New Caledonia. The experiments that were based on GO analysis and functional genetic tools suggest also the role of these genes as (i) biomarkers of tolerance to nickel and (ii) putative adaptive mechanisms of nickel tolerance in *P. albus*. Among the sequences obtained from the pyrosequencing analysis of *P. albus* grown in the presence and absence of nickel, only 25% and 23%, respectively, were assigned to one or more GO categories. Comparison of the results showed that the global distributions of GO terms for the two nickel conditions were similar for the category of 'molecular function', but not for the categories of 'biological process' and 'cellular component'. Moreover, a large number of orphan genes were present in the two databanks, which suggests that the accuracy of the functional annotation was limited. This limited accuracy can probably be explained by the fact there is no reference transcriptome available for *P. albus*, and there is no transcriptome available for related species under similar conditions. Indeed, our database is the first to be based on the full transcriptome from an organism isolated from an environment that naturally contains high concentrations of a heavy metal.

Most previous studies that involved comparative transcriptomic approaches have focussed initially on genes up-regulated by environmental stimuli or developmental cues (Chelaifa *et al.* 2010). In our study, we focussed on the GO distribution of the genes for which expression was up-regulated by nickel. The majority of genes up-regulated by nickel belonged to the GO category 'cellular component'. Information on the annotations of these genes is valuable for the further investigation of gene functions, cellular structures and biological processes that might be involved in the tolerance of fungi to nickel via extracellular and intracellular mechanisms, as suggested by Bellion *et al.* (2006).

Predicted function of the specific biomarkers

In previous studies involving fungi, many genes involved in the response to stress induced by heavy metals were found to encode proteins that function as metal transporters or metal-binding proteins (Jacob *et al.* 2004; Bellion *et al.* 2006; Bolchi *et al.* 2011). However, in the present study, most of the nickel biomarkers did not belong to classes of proteins that are typically involved in response mechanisms to stress induced by heavy metals. This suggests that the mechanisms that underlie the tolerance to nickel in *P. albus* from ultramafic soils differ from those of other fungi.

Two of the biomarkers identified, namely levels of mRNA transcripts that encode chitinase-like and glycosylphosphatidylinositol (GPI) cell-wall structural proteins, are involved in extracellular processes and encode putative cell-wall proteins. Recently, it was suggested that modifications of structural elements of the cell wall, such as the rearrangement of chitin and biosynthesis of glucan- or galactosamine-containing polymers, might play a key role in modulating the integrity of the cell wall and its capacity to immobilize heavy metals. In this way, such modifications could confer tolerance to metals and affect the ability of fungi to survive in stressful environments (Meharg 2003; Bellion *et al.* 2006; Fuchs & Mylonakis 2009). Our findings regarding the expression of genes that are involved in the rearrangement of the cell wall in the presence of nickel suggest that these genes play a substantial role in modifying the chemical composition and hence the integrity of the cell wall in *P. albus*. This provides an effective barrier that can chelate the heavy metal.

The other biomarkers that were up-regulated in the presence of nickel were predicted to function in intracellular mechanisms such as resistance to oxidative stress, transcriptional regulation and subcellular compartmentalization. One biomarker encodes a putative S-adenosylmethionine-methyl (SAM) transferase. In the fungus *Podospora anserina*, overexpression of SAM transferase increases resistance to metal-induced oxidative stress by counteracting the pro-oxidant effect of phenolic compounds in the presence of heavy metal. This overexpression of SAM transferase leads to a decrease in the formation of reactive oxygen species (ROS) (Kunstmann & Osiewacz 2009), which are known to cause irreversible and lethal oxidative damage to cells (Gill & Tuteja 2010). Our results in *P. albus* suggest that SAM transferase is also overexpressed in the presence of nickel and thus might increase fungal resistance to metal-dependent oxidative stress by methylating phenolic compounds, which are known to accumulate in *P. albus*.

Another biomarker encodes a basic leucine zipper (bZIP) transcription factor. Previous studies on *Saccharomyces cerevisiae* have characterized AP-1 as a master regulator that orchestrates responses to metal/metalloid stress (Wysocki & Tamas 2010). Another study showed that a transcription factor related to AP-1-like (a member of the bZIP family) is up-regulated in the fungus *Paxillus involutus* upon exposure to cadmium. This suggests that Zip1-like proteins could play a critical role in the transcription of genes that are essential for protection against cadmium stress (Jacob *et al.* 2004). The overexpression of a bZip transcription factor in *P. albus* in the presence of nickel suggests that members of the bZIP family might act as regulators of genes involved in tolerance of metals.

Another of the selected biomarkers encodes a putative vacuole targeting-related protein. A previous study on vacuolar-defective mutants of *S. cerevisiae* confirmed the essential role that the vacuole plays in the detoxification of metals as such as nickel, zinc, cobalt and manganese. It was hypothesized that compartmentalization of these toxic metals in the vacuole prevents them from harming the cell (Ramsay & Gadd 1997). In the present study, the overexpression of a vacuole targeting-related protein in the presence of nickel suggests that the vacuole may play a key role in metal detoxification in *P. albus*.

Regarding the other biomarkers, no known function related to nickel tolerance could be identified. Taken together, the results for predicted protein function agree with the results of the GO analyses, in which the genes that were up-regulated by nickel were classified into the established GO categories 'cellular components', 'molecular function' and 'biological processes'.

Characterization of biomarker expression in Pisolithus albus

Comparison of the levels of expression of the 10 genes that were up-regulated most strongly in response to nickel (Fig. 3) revealed marked differences between the nickel-tolerant and nickel-sensitive phenotypes of different *P. albus* isolates from ultramafic soils. It also enabled us to identify five genes that were expressed exclusively in nickel-tolerant isolates. The remaining five genes were not selected as candidate biomarkers because they were expressed in both phenotypes. However, these genes might also be of value in predicting nickel tolerance because their level of expression was higher in the nickel-tolerant isolate than in the nickel-sensitive one, and their induction by nickel was observed only in the nickel-tolerant isolate. To strengthen the robustness of the predictive value of these final five biomarkers, levels of the appropriate transcripts were analysed in additional nickel-tolerant and nickel-sensitive isolates of *P. albus*. The data (Fig. 4) confirmed that these five final selected biomarkers of *P. albus* are suitable for identifying nickel-tolerant fungal isolates.

Given that ECM symbioses are known to play a major role in the fitness of plants in the presence of heavy metals (Jentschke & Godbold 2000), these biomarkers should also be analysed in the context of symbiotic interactions between ECM fungi and plants. Preliminary results from *in vitro* assays of nickel-tolerant *P. albus* (MD06-337) and nickel-sensitive *P. albus* (MD09-078) in symbioses with two members of the *Myrtaceae*, the model species *Eucalyptus globulus* and *Tristaniopsis* sp., which is endemic to New Caledonia, show that these biomarkers are expressed exclusively in nickel-tolerant ECM symbioses (data not shown). This confirms their suitability for monitoring the adaptation of ECM symbioses to nickel both *in vitro* and *in situ*.

Such candidate gene approaches, which involve the identification of individual genes linked to nickel tolerance, provide a starting point for investigating the molecular basis of adaptation to heavy metal content in soils and contribute to understanding tolerance for ultramafic soils in natural fungal populations (Brady *et al.* 2005). Interestingly, for each gene, the level of expression that was induced by nickel varied among the isolates tested. This intraspecific variation in transcript profiles was also observed in cadmium-tolerant *Suillus luteus* isolates, which suggests that such variation helps fungal species to persist in novel environments (Ruytinx *et al.* 2011). Interestingly, for each gene, the level of expression that was induced by nickel varied among the isolates tested, for both nickel-tolerant and nickel-sensitive isolates of *P. albus*.

The presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in the same ultramafic habitat could be explained by variation in the effective concentration of nickel in such soils. Recent studies have shown that the ecotoxicological risk of nickel to organisms in ultramafic soils depends on its availability and that nickel shows local concentration gradients and variation in metal mobility (Becquer *et al.* 2006; Echevarria *et al.* 2006; Wright & Stanton 2011). Edaphic variation could have induced a variety of responses to cope with nickel toxicity in ultramafic soils (Branco 2009). Alternatively, elevated levels of magnesium in the soils could explain the presence of the nickel-sensitive isolates. Indeed, high concentrations of magnesium were found exclusively in the soils at the sites where the two sensitive isolates MD09-078 and MD09-063 were sampled (Table S3, Supporting information). Previous studies have shown that in yeast, the magnesium transporter allows low-affinity uptake of other divalent cations such as nickel, manganese, zinc and cobalt and that the *GAI* mutant of the yeast *Schizosaccharomyces pombe,* which is defective in the transport of magnesium, is resistant to nickel and cobalt (Sarikaya *et al.* 2006). In addition, magnesium is known to be an antagonist of nickel (Sarikaya *et al.* 2006). These observations could indicate that magnesium prevents the nickel uptake, thereby alleviating nickel toxicity in ultramafic soils and allowing the growth of fungi with a nickel-sensitive phenotype. These fungal responses to nickel could result from environment-dependent phenotypic expression or from genetic adaptation (or specialization) to local biotic and abiotic conditions (Branco 2009; Wolfe *et al.* 2009). Selection in ultramafic environments is associated with multiple constraints in addition to nickel concentration. These include pH, metal abundance, shortage of macronutrients, cation exchange capacity and water deficit. These constraints might have favoured genetic differentiation of *P. albus* between ultramafic and non-ultramafic sites as previously described (Jourand *et al.* 2010a,b). Further experiments should be carried out to assess the existence of local adaptation or phenotypic plasticity of *P. albus* to nickel in ultramafic soils. These experiments should include comparison of fitness parameters for isolates of *P. albus* that have been collected from their native soils and cultivated under different nickel conditions, and analysis of the physiological and molecular responses to nickel in the presence of the other metals that are found in ultramafic soils.

Conclusion

We have described transcriptomic differences in the molecular response to nickel between nickel-tolerant and nickel-sensitive isolates of *P. albus* from ultramafic soils. As a result, we have been able to identify specific biomarkers of nickel tolerance, which are expressed in nickel-tolerant isolates *in vitro* as well as *in situ* in ECM isolates. We suggest (i) that these results reflect a positive transcriptomic response of the fungus to nickel-rich environments and (ii) the presence of both nickel-tolerant and nickel-sensitive fungal phenotypes in ultramafic soils might reflect environment-dependent phenotypic responses to variations in the effective concentrations of nickel in heterogeneous ultramafic habitats. In addition, the results of our study suggest new directions for genetic research in terms of the selection of novel isolates that are tolerant to nickel or various other ultramafic conditions such as elevated levels of chromium, cobalt, or manganese, a low calcium:magnesium ratio, or low levels of essential macro-/micronutrients and drought stress.

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References

Addinsoft (2005) XLSTAT software version 7.5.2. Addinsoft. http://www.xlstat.com.

Becquer T, Quantin C, Rotte-Capet S, Ghanbaja J, Mustin C, Herbillon AJ (2006) Sources of trace metals in Ferralsols in New Caledonia. *European Journal of Soil Science*, **57**, 200–213.

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS Microbiology Letter*, **254**, 173–181.

Blaudez D, Botton B, Chalot M (2000a) Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. *Microbiology*, **146**, 1109–1117.

Blaudez D, Jacob C, Turnau K *et al.* (2000b) Differential responses of ectomycorrhizal fungi to heavy metals *in vitro*. *Mycological Research*, **104**, 1366–1371.

Bolchi A, Ruotolo R, Marchini G *et al.* (2011) Genome-wide inventory of metal homeostasis-related gene products including a functional phytochelatin synthase in the hypogeous mycorrhizal fungus *Bolchi melanosporum*. *Fungal Genetics and Biology*, **48**, 573–584.

Brady K.U., Kruckeberg A.R., Bradshaw H.D. Jr (2005) Evolutionary ecology of plant adaptation to serpentine soils. *Annual Review of Ecology, Evolution, and Systematics*, **36**, 243–266.

Branco S. (2009) Are oaks locally adapted to serpentine soils. *Northeast Naturalist*, **16**(sp5), 329–340.

Branco S, Ree RH (2010) Serpentine soils do not limit Mycorrhizal fungal diversity. *PLoS One*, **5**, e11757.

Brooks RR (1987) *Serpentine and its Vegetation: A Multidisciplinary Approach*. Dioscorides Press, Portland, OR.

Chelaifa H, Mahé F, Ainouche M (2010) Transcriptome divergence between the hexaploid salt-marsh sister species *Spartina maritima* and *Spartina alterniflora* (Poaceae). *Molecular Ecology*, **19**, 2050–2063.

Colpaert J, Wevers J, Krznaric E, Adriaensen K (2011) How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. *Annals of Forest Science*, **68**, 17–24.

Echevarria G, Massoura S, Sterckeman T, Becquer T, Schwartz C, Morel JL (2006) Assessment and control of the bioavailability of Ni in soils. *Environmental Toxicology and Chemistry*, **25**, 643–651.

Fuchs BB, Mylonakis E (2009) Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. *Eukaryotic Cell*, **8**, 1616–1625.

Gibson G (2008) The environmental contribution to gene expression profiles. *Nature Reviews Genetics*, **9**, 575–581.

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, **48**, 909–930.

Gonçalves SC, Martins-Louçao MA, Freitas H (2009) Evidence of adaptative tolerance to nickel in isolates of *Cenococcum geophilum* from serpentine soils. *Mycorrhiza*, **19**, 221–230.

Götz S, García-Gómez JM, Terol J *et al.* (2008) High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, **36**, 3420–3435.

Hartley J, Cairney JWG, Meharg AA (1997) Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? *Plant and Soil*, **189**, 303–319.

Jacob C, Courbot M, Martin F, Brun A, Chalot M (2004) Transcriptomic responses to cadmium in the ectomycorrhizal fungus *Paxillus involutus*. *FEBS Letters*, **576**, 423–427.

Jentschke G, Godbold DL (2000) Metal toxicity and ectomycorrhizas. *Physiologia Plantarum*, **109**, 107–116.

Jourand P, Ducousso M, Loulergue-Majorel C *et al.* (2010a) Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype. *FEMS Microbiology Ecology*, **72**, 238–249.

Jourand P, Ducousso M, Reid R *et al.* (2010b) Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant *Eucalyptus globulus* at toxic nickel concentrations. *Tree Physiology*, **30**, 1311–1319.

Kazakou E, Dimitrakopoulos PG, Baker AJ, Reeves RD, Troumbis AY (2008) Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. *Biological Reviews*, **83**, 495–508.

Kunstmann B, Osiewacz HD (2009) The S-adenosylmethionine dependent O-methyltransferase PaMTH1: a longevity assurance factor protecting *Podospora anserina* against oxidative stress. *Aging*, **1**, 328–334.

Lanfranco L (2007) The fine-tuning of heavy metals in mycorrhizal fungi. *New Phytologist*, **174**, 3–6.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, **25**, 402–408.

Martin F, Díez J, Dell B, Delaruelle C (2002) Phylogeography of the ectomycorrhizal Pisolithus species as inferred from nuclear ribosomal DNA ITS sequences. *New Phytologist*, **153**, 345–357.

Martin F, Aerts A, Ahrén D *et al.* (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature*, **452**, 88–92.

Marx DH (1977) Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. *Canadian Journal of Microbiology*, **23**, 217–223.

Meharg AA (2003) The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycological Research*, **107**, 1253–1265.

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods*, **5**, 621–628.

Muller LAH, Craciun AR, Ruytinx J *et al.* (2007a) Gene expression profiling of a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate exposed to increased external zinc concentrations. *Mycorrhiza*, **17**, 571–580.

Muller LA, Vangronsveld J, Colpaert JV (2007b) Genetic structure of *Suillus luteus* populations in heavy metal polluted and nonpolluted habitats. *Molecular Ecology*, **16**, 4728–4737.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853–858.

Perrier N, Amir H, Colin F (2006) Occurrence of mycorrhizal symbioses in the metal-rich lateritic soils of the Koniambo Massif, New Caledonia. *Mycorrhiza*, **16**, 449–458.

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, **30**, e36.

Rajkumar M, Ae N, Freitas H (2009) Endophytic bacterial and their potential to enhance heavy metal phytoextraction. *Chemosphere*, **77**, 153–160.

Ramesh G, Podila GK, Gay G, Marmeisse R, Reddy MS (2009) Different patterns of regulation for the copper and cadmium metallothioneins of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *Applied Environmental Microbiology*, **75**, 2266–2274.

Ramsay LM, Gadd GM (1997) Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirms a role for the vacuole in toxic metal ion detoxification. *FEMS Microbiology Letters*, **152**, 293–298.

Ruytinx J, Craciun AR, Verstraelen K, Vangronsveld J, Colpaert JV, Verbruggen N (2011) Transcriptome analysis by cDNA-AFLP of *Suillus luteus* Cd-tolerant and Cd-sensitive isolates. *Mycorrhiza*, **21**, 145–154.

Salzman RA, Fujita T, Salzman Z, Hasegawa PM, Bressan RA (1999) An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. *Plant Molecular Biology Reporter*, **17**, 11–17.

Sarikaya A, Akman G, Temizkan G (2006) Nickel resistance in fission yeast associated with the magnesium transport system. *Molecular Biotechnology*, **32**, 139–145.

Schmid J, Müller-Hagen D, Bekel T *et al.* (2010) Transcriptome sequencing and comparative transcriptome analysis of the scleroglucan producer *Sclerotium rolfsii*. *BMC Genomics*, **11**, 329.

Wolfe BE, Parrent JL, Koch AM *et al.* (2009) Spatial heterogeneity in mycorrhizal populations and communities: scales and mechanisms. In: *Mycorrhizas – Functional Processes and Ecological Impact* (eds Azcón-Aguilar C, Barea JM, Gianinazzi S and Gianinazzi-Pearson V), pp. 167–186. Springer-Verlag Berlin Heidelberg, Germany.

Wright JW, Stanton ML (2011) Local adaptation in heterogeneous landscapes. In: *Serpentine: the Evolution and Ecology of a Model System* (eds Harrison S and Rajakaruna N), pp. 155–181. The University of California Press, Berkeley and Los Angeles, CA.

Wysocki R, Tamas MJ (2010) How *Saccharomyces cerevisiae* copes with toxic metals and metalloids. *FEMS Microbiology Reviews*, **34**, 925–951.

The main research interest of the authors focuse on the role of ectomycorrhizal symbiosis in plant adaptation to extreme edaphic conditions as found in ultramafic soils (deficiency in major plant nutrients, unbalanced ratio Ca/Mg and high contents in heavy metals such as Co, Cr, Mn and Ni).

Data accessibility

Internal transcribed spacer DNA sequences: EMBL accessions FR852890, FR852891, FR852892, FR852893. The trimmed pyrosequencing contig sequences (Table 2) are available in the EMBL Database under the following accession numbers: contig C10864: FR852894; contig C6406: FR852895; contig C9200: FR852896; contig C7318: FR852897; contig C10795: FR852898; contig C15327: FR852899; contig C17653: FR852900; contig C18133: FR852901; contig C10491: FR852902; contig C4552: FR852903. mRNA accumulation data: The data used for Fig. 3 are included in the figure. The data used for Fig. 4 are available in Table S4 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 primer sequences (Primer express software), amplification size and efficiency of the selected ESTs and two reference genes (GAPDH and EF4) used for qPCR analysis.

Table S2 Pyrosequencing 454 data from *P. albus* MD06-337 isolate.

Table S3 Geochemical characteristics of rhizospheric soils site: major element content.

Table S4 Comparison of mRNA accumulation profiles for the six selected up-regulated genes in five *P. albus* isolates from ultramafic soil.

Fig. S1 General geographical map describing the New Caledonian archipelago with location of ultramafic massifs (in grey) and sites where *P. albus* isolates were collected (1, 2, 3, 4 & 5).

Fig. S2 Phylogenetic synthetic relationships among representative *Pisolithus* sp., in particular *P. albus* isolates from New Caledonia included in this study, i.e. MD06-337, MD09-078, MD09-045, MD09-063 and MD09-001 and worldwide reference isolates.

Fig. S3 Functional GO terms assignment and distribution of sequences GO terms of 4211 genes nickel up regulated from the differential transcriptome of MD06-337 nickel tolerant isolate from *P. albus* with +250 μM of nickel and without nickel, among Gene Ontology (GO) biological process, molecular function and cellular component.

Fig. S4 Relative expression patterns of 10 nickel selected genes from nickel tolerant *P. albus* MD06-337 isolate with two reference genes GAPDH and EIF4a. Panel A and B show respectively, relative expression patterns of 10 nickel with 250 or 50 μM of nickel, after 15 days of growth.

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Evidence of nickel (Ni) efflux in Ni-tolerant ectomycorhizal *Pisolithus albus* isolated from ultramafic soil

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Summary

Nickel (Ni)-tolerant ectomycorrhizal *Pisolithus albus* was isolated from extreme ultramafic soils that are naturally rich in heavy metals. This study aimed to identify the specific molecular mechanisms associated with the response of *P. albus* to nickel. In presence of high concentration of nickel, *P. albus* Ni-tolerant isolate showed a low basal accumulation of nickel in its fungal tissues and was able to perform a metal efflux mechanism. Three genes putatively involved in metal efflux were identified from the *P. albus* transcriptome, and their overexpression was confirmed in the mycelium that was cultivated *in vitro* in the presence of nickel and in fungal tissues that were sampled *in situ*. Cloning these genes in yeast provided significant advantages in terms of nickel tolerance (+ 31% Ni EC_{50}) and growth (+ 83% μ) compared with controls. Furthermore, nickel efflux was also detected in the transformed yeast cells. Protein sequence analysis indicated that the genes encoded a P-type-ATPase, an ABC transporter and a major facilitator superfamily permease (MFS). This study sheds light on a global mechanism of metal efflux by *P. albus* cells that supports nickel tolerance. These specific responses to nickel might contribute to the fungal adaptation in ultramafic soil.

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Introduction

Various heavy metals are essential at low concentrations in plant and microorganisms nutrition but become toxic at increasing concentrations (Tyler *et al*., 1989; Marschner, 2012). As a consequence, soils that contain high concentrations of heavy metals, whether from natural origins or anthropogenic activity, may be toxic for plants and soil microorganisms (Giller *et al*., 2009; Marschner, 2012). To avoid heavy metal toxicity in soils, plants and microorganisms have developed many strategies based on molecular and physiological responses (Haferburg and Kothe, 2007; Manara, 2012). Ectomycorrhizal fungi (ECM) are also known to tolerate heavy metals (Hartley *et al*., 1997; Colpaert *et al*., 2011). These ECM participate in crucial symbiotic relationships with plants that grow in soils with high concentrations of heavy metals and alleviate metal toxicity for their host plants (Gadd, 1993; Leyval *et al*., 1997; Jentschke and Godbold, 2000; Jourand *et al*., 2014). The ECM fungal metal tolerance is supported by various mechanisms that include (i) reduction of uptake of metals into the cytosol by extracellular chelation through extruded ligands and binding onto cell-wall components, (ii) intracellular chelation of metals in the cytosol by a range of ligands (glutathione and metallothioneins) and (iii) increase of efflux from the cytosol out of the cell or into sequestering compartments (Bellion *et al*., 2006). In addition, metal tolerance may involve supplementary mechanisms, such as modification of a magnesium transport system, as reported for nickel tolerance in yeast and fungi (Joho *et al*., 1995).

