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Arbuscular mycorrhizal fungi and sewage sludge enhance growth and adaptation of *Metrosideros laurifolia* on ultramafic soil in New Caledonia: a field experiment.

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ABSTRACT

The ecological restoration of nickel mining-degraded areas in New Caledonia is strongly limited by soil mineral nutrient deficiencies, heavy metal toxicity and slow growth rate of the native plant species. The improvement of revegetation technics needs multidisciplinary knowledge. A field experiment with relocated topsoil was assessed to test plant inoculation with a mix of three selected arbuscular mycorrhizal fungi (AMF) combined with sewage sludge amendment of the soil. *Metrosideros lauriflora* seedlings, an endemic Myrtaceae, were inoculated with the mixed AMF isolates and grown in a nursery for 18 months before being planted. 528 days after plantation, the dry weight of inoculated plants was 4 times higher than non-inoculated

ones. AMF inoculated plants growing in sewage sludge amended soil showed a dry weight more than seven times higher than control plants. These differences were positively correlated with mycorrhizal colonization. However at this stage, AMF inoculated isolates were reduced in roots of *M. laurifolia* and replaced by several AMF indigenous species. This AMF diversity was higher in inoculated plants and in non-amended plots. Inoculated plants were characterized by a better mineral nutrition, a higher Ca/Mg ratio and a lower heavy metal translocation. In conclusion, this study showed that AMF inoculation combined with sewage sludge amendment of soil can improve ecological restoration of ultramafic mine-degraded areas.

HIGHLIGHTS

- First field experiment showing effects of AMF on plant growth in ultramafic soil.
- Strong cumulative effects of AMF and sewage sludge amendment on plant growth.
- Clear improvement by AMF of Ca/Mg ratio of the plant (adaptive mechanism).
- Clear reduction by AMF of heavy metal translocation in plant (tolerance mechanism).
- Decline of inoculated AMF isolates in roots, coupled with increase of AMF diversity.

Keywords : Serpentine soil, AMF, sewage sludge, field experiment, plant nutrition, heavy metals.

1. Introduction

New Caledonian ultramafic ecosystems, which cover one third of the main island, are characterized by poor and toxic soils, with very low concentrations of P, K and N, a low Ca/Mg ratio, along with high levels of heavy metals particularly Ni, Co, Cr and Mn (Jaffré and L'Huillier, 2010a; Proctor, 2003). Most of these soils are occupied by ultramafic maquis (a shrub heathland) subject to large fluctuations of water availability ranging from waterlogging to drought (Jaffré and L'Huillier, 2010a, 2010b). The adaptive traits of the plants to these constraints (low growth rate, xenomorphic foliage, etc.) are termed "serpentine syndrome" (Bini and Maleci, 2014; Jenny, 1980). Due to these extreme conditions (but also to insularity), about 89% of the vascular plants of New Caledonian maquis are endemic (Jaffré et al., 2004) and have developed different specific mechanisms to cope with these constraints (Baker, 1981; Bini and Maleci, 2014). *Metrosideros laurifolia* (Myrtaceae) is an endemic shrub (sometimes becomes a tree), with coriaceous leaves, that is considered often a pioneer species in these extreme ultramafic soils and frequently used for ecological restoration of mining-degraded areas (Wulff et al., 2010).

Arbuscular mycorrhizal fungi (AMF) are known to be involved in plant mineral nutrition by increasing the soil volume explored by the roots (Smith and Smith, 2011). They are also involved in plant adaptation to water stress (Augé, 2001; Smith et al., 2010) and to heavy metal toxicity in metal polluted soils (Amir et al., 2014; Ferrol et al., 2016; Khade and Adoleya, 2007; Wang, 2017). AMF are abundant in New Caledonian ultramafic soils (Amir and Ducouso, 2010; Perrier et al., 2006) and contribute to plant adaptation to ultramafic conditions, particularly by improving P

absorption and increasing plant metal tolerance (Amir et al., 2013; Lagrange et al., 2011).

In New Caledonian ultramafic maquis, the soils are generally poor in organic matter (Jaffré and L'Huillier, 2010a). Sewage sludge is one of the amendments used to enrich the topsoil (or the lateritic subsoil, when there is no topsoil available) before the revegetation of degraded areas after mining (L'Huillier et al., 2010). It is known to improve soil fertility, especially in forest soils (Henri and Cole, 1986) and degraded areas (Delschen, 1999; Petersen et al., 2003). Different positive effects of this sludge have been reported: improvement of soil structure and soil stabilization (Mata-Gonzalez et al., 2002; Ojeda et al., 2008), soil enrichment with phosphorus and other elements (Garcia et al., 1991), stimulation of microbial activity (Petersen et al., 2003; Selivanovskaya and Latypova, 2006), inducing clear increase of plant growth (Shen et al., 2007; Wang et al., 2004) and stimulating plant recolonization of former mine sites (Rate et al., 2004).

Only few field experiments have been reported on ultramafic soils. Some concerned the effects of soil fertilization on plant growth and species dynamics (Chiarucci and Maccherini, 2007). As far as we know, the only field experiment on mycorrhizal symbiosis (Lagrange et al., 2013) was about the effects of P fertilisation on mycorrhizal status of *Costularia comosa* (Cyperaceae). The field experiment presented here was the first using AMF inoculation.

Different experiments combining AMF inoculation and sewage sludge in non-ultramafic soils have been reported. Some of them showed complementary effects between this organic input and AMF inoculation on plant growth and nutrition in greenhouse (Arriagada et al., 2009; Mackay et al., 2017), or in field conditions (Algacil

et al., 2004). Another experiment recorded an inhibition effect of sewage sludge on AMF root colonization (Jacquot et al., 2000).

We inoculated *M. Laurifolia* seedlings with AMF in a nursery and then transferred them to a test site consisting of a degraded laydown platform area that had been covered with a stored topsoil layer ready for revegetation (the topsoil is frequently stored during mining activities until revegetation space available). A combination of AMF inoculation and sewage sludge amendment was also tested. The aims of the experiment were : i) to test the effects of AMF on growth and adaptation parameters in field conditions; ii) to assess the effects of this inoculation on natural AMF diversity; iii) to test the influence of sewage sludge on mycorrhizal symbiosis and plant growth in these conditions.

2. Materials and methods

2.1. Experimental site description.

New Caledonia is located in the South Pacific, approximately 1,500 km east of Australia and 1,800 km north of New Zealand (Fig. S1). The climate is subtropical to tropical, with a rainy season from December to May and a dry season from September to November that can vary in duration and severity.

The experimental site is located in Goro, in the south of the main land, inside the VALE New Caledonia mining company area (22°19'13.2"S 166°54'16.0"E) (Fig. S1). The rainfall level is high: average of 3490 mm/year. Mean monthly temperature in the area of Goro is highest in February with 25.0°C and lowest in August with 19.2°C. The area was situated at an altitude of 180 m and was an ultramafic maquis before the soil and

vegetation were stripped. The experimental field was plane and homogeneous, with a slight slope (2%).

Platform used for the test was cleared of vegetation and topsoil, and the degraded topsoil, stored for 4 years, was then spread on the area as a 20 cm thick layer before the experiment, with grader and frond end loader to reduce soil compaction. The use of a degraded topsoil has two advantages: i) topsoil storage by mining companies is a frequent practice and AMF inoculation is then particularly useful, because of the strong reduction of natural AMF populations in these conditions (Bordez, 2014) ; ii) the stored topsoil has then a low density of AMF spores, so that the difference between the non-inoculated plots (control) and the inoculated ones was sufficient to highlight the effects of the mycorrhization. Preliminary analyses before the experiment showed that this topsoil contained only 22 AMF spores per 100 g, whereas the soil collected in the non-perturbed maquis close to experimental field showed 4489 spores per 100 g.

The topsoil was a ferralsol (Becquer et al., 2001); its main physico-chemical characteristics are as follow : organic carbon (%): 1.04 ; total N (%): 0.045 ; C/N: 23.48 ; pH (H₂O): 5.75 ; available P (Olsen, mg kg⁻¹): 1.63 ; total P₂O₅ (%): 0.01 ; CaO (%): 0.02 ; K₂O (%) : 0.01 ; Na₂O (%) : 0.01 ; MgO (%) : 0.30 ; SiO₂ (%) : 0.89 ; Fe₂O₃ (%) : 91.28.8 ; Al₂O₃ (%) : 5.37 ; total Mn (g kg⁻¹): 4.74 ; total Ni (g kg⁻¹): 5.62 ; total Co (g kg⁻¹): 0.47 ; total Cr (g kg⁻¹): 32.25 ; DTPA extractable Mn (mg kg⁻¹): 282.9 ; DTPA extractable Ni (mg kg⁻¹): 19.73; DTPA extractable Co (mg kg⁻¹): 79 ; DTPA extractable Cr (mg kg⁻¹): 0.6.

