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Full Length Article

Nitrogen and Phosphorus Flow Stimulated by Bacterial Grazer Nematodes in Mycorrhizosphere of *Pinus pinaster*

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Abstract

The microbial loop based on the grazing of bacteria by bacterial-feeding nematodes is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. This study was undertaken to quantify the effect of bacterial grazing nematodes on root architecture, growth and mineral nutrition (N and P) in the mycorrhizosphere of woody species (*Pinus pinaster*). Thirty five days simplified sterile experiment was conducted with young mycorrhizal *P. pinaster* seedlings (inoculated with *Hebeloma cylindrosporum*) along with bacteria (*Bacillus subtilis*) and bacterivorous nematodes (*Rhabditis* sp.). To know better the role of microbial complexity on N nutrition, two N sources, bacterial N labeled with ^{15}N and nitrate were supplied in the medium. Phosphorus was supplied as insoluble inorganic tri-calcium phosphate (TCP). The results showed that the ^{15}N flow (4 µg plant^{-1}) from the bacteria to the plant shoots was only significant when nematodes were present. Plants cultivated with bacteria or nematodes also accumulated higher total N and P in their shoots than mycorrihzal ones. These results revealed that the presence of bacterial-feeding nematodes significantly enhanced N and P availability to mycorrihzal *P. pinaster* seedlings, probably by improving plant use of nitrate and insoluble P supplied in the medium.

Keywords: Mycorrhizosphere; Bacterial grazers; Isotopic ^{15}N; Nutrient flow; Plant growth

Introduction

Microorganisms found in the soil are vital to many of the ecological processes that sustain life such as nutrient cycling and decay of plant matter (Panikov, 1999). In terrestrial ecosystems, and more particularly forest ecosystems, the availability of macronutrients such as N and P is often limiting for plant growth. Generally, plants absorb 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.

Hence, plant roots in natural and semi-natural ecosystems are commonly mycorrhizal, the rhizosphere concept has been progressed to include the fungal component of the symbiosis, resulting in the term "mycorrhizosphere" (Rambelli, 1973).

In mycorrhizosphere, mycorrhizal fungi are considered as a key component for improving mineral nutrition of the host-plant (Smith and Read, 1997). In addition to increasing the absorptive surface area of their host plant root systems, the hyphae of these symbiotic fungi provide an increased area for interactions with other microorganisms. Besides the positive effect of the mycorrhizal symbiosis almost always observed for plant phosphorus (P) nutrition, the effect of this association on the nitrogen (N) nutrition has been questioned (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).

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 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

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microbial population is grazed.

However, beside this work, no study has been carried out so far on the effect of interactions occurring between the ectomycorrhizal symbiosis, bacteria and bacteria-feeding nematodes on N nutrition of pine seedlings. Given the potential importance of such interactions the present study was carried out to assess whether the presence of mycorrhizae, bacteria and bacteria-grazing nematodes alone or in combination could improve the mineral nutrition (N, P) in pine seedlings. We tried to evaluate whether these microbial relationships in fact complement each other or not.

Materials and Methods

Seed Germination and Mycorrhizal Synthesis

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 on agar medium (15 g/L) supplemented with glucose (2 g/L) and grown for two weeks at 24°C in the dark. The germination took place after two weeks at 24°C, in the dark. 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 root. Each tube received 10 mL of a sterile nutritive solution, which was renewed once in a week in sterile conditions. Plants were placed in a growth chamber under a 16 h light/8 h dark cycle at 25/18°C, 80% RH, c. 350 mm^3/L CO_2 concentration and a PAR of ca. 400 µmol/m^2/sec (400-700 nm).

Bacteria and Isotopic ^{15}N Labelling

A bacterial strain 111b (*Bacillus subtilis*) previously isolated from ectomycorrhizal roots was maintained in LB medium used in co-inoculation experiment. In addition this strain was shown to be able to dissolve insoluble tricalcium phosphate when grown in pure culture with nitrate as sole source of N.

Labelling of organic N was carried out by growing the bacterial cells in a synthetic medium. The ^{15}N source (77% label) was added as $(^{15}NH_4)_2SO_4$. 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, 32 mg of ^{15}N was supplied into the culture medium. Bacteria were grown at 26°C in shaking conditions. Ammonium concentration remained in the medium was assessed in order to determine the duration of growth. Bacteria were centrifuged and washed twice in 10 mM $CaCl_2$ solution to eliminate any remaining $^{15}N\text{-}NH_4^+$ and re-suspended in 16 mL of liquid medium for further use in co-inoculation experiment.

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 (S'Jacobb and van Bezooijen, 1986). Nematodes were then inoculated one by one on Petri plates containing T.S.A medium [Tri Syptic agar 0.3% (w/w), agar 1% (w/w)] supplemented with cholesