Trophic relations in the rhizosphere: Effect of fungal, bacterial and Nematodes community on nutrient uptake by plants.

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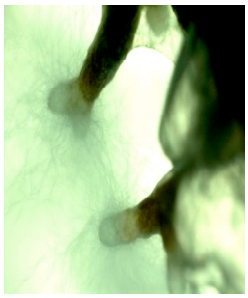
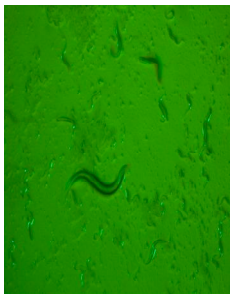
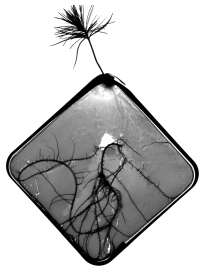
By Usman IRSHAD



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Abstract: The most common responses attributed to microfloral grazers (protozoa, nematodes, microarthropods) in the literature are increased plant growth, increased N uptake by plants, decreased or increased bacterial populations, increased CO_2 evolution in soil, and increased N and P mineralization. Based on this evidence in the literature, an experiment was conducted in which micro-faunal grazers were used. The effects of microbivorous nematodes on nutrient cycling, plant growth, and nutrient uptake were examined with reference to activities within the rhizosphere of *Pinus pinaster* seedlings. Two-month old seedlings, whether or not associated with the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* were grown in gnotobiotic microcosms containing agar medium with insoluble mineral P and nitrate. An additional source of N was supplied as labelled ^{15}N in the bacterial biomass of *Pseudomonas fluorescens*, whether or not with nematodes. After 35 d of co-culture, the treatments containing nematodes and bacteria increased shoot biomass and root surface area compared to treatment without nematodes. Non mycorrhizal plants growing with bacteria and bacteria-feeding nematodes took up more N from nitrate than plants grown alone or with only bacteria, because of increased N mineralization from bacteria, $NH4^+$-N excretion by nematodes, and greater initial exploitation of media by plant roots. Addition of fungal treatment did not increase plant growth or N uptake because the N taken up by the fungus alone was sufficient for plant growth.

Key words: bacterial-feeding nematodes, microbial-faunal interactions, nutrient cycling, plant nitrogen uptake, rhizosphère, woody plant.

Résumé: Dans la littérature, il est communément admis que la prédation de la microflore par la microfaune du sol (protozoaires, nématodes, microarthropodes) améliore la croissance des plantes et leur absorption d'azote, modifie les ppopulations bactériennes, stimule la respiration du sol et entraîne une minéralisation accrue de N et P. Dans ce contexte, nous avons mis en place une expérimentation afin d'étudier les relations multitrophiques de la rhizosphère des arbres forestiers. L'effet de la présence de nématodes bactérivores sur le cycle des éléments minéraux, la croissance végétale et l'absorption des éléments minéraux a été étudiée sur de jeunes plantes de Pin maritime (*Pinus pinaster*). Des plantes âgées de 2 mois, associées ou non avec le basidiomycète ectomycorhizien *Hebeloma cylindrosporum* ont été cultivées en conditions contrôlées en présence de P minéral insoluble et de nitrate comme macro-éléments. Une source supplémentaire d'azote a été ajoutée sous la forme d'azote organique marqué au ^{15}N contenu dans les bactéries *Pseudomonas fluorescens*. Après 35 jours de co-culture, les traitements contenant des nématodes ont augmenté la biomasse de la partie aérienne et la surface des racines par comparaison aux mêmes traitements sans nématodes. Les plantes non mycorhizées cultivées en présence de bactéries et nématodes ont absorbé plus d'azote du milieu que les plantes cultivées seules ou en présence de bactéries seulement, due à une minéralisation accrue de l'azote contenu dans les bactéries, une excrétion d'azote ammoniacal par les nématodes et une meilleure exploitation du milieu par les racines. La présence du champignon ectomycorhizien et des nématodes n'a pas augmenté la croissance ni l'accumulation d'azote dans les parties aériennes des plantes car l'azote absorbé par le champignon seul était suffisant pour la croissance de la plante.

Mots clés : nématodes bactérivores, interactions faune-micro-organismes, cycles des éléments minéraux, absorption d'azote par la plante, rhizosphère, plante ligneuse.

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1-INTRODUCTION

In terrestrial ecosystems, and more particularly forest ecosystems, the availability of macro nutrients such as N and P is often limiting the plant growth. Generally, plants take up these macronutrients as mineral ions from the soil solution. During plant growth, these mineral nutrients are assimilated into complex, organic molecules that come back to the soil to be mineralized by the soil microbial populations. This alternance of mineral and organic forms constitutes the bio-geo-chemical cycle of a given element. In natural conditions, the development of microbial populations can be stimulated by root exudates in the space named a century ago “rhizosphere” by Hiltner (1904). This term was introduced to describe the stimulation of biomass and activity of microorganisms in soil around plant roots. However, even today it is often not fully acknowledged that all nutrients taken up by plants from soil solution pass through a region of intense microbial and faunal activity.

Recent studies have highlighted that nutrient cycling of N and P depends on the activities of microbial populations but also on those of their microbial grazers, such as protozoa and nematodes as it was observed that the presence of microbial grazers was able to stimulate the plant growth. The mechanism behind the effect of grazing is that due to their lower C/N ratio than their preys, excess of mineral N can be released into the soil solution. This hypothesis was confirmed by Ingham *et al*., (1985), who found that most of the bacterial nitrogen ingested by the nematodes was excreted as ammonia after ingestion. Mercer and Cairns (1974) reached a similar conclusion for bacterial-feeding nematodes. Johannes (1968) experimentally demonstrated that bacteria do not always directly mineralize nutrients from organic compounds and that nutrients are released at an accelerated rate when the microbial population is grazed. Coleman *et al*. (1977) found that almost all soil NH_4^+-N, and 40% of the initial P, were immobilized 21 h after inoculation of bacteria into glucose-amended sterile soils. When either amoebae or bacteriophage nematodes were introduced to some of the microcosms, nearly the entire immobilized N was re-mineralized, while less than one-third of the NH_4^+-N was returned in the treatment with bacteria alone. After 24 d, significantly more P, was re-mineralized in the bacteria-and-nematode treatment than in either the treatment with bacteria alone or the bacteria and amoebae treatment.

In addition to the acceleration of mineralization rates, it has been shown that grazing on bacteria may induce dramatic changes in root morphology, with a more branching root system and an increased root surface area (Bonkowski *et al*., 2004; Mao *et al*., 2006). It was hypothesized that the grazing may modifiy the composition of bacterial populations by favouring the ones able to release hormones such as auxin, inducing the modifications of root morphology. This hypothesis was confirmed by the study of Kreuzer *et al*. (2006) on rice. These authors observed that the presence of amoebae increased bacterial activity and significantly changed the community composition and spatial distribution of bacteria in the rhizosphere. In particular, *Betaproteobacteria* did benefit from protozoan grazing. He hypothesize that the changes in bacterial community composition affected the root architecture of rice plants. Thus, grazing creates more complex patterns rather than simply liberating nutrients from grazed bacterial biomass. Particularly, the development of a greater root surface in the presence of protozoa, which enabled efficient nutrient uptake, is a common pattern observed that merits special attention.

