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Inoculation of *Mimosa latispinosa* Lam with the Commercial Arbuscular Mycorrhizal Fungus *Rhizophagus irregularis* DAOM 197198, and *Bradyrhizobium* spp. under Nursery Production Conditions in South-East Madagascar

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Summary

Qit Madagascar Minerals (QMM) has planned several actions to reduce the environmental footprint of its mining project located near the city of Fort-Dauphin (Madagascar). One of these actions is the reclamation of a portion of its mined sites. Different symbiotic strains were tested as bio-enhancers for the ecological restoration using Mimosa latispinosa *Lam, a native and pioneer shrub. The symbiotic strains tested in nursery were the commercial strain of arbuscular mycorrhizal fungus,* Rhizophagus irregularis *DAOM197198, and two local strains of* Bradyrhizobium spp.*, STM1415 and STM1447, inoculated alone or dually with the arbuscular mycorrhiza. Treatments did not significantly increase the plant height and dry mass. However, plants grown in tyndallized soil had better growth than those in unsterilized soil. Results obtained twenty weeks after inoculation suggest that soil tyndallization (heating at 100 °C and at atmospheric pressure of 700 kPa during three hours) is an effective method for nursery production of high quality seedlings of* M. latispinosa*.*

Résumé

Inoculation en pépinière de *Mimosa latispinosa* Lam. avec le symbionte mycorhizien arbusculaire, *Rhizophagus irregularis* DAOM 197198, et *Bradyrhizobium* spp. dans le sud-est de Madagascar

La compagnie Qit Madagascar Minerals (QMM) a pris plusieurs engagements environnementaux dans son projet minier basé près de la ville de Fort-Dauphin (Madagascar), dont celui de restaurer une partie de ses sites après exploitation. Différentes souches symbiotiques ont été testées en tant que bio-stimulateurs pour la restauration écologique de Mimosa latispinosa *Lam. un arbuste pionnier local. Les souches symbiotiques testées en pépinière étaient la souche de l'inoculum commercial,* Rhizophagus irregularis *DAOM197198 et deux souches locales de* Bradyrhizobium *spp., STM1415 et STM1447, inoculées seules ou en combinaison avec la souche de mycorhize arbusculaire. Les traitements n'ont pas montré de différences significatives au niveau de la hauteur et de la biomasse sèche des plantes. Les plantes cultivées en sol stérilisé ont toutefois connu une croissance significativement supérieure à celles produites en sol non-stérilisé. Vingt semaines après inoculation, les résultats suggèrent que la tyndallisation du sol (chauffage à 100 °C et à pression atmosphérique de 700 kPa pendant trois heures) est une méthode efficace pour la production en pépinière de plants de bonne qualité de* M. latispinosa *issus de semis.*

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Introduction

Qit Madagascar Minerals (QMM), a Rio Tinto subsidiary, started an important mining project of ilmenite extraction in 2009 in southeast Madagascar, near the city of Fort- Dauphin (22).

The Anosy region, where the mining project is located, contains one of Madagascar's last remaining littoral forests growing on sandy soils with high level of endemic species (83% of plants) (22, 27). To ensure environmental sustainability, QMM has built two nurseries able to produce more than 250,000 plants a year for rehabilitation and restoration purposes (28). The mining process does not require chemicals but does involve digging to a depth of twenty meters, resulting in the destruction of the mycelium network and other symbiotic microorganisms found in the soil (22).

Hence, it is necessary to restore this mycorhizosphere, which is in essential and interdependent relation with the plants.

Mycorrhizae are symbiotic associations between fungi and the roots of the host plant where the plant provides sugars to the fungi in exchange for nutrients, mainly phosphorus, and water (32). Arbuscular mycorrhizal fungi (AMF) are by far the most widely distributed and the oldest type of mycorrhizal symbionts (10). AMF are found in most soil ecosystems and plants and should be of major concern in ecological restoration (18).

