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.

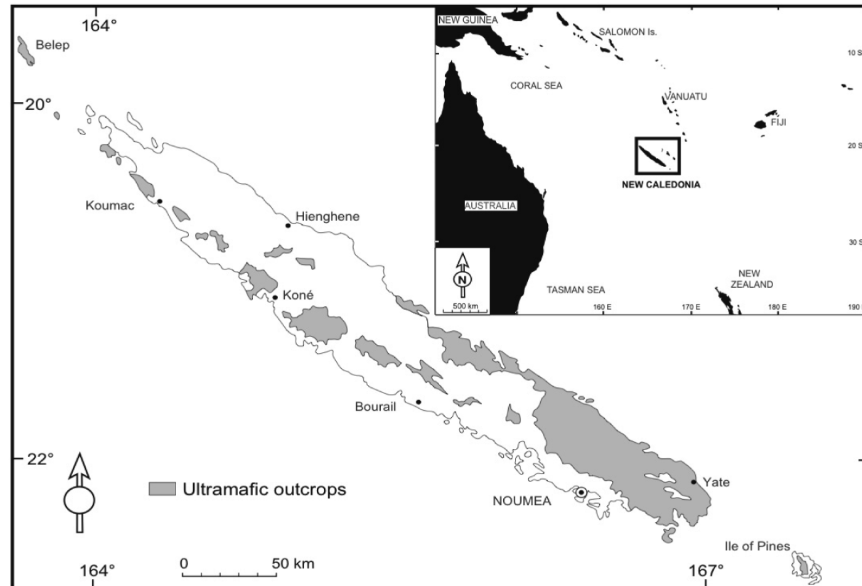

Fig. 1. Geographical map of the New Caledonian archipelago in the South Pacific Ocean with location of ultramafic massifs (in grey). Data from Perrier et al. (2006a).

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).

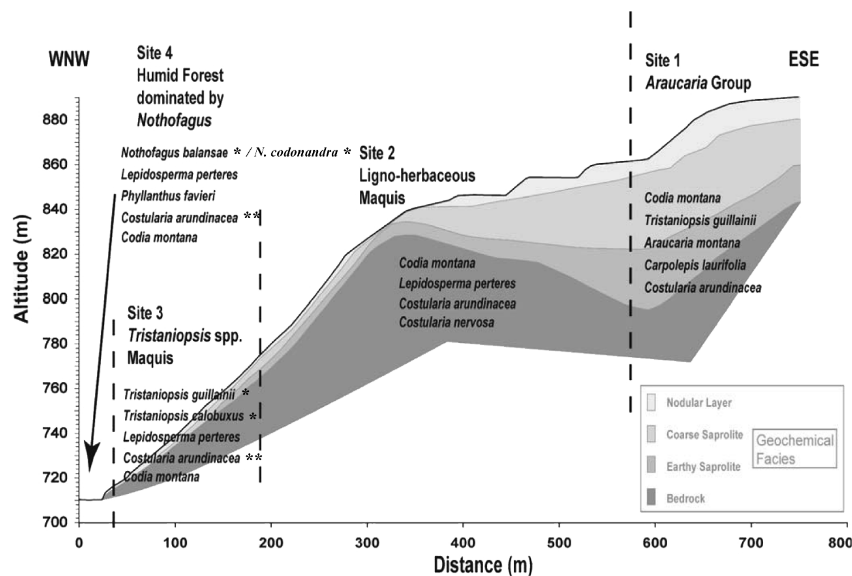

Fig. 2. Repartition of the four distinct plant formations (sites 1 to 4) on the topographic sequence studied by Perrier et al. (2006) at the Koniambo Massif. Most abundant and potential species for restoration purpose within each vegetation type are shown. Plants with ECM structures or ECM-like-structures 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

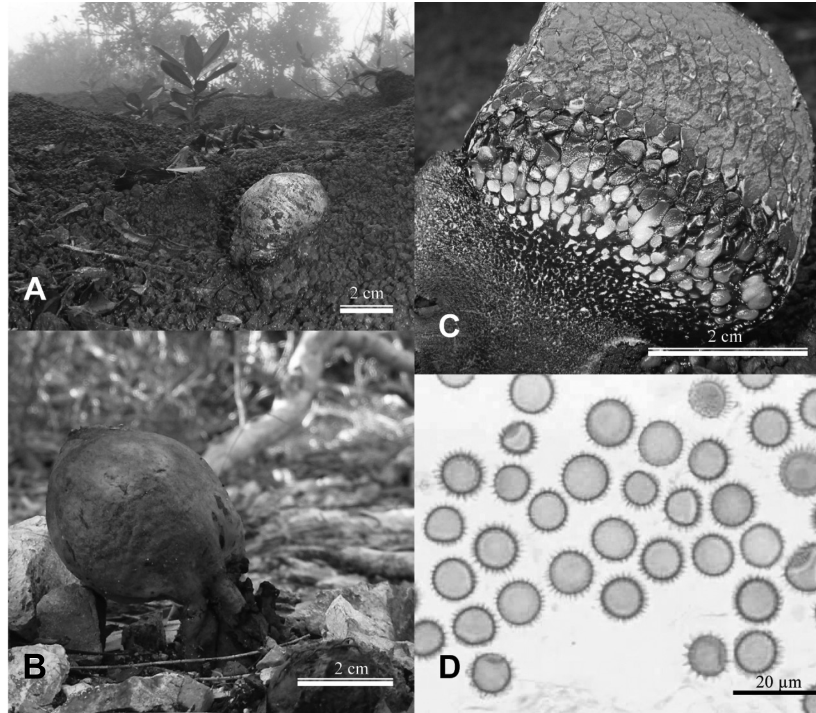

Fig. 4. *Pisolithus albus* from New Caledonia. A: *Pisolithus albus* MD07-117 from the Koniambo massif; B: *Pisolithus albus* MD07-228 from the Ouen-Toro, Noumea; C: cross section of *Pisolithus albus* MD07-166 from Pindjen water-fall and D: globose spores (8.77 to 9.62 µm) of *Pisolithus albus* MD06-379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

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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,

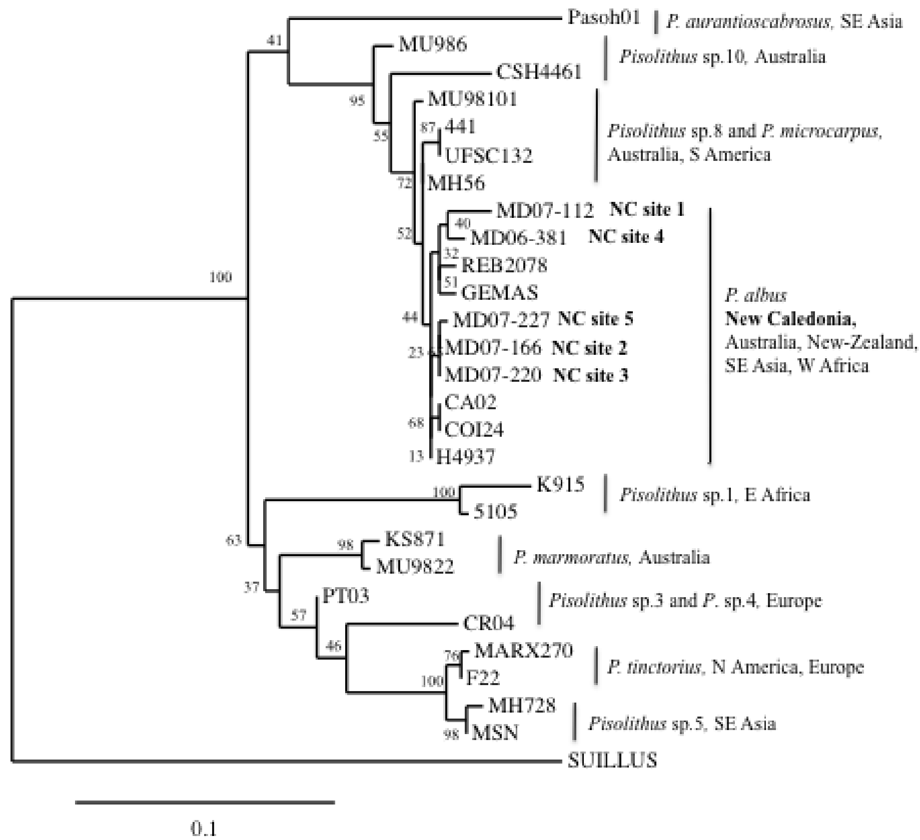
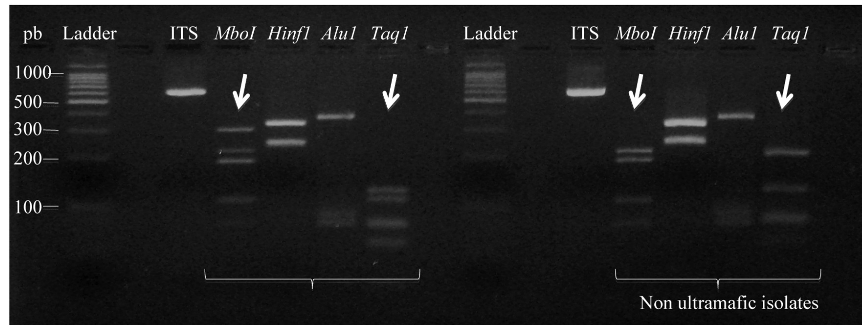


Fig. 5. Phylogenetic synthetic relationships among representative *Pisolithus* sp. from New Caledonia collection sites and worldwide reference isolates. The phylogeny is based on the analysis of the rDNA ITS1, 5.8S and ITS2 sequences. The tree was rooted with *Suillus luteus* ITS sequences. Significant 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

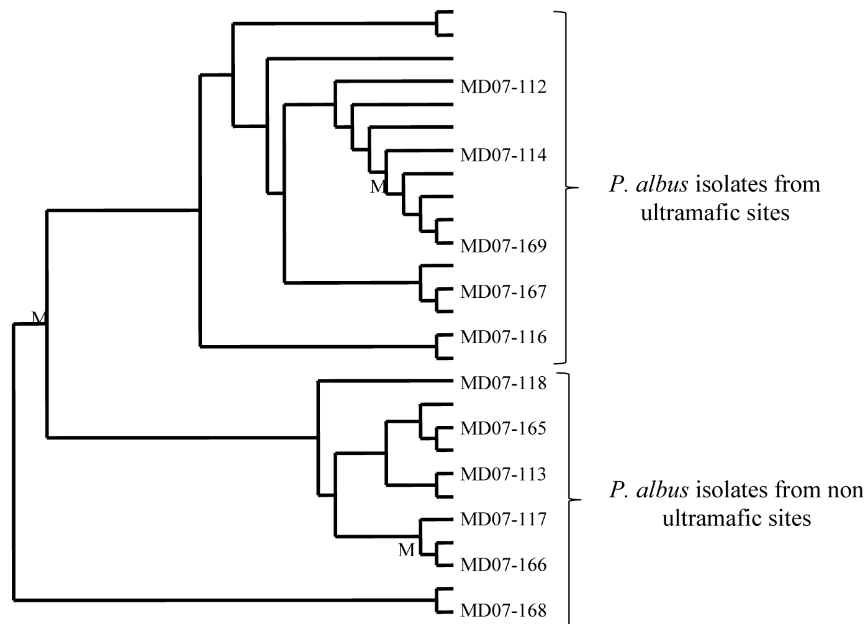


Fig. 6. A) Representative patterns of ITS restriction fragment length polymorphism (RFLP) profiles of *Pisolithus albus* isolates from both ultramafic and volcano sedimentary soils compared to both undigested amplified ITS and 100 pb DNA Ladder (Promega). Arrows highlight major differences between profiles. 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).