Among the microbes living in the rhizosphere, mycorrhizal fungi are also considered as a key component for improving mineral nutrition of the host-plant (Smith and Read, 1977). Besides the positive effect of the mycorrhizal symbiosis almost always observed for plant P nutrition, the effect of this association on the N nutrition has been questionned (Gobert and Plassard, 2008). To our knowledge, only the impact of protozoa

and endomycorrhizal association on plant performance were investigated by Bonkowski *et al*. (2001).

Their results showed that the presence of protozoa resulted in the development of a more complex root system by increasing root length (51%), length of fine roots (64%) and number of root tips (43%). The effects of protozoa were more pronounced in the absence of mycorrhiza. In contrast to protozoa, the presence of mycorrhiza resulted in a less complex root system, i.e. root length, length of fine roots and number of root tips were reduced by 47, 47 and 40%, respectively. Shoot height and shoot biomass were at a maximum in the combined treatment with both mycorrhiza and protozoa. However, beside this work, no study have been carried out so far on the effect of interactions occurring between the ectomycorrhizal symbiosis, bacteria and bacteria-feeding nematodes.

Given the potential importance of such interactions for the supply of N and P to woody plants, the study presented in this report addresses the following questions in a woody species of great economical importance in France, *Pinus pinaster*:

1) Does it exist interactions between bacteria or fungi and nematodes that feed upon ?
2) What are the effect of such interactions on root growth and root architecture ?
3) Do these relationships influence nutrient availability and plant growth ?

In a first step, we used a very simplified system with young seedlings of *P. pinaster*, whether or not associated with the ectomycorrhizal fungus *Hebeloma cylindrosporum*. These seedlings were grown alone or with *Pseudomonas fluorescens* previously labelled with ^{15}N, with or without nematodes isolated from soil sampled in a *P. pinaster* forest.

2- Material and Methods

2.1- Plant and fungal material

2.1.1- Germination of seeds

Seedlings of Maritime pine (*Pinus pinaster* Soland in Ait. from Medoc, Landes-Sore-VG source, France) were grown from seeds that were surface sterilized for 30 min. in a 30% (w/w) H_2O_2) solution. After rinsing with sterile distilled water, the seeds were kept at 4°C for 3 days. These seeds were germinated in Petri plates containing agar medium (15g/L) supplemented with glucose 2g/L. The germination took place after two weeks at 24°C, in the dark.

2.1.2- Mycorrhizal synthesis

Mycorrhizal synthesis was carried out using young germinated seedlings placed in test tubes as described in Plassard *et al*. (1994). For plant inoculation, three agar plugs of *Hebeloma cylindrosporum* (strain D2) (8 mm diameter) were taken from the margin of an active culture and placed in the test tube, in the vicinity of the root (see Appendix A1-1). Non mycorrhizal plants were prepared by replacing fungal agar plugs with simple agar plugs without any fungus. Each tube received 10 ml of a sterile nutritive solution (see Appendix A2-1) which was renewed once a week in sterile conditions. Plants, whether inoculated or not, were placed in a growth chamber under a 16/8h light/dark cycle at 25/18°C, 80% rh, CO_2 concentration of c. 350 mm^3/l and a PAR of c. 400 µmol/m^2/sec (400-700 nm).

2.2- Bacteria

Escherichia coli was used to select bacteria-feeding nematodes from soil. To do that, fresh culture of *E.coli* was prepared by shaking LB medium (see Appendix A2-2) previously inoculated by the bacterial strain DH10B for 24h at 37°C.

A collection of bacterial strains previously isolated from ectomycorrhizal roots was then used to choose a *Pseudomonas fluorescens* strain for the co-inoculation experiment.

This collection was kindly supplied by the UMR LSTM (Laboratoire d'Etude des Symbioses Méditerranéennes, Baillarguet, Hérault). Ectomycorrhizal roots were extracted from soil cores collected in a 14-year old *P. pinaster* plot in the Landes Region, near Bordeaux. The plot belongs to an experimental design comprising three different fertilisation regimes that were i) without fertilisation, ii) P fertilisation, iii) N & P fertilisation, whether irrigated or not. We selected the plot which was irrigated together with N & P fertilisation. Among the 22 individual bacterial colonies available, we chose the strain 111b displaying morphological characters of *P. fluorescens* (a test was made against King B medium, See appendix A2-4). In addition this strain was shown to be able to dissolve insoluble tricalcium phosphate when grown in pure culture with nitrate as the sole source of N (O. Domergue, LSTM, personal communication).

The strain 111b was maintained in LB medium. Labelling of organic N was carried out by growing the bacterial cells in a synthetic medium (see Appendix A2-3 for the composition). The ^{15}N source was added as $(^{15}NH_4)_2SO_4$ labelled at 77%. The amount of ^{15}N used in the co-inoculation treatment was calculated to bring 2 mg of ^{15}N per plant representing the total N contents of plants at the time of inoculation. Thus, in total, 64 mg of ^{15}N was supplied into the culture medium. Bacteria were grown at 26°C in shaking conditions. Ammonium concentration remaining in the medium was assessed in order to determine the duration of growth (see results). Bacteria were centrifuged at 5000 g, 10 min and the bacterial pellet was washed twice in 10 mM $CaCl_2$ solution to eliminate any remaining ^{15}N-NH_4+. Bacteria were then re-suspended in 16 ml of liquid medium which was further used for filling Petri dishes in co-inoculation experiment (see appendix A2-5 for the composition).

2.3-Nematodes

2.3.1-Isolation and multiplication of Nematodes

Nematodes were isolated from a soil sample collected in November 2007 in the same plot as the one used to isolate the bacteria associated with ectomycorrhizal roots of *P. pinaster* trees.

Nematodes were extracted following the Cobb method (see appendix A3-1; s'Jacobb and van Bezooijen, 1986). Nematodes were then inoculated one by one on Petri plates containing T.S.B medium (Tri Syptic Broth 0.3% (w/w), agar 1% (w/w)) supplemented with cholesterol (final concentration of 5 mg/L) added in cooled, autoclaved medium (see appendix A2-5 for the concentrated solution) and already inoculated with 0.4ml culture of *E.coli*.

After 4 days of inoculation, bacterial-feeding nematodes have grown on the agar plates whereas nematodes with other feeding behaviour died. Female nematodes were recognised with plenty of eggs and inoculated on new plates to be sure that we will start monospecific breading. Twenty plates were prepared with one female having plenty of eggs, at this step, the nematode family was unknown.

2.3.2-Sterilization and testing of Nematodes with *Pseudomonas fluorescens* (111b)

In order to have monoxenic breeding of nematodes, sterilization of nematodes was carried out (using the procedure given in Appendix A3-2).

The ability of each sterilized nematode population to feed on the *Pseudomonas fluorescens* strain 111b was checked in sterile conditions.

A nematode population able to eat the *P. fluorescens* strain 111b and multiply on it was then multiplied individually with the same bacterial culture for further use. This nematode belongs to the *Rhabditidae* family; the species determination has not yet been done.