Recently, nickel-tolerant ECM *Pisolithus albus* (Cooke, 1891) has been isolated from ultramafic soils in New Caledonia (Jourand *et al*., 2010a). Ultramafic soils are a weathered product from ultramafic bedrock that covers less than 1% of the earth's surface (Coleman and Jove, 1992). These soils are characterized by high concentrations of iron oxides (up to 85% w/w), unbalanced calcium (Ca)-to-magnesium (Mg) ratios (up to 1:30 that consequently may influence Ca and Mg plant nutrition) and the presence of various heavy metals at high concentrations, such as chromium, cobalt, manganese and nickel, all of which are toxic for many plants (Brooks, 1987; Harrison and Rajakaruna, 2011). These soils are also extremely deficient in elements that are essential for plant nutrition,

including nitrogen, phosphorus and potassium (Brooks, 1987; Chiarucci and Baker, 2007). In New Caledonia, the ultramafic soils cover one third of the main island because of natural geological evolution. As a result of the presence of such ultramafic outcrops, specific biological endemic ecosystems have developed (Jaffré, 1992), making the main island a biodiversity hot spot (Myers *et al*., 2000). In addition, in neo-Caledonian ultramafic ecosystems, ectotrophic mycorrhizal symbioses are dominant and contribute to plant adaptation and fitness to the extreme soil conditions (Jentschke and Godbold, 2000; Prin *et al*., 2012). Thus, the dominant ECM *P. albus* was isolated from ultramafic soils in New Caledonia, and Ni-tolerant and Ni-sensitive isolates were reported in one ecotype (Jourand *et al*., 2010a). To track biomarkers of nickel tolerance in *P. albus*, the Ni differential transcriptomes with or without nickel were determined. The expression of genes involved in the specific molecular response to nickel was monitored in a nickel-tolerant *P. albus* isolate (Majorel *et al*., 2012). Comparison of expression levels revealed that 30% of the identified genes were modulated by nickel treatment.

The present study aimed to investigate the molecular mechanisms supporting nickel tolerance in this *P. albus* transcriptome, particularly the metal efflux mechanisms recently reported by Ruytinx and colleagues (2013) in *Suillus bovinus* in response to zinc. To test this hypothesis, (i) nickel efflux and accumulation were analysed in *P. albus*, (ii) genes potentially involved in the metal efflux were investigated, (iii) and cloning of these genes *in levuro* was achieved to assess their expression in the presence of nickel and their role in metal efflux.

Results and discussion

Nickel efflux and accumulation in P. albus

Ectomycorrhizal fungi *P. albus* fungal isolates used in the study are described in Table 1. These fungal isolates were sampled from ultramafic soils in New Caledonia: the Ni-tolerant isolate (MD06-337) and the Ni-sensitive isolate (MD09-078) as reported in Jourand and colleagues (2010b) and Majorel and colleagues (2012) respectively. To test the nickel accumulation in *P. albus*, the fungal isolates were cultivated in liquid in presence of nickel at different concentrations, and then nickel was analysed in the mycelial tissue. As shown in Fig. 1 (A and A'), the mycelium of *P. albus* presented a linear relationship between (i) the nickel concentrations tested in the liquid medium and (ii) the residual nickel content in its tissues. For the *P. albus* Ni-tolerant isolate (Fig. 1A), the slope of the linear regression was 0.0173 ($R^2 = 0.98734$), and the slope for the Ni-sensitive isolate (Fig. 1A'), was 0.025 ($R^2 = 0.99881$). At nickel concentrations of 50 μM and 100 μM, the residual nickel content in the mycelia of Ni-sensitive *P. albus* was 34% and 25% higher than the nickel content in Ni-tolerant *P. albus* respectively. To test the existence of a nickel efflux, the fungal isolates were cultivated for 1 week to generate a suitable quantity of biomass. Then, the kinetics of the metal efflux were tested by adding nickel at different concentrations, and residual nickel in mycelial tissue was measured at different times. Figures 1B and 1B present a comparison of the time-courses of residual nickel in mycelium between *P. albus* Ni-tolerant and Ni-sensitive isolates when exposed in liquid medium to 50 μM Ni (B) or 250 μM Ni (B'). After 48 h of nickel exposure and compared with nickel content at time 2 h, the residual nickel content in the mycelium of Ni-tolerant *P. albus* was increased by 25.7% at Ni = 50 μM and by 6.9% at Ni = 250 μM. In contrast, at the same time exposure (48 h) and compared with the nickel content at time 2 h, the residual nickel content in the mycelium of Ni-sensitive *P. albus* increased by 46.3% at Ni = 50 μM and 27.4% at Ni = 250 μM.

These data showed that the Ni-tolerant *P. albus* isolate accumulates a low basal level of nickel in its mycelial tissue, notwithstanding the nickel concentration applied in the medium. In liquid medium, a linear relationship exists between the nickel concentrations applied in the medium and the residual nickel content in the fungal tissue for Ni-tolerant and Ni-sensitive isolates *P. albus*. A comparison of the slopes (Fig. 1A and 1A') highlighted that the Ni-sensitive isolate presents faster nickel uptake than the Ni-tolerant isolate. Correlated with

Table 1. Isolates of *Pisolithus albus* used in this study. A description of the sites where isolates were sampled (map location, global positioning system (GPS) coordinates); isolate codes, DNA internal transcribed spacer (ITS) sequences accession number in the EMBL database and *in vitro* tolerance to nickel (phenotype and Ni EC_{50}) are provided for each fungal isolate.

Site description (ultramafic soils)	*Pisolithus albus* isolates				
Site location and GPS coordinates	Isolate code	DNA-ITS EMBL accession code	Nickel phenotype	Ni EC_{50} μM in MMN medium	References
Trazy-Guerioum. Koniambo Massif (21°00′28″ S; 164°49′50″ E)	MD06-337	AM947121	Tolerant	569 ± 3	Jourand *et al*., 2010a
Pindjen Waterfall Road Koné (21°02′19′ S; 164°46′26′ E)	MD09-078	FR852890	Sensitive	42 ± 4	Majorel *et al*., 2012

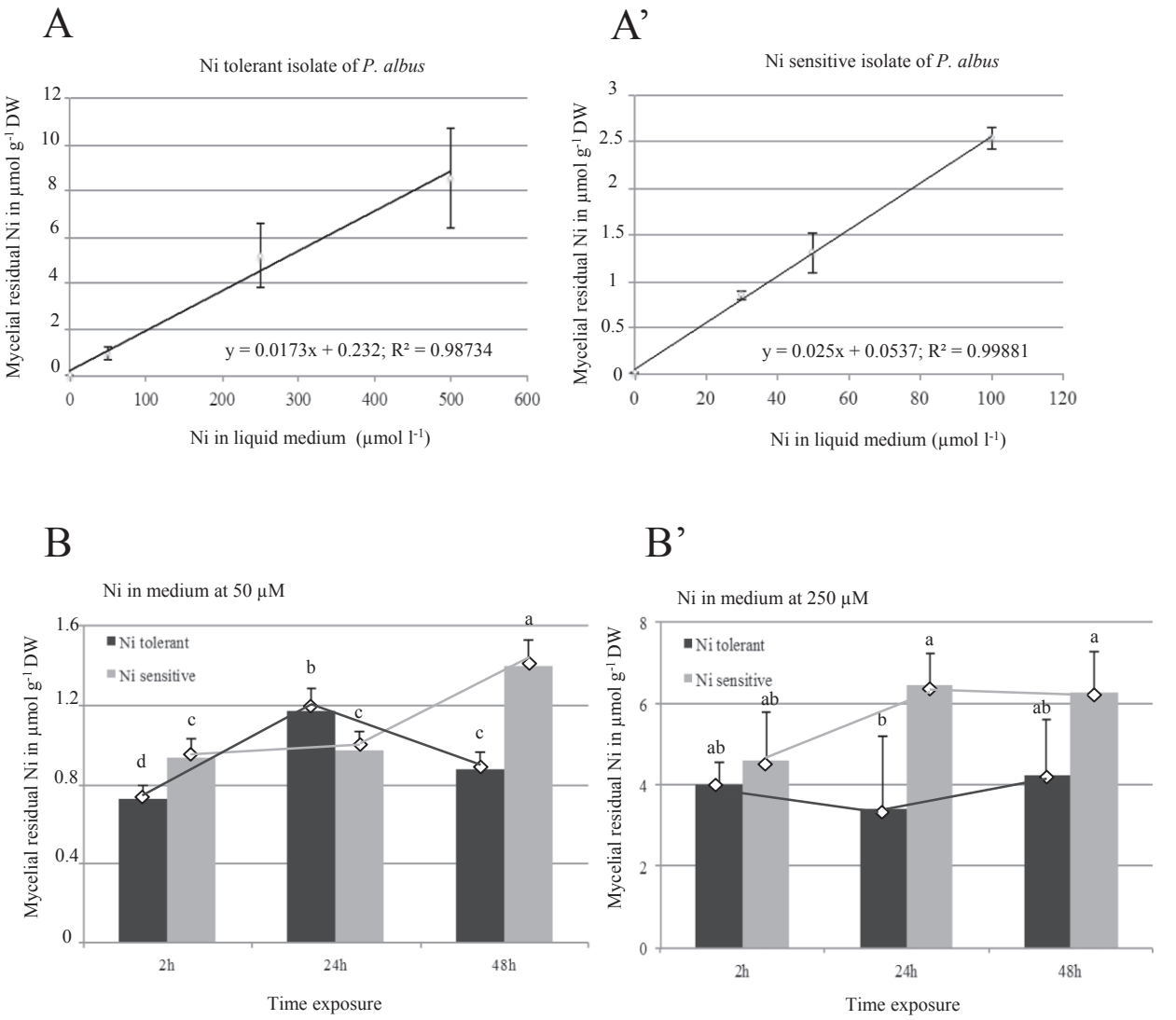

Fig. 1. Nickel content in *Pisolithus albus* mycelial tissue, expressed in µmol of Ni per g of DW mycelium. A and A'. Residual nickel in mycelium of the *P. albus* Ni-tolerant (A) or Ni-sensitive (A') isolates after 15 days of culture in liquid medium containing increasing nickel concentrations. B and B'. Time-course comparison between *P. albus* Ni-tolerant and Ni-sensitive isolates when exposed in liquid medium to 50 µM Ni (B) or 250 µM Ni (B'). Bars represent the means, and error bars represent the standard deviation of the mean ($n = 3$) of three independent experiments. The different letters above the columns indicate significant differences as determined by Tukey's honest significant difference test ($P \leq 0.05$). Abbreviation: DW: dry weight.

these data, the residual nickel content in the Ni-sensitive isolate was higher (+ 29%) than in the Ni-tolerant isolate. The profiles of the time-courses of residual nickel in the mycelia (Fig. 1B and 1B') were significantly different for both isolates. Indeed, the Ni-tolerant isolate was able to manage the nickel flux according to time, but the Ni-sensitive isolate could not. Altogether, these data clearly demonstrate that metal does not accumulate in the fungal tissue of Ni-tolerant *P. albus*, which suggests a metal efflux. Similar fungal behaviour in the presence of heavy metal has been recently reported in other ectomycorrhizal fungi, such as in *Suillus* and its tolerance to cadmium (Cd) and zinc (Zn) (Colpaert *et al.*, 2011; Ruytinx *et al.*, 2013). To argue this efflux hypothesis, we investigated the genes in the transcriptome of the Ni-tolerant *P. albus* isolate (i) that showed Ni-induced expression (Majorel *et al.*, 2012) and (ii) that were potentially involved in the metal efflux, such as the P-type ATPase (Portillo, 2000; Coleman and Mylonakis, 2009).

Expression and selection of putative genes involved in nickel *efflux in* P. albus

The contigs of putative genes involved in nickel efflux were selected from functional data obtained using the gene ontology (GO) analysis of the *Pisolithus albus*

Table 2. *Pisolithus albus* contigs selected in the transcriptomic database (Majorel *et al*., 2012) which are functionally identified as involved in the nickel efflux. Data come from pyrosequencing analysis, and they are showing differential expression in *P. albus* nickel-tolerant isolate MD06-337. Data of the Blast X functional identification were collected from the NCBI database (http://blast.ncbi.nlm.nih.gov).

Pyrosequencing analysis				Blast X functional identification	
N° Contig	Size (bp)	RPKM 0 μM Ni	RPKM 250 μM Ni	Functional classification	Species
C2515	464	0	114	Aminophospholipid-transporting P-type ATPase	*Laccaria bicolor*
C2624	295	0	102	Protein transporter	*Coprinopsis cinerea*
C3578	2428	101	134	0H+-transporting ATPase	*Laccaria bicolor*
C5339	2324	219	334	ABC transporter	*Coprinopsis cinerea*
C6943	578	6	46	Aminophospholipid-transporting P-type ATPase	*Laccaria bicolor*
C7177	1284	6	72	Phospholipid-translocating P-type ATPase	*Coniophora puteana*
C9915	446	0	11	Cu-transporting P-type ATPase	*Laccaria bicolor*
C16623	419	9	58	Minophospholipid-transporting P-type ATPase	*Laccaria bicolor*
C17235	2224	10	55	MFS general substrate transporter	*Coniophora puteana*

bp, base pair; RPKM, reads per kilobase of exon model per million mapped reads (Mortazavi *et al*., 2008).

cDNA library (Majorel *et al*., 2012). The list of the selected Ni-efflux genes (contigs) obtained from the GO analysis of the *P. albus* cDNA library is presented in Table 2 including the DNA sequences deposited into the EMBL database (from HG530057 to HG530065) and their BlastX functional identification. Their expression was tested by quantitative polymerase chain reaction (PCR) on either the fungal isolates *in vitro* cultivated with or without nickel and on mycelial tissue *in situ* sampled. The results of quantitative PCR analysis of the expression of these genes in *P. albus* are presented in Fig. 2. In mycelium cultivated *in vitro*, most of the genes were mainly expressed in the *P. albus* Ni-tolerant isolate (Fig. 2A). In particular, the contigs C3578 and C5339 showed the highest expression levels. Moreover, the contigs expression showed a clear induction at 250 μM of nickel. Furthermore, most of the genes were expressed in the mycelia and ectomycorrhizal tissue of Ni-tolerant *P. albus* sampled *in situ* (Fig. 2B). Contig C17235 showed the highest level of expression in *in situ*-sampled mycelium and the second highest level of expression in ECM. By combining the data from Table 2 and Fig. 2, contigs C3578, C5339 and C17235 were selected as the best candidate genes putatively involved in nickel efflux. The contigs C3578, C5339 and C17235 belong to the P-type ATPase family, the ATP-binding cassette (ABC) transporter family and a major facilitator superfamily (MSF) respectively. As shown in Fig. 2, these genes were expressed and induced by nickel in the Ni-tolerant *P. albus* isolate but not in the Ni-sensitive isolate. The expression of these genes was also detected in mycelia and ECM tissues that were sampled *in situ*. Both *in vitro* and *in situ* gene expression argue in favour of their role in nickel tolerance by metal efflux in *P. albus*. To test this hypothesis, these contigs were further used in heterologous expression experiments in yeast.

RACE and gene expression in yeast

Contigs 17235 already showed a full-length sequence, while full sequences contigs of C3578 and C5339 were investigated and submitted into the EMBL database with accession numbers HG530066 and HG530067 respectively. Transformed yeast containing one of the three full-length contigs included transformant A: *Pa-MFS* (contig 17235), transformant B: *Pa-ATPase* (contig 3578) and transformant C: *Pa-ABCtr* (contig 5339). They were tested for their tolerance to nickel (i) using a drop test on solid medium and (ii) by following their kinetics in liquid medium. Figure 3 presents their responses to nickel on yeast nitrogen base (YNB) selective medium with and without nickel (1 mM). The data showed that transformants A, B, and C presented a gain of growth with nickel at 1 mM up to dilution $10E^{-3}$ compared with controls. The kinetics of growth in liquid medium at different nickel concentrations are presented in Fig. 4. Growth data analyses are summarized in Table 3 as growth rates (μ), the relationship between μ with nickel concentrations, and the determination of the nickel concentration that inhibited growth by 50% (Ni EC_{50}). All of these data showed that the expression of each contig provided a significant advantage in terms of growth rates (μ) in the presence of nickel; at 1 mM Ni, the mean of μ was enhanced significantly by 83% compared with controls (mean of μ of transformants A, B and C as reported in Table 3 compared with the mean of μ of the controls). In addition, the tolerance of the yeast transformants to nickel was improved; the mean of the Ni EC_{50} was enhanced by 30.7% compared with controls. The nickel efflux of each transformant was also investigated, as shown in Fig. 5. Compared with controls, transformants A, B and C did not accumulate nickel in cells; at $t = 90$ min, a comparison of the means of nickel content in transformants A, B and C (3.05 ± 0.15 μmol Ni per

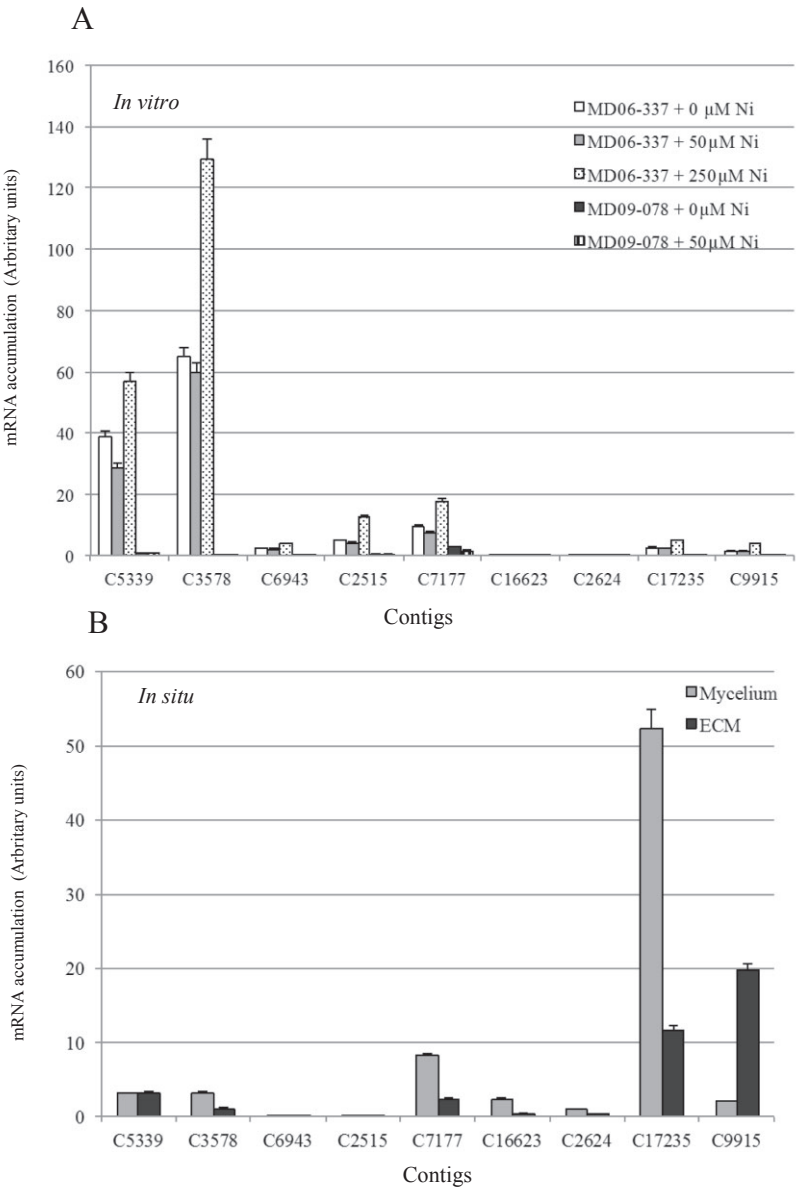

Fig. 2. mRNA accumulation patterns of the nine genes putatively involved in metal efflux that were upregulated by nickel and selected using the 454 data (Table 2). Q-PCR mRNA patterns were tested in the Ni-tolerant *P. albus* isolate MD06-337; A: in mycelium cultivated *in vitro* without nickel or with nickel (+ 50 or + 250 µM) and compared Ni-sensitive *P. albus* isolate (MD09-078); B: in mycelium and ECM sampled *in situ*. Transcript accumulation was quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and EF4α as reference genes for normalization and expressed as arbitrary units. The mean values were calculated from three technical replicates for each triplicate biological sample ± stdv and are indicated by bars.

OD_{600nm} unit) with the means of the nickel content of the controls (7.20 ± 0.36 µmol Ni per OD_{600nm} unit) showed that the residual nickel content in yeast cells of the transformants (A, B and C) was significantly reduced by 57.6%. In addition, a comparison of the means of the residual nickel contents between time $t = 0$ min (3.89 ± 0.19 µmol Ni per OD_{600nm} unit) and $t = 90$ min (3.05 ± 0.15 µmol Ni per OD_{600nm} unit) in the transformants (A, B and C) were significantly reduced by 21.6%. In contrast, the mean of the residual nickel content for the controls was enhanced significantly by 92%.

The results of phenotypic tests and kinetics of the transformed yeast cells showed significant advantages in terms of tolerance to nickel and growth compared with controls (Figs 3 and 4). In addition, the nickel efflux rates detected in the transformed yeast cells (Fig. 5) were similar to the nickel efflux rates detected in *P. albus*. Together, the transforming results of these genes in yeast support the metal efflux hypothesis. Various mechanisms were expected regarding metal tolerance, such as metal binding on the fungal wall and metal accumulation in the tissue or extra cellular chelation (Bellion *et al.*, 2006). Our data suggest nickel efflux as a main mechanism supporting nickel tolerance in *P. albus*, as recently reported in other ectomycorrhizal fungi, such as *Suillus bovinus* in response to Zn (Ruytinx *et al.*, 2013).

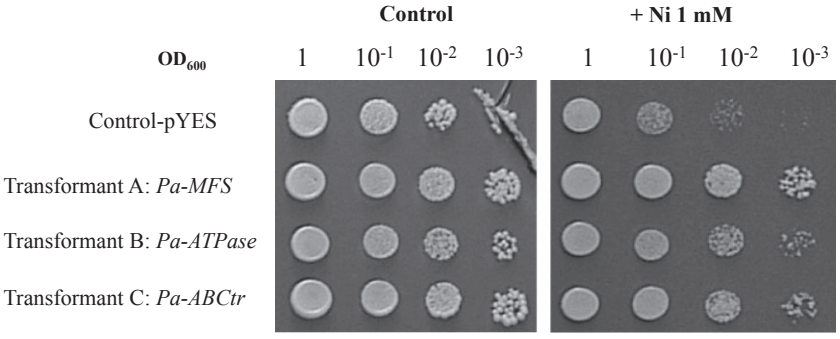

Fig. 3. Sensitivity of transformed *Saccharomyces cerevisae* strains to nickel after 6 days of exposure. Each yeast strain was transformed with the empty vector (pYES) or the transformant constructions and plated in four serial dilutions onto YNB medium with (right column) or without (control, left column) $NiSO_4$. The concentration of nickel that was used in the spot assays is indicated above each row (1 mM). Plates were incubated at 30°C for 6 days.
A. The BY4741 wild-type strain and three transformed yeast strains (transformant A: *Pa-MFS*, transformant B: *PA-ATPase* and transformant C: *Pa-ABCtr*) on control and nickel-amended medium with 2% glucose. Abbreviation: Pa: *Pisolithus albus*.

