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Tolerance and induction of tolerance to Ni of arbuscular mycorrhizal fungi from New Caledonian ultramafic soils

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Abstract The influence of Ni on arbuscular mycorrhizal fungi (AMF) has not been studied yet. We tested the tolerance to Ni of five AMF isolates from New Caledonian ultramafic soils. Spore germination indicated that these isolates were clearly more tolerant to Ni than three other isolates from non-ultramafic soils. They were able to germinate at 30 μg g^{-1} Ni, whereas spores of the non-ultramafic isolates were totally inhibited at 15 μg g^{-1} Ni. Among the ultramafic isolates, two were obtained from roots of Ni-hyperaccumulating plants. Their tolerance to Ni was clearly higher than all the other isolates. The proportion of germinated spores of the different isolates in contact with ultramafic soils showed the same tendencies as those observed with Ni solutions. Tolerance to Ni increased when spores were produced from mycorrhiza on plants grown on sand containing 20 μg g^{-1} Ni, in comparison with those produced on sand without Ni. These results indicate that the tolerance to Ni of AMF spores can be induced by the presence of this metal in the substrate.

Keywords Arbuscular mycorrhiza · New Caledonia · Ultramafic soils · Spore germination · Nickel · Induced tolerance

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Introduction

New Caledonian serpentine ecosystems developed on ultramafic rocks constitute original landscapes with a very high number of endemic plant species (Jaffré 1980; Proctor 2003). These ecosystems are threatened by nickel mining and other anthropogenic influences that have led to degradation of large areas of soil and vegetation. The restoration of these areas needs a better understanding of how plant growth can be stimulated. Indeed, ultramafic soils are characterised by high metal contents and phosphorus deficiency (Brooks 1987), and it is well known that arbuscular mycorrhizal fungi (AMF) can contribute to a better adaptation of plant species to such unfavourable soils (Jasper et al. 1989; Hildebrandt et al. 1999; Gupta and Kumar 2000). Our previous studies indicated that the fungal flora from New Caledonian ultramafic soils is tolerant to Ni and other metals (Amir and Pineau 1998) and that AMF are abundant in these soils (Amir et al. 1997; Perrier et al. 2006; Amir et al. 2007). Many recent publications have shown the capacity of AMF to reduce the absorption of toxic metals by plants (Vogel-Mikus et al. 2006; Janouskova et al. 2006; Audet and Charest 2007; Hildebrandt et al. 2007; Leung et al. 2007). Only two studies reported a few results on the effects of AMF on the absorption of Ni by plants (Guo et al. 1996; Vivas et al. 2005): the symbionts reduced Ni concentration in the shoots of bean and clover.

Studies on the tolerance of AMF to metals have mainly concerned Cd, Zn, Pb and Mn (Gildon and Tinker 1981; Weissenhorn et al. 1994; Tullio et al. 2003; Malcova et al. 2003; Pawlowska and Charvat 2004), but the influence of Ni on AMF activity has not yet been reported. The unique study (Amir et al. 2007), which concerned the mycorrhizal status of Ni-hyperaccumulating plants, showed that spores

of AMF isolated from roots of those plants are more tolerant to Ni than spores of other isolates. But are isolates from ultramafic soils generally more adapted to Ni than non-ultramafic isolates? The first part of the present study aims to answer this question.

Malcova et al. (2003) showed that cultivation of a *Glomus* spp isolate from Mn-contaminated soil for 2 years on a metal-free substrate reduced its tolerance to Mn. Are AMF grown in the presence of relatively high Ni concentration more tolerant to this metal than those obtained in metal-free substrate? The induction of tolerance by the presence of high concentrations of metals is known for a number of fungi (Valix et al. 2001; Averbeck et al. 2001; Clemens and Simm 2003; Courbot et al. 2004) but has not been reported yet for AMF.

We first evaluated the tolerance to Ni of spore germination of five AMF isolates from New Caledonian ultramafic soils in comparison with three other isolates from non-ultramafic soils. In a second experiment aiming to assess the induction process, we evaluated the level of Ni-tolerance of spores of two AMF isolates previously grown in sand containing 0 μg g^{-1} or 20 μg g^{-1} available Ni.