Additionally, rhizobium are bacteria known to colonize plants belonging to the *Fabaceae* family, and to fix atmospheric nitrogen in root nodules (9, 35). While the mass production of clean arbuscular mycorrhizal inoculum is challenging, inoculum of nitrogen fixing bacteria is easy to produce, technically accessible in "less industrialized countries" and hence, widely available as a tool to facilitate ecological restoration (7, 34).

From a restoration perspective, nitrogen-fixing trees must be targeted to start the process as they bring back nitrogen to the soil and help the reestablishment of other plant species (5, 11). The use of this biotechnology to successfully restore disturbed sites makes it an appropriate bio-tool for the mining industry to minimize its environmental footprint (12, 23).

The aim of the present study was to evaluate the potential of using root symbioses as a bio-tool to improve the efficiency of QMM's restoration activities of mined areas. Current state of knowledge supports the importance of considering mycorrhizal biotechnology in the restoration process of degraded land (23). While QMM has achieved a number of experiments to support their ecological restoration process, this study is the first specifically targeted on soil microsymbiots.

By using strains of AMF and rhizobium, the study aimed to evaluate the plant performance in nursery, which could lead to a greater efficiency of the restoration efforts through reduced time in nursery and lower mortality rates after planting.

Materials and methods

Study site

The nursery owned by QMM is located close to Mandena mining site, in the Tolagnaro (Fort-Dauphin) area (UTM 38 J 703062.00 m E 7238115.00 m S) (Figure 1). This littoral region, located on the southeast coastline of Madagascar, is mostly composed of littoral sands and is characterized by a humid and warm climate (39). The soil is sandy and characterized by low nutrients content and acidic pH (in H_2O) of around 5.03 and 5.44 for topsoil and demineralized soil, respectively (Table 1) (Photo 1).

Seedling production method

The substrate used was the stockpiled topsoil removed from the mining site before exploitation (Figure 2). This practice is known to support viability of AMF in the soil (14). This soil was used to fill polythene bags in the nursery. Half of those bags contained soil sterilized by tyndallization at a pressure of 700 kPa (6,9 atm) and a temperature of 100 °C for one hour (38) and the other half contained untyndallized soil. No fertilization was applied to the soil during the experiment in order to be consistent with the current nursery practices of seedling production used by QMM (Photo 2).

The species used for the experiment was *Mimosa latispinosa*, a local shrub found in the coastal forest of Fort-Dauphin.

It is locally named Rakaraka and is used by local populations for medicinal purposes. *Mimosa latispinosa* is a nitrogen fixing, pioneer and sun-loving species that belongs to the *Mimosoideae* sub-family (*Fabaceae* family) (8). Seeds were collected from two sites around the village of Mangaika (M1925 and M1920) (UTM 38 J 700779.06 m E 7240625.93 m S) on June 29-30th, 2009. A broadcast seeding was done in two separate plots, one sterilized and another one unsterilized (3,000 seeds/plot). Seeds generally germinated ten days after seeding. Seedlings were transplanted in nursery bags filled with either sterilized or unsterilized soil.

Inoculation

Inoculation was done four weeks after seeding using one arbuscular mycorrhiza, *Rhizophagus irregularis* DAOM197198 (formerly *Glomus irregulare*) (37), and two strains of rhizobium, *Bradyrhizobium* spp. (STM1415 & STM1447). *Rhizophagus irregularis* was provided by Premier Tech Biotechnologies Ltd (Rivière-du-Loup, Québec, Canada).

This company produces *R. irregularis* under the commercial name «MYKE® PRO PS3».