2.2. Plant production in nursery

Metrosideros laurifolia is an endemic Myrtaceae. Seeds were surface-disinfected by soaking in hot water (80°C for 5 minutes) before being sown on sterilised substrate

constituted with a mix of equal volumes of compost and vermiculite. After 3 months, the seedlings were transferred on 750 mL plastic bags with a sterilised mix of equal volumes of sand, compost and vermiculite. At this stage, the stem of the seedlings was only 2-3 cm height.

2.3. Plant inoculation with AMF in nursery

Two hundred seedlings were inoculated with AMF three months after germination, during the transfer to the plastic bags. We used a mix of three selected isolates from ultramafic soils, previously tested in greenhouse conditions and described (Gensous, 2014). They were isolated from soil taken under plants in ultramafic maquis in the south of Grande Terre (Demazures), by trap culture with sorghum. They were selected for their tolerance to Ni, their spore production and their effect on sorghum growth. These isolates have been characterized by a sequence analysis of a part of the SSU rDNA: PSB1 (98% of similarity with *Claroideoglossum etunicatum*), PSO1 and RARC (the two showed 98% of similarity with *Acaulospora rugosa*). The accession numbers are as follow: PSB1: Gb_MH424515 ; PSO1: Gb_MH424516; RARC: Gb_MH424517. Their morphological characteristics confirmed this identification. The plants were inoculated with a mix of spore suspensions of the three isolates at the following proportions (in relation to the spore production of each isolate): PSB1: 60%; PSO1: 20%; RARC: 20%. Spores of the AMF isolates were produced on a sorghum culture grown for 5 months, on a non-ultramafic soil (sorghum does not grow correctly in ultramafic soil), autoclaved three times at 120°C for 1 hour, with an interval of 24h. The spores were isolated by wet sieving (50 µm) and centrifugation using a 50% sucrose solution and thoroughly rinsed with water. For each plant, two mL

of suspension containing 100 spores were applied on the root system. For the two hundred non-inoculated plants, the same treatment was assessed but the spore suspension was autoclaved 30 min at 120°C before the application on root system. All the plants were then grown for 15 months in the nursery to reach a sufficient size to resist environmental stresses: plants of New Caledonian ultramafic maquis have a low growth rate and small plants could be killed by the water stress.

2.4. Field experiment

The total surface of the experimental field was about 450 m². Four treatments were assessed: non-inoculated plants growing in non-amended topsoil (control); plants inoculated with AMF growing in non-amended topsoil; non-inoculated plants growing in a topsoil amended with sewage sludge; and combined treatment: inoculated plants, in topsoil amended with sewage sludge. For each treatment, four plots of 16 m² representing experimental units were planted with 25 plants separated each from other by 80 cm to reduce contamination. Two-meter bands separated adjacent plots and allowed for easier evaluation of plants while at the same time reducing contamination. To avoid any substrate influences between adjacent plots of different treatments, we chose to place the plots of each treatment on a line following the direction of the slight slope. As the topsoil was carefully homogenised before being spread on the site, a randomized block was not necessary here and would create more difficulties to control the influences between adjacent plots of different treatments.

The plantation was performed in May 2012. At this stage, the height of the plant stems varied from 6 to 18 cm. Holes of 25 cm diameter and 30 cm depth were prepared for the plantation. About 100 g of a water retention agent (biopolymer of

hyaluronic acid) were mixed with soil in the bottom of the hole to ensure greater moisture availability during post seedling plantation acclimatization. For the treatments without sewage sludge, each plant was deposited inside base of the hole which was then mixed with surrounding topsoil. For the amended treatments, 158 g of sewage sludge were carefully mixed with the topsoil inside the hole. This amount of sludge corresponds globally to about 1.5 t DW.ha⁻¹, but the content inside each hole is a lot higher (about 20 t DW. ha⁻¹). The characteristics of the sludge are as follow: dryness: 57.3%; mineral matter: 87.2%; organic matter: 12.8%; pH: 6.2; total N: 22.73 mg.g⁻¹; total P: 4.44 mg.g⁻¹; total Cu: 90 mg.kg⁻¹ ; total Zn: 960 mg.kg⁻¹ ; total Cd: < 0.5 mg.kg⁻¹ ; total Hg: 0.7 mg.kg⁻¹ ; total Pb: 23 mg.kg⁻¹.

2.5. Plant growth evaluation

After plantation, plant height was measured each 3 to 5 months during 23 months and a final measure was performed after 41 month to check the persistence of the observed effects. After 17.3 months (528 days), 12 plants randomly selected inside the 4 plots were removed for each treatment. The fresh and dry weight (at 45°C) of the shoot biomass was also determined. The roots were not included because at this stage, the root system was large and very difficult to remove totally.

2.6. Mycorrhizal colonization

Mycorrhizal colonization was estimated 528 days after plantation. For each treatment, fine roots of 6 randomly sampled plants were taken for mycorrhizal colonization assessment (for each plant, the roots were collected from different parts

of the root system). The roots were stained with Trypan blue (Koske & Gemma, 1989). Root fragments were placed in a 10% KOH bath at 90°C for 90 minutes. A few drops of hydrogen peroxide (H₂O₂) were added after one hour to lighten the brown colour. The roots were then rinsed in distilled water and stained with Trypan blue (15 minutes at 90°C). For each plant, at least 25 root segments of 1-2 cm length were mounted on glass slides in 10% glycerol and observed under an optical microscope. A root segment was considered to be mycorrhized when arbuscules, vesicles or intracellular hyphal coils were clearly identified. The intensity of arbuscular mycorrhizal colonization, i.e. mycorrhizal density (M%), was determined according to Trouvelot et al. (1986).

$$M (\%) = \frac{95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1}{\text{total number of root fragments}} \quad \text{where } n_5 \text{ is}$$

the number of root fragments with more than 90% infection, n_4 between 90% and 50% infection, n_3 between 50% and 10% infection, n_2 between 10% and 1% infection, n_1 less than 1% infection.

2.7. Chemical analyses

To assess the plant mineral nutrition, for each treatment, five samples of washed roots, stems and leaves were dried and ground. The roots (only fine) were cleaned thoroughly with water in a small colander and checked meticulously to avoid any soil particles. The ground samples were then used to determine total P, total N, K, Ca and Mg contents, after digestion in microwave with potassium persulfate (K₂S₂O₈) and H₂SO₄ and analysed by ICP-OES and by CHN, mass spectrometry using an Integra2 spectrometer (Sercon Instruments, www.sercongroup.com) linked to a Sercon elemental analyzer. These analyses were performed by the Laboratoire des Moyens Analytiques (LAMA, IRD-Noumea). Ca/Mg ratio was then calculated. Metal contents (Fe, Mn, Co,

Ni, Cr) in roots, stems and leaves were also determined after microwave acidic mineralization in aqua regia (means of 4 samples) and analysis with an atomic absorption spectrometer (Perkin Elmer Analyst 800).

2.8. DNA extraction, PCR and sequencing.

Root samples of *M. laurifolia* were collected 335 days after plantation. Three plants of each treatment were sampled. For each plant, fine root tips collected on two sides of the root system, were gathered and grinded in liquid nitrogen with a mortar and a pestle. The resulting powder was used to extract genomic DNA using the Ultraclean Plant DNA extraction kit (MoBio - ref # 13000-250) according to the manufacturer's instructions.

A 550 bp partial fragment of SSU rDNA was produced using a nested PCR approach in order to avoid cross amplification of non-AMF DNA. First PCR was performed using the AML1 and AML2 primers (Lee et al., 2008), in a 25 μ l reaction containing 1X AmpliTaq[®] 360 PCR buffer (ABI), 2.5 mM MgCl₂, 200 μ M dNTP, 0.4 μ M of each primer and 0.5 U AmpliTaq[®] 360 DNA polymerase. Reaction conditions were as follow: one initial denaturation step at 95 °C for 3 min; 30 cycles of: 94 °C for 45s, 50 °C for 45s, 72 °C for 45 s; 72°C for 10min. One microliter of the PCR product was used to perform the second PCR amplification with primer pair AM1 (Helgason et al., 1998) and NS31 (Simon et al., 1992). The PCR was performed in the same conditions except the annealing temperature that was set to 55°C.