Contig translation into protein

Table 4 summarizes the data on cDNA translation of the contigs into protein. The contig C3578 encodes for a protein belonging to the P-type ATPase family. As most of typical members of this protein family, this *P. albus* P-type ATPase contains 10 predicted trans-membrane segments and conserved sequence motifs such as TGES, CSDKTGT, MLTGD and GDGVN, within the catalytic region (see ESM Table 1). In plant and fungi, such protein, which contains a potential metal-sensitive enzyme system such as ATPase, is the first living functional structure that comes in contact with toxic heavy metals (Hall, 2002). The P-type ATPase proteins are described to form phosphorylated intermediate substrates and to pump a range of cations across cellular membrane including heavy metals (Hall and Williams, 2003). The contig C5339 encodes for an ABC transporter family member. In plants, such transporters are membrane-intrinsic primary-active pumps and are known to play a key role in many biological functions, such as heavy metal cell detoxification by sequestration in the vacuole or exportation out of the cell (Wanke and Kolukisaoglu, 2010; Kretzschmar *et al*., 2011; Lee *et al*., 2014). In fungi, we can assume that the ABC protein acts as trans-membrane shuttle exporting heavy metals while transporting substrates into the cell. Contig 17235 encodes for a major facilitator protein permease (MFS). Major facilitator superfamily is one of the largest groups of secondary active transporters that are conserved from bacteria to humans. They transport a wide spectrum of substrates across bio-membranes and play a pivotal role in multiple physiological processes (Yan, 2013). In plants, MFS transporters respond to plant stresses, such as defending against infection by pathogens and exporting toxic compounds and heavy metals (Peng *et al*., 2011). In this study, we noticed that the gene expression level of the contig C17235 was higher in mycelium sampled *in situ* than in the mycelium cultivated *in vitro* in presence of nickel. We hypothesize that C17235 gene expression might not specifically be upregulated by nickel but by all metals as found in the ultramafic substrate, i.e. cobalt, chromium, iron, nickel and manganese as reported in Majorel and colleagues (2012).

Table 3. Calculated exponential growth rates (10^{-3} μ are expressed in min^{-1}) based on the kinetics of the five *Saccharomyces cerevisiae* strains as shown in Fig. 4: BY4741 WT) strain and four yeast transformants: transformant A: *Pa-MFS*; transformant B: *Pa-ATPase*; transformant C: *Pa-ABCtr*, and the yeast transformed with the empty vector (pYES) and used as a control (Control-pYES).

Yeast transformants	Growth rates (10^{-3} μ in min^{-1}) at different nickel concentrations tested (μM)				Exponential regression parameters μ = f [(Ni)]	Ni EC_{50} (μM)
	Ni = 0	Ni = 250	Ni = 500	Ni = 1000		
BY4741 WT	4.8 ± 0.2ab	3.3 ± 0.2de	2.6 ± 0.8e	1.3 ± 0.9gh	$y = 0.0047e^{-0.001x}$, $r^2 = 0.99597$	672 ± 31b
Transformant A: *Pa-MFS*	4.8 ± 0.3ab	3.9 ± 0.3cd	2.8 ± 0.6e	2.1 ± 0.4g	$y = 0.0054e^{-0.001x}$, $r^2 = 0.92556$	811 ± 72ab
Transformant B: *Pa-ATPase*	5.1 ± 0.9a	4.8 ± 0.4ab	3.6 ± 0.2cd	2.4 ± 0.2f	$y = 0.0060e^{-9E-04x}$, $r^2 = 0.9946$	950 ± 192a
Transformant C: *Pa-ABCtr*	5.1 ± 0.7a	4.0 ± 0.2b	3.3 ± 0.4de	2.1 ± 0.2g	$y = 0.0051e^{-9E-04x}$, $r^2 = 0.99896$	770 ± 161ab
Control-pYES	4.9 ± 0.3ab	3.7 ± 0.2bc	2.2 ± 0.3fg	1.1 ± 0.3h	$y = 0.0046e^{-0.001x}$, $r^2 = 0.97492$	630 ± 62b

The data are presented as the means ± standard deviation from three independent experiments. The different letters after the data indicate significant differences as determined by a Tukey's HSD test ($P \leq 0.05$).

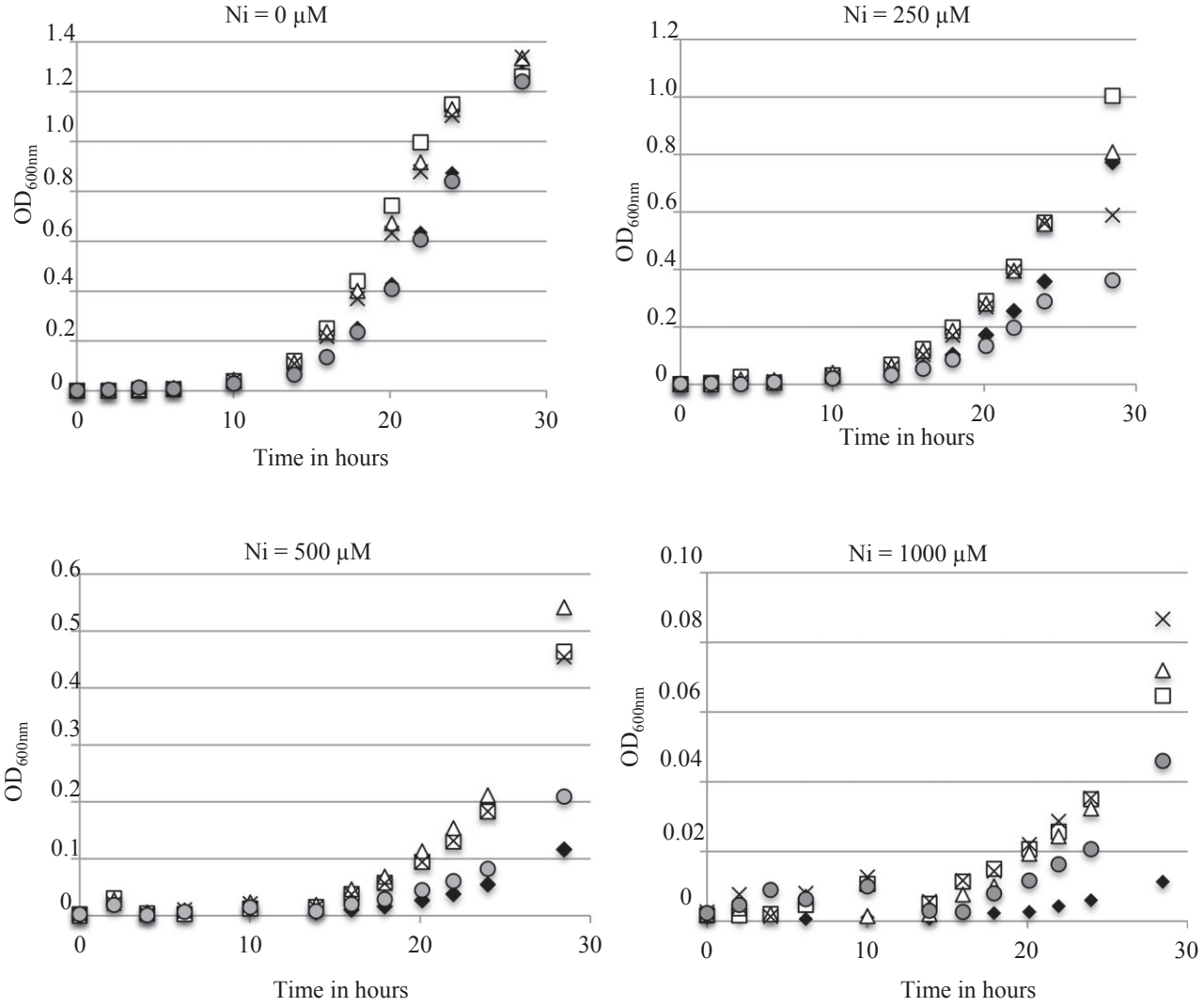

Fig. 4. Growth of *Saccharomyces cerevisiae* cultures exposed to different nickel concentrations. Time-course (in h) of the optical density at 600 nm. The studied *S. cerevisiae* strains included the wild-type (WT) strain BY4741 (◆), four-yeast transformed cells, i.e. transformant A: *Pa-MFS* (□), transformant B: *Pa-ATPase* (△), transformant C: *Pa-ABCtr* (×) and the wild-type strain BY4741 that contained the empty plasmid pYES and was used as control (control-pYES) (●).

Altogether, our data and the protein structure analyses indicated that the three systems (i.e. P-type ATPase, ABC transporter and MFS) (i) are induced in the presence of nickel *in vitro*, (ii) expressed in *in situ* fungal tissues and (iii) are involved in nickel efflux in transformed yeast. They may act simultaneously in the fungi *P. albus* to export the metal. These distinct and various mechanisms of metal efflux in *P. albus* could be involved in nickel tolerance and may contribute to the global metal homeostasis as an adaptive response to the ultramafic edaphic constraint, as suggested by Majorel and colleagues (2012). Further experiments such as (i) a comparison of metal accumu-

Table 4. Deoxyribonucleic acid full-length encoding contig sequences with their accession numbers in the EMBL database, translation into amino acids and prediction of (i) protein family identification, (ii) number of transmembrane domains and (iii) putative transport processes.

Contig code	EMBL accession number	Size translation in amino acids	Protein family identification	Number of trans membrane domains	Putative transport process
C17235	HG530065	515 aa	MFS family	14	Proton motive force (H+ anti-porter)
C3578	HG530066	940 aa	P-type ATPase	10	Electrochemical gradient
C5339	HG530067	727 aa	ABC transporter	6	Hydrolysis of ATP

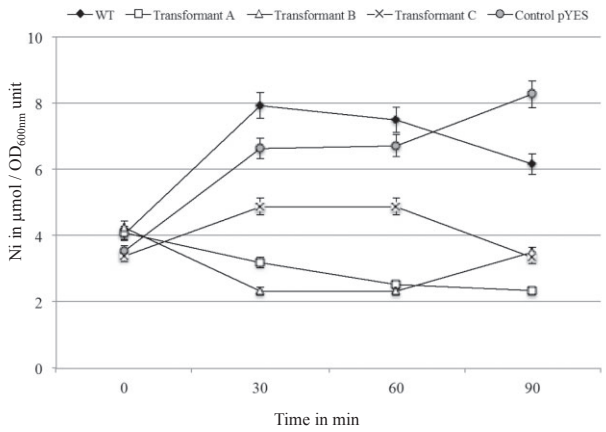

Fig. 5. Nickel efflux in *Saccharomyces cerevisiae* cultures exposed to nickel at 1 mM. The time-course (in min) of residual nickel content is expressed in µmol of Ni per unit of optical density (OD) and was measured at 600 nm. The studied *S. cerevisiae* strains included the wild-type (WT) strain BY4741, four yeast transformed cells, i.e. transformant A: *Pa-MFS*, transformant B: *Pa-ATPase*, transformant C: *Pa-ABCtr* and the WT strain BY4741 that contained the empty plasmid pYES and was used as the control (control-pYES). Bars represent the means, and error bars represent the standard deviation of the mean ($n = 3$) of three independent experiments.

lation and efflux in fungal tissues among metal tolerant and non-tolerant of other *P. albus* isolates, (ii) the construction of a GFP-fusion protein and its expression in yeast for each of sequences of the gene putatively involved in the metal efflux, and (iii) the use of metal radioelement when available (such as ^{63}Ni), should be carried out to confirm the metal efflux hypothesis as major mechanism in the fungal metal tolerance.

Acknowledgements

This work was supported by (i) the GIP CNRT 'Nickel and its Environment' [grant number GIPCNRT98] and (ii) the National Agency for Research (ANR) Financial Support through the BIOADAPT Program 2012 [grant number ANR-12-ADAP-0017 ADASPIR]. We wish to thank L. Jamet and its colleagues for the chemical analyses (ICP-EOS) conducted at Laboratoire d'Analyses Chimiques (LAMA), Centre IRD de Nouméa, Nouvelle-Calédonie.

Conflict of interest: The authors declare having no conflict of interest.

References

Bellion, M., Courbot, M., Jacob, C., Blaudez, D., and Chalot, M. (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS Microbiol Lett* **254:** 173–181.

Brooks, R.R. (1987) *Serpentine and its Vegetation: A Multidisciplinary Approach*. Portland, OR, USA: Dioscorides Press.

Chiarucci, A., and Baker, A.J.M. (2007) Advances in the ecology of serpentine soils. *Plant Soil* **293:** 1–217.

Coleman, J.J., and Mylonakis, E. (2009) Efflux in Fungi: La Pièce de Résistance. *PLoS Pathog* **5:** e1000486.

Coleman, R.G., and Jove, C. (1992) Geological origin of serpentinites. In *The Vegetation of Ultramafic (Serpentine) Soils*. Baker, A.J.M., Proctor, J., and Reeves, R.D. (eds). Hampshire, UK: Intercept Limited, pp. 1–17.

Colpaert, J.V., Wevers, J.H., Krznaric, E., and Adriaensen, K. (2011) How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. *Ann Forest Sci* **68:** 17–24.

Cooke, M.C. (1891) Australian fungi. *Grevillea* **20:** 4–7.

Gadd, G.M. (1993) Interaction of fungi with toxic metals. *New Phytol* **124:** 25–60.

Giller, K.E., Witter, E., and McGrath, S.P. (2009) Heavy metals and soil microbes. *Soil Biol Biochem* **41:** 2031–2037.

Haferburg, G., and Kothe, E. (2007) Microbes and metals: interactions in the environment. *J Basic Microbiol* **47:** 453–467.

Hall, J.L. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *J Exp Bot* **53:** 1–11.

Hall, J.L., and Williams, L.E. (2003) Transition metal transporters in plants. *J Exp Bot* **54:** 2601–2613.

Harrison, S., and Rajakaruna, N. (2011) *Serpentine: The Evolution and Ecology of a Model System*. Berkeley and Los Angeles, CA, USA: The University of California Press.

Hartley, J., Cairney, J.W.G., and Meharg, A.A. (1997) Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? *Plant Soil* **189:** 303–319.

Jaffré, T. (1992) Floristic and ecological diversity of the vegetation on ultramafic rocks in New Caledonia. In *The Vegetation of Ultramafic (Serpentines) Soils*. Baker, A.J.M., Proctor, J., and Reeves, R.D. (eds). Andover, UK: Intercept Ltd, pp. 101–107.

Jentschke, G., and Godbold, D.L. (2000) Metal toxicity and ectomycorrhizas. *Physiol Plant* **109:** 107–116.

Joho, M., Inouhe, M., Tohoyama, H., and Murayama, T. (1995) Nickel resistance mechanisms in yeasts and other fungi. *J Ind Microbiol* **14:** 164–168.

Jourand, P., Ducousso, M., Loulergue-Majorel, C., Hannibal, L., Santoni, S., Prin, Y., and Lebrun, M. (2010a) Ultramafic soils from New Caledonia structure *Pisolithus albus* in ecotype. *FEMS Microbiol Ecol* **72:** 238–249.

Jourand, P., Ducousso, M., Reid, R., Majorel, C., Richert, C., Riss, J., and Lebrun, M. (2010b) Nickel-tolerant ectomycorrhizal *Pisolithus albus* ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant Eucalyptus globulus at toxic nickel concentrations. *Tree Physiol* **30:** 1311–1319.

Jourand, P., Hannibal, L., Majorel, C., Mengant, S., Ducousso, M., and Lebrun, M. (2014) Ectomycorrhizal *Pisolitus albus* inoculation of *Acacia spirorbis* and *Eucalyptus globulus* grown in ultramafic topsoil enhances plant growth and mineral nutrition while limits metal uptake. *J Plant Physiol* **171:** 164–172.

Kretzschmar, T., Burla, B., Lee, Y., Martinoia, E., and Nagy, R. (2011) Functions of ABC transporters in plants. *Essays Biochem* **50:** 145–160.

Lee, J.Y., Yang, J.G., Zhitnitsky, D., Lewinson, O., and Rees, D.C. (2014) Structural basis for heavy metal detoxification by an Atm1-type ABC exporter. *Science* **343:** 1133–1136.

Leyval, C., Turnau, K., and Haselwandter, K. (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza* **7:** 139–153.

Majorel, C., Hannibal, L., Soupe, M.E., Carriconde, F., Ducousso, M., Lebrun, M., and Jourand, P. (2012) Tracking nickel-adaptive biomarkers in *Pisolithus albus* from New Caledonia using a transcriptomic approach. *Mol Ecol* **21:** 2208–2223.

Manara, A. (2012) Plant responses to heavy metal toxicity. In *Plants and Heavy Metals*. Furini, A. (ed.). Berlin, Germany: Springer, pp. 27–53.

Marschner, P. (2012) *Mineral Nutrition of Higher Plants*. London, UK: Academic Press Limited.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5:** 621–628.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A., and Kent, J. (2000) Biodiversity hotspots for conservation priorities. *Nature* **403:** 853–858.

Peng, H., Han, S., Luo, M., Gao, J., Liu, X., and Zhao, M. (2011) Roles of multidrug transporters of MFS in plant stress responses. *Int J Biosci Biochem Bioinform* **1:** 109–113.

Portillo, F. (2000) Regulation of plasma membrane H^+-ATPase in fungi and plants. *Biochim Biophys Acta* **1469:** 31–42.

Prin, Y., Ducousso, M., Tassin, J., Béna, G., Jourand, P., Dumontet, V., *et al*. (2012) Ectotrophic mycorrhizal symbioses are dominant in natural ultramafic forest ecosystems of New Caledonia. In *The Mycorrhizal Symbiosis in Mediterranean Environment: Importance in Ecosystem Stability and in Soil Rehabilitation Strategies*. Hafidi, M., and Duponnois, R. (eds). New York, NY, USA: Nova Science Publishers, pp. 25–48.

Ruytinx, J., Nguyen, H., Van Hees, M., Op De Beeck, M., Vangronsveld, J., Carleer, R., *et al*. (2013) Zinc export results in adaptive zinc tolerance in the ectomycorrhizal basidiomycete Suillus bovinus. *Metallomics* **5:** 1225–1233.

Tyler, G., Balsberg Påhlsson, A.M., Bengtsson, G., Bååth, E., and Tranvik, L. (1989) Heavy-metal ecology of terrestrial plants, microorganisms and invertebrates. *Water Air Soil Pollut* **47:** 189–215.

Wanke, D., and Kolukisaoglu, H.Ü. (2010) An update on the ABCC/MRP transporter family in plants – many genes, many proteins, but how many functions? *Plant Biol* **12:** 15–25.

Yan, N. (2013) Structural advances for the major facilitator superfamily (MFS) transporters. *Trends Biochem Sci* **38:** 151–159.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Amino acid (aa) sequence of the translated contigs. In bold red: the domains that are conserved. Underlined in green: the trans-membrane domains. In blue: the putative walker B, ABC signature. Bold underlined: the metal binding site. Bold in black: the histidine (H) and the cysteine (C).

Table S2A. Primer sequences (Primer Express software), efficiency of the selected ESTs and the two reference genes (GAPDH and EF4α) that were used for qPCR analysis.

Table S2B. RACE primer DNA sequences.

Table S2C. Primer cloning DNA sequences.

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Chapter II

Ectotrophic Mycorrhizal Symbioses Are Dominant in Natural Ultramafic Forest Ecosystems of New Caledonia

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Dedicated to the memory of our friend Nicolas Perrier

Abstract

Insularity, geological history and biogeography have made from New-Caledonia a hot spot of biodiversity where extremely diversified ecosystems occupies ultramafic terrains with drastic edaphic conditions in terms of fertility and metallic toxicity. In the framework of the mine project of the Koniambo Massif, a large nickel deposit, we tried to explore the diversity of ectomycorrhizal symbioses within these poorly explored natural ultramafic ecosystems.

Floristic inventories along an altitudinal gradient ranging from 700 to 900 m evidenced 4 different plant communities. The 2 lower plant communities, 3 and 4, were dominated by 2 endemic tree genera, *Tristaniopsis* (Leptospermoideae) and *Nothofagus*

(Nothofagaceae) respectively, whose ectomycorrhizal (ECM) status was shown and explored through molecular methods on sporocarps, mycorrhizae and soil mycelium.

We evidenced a diversified fungal community in the basal plant community dominated by two tree species of the genus *Nothofagus*. The molecular characterization of these ECM fungi was established on the total ribosomal inter transcribed spacer (ITS) by PCR-sequencing and BLASTn analysis, revealing the relative abundance of the Cortinariaceae among our samples. Samples belonging to this fungal family were phylogenetically analyzed on the same ITS, in reference to sequences of samples with geographically different origins, including countries derived from the Gondwanaland fragmentation. If no clear phylogenetical relationships were evidenced, our study confirmed the same relative dominance of ECM Nothofagaceae, as well as the relative abundance of associated Cortinariaceae, in New Caledonia as in several of the Gondwanaland-originating countries.

Keywords: Ectomycorrhiza, New Caledonia, *Cortinarius*, *Nothofagus*, Metal toxicity

Introduction

New Caledonia is a relatively small group of islands (20,000 Km^2) located in the South Pacific Ocean. Its geological history is directly related to that of the Gondwana super continent until 80 million years ago when the Eastern margin of the Gondwana started to break up (Veevers 1986, Veevers et al. 1991). A complex geological history has led to the presence of large nickel ore deposits located within the lateritic regolith, making, according to the International Nickel Study Group, this island the 4th world producer of nickel ore for the period 1993-2001.

The Koniambo Massif is one of these ore deposits, relatively isolated from each other's, in the Northern Province. It is in exploration by the mining group KNS (Xtrata and SMSP consortium) to establish an open pit nickel mine and smelter a high grade nickel ore located in the thick regolith. The soils present on the Koniambo massif can be classified as highly weathered oxisols formed of a majority (50 to 85%) of nodular iron oxides. These soils are characterised by a deficiency in the main plant nutrients (N, P, K), high concentrations of toxic heavy metals: Fe, Ni, Cr, Co, Mn (Perrier et al. 2006a) and an unbalanced Ca/Mg ratio.

Specific vegetation conditions have risen as a result of the specific geological history of New Caledonia. The local flora comprises approximately 3,300 species of which 74.5 % are endemic to the island (Jaffré et al. 2001). This endemism increases to 90 % on the ultramafic terrains, which count approximately 1,840 species (Jaffré 1974).

In these particularly drastic edaphic conditions, mining companies have the constraint to proceed to the ecological restoration of mine sites after exploitation, which is a big challenge considering the lack of knowledge on these ecosystems, their flora and associated symbioses. Symbioses, be they ectomycorrhizal (ECM) or arbuscular (AM) are known to be essential in plant adaptation to soil conditions (Jentschke and Godbold 2000, Stahl et al. 1988). Concerning AM symbioses, Perrier et al. (2006b) described the status (mycorrhizal colonization frequency and intensity) of 10 different plant species in the Koniambo Massif. For ECM in these ecosystems, only partial information were available either for some of the plant species studied by Perrier et al. (2006b) or through the description of a new *Cantharellus* species by Ducousso et al. (2004). However, the diversity and the distribution of

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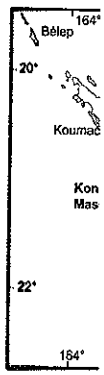

Figure 1. (A
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ECM symbioses in the different endemic vegetation ecosystems using molecular typing were not known.

The aim of this study is (i) to explore the floristic composition of the ecosystems in a zone representative of those targeted by the mining group as prioritized mining sites (ie over 700 m) (ii) to characterize the diversity of the ectomycorrhizal (ECM) fungi naturally associated to ECM plants within these inventories and (iii) to replace these data in the evolutionary and bio-geographical context of New Caledonia. Additionally, these data should later allow giving to the mining consortium recommendations on the use of ECM plants and management of topsoils in future revegetation processes.