2.4- Co- inoculation experiment

Experiment of co-inoculation consisted of 6 treatments that are:

a: Non-mycorrhizal (NM) *P. pinaster* seedlings (n=6)
b: NM *P. pinaster* seedlings + *P. fluorescens*, strain 111b (n=6)
c: NM *P. pinaster* seedlings + *P. fluorescens* + Nematodes (n=7)
d: Mycorrhizal (M) *P. pinaster* seedlings associated with the ectomycorrhizal fungus, *H. cylindrosporum*, strain D2 (n= 8)
e: M *P. pinaster* seedlings+ *P. fluorescens*, strain 111b (n=8)
f: M *P. pinaster* seedlings+ *P. fluorescens* + Nematodes (n=8)

Co-inoculation experiment was carried out in square Petri dishes (12 x 12 cm, see appendix A1-2) and filled with a solid medium containing nitrate as the sole source of N and insoluble P as the sole source of P (see the composition in appendix A2-5). Petri dishes were manufactured by making a hole to enable us to place the shoots outside. The solid medium was then poured at an angle of 10° to give more support to roots in the opposite direction of the hole. ^{15}N labelled *P. fluorescens* culture (0.5 ml/plant) were spread over the surface of agar medium, together with 0.5ml nematode culture (representing approximately 40 nematodes/plate). The root system of 2-month old pine seedling grown in test-tubes was then placed on the top of the solid medium. A support was given to the plant stem with a cotton plug sterilized twice (120°C, 30 min). Finally the plates were sealed with a sticky tape to protect the plant from contamination. All the plates were placed horizontally in the growth chamber with the same conditions as those previously described in 2.1-2. They were covered with aluminium foil to minimize root system lighting.

2.4- Measurements

2.4.1- Root parameters

The effects of various modes of inoculation on root development were measured after each 15 days interval during the 7 weeks of growth. The root system was scanned without disturbing the plant. Root length, root branching (forks and tips) and surface area were determined with the help of the special software (WinRHIZO).

2.4.2- Nematodes counting

Nematodes were removed from the medium by placing it on a filter for 48 hours. An aliquot (1/10) of the suspension obtained was placed in a counting dish and nematodes were counted under a binocular.

2.4.3- Nitrogen cycling

For chemical analysis, plants were harvested at the end of the co-inoculation period (35 days) and separated into roots and shoots. After weighing fresh weight, the different parts of plants were freeze-dried and dry weights of these samples were recorded before grinding them. Total nitrogen contents and ^{15}N abundance were measured on powder using a mass spectrometer (Tracer Mass; Europa Scientific, Crewe, UK).

Concentration of ammonium remaining in nutrient solution after culture of *P. fluorescens* was assayed using the phenolic colorimetric of Berthelot (Martin *et al*., 1983).

2.5- Presentation of results and statistical analysis

The data obtained were first visualized using R software by tracing box plots. The differences between treatments were then analysed using the software ExcelStat. One way ANOVA followed by multiple comparisons of means (Fisher test) were performed at $p \leq 0.05$.

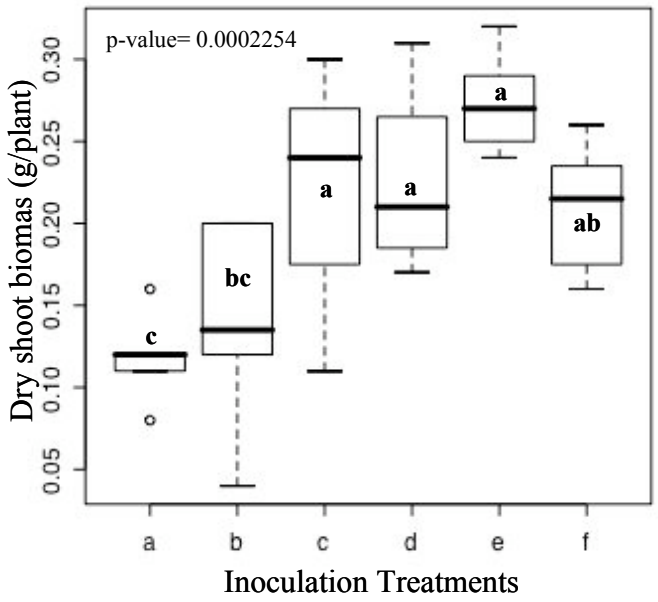

Figure 2. Shoot biomass measured in *P. pinaster* seedlings after 35 d of co-inoculation with an ectomycorrhizal fungus, a bacterial strain and nematodes. The treatments are: a/ non mycorrhizal (NM) b/ NM+bacteria, c/. NM+B+nematodes (NMT) d/ myccorhizal (M), e/ M+B and f/ M+B+NMT. Whisker boxes were calculated from 6 to 8 values. Different letters in boxes indicate that the corresponding means are significantly different at p≤ 0, 05 (ANOVA, Fisher test). Circles out of the boxes represent aberrant values.

3- Results

3.1-Assimilation of $^{15}NH_4$ in bacterial biomass

The strain 111b was grown in pure culture and the evolution of [NH_4^+] remaining in the culture medium is given in Fig. 1. After 42h of culture, most of ammonium had disappeared from the medium. Assuming that all ammonium depleted from the medium was assimilated by the bacteria, and N contained in bacteria at the time of inoculation was close to zero, we calculated that the bacteria assimilated 74.2 mg NH_4^+ from the medium. Bacterial cells were grown until the [NH_4^+] remaining in the culture medium was very close from zero. This was observed after 83h of culture (Fig. 1). After this time, bacteria accumulated 82.3 mg of total N corresponding to 63.4 mg of ^{15}N.

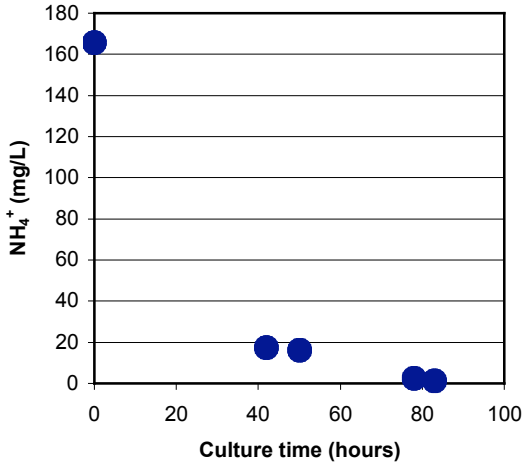

Figure 1. Depletion of ammonium concentration during the culture of *Pseudomonas fluorescens*, strain 111b. Ammonium was supplied as $(^{15}NH_4)_2SO_4$ labelled at 77%.

3.2-Plant parameters

3.2.1- Shoot biomass

The effect of inoculation on dry biomass accumulation of shoot of *P. pinaster* seedlings is shown in Figure 2. Shoots from non mycorrhizal plants (treatment a) accumulated significantly lower amounts of biomass than NM plants grown with bacteria (treatment c) and than mycorrhizal plants alone or grown with bacteria, bacteria and nematodes (treatments d, e and f). Culture of NM plants with *P. fluorescens* slightly increased the shoot biomass by 17% relative to the NM plants but this increase was not significant at $p \leq 0.05$. In contrast, the co-inoculation of NM plants with *P. fluorescens* and nematodes strongly promoted shoot biomass by 85%. When the plants are associated with the ectomycorrhizal fungus *H. cylindrosporum*, shoot biomass significantly increased by 76% compared to the NM plants. The presence of *P. fluorescens* gave rise to the maximal shoot biomass of M plants that was increased by 131% compared to control plants. However, the addition of nematodes to M plants grown with bacteria (treatment f) tended to decrease shoot biomass compared to M plants with bacteria only (treatment e), although this effect was not significant. Finally, this last treatment (e) caused a significant increase of shoot biomass by 85% compared to the control (Fig. 2).

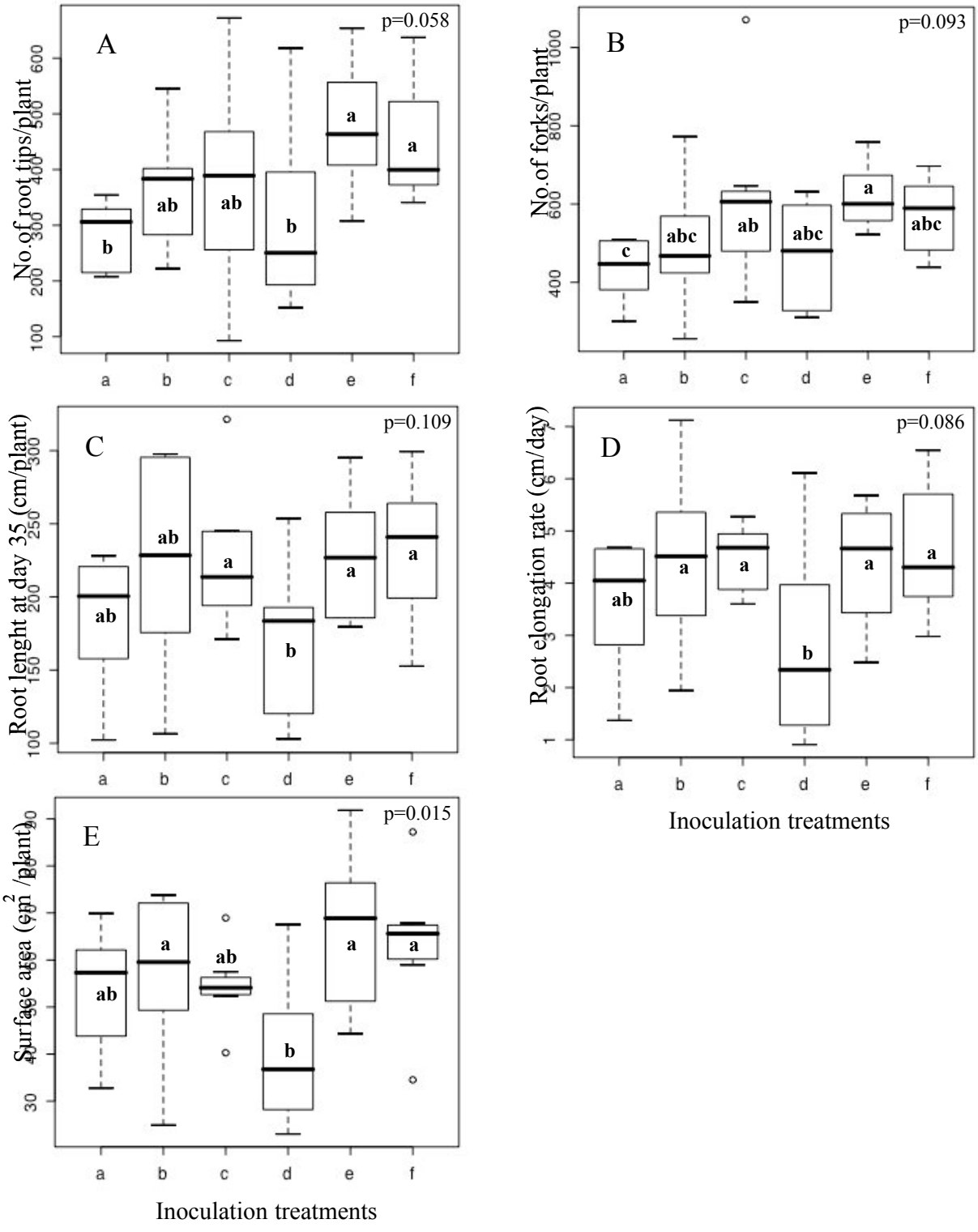

Figure 3. Effect of different treatments as described in figure 2 on root parameters i.e A/ number of root tips, B/ number of forks, C/ root length, D/ root elongation rate and E/ root surface area measured in *P. pinaster* seedlings after 35 days of co-inoculation with an ectomycorrhizal fungus, soil bacteria and bacteria-feeding nematodes. Box plots were calculated from 6 to 8 values. Different letters in boxes indicate that the corresponding means are significantly different at p≤ 0, 05 (ANOVA, Fisher test). Circles out of the boxes represent aberrant values.

3.2.2- Root architecture

Five parameters were recorded to study the effect of the inoculation treatments on root architecture (Fig. 3). Regarding the number of tips recorded after 35 d of inoculation treatments (Fig. 3A), NM and M plants presented lower average values amounting respectively to 286 and 306 tips/plant than those measured in the corresponding treatment with bacteria, whether or not associated with nematodes. However, compared to NM and M treatments, only M plants grown with bacteria (e) and bacteria with nematodes (f) had significantly more root tips per plant, with average values of 477 and 445 tips per plant, respectively. The same trend was observed regarding the number of forks but only treatment e (M+Bac) was significantly higher than the control roots, with values of 618 and 432 forks/plant, respectively (Fig. 3B).

Root length measured at 35 d was found to be highly variable between the plants of a same treatment, excepted when NM plants were grown with bacteria and nematodes (Fig. 3C). Mycorrhizal plants presented lower root length than the same plants grown with bacteria, whether or not with nematodes as well as NM plants with bacteria and nematodes. The same trend was observed regarding the root elongation rate which was about 4 cm/d in all the treatments (Fig. 3D). The lowest value (2.77 cm/d) was calculated in mycorrhizal plants (Fig. 3D).

The most pronounced effect of inoculation treatments was found on the root surface area ($p=0.015$) (Fig. 3E). The highest averages were recorded for treatments e (M+Bac), f (M+Bac+N) and b (NM+Bac) with values of 66.15, 63.5 and 56.53 cm^2 per plant respectively. The two other treatments with NM plants, treatment a (NM) and c (NM+Bac+N) remain close to each other with values of 54.5 and 58.5cm^2 per plant, respectively. Finally, the mycorrhizal association alone (treatment d) gave the minimum surface area amounting to 39.7cm^2 per plant.

3.4-Number of nematodes

The number of nematodes estimated after 35 d of treatment was not significantly different between NM and M plants, with mean values of 140 and 117 nematodes/plant in treatment c and f, respectively (for details, see appendix 1). It should be noticed that the nematode population increased during the experiment, the numbers of nematodes were 3 times greater at the end of experiment as compared to initial numbers.

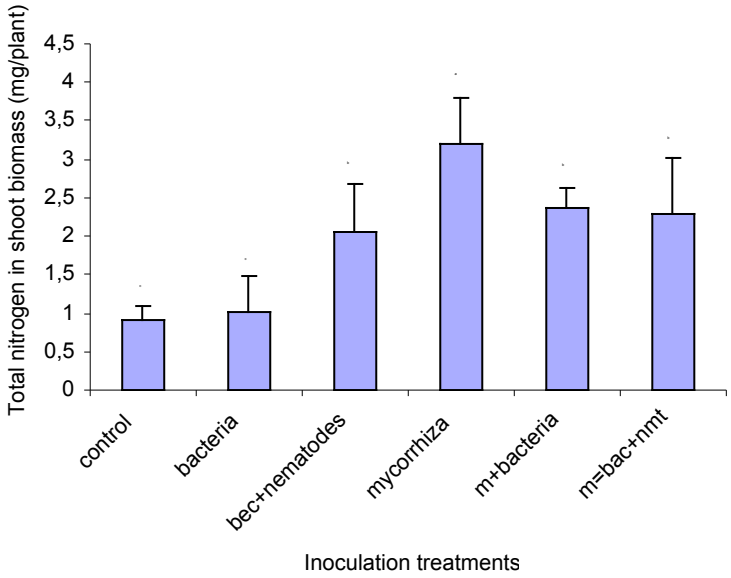

Figure 4. Effect of different treatments on total nitrogen accumulation in shoots of *P. pinaster* seedlings, wether or not associated with an ectomycorrhizal fungus, after 35 days of co-inoculation with a bacterial strain and bacteria-feeding nematodes. The treatments are: NM plants (control), NM plants + bacteria (bac), NM plants + bacteria + nematodes (bac+nmt), M plants (mycorrhiza), M plants + bacteria (m+bac), M plants + bacteria + nematodes (m+bac+nmt). Given values are the means (n=6 to 8) with standard variation and the bars with different letters are significantly different at $p \leq 0.05$ (ANOVA, Fisher test).

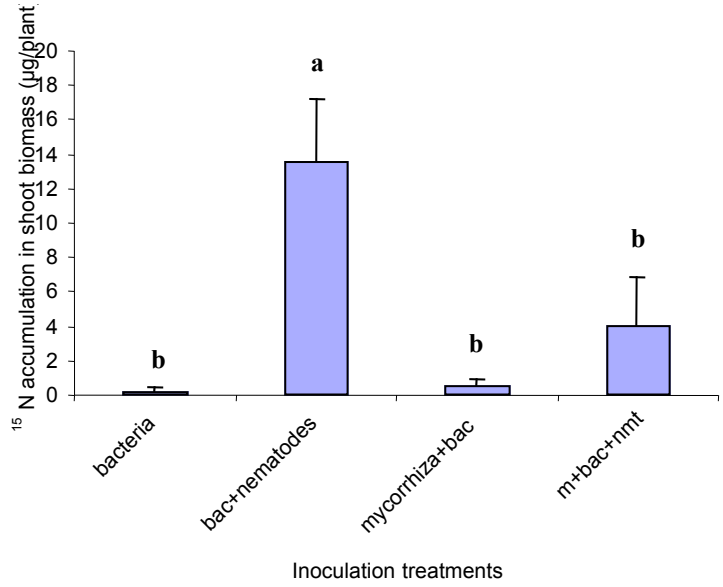

Figure 5. Effect of different treatments on ^{15}N accumulation in shoots of *P. pinaster* seedlings, whether or not associated with an ectomycorrhizal fungus, after 35 days of co-inoculation with a bacterial strain and bacteria-feeding nematodes. The treatments are: NM plants + bacteria (bac), NM plants + bacteria + nematodes (bac+nmt), M plants + bacteria (m+bac), M plants + bacteria + nematodes (m+bac+nmt). Given values are the means (n=6 to 8) with standard variation and the bars with different letters are significantly different at $p \leq 0.05$ (ANOVA, Fisher test).

3.5- Nitrogen accumulation in shoots

Figure 4 shows that the total amounts of nitrogen accumulated in shoots vary significantly according to the inoculation treatments. Maximum accumulation of total nitrogen (3.2 mg/plant) occurred in mycorrhizal *P. pinaster* seedlings (treatment d = mycorrhiza). In contrast, NM plants, grown alone (treatment a = control) or with bacteria (treatment b = bac), accumulated the lowest amounts of total N in their shoots. The presence of bacteria and nematodes significantly increased the amount of N in NM plants (treatment c = bac+nmt) that becomes not significantly different from the values measured in M plants with bacteria (treatment e = m+bac) and bacteria + nematodes (treatment f = m+bac+nmt), around 2 mg/plant (Fig. 4).

3.6- ^{15}N accumulation in shoot biomass

Figure 5 reports the average values of ^{15}N accumulation in shoots from ^{15}N contained in bacterial biomass. Differences between treatments were highly significant with the greatest accumulation occurring in NM plants grown with bacteria and nematodes (treatment c = bac+nmt) with a value close to 14 µg/plant. In contrast, plants grown with bacteria, whether inoculated or not with *H. cylindrosporum* (treatment b = bac; treatment e = m+bac), accumulated very low amounts of ^{15}N. Accumulation of ^{15}N by mycorrhizal plants grown with nematodes (treatment f = m+bac+nmt) was highly variable between plants and generally lower than those measured in NM plants. However, due to this great variability, the means were not significantly different between them (Fig. 5).

4- Discussion

4.1- Effects of co-inoculation treatments on shoot biomass and root development of *P. pinaster* seedlings

The effect of the presence of bacteria, whether or not accompanied by nematodes that are able to feed on them, induced contrasted effects on shoot biomass of *P. pinaster* seedlings after only 35 d of contact. As shown in figure 2, amounts of biomass accumulated in shoots of NM plants were the lowest ones, suggesting that these plants grew very slowly. The addition of bacteria did not significantly improve shoot growth of NM plants, suggesting that the additional nutrient supply contained in bacteria remained inaccessible to the NM plants. These results also suggest that this bacterial strain has a low PGPR effect per se. In contrast, the presence of the ectomycorhizal fungus *H. cylindroporum* strongly stimulated the shoot growth compared to the NM plants (Fig. 2). In M plants, the presence of *P. fluorescens* tended to reinforce this positive effect, although it was not significant (Fig. 2). This positive effect could be due to specific relationships occurring between the hyphae of *H. cylindrosporum* and this bacterial strain, which was isolated from ectomycorrhizal roots collected in the field. These results are in the same line as those of Vosatka (1994) who reported that the dual inoculation (bacteria and fungi) increased the shoot biomass of plants by approximately 30% as compared with control and individual inoculation.