The PCR products were cloned into the pGEM-T vector (Promega) following the manufacturer's instructions. Ligated plasmids were transformed into CaCl₂ competent *E. coli* DH5 α cells using a heat-shock approach. The transformed bacteria were plated

into Luria-Bertani (LB) solid medium containing ampicillin ($50 \mu\text{g mL}^{-1}$) and grown overnight at 37°C . A PCR using the universal M13F and M13R primers was performed directly on bacterial colonies to screen for positive clones. Clones that exhibited fragments with the expected size were Sanger sequenced on an Applied Biosystems 3730xl capillary sequencer with the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with PCR amplicons obtained from positive clones.

2.9. Sequence analyses and construction of phylogenetic tree

DNA sequences were proofread and trimmed to remove primer sequences before being aligned using MAFFTv7 (Kato & Standley, 2013) and grouped into Operational Taxonomic Units (OTUs) using the average-neighbour algorithm of the MOTHUR program with a 97% sequence similarity cut off (Schloss et al., 2009). A representative sequence for each OTU, reference sequences isolated from the *MaarjAM* database (Öpik et al., 2010) and consensus sequences as determined by Krüger *et al.* (2012) were subsequently aligned as described above. The alignment was used to construct a neighbour-joining tree with MEGA6 using a Kimura 2-parameter sequence evolution model. Statistical confidence of branching was determined based on 1000 bootstrap resamplings (Felsenstein, 1985).

The representative rDNA sequences for each OTU were submitted to GenBank and can be retrieved under the accession numbers MH150839 – MH150870.

2.10. Statistical treatments

Data were expressed as means with standard errors. The data were compared by ANOVA using the statistical software R, with a post-hoc Bonferroni corrected Mann-

Withney test. Total correlation test was also used to analyse the correlations between mycorrhizal colonization parameters and plant growth.

3. Results

3.1. Plant growth

At the beginning of the field experiment, plants of *M. laurifolia* that have been inoculated with AMF in nursery were significantly bigger than non-inoculated ones (Fig. 1). This difference remained constant all along the experiment period, while the plants height increased progressively. After 41 months, the mean values of plant height reached 14.3 cm and 29.7 cm respectively for non-inoculated and inoculated plants. In soil amended with sewage sludge, the plants grew faster than the others and finally caught up with the height of the inoculated plants. The combination “AMF + sludge” showed the better effect, with plant height being 2.6 times higher than the control after 528 days and 3.1 higher after 41 months. The visual differences between the tested treatments are very clear (Figure S2).

The dry weight of the shoots, measured 528 days after plantation, attained a mean of 15.4 g for the control plots (Fig. 2). The control was significantly different from the other treatments ($P = 0.002$). The weight was four times greater for AMF inoculated plants. The mean value in plots amended with sludge was 3.1 times greater than the control. The combination “AMF + sludge” was significantly different from the two separate corresponding treatments ($P = 0.018$ and 0.005). It showed mean values of shoot biomass 7.7 times greater than the control (119.4 g).

3.2. Mycorrhizal colonization

Mycorrhizal colonization (M) expresses the percentage of root cortex occupied by AMF. After 528 days the mean value of M for non-inoculated plants was relatively weak (3.3% for the control) (Fig. 2). Preliminary analyses before the experiment showed that the stockpiled topsoil of the experimental field contained few viable spores before plantation (22 AMF spores per 100 g). Even if sewage sludge treatment with non-inoculated plants showed M value higher than the control (10.9%), the difference was not significant ($P = 0.103$). AMF inoculation significantly enhanced mycorrhizal colonization, in comparison with control ($P = 0.009$), but the values stayed moderate (12.4%). The values of mycorrhizal colonization were higher for the combination "AMF + sludge" (22.8%), but non-significantly different from AMF treatment, because of the high variability of the mycorrhization in sewage sludge amended plots.

A significant positive correlation between shoot biomass and mycorrhizal colonization was detected ($r = 0.378$; $p < 0.05$).

Mycorrhizal frequency values (F), expressing the percentage of root fragments containing AMF, were: $35.6 \pm 12.7\%$ (control), $47.2 \pm 14.1\%$ (sludge), $72.2 \pm 5.6\%$ (AMF) and $85.0 \pm 9.8\%$ (AMF+ sludge). The values of F for the two treatments with inoculated plants were significantly different from the non-inoculated ones ($P < 0.05$), but not different from each other. Microscopic aspects of the mycorrhizal colonization are showed in Figure S3.

3.3. Mineral nutrition

Contents of the main mineral elements in *M. laurifolia* leaves 528 days after plantation were relatively low (Table 1). P content of leaves was significantly enhanced

by sludge amendment ($P < 0.05$). AMF inoculation did not increase this value; but the combination of sludge and AMF inoculation showed the best effect on this element (1.8 times higher than the control; ($P < 0.01$). P content of the stems was significantly higher than the control for the three tested treatments ($P < 0.05$; $P < 0.01$ for “AMF+sludge”). Values of P total uptake per plant were a lot higher than the control for the three treatments, in relation to the biomass increase ($P < 0.01$). For example, inoculated plants in plots amended with sludge showed values of P per plant 14 times higher than control plants.

Contents of N, K, and Ca in leaves of *M. laurifolia* plants were not enhanced by the three tested treatments. However the quantities of these elements absorbed for each plant (total uptake per plant) were clearly higher than those of the control ($P < 0.01$). Unlike leaves, content of K in stems and roots was higher for inoculated plants ($P < 0.05$). Ca content was enhanced in roots by sludge amendment and AMF inoculation ($P < 0.05$).

The results also indicated that the three treatments did not influence significantly the water content of the plants (Fig. S4).

3.4. Ca/Mg ratio

Ca/Mg ratio in leaves was 3.5 ± 0.3 for control plants (Fig. 3). Sludge amendment significantly reduced this ratio even in inoculated plants ($P < 0.05$), while AMF inoculation without amendment increased it ($P < 0.05$).

Ca/Mg ratio in roots of control plants was 5.2 ± 0.5 . Sludge amendment and AMF inoculation significantly enhanced this ratio ($P < 0.05$), whereas their combination reduced it ($P < 0.01$).

3.5. Metal contents in plant parts

Except for Co, no significant differences between the treatments were noted for the tested metal contents in leaves (Table 2). Co content was reduced in this part of the plant by sludge amendment and AMF inoculation ($P < 0.05$). No significant differences were found in stems for all metals. In roots, large differences were noted between the treatments, but standard errors were very high. However Fe and Mn contents of inoculated plants were significantly higher than the control plants ($P < 0.05$). Root Ni content was also increased by AMF inoculation ($P < 0.05$).

Metal translocation factor (leave/root contents) for Mn and Co was significantly lower for the three treatments in comparison with control (respectively $P < 0.05$ and $P < 0.01$). For Fe and Ni, the values of this factor were significantly lower only for AMF inoculated plants (respectively $P < 0.05$ and $P < 0.01$). Cr translocation was not influenced by the three treatments

DTPA-extractable concentrations of each metal in soil under plants were not significantly different (Table 2), except for Fe and Ni values in AMF inoculated plots which were significantly lower than the control ($p < 0.05$).

3.6. AMF diversity in roots

The neighbour-joining tree (Fig. S5) allowed us to identify the phylogenetic relationships between the OTUs obtained in plant roots and the three AMF isolates used for plant inoculation. Strikingly, only two OTUs out of the 32 identified (6.25%) were similar to those of the inoculum. AMF diversity in roots of *M. laurifolia* 335 days after plantation showed the presence of several taxa. The total amount of obtained

DNA sequences was 157; these sequences have been clustered in 32 different OTUs (Table 3). Nine OTUs have been found in control plants, for a total amount of 37 sequences. 8 OTUs were found in plants amended with sewage sludge (31 sequences), whereas AMF-inoculated plants showed 25 OTUs for 58 sequences. Inoculated plants showed higher AMF diversity within roots as indicated by the clear increase of the amount of identified OTUs and the Shannon Index. However, this positive effect of inoculation on AMF diversity did not occur in plants amended with sewage sludge.

Eight different genera (*Diversispora*, *Redeckera*, *Scutellospora*, *Acaulospora*, *Claroideoglossum*, *Rhizophagus*, *Glomus* and *Sclerocystis*), corresponding to five families were detected for the entire experimental field, six genera in roots of control plants and in AMF-inoculated plants, and four genera in sludge amended plots (Fig. 4). Glomeraceae were the most frequent taxa, with 118 sequences out of 157 (75%).