Materials and Methods

- Study Site

The Koniambo massif is one of the isolated ultramafic massifs of the West coast (Figure 1 A and B). It has a total area of 381 km^2 and peaks at 930 m. The climate on the Koniambo Massif can be defined by two different seasons, a humid season ranging from January to April (an average of 250 mm monthly precipitation) and a drier season from May to December (an average of 100 mm monthly precipitation with a 50 mm low peak in September). The wind regime is governed generally by the trade winds blowing from an ESE direction.

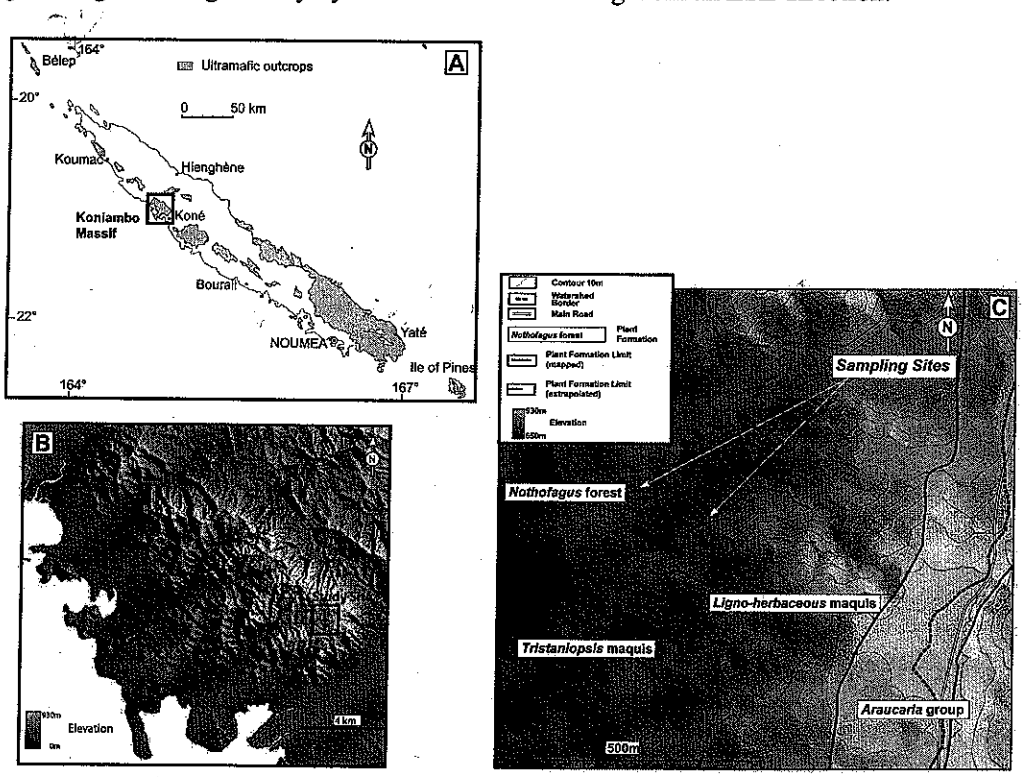

Figure 1. (A) Map of the ultramafic bodies of New Caledonia (in grey), (B) Shaded relief map of the Koniambo Massif with study site location, (C) Partial shaded relief map of the Pandanus river watershed zone of the Koniambo Massif with location of plant communities and sampling sites.

The average daily temperature on Koniambo ranges from 22°C (at 900 m altitude) in the summer months (January-March) to 14°C in the winter months (July to September). This massif is mainly composed of a harzburgite substrate (a type of peridotite formed of the minerals olivine and orthopyroxene) with some dunite and gabbro inclusions, the lateritic weathering of this body has led to the presence of a thick nickel rich regolith. The sampling site was located in the watershed of the Pandanus river on a slope ranging from 883 to 700 m (21°00'32"S; 164°50'17"E and 21°00'25"S; 164°49'45"E). In a floristic analysis of the whole massif, Jaffré (1974) identified 650 plant species and described, according to plant abundance, 12 different plant communities. The vegetation is an assemblage of maquis and rainforests. The maquis can be of different types, dominated, below 500 m, by the shrubby/arborescent angiosperm species (height of 6-10 m) *Gymnostoma chamaecyparis* Poiss. L.A.S. Johnson or, from 400 to 900 m, by species of the more shrubby genera *Tristaniopsis*. In the valleys above 600 m, rainforests are dominated by *Nothofagus balansae* (Baill.) Steenis and *Nothofagus codonandra* (Baill.) Steenis. Botanical nomenclature follows The International Plant Index (2008).

- Floristic analyses

Floristic data were collected from 80 randomly located 100 m^2 plots placed along a topological sequence ranging from the plateau at 882 m to the talweg at 700 m and spanning different vegetation types. Sixty plots were located in the maquis and 20 in the forest. The number of plots in each vegetation type was approximately proportional to the area represented in the landscape (Figure 1C). The Braun-Blanquet cover-abundance scale was used to describe the species abundance in each plot. Frequencies percentages were inferred from each Braun-Blanquet class (Braun-Blanquet et al. 1932). Data were analyzed using correspondence analysis (Greenacre 1984). They were processed using ADE4 software (Thioulouse et al. 1997).

- Ectomycorrhizas surveys

Sporocarps of ectomycorrhizal (ECM) fungi were collected at 4 different dates (June and July 2002, July and December 2003) under identified tree species, all located in plant communities 3 and 4 in the same site of the Pandanus river watershed. A photograph of each sampled sporocarp was taken and a sample number was given. A small portion of the flesh (≈ 0.5 cm^3) of each sample was placed on a cotton layer in 10 ml plastic tubes half filled with Silicagel (Prolabo, France) for rapid drying and kept at room temperature for subsequent DNA extraction. The sporocarps were air-dried at 40°C, and they were deposited in Paris Cryptogamie (PC) herbarium (Paris, France). Identification was based on macroscopic and microscopic characters of basidiomata. For most specimens, identification was not possible at the species level (restricted to the genus level) because the study was conducted in ecosystems that have been very poorly studied by taxonomists (Horak and Mouchacca 1998) and where many species remain to be described. Complementarily to sporocarps, some morphotypically different (color, shape) ECM apices from each of the four ECM plant species were randomly collected from superficial roots excavated all the way from the trunk to the ultimate fine ECM roots to determine their host-plant without any error, ECM. For DNA extraction, ECM samples were rapidly dried in Silicagel and kept at room temperature. Other samples of the same origin and morphotype were fixed in glycerol, ethanol and water (1:1:1, v/v/v) for morphological and microscopic observations. Fine roots with

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Cortinarius amoenus
Cortinarius anomalus 1
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Cortinarius armeniacus
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ectomycorrhizas were gently washed under tap water. Freehand sections were cleared with a 20% sodium hypochlorite solution, rinsed in water, stained with Congo red and observed under the microscope. Roots were considered to be ECM when they showed a distinctive mantle and a Hartig net. Soil samples aggregated by visible mycelium were also collected and kept fresh at 4°C in the soil until DNA extraction. Other biological material included nodular iron oxide soil particles with their colonizing hyphae, and soil mycelial fragments.

- Molecular characterizations

DNA was extracted from the dried sporocarp flesh, from a single ECM tip, from soil mycelium or from a single iron oxide particle (or bead), using a Dneasy Plant Mini kit following the manufacturer's recommendations (Qiagen, Courtaboeuf, France). An approximately 600-bp fragment of the nuclear ribosomal internal transcribed spacer (ITS) rDNA containing the 5.8S region was amplified using the specific primers ITS1 (5'-TCCGTAGGTGAA CCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990). PCR reaction was made in 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), 200 µM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. Amplification was performed with a DNA thermal-cycler (GenAmp PCR System 2400, Perkin Elmer) programmed as follows: 1 cycle for 5 min at 95°C followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 30 s, and a final extension at 72°C for 7 min. PCR products were separated by electrophoresis in 1 % (wt/vol) agarose gels (Sigma) in 1x TAE with ethidium bromide at 10 µg/ml in the running buffer. DNA bands were visualised by fluorescence under UV light and photographed.

- Sequencing and phylogeny

Sequencing was performed with each ITS1/ITS4 primers. Each PCR product was extracted from agarose gel and purified using a QIA (Quick Gel Extraction) kit following the manufacturer's recommendations. Sequencing was performed with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, California) and analysed on an Applied Biosystems model 310 DNA sequencer (Applied Biosystems, Foster City, California). DNA sequences were submitted to the NCBI database (http://www.ncbi.nlm.nih.gov/) under the accession numbers FJ 656000 to FJ 656047.

Table 1. List of reference material and GenBank accession numbers included in this study. Names and subgenus classification after Peintner et al. (2001, 2004)

Genus / Species	Geogr. origin	Subgenus	GenBank access.
Cortinarius acutus	Europe	*Acutus*	AF325578
Cortinarius albocanus	Chile	*Myxotelamonia*	AF325599
Cortinarius alboviolaceus 1	USA	*Telamonia*	AF325597
Cortinarius alboviolaceus 2	Europe	*Telamonia*	AF325596
Cortinarius allutus	Europe	*Allutus*	AF325585
Cortinarius amoenus	Chile	*Icterinula*	AF539721
Cortinarius anomalus 1	Europe	*Anomali*	AF325581
Cortinarius anomalus 2	Europe	*Anomali*	AJ236071
Cortinarius armeniacus 1	Europe	*Telamonia*	AF325595
Cortinarius armeniacus 2	Europe	*Telamonia*	AJ236074

Table 1. (Continued)

Genus / Species	Geogr. origin	Subgenus	GenBank access.
Cortinarius armillatus	Europe	*Telamonia*	AJ236075
Cortinarius austrovenetus	Europe	*Dermocybe*	AF112147h
Cortinarius bigelowii	USA	*Phlegmacium*	AF325617
Cortinarius brunneus 1	Europe	*Telamonia*	AF325590
Cortinarius brunneus 2	Europe	*Telamonia*	AJ236076
Cortinarius caelicolor	Chile	*Phlegmacium*	AF539715
Cortinarius callochrous	Europe	*Calochroi*	AF325619
Cortinarius campbellae 1	Australia	*Purpurascentes*	AF325558
Cortinarius campbellae 2	Australia	*Phlegmacium*	Specimen ref Tr 18323
Cortinarius caninus	USA	*Anomali*	U56024
Cortinarius caperatus 1	Europe	*Rozites*	AJ238033
Cortinarius caperatus 2	Europe	*Rozites*	AF325614
Cortinarius carneolus	Chile	*Telamonia*	AF539712
Cortinarius cinereobrunneus	Argentina	*Myxotelamonia*	AF325600
Cortinarius citriolens	USA	*Phlegmacium*	AF325607
Cortinarius collinitus	Europe	*Myxacium*	AF325565
Cortinarius corrosus	Europe	*Phlegmacium*	AF325618
Cortinarius corrugatus	USA	*Corrugatus*	AF325611
Cortinarius cupreorufus	Europe	*Phlegmacium*	AY174831
Cortinarius delibutus 1	Europe	*Delibuti*	AJ236065
Cortinarius delibutus 2	Europe	*Delibuti*	AF325580
Cortinarius elaiochrous	New Zealand	*Cuphocybe*	AY033100
Cortinarius elaphinus	Chile	*Telamonia*	AF539725
Cortinarius elegantior	Europe	*Calochroi*	AF325622
Cortinarius evernius	Europe	*Telamonia*	AJ236077
Cortinarius favrei	Europe	*Myxacium*	AF325575
Cortinarius flavaurora	USA	*Calochroi*	AF325621
Cortinarius fragilis	Australia	*Purpurascentes*	AF325559
Cortinarius fraudulosus	Europe	*Phlegmacium*	AF325605
Cortinarius gentilis 1	Europe	*Telamonia*	AF325589
Cortinarius gentilis 2	USA	*Telamonia*	U56026
Cortinarius glaucopus	Europe	*Phlegmacium*	AF325604
Cortinarius globuliformis	Australia	*Dermocybe*	AF325582
Cortinarius hercynicus	Europe	*Cortinarius*	AF062631
Cortinarius humicola	Europe	*Telamonia*	AF325594
Cortinarius laniger 1	Europe	*Telamonia*	AF325592
Cortinarius laniger 2	Europe	*Telamonia*	AF325591
Cortinarius leucopus	Europe	*Telamonia*	AF325593
Cortinarius limonius 1	Europe	*Limonius*	U56028
Cortinarius limonius 2	Europe	*Limonius*	AF325588
Cortinarius lividoochrascens	Europe	*Myxacium*	AF325565
Cortinarius luteistriatulus	Chile	*Dermocybe*	AF539707
Cortinarius magnivelatus	USA	*Calochroi*	AF325615
Cortinarius mucifluus	USA	*Myxacium*	AF182795
Cortinarius mucosus	USA	*Myxacium*	AF325574
Cortinarius muscigenus	USA	*Myxacium*	AF182800
Cortinarius obscurooliveus	Chile	*Dermocybe*	AF539708
Cortinarius obtusus	Europe	*Acutus*	AJ238035
Cortinarius odorifer	Europe	*Calochroi*	AF325620
Cortinarius olivaceobubalinus	Chile	*Dermocybe*	AF539736
Cortinarius olivaceopictus 1	USA	*Dermocybe*	U56049
Cortinarius olivaceopictus 2	USA	*Dermocybe*	U56050

GenBank access.
AJ236075
AF112147h
AF325617
AF325590
AJ236076
AF539715
AF325619
AF325558
Specimen ref Tr 18323
U56024
AJ238033
AF325614
AF539712
AF325600
AF325607
AF325565
AF325618
AF325611
AY174831
AJ236065
AF325580
AY033100
AF539725
AF325622
AJ236077
AF325575
AF325621
AF325559
AF325605
AF325589
U56026
AF325604
AF325582
AF062631
AF325594
AF325592
AF325591
AF325593
U56028
AF325588
AF325565
AF539707
AF325615
AF182795
AF325574
AF182800
AF539708
AJ238035
AF325620
AF539736
U56049
U56050

Genus / Species	Geogr. origin	*Subgenus*	GenBank access.
Cortinarius paragaudis	USA	*Telamonia*	U56030
Cortinarius parahumilis	Chile	*Telamonia*	AF539731
Cortinarius pavelekii	USA	*Myxacium*	AF325564
Cortinarius pholideus	Europe	*Sericeocybe*	AJ236072
Cortinarius pingue 1	USA	*Myxacium*	AF325570
Cortinarius pingue 2	USA	*Myxacium*	AF325571
Cortinarius piriforme	Australia	*Myxacium*	AF325569
Cortinarius porphyroides 1	New Zealand	*Thaxterogaster*	AF325576
Cortinarius porphyroides 2	New Zealand	*Myxacium*	AF325577
Cortinarius porphyropus 1	Europe	*Purpurascentes*	AF325560
Cortinarius porphyropus 2	Europe	*Purpurascentes*	AJ236069
Cortinarius pseudosalor	USA	*Myxacium*	AF182792
Cortinarius pugionipes	Chile	*Phlegmacium*	AF539713
Cortinarius rapaceus 1	Chile	*Phlegmacium*	AF539723
Cortinarius rapaceus 2	Chile	*Phlegmacium*	AF539724
Cortinarius saginus	Europe	*Phlegmacium*	AF325608
Cortinarius salor	Europe	*Delibuti*	AF325579
Cortinarius scaurus 1	Europe	*Purpurascentes*	AJ236070
Cortinarius scaurus 2	Europe	*Purpurascentes*	AF325562
Cortinarius scaurus 3	Europe	*Purpurascentes*	AF325563
Cortinarius sp.	USA	*Phlegmacium*	AF325606
Cortinarius squamiger	Chile	*Telamonia*	AF539729
Cortinarius subfoetidus	USA	*Phlegmacium*	AF325609
Cortinarius talus	Europe	*Allutus*	AF325586
Cortinarius tenellus	Chile	*Telamonia*	AF539728
Cortinarius traganus 1	Europe	*Telamonia*	AF037224
Cortinarius traganus 2	Europe	*Telamonia*	AF325598
Cortinarius trivialis	Europe	*Myxacium*	AJ236066
Cortinarius umbilicatus	USA	*Telamonia*	U56032
Cortinarius vanduzerensis	USA	*Myxacium*	AF182793
Cortinarius variecolor	Europe	*Phlegmacium*	AJ238082
Cortinarius vernicosus	USA	*Myxacium*	AF182799
Cortinarius verrucisporus	USA	*Phlegmacium*	AF325616
Cortinarius vibratilis 1	Europe	*Ochroleuci*	AJ238032
Cortinarius vibratilis 2	USA	*Ochroleuci*	AF325584
Cortinarius violaceus 1	Europe	*Cortinarius*	AJ236059
Cortinarius violaceus 2	Europe	*Cortinarius*	AF325601
Cortinarius violaceus 3	USA	*Cortinarius*	AF389130
Cortinarius viridibasalis	Chile	*Telamonia*	AF539717
Cuphocybe melliolens	New Zealand	*Cuphocybe*	AF325610
Dermocybe cinnamomea	Europe	*Dermocybe*	AJ238030
Dermocybe crocea	Europe	*Dermocybe*	AJ238031
Dermocybe malicoria	USA	*Dermocybe*	U56045
Dermocybe phoenicea	Europe	*Dermocybe*	U56055
Dermocybe splendid	New Zealand	*Dermocybe*	AF325583
Hebeloma circinans			AF124699
Hebeloma crustuliniforme			AF124716
Hebeloma fastibile			AF325643
Hymenogaster remyi	Europe	*Phlegmacium*	AF325602
Hymenogaster sublilacinus	USA	*Phlegmacium*	AF325603
Protoglossum luteum	Australia	*Corrugatus*	AF325612
Protoglossum sp. 1	New Zealand		AF325613
Protoglossum sp. 2	Australia	*Acutus*	AF325561
Quadrispora oblongispora	Australia	*Myxacium*	AF325566
Quadrispora sp.	Australia	*Myxacium*	AF325567

Table 1. (Continued)

Genus / Species	Geogr. origin	Subgenus	GenBank access.
Thaxterogaster redactus	Australia	*Myxacium*	AF325568
Thaxterogaster sp.	USA	*Myxacium*	AF325572
Thaxterogaster violaceum 1	Argentina	*Thaxterogaster*	AF325556
Thaxterogaster violaceum 2	Argentina	*Thaxterogaster*	AF325557

- Phylogenetic analyses

New ITS sequences were compared to the Genbank database using the BLASTn algorithm (http://www.ncbi.nlm.nih.gov/blast). Multiple alignments were performed with Clustal X (Thompson et al. 1997) or Muscle 3.6 (Edgar 2004), and manually cured under Genedoc software (Nicholas and Nicholas 1997). The phylogenetic analysis was focused on *Cortinarius* species (as it was the most represented genus) by using the full spacer sequences (ITS1-5.8S-ITS2) hand-aligned with the Peintner et al. (2001) alignment (Treebase, accession number S636: M988-990), also enriched with some *Cortinarius* sequences from the southern hemisphere (Table 1). A Maximum Likelihood (ML) phylogeny was established using the best-fit model detected by Model test (Posada and Crandall 1998) under the Akaike information criterion (TrN+I+G: transition only, estimation of invariant sites, gamma distribution of substitution). Internal gaps were treated as effective data (fifth base). Maximum-likelihood (ML) analyses were carried out with the computer program PAUP 4.0b5 under heuristic searches with "asis" addition sequence and TBR branch swapping. Bootstrapping replicates were performed using PHYML on-line using 100 replicates under a GTR+I+G model (Nst=6, Invariant sites, Gamma distribution) (20). The Cortinariaceae phylogeny was also inferred by a Bayesian approach (Huelsenbeck et al. 2001), using MrBayes 3.1.2 (Ronquiste and Huelsenbeck 2003) with four Monte Carlo Markov Chains (MCMC) run on 3 millions generations, with print frequency and chain temperature set to 50,000 and 0.2, respectively, and saving branch lengths. A 50% majority rule consensus analysis was performed on trees sampled after the chains had reached stationarity. Priors used to run the chains were the parameters estimated by Modeltest (TrN+I+G). Trees topology congruence between ML and Bayesian inferences of Cortinariaceae ITS sequences was assessed using the Shimodaira-Hasegawa Likelihood based test (S-H test using 1000 RELL bootstrap replicates) (Shimodaira and Hasegawa 1999), implemented in PAUP4. Congruence was found between trees obtained by both methods (-ln L = 12183 by ML; -ln L=12200 by MrBayes; S-H test P value=0.254)

Results

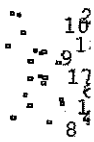
- **Vegetation distribution along** the topographic gradient

From the plateau located at 882 m to the talweg at 700 m, the site presented four different plant communities among **the twelve** groups defined by Jaffré (1974). Statistical analyses confirmed the drastic changes in **floristic** composition between the different plant communities, excepted between plant communities 2 and 3 where the discontinuity was less strongly marked.

The *Araucaria mo*
strongly isolated on t
downwards the toposequ

Plant community 1:
Araucaria montana, and
most abundant in terms
Forst. (15.7 %), *Trista*
montana (4.3 %) and *D*
less abundant (27.3 %),
and *Pteridium esculentu*
the dominant plant com
numbered. The herbacec
Raynal (60.1 %), *Dicran*
neocaledonicus C.B. Cla

Plant community
Araucaria ligneous n



Plant community 3:
Tristaniopsis ligneous

Plant commu
ligno-herbac

Figure 2. Ordination of recor
changes in plant communitie
thalweg (plant community 4)

The ligneous stratun
montana (35.9 %), *Drac*
Myodocarpus crassifolius
part of the slope, the domi

The ligneous stratu
Tristaniopsis guillainii (5

GenBank access.
AF325568
AF325572
AF325556
AF325557

ɔase using the BLASTn
ıts were performed with
ınd manually cured under
ɔ analysis was focused on
ı the full spacer sequences
ıment (Treebase, accession
quences from the southern
was established using the
1998) under the Akaike
f invariant sites, gamma
fective data (fifth base).
computer program PAUP
ıd TBR branch swapping.
sing 100 replicates under a
(20). The Cortinariaceae
nbeck et al. 2001), using
onte Carlo Markov Chains
ıd chain temperature set to
% majority rule consensus
hed stationarity. Priors used
TrN+I+G). Trees topology
iaceae ITS sequences was
S-H test using 1000 RELL
ıted in PAUP4. Congruence
83 by ML; -ln L=12200 by

; site presented four different
(1974). Statistical analyses
ɛtween the different plant
re the discontinuity was less

The *Araucaria montana* Brongn. and Gris and *Nothofagus* plant communities are strongly isolated on the ordination graph (Figure 2). Floristic biodiversity increases downwards the toposequence.

Plant community 1: On the plateau, the dominant plant species of the plant community is *Araucaria montana*, and 53 other plant species were numbered. The ligneous stratum is the most abundant in terms of cover abundance (33.9 %), with *Codia montana* J.R. Forst. and G. Forst. (15.7 %), *Tristaniopsis guillainii* Viell. ex Brongn. and Gris (5.7 %) *Araucaria montana* (4.3 %) and *Dracophyllum verticillatum* Labill. (3.5 %). The herbaceous stratum is less abundant (27.3 %), with the pteridophytes *Dicranopteris linearis* J. Underw. (15.0 %) and *Pteridium esculentum* (G. Forster) Cockayne (13.9 %). Plant community 2: On the slope, the dominant plant community is a ligno-herbaceous maquis where 58 plant species were numbered. The herbaceous stratum is the most abundant (85.0 %) with *Costularia nervosa* Raynal (60.1 %), *Dicranopteris linearis* (24.8 %), *Pteridium esculentum* (15.0 %), *Schoenus neocaledonicus* C.B. Clarke (11.8 %) and *Lepidosperma perteres* C.B. Clarke (9.8 %).