Materials and methods

Ultramafic soils

The main soils used here for AMF isolation and for germination tests are ultramafic soils with serpentine vegetation in the north and south mainland of New Caledonia. Three of them are ferralsols (lateritic soils) characterised by low organic matter content, low levels of the main mineral elements and very high iron content. The main physico-chemical characteristics of a representative sample were: pH H_2O 4.4; organic carbon 1.94%; total N 0.079%; Ca 0.02%; K, traces: Na, traces; Mg 1.68%; P 153 μg g^{-1}; Fe 62.16%; Al 7.43%; Mn 0.21%; Ni 0.32%; Cr 12.45%; Co 0.03%. The third soil was a hypermagnesian brown soil (pH H_2O 6.9; organic carbon 3.62%; total N 0.25%; Ca 0.49% K 0.03; Na 0.05; Mg 8.89%; P 123 μg g^{-1}; Fe 25.89%; Al 3.97%; Mn 0.57%; Ni 0.72%; Cr 1.90%; Co 0.07%).

AMF isolates

For AMF isolation, soils were collected under plants in non-serpentine dry forests (for control isolates), and in "maquis" and humid forest with serpentine vegetation. Spores were produced from these soils via trap cultures of sorghum. After wet sieving (53 μm) and centrifugation in 50% sucrose solution, five identical spores, selected after meticulous microscopic observation, were inoculated to a sorghum plantlet (monosporic isolation is difficult because the percentage of germination is relatively low). Plants were grown in a greenhouse for 5 months in a non-ultramafic soil poor in phosphorus, autoclaved at 120°C for 1 h. This soil contained 3 μg g^{-1} diethylene triaminopentaacetic acid (DTPA)[1] extractable Ni. The same procedure of purification was repeated twice after the development of mycorrhiza. Thus, all the AMF isolates were cultivated about 1 year on this non-ultramafic soil. All isolates are maintained at the University of New Caledonia.

Five *Glomus* spp. isolates from ultramafic soils were tested here: SFOCNL, isolated from Ouenarou region, in the south of the mainland (colluvial ferralsol, with 65 μg g^{-1} DTPA extractable Ni), SBH56, isolated from Plum region (hypermagnesian brown soil, with 175 μg g^{-1} DTPA extractable Ni), T2R3, isolated from Koniambo massif, in the north of the mainland (ferralsol, with 118 μg g^{-1} DTPA extractable Ni), Psychot1 and Sebert1, isolated from roots of *Psychotria douarrei* (Rubiaceae) and *Sebertia acuminata* (Sapotaceae), respectively, two strong Ni-hyperaccumulating plants (Jaffré et al. 1976; Boyd et al. 1999), in "Rivière Bleue" natural park in the south of the main land (ferralsol soil with 278 μg g^{-1} DTPA extractable Ni).

The three non-ultramafic *Glomus* spp isolates, used for the comparison, were obtained from three different sites. FSGL1 and FSCtAc were obtained from two soils under tropical dry forests situated, respectively, in the west mainland of New Caledonia, near Pouembout and in Pointe Maa about 30 km northwest of Noumea. The two are brown muddy soils with 0.01% total Ni, 4–7 μg g^{-1} DTPA extractable Ni. The third isolate FM2-1 was obtained from soil collected under shrubs near Nouville University in Noumea (calcareous brown soil with 0.008% total Ni, 5 μg g^{-1} DTPA extractable Ni).

Bioassay of Ni-tolerance of AMF spore germination

The spore germination test was adapted from Weissenhorn et al. (1993). Aliquots of 30 g of fine sand autoclaved 1 h at 120°C were placed into 50-mm diameter Petri dishes. A cellulose filter membrane (0.45 μm, 47 mm diameter) was applied on the surface of the sand of each plate. Eighty AMF spores obtained after wet sieving and centrifugation (see above), were placed on the membrane. Distilled water or Ni solution (NiSO4) was then added to the sand until saturation caused the membrane to stick to the sand by water capillarity. Seven Ni concentrations were used: 0, 5, 10, 15, 20, 30, 40 and 50 μg g^{-1} depending on experiments.

[1] The DTPA extractable concentration reflects the potentially mobilisable concentration of the element (potentially toxic), but not the available concentration at the present time, which is generally too low to allow a good comparison between samples.

Three replicates of each treatment were prepared. The Petri dishes were then sealed with parafilm. After 4 weeks incubation in the dark at 25°C, the membranes were removed, and flooded with acid glycerol trypan blue for 15 min. The membranes were then examined under dissecting microscope (×45) and the number of germinated spores was determined. The same technique was used to test the influence of soils on spore germination, but the sand was then replaced by the tested soil.