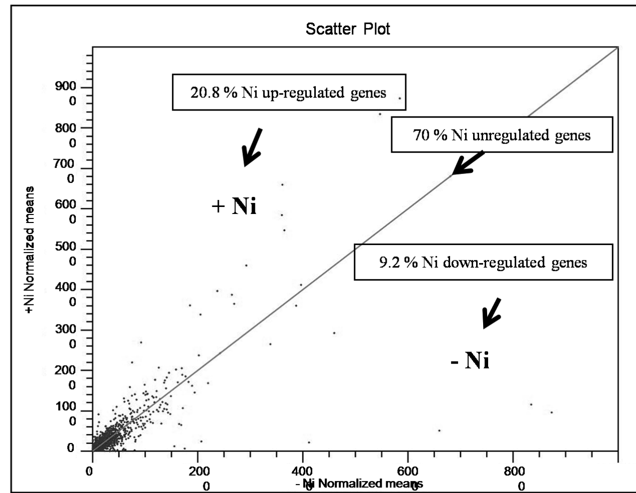
3.2.2 Pisolithus albus transcriptomic response to nickel

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*

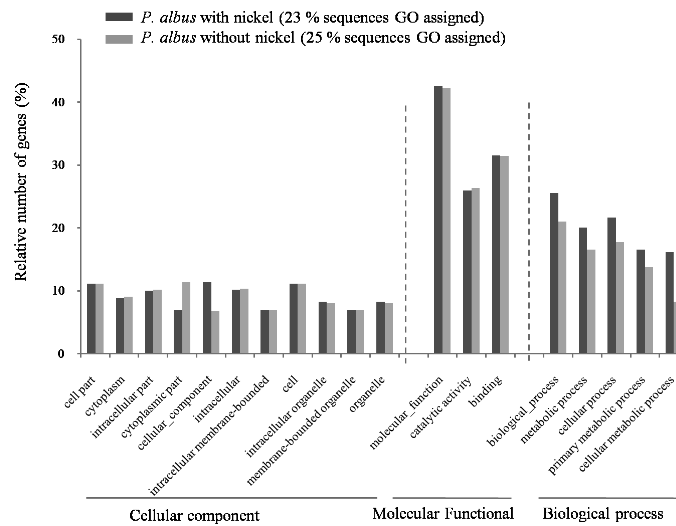


Fig. 7. A) Scatter plot presenting gene expression levels in *Pisolithus albus* Ni-tolerant ecotypefree-living mycelium grown without or with Ni at 250 µM. The expression levels of genes were normalized using a scale of 0 to 10,000. Each circle in the plot represents expression of one gene. B) Functional 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).