The addition of nematodes in NM plants had a spectacular effect on shoot growth, suggesting that their improved growth could be due to a better nutrient uptake. This improvement of nutrient status could be due, in turn, to the effect of nematodes on mineralization of nutrients accumulated in the bacteria, as suggested by Bonkowski *et al*. (2004) and Ingham *et al*., (1985). In contrast, the addition of nematodes to M plants did not change shoot biomass of *P. pinaster* plants. Despite the fact that no data are

available regarding the effect of the triple inoculation (bacteria + fungus + nematodes), our results are different from other studies reported so far with other microbivorous organisms such as protozoa. As an example, Bonkowski *et al*., (2001) noted that protozoa and mycorrhiza synergistically affected plant performance, i.e. stem mass was at a maximum and specific root length at a minimum in the combined treatment with protozoa and mycorrhiza.

Regarding the root architecture of *P. pinaster* seedlings, we found generally that the presence of *H. cylindrosporum* decreased most of the parameters measured (Fig. 3). These results are in agreement with those obtained by Torres-Aquino & Plassard (2004) who reported a strong negative effect on the growth of *P. pinaster* roots associated with the same fungal strain. Former studies dealing with the effect of mycorrhizal symbiosis on root growth reported negative, positive or no effects. Although the factors responsible for the fungal effects on root growth and development remain largely unknown, a competition for the carbon compounds between the roots and the fungal cells gained by the fungus could explain the decrease in root growth, as proposed by Torres-Aquino & Plassard (2004).

On the other hand, the treatments with bacteria + nematodes and bacteria + nematodes + fungus caused the development of more branched root system (Fig. 3), probably as a result of the micro-faunal grazing as compared to treatments with the fungus alone and NM plants. These results agree with former studies (Jentschke *et al*., 1995; Bonkowski *et al*., 2000; Bonkowski and Brandt, 2002; Bonkowski, 2004) that showed that the grazing of protozoa on bacteria stimulated changes in root morphology, such as a more branched root system with longer, thinner roots. This positive effect of bacteria and bacterial grazing could be due to a PGPR effect via the release of auxin by the bacteria, as proposed by Bonkowski *et al*. (2004). However, this hypothesis should be checked by the ability of this bacterial strain to release auxin in its environment.

4.2- Effects of bacteria alone and with fungi on nematodes population in rhizosphere of pine plant

At the end of the experiment, nematode populations had increased whatever the mycorrhizal status of the plant. Although not significantly different, the lower number of nematodes in mycorrhizal plants (treatment f) may be due to a competition between bacteria and fungi for root exudates as shown by Olsson *et al*. (1996) who found that bacterial production in the rhizosphere of pine was strongly reduced by the presence of ectomycorrhiza. As a result of this nutritional competition the bacteria started to die. The reduction in living bacteria (*P. fluorescens*) ultimately reduced the nematode population as the living bacteria act as a food source for nematodes. This hypothesis is also supported by the study carried out by Andrew & Nicholas (1976) who showed that living *Pseudomonas spp.* attracted bacterial-feeding nematodes, but dead bacteria did not. Therefore, the conclusion that root exudation was reduced in the presence of mycorrhiza is further supported by data on nematodes numbers.

Nematode numbers may be taken as an integrative parameter for the bacterial turnover in the rhizosphere during the experiment. The presence of mycorrhiza caused a decrease in the number of Nematodes indicating that in fact less carbon was available for rhizosphere bacterial production.

4.3- Effect of inoculation treatments on total N and ^{15}N accumulation by shoot biomass

Pine seedlings grown in presence of fungi, bacteria and nematodes as a bacterial grazers accumulated strikingly different amounts of total N (Fig. 4) and nitrogen marked as ^{15}N (Fig. 5) in their shoots. Amounts of total N accumulation in shoots of NM plants, whether grown or not with bacteria, were the lowest ones compared to other treatments. This low accumulation can be due to the unability of plants to take up N supplied as KNO_3 1mM in the agar medium. This could be due, in turn, to the absence of soluble orthophosphate (Pi) available for uptake as all phosphorus was supplied as insoluble mineral P (Tri Calcium Phosphate). Previous studies have shown that maritime pine is not able to taken up NO_3^- from the solution in absence of Pi (C. Plassard, unpublished data). Therefore, this very low Pi availability in the culture medium could have hampered nitrate uptake by NM plants. The presence of bacteria did not change this situation. In contrast, the addition of nematodes strongly increased total N accumulation in the shoots of Pine seedlings by around 1 mg/plant that could come from nitrate in agar medium or from organic ^{15}N supplied with bacteria. The magnitude of ^{15}N accumulation in shoots given in Figure 5 (treatment bac+nmt) amounting to 13 µg of ^{15}N clearly indicate that most of N is coming from NO_3^-. Therefore, these results strongly suggest that the P nutrition of the NM plant was also improved by the presence of nematodes. Thus, our results confirmed the functional role of nematodes in N mineralization as previously shown in other situations (for example, see Stout 1973, 1980 and Bonkowski 2001, 2004; Anju Kamra *et al*., 2004; Anderson *et al*., 1979, 1983; Ingham *et al*., 1985). However, in our experimental conditions, the positive effect of nematodes on shoot growth and N nutrition of NM plants seems to be due to an indirect effect via improved P nutrition and improved nitrate uptake rather than to a direct uptake of ^{15}N.

In contrast to NM plants, Pine seedlings associated with the ectomycorrhizal fungus *H. cylindrosporum* accumulated the highest amount of total N in their shoots (Fig. 4). This supplementary amount of N is greater than the theoretical amount of N that can be supplied during the 35 d period of co-inoculation, calculated as 1 mg of N/plant. Thus, this excess of N accumulated in shoots could be due also to enhanced nitrate uptake from the medium that have occurred before the inoculation treatment (plants were supplied with a complete nutritive solution). This could also result from a higher ratio of shoot N/root N due to the mycorrhizal symbiosis. However, in any cases, our results strongly suggest that the ectomycorrhizal association lead to a positive effect on N accumulation in the shoots of the host plant. This higher accumulation of N in shoots was also observed in the two other treatments (Fig. 4). As observed in NM plants, the addition of ^{15}N labelled N in bacteria did not result in a significant accumulation of ^{15}N in shoots of M plants. The presence of nematodes resulted in a highly variable ^{15}N accumulation in shoots of M plant (Fig. 5). Our hypothesis is that a competition could occur between the hyphae and the roots to take up the mineralized ^{15}N from the bacteria. Thus, this high variability of ^{15}N accumulation could be due to variable degrees of mycorrhizal infection, i.e. plants with a low degree of mycorrhizal roots took up more ^{15}N than well mycorrhized plants.

5- Concluding remarks and perspectives

In this study, we demonstrated that nematodes were able to exert a positive effect for the mineralisation of ^{15}N contained in bacteria, specially in non-mycorrhizal *P. pinaster* seedlings. However, our results suggest that this positive effect is not simply due to ^{15}N uptake but also probably to a better exploitation of nitrate from the medium that depends on P availability. The results were different when the plant was associated with *H. cylindropsporum*, suggesting also that the P nutrition of the host-plant could be enhanced by the mycorrhizal symbiosis. These first results and our hypothesis need to be confirmed by establishing a complete balance of P and N accumulation in roots and shoots of the plants. This, together with the estimation of the ectomycorrhizal degree of mycorrhizal plants, will be carried out in the future.