To study the induction of Ni tolerance, two AMF isolates from ultramafic soils (SFOCNL and SBH56) were previously inoculated on sorghum seedlings in 700-ml pots containing sterile sand. The pots, placed in greenhouse, were then watered with Long Ashton mineral solution (Hewitt 1966) with only 30% of the phosphorus content, and with 20 μg g^{-1} Ni solution added as sulfate salt (50 ml once a week). At the end of the culture, the sand in the pots contained an average of 32 μg g^{-1} Ni. Control pots without Ni were watered with Long Ashton solution and water. After 5 months, AMF spores were extracted as described before, and used for the same germination test.

Results

The percentages of AMF spore germination, after 4 weeks in sand with distilled water (control), ranged from 23% to 84% (Table 1). Except for the isolates collected from Ni-hyperaccumulator roots, germination decreased in the presence of nickel. The spores of the three non-ultramafic isolates germinated weakly at 10 μg g^{-1} Ni (3% to 30% of the control) and were totally inhibited at 20 μg g^{-1} Ni. Spore germination of the three isolates from ultramafic soils tolerated higher concentrations of Ni. Their values ranged from 58% to 77% of the control at 10 μg g^{-1} Ni, from 14% to 38% of the control at 15 μg g^{-1}, from 8% to 18% of the control at 20 μg g^{-1}, and from 1.8% to 12% of the control at 30 μg g^{-1}. These isolates were totally inhibited at 50 μg g^{-1} Ni. The two AMF isolates from Ni-hyperaccumulators showed a better tolerance to Ni than the other ultramafic isolates. They were not influenced by Ni until 30 μg g^{-1}. At 50 μg g^{-1} Ni, their germination percentages were 36.8% and 41.3% of the control.

The differences between three isolates chosen among each of the three defined groups were also apparent when the spores were germinated in contact with soils (Fig. 1). The non-ultramafic isolate (FSCtAc) was strongly inhibited in contact with an ultramafic soil, especially the soil taken under Ni-hyperaccumulator (over 10% of the control). The isolate from ultramafic soil (SFOCNL) germinated better than FSCtAc in contact with ultramafic soils (the values were two times higher), whereas the isolate from Ni-hyperaccumulator roots (Psychot 1) germinated on ultramafic soil as well as on non-ultramafic soil and was inhibited moderately in contact with soil under Ni-hyperaccumulator (53% of the control value).

Concerning the experiment on the induction of Ni-tolerance, the spores extracted from pots watered with Ni solution appeared to be more tolerant to Ni than those extracted from pots without Ni (Table 2). For the two native isolates, spore germination, in the presence of 10 to 40 μg g^{-1} Ni, were significantly higher when the spores were previously grown with Ni. The spores produced on plants grown without Ni had a very low germination percentage (5% of the control or less) at 30 μg g^{-1} Ni and were totally inhibited at 40 μg g^{-1}, whereas spores grown with Ni exhibited germination values higher than 29% of the control at 40 μg g^{-1}.

Table 1 Influence of different nickel concentrations on spore germination of 8 AMF isolates from New Caledonian ultramafic soils and non-ultramafic soils (percent of the control without Ni; values in brackets: effective percentage of germinated spores)

Ni (μg g^{-1})	Isolates from non-ultramafic soils			Isolates from ultramafic soils			Isolates from ultramafic soils, under Ni-hyperaccumulators[a]	
	FM2-1	FSCtAc	FSGL1	T2R3	SFOCNL	SBH56	Psychot1	Sebert1
0 (Control)	100 (53.2) a	100 (53.7) a	100 (84.3) a	100 (75.8) a	100 (33.3) a	100 (47.9) a	100 (29.8) a	100 (22.7) a
5	42.1 (22.4) b	56.1 (30.1) b	53.6 (45.2) b	86.3 (65.4) b	78.6 (26.2) b	71.4 (34.2) b	/	/
10	3.2 (1.7) c	29.9 (16.1) c	14.9 (12.6) c	77.2 (58.5) b	71.2 (23.7) b	58.0 (27.8) c	122.1 (36.4) a	99.4 (22.6) a
15	0 d	8.6 (4.6) d	0 d	37.7 (28.6) c	22.2 (7.4) c	13.8 (6.6) d	97.9 (29.2) a	125.1 (28.4) a
20	0 d	0 e	0 d	18.3 (13.9) d	8.7 (2.9) d	7.7 (3.7) d	104.4 (31.1) a	122.4 (27.8) a
30	0 d	0 e	0 d	11.8 (8.9) d	5.2 (1.7) d	1.8 (0.9) e	118.1 (35.2) a	99.1 (22.5) a
50	/	/	/	0 e	0 e	0 e	41.3 (12.3) b	36.8 (8.4) b

For each column, different letters indicate significant difference (Tukey–Kramer test). The horizontal comparison, for each Ni concentration, between the values of the three isolate groups (non-ultramafic, ultramafic and ultramafic under Ni-hyperaccumulators), indicates significant differences (for $p \leq 0.05$) between the three groups (ANOVA).