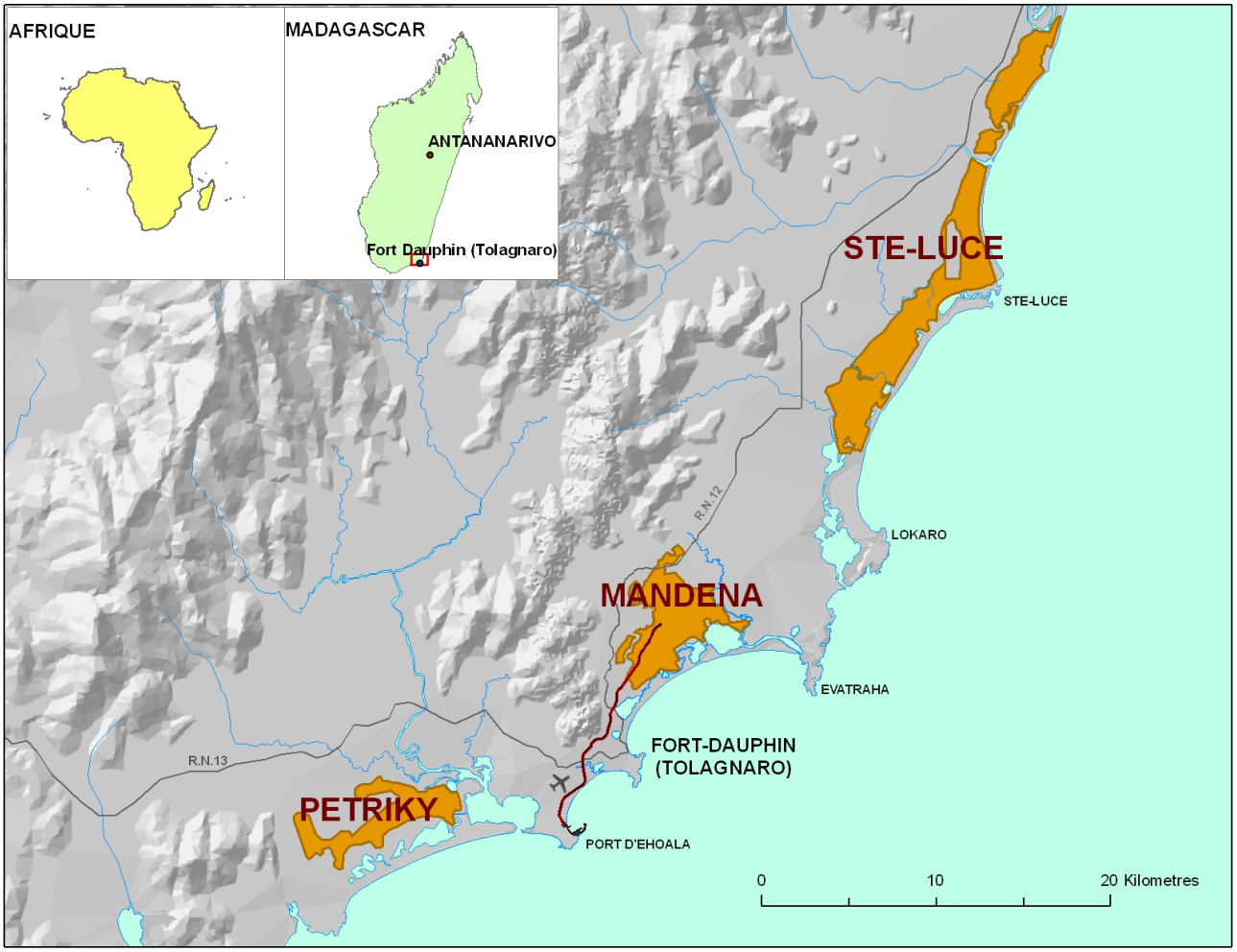


Figure 1: Location of the study site (Mandena) (QMM, 2001).

Table 1
Chemical characteristics of untyndallized topsoil collected prior to mining operations and untyndallized soil collected after mining operations.