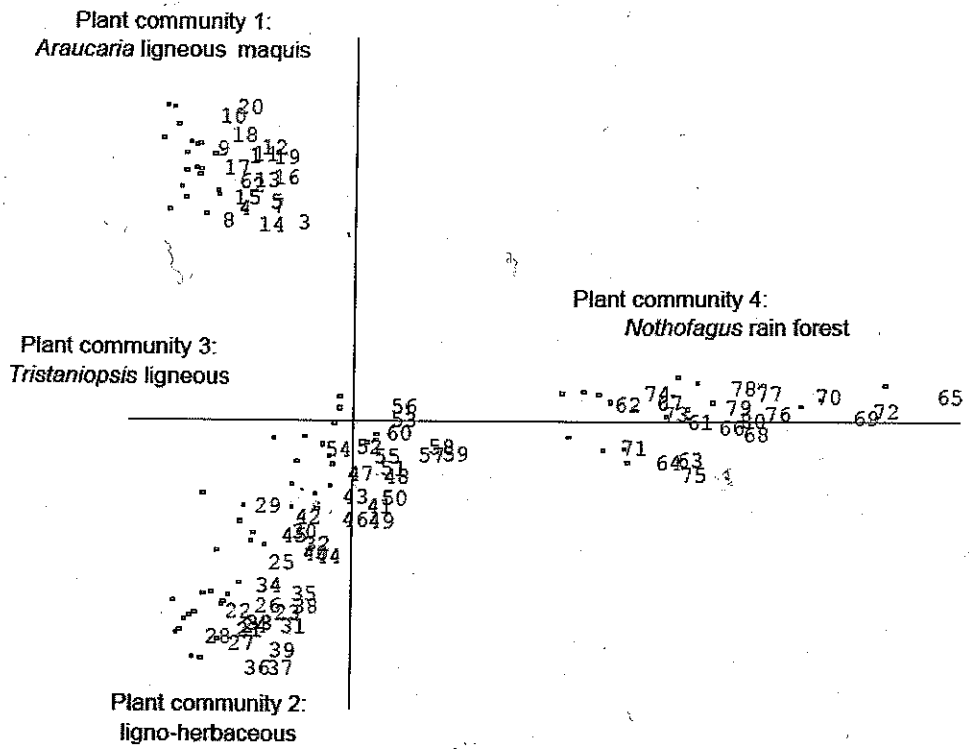


Figure 2. Ordination of records along the two main factors of correspondence analysis, showing drastic changes in plant communities within the toposequence, from the plateau (plant community 1) to the thalweg (plant community 4).

The ligneous stratum is less abundant (55.1 %), and it is mainly formed of *Codia montana* (35.9 %), *Dracophyllum ramosum* Pancher ex Brongn. and Gris (9.7 %) and *Podocarpus crassifolius* Dubard and R. Viguier (7.0 %). Plant community 3: On the lower part of the slope, the dominant plant community is a maquis, with 72 plant species.

The ligneous stratum is the most abundant (80.0 %), and is mainly formed of *Tristaniopsis guillainii* (50.5 %), *Tristaniopsis callobuxus* Brongn. and Gris (27.0 %) and

Codia montana (23.5 %). The herbaceous stratum is less abundant (34.5%) with *Costularia nervosa* (22.2 %) and *Lepidosperma perteres* (12.13%). Plant community 4: In the talweg, the plant community is a rainforest dominated by 2 *Nothofagus* species and 81 other species were numbered. The dominant plant species is *Nothofagus balansae* (62.5%), which represents the tallest stratum, *Callophylum caledonicum* Vieill. (8.9 %) is another member of the tallest stratum.

The species *Nothofagus codonandra* is also present in this forest but forms monodominant patches, with only a small overlap between the two *Nothofagus* species, not illustrated in this paper. The medium stratum is heterogeneous; the most abundant species are *Styphelia pancheri* (Brongn. and Gris) F. Muell. (11.7 %), *Basselinia gracilis* (Brongn. and Gris) Vieill. (6.45 %) *Pancheria ferruginea* Brongn. (3.3 %) and *Rapanea assymetrica* Mez. The herbaceous stratum is represented essentially by *Lepidosperma perteres* (7.23 %) and *Costularia arundinacea* (Soland. ex Vahl) Kük. (1.5 %).

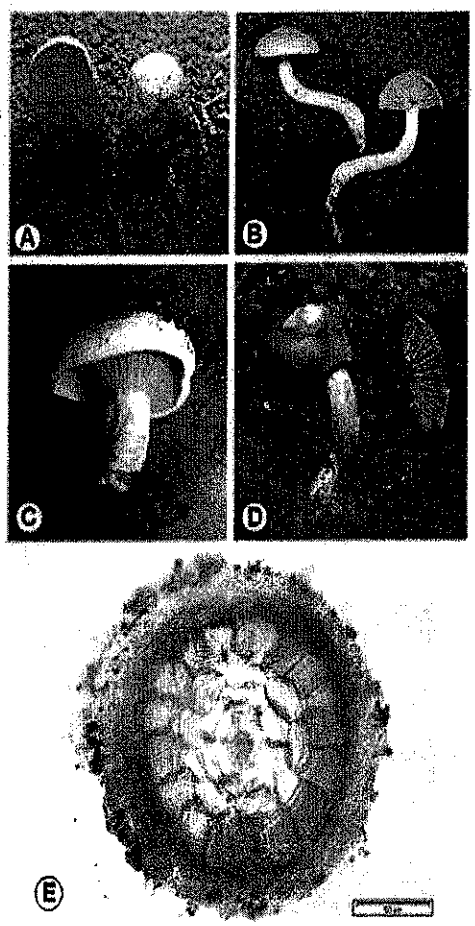


Figure 3. A to D: aspect of some ECM sporocarps from plant communities 3 and 4. A: *Pisolithus albus* K66C, associated to *Tristaniopsis guillainii*, in plant community 3. B: *Cortinarius* sp *nov.* K12C, associated to *Nothofagus balansae* in plant community 4. C: *Multifurca auranthiophylla* K18C, associated to *Nothofagus balansae* in plant community 4. D: *Tricholoma* sp *nov. aff ustale* K10C, associated to *Nothofagus balansae* in plant community 4. E: Cross section through an ECM apex of *Tristaniopsis guillainii* (plant community 3), after staining with Trypan Blue, showing the fungal mantle and the Hartig net.

• Ectomycorrhizal ti

Roots of the plant spe
in order to characterize th
plateau (plant community
2), all plants were endomy
ECM symbiosis was obs
community 3) and *Nothofa*
species (*Tristaniopsis gui*
respectively) presenting ty
microscope.

Figure 4. A: Aspect of a soil ag
community 3 under *Pisolithus*
compose the soil is clearly visil
stereomicroscope, with numero

(34.5%) with *Costularia*
nity 4: In the talweg, the
nd 81 other species were
%), which represents the
er member of the tallest

this forest but forms
Nothofagus species, not
most abundant species are
ia gracilis (Brongn. and
apanea assymetrica Mez.
a perteres (7.23 %) and

- Ectomycorrhizal tree species

Roots of the plant species of each plant community were observed under the microscope in order to characterize the symbiotic associations. In the *Araucaria montana* group on the plateau (plant community 1) and the ligno-herbaceous maquis on the slope (plant community 2), all plants were endomycorrhizal confirming observations by Perrier et al. (2006b) and no ECM symbiosis was observed. Ectomycorrhization in the *Tristaniopsis* maquis (plant community 3) and *Nothofagus* forest (plant community 4) was limited to the dominant plant species (*Tristaniopsis guillainii*, *T. calobuxus*, *Nothofagus balansae* and *N. codonandra*, respectively) presenting typical fungal mantle around the roots and a Hartig net under the microscope.

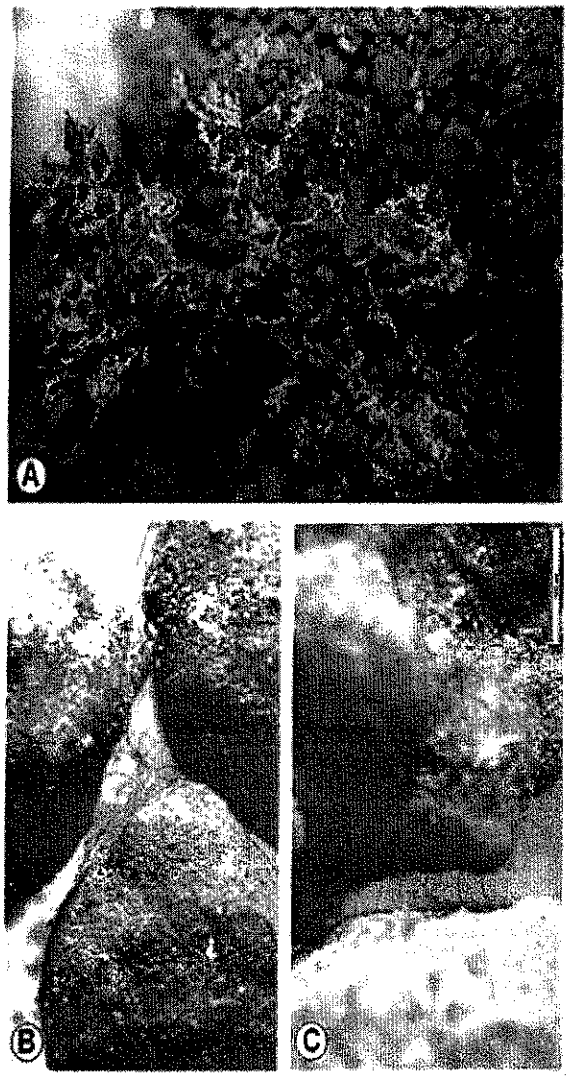


Figure 4. A: Aspect of a soil aggregate with yellow gold mycelium and rhizomorphs, harvested in plant community 3 under *Pisolithus albus* K66C sporocarp. The spherical aspect of iron oxide beads that compose the soil is clearly visible. B and C: High magnification of iron oxide beads under the stereomicroscope, with numerous non-identified hyphal links between beads.

ies 3 and 4. A: *Pisolithus albus*
B: *Cortinarius* sp *nov.* K12C,
ifurca auranthiophylla K18C,
oma sp *nov. aff ustale* K10C,
ction through an ECM apex of
pan Blue, showing the fungal

- Fungal diversity

A total of 48 samples were analyzed, 11 from plant community 3 and 37 from plant community 4. Twenty-eight samples were sporocarps among which only 2 from plant community 3. Figure 3A to D illustrates some of the sporocarps harvested in plant communities 3 and 4. Twelve samples were mycorrhizal apices with 3 and 9 collected from plant communities 3 and 4, respectively. The last type of samples was from soil aggregates as illustrated in Figure 4A with yellow-gold mycelium harvested in plant community 3 under *Pisolithus albus* K66C sporocarp. The spherical aspect of iron oxide beads that compose the soil is clearly visible. A closer examination of these iron oxide beads under the stereomicroscope is presented Figure 4B and C, with unidentified hyphal links between beads. Other soil samples, with different mycelial colors, were also harvested in plant communities 3 and 4. Six samples were analyzed from plant community 3 and 2 from plant community 4. The material used for extraction was a fragment of mycelium or rhizomorph and in plant community 3, 4 samples were individual iron oxide beads, with their colonizing hyphae as illustrated by Figure 4B and C. Table 2 lists the different ECM samples collected in the two plant communities, indicating the morphological determination of sporocarps, ITS sequence length and BLASTn identity.

Table 2. Ectomycorrhizal samples collected in the different plant communities, submitted ITS sequence length, closest match with GenBank genera, query coverage, maximum identity and e-value (www.ncbi.nlm.nih.gov/blast/)

Ref*	Plant community	Host plant	Macroscopy	ITS1/ITS4 submitted length	BLAST closest genera	Query Coverage	Max identity	e-value
K01C	4	*Nothofagus balansae*	*Tricholoma* sp *nov*	701	*Tricholoma*	98%	88%	0,0
K02 C	4	*Nothofagus balansae*	*Boletus* sp	609	*Boletus*	91%	76%	2e-120
K05 C	4	*Nothofagus balansae*	*Cortinarius* sp *nov*	551	*Cortinarius*	99%	83%	3e-155
K06 C	4	*Nothofagus balansae*	*Phellodon* sp	605	*Phellodon*	81%	85%	4e-155
K09 C	4	*Nothofagus balansae*	nd**	547	*Clavariadelphus*	70%	90%	8e-87
K10 C	4	*Nothofagus balansae*	*Tricholoma* sp *nov aff ustale*	670	*Tricholoma*	98%	93%	0.0
K12 C	4	*Nothofagus balansae*	*Cortinarius* sp. *nov*	572	*Cortinarius*	99%	92%	0.0
K14 C	4	*Nothofagus balansae*	*Cortinarius* sp.	506	*Cortinarius*	98%	95%	0.0
K16 C	4	*Nothofagus balansae*	*Lactarius* sp	706	*Lactarius*	99%	91%	0.0
K18 C	4	*Nothofagus balansae*	*Multifurca auranthiophylla*	598	*Lactarius*	98%	87%	0.0
K22 C	4	*Nothofagus balansae*	*Inocybe* sp	582	*Inocybe*	99%	83%	2e-159
K66 C	3	*Tristaniopsis guillainii*	*Pisolithus albus*	527	*Pisolithus*	99%	98%	0.0
KC02 C	4	*Nothofagus balansae*	*Cortinarius* sp *nov aff lividoochrascens*	436	*Cortinarius*	97%	89%	3e-154
KC03 C	4	*Nothofagus balansae*	*Russula* sp	612	*Russula*	100%	93%	0.0

Ref*	Plant community	Host pl
K'05 C	4	*Nothofa balansa*
K'06 C	4	*Nothofa balansa*
K'08 C	4	*Nothofa balansa*
K'10 C	4	*Nothofa balansa*
K'11 C	4	*Nothofa balansa*
K'12 C	4	*Nothofa balansa*
K'16 C	4	*Nothofa balansa*
K'17 C	4	*Nothofa balansa*
K'19 C	4	*Nothofa balansa*
K'22 C	4	*Nothofa balansa*
K'23 C	3	*Tristanic guillaini*
KD10 M	4	*Nothofa balansa*
KD18 M	4	*Nothofa codonan*
KD19-1	3	*Tristanic guillaini*
KD19-2	3	*Tristanic guillaini*
KD19-9	3	*Tristanio guillaini*
KD20-2	3	*Tristanio guillaini*
KD20-5	3	*Tristanio guillaini*
KD20-6	3	*Tristanio guillaini*
KD29-2 M	4	*Nothofag codonan*
KD31"-2 M	4	*Nothofag codonan*
KD31' M	4	*Nothofag codonan*
KD36-2 M	4	*Nothofag codonan*
KD36 C	4	*Nothofag codonan*
KD37 C	4	*Nothofag codonan*
KD42 C	4	*Nothofag codonan*
KF01-2 M	3	*Tristanio guillaini*
KF02 M	3	*Tristanio guillaini*
KF04 M	3	*Tristanio guillaini*
KF06 M	4	*Nothofag balansae*

nity 3 and 37 from plant which only 2 from plant carps harvested in plant ith 3 and 9 collected from was from soil aggregates as plant community 3 under ide beads that compose the oxide beads under the fied hyphal links between re also harvested in plant mmunity 3 and 2 from plant f mycelium or rhizomorph, beads, with their colonizing t ECM samples collected in lination of sporocarps, ITS

nt plant communities, genera, query coverage, ih.gov/blast/)

st	Query Coverage	Max identity	e-value
	98%	88%	0,0
	91%	76%	2e-120
	99%	83%	3e-155
	81%	85%	4e-155
hus	70%	90%	8e-87
	98%	93%	0.0
	99%	92%	0.0
	98%	95%	0.0
	99%	91%	0.0
	98%	87%	0.0
	99%	83%	2e-159
	99%	98%	0.0
	97%	89%	3e-154
	100%	93%	0.0

Ref*	Plant community	Host plant	Macroscopy	ITS1/ITS4 submitted length	BLAST closest genera	Query Coverage	Max identity	e-value
KC05 C	4	*Nothofagus balansae*	*Inocybe* sp	580	*Inocybe*	94%	84%	4e-161
KC06 C	4	*Nothofagus balansae*	nd	436	*Phaeocollybia*	94%	95%	6e-176
KC08 C	4	*Nothofagus balansae*	*Lactarius* sp *nov*	670	*Lactarius*	98%	91%	0.0
KC10 C	4	*Nothofagus balansae*	*Cortinarius* sp *nov aff teraturgus*	447	*Thaxterogaster*	99%	93%	0.0
KC11 C	4	*Nothofagus balansae*	*Russula* sp *nov aff occidentalis*	609	*Russula*	99%	93%	0.0
KC12 C	4	*Nothofagus balansae*	*Cortinarius* sp	518	*Cortinarius*	100%	84%	9e-143
KC16 C	4	*Nothofagus balansae*	*Cortinarius* sp	574	*Cortinarius*	100%	86%	0.0
KC17 C	4	*Nothofagus balansae*	*Cortinarius* sp.	574	*Cortinarius*	99%	83%	1e-147
KC19 C	4	*Nothofagus balansae*	*Cortinarius* np *perrierii*	583	*Cortinarius*	99%	86%	0.0
KC22 C	4	*Nothofagus balansae*	nd	528	*Clavariadelphus*	74%	94%	2e-93
KC23 C	3	*Tristaniopsis guillainii*	nd	414	*Cortinarius*	71%	88%	6e-100
KD10 M	4	*Nothofagus balansae*	-	552	*Oidiodendron*	91%	92%	0.0
KD18 M	4	*Nothofagus codonandra*	-	474	*Cortinarius*	98%	89%	3e-155
KD19-1 fin	3	*Tristaniopsis guillainii*	-	698	*Lycoperdon*	94%	93%	0.0
KD19-2 fin	3	*Tristaniopsis guillainii*	-	592	*Cortinarius*	99%	92%	0.0
KD19-9 fin	3	*Tristaniopsis guillainii*	-	410	*Cortinarius*	97%	85%	6e-106
KD20-2 fin	3	*Tristaniopsis guillainii*	-	616	*Cortinarius*	100%	85%	0.0
KD20-5 fin	3	*Tristaniopsis guillainii*	-	585	*Cortinarius*	100%	85%	0.0
KD20-6 fin	3	*Tristaniopsis guillainii*	-	615	*Cortinarius*	100%	86%	0.0
KD29-2 M	4	*Nothofagus codonandra*	-	608	*Cortinarius*	99%	91%	0.0
KD31"-2 M	4	*Nothofagus codonandra*	-	570	*Cortinarius*	99%	85%	1e-172
KD31' M	4	*Nothofagus codonandra*	-	682	*Tomentella*	98%	93%	0.0
KD36-2 M	4	*Nothofagus codonandra*	-	584	*Cortinarius*	99%	86%	0.0
KD36 C	4	*Nothofagus codonandra*	*Cortinarius* sp *nov*	585	*Cortinarius*	99%	93%	0.0
KD37 C	4	*Nothofagus codonandra*	*Cortinarius* sp *nov*	582	*Cortinarius*	98%	94%	0.0
KD42 C	4	*Nothofagus codonandra*	*Tricholoma* sp *nov*	458	*Tricholoma*	94%	88%	3e-154
KE01-2 M	3	*Tristaniopsis guillainii*	-	457	*Piloderma*	98%	88%	2e-150
KE02 M	3	*Tristaniopsis guillainii*	-	511	*Cortinarius*	98%	79%	2e-126
KE04 M	3	*Tristaniopsis guillainii*	-	438	*Piloderma*	97%	88%	2e-143
KE06 M	4	*Nothofagus balansae*	-	584	*Cortinarius*	100%	91%	0.0

Table 2. (Continued)

Ref*	Plant community	Host plant	Macroscopy	ITS1/ITS4 submitted length	BLAST closest genera	Query Coverage	Max identity	e-value
KE10 M	4	*Nothofagus balansae*	-	572	*Thaxterogaster*	100%	86%	0.0
KE12-1 M	4	*Nothofagus balansae*	-	620	*Tricholoma*	99%	86%	0.0
KE12-2 Sm	4	*Nothofagus balansae*	-	608	*Tricholoma*	99%	87%	0.0
KE18-2 Sm	4	*Nothofagus codonandra*	-	540	*Cortinarius*	98%	83%	1.9e-150

*Suffixes refer to the type of material: C: sporocarp, M: mycorrhizal apex, S: soil aggregate with hyphal mats, with two different types: Sn: one single iron oxyde bead, Sm: one mycelial or rhizomorph fragment.

**: nd: not determined.

Most sporocarps (suffix "C") were identified on their macroscopic and microscopic characteristics at least at the genus level, only four of them ("nd", not determined) were not attributed any taxonomic name. Mycorrhizal root apices (suffix "M") and soil samples (suffix "S") were obviously left without taxonomic name. None of the sequences compared to the Genbank database gave 100 % similarities with previously described species. On the number of samples analyzed and considering the relatively small area of the prospected site (roughly estimated to represent 5 ha, in a massif of more than 38,000 ha) a large diversity of fungal taxons was found. Among sporocarps, there was generally a good correspondence between macroscopic and molecular determinations. In plant community 3, 4 different genera were detected among 11 different samples. Seven of these samples belonged to the genus *Cortinarius*, 2 to *Piloderma*, 1 to *Pisolithus,* sampled as a sporocarp, and 1 to *Lycoperdon,* detected in one soil iron oxide bead and generally considered as non-mycorrhizal. The genus *Cortinarius* was detected in one sporocarp, in ECM, mycelium and soil beads. In plant community 4, the diversity is much greater with 12 different genera recorded from ITS sequences, corresponding to 37 samples. The dominant genus is *Cortinarius* with a total of 18 samples (including 2 species formerly identified as *Thaxterogaster*) thus reaching almost 50 % of the samples for Cortinariaceae. Genera *Tricholoma* and *Lactarius-Russula* followed with 5 samples each. Dominance of the Cortinariaceae is observed for the different forms of fungal samples. Out of 26 collected sporocarps, 11 belonged to this group. Among ECM, 9 different samples were successfully analyzed, belonging to 5 different genera. The dominant genus also is *Cortinarius* with 5 different samples. In the soil, only 2 samples could be analyzed and they belonged to genera *Cortinarius* and *Tricholoma.*

- Phylogenetic grouping

The analysis (Figure 5) of the Cortinariaceae family was focused on the whole 18S-28S portion (ie including both ITS and 5.8S sequences) and referred to 124 database sequences (121 from Cortinariaceae and 3 from *Hebeloma*) as listed in Table 1. On the 18 different samples analyzed, 5 were from plant community 3 and 13 from plant community 4. The different forms of the 18 samples were sporocarps (8), mycorrhizal tips (5) and soil mycelium (5). On this limited number of samples, the diversity appears to be high, all samples being distinct from each other and from the database sequences. New Caledonian Cortinariaceae are

t	Query Coverage	Max identity	e-value
r	100%	86%	0.0
	99%	86%	0.0
	99%	87%	0.0
	98%	83%	1.9e-150

. apex, S: soil aggregate with
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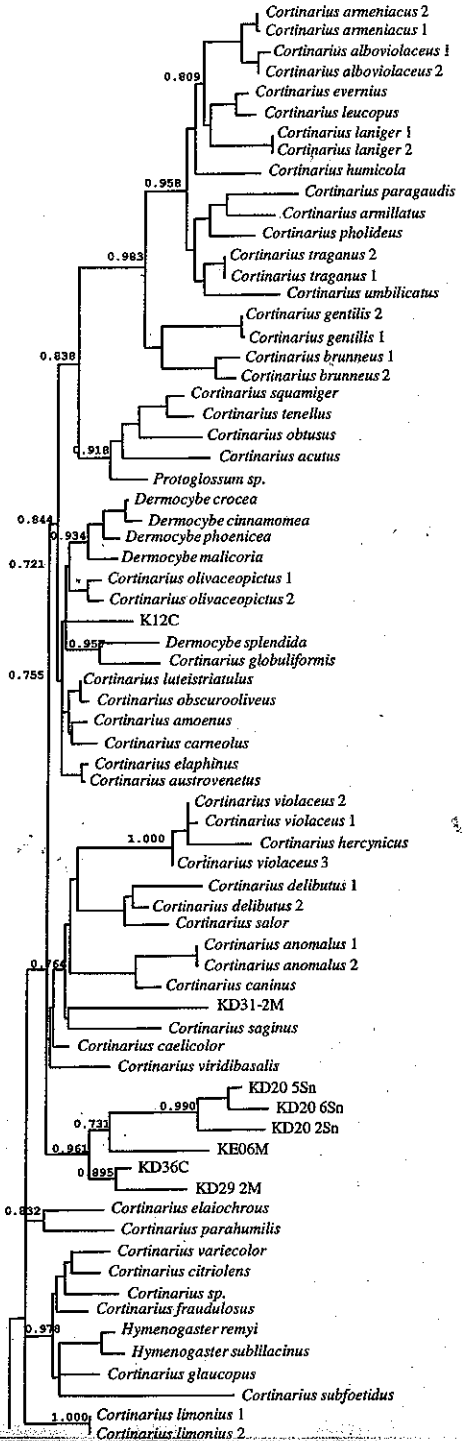
distributed in 6 different clades. A few clades mix sequences from plant community 3 and 4, but there obviously is a need for more samples analyses to evaluate a putative link between fungal communities, as for example there is no grouping between ECM apices from both plant communities and no total sequence identity between any sample.