/ not determined

[a] Isolated from roots of *Psychotria douarrei* and *Sebertia acuminata*

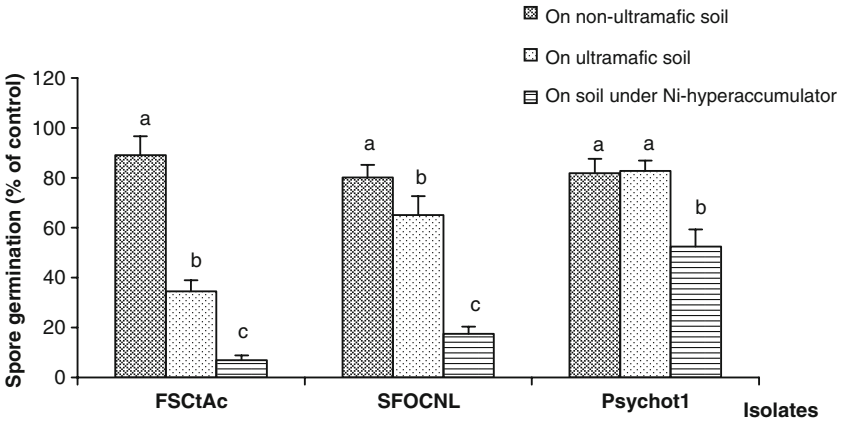

Fig. 1 Influence of different soils on spore germination of three AMF isolates from New Caledonian ultramafic or non-ultramafic soils. Spore germination is expressed as percent of the control (sand with distilled water). Non-ultramafic soil: Pouembout dry forest soil with 4 μg g^{-1} DTPA Ni; ultramafic soil: Ouenarou soil with 65 μg g^{-1} DTPA Ni; soil under Ni-hyperaccumulator: Rivière Bleue soil under *Psychotria douarrei* with 278 μg g^{-1} DTPA Ni. Different letters indicate significant differences at $p \leq 0.05$ (Tukey–Kramer test)

Discussion

Spore germination tests showed that the five AMF isolates from ultramafic soils were clearly more tolerant to Ni than those from non-ultramafic soils. The isolates collected from roots of Ni-hyperaccumulating plants were highly tolerant; indeed, spore germination was not inhibited up to 30 μg g^{-1} Ni and continued at 50 μg g^{-1} when all the other isolates were totally inhibited. Weissenhorn et al. (1994) also reported the relative high tolerance to Cd and Zn of spore germination of AMF isolates from metal-polluted soils. The limiting concentrations of Cd and Zn supported in that case were 10 to 30 μg g^{-1}. Tullio et al. (2003) found that AMF spores isolated from a Cd-polluted soil were more tolerant to this metal than spores from unpolluted soil. McCreight and Schroeder (1982) working on the effects of Ni on mycelial growth of ectomycorrhizal fungi, recorded tolerance limits ranging from 10 to 40 μg g^{-1}.

Generally, the DTPA extractable concentrations of Ni in New Caledonian ultramafic soils vary from 1 to 300 μg g^{-1} (Amir and Pineau 2003; Perrier et al. 2006). These values do not reflect the concentrations of this element in the soil solution, but more closely reflect the potentially mobilisable Ni. For example, one of the soils used here contained 118 μg g^{-1} Ni-DTPA, but had only 12 μg g^{-1} Ni-KCl (Ni-KCl reflects better the available Ni, but is generally too low to allow comparisons). Therefore, the tolerance of AMF to 20–40 μg g^{-1} Ni can be sufficient for their adaptation to this environment. However, under Ni-hyperaccumulator plants, the concentration of available Ni can be clearly higher.