Soil conditions	pH (w ater)	N Kjeldahl (ppm)	C (µg/g)	P (soluble) (ppm)	K (assimilable) (ppm)	C/N
Topsoil	5.44	618.33	14 740,00	7.92	19.5	25.6
Demineralized soil	5.03	186.66	3 211,67	12.43	7.8	26.83

Photo 1: Mining site to be restored (on the left - with topsoil, on the right - without topsoil).

Photo 2: QMM's nursery, the location of the experiment.

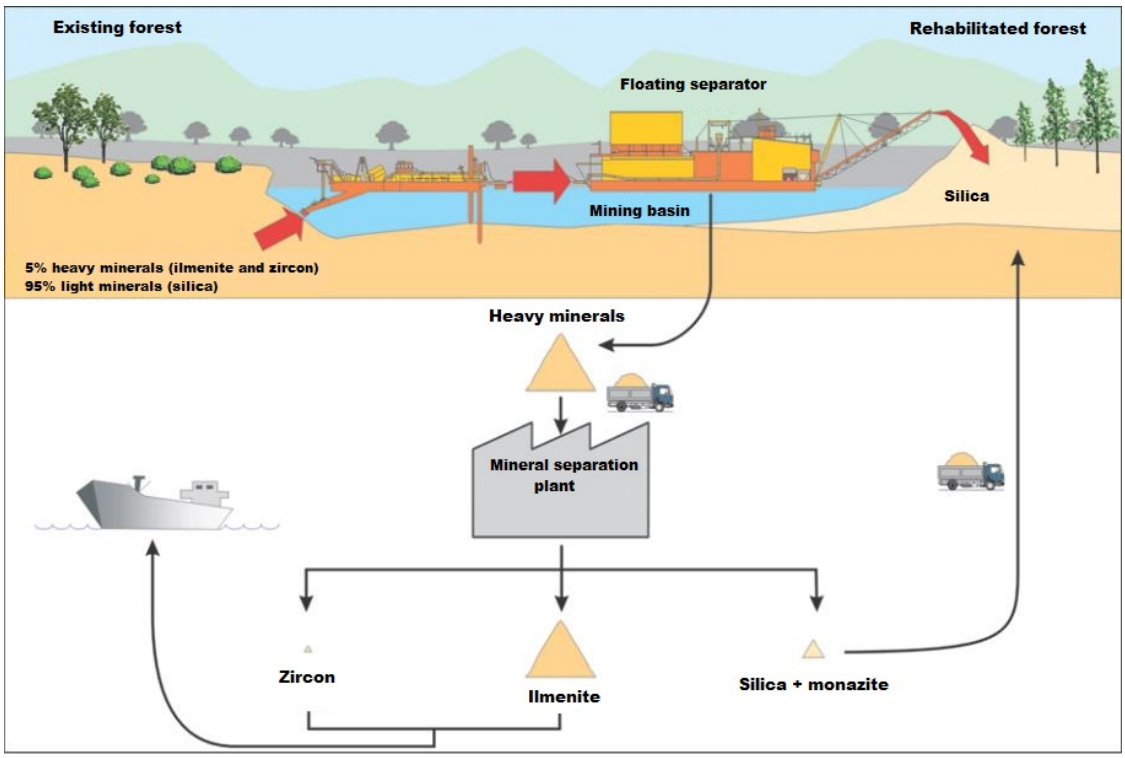


Figure 2: Scheme of the mining operations process used for the extraction of ilmenite by QMM (QMM, 2001).

This inoculum contains eight hundreds spores/g, in a substrate composed of silica, peat and clay. For each plant inoculated with *R. irregularis*, one gram of solid inoculum (800 spores) was spread around the collar of the plants. To allow infiltration of the spores into the soil, fifteen milliliters of water were spread twice over the solid inoculum.