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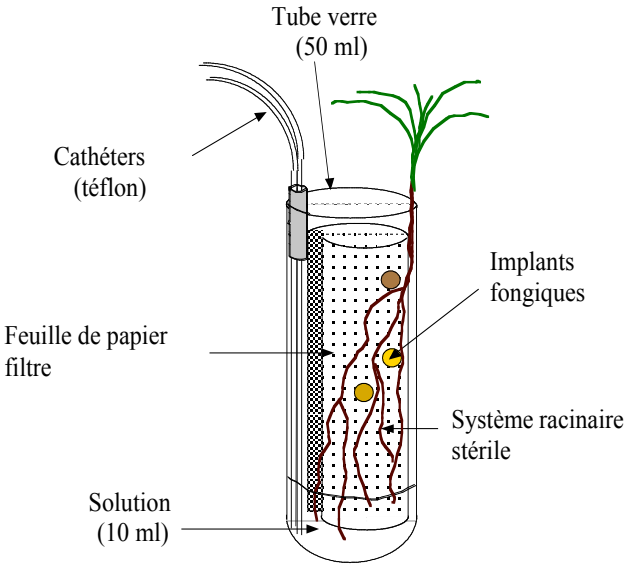
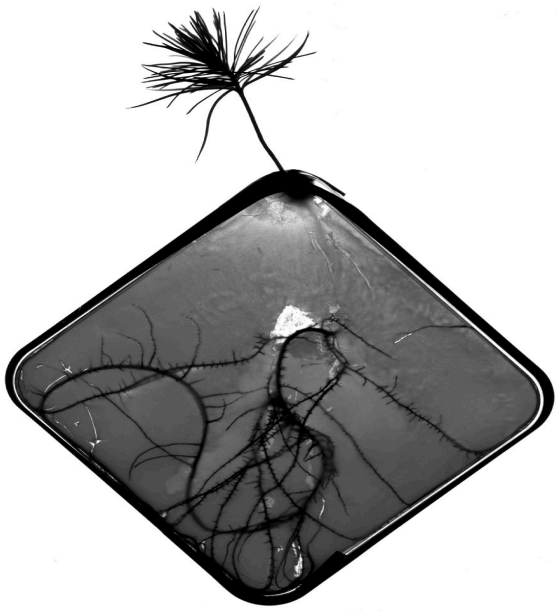
Table of Appendix

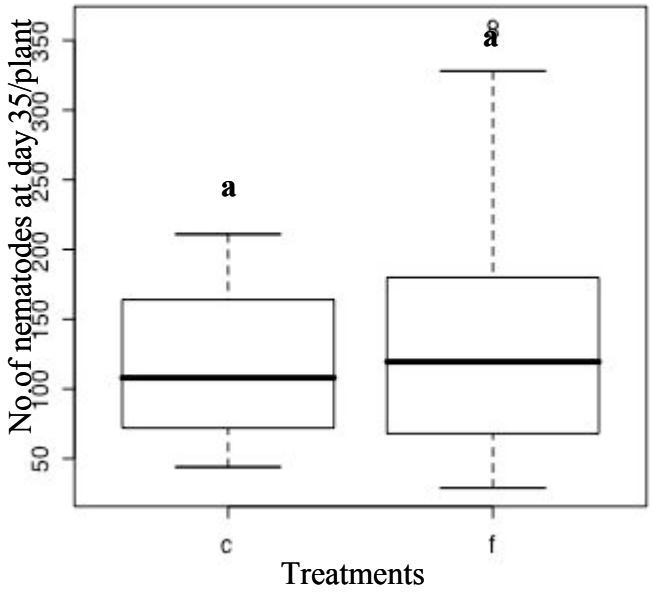
Appendix 1 : Plant culture devices



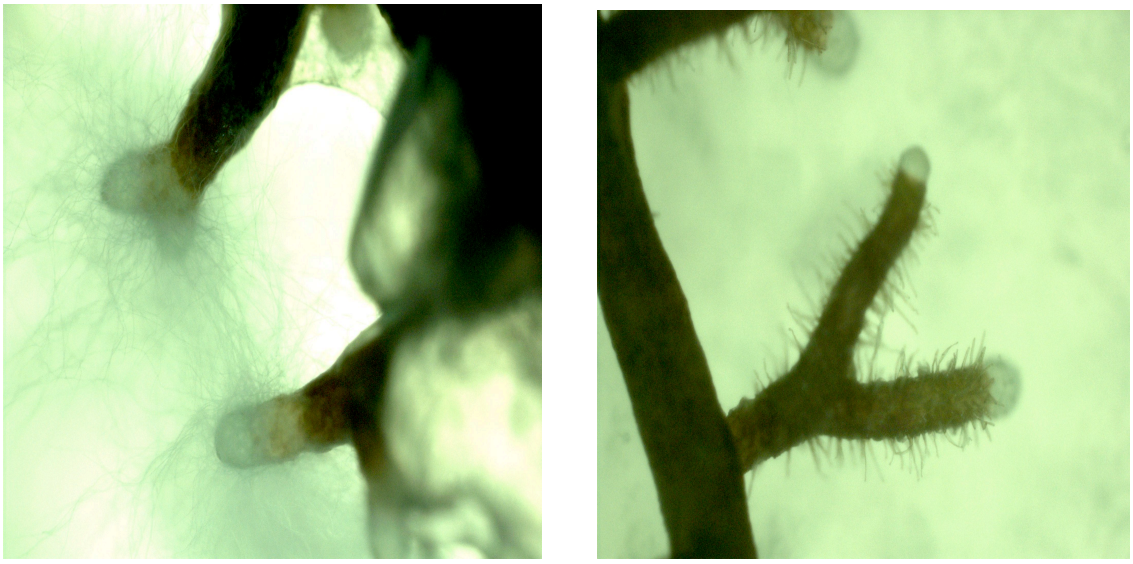
A1-1. Schematic presentation of test-tube used for mycorrhizal synthesis