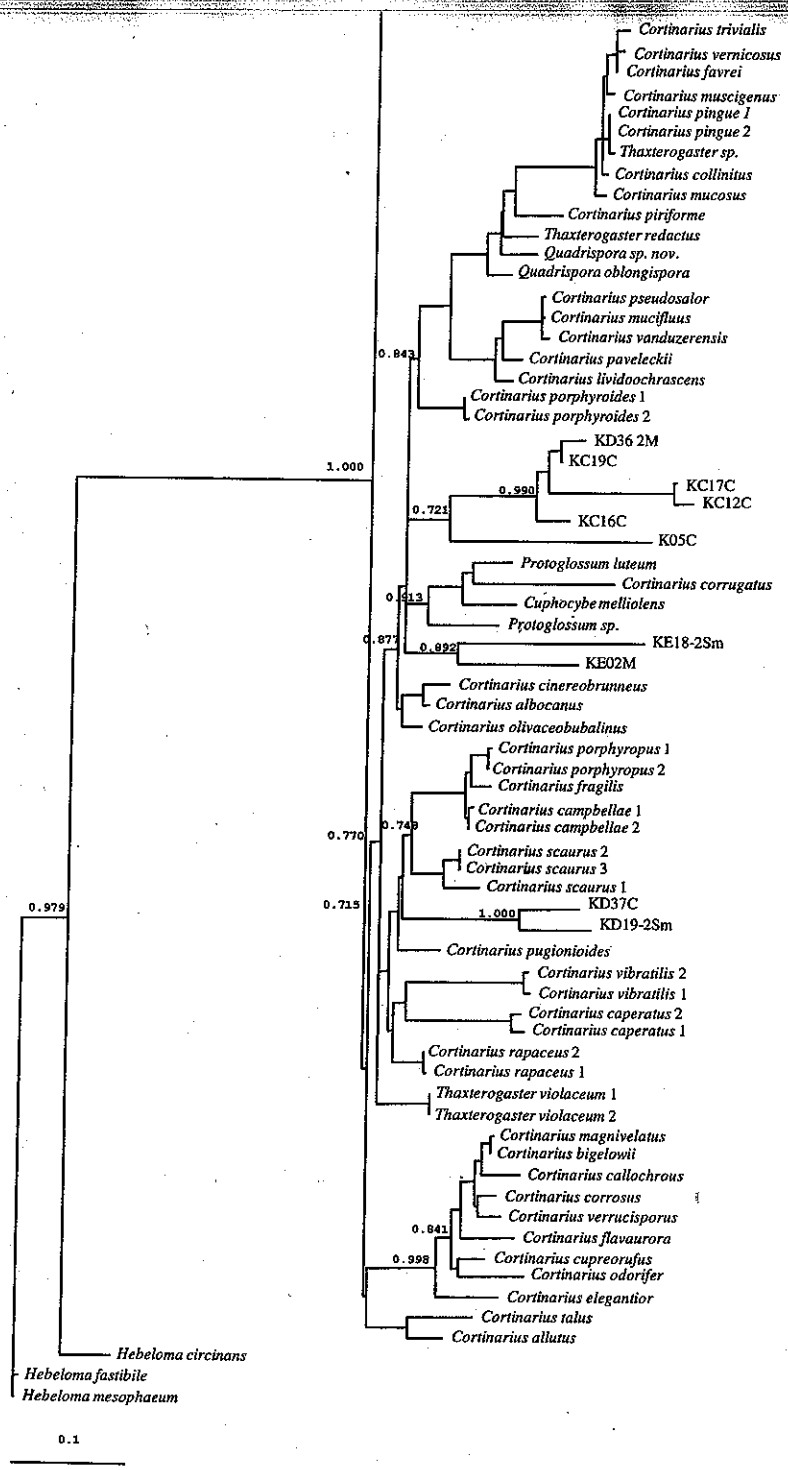


Figure 5. Maximum likelihood phylogram (congruent with Bayesian tree, see Material & Methods) of 136 Cortinariaceae rDNA ITS sequences, including 18 New Caledonian samples harvested in the Pandanus river watershed, in the Koniambo massif. Outgroup was made with 3 *Hebeloma* spp sequences. Only bootstrap values superior to 70% and also supported by Bayesian Posterior Probabilities superior to 70% are indicated. The ML model used is described in Material and Methods.

igure 6. Schematic representat **a cladogram**, and illustrated **de tree)** or the taxonomic gro **de tree).** European, Russian a **the same** clusters.

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rtinarius pingue 1
rtinarius pingue 2
iaxterogaster sp.
rtinarius collinitus
rtinarius mucosus
us piriforme
er redactus
sp. nov.
ongispora
pseudosalor
mucifluus
s vanduzerensis
veleckii
loochrascens
es 1
les 2
36 2M

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· Cortinarius corrugatus
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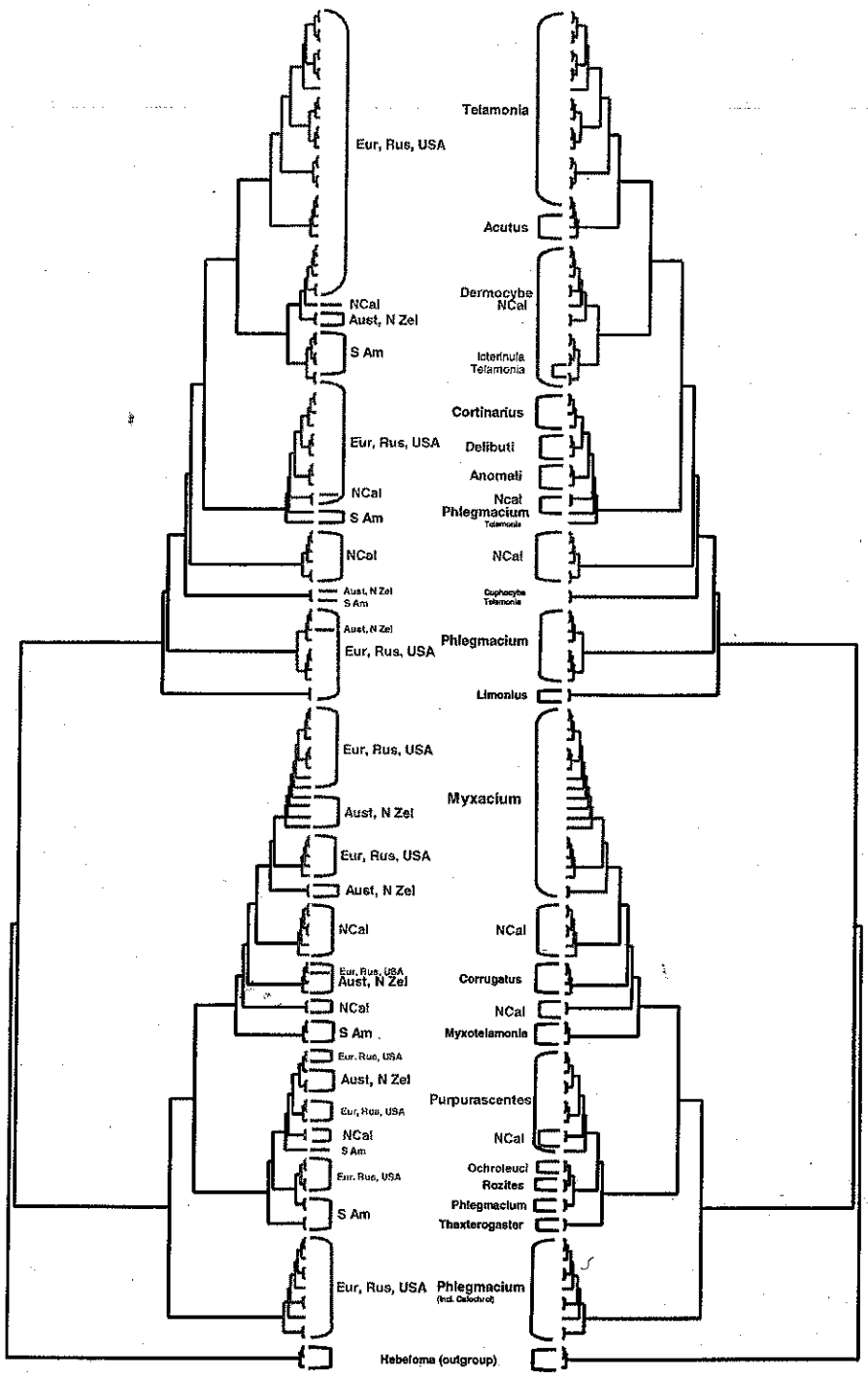

tree, see Material & Methods) of
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Figure 6. Schematic representation of the phylogenetic tree presented in Fig. 5A and B, but converted to a cladogram, and illustrated with a colour code for the geographical origin of the Cortinariaceae (left side tree) or the taxonomic grouping (genera and subgenera) according to Peintner et al. (2004) (right side tree). European, Russian and North American Cortinariaceae were arbitrarily considered as fitting in the same clusters.

New Caledonian samples fit sometime in distinctive clusters, among the 118 referen sequences from the Cortinariaceae, whatever their origin, including geographically link countries like Australia, New Zealand, Chile or Papua New Guinea, indicating the presence several new taxons. Reporting on the tree (drawn as a cladogram) the phyto-geographic origin of the samples from sequences taken as references (Figure 6) the existence of sever New Caledonian clades, but not always positioned closer to clades from Gondwanalan fragments than to clades from Northern hemisphere. This study did not aim at giving taxonomical description of New Caledonian Cortinariaceae due to the heterogeneity of ou material (not only sporocarps).

However, after reporting on the cladogram (Figure 6) most of the different genera an subgenera described by Peintner et al. (2004) with a colour code, the New Caledonian clade seemed to either fit within existing clades like *Dermocybe*, *Purpurascentes* or constitute separated and potentially new taxons, one of them being possibly related to *Myxacium*. *Descolea* sequences that we first included in our phylogeny were finally removed as these sequences fitted in a separated cluster (Moncalvo et al. 2002) with no affinity with any of ou New Caledonian species.

Discussion

- Floristic diversity, dominance and symbioses

As stated by Jaffré (1974), the floristic diversity of the Koniambo massif is quite high as referred to those of New Caledonia: the 453 species inventoried by this author represented 15% of the diversity of the island, for a geographic surface ratio of less than 1%. Our floristic inventories, limited to 4 of the 12 phyto-ecological plant communities listed by Jaffré (1974) on the massif, are consistent in terms of species number with those given by this author. Dominant plant species of plant communities 3 and 4 were found ECM. Such a situation has often been described in tropical ecosystems (ie Dipterocarps in Asian forests), although it is far from being systematic (Torti and Coley 1999). In New Caledonia, it has been hypothesized that this dominance could be only a transient stage linked to exogenous disturbance, like fire or cyclones (Read et al. 1995). This study confirmed the description (Perrier et al. 2006b) of ectomycorrhizas on the roots of *Nothofagus balansae* (Nothofagaceae) and *Tristaniopsis*, from the Myrtaceae (Leptospermoideae) family. The genus *Tristania*, close to *Tristaniopsis*, has already been described as ECM in Brunei (Moyersoen et al. 2001). *Nothofagus* is the well-known Southern beech, reported as naturally ECM in Australia, New Zealand and South America (Halling 2001, Tedersoo et al. 2008, McKenzie et al. 2000, Valenzuela et al. 1999). Mycorrhizal species were present as ECM roots, soil mycelia and sporocarps enabling us to get a picture of the general diversity since several studies have shown that there was little correlation between diversity of sporocarps and ectomycorrhizal roots (Buscot et al. 2000, Dahlberg et al. 1996, Gardes and Bruns 1996, Horton and Bruns 2001). Furthermore, Landeweert et al. (2003) showed that using internal transcript spacers on mycelium from soils gave an extra insight on the diversity of the fungal communities.

Only 1 sporocarp was collected in plant community 3, which again shows the divergence between the above- and below-ground fungal communities. This probably resulted from the

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environmental conditions of the maquis, which could be not optimal for fungal fruiting as very dry conditions prevail and as the soil contains little organic matter (Brundrett 1991, Slankis 1974, Smith and Read 1997). Furthermore, the presence of typical sporocarps of *Pisolithus albus* (Figure S3A), which presented identical morphological and anatomical features to those described by Anderson et al. (1998, 2001) in Australia and Aggangan et al. (1998) in New Caledonia, is not surprising in the maquis-type vegetation dominated by sclerophyllous plants, since this genus is known to be well-adapted to growth in mineral soils as an "early stage" mycorrhizal fungus, until forest soil conditions develop (Brundrett 1991, Gardner and Malajczuk 1988, Stahl et al. 1988). The presence of *Cortinarius* in this ecosystem is also of prime importance: it is one of the taxonomically most complex genera in the Basidiomycetes (with ca. 2000 species described so far), being the most frequently recorded ectomycorrhizal fungal genus in many European and North American conifer forests (Alexander and Watling 1987, Hoiland and Holst-Jensen 2000, Villeneuve et al. 1989). Its occurrence in the Southern hemisphere is still poorly documented (Garnica et al. 2005, Tedersoo et al 2008). In the tropics, the genus was reported in tropical North America (Murrill 1912), South America (Singer et al. 1983, Garnica et al. 2003), India (Peintner et al. 2003, Natarajan et al. 2005) along with Australian eucalypt forests (Chambers et al. 1999, Malajczuk et al. 1987, Sawyer et al. 1999, 60). Cortinariaceae seem to be poorly represented in African rainforests as stated by Rivière et al. (2007) in Southern Guinea who did not identify any Cortinariaceae within sequences of 119 sporocarps and 55 ECM and Onguene and Kuyper (2001) in Cameroon.

In plant community 4, ECM symbioses were found with a much greater diversity of genera, confirmed by molecular methods. This diversity mostly included ECM taxons, like *Russula, Lactarius, Tricholoma, Boletus, Inocybe,* etc, and was already reported in other tropical forest ecosystems like India, Guinea (Rivière et al. 2007) or Cameroon (Onguéné and Kuyper 20001). However, in each type of sample, the presence of the genus *Cortinarius* and other genera of the Cortinariaceae is noted and this taxon is dominant among the sporocarps and ectomycorrhizas. If the dominance of the genus *Cortinarius* and family Cortinariaceae in *Nothofagus* forests is well documented in South America (Garnica et al. 2003, Garnica et al. 2005, Valenzuela et al. 1999), Australia (Bougher et al. 1994, Halling 2001) and New Zealand (McKenzie et al. 2002), this study represents the first record its dominance in New Caledonia.

Biogeography and Evolution

The dominance of Cortinariaceae in the *Nothofagus* forest of the Koniambo massif gives a new insight into the phylogeography of this fungal family in the Southern hemisphere. *Nothofagus* incorporates 4 subgenera and 35 species in 6 isolated landmasses: Australia, New Caledonia, New Guinea, New Zealand and South America (Hill and Dettman 1996, Swenson et al. 2000). Swenson et al. (2001b) showed that the biogeography of *Nothofagus* supports the Gondwanaland break-up sequence and that the four major lineages were already present 80 Mya ago prior to the break up of the Gondwanaland eastern margin. The *Nothofagus* members of New Caledonia all belong to *Brassospora* subgenus, which also encompasses species from New Guinea and fossil species from New Zealand, Antarctica, South America and Australia. The presence of species belonging to *Brassospora* subgenus in Papua New Guinea and New

Caledonia can be explained by dramatic tectonic events since the mid-Cenozoic (Halla 1994; Veevers et al. 1991), which provided habitable niches in tropical latitudes (Swenson al. 2001a). Strong evidence exists to support the possible co-evolution of *Nothofagus* a ECM fungi from the Cortinariaceae family (Bougher et al. 1994, Halling 2001, Halling a Mueller 2002) in the Southern Hemisphere. The data accumulated in the *Nothofagus* forest the Koniambo massif identifies several fungal clades specific to New Caledonia, inferri that both plant and fungal ancestors already co-existed at the time they colonized the island.

Along with Nothofagaceae, Leptospermoideae (dry-fruit Myrtaceae) like *Tristaniops* have their geographical distribution closely linked to the fragmentation of Gondwanaland The presence of *Cortinarius* species in the *Tristaniopsis* plant community concern 6 amon 10 samples, among those only one was an ECM apex, the other being soil samples, i.e. eith mycelium or iron oxyde beads from soil mycelia aggregates, whose actual symbiosis wit *Tristaniopsis* was not attested. Sawyer et al. (1999) characterized genets of *Cortinariu rotundisporus* reaching 9 to 30 m in diameter in Australian schlerophyll forests. It cannot b rejected that mycelium from *Nothofagus*-associated Cortinariaceae from plant community explore soil from under *Tristianopsis* in plant community 3. The ability of Cortinariaceae t switch hosts was demonstrated in the laboratory by pure culture synthesis of ECM wit *Nothofagus* and *Eucalyptus* (Bougher 1987, Bougher et al. 1994) opening the door to putativ exchange of ECM fungi between plant communities 3 and 4. Phylogenetic analyses showe that New Caledonian Cortinariaceae did not constitute a unique separated clade but were rather dispersed among or close to several different taxonomical groups. They neither constituted a systematic sister clade of other Cortinariaceae from countries derived from the Gondwanaland break up, although the size of our sampling, and its relatively confined area (only a few hundred square meters, on one watershed, in one isolated ultramafic massif in New Caledonia) might clearly not represent the whole diversity of New Caledonian Cortinariaceae. It is remarkable that all the New Caledonian Cortinariaceae were different from molecular databases existing taxons, and from each other, as if this study only scratched the surface of the diversity present. More systematic sampling of Cortinariaceae and ECM root apices within *Nothofagus* forests in all regional Gondwanaland countries would be necessary to further analyse the possible phylogeography and co-evolution of both plant and fungal partners.

The dominance of the ECM symbioses in two plant communities shows the necessity to consider these partners in the early stages of restoration processes, e.g. when choosing plant species to be planted or when optimizing the management of topsoils. We also showed the existence of soil fungal mats and hyphal networks, with several ECM taxons, that could be of key interest to stabilize the iron oxide spherical beads that almost exclusively compose the soil, in often steep slopes and under cyclonic rains.

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hlberg A, Jonsson L,
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dgar RC (2004) MUSC
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References

Aggangan NS, Dell B, Malajczuk N (1998) Effects of chromium and nickel on growth of the ectomycorrhizal fungus *Pisolithus* and formation of ectomycorrhizas on *Eucalyptus urophylla* S.T. Blake. *Geoderma*, 84,15-27.

Alexander IJ, Watling R (1987) Macrofungi of Sitka spruce in Scotland. *Proceedings of the Royal Society Edinburgh*, 93B,107-115.

Anderson IC, Chambers SM, Cairney JWG (2001) Distribution and persistence of Australian *Pisolithus* species genets at native sclerophyll forest field sites. *Mycological Research*, 105, 971-976.

Anderson IC, Chambers SM, Cairney JWG (1998) Molecular determination of genetic variation in *Pisolithus* isolates from a defined region in New South Wales, Australia. *New Phytologist*, 138,151-162.

Bougher NL (1987) *The systematic position and ectomycorrhizal status of the fungal genus Descolea*. Ph.D. thesis. University of Western Australia, Perth, WA.

Bougher NL, Fuhrer BA, Horak E (1994) Taxonomy and biogeography of Australian *Rozites* species mycorrhizal with *Nothofagus* and Myrtaceae. *Australian Systematic Botany*, 7, 353-375.

Braun-Blanquet G, Fuller GD, Conard HS (1932) *Plant sociology: the study of plant communities*. McGraw-Hill book, New York, USA.

Brundrett M (1991) Mycorrhizas in natural ecosystems. *Advances in Ecological Research*, 21, 171-313.

Buscot F, Munch JC, Charcosset JY, Gardes M, Nehls U, Hampp R (2000) Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. *FEMS Microbiology Reviews*, 24, 601-614.

Chambers SM, Sawyer NA, Cairney JWG (1999) Molecular identification of co-occurring *Cortinarius* and *Dermocybe* species from Southeastern Australian sclerophyll forests. *Mycorrhiza*, 9, 85-90.

Dahlberg A, Jonsson L, Nylund JE (1996) Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in South Sweden. *Canadian Journal of Botany*, 75, 1323-1335.

Garcia-Dousso M, Contesto C, Cossegal M, Prin Y, Rigault F, Eyssartier G (2004) *Cantharellus garnierii* sp. *nov.*, une nouvelle chanterelle de Nouvelle-Calédonie. *Cryptogamie Mycologie*, 25, 115-125.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792-1797.

Gardes M, Bruns TD (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above and below ground views. *Canadian Journal of Botany*, 74, 1572-1583.

Gardner JH, Malajczuk N (1988) Recolonisation of rehabilitated bauxite mine sites in Western Australia by mycorrhizal fungi. *Forest Ecology and Management*, 24, 27-42.

Garnica S, Weiss M, Oberwinkler F (2002) New *Cortinarius* species from *Nothofagus* forests in South Chile. *Mycologia*, 94, 136-145.

Garnica S, Weiss M, Oberwinkler F (2003) Morphological and molecular phylogenetic studies in South American *Cortinarius* species. *Mycological Research*, 107, 1143-1156.