The two strains of *Bradyrhizobium* spp. were isolated from the eastern littoral forest of Madagascar by the National Centre for Environmental Research of Madagascar (CNRE). *Bradyrhizobium* spp. STM1415 was originally isolated from nodules of *Dalbergia louveli*, collected in Ambila Lemaintso, and *Bradyrhizobium* spp. STM1447 was originally isolated from nodules of *Dalbergia monticola*, collected in Andasibe Perinet (29), on the eastern littoral region of Madagascar. These strains were characterized and preserved at the Laboratory of Tropical and Mediterranean Symbioses (LSTM) in Montpellier. *Bradyrhizobium* spp. strains were grown in liquid Yeast extract-Malt extract Agar (YMA) medium at the CNRE headquarters located in Antananarivo. For this inoculation, one milliliter of this liquid inoculum was spread around the collar of the plant, four weeks after seeding.

Experimental design

The experiment was set up as a split-plot experimental design (36). It was composed of four blocks, two main plots and six subplots.

Both soil types formed the main plots (sterilized and unsterilized) and the five treatments and one control formed the subplots.

The treatments tested were:

1) *R. irregularis* DAOM197198;
2) *Bradyrhizobium* spp. STM1415;
3) *Bradyrhizobium* spp. STM1447;
4) *R. irregularis* + *Bradyrhizobium spp. STM1415;*
5) *R. irregularis* + *Bradyrhizobium* spp. STM1447;
and 6) control.

The treatments were randomly applied within the subplots.

Sampling and analysis

Samples were collected 20 weeks after inoculation. The root systems were placed in hermetic plastic tubes in a mix of ethanol: water (1:1) until analysis. Height, root, shoot and total dry mass along with root: shoot dry mass ratios were measured to assess growth, in accordance with previous similar experiments (24, 25, 26). Height was measured with an accuracy of 0.5 cm. To obtain the dry mass, plants were dried at 65 °C for 48 hours. Mycorrhizal colonization was assessed by hyphal and vesicle observations using the gridline intersection method (13) (Photo 3).

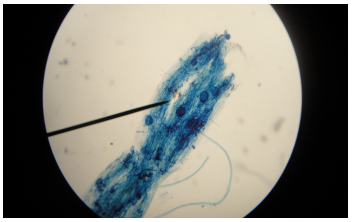


Photo 3: Portion of root of *Mimosa latispinosa* colonized with arbuscular mycorhizae.

Nodulation was assessed by the number of nodules on each root system and the dry mass of nodules. Statistical analysis was conducted using SAS 9.2 for Windows (SAS Institute Inc. 2010). The MIXED procedure was used for the ANOVA (analysis of variance) of the split-plot design.

Growth enhancement was calculated using the same methodology as in previous studies (16).

The Tukey's multiple comparison test was used to compare means for the height (n=32), dry mass (n=12), mycorrhizal colonization (n=8) and nodulation parameters (n=40).

The results of this experiment are presented in table 2 showing the effects of each treatment on plant height, total dry mass, shoot dry mass, root dry mass and shoot/root ratio. This table is provided along with two graphs showing the effects of each treatment on root colonization (Figure 3) and on nodule number and dry mass (Figure 4) 20 weeks after inoculation.

Results

Growth assessment

No significant differences were observed between inoculation treatments for the growth parameters considered (height and whole plant dry mass). However, the overall growth in sterilized soil is significantly greater than in unsterilized soil (Table 2).

Mycorrhizal fungi colonization assessment

In unsterilized soils, there was no significant differences among treatments regarding colonization, including plants inoculated with *R. irregularis*. In sterilized soil, plants inoculated with *R. irregularis* were all significantly more colonized than the non-inoculated ones. Still in sterilized soil, dual inoculation, AMF and *Rhizobium*, did not significantly change the colonization level compared to plants inoculated with the AMF alone (Figure 3).