Inoculation with AMF has induced changes in the structure of AMF populations inside the roots (Fig. 4). Two genera present in control plants (*Diversispora* and *Redeckera*) were not detected in inoculated plants. *Claroideoglossum* was abundant in control plants (27% of the identified sequences) and seemed to be marginalized in inoculated plants (1.7%). Conversely, *Scutellospora* and *Glomus* were present in inoculated plants but not found in control ones. In sewage sludge amended plots, *Diversispora* and *Redeckera* were also absent in the roots.

4. Discussion

4.1. Effects of the treatments on plant growth and mycorrhizal colonization

Only few studies have been reported on the effects of AMF inoculation on growth and adaptation of endemic plants to ultramafic soils in New Caledonia (Amir et al.,

2013; Lagrange et al., 2011). Some native AMF isolates highly tolerant to Ni (Amir et al., 2008), tested in greenhouse conditions, showed positive effects on growth and tolerance to Ni of endemic plants species (Amir et al., 2013). However, the effects of AMF inoculation on ultramafic plant species in field conditions have not been reported until now, even in other countries.

The mixture of the three AMF isolates studied here has been tested in greenhouse conditions with the same plant species (Gensous, 2014). The results obtained here confirm their highly positive effect on plant growth, in field conditions: the shoot dry weight, 528 days after plantation, was 4.1 times higher than the control. These results are similar to those obtained in greenhouse despite the presence of AMF viable spores in the field topsoil before the experiment (as revealed also by mycorrhizal colonization and AMF diversity analyses). In comparison, Emam (2016), working in mercury mine restoration site in field conditions, compared the effects of inoculation of *Stipa pulchra* with commercial AMF or topsoil. Commercial inoculum was inefficient, whereas topsoil inoculation induced a biomass increase of about 230%. In a review article, Berruti et al. (2015) reported field effects of AMF on growth of different plants. The values of biomass increase were 70% to 90%. Studies on agricultural crops in field conditions showed that yields of AMF-inoculated plants were about two times higher for cucumber (Ortas, 2010) and soy bean (Cely et al., 2016). Saia et al. (2015) reported an increase of 20-30% of wheat growth inoculated with AMF. The mixed AMF isolates tested here showed then a great influence on *M. laurilolia* growth, in mine restoration conditions.

The evaluation of the mycorrhizal colonization showed that control plants were colonized at a low level (M: 3.3%); but the mycorrhizal colonization of AMF-inoculated

plant was also moderate (M: 12.4% for AMF inoculated plants; M: 22.8% for inoculated plants in amended plots). Low levels of mycorrhization are frequent in New Caledonian ultramafic soils (Perrier et al., 2006) and this seems to not limit the positive effects of the symbiosis (Lagrange et al., 2011). This limitation of mycorrhizal colonization is partly related to the low level of plant productivity in ultramafic maquis and to soil nutrient deficiency, especially P (Koide, 1991; Smith and Smith, 2012). Nevertheless a significant positive correlation between shoot dry weight and mycorrhizal colonization was detected.

The content of sewage sludge amendment used here is about 10 times lower than that used by Pitchel et al. (1994) for an abandoned mine land, and similar to that used by Rate et al. (2004) for mine rehabilitation with stockpiled topsoil. The effect of this amendment on plant growth was clearly positive and similar to that observed for AMF inoculated plants, whether considering plant height progression or plant weight 528 days after plantation. The positive effects of sewage sludge on plant growth have been reported by many studies concerning forest trees and shrubs (Bramryd, 2002; Velayoudon et al., 2014) or herbaceous plants (Sabey et al., 1990; Velayoudon et al., 2014), in relation to different soil characteristics improvements: soil structure and stabilization (Mata-Gonzalez et al., 2002; Ojeda et al., 2008) microbial activity (Petersen et al., 2003; Selivanovskaya & Latypova, 2006) and mineral composition (Garcia et al., 1991). But few studies have been reported on the combination effects of sewage sludge and AMF. Alguacil et al. (2004), working with *Retama sphaerocarpa* in field experiment with semiarid soil, observed about 15% more shoot dry weight for the combined treatment (AMF plus sewage sludge) in comparison with AMF in absence of sludge. Arriagada et al. (2009), testing these two treatments in greenhouse condition

with *Eucalyptus globulus*, showed an increase of 45% of shoot dry weight in plants inoculated with *Glomus deserticola*, whereas this increase reached 285% when AMF inoculation was combined with sewage sludge amendment. These results are consistent with ours; however, contrary to our findings, the mycorrhizal colonization was reduced by the sludge amendment. Field experiment testing combining AMF inoculation with other organic amendment has been reported by Caravaca et al. (2002). These authors did not obtain differences on growth and mycorrhizal colonization of *Olea europea* plants, between AMF inoculation and a combination of AMF and a compost of solid municipal waste.

4.2. Effects of AMF inoculation and sewage sludge on plant mineral nutrition

The increase in growth of inoculated plants was coupled with a better mineral nutrition, especially for phosphorus. P is rare and weakly available in ultramafic soils, as it is strongly adsorbed to iron oxides, particularly in New Caledonia (Dubus and Becquer, 2001; Jaffré and L'Huillier, 2010a). The role of mycorrhiza can be important in these conditions, particularly in field conditions with large fluctuations of environmental variables in comparison with greenhouse conditions. The soil water content was generally sufficient, because there is a high rainfall level in Goro area (see site description), but there are large fluctuations with frequent saturations and soil surface drying and the temperatures are more variable than in greenhouse (12°C-34°C). The effect of AMF inoculation was not very clear when we consider only the element contents in plant organs, even if differences for stem or root contents were significant for P and K. As stressed by Smith and Smith (2013), there is "a highly significant role for the fungi, not only in P uptake via the AM pathway but also in

reducing the contribution of the direct pathway" (by root hairs) inducing an energy gain. Nevertheless, when we consider the total uptake of each AMF-inoculated plant, the absorption of major elements (i.e. P, N, K and Ca) was clearly stimulated by mycorrhizal symbiosis, in relation to the high biomass increase. The effect of sewage sludge on major elements absorption is similar to the AMF effect.

Ca/Mg ratio was higher in leaves and roots of AMF inoculated plants. As Ca is rare and Mg abundant in ultramafic soils (Ca/Mg low), this increase is an important contribution of AMF symbiosis to plant adaptation to ultramafic soils. Indeed, this low ratio is a limiting factor in these soils because of the competition between Mg and Ca (Jaffré and L'Huillier, 2010a; Proctor and Nagy, 1992). Doubkova et al. (2011) reported a significant positive correlation between mycorrhizal colonization and Ca/Mg ratio of *Knautia arvensis* plants in different ultramafic and non-ultramafic areas.

Sewage sludge showed a negative effect on Ca/Mg ratio, particularly for leaves. It is possible that this organic matter has induced a complexation of Ca ions or/and microbial immobilization of Ca by the microorganisms in the rhizosphere.

4.3. Effects of AMF inoculation and sewage sludge on heavy metal translocation

Metal contents in leaves were not significantly reduced by the three treatments. In fact, the values obtained here for leaves were lower than those generally observed for *M. laurifolia* in natural or greenhouse conditions (Crossay et al., submitted; Wulff et al., 2010), which means that this species is not inhibited by these amounts of metals in its leaves.

By contrast, the translocation factors for Fe, Mn, Co and Ni were all significantly lower in AMF inoculated plants in comparison with control ones. As heavy metal

contents in mycorrhizal roots are generally higher than in control ones, it seems clear that the mycorrhizal root system retained a large part of the entering heavy metals. These results in field conditions confirm those reported by Amir et al. (2013) in greenhouse conditions for Ni and confirm the important role of AMF in the alleviation of heavy metal toxicity in plants in ultramafic soils. Different studies reported a lower translocation of heavy metals by plants inoculated with AMF in metal polluted soils (Hildebrandt et al., 2007; Amir et al., 2014); but very few concerned ultramafic soils (Orlowska et al., 2011; Doubkova and Sudova, 2014).

Sewage sludge reduced the translocation of Mn and Co but not Fe, Ni and Cr. Velayoudon et al. (2014) have reported a reduction effect of sewage sludge on Mn, Co and Cr contents in pines planted in a New Caledonian ultramafic soil.

4.4. Effects of AMF inoculation on plant water content

AMF have frequently a positive effect on plant water content in relation to the increase of the absorbing surface (Augé, 2001; Smith et al., 2010). In our experiment no effect of AMF inoculation on plant water content was observed 528 days after plantation. The climate in the experimental zone (Goro) is very humid and the soil was frequently saturated. The drought stress is therefore negligible, which can explain the absence of differences between inoculated and control plants for water content. Gensous (2014), working with the same plant species in more fluctuating conditions of water availability, reported a clear increase of water content of AMF inoculated plants in relation to their tolerance to drought stress. Similar conclusion has been reported by Doubkova et al. (2013) for *Knautia arvensis* in ultramafic soil.