Garnica S, Weiss M, Oertel B, Oberwinkler F (2005) A framework for a phylogenet
classification in the genus *Cortinarius* (Basidiomycota, Agaricales) derived fro
morphological and molecular data. *Canadian Journal of Botany,* 83, 1457-1477.
Greenacre MJ (1984) *Theory and applications of correspondence analysis*, Academic Pres
London.
Guindon S, Lethiec F, Duroux P, Gascuel O (2005) PHYML Online--a web server for fa
maximum likelihood-based phylogenetic inference. *Nucleic Acids Research,* 33, W557-9
Hallam A (1994) *An outline of Phanerozoic biogeography.* Oxford University Press, Oxford
Halling RE (2001) Ectomycorrhizae: co-evolution, significance, and biogeography. *Annals o
the Missoury Botanical Garden,* 88, 5-13.
Halling RE, Mueller GM (2002) Agarics and Boletes of neotropical oakwoods. In: *Tropica
Mycology, Vol 1, Macromycetes* (ed. Watling R, Frankland JC, Ainsworth AM, Isaac S
Robinson CH). CAB International, New York, USA.
Hill RS, Dettmann ME (1996) Origin and diversification of the genus *Nothofagus*. In: *Th
Ecology and Biogeography of Nothofagus forest* (ed. Veblen TT, Hill RS, Read J) pp. 1
24. Yale University Press, New Haven, CT.
Hoiland K, Holst-Jensen A (2000) *Cortinarius* phylogeny and possible taxonomi
implications of ITS rDNA sequences. *Mycologia*, 92, 694-710.
Horak E, Mouchacca J (1998) Annotated checklist of New Caledonian Basidiomycota. I
Holobasidiomycetes. *Mycotaxon,* 68, 75-129.
Horton R, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peekin
into the black-box. *Molecular Ecology*, 10, 1855-1871.
Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP (2001) Bayesian inference of phylogen
and its impact on evolutionary biology. *Science*, 294, 2310-4.
Jaffré T (1992) Floristic and ecological diversity of the vegetation on ultramafic rocks in New
Caledonia. In: *The vegetation of ultramafic (serpentine) soils* (ed. Baker AJM, Proctor J
Reeves RD) pp. 101-107. Intercept Ltd, Andover, NL.
Jaffré T (1974) La végétation d'un massif de roches ultrabasiques de Nouvelle-Calédonie: le
Koniambo. *Candollea,* 29, 427-456.
Jaffré T, Morat P, Veillon JM, Rigault F, Dagostini G (2001) Composition et caractérisation
de la flore indigène de Nouvelle-Calédonie. (ed. IRD Centre de Noumea) Documents
Scientifiques et Techniques vol. II 4. IRD Nouméa.
Jentschke G, Godbold DL (2000) Metal toxicity and ectomycorrhiza. *Physiologia Plantarum,*
109, 107-116.
Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E (2003)
Molecular identification of eetomycorrhizal mycelium in soil horizons. *Applied
Environmental Microbiology*, 69, 327-333.
Malajczuk N, Dell B, Bougher NL (1987) Ectomycorrhiza formation in *Eucalyptus* III-
superficial ectomycorrhizas initiated by *Hysterangium* and *Cortinarius* species. *New
Phytologist*, 105, 421-428.
McKenzie EHC, Buchanan PK, Johnston PR (2000) Checklist of fungi on *Nothofagus* species
in New Zealand. *New Zealand Journal of Botany*, 38, 635-720.
Moncalvo JM, Vilgalys R, Redhead SA, et al. (2002) One hundred and seventeen clades of
euagarics. *Molecular Phylogenetics and Evolution*, 23, 357-400.
Moyersoen B, Becker P, Alexander IJ. 2001. Are ectomycorrhizas more abundant than
arbuscular mycorrhizas in tropical heath forests? *New Phytologist,* 150, 591-599.

rill WA (1912) *Agaric*
larajan K, Senthilarasu
fungi of a dipterocarp f
holas KB, Nicholas B
sequence alignments. I
guéné NA, Kuyper TV
Cameroon. *Forest Ecol*
tner U, Bougher N, C
related to *Cortinarius* (
tner U, Moser MM, TI
Cortinarius species
phylogenetic position b
494.
tner U, Moncalvo JM
infrageneric relationshi
1042-1058.
ier N, Ambrosi JP, Col
Caledonian Koniambo
58.
ier N, Amir H, Colin F
lateritic soils of the Kor
da D, Crandall KA
Bioinformatics, 14, 817
J. Hallam P, Cherrier
some preliminary obs
Caledonia. *Journal of T*
ère T, Diédhiou AG,
Basidiomycetes from A
nquist F, Huelsenbeck JP
models. *Bioinformatics,*
wyer NA, Chambers S
distribution and geneti
sclerophyll forests. *New*
himodaira H, Hasegawa
Applications to Phylog
1116.
inger R, Araujo I, Ivory M
lowlands, especially Ce
lankis V (1974) Soil fac
Phytopathology, 12, 43
mith SE, Read DJ (1997) *M*
Stahl PD, Williams SE, (
mycorrhizal fungi after
wenson U, Backlund A, M
with special emphasis
Caledonia. *Cladistics,* 1

Murrill WA (1912) *Agaricaceae* of tropical North America. *Mycologia*, 4, 72-83.
Natarajan K, Senthilarasu G, Kumaseran V, Rivière T (2005) Diversity in ectomycorrhizal fungi of a dipterocarp forest in Western Ghats. *Current Science*, 88, 1893-1895.
Nicholas KB, Nicholas BJ (1997) Genedoc: a tool for editing and annotating multiple sequence alignments. Distributed by the author.
Onguéné NA, Kuyper TW (2001) Mycorrhizal associations in the rainforest of South Cameroon. *Forest Ecology and Management*, 40, 277-287.
Peintner U, Bougher N, Castellano M et al. (2001) Multiple origins of sequestrate fungi related to *Cortinarius* (Cortinariaceae). *American Journal of Botany*, 88, 177-184.
Peintner U, Moser MM, Thomas KA, Manimohan P (2003) First record of ectomycorrhizal *Cortinarius* species (Agaricales, Basidiomycetes) from tropical India and their phylogenetic position based on rDNA ITS sequences. *Mycological Research*, 107, 485-494.
Peintner U, Moncalvo JM, Vilgalys R (2004) Toward a better understanding of the infrageneric relationships in *Cortinarius* (Agaricales, Basidiomycota). *Mycologia*, 96, 1042-1058.
Perrier N, Ambrosi JP, Colin F, Gilkes RJ (2006a) Biogeochemistry of a regolith: The New Caledonian Koniambo ultramafic massif. *Journal of Geochemical Exploration*, 88, 54-58.
Perrier N, Amir H, Colin F (2006b) Occurrence of mycorrhizal symbioses in the metal rich lateritic soils of the Koniambo Massif, New Caledonia. *Mycorrhiza*, 16, 449-458.
Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14, 817-818.
Read J, Hallam P, Cherrier JF (1995) The anomaly of mono-dominant tropical rainforests: some preliminary observations in the *Nothofagus*-dominated rainforests of New Caledonia. *Journal of Tropical Ecology*, 11, 359-389.
Rivière T, Diédhiou AG, Diabaté M et al. (2007) Genetic diversity of ectomycorrhizal Basidiomycetes from African and Indian tropical rainforests. *Mycorrhiza*, 17, 415-428.
Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572-4.
Sawyer NA, Chambers SM, Cairney JWG (1999) Molecular investigation of genet distribution and genetic variation of *Cortinarius rotundisporus* in eastern Australian sclerophyll forests. *New Phytologist*, 142, 561-568.
Shimodaira H, Hasegawa M (1999) Multiple Comparisons of Log-Likelihoods with Applications to Phylogenetic Inference. *Molecular Biology and Evolution*, 16, 1114-1116.
Singer R, Araujo I, Ivory MH (1983) The ectotrophically mycorrhizal fungi of the neotropical lowlands, especially Central Amazonia. *Beiheft zur Nova Hedwigia*, 77, 1-339.
Slankis V (1974) Soil factors influencing formation of mycorrhizae. *Annual Review of Phytopathology*, 12, 437-457.
Smith SE, Read DJ (1997) *Mycorrhizal Symbiosis*, 2nd Edition. Academic Press, London.
Stahl PD, Williams SE, Christensen M (1988) Efficacy of native vesicular-arbuscular mycorrhizal fungi after severe soil disturbance. *New Phytologist*, 110, 347-354.
Swenson U, Backlund A, McLoughlin S, Hill RS (2001a) *Nothofagus* biogeography revisited with special emphasis on the enigmatic distribution of subgenus *Brassospora* in New Caledonia. *Cladistics*, 17, 28-47.

Swenson U, Hill RS, McLoughlin S (2000) Ancestral area analysis of *Nothofag* (Nothofagaceae) and its congruence with fossil record. *Australian Systematic Botany,* 469-478.

Swenson U, Hill RS, McLoughlin S (2001b) Biogeography of *Nothofagus* supports sequence of Gondwana break-up. *Taxon,* 50, 1025-1041.

Tedersoo L, Jairus T, Horton BM et al. (2008) Strong host preference of ectomycorrhiz fungi in a Tasmanian wet schlerophyll forest as revealed by DNA barcoding and taxo specific primers. *New Phytologist,* 180, 479-490.

The International Plant Index (2008) Published on the Internet http://www.ipni.org [accesse 20 October 2008].

Thioulouse J, Chessel D, Dolédec S, Olivier JM (1997) ADE4: a multivariate analysis an graphical display software. *Statistics and Computing,* 7, 75-83.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The clustal- windows interface: flexible strategies for multiple sequence alignment aided by quali tools. *Nucleic Acids Research,* 25, 4876-4882.

Torti SD, Coley PD (1999) Tropical monodominance: A preliminary test of th ectomycorrhizal hypothesis. *Biotropica,* 31, 220-228.

Valenzuela E, Moreno G, Garnica S, Godoy R, Ramirez C (1999) Mycosociology in nativ forests of *Nothofagus* of the X region of Chile, diversity and ecological role. *Mycotaxon* 72, 217-226.

Veevers JJ (1986) Breakup of Australia and Antarctica estimated as Mid-Cretaceous (95± Ma) from magnetic and seismic data at the continental margin. *Earth and Planetar Science Letters*, 77, 91-99.

Veevers JJ, Powell CM, Roots SR (1991) Review of seafloor spreading around Australia. Synthesis of the patterns of spreading. *Australian Journal of Earth Science*, 38, 373-389.

Villeneuve NM, Grandtner M, Fortin JA (1989) Frequency and diversity of ectomycorrhiza and saprophytic macrofungi in the Laurentide Moutains of Quebec. *Canadian Journal o Botany*, 67, 2616-2629.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A guide to methods and applications* (ed. Innis MA, Gelfand H, Sninsky JS, White TJ) pp.185-189. Academic Press, New York.

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8.3. VOLET 3 : DISTRIBUTION DES ÉLÉMENTS MÉTALLIQUES ; VARIATION DU POTENTIEL REDOX DU PH ET DE LA CONDUCTIVITÉ ÉLECTRIQUE AU COURS DU STOCKAGE DU TOPSOIL.

1- pH, conductivité électrique et potentiel d'oxydoréduction du topsoil de Goro stocké en andain et prélevé à 0, 50, 100 ou 150 cm sous la surface et stocké étalé (*)

Mois		*pH*	*CE(µS/cm)*	*E (mV)*
t=0	BS6 (0-20)	4,2	152,6	265
	BS6(20-40)	4,1	227,0	273
	tas	4,8	112,1	104
t=3	0	4,9	117,2	207
	50	5,1	74,9	189
	100	5,1	90,0	215
	150	5,1	94,3	197
	50*	5,2	77,3	207
t=6	0	5,1	74,2	103
	50	5,2	66,3	113
	100	5,0	114,6	139
	150	5,2	111,6	163
	50*	5,0	78,1	113
t=9	0	5,3	79,7	76
	50	5,2	74,0	74
	100	4,9	95,2	86
	150	5,2	81,9	88
	50*	5,1	61,2	74
t=12	0	4,9	70,7	76
	50	4,9	101,5	80
	100	4,8	237,0	87
	150	4,9	120,9	118
	50*	5,1	84,3	108
t=18	0	5,0	61,1	96
	50	5,1	53,6	153
	100	5,0	155,6	134
	150	5,0	76,5	108
	50*	4,8	68,7	90
t=24	0	5,2	105,9	101
	50	4,6	103,7	97
	100	4,7	85,9	99
	150	4,5	101,2	109
	50*	5,3	71,3	91
t=30	0	4,9	67,1	109
	50	4,4	113,5	170
	100	4,4	118,3	127
	150	4,3	212,0	134
	50*	4,6	52,9	162
t=36	0	4,8	53,5	129,3
	50	4,6	118,3	173,3
	100	4,4	79,0	128,1
	150	4,8	45,7	142,5
	50*	4,4	111,9	160,5

2- pH, conductivité électrique et potentiel d'oxydoréduction du topsoil de Tontouta stocké en andain et prélevé à 0, 50, 100 ou 150 cm sous la surface et stocké étalé (*)

Mois		*pH*	*CE(µS/cm)*	*E(mV)*
t=0	TS:0-20 cm	5,58	56,4	172
	TS :40-50cm	5,88	55,6	166
	t=0 (E1-0)	5,87	90,2	168
t=3	0	6,03	105,3	227
	50	5,57	104,7	180
	100	5,73	108,8	203
	150	5,97	106	219
	50*	6,1	152,4	219
t=6	0	6,397	97,6	177
	50	5,42	83,1	208
	100	5,82	78,8	204
	150	5,71	73,1	201
	50*	6,399	178,1	174
t=9	0	5,96	88	196
	50	6,41	116,2	228
	100	6,33	107	222
	150	5,85	105,8	210
	50*	6,19	91,8	214
t=12	0	5,6	97,8	220
	50	5,72	85,4	179
	100	5,7	94,7	226
	150	5,71	111,8	212
	50*	5,85	112,7	187
t=18	0	5,72	64,5	105
	50	5,71	56,5	74
	100	5,76	59,6	102
	150	5,67	85,8	105
	50*	6,1	57,7	101
t=24	0	5,49	37,7	126
	50	5,69	78,3	116
	100	6	59,4	76
	150	5,57	46,2	89
	50*	5,65	66,9	75

3- Concentrations en Cr(VI), Ni et Co soluble dans l'eau pour le topsoil de Goro stocké en andain et prélevé à 0, 50, 100 ou 150 cm sous la surface et stocké étalé (*).

Mois		Cr(VI) en ng/g	Ni en ng/g	Co en ng/g
t=0	BS6 (0-20cm)	3,8	23,8	nd
	BS6 (20-40cm)	11,2	26,6	nd
	tas	1,0	37,3	nd
t=3	0	14,0	36,2	8,7
	50	40,6	31,6	2,2
	100	6,7	30,5	3,4
	150	12,7	11,4	2,2
	50*	25,7	5,9	2,9
t=6	0	11,4	37,4	nd
	50	14,6	19,8	nd
	100	4,1	27,1	nd
	150	14,1	20,8	0,2
	50*	9,1	26,0	0,04
t=9	0	3,3	9,8	nd
	50	16,9	21,5	6,6
	100	18,7	23,8	1,8
	150	49,4	46,0	nd
	50*	23,6	12,8	nd
t=12	0	9,3	8,8	0,6
	50	9,5	8,1	0,1
	100	41,3	18,6	5,8
	150	51,5	12,3	0,3
	50*	18,2	9,9	nd
t=18	0	24,5	20,7	nd
	50	77,1	22,1	nd
	100	300,5	15,4	nd
	150	126,9	29,8	nd
	50*	102,5	26,1	nd
t=24	0	0,7	32,5	nd
	50	27,4	36,6	nd
	100	23,4	39,3	nd
	150	1,1	39,0	nd
	50*	10,5	24,0	0,2
t=30	0	102,0	18,2	nd
	50	100,9	9,2	n.d
	100	127,6	40,9	nd
	150	51,1	24,3	nd
	50*	0,0	17,1	n.d
t=36	0	63,7	32,0	nd
	50	79,6	37,0	nd
	100	74,7	49,9	nd
	150	0,0	12,4	nd
	50*	0,0	4,1	nd

n.d pour non détecté

4- Concentrations en Cr(VI), Ni et Co soluble dans l'eau pour le topsoil de Tontouta stocké en andain et prélevé à 0, 50, 100 ou 150 cm sous la surface et stocké étalé (*). n.d pour non détecté et n.a pour non analysé

Mois		Cr(VI) en ng/g	Ni en ng/g	Co en ng/g
t=0	tas	650,7	291,2	0,2
t=3	0	413,2	376,8	1,7
	50	534,1	196,2	26,4
	100	576,5	127,9	18,1
	150	489,8	133,6	4,2
	50*	1213,6	193,2	n.d
t=6	0	589,7	146,6	3,7
	50	463,8	77,4	1,2
	100	682,1	62,1	0,9
	150	641,1	97,9	1,5
	50*	502,4	311,3	3,1
t=9	0	558,6	283,0	1,7
	50	1274,3	129,1	8,3
	100	516,3	944,4	55,6
	150	416,2	258,5	30,9
	50*	520,6	195,7	11,0
t=12	0	666,8	233,0	14,2
	50	553,0	151,1	12,3
	100	313,0	312,4	6,0
	150	510,8	210,7	8,7
	50*	489,7	160,8	18,4
t=18	0	303,2	290,4	0,1
	50	439,3	356,3	0,6
	100	2155,5	207,1	1,3
	150	209,0	328,1	0,2
	50*	260,8	131,8	0,2
t=24	0	367,4	239,9	n.d
	50	332,7	221,3	n.d
	100	467,9	439,0	n.d
	150	453,2	520,5	n.d
	50*	426,6	433,9	n.d
t=30	0	n.a		
	50			
	100			
	150			
	50*			
t=36	0			
	50			
	100			
	150			
	50*			

5- Détermination des concentrations en éléments métalliques "biodisponibles" dans le sol des parcelles expérimentales - VOLET 3-Dynamique des métaux et de la matière organique in situ

Des échantillons de sol ont été prélevés sur le dispositif expérimental de GORO lors de 2 campagnes d'échantillonnage

Les mottes sont prélevées à la bêche, emballées et transportées au laboratoire. Les sacs sont ouverts et le sol rhizosphérique (adhérent aux racines et à proximité du système racinaire (moins de 1 cm)

1ère campagne le 27/05/2013, prélèvement sur les parcelles contenant les plants de Capolepis laurifolia (les plants de Tristaniopsis n'ont pas suffisamment poussé pour être prélevés) et prélèvement de sol non racinaire

n=4 pour chaque catégorie de sol rhizosphérique ("témoin" non inoculés/ plants endomycohrizés/"témoins avec boues/ endo+boues)

2ème campagne le 29/10/2013, prélèvement sur les parcelles contenant les plants de Carpolepis laurifolia, les plants de Tristaniopsis glauca et guillaini et prélèvement de sol non rhizosphérique

n=8 pour chaque catégorie de sol rhizosphérique ("témoin" non inoculés/ plants endomycohrizés/"témoins avec boues/endo+boues/ecto/ecto+boues)

La solution de lixiviation est préparée avec 1,967 g de DTPA+1,470g de CaCl2 pour un volume de 1L. pH ajusté à 7,3 par ajout de NaOH

20g de sol sont agités sur table ping-pong en présence de 40 mL de la solution de DTPA pendant 2h

Analyses des oncentrations en éléments métalliques dans les lixiviats de sol

Les concentrations sont déterminées par spectroscopie atomique flamme ou four (AA800 PerkinElmer) selon les gammes de concentrations

Les gammes d'étalonnage sont établies à partir de solution standards certifiées (PerkinElmer)

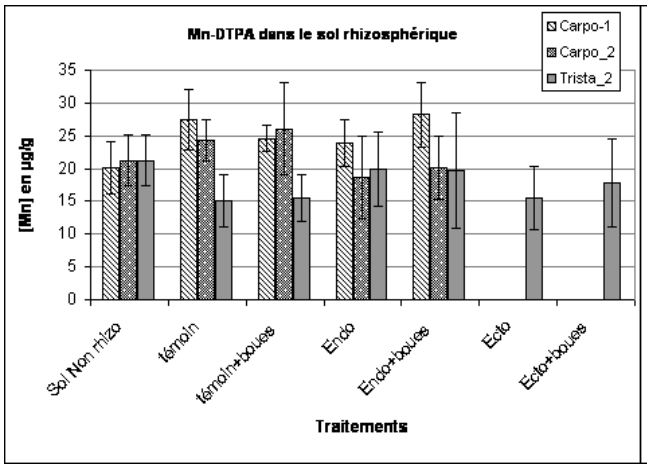
Les concentrations sont exprimées en µg/g (= mg/kg) de sol sec ou en ng/g (=µg/kg) de sol sec. IC sont les intervalles de confiance (alpha=0,05).