Nodulation assessment

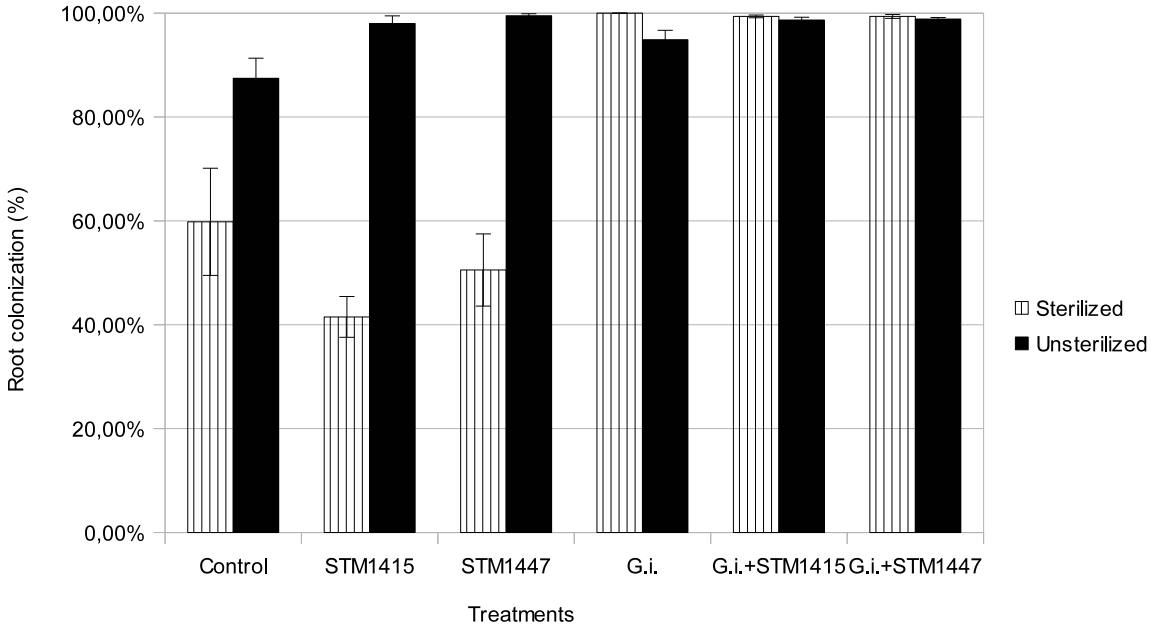
The soil sterilization process did not produce any significant differences for plant nodulation between sterilized and unsterilized soils.

Only *Bradyrhizobium* spp. strain STM1447, when inoculated alone, significantly increased nodulation compared to control and *R. irregularis* treatments. Plants dually inoculated with both *Rhizobium* strains and *R. irregularis* and those inoculated with STM1447 alone had similar level of nodulation compared to each other (Figure 4).