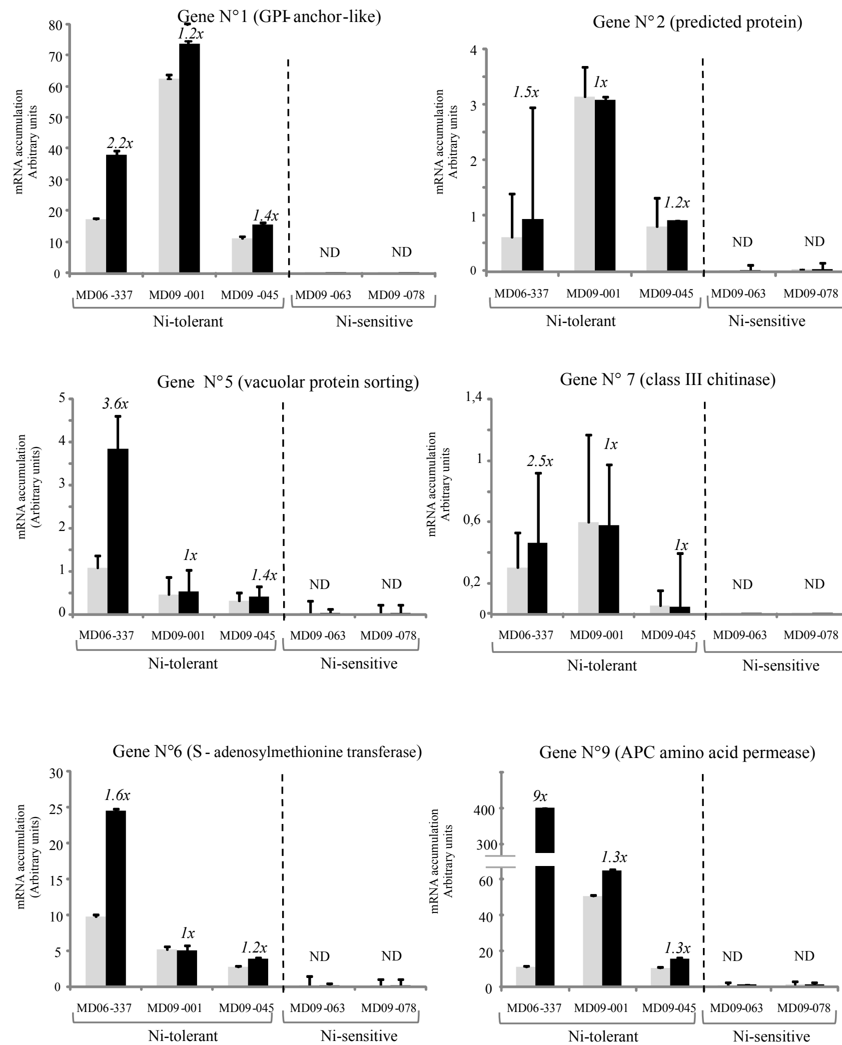


Fig. 8. Comparison of mRNA accumulation profiles for six selected Ni up-regulated genes in five *P. albus* isolates from ultramafic soil in presence of nickel 50 µM (black columns) and in absence of nickel (grey columns). Three nickel-tolerant isolates (MD06-337, MD09-045, and MD07-001) and two nickel-sensitive 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

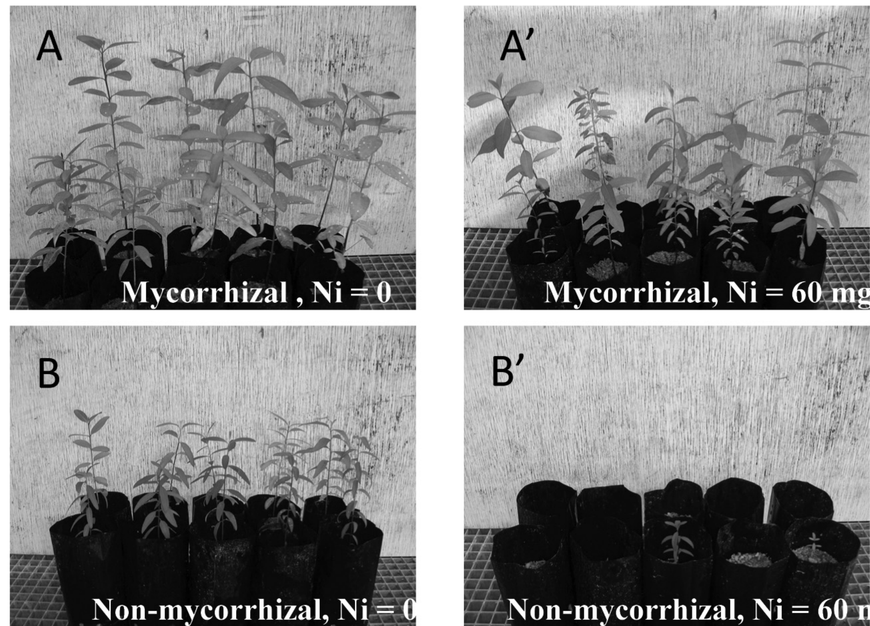


Fig. 9. *Eucalyptus globulus* seedlings after 12-weeks growth. A and A' mycorrhizal; B and B': non-mycorrhizal (controls). A and B: no nickel added; A' and B' seedlings treated with Ni. From Jourand et al. (2010b).

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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