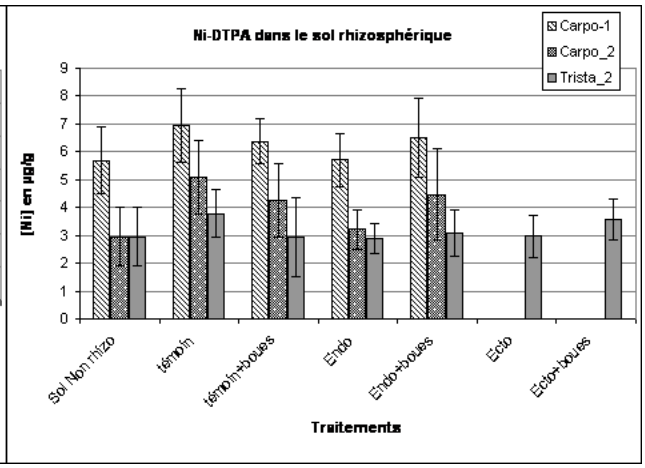
Carpo_1=résultats de la 1ère campagne pour le sol prélevé dans la rhizosphère de Carpolepis laurifolia

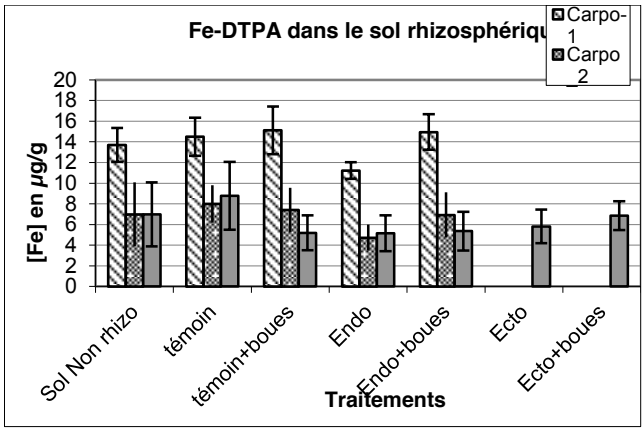
Carpo_2=résultats de la 2ème campagne pour le sol prélevé dans la rhizosphère de *Carpolepis laurifolia*

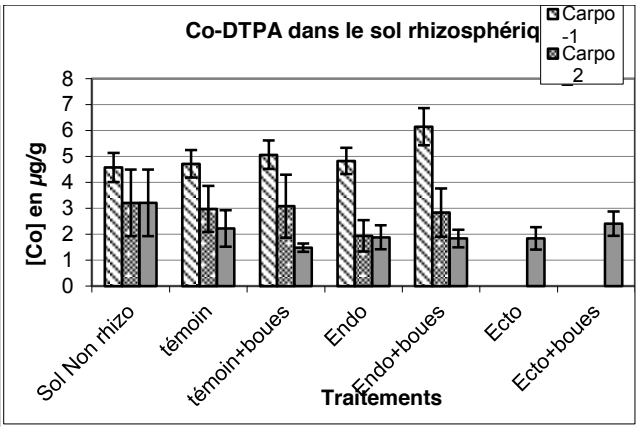
Trista_2=résultats de la 2ème campagne pour le sol prélevé dans le rhizosphère de *Tristaniopsis glauca* et *guillaini*

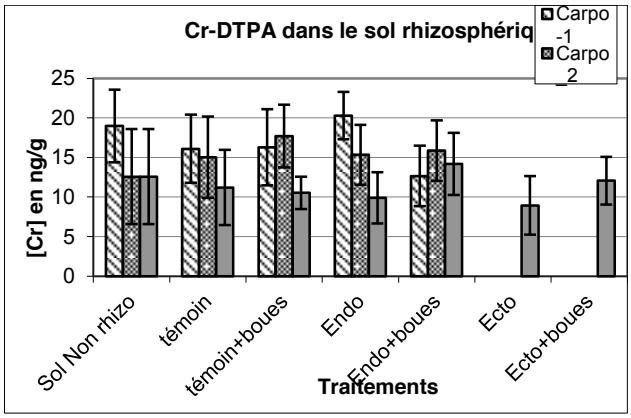
Mn µg/g	Carpo-1	IC	Carpo_2	IC	Trista_2	IC
Sol Non rhizo	**20,1**	4,0	**21,2**	3,9	**21,2**	3,9
témoin	**27,5**	4,6	**24,2**	3,2	**15,1**	3,9
témoin+boues	**24,6**	2,0	**26,1**	7,0	**15,5**	3,5
Endo	**23,9**	3,5	**18,6**	6,3	**19,9**	5,7
Endo+boues	**28,2**	5,0	**20,2**	4,8	**19,7**	8,7
Ecto					**15,6**	4,8
Ecto+boues					**17,8**	6,7
Fe µg/g	**Carpo_1**	IC	**Carpo_2**	IC	**Trista_2**	IC
Sol Non rhizo	**13,7**	1,7	**7,0**	3,1	**7,0**	3,1
témoin	**14,5**	1,8	**8,0**	1,8	**8,8**	3,3
témoin+boues	**15,1**	2,3	**7,4**	2,1	**5,2**	1,7
Endo	**11,2**	0,8	**4,7**	1,2	**5,2**	1,7
Endo+boues	**15,0**	1,7	**6,9**	2,2	**5,4**	1,9
Ecto					**5,8**	1,6
Ecto+boues					**6,9**	1,4
Cr ng/g	**Carpo_1**	IC	**Carpo_2**	IC	**Trista_2**	IC
Sol Non rhizo	**19,0**	4,6	**12,6**	6,0	**12,6**	6,0
témoin	**16,1**	4,3	**15,0**	5,2	**11,2**	4,8
témoin+boues	**16,3**	4,8	**17,7**	3,9	**10,5**	2,0
Endo	**20,3**	3,0	**15,3**	3,8	**9,9**	3,2
Endo+boues	**12,7**	3,8	**15,9**	3,8	**14,2**	3,9
Ecto					**8,9**	3,7
Ecto+boues					**12,1**	3,0
Ni µg/g	**Carpo_1**	IC	**Carpo_2**	IC	**Trista_2**	IC
Sol Non rhizo	**5,7**	1,2	**3,0**	1,0	**3,0**	1,0
témoin	**6,9**	1,3	**5,1**	1,3	**3,8**	0,9
témoin+boues	**6,4**	0,8	**4,3**	1,3	**2,9**	1,4
Endo	**5,7**	1,0	**3,2**	0,7	**2,9**	0,5
Endo+boues	**6,5**	1,4	**4,5**	1,7	**3,1**	0,8
Ecto					**3,0**	0,7
Ecto+boues					**3,6**	0,7
Co µg/g	**Carpo_1**	IC	**Carpo_2**	IC	**Trista_2**	IC
Sol Non rhizo	**4,6**	0,6	**3,2**	1,3	**3,2**	1,3
témoin	**4,7**	0,5	**3,0**	0,9	**2,2**	0,7
témoin+boues	**5,1**	0,5	**3,1**	1,2	**1,5**	0,2
Endo	**4,8**	0,5	**1,9**	0,6	**1,9**	0,5
Endo+boues	**6,1**	0,7	**2,8**	0,9	**1,8**	0,3
Ecto					**1,8**	0,4
Ecto+boues					**2,4**	0,5
Mg µg/g	**Carpo_1**	IC	**Carpo_2**	IC	**Trista_2**	IC
Sol Non rhizo	**1,3**	0,1	**2,0**	0,5	**2,0**	0,5
témoin	**1,2**	0,1	**2,9**	0,7	**2,4**	0,9
témoin+boues	**1,2**	0,1	**2,3**	0,6	**3,3**	0,7
Endo	**1,1**	0,1	**2,5**	0,8	**3,2**	0,6
Endo+boues	**1,3**	0,1	**2,1**	0,3	**2,2**	0,6
Ecto					**2,4**	0,7
Ecto+boues					**2,5**	1,0

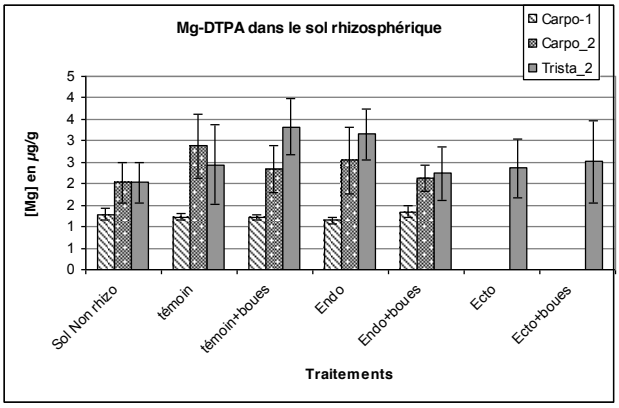

Mn-DTPA dans le sol rhizosphérique
Carpo-1
Carpo_2
Trista_2
[Mn] en µg/g
Sol Non rhizo
témoin
témoin+boues
Endo
Endo+boues
Ecto
Ecto+boues
Traitements


Ni-DTPA dans le sol rhizosphérique
Carpo-1
Carpo_2
Trista_2
[Ni] en µg/g
Sol Non rhizo
témoin
témoin+boues
Endo
Endo+boues
Ecto
Ecto+boues
Traitements


Fe-DTPA dans le sol rhizosphériqu
Carpo-1
Carpo_2
[Fe] en µg/g
Sol Non rhizo
témoin
témoin+boues
Endo
Endo+boues
Ecto
Ecto+boues
Traitements


Co-DTPA dans le sol rhizosphériq
Carpo-1
Carpo_2
[Co] en µg/g
Sol Non rhizo
témoin
témoin+boues
Endo
Endo+boues
Ecto
Ecto+boues
Traitements


Cr-DTPA dans le sol rhizosphériq
Carpo-1
Carpo_2
[Cr] en ng/g
Sol Non rhizo
témoin
témoin+boues
Endo
Endo+boues
Ecto
Ecto+boues
Traitements


Mg-DTPA dans le sol rhizosphérique
Carpo-1
Carpo_2
Trista_2
[Mg] en µg/g
Sol Non rhizo
témoin
témoin+boues
Endo
Endo+boues
Ecto
Ecto+boues
Traitements

Fonctionnement géochimique des topsoils

Eléments majeurs en wt% d'oxydes dans les échantillons.

< L.D. : teneur inférieure à la limite de détection. P.F. : perte au feu. SBH : sols bruns hypermagnésiens de Tontouta ; les autres sont des sols latéritiques de Goro.

Echantillon	SiO2	Al2O3	Fe2O3	MnO	MgO	CaO	Na2O	K2O	P2O5	PF	Total
	%	%	%	%	%	%	%	%	%	%	%
TOP A	0,43	2,06	23,01	0,05	0,51	0,20	0,02	0,04	0,05	70,69	97,19
TOP B	0,62	5,77	60,83	0,11	0,54	< L.D.	< L.D.	0,03	0,08	25,73	94,15
SFFC 1	1,09	6,94	71,41	0,79	0,25	< L.D.	< L.D.	0,01	0,06	15,32	96,06
SFFC 2	1,36	6,58	70,89	0,58	0,38	< L.D.	< L.D.	0,02	0,05	15,21	95,18
SFFC 3	1,83	5,86	68,74	1,84	0,47	< L.D.	< L.D.	0,01	0,04	16,54	95,54
SFFP 1	3,42	8,15	65,44	0,44	0,82	0,04	< L.D.	0,02	0,06	17,28	95,82
SFFP 2	2,18	9,95	66,30	0,28	0,75	< L.D.	< L.D.	0,01	0,06	15,88	95,57
SFFP 3	10,81	5,54	55,85	1,08	3,64	< L.D.	0,02	0,01	0,03	18,18	95,21
GORO B	1,84	5,18	70,66	0,57	0,70	< L.D.	< L.D.	0,01	0,06	15,21	94,41
TOP T0 1	0,53	5,81	48,59	0,15	0,61	0,17	0,01	0,03	0,07	38,06	94,38
TOP T0 2	0,41	4,68	60,12	0,11	0,51	0,09	< L.D.	0,03	0,07	27,77	94,11
TOP T0 3	0,60	4,65	55,61	0,12	0,68	0,15	0,01	0,04	0,06	32,53	94,76
TOP T0 4	0,68	4,73	61,02	0,18	0,71	0,11	< L.D.	0,02	0,06	25,04	92,87
TOP T0 5	0,48	4,57	59,94	0,14	0,61	0,10	< L.D.	0,02	0,06	27,17	93,41
SBH 1	41,30	2,87	28,40	0,85	7,64	0,53	0,06	0,03	0,03	15,33	97,14
SBH 2	47,34	2,57	23,06	0,69	9,72	0,47	0,05	0,01	< L.D.	12,36	96,35
SBH 3	49,02	0,76	13,42	0,20	15,27	0,44	0,02	0,02	< L.D.	19,50	98,66

Eléments en traces (ET) les plus importants en ppm (µg/g) dans les échantillons et total de tous les ET analysés (non représentés) + MnO en wt%.

Echantillon	Co	Cr	Ni	MnO	Total des ET
	mg kg^{-1}			%	%
TOP A	47,04	13410	452,3	0,05	1,42
TOP B	130,7	40790	1126	0,11	4,28
SFFC 1	540	25412,5	4216	0,79	3,09
SFFC 2	672,4	22265	5551	0,58	2,92
SFFC 3	3184	22220	8038	1,84	3,43
SFFP 1	606,1	25495	5597	0,44	3,24
SFFP 2	615,5	22662,5	5922	0,28	2,99
SFFP 3	3405	24550	11780	1,08	4,05
GORO B	539,5	35310	5076	0,57	4,17
TOP T0 1	197,9	40780	1681	0,15	4,34
TOP T0 2	153	38830	1309	0,11	4,11
TOP T0 3	161,8	39510	1388	0,12	4,18
TOP T0 4	232,4	52860	1943	0,18	5,59
TOP T0 5	176,7	45540	1462	0,14	4,80
SBH 1	698,7	16625	4882	0,85	2,29
SBH 2	742,4	19177,5	3826	0,69	2,42
SBH 3	200,5	4559	5813	0,20	1,07

Mesures de CEC en mEq/100g de sol = cmol.kg-1 de sol.

Echantillon	Masse (g)	Volume (ml)	Normalité de l'échantillon (N)	CEC en mEq/100g	Moyenne CEC mEq/100g sur poids humide
TOPA	7,010	30,000	6,0570	19,528	20,078
TOPA'	7,010	30,000	2,5640	20,628	
TOPB	7,038	30,000	0,0360	5,953	5,964
TOPB'	7,037	30,000	0,0360	5,974	
SFFC1	7,001	30,000	0,0481	0,825	0,773
SFFC1'	7,006	30,000	0,0483	0,721	
SFFC2	7,001	30,000	0,0499	0,063	0,067
SFFC2'	7,001	30,000	0,0498	0,072	
SFFC3	7,000	30,000	0,0476	1,043	1,028
SFFC3'	7,000	30,000	0,0476	1,014	
SFFP1	7,001	30,000	0,0411	3,828	3,909
SFFP1'	7,004	30,000	0,0407	3,990	
SFFP2	7,002	30,000	0,0478	0,922	0,925
SFFP2'	7,001	30,000	0,0478	0,927	
SFFP3	7,010	30,000	0,0459	1,770	1,748
SFFP3'	7,006	30,000	0,0460	1,726	
GORO B	7,003	30,000	0,0464	1,528	1,545
GORO B'	7,001	30,000	0,0464	1,563	
TOPT01	7,003	30,000	0,0189	13,323	13,232
TOPT01'	7,003	30,000	0,0193	13,142	
TOPT02	7,015	30,000	0,0308	8,230	8,377
TOPT02'	7,016	30,000	0,0301	8,524	
TOPT03	7,012	30,000	0,0248	10,781	10,979
TOPT03'	7,013	30,000	0,0239	11,177	
TOPT04	7,001	30,000	0,0308	8,214	8,413
TOPT04'	7,001	30,000	0,0299	8,613	
TOPT05	7,000	30,000	0,0291	8,944	8,990
TOPT05'	7,001	30,000	0,0289	9,036	
SBH1	5,026	30,000	0,0098	24,009	24,120
SBH1'	5,026	30,000	0,0094	24,231	
SBH2	5,009	30,000	0,0126	22,411	22,447
SBH2'	5,010	30,000	0,0125	22,482	
SBH3	5,154	30,000	0,0002	28,979	28,987
SBH3'	5,153	30,000	0,0002	28,994	

Matrices des corrélation pour la paramètres des solutions d'extraction :

- NaNO3 (0,01 M)

	pH incubé	Al	Ca	Co	Cr	Fe	K	Mg	Mn	Ni	S	Si	Zn	TOC (mg L-1)	TIC (mg L-1)
pH incubé	1	-0,21	-0,41	-0,17	0,21	0,29	-0,03	**-0,51**	**-0,48**	-0,45	**0,55**	-0,06	**-0,57**	-0,11	0,42
Al	-0,21	1	-0,34	-0,33	**0,49**	0,32	0,27	**0,64**	-0,21	**0,77**	-0,29	0,27	0,04	**0,87**	0,14
Ca	-0,41	-0,34	1	**0,65**	-0,15	-0,30	-0,14	0,45	**0,92**	-0,21	0,04	0,27	**0,55**	-0,26	-0,37
Co	-0,17	-0,33	**0,65**	1	-0,29	-0,40	-0,15	0,23	0,43	-0,09	0,10	-0,04	0,33	-0,29	-0,18
Cr	0,21	**0,49**	-0,15	-0,29	1	**0,93**	0,38	0,28	-0,16	0,10	0,09	0,44	-0,08	**0,70**	**0,53**
Fe	0,29	0,32	-0,30	-0,40	**0,93**	1	0,18	0,00	-0,31	-0,05	0,03	0,29	-0,20	**0,48**	**0,53**
K	-0,03	0,27	-0,14	-0,15	0,38	0,18	1	0,11	-0,18	0,25	0,14	0,14	0,13	**0,53**	**0,48**
Mg	**-0,51**	**0,64**	0,45	0,23	0,28	0,00	0,11	1	**0,47**	**0,59**	-0,23	0,43	**0,53**	**0,56**	-0,24
Mn	**-0,48**	-0,21	**0,92**	0,43	-0,16	-0,31	-0,18	**0,47**	1	-0,07	-0,09	0,32	0,45	-0,17	-0,45
Ni	-0,45	**0,77**	-0,21	-0,09	0,10	-0,05	0,25	**0,59**	-0,07	1	**-0,53**	-0,03	0,20	**0,65**	-0,04
S	**0,55**	-0,29	0,04	0,10	0,09	0,03	0,14	-0,23	-0,09	**-0,53**	1	0,44	-0,13	-0,10	0,10
Si	-0,06	0,27	0,27	-0,04	0,44	0,29	0,14	0,43	0,32	-0,03	0,44	1	0,32	0,35	-0,26
Zn	**-0,57**	0,04	**0,55**	0,33	-0,08	-0,20	0,13	**0,53**	0,45	0,20	-0,13	0,32	1	-0,02	-0,35
TOC (mg L-1)	-0,11	**0,87**	-0,26	-0,29	**0,70**	**0,48**	**0,53**	**0,56**	-0,17	**0,65**	-0,10	0,35	-0,02	1	0,36
TIC (mg L-1)	0,42	0,14	-0,37	-0,18	**0,53**	**0,53**	**0,48**	-0,24	-0,45	-0,04	0,10	-0,26	-0,35	0,36	1

En gras, valeurs significatives (hors diagonale) au seuil alpha=0,05 (test bilatéral)

- Citrate (0,01 M)

	pH incubé	Al	Ca	Co	Cr	Cu	Fe	K	Mg	Mn	Ni	P	S	Zn	TOC (mg L-1)	TIC (mg L-1)
pH incubé	1	0,06	0,04	-0,05	0,20	0,34	0,25	0,01	0,06	0,10	0,32	-0,41	0,09	-0,07	**0,51**	0,28
Al	0,06	1	**-0,80**	-0,40	0,45	**0,66**	0,40	**-0,56**	-0,07	-0,23	**0,84**	0,42	**0,50**	0,00	-0,21	0,04
Ca	0,04	**-0,80**	1	0,46	**-0,51**	**-0,62**	-0,33	**0,67**	0,21	0,44	**-0,64**	-0,36	**-0,53**	0,28	0,21	0,18
Co	-0,05	-0,40	0,46	1	-0,25	-0,45	-0,08	0,02	-0,17	0,02	-0,41	-0,26	-0,20	0,12	-0,07	-0,06
Cr	0,20	0,45	**-0,51**	-0,25	1	0,21	**0,85**	**-0,55**	**-0,60**	0,29	**0,47**	-0,25	**0,98**	0,15	**-0,51**	-0,07
Cu	0,34	**0,66**	**-0,62**	-0,45	0,21	1	0,20	-0,29	0,20	-0,29	**0,72**	0,31	0,20	-0,19	0,24	-0,19
Fe	0,25	0,40	-0,33	-0,08	**0,85**	0,20	1	-0,40	**-0,49**	0,08	**0,54**	-0,18	**0,78**	0,29	-0,39	-0,09
K	0,01	**-0,56**	**0,67**	0,02	**-0,55**	-0,29	-0,40	1	**0,64**	0,16	-0,39	0,05	**-0,61**	0,02	**0,60**	0,05
Mg	0,06	-0,07	0,21	-0,17	**-0,60**	0,20	**-0,49**	**0,64**	1	-0,14	0,20	0,43	**-0,60**	-0,02	**0,72**	-0,09
Mn	0,10	-0,23	0,44	0,02	0,29	-0,29	0,08	0,16	-0,14	1	-0,19	-0,46	0,35	0,12	-0,18	0,00
Ni	0,32	**0,84**	**-0,64**	-0,41	**0,47**	**0,72**	**0,54**	-0,39	0,20	-0,19	1	0,31	0,46	0,15	0,03	-0,06
P	-0,41	0,42	-0,36	-0,26	-0,25	0,31	-0,18	0,05	0,43	-0,46	0,31	1	-0,21	-0,05	0,17	-0,10
S	0,09	**0,50**	**-0,53**	-0,20	**0,98**	0,20	**0,78**	**-0,61**	**-0,60**	0,35	0,46	-0,21	1	0,16	**-0,59**	-0,10
Zn	-0,07	0,00	0,28	0,12	0,15	-0,19	0,29	0,02	-0,02	0,12	0,15	-0,05	0,16	1	-0,39	**0,49**
TOC (mg L-1)	**0,51**	-0,21	0,21	-0,07	**-0,51**	0,24	-0,39	**0,60**	**0,72**	-0,18	0,03	0,17	**-0,59**	-0,39	1	-0,01
TIC (mg L-1)	0,28	0,04	0,18	-0,06	-0,07	-0,19	-0,09	0,05	-0,09	0,00	-0,06	-0,10	-0,10	**0,49**	-0,01	1

En gras, valeurs significatives (hors diagonale) au seuil alpha=0,05 (test bilatéral)

- EDTA (0,01 M)

	pH incubé	Al	Ca	Co	Cr	Cu	K	Mg	Mn	Na	Ni	S	Si	Zn	TOC (mg L-1)	TIC (mg L-1)
pH incubé	1	-0,32	-0,34	0,13	**0,58**	**0,52**	0,32	**-0,64**	-0,30	-0,32	-0,19	0,21	0,22	0,43	0,41	0,31
Al	-0,32	1	-0,35	-0,39	-0,14	0,15	-0,15	0,08	-0,23	**0,98**	0,37	-0,08	-0,16	-0,17	-0,02	-0,04
Ca	-0,34	-0,35	1	**0,52**	-0,32	-0,32	0,07	**0,55**	**0,79**	-0,24	-0,26	-0,30	-0,10	-0,10	-0,30	-0,25
Co	0,13	-0,39	**0,52**	1	-0,36	-0,14	0,02	0,16	0,16	-0,28	-0,35	-0,14	-0,22	-0,07	-0,21	-0,28
Cr	**0,58**	-0,14	-0,32	-0,36	1	0,37	0,41	-0,31	-0,24	-0,16	0,05	-0,13	0,03	**0,48**	**0,65**	**0,63**
Cu	**0,52**	0,15	-0,32	-0,14	0,37	1	0,34	-0,09	-0,28	0,10	0,39	0,01	0,26	0,33	**0,64**	-0,08
K	0,32	-0,15	0,07	0,02	0,41	0,34	1	0,21	-0,02	-0,15	0,17	-0,21	0,17	**0,70**	0,41	-0,17
Mg	**-0,64**	0,08	**0,55**	0,16	-0,31	-0,09	0,21	1	0,34	0,07	**0,48**	**-0,54**	-0,36	0,09	0,07	-0,39
Mn	-0,30	-0,23	**0,79**	0,16	-0,24	-0,28	-0,02	0,34	1	-0,17	-0,30	-0,14	0,16	-0,23	-0,42	-0,17
Na	-0,32	**0,98**	-0,24	-0,28	-0,16	0,10	-0,15	0,07	-0,17	1	0,21	-0,07	-0,18	-0,25	-0,09	-0,02
Ni	-0,19	0,37	-0,26	-0,35	0,05	0,39	0,17	**0,48**	-0,30	0,21	1	-0,36	-0,21	0,46	**0,57**	-0,23
S	0,21	-0,08	-0,30	-0,14	-0,13	0,01	-0,21	**-0,54**	-0,14	-0,07	-0,36	1	**0,79**	-0,31	-0,44	0,29
Si	0,22	-0,16	-0,10	-0,22	0,03	0,26	0,17	-0,36	0,16	-0,18	-0,21	**0,79**	1	-0,05	-0,25	0,08
Zn	0,43	-0,17	-0,10	-0,07	**0,48**	0,33	**0,70**	0,09	-0,23	-0,25	0,46	-0,31	-0,05	1	**0,65**	-0,02
TOC (mg L-1)	0,41	-0,02	-0,30	-0,21	**0,65**	**0,64**	0,41	0,07	-0,42	-0,09	**0,57**	-0,44	-0,25	**0,65**	1	0,08
TIC (mg L-1)	0,31	-0,04	-0,25	-0,28	**0,63**	-0,08	-0,17	-0,39	-0,17	-0,02	-0,23	0,29	0,08	-0,02	0,08	1

En gras, valeurs significatives (hors diagonale) au seuil alpha=0,05 (test bilatéral)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