Table 2

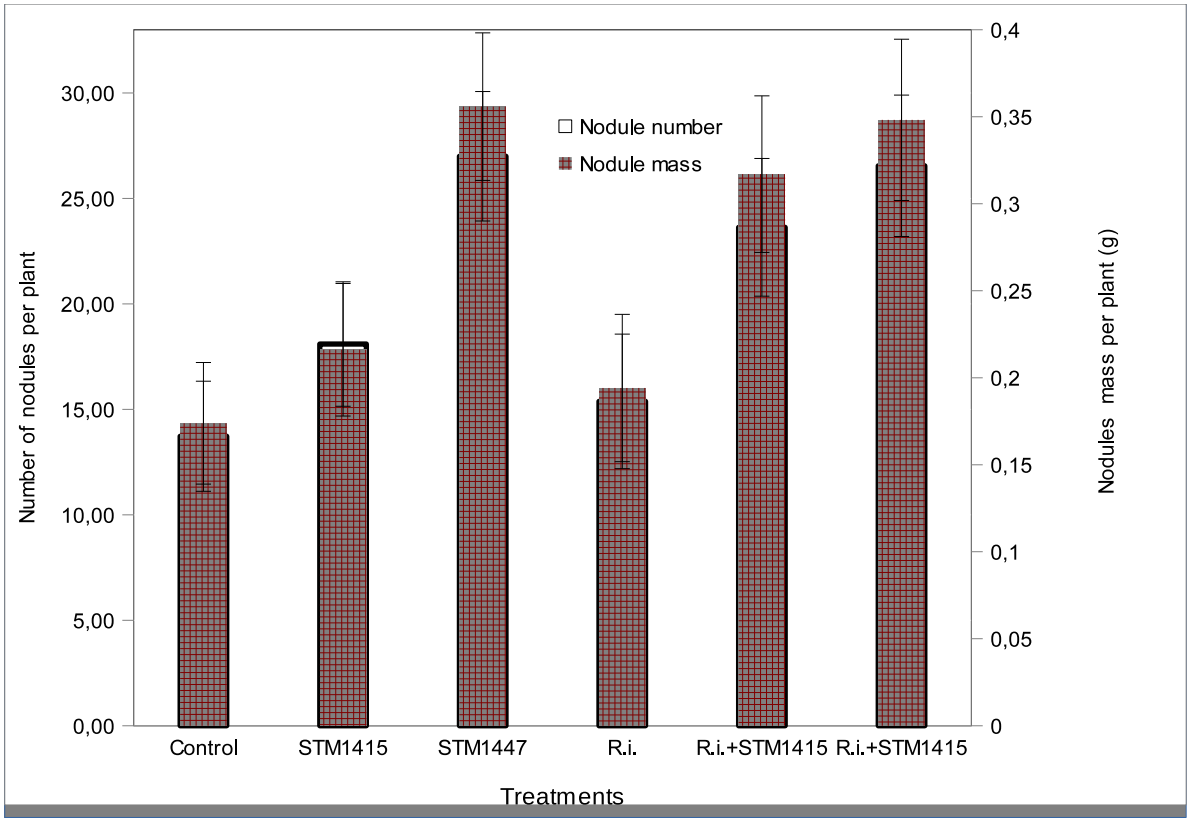
Effects of Bradyrhizobium strains (STM1415 and STM1447) and AM fungi *R. irregularis* (R.i.) inoculation on height (cm), total dry mass (g), shoot dry mass (g), root dry mass(g) and Shoot/Root ratio of *M. latispinosa* 20 weeks after inoculation in unsterilized (US) and sterilized (S) soil.

Soil	Treatment	Height (cm)		Total dry mass (g)		Shoot dry mass (g)		Root dry mass (g)		Shoot:Root ratio	
US	Control	6.90	b	0.57	b	0.22	b	0.36	b	0.61	b
	STM1415	6.25	b	0.57	b	0.20	b	0.37	b	0.54	b
	STM1447	8.03	b	0.78	b	0.30	b	0.48	b	0.63	b
	R.i.	6.63	b	0.57	b	0.21	b	0.36	b	0.58	b
	R.i.+STM1415	7.03	b	0.66	b	0.28	b	0.38	b	0.74	b
	R.i.+STM1447	7.09	b	0.63	b	0.24	b	0.40	b	0.60	b
	Means	6.99	b	0.63	b	0.24	b	0.39	b	0.62	b
S	Control	9.59	a	1.39	a	0.58	a	0.81	a	0.72	a
	STM1415	9.45	a	1.04	a	0.45	a	0.59	a	0.76	a
	STM1447	9.78	a	0.99	a	0.40	a	0.58	a	0.69	a
	R.i.	10.84	a	1.54	a	0.66	a	0.88	a	0.75	a
	R.i.+STM1415	9.64	a	1.10	a	0.47	a	0.63	a	0.75	a
	R.i.+STM1447	11.11	a	1.38	a	0.56	a	0.83	a	0.67	a
	Means	10.07	a	1.24	a	0.52	a	0.72	a	0.72	a



Means (n= 8) with different letters are significantly different according to Tukey's test (P < 0.05). Error bars correspond to standard errors.