4.5. Relationships with AMF diversity

Despite the positive effect of AMF inoculation on the growth of plants in field, we found that the inoculated AMF isolates were under represented in roots of *M. laurifolia* 335 days after plantation, compared to the other identified taxa. Indeed the OTUs corresponding to the genera of the three isolates represented at most 9% of the detected OTUs in the roots of inoculated plants. As plant biomass was measured more than 6 months later (after 528 days), the inoculated isolates were probably less abundant at this time. How then can we explain the large differences of growth, persisting for 41 months after plantation, between inoculated and non-inoculated plants? It is important to first point out that AMF inoculation was assessed in a nursery 18 months before plantation in the field. Differences between inoculated and control plants were then clearly visible when AMF inoculated isolates were alone to colonize the roots. After plantation, autochthon AMF progressively replaced inoculated isolates; but we could clearly observe that AMF inoculation stimulated AMF diversity in roots: 25 OTU were detected for inoculated plants and only 9 OTU for control plants. Thus the most probable mechanism explaining the positive effect of AMF inoculation is that the higher growth rate of AMF-inoculated plants, at the beginning, induced more sugar photosynthesis and then more carbon allocated to symbiosis. This promoted AMF diversity in roots which again stimulated plant growth. Similar results were obtained by Alguacil et al. (2011). These authors observed that plant inoculation with AMF stimulated AMF diversity and this stimulation was higher when plants were inoculated with two isolates in comparison with one.

Inoculation with AMF has also induced changes in the structure of AMF populations in the roots. The techniques used (DNA sequencing after nested PCR) are not adapted

to quantify the abundance of each taxa. However the changes, considered globally, are sufficiently clear to be taken into account. These changes concerned 22 OTUs as seen in the phylogenetic tree (Fig. S5); two genera present in the roots of control plants were absent in inoculated plants and two other genera found in inoculated plants were not found in control plants (Fig. 4). Very few studies have been reported on the effects of AMF inoculation on AMF population structure. Janoušková et al. (2013), using a clayey spoil mixed with sand, have reported the competition between initial AMF community and inoculated isolates, inducing reduction of the latter abundance in roots. Cano and Bago (2005), using Ri T-DNA transformed carrot roots *in vitro*, reported a strong competition between AMF species for root colonization. Werner and Kiers (2014) tested the competition between two AMF species (*Glomus aggregatum* and *Rhizophagus irregularis*) in roots of *Medicago truncatula*, in glasshouse conditions, with pots filled with sand dune. When the roots were colonized by one of the two species, the inoculation of the second species induced a reduction of the abundance of the first species.

In our experiment, sewage sludge has significantly stimulated mycorrhizal colonization after 528 days, both in non-inoculated plants and inoculated ones. Different studies reported the effects of sewage sludge on AMF. Weissenhorn et al. (1995) found that this amendment stimulated mycorrhizal colonization of maize, but reduced spore abundance. Other studies reported negative effects on spore abundance only (Del Val et al., 1999; Moreira et al., 2015). We also observed that AMF diversity was reduced by sewage sludge after 335 days, especially in the roots of inoculated plants (8 OTUs instead of 25). Moreira et al. (2015) reported equally a reduction of AMF diversity by this amendment, but this reduction was linked to the

increase in heavy metal concentrations. In our case, the reduction of AMF diversity could not be explained by heavy metal toxicity as their extractable concentration in soil was not increased by sludge amendment (Table 2). The differences between measurement periods between AMF diversity (335 days) and mycorrhizal colonization (528 days) make interpretation more difficult; however a probable explanation is that the soil enriched with sewage sludge promoted few taxa more competitive in these conditions for root colonization, which induced the elimination (or rarefaction) of others.

In conclusion, the field experiment, assessed in mine restoration conditions in south New Caledonia, clearly demonstrated the importance of AMF functions in an ultramafic soil. Growth of *Metrosideros Laurifolia* plants was greatly increased by AMF symbiosis and the combination of AMF with sewage sludge showed complementary effects with a higher growth rate. Plant adaptation to ultramafic constraints has been also enhanced, with a higher Ca/Mg ratio and a reduction of heavy metal translocation. Application of these results to ecological restoration of ultramafic degraded areas is now under consideration.

Supplementary data

Supplementary data associated with this article can be found online at: doi...

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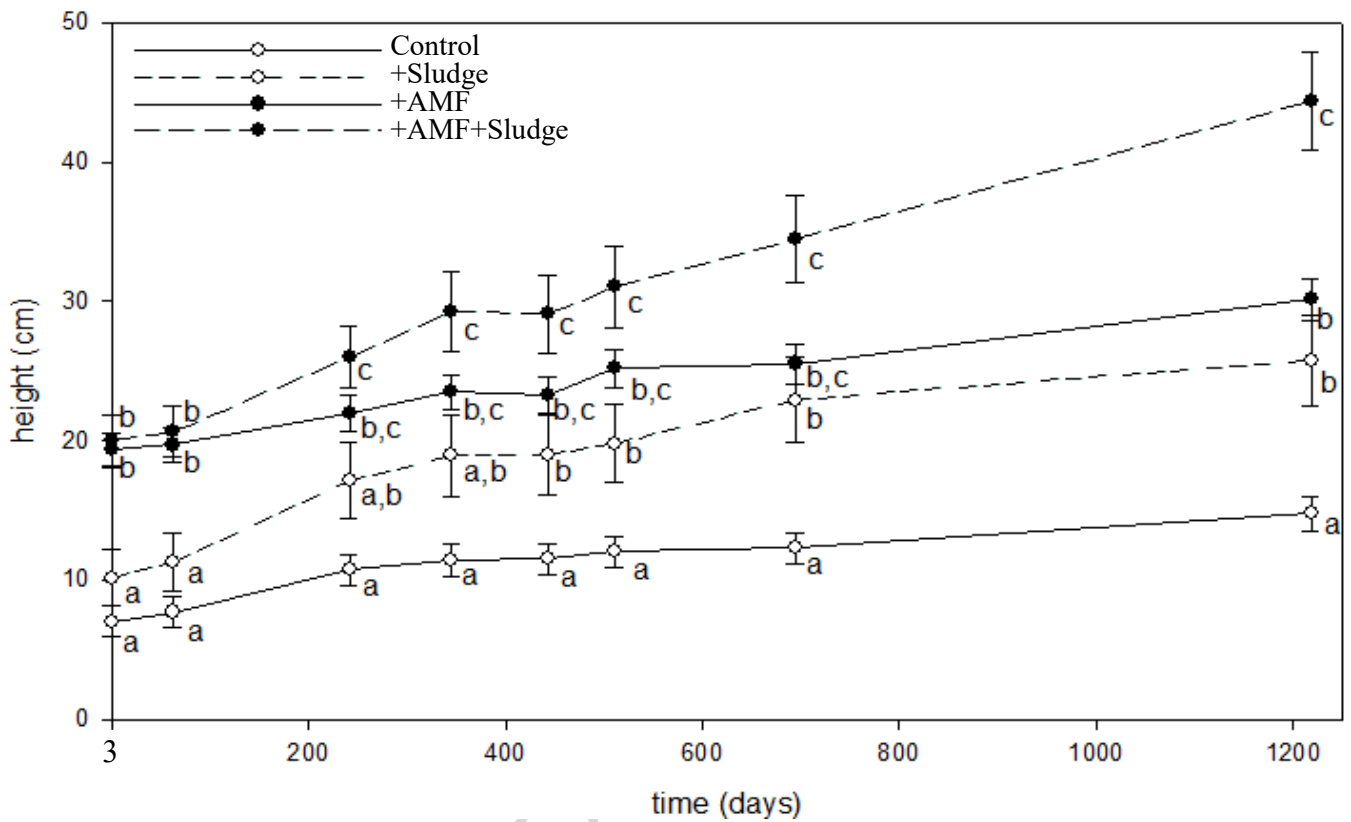
Figures and tables

Fig. 1. Influence of AMF inoculation, sewage sludge and their combination on *Metrosideros laurifolia* plant growth (means values of height with standard errors) in field conditions, with ultramafic topsoil. For each time period, values followed by the same letters are non-significantly different (post-hoc Bonferroni corrected Mann-Whitney test).