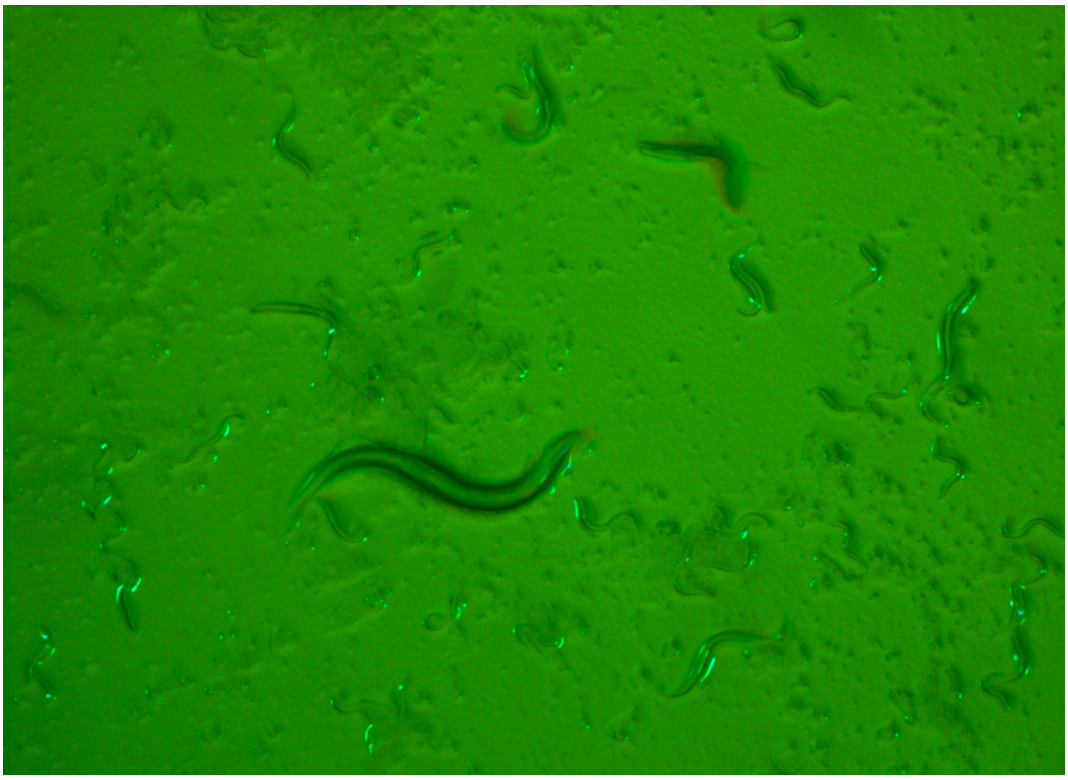
A1-2. Picture of a seedling placed in Petri plate in the co-inoculation experiment



A1-3. Figure of nematodes density at day 35 per plant



A1-4. Roots of *pinus pinuster* with and without mycorrhizae development



A1-5 Picture of nematodes isolated from soil samples from pine forest

Appendix 2: Composition of nutritive solutions

A2-1. N1 medium

Salt	Final concentration
$Ca(NO_3)_2$	0.2 mM
KNO_3	0.6 mM
KH_2PO_4	0.2 mM
$MgSO_4,7H_20$	1 mM
KCl	0.2 mM
Fe citrate 1% (W/V)	0.5 ml/l
Micro-nutrients[a]	0.2 ml/l
Thiamine-HCl (100 µg/L)	1 ml/l

[a]Micro-nutrient solution composition, per liter : 2.82 g H_3BO_3, 98 mg $CuSO_4$ $5H_2O$, 3.08 g $MnSO_4$ H_2O, 0.29 g $NaMoO_4$ $2H_2O$, 4.41 g $ZnSO_4$ $7H_2O$ (Morizet & Mingeau, 1976). This solution is kept at 4°C.

The pH of N1 medium is adjusted to 5.5 with $Ca(OH)_2$ or H_2SO_4 before sterilization by autoclaving 120°C, 20 min.

A2-2. Luria Broth (LB) medium

Compound	Concentration (g/L)
Metapetone	10
NaCl	5
Yeast extract	5
Agar	15

Sterilization by autoclaving 120°C, 20 min

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A2-3. Synthetic medium for *Pseudomonas*

	Final concentration	
KCl	0.8 g/L	11 mM
MgSO4, $7H_2O$	0.5 g/L	2 mM
$CaCl_2$	0.5 g/L	5 mM
$(NH4)_2SO_4$	1.08 g/L	8 mM
KH_2PO_4	0.175 g/L	1 mM
Glucose	5 g/L	
Micro-nutrient solution[a]	4 ml/L	
Vitamin solution[b]	1 ml/L	

[a]Micro-nutrient solution composition, per liter : 125 mg H_3BO_3, 67.5 mg $CuSO_4$ $5H_2O$, 25 mg KI, 500 mg $MnSO_4$ H_2O, 50 mg $NaMoO_4$ $2H_2O$, 650 mg $Fe_2(SO_4)_3$, 100 mg $ZnSO_4$ $7H_2O$, 30 mg $CoCl_2$ $6H_2O$, 25 mg $NiSO_4$ $6H_2O$. This solution is autoclaved 40 min at 110°C.

[b]Vitamin solution composition, per liter: 5 g panthotenate, 20 g inositol, 2 g nicotinic acid, 250 mg pyridoxal hydrochloride, 250 mg thiamine hydrochloride, 10 mg of biotine. This solution is sterilized by filtration at 0.2 µm.

A2-4. King B medium for *Pseudomonas fluorescens*

Compounds	Concentration
Proteose peptone n°3 (DIFCO)	20g/L
K_2HPO_4	8.5mM
$MgSO_4$, $7H_2O$	6mM
Glycerol	8ml/L
Agar	15g/L

pH adjusted to 7.2 and sterilization by autoclaving 110°C, 40 min.

A2-5. Co-inoculation media in one litre

Compounds	Concentration
TCP (tri calcium phosphate)	4g/L
$MgSO_4$	0.5mM
KNO_3	1mM
Micro-nutrients solution[a]	0.2ml/L
Fe citrate 1% (W/V)	0.5ml/L
Thiamine-HCl (100 µg/L)	1ml/L
Agar	7g/L
Cholesterol (5g/L)[b]	1ml/L

[a]Micro-nutrient solution as in A2-1.
[b]Cholesterol dissolved in pure ethanol.
Sterilize the medium by autoclaving at 120°C, 20 min.
Add cholesterol solution in cooled medium at 50°C-60°

Appendix 3: protocols for Nematodes

A3-1. Cobb method to extract nematodes from a soil sample(s'Jacobb and van Bezooijen, 1986)

1- Add 200g of soil in a plastic bottle
2- Add 400ml of tap water
3- Shake it for 3 minutes
4- Leave it for 30 sec for settling down of particles
5- Pass the supernatant from a sieve of 1mm and wash the impurities
6- Add again 400ml of tap water and repeat same operation twice
7- Remove big particles from the sieve of 1 mm
8- Then successively pass the soil solution from sieves of 500, 250, 200, 100 and 50 µm respectively (repeat same operation 3 times with sieve of 50 µm)
9- Collect all material passed through sieves and leave it for 5 minutes for particles to settle down
10- Then pour the final solution on filter paper placed in Petri plate
11- Then leave it for 48 h

A3-2. Sterilization procedure for Nematodes

1- Select 4 Petri plates with a lot of eggs or a lot of females with eggs
2- Add 5ml of sterilized water to collect all the nematodes and their eggs from plate
3- Transfer this material into a centrifugation tube of 15ml
4- Centrifuge it for 8 minutes at 4000rpm$ and at 4°C
5- Remove the supernatant from the tube
6- Add successively and rapidly the following solutions:
   6ml distilled water
   3ml NaOCl (26%)
   0.5ml 1M NaOH
7- Incubate the tube for 22 minutes to be sure that all nematodes are dead
8- Centrifuge it again 4000rpm and at 4°C
9- Remove the supernatant
10- Wash immediately the eggs by adding 10ml of sterilized water
11- Centrifuge it again 4000rpm and at 4°C and repeat twice the washing procedure
12- Put the water solution with in a Petri plate and leave it for 24 hours