Figure 3: Effects of *Bradyrhizobium* spp. strains (STM1415 and STM1447) and AMF *R. irregularis* (R.i.) inoculation on root colonization (%) assessed by hyphal and vesicle structures on *M. latispinosa* 20 weeks after inoculation.



Means (n= 40) with different letters are significantly different according to Tukey's test (P<0.05). Error bars correspond to standard errors.

Figure 4: Effect of *Bradyrhizobium* strains (STM1415 and STM1447) and AMF *R. irregularis* (R.i.) inoculation on nodule number and nodule mass per plant of *M. latispinosa* 20 weeks after inoculation.

Discussion

Results show that plants in tyndallized soil grow better than in unsterilized soil, which are corroborated by other studies (33, 40).

Although this was not investigated in the present study, it is known that soil tyndallization kills pathogens and other harmful organisms and may also release nutrients such as potassium resulting in improved plant performance as reported by other investigators (4, 38). On the other hand, *R. irregularis* is a strain isolated from a boreal environment in Quebec, Canada. Despite its wide ecological valence it is possible that this strain is less efficient in a tropical environment.

It is well known that there are significant phenotypic variations in the efficiency of arbuscular mycorrhizal strains within a species depending on the original growth conditions of those strains and on the population from which it has been isolated (3, 6, 17, 20).

At the colonization level, plants inoculated with *R. irregularis* in tyndallized soil are significantly more colonized than non-inoculated plants, indicating that the inoculated AMF has developed and entered the roots of *M. latispinosa*.

Because of technical problems, however, it was not possible to use refined molecular techniques to monitor the persistence of the introduced microbial inoculants and assess their competitiveness with the native inoculum and the possible contamination during the 20-week period of the experiment. Other studies have also reported cross-contamination through arbuscular mycorrhizal spore dispersal by zoochory, hydrochory or anemochory (19, 32).

At the nodulation level, absence of significant differences between tyndallized and untyndallized soils may indicate that the soil rhizobial inoculum was too low or that sterilization was incomplete. On the other hand, it could also mean that the contamination was important in tyndallized soil. Moreover, non-inoculated plants had an important nodulation, indicating a possible cross-contamination of nodulating bacteria from the surrounding inoculated plants, or from the seeds themselves. Although differences were not significant, nodulation resulting from inoculation with STM1415 was improved upon dual inoculation with *R. irregularis*. This suggests a potential synergistic effect between those two strains for nodulation (3, 21, 31). Other studies also have reported that increase of nodulation with co-inoculation of *arbuscular mycorrhizas* and Rhizobium could result from improved phosphorus nutrition (3, 15, 30). However, our results also have clearly shown that the synergistic effect appears to be strain-dependent since the double and single inoculation of STM 1447 and *R. irregularis* yielded the same nodulation effect (Figure 4).

Conclusion

Our results have shown that neither of the strains of AMF nor *Bradyrhizobium* spp. inoculated alone or dually promoted significantly the growth of *M. latispinosa*. This nursery study shows that tyndallization of the substrate significantly increased plant growth compared to untyndallized soil. It is necessary to pursue the monitoring after outplanting to determine if the trends observed in nursery are sustained in the impoverished demineralized soil after mining operations (Table 1). Future research should continue to isolate and identify native symbiotic strains found into the soil in order to test them in nursery and to select the most ecologically adapted and effective ones in the field. Also, other nitrogen-fixing bacteria that have already been isolated from the eastern part of Madagascar by the National Center for Environmental Research (CNRE) of Madagascar could be tested in nursery (29).

Given the current status of knowledge, results from the present study suggests to sterilize the topsoil in order to improve growth and to proceed with further investigations on native mycorrhizal and rhizobial strains for inoculation on *M. latispinosa*.

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