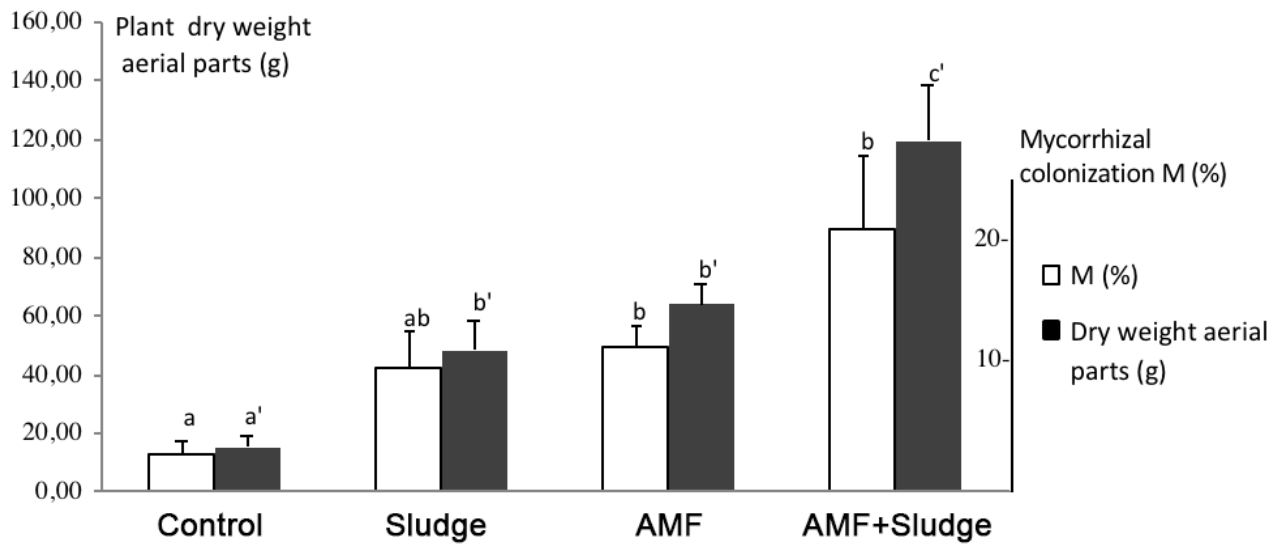


Fig. 2. Influence of AMF inoculation, sewage sludge and their combination on shoot biomass (dry weight) and mycorrhizal colonization (M) of *Metrosideros laurifolia* plants, 528 days after plantation (means values with standard error). Bars with the same letters are non-significantly different (post-hoc Bonferroni corrected Mann-Whitney test).

Table 1

Influence of AMF inoculation, sewage sludge and their combination, on mineral nutrition of *Metrosideros laurifolia* plants, 528 days after plantation (means values with standard error). For each parameter, values followed by the same letters are non-significantly different (post-hoc Bonferroni corrected Mann-Whitney test).

| | Leaves (mg/g) | Stem (mg/g) | Total uptake for aerial parts (mg/ plant)* | Roots (mg/g) |
|-------------|------------------|----------------|--|--------------|
| P | | | | |
| Control | 0.17 ± 0.01 a | 0.12 ± 0.03 a | 4.47 ± 0.62 a | / |
| +Sludge | 0.23 ± 0.02 b | 0.18 ± 0.03 b | 19.71 ± 2.40 b | / |
| +AMF | 0.15 ± 0.02 a | 0.20 ± 0.01 b | 22.37 ± 1.92 b | / |
| +AMF+Sludge | 0.31 ± 0.03 c | 0.53 ± 0.11 c | 100.57 ± 16.76 c | / |
| N | | | | |
| Control | 4.1 ± 0.7 a | 1.7 ± 0.4 a | 90.1 ± 17.2 a | / |
| +Sludge | 4.9 ± 1.3 a | 2.8 ± 0.7 b | 366.8 ± 93.7 b | / |
| +AMF | 3.5 ± 0.9 a | 2.7 ± 0.7 a | 395.7 ± 107.4 b | / |
| +AMF+Sludge | 3.5 ± 1.3 a | 2.8 ± 0.8 a | 750.7 ± 249.0 c | / |
| K | | | | |
| Control | 1.6 ± 0.5 a | 1.0 ± 0.2 a | 40.0 ± 10.8 a | 0.5 ± 0.2 a |
| +Sludge | 2.7 ± 1.2 a | 1.5 ± 0.2 b | 201.9 ± 67.3 b | 1.3 ± 0.7 ab |
| +AMF | 2.2 ± 1.0 a | 1.7 ± 0.5 b | 249.3 ± 95.9 b | 1.1 ± 0.3 b |
| +AMF+Sludge | 2.5 ± 2.0 a | 2.9 ± 1.0 c | 646.5 ± 359.2 b | 2.0 ± 1.3 b |
| Ca | | | | |
| Control | 12.8 ± 5.5 a | 12.9 ± 4.1 a | 395.8 ± 147.8 a | 3.0 ± 0.1 a |
| +Sludge | 9.9 ± 5.2 a | 11.7 ± 2.7 a | 1038.3 ± 379.7 b | 6.7 ± 3.2 b |
| +AMF | 12.2 ± 6.7 a | 13.2 ± 4.4 a | 1623.8 ± 709.6 bc | 5.5 ± 2.0 b |
| +AMF+Sludge | 8.3 ± 2.2 a | 12.3 ± 3.1 a | 2466.4 ± 643.6 c | 3.7 ± 2.0 ab |

*: Only the aerial parts are considered because the roots were difficult to collect completely at this stage; /: value not available.

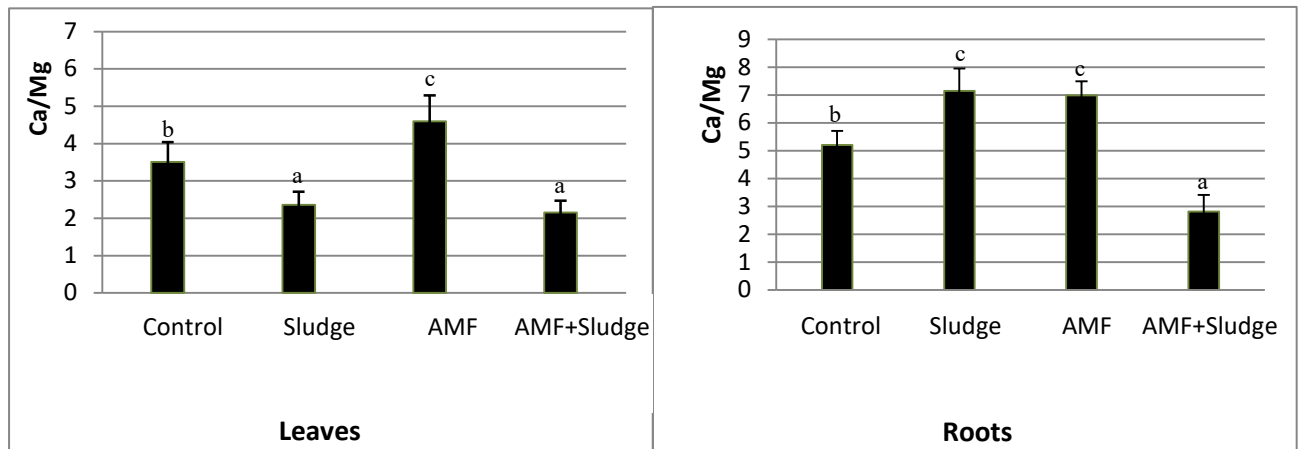


Fig. 3. Influence of AMF inoculation, sewage sludge and their combination on Ca/Mg ratio of *Metrosideros laurifolia* plants 528 days after plantation (means values with standard error. Bars with the same letters are non-significantly different (post-hoc Bonferroni corrected Mann-Whitney test)).