Viable *Glomus* spores were found in soils containing more than 1,000 μg g^{-1} Ni-DTPA under Ni-hyperaccumulating plants; roots of *Psychotria douarrei*, generally colonised by AMF, contained 7,700 μg g^{-1} Ni-DTPA (Amir et al. 2007). It is therefore clear that this highly toxic microenvironment creates conditions, which could cause selection, thereby explaining why the isolates from the two Ni-hyperaccumulators studied here were far more tolerant than the other isolates. The relatively high tolerance of

Table 2 Influence of different nickel concentrations on spore germination of two AMF isolates from New Caledonian ultramafic soils, previously grown on sand with Ni or without Ni (percent of the control without Ni; values in brackets: effective percentage of germinated spores)

Ni (μg g^{-1})	SFOCNL		SBH56	
	Grown without Ni[a]	Grown with Ni[a]	Grown without Ni[a]	Grown with Ni[a]
0	100 (56.2)	100 (46.3)	100 (62.7)	100 (55.7)
10	61.2 (34.4)	90.2* (41.8)	59.6 (37.4)	71.8* (40.0)
20	18.9 (10.6)	89.8* (41.6)	11.6 (7.3)	66.7* (37.2)
30	5.1 (2.9)	50.3* (23.3)	2.9 (1.8)	35.2* (19.6)
40	0	42.7 * (19.8)	0	29.4* (16.4)

[a] The spores of the two isolates were obtained from pots grown in greenhouse with sorghum, in sand watered with 0 μg g^{-1} Ni, or 20 μg g^{-1} Ni solution (at the end of the culture the sand contained 33 μg g^{-1} and 31 μg g^{-1} Ni, respectively, for SFOCNL and SBH56). The asterisks (*) indicate significant differences (for $p \leq 0.05$) between these values and the corresponding values for the isolate grown without Ni, for each Ni concentration (ANOVA).

AMF isolates from ultramafic soils compared with those from non-ultramafic soils suggests that native isolates could be more efficient for ecological restoration of degraded mining areas.

The spores used here in the first experiment were obtained from mycorrhiza maintained in sand devoid of metals. This was sufficient to show the better adaptation to Ni of the isolates from ultramafic soils, but not sufficient to determine the maximal level of Ni-tolerance of these isolates. Indeed, it is important to know if spores formed in contact with Ni could develop a better tolerance to this metal (induction). Results clearly support this hypothesis: Ni-tolerance of AMF isolates changed in response to different Ni concentrations in the cultivation substrate. Malcova et al. (2003) reported a reduction in Mn-tolerance of a *Glomus* spp isolate kept 2 years on maize in a metal-free substrate. In our experiments, AMF isolates were maintained about 1 year in a non-ultramafic soil containing a low concentration of extractable Ni, but not totally metal-free.

The level of Ni-tolerance of some isolates could have been reduced, which would explain the apparent induction of metal tolerance in spores obtained from mycorrhizal plants growing on Ni-supplied sand. However, the three groups of AMF isolates (non-ultramafic, ultramafic, isolated from hyperaccumulating plants) still showed clear differences in their Ni-tolerance level.

The induction of metal tolerance reported for yeasts and other fungi has been related to the activation by high metal concentrations of metallothionein or/and phytochelatin synthesis (Averbeck et al. 2001; Clemens and Simm 2003; Meharg 2003; Jaeckel et al. 2005). The mechanisms were studied mainly for Cd, Zn and Cu, but not for Ni. Few studies have considered the mechanisms of metal-tolerance in AMF. Joner et al. (2000) and Gonzales-Chavez et al. (2002) reported that AMF isolates from metal-contaminated soils have the ability to concentrate metals on external parts of hyphal walls. The metals are also accumulated inside vacuoles (Meharg 2003; Göhre and Paszkowski 2006). Lanfranco et al. (2002) showed the existence of a metallothionein-like polypeptide in *Gigaspora margarita*. Meharg (2003) reported the presence of metallothioneins in endomycorrhizal systems. According to Ouziad et al. (2005) and Hildebrandt et al. (2007) metallothioneins, glutathione and heat shock proteins could be involved in mechanisms of metal tolerance in AMF; some of their genes have been identified.

The regulation of metal transport through cell membranes has also been investigated in AMF. In particular, a Zn transporter gene (*GintZnT1*) and an ABC transporter gene (*GintABC1*) involved in Cd and Cu detoxification have been found in *Glomus intraradices* (Gonzalez-Guerrero et al. 2005; Hildebrandt et al. 2007). However, no studies have focused on Ni; for this reason, our current and future experiments concern especially the molecular mechanisms of Ni tolerance in AMF.

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