Table 2

Influence of AMF inoculation, sewage sludge and their combination, on metal contents in *Metrosideros laurifolia* organs and in soil under plants, 528 days after plantation (means values with standard error). Values followed by the same letters are non-significantly different (post-hoc Bonferroni corrected Mann-Whitney test).

| | Leaves (L) | Stem | Roots (R) | Translocation factor (L/R) | Soil* |
|--|---------------------|---------------------|-----------------------|----------------------------|------------------|
| Fe ($\mu\text{g/g}$) | | | | | |
| Control | 232.3 \pm 112.9 a | 493.1 \pm 187.7 a | 331.1 \pm 66.7 a | 0.70 \pm 0.38 a | 8.0 \pm 1.8 a |
| +Sludge | 210.8 \pm 72.7 a | 846.1 \pm 624.4 a | 596.2 \pm 297.6 a | 0.35 \pm 0.21 a | 7.4 \pm 2.1 ab |
| +AMF | 172.6 \pm 98.04 a | 251.1 \pm 99.0 a | 2169.8 \pm 1668.1 b | 0.08 \pm 0.06 b | 4.7 \pm 1.2 b |
| +AMF+Sludge | 202.5 \pm 64.9 a | 340.7 \pm 173.3 a | 4785.0 \pm 4179.2 b | 0.04 \pm 0.03 b | 6.9 \pm 2.2 ab |
| Mn ($\mu\text{g/g}$) | | | | | |
| Control | 497.0 \pm 178.8 a | 255.9 \pm 67.5 a | 62.2 \pm 26.3 a | 7.99 \pm 3.34 a | 24.2 \pm 3.2 a |
| +Sludge | 390.4 \pm 183.7 a | 256.3 \pm 60.8 a | 138.4 \pm 91.4 ab | 2.82 \pm 0.98 b | 26.1 \pm 7.0 a |
| +AMF | 397.3 \pm 114.4 a | 269.2 \pm 56.1 a | 121.9 \pm 26.0 b | 3.26 \pm 0.77 b | 18.6 \pm 6.3 a |
| +AMF+Sludge | 487.9 \pm 186.9 a | 332.8 \pm 134.0 a | 247.1 \pm 164.0 b | 1.97 \pm 1.23 b | 20.2 \pm 4.8 a |
| Co ($\mu\text{g/g}$) | | | | | |
| Control | 1.1 \pm 0.4 b | 1.1 \pm 0.4 a | 1.3 \pm 0.2 a | 0.85 \pm 0.29 a | 3.0 \pm 0.9 a |
| +Sludge | 0.3 \pm 0.1 a | 1.0 \pm 0.7 a | 1.6 \pm 0.6 a | 0.19 \pm 0.09 b | 3.1 \pm 1.2 a |
| +AMF | 0.4 \pm 0.2 a | 0.8 \pm 0.2 a | 4.2 \pm 3.0 a | 0.09 \pm 0.06 b | 1.9 \pm 0.6 a |
| +AMF+Sludge | 0.6 \pm 0.3 ab | 0.9 \pm 0.2 a | 17.9 \pm 16.0 a | 0.03 \pm 0.03 b | 2.8 \pm 0.9 a |
| Ni ($\mu\text{g/g}$) | | | | | |
| Control | 8.7 \pm 3.1 a | 8.1 \pm 1.5 a | 9.0 \pm 4.0 a | 0.97 \pm 0.33 a | 5.1 \pm 1.3 a |
| +Sludge | 7.6 \pm 2.7 a | 9.8 \pm 1.2 a | 9.6 \pm 3.8 a | 0.79 \pm 0.30 a | 4.3 \pm 1.3 ab |
| +AMF | 6.5 \pm 2.3 a | 12.4 \pm 6.5 a | 19.9 \pm 4.5 b | 0.32 \pm 0.13 b | 3.2 \pm 0.7 b |
| +AMF+Sludge | 9.8 \pm 4.0 a | 10.3 \pm 4.4 a | 83.7 \pm 80.2 ab | 0.11 \pm 0.09 b | 4.5 \pm 1.7 ab |
| Cr ($\mu\text{g/g}$) | | | | | |
| Control | 12.8 \pm 4.1 a | 8.1 \pm 4.5 a | 13.6 \pm 4.5 a | 0.94 \pm 0.48 a | 15.0 \pm 5.2 a |
| +Sludge | 15.2 \pm 4.1 a | 8.9 \pm 0.5 a | 17.7 \pm 3.0 a | 0.86 \pm 0.35 a | 17.7 \pm 3.9 a |
| +AMF | 15.4 \pm 2.2 a | 13.2 \pm 5.6 a | 24.2 \pm 15.1 a | 0.64 \pm 0.31 a | 15.3 \pm 3.8 a |
| +AMF+Sludge | 12.0 \pm 3.9 a | 9.6 \pm 3.5 a | 21.0 \pm 5.6 a | 0.57 \pm 0.29 a | 15.9 \pm 3.8 a |

* DTPA-extractable metals in soil sampled under plants, between roots; DTPA: Dimethylene triaminopentaacetic acid.

Table 3

AMF diversity associated with roots of *Metrosideros laurifolia*, 335 days after plantation.

| | Treatments | | | | Total |
|---------------------|------------|--------|------|--------------|--------------|
| | Control | Sludge | AMF | AMF + Sludge | |
| Number of sequences | 37 | 31 | 58 | 31 | 157 |
| Number of OTUs | 9 | 8 | 25 | 8 | 32 |
| Sequences / OTUs | 4.11 | 3.88 | 2.32 | 3.88 | 4.91 |
| Shannon index | 1.46 | 1.14 | 2.42 | 1.15 | |

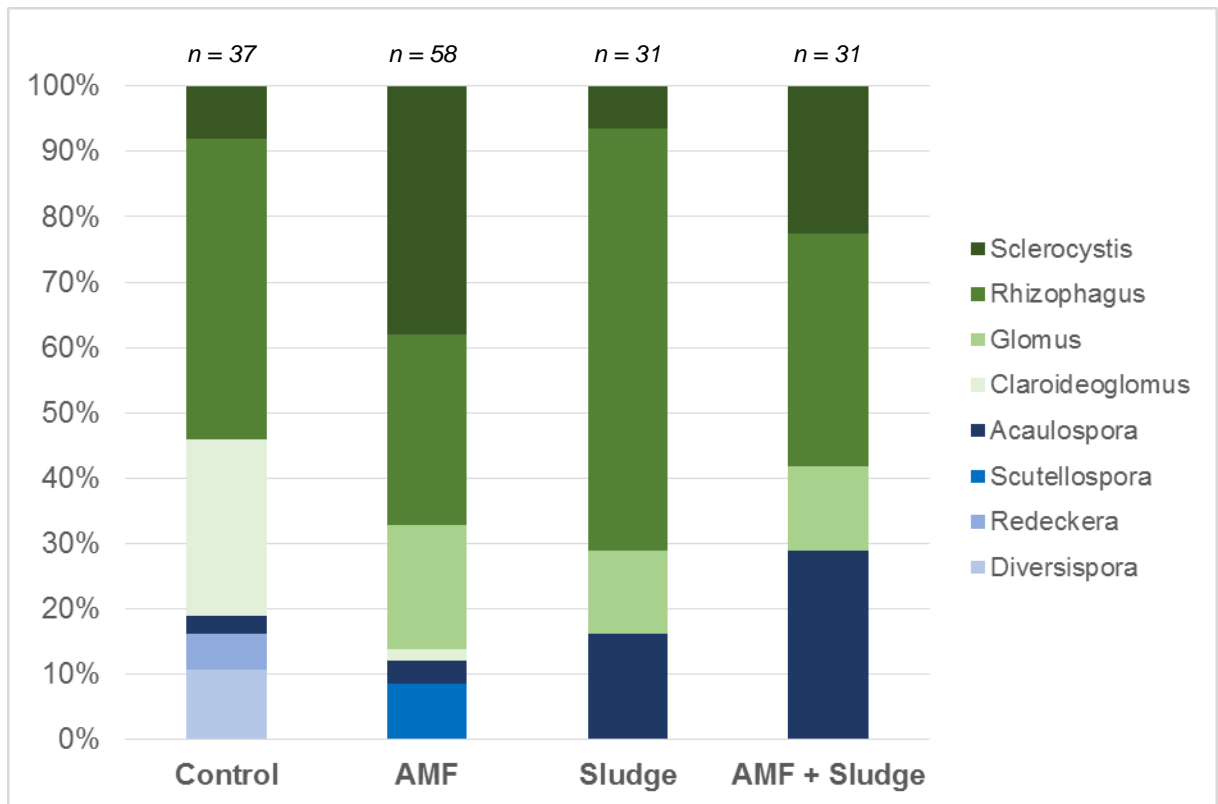
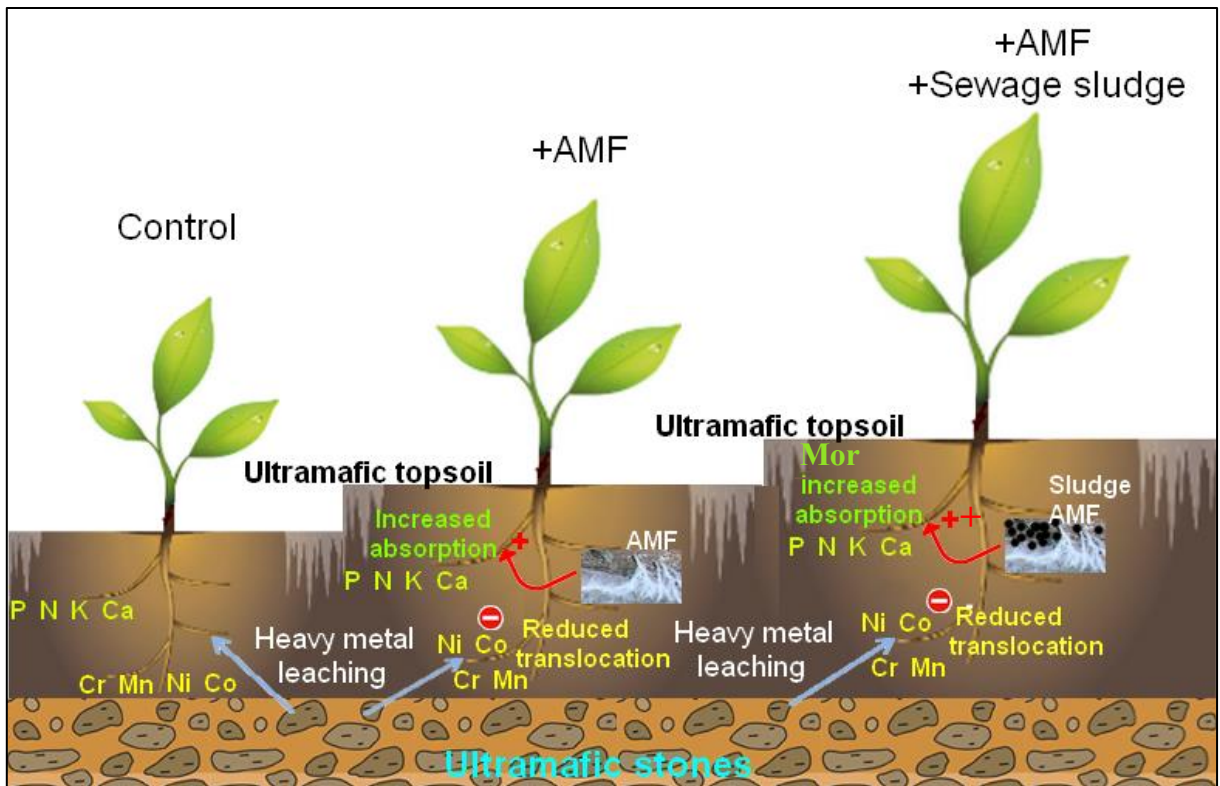


Fig. 4. Distribution of AMF genera identified in the roots of *Metrosideros laurifolia* 335 days after plantation. For each experimental condition, the distribution is represented as a percentage in accumulated columns. The total number of AMF sequences obtained (n) is indicated for each treatment.

GRAPHICAL ABSTRACT

A valuable improve of ecological restoration of mining-degraded areas with arbuscular mycorrhizal fungi inoculation and sewage sludge recycling to fertilize the degraded topsoil.

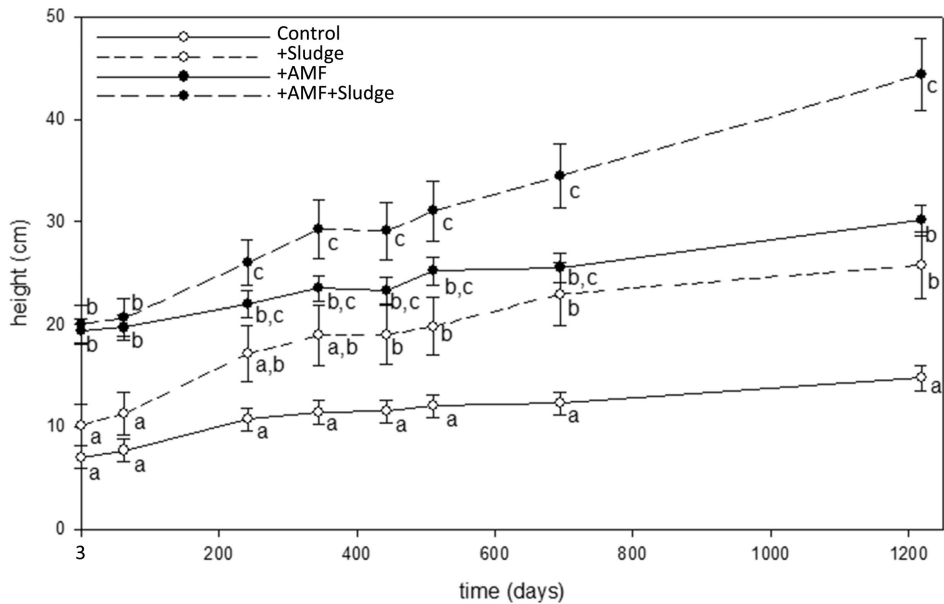


Figure 1

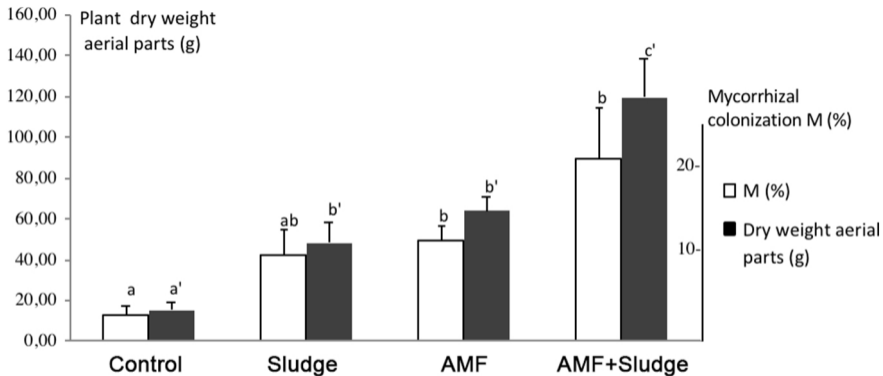


Figure 2

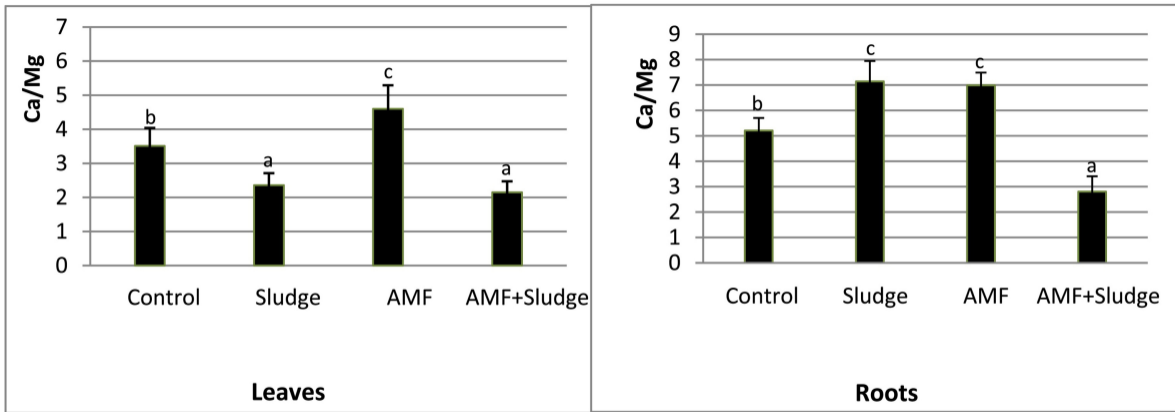


Figure 3

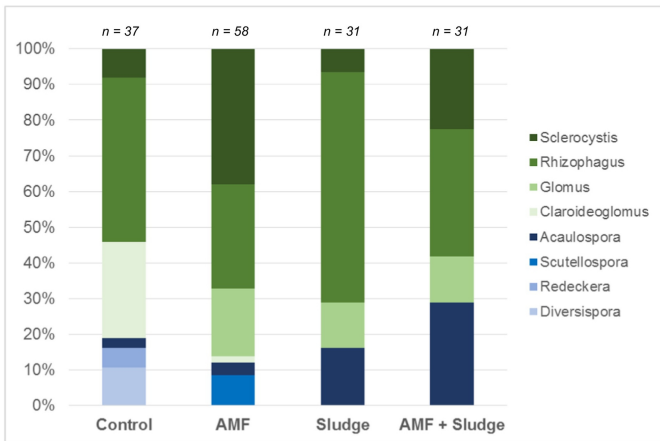


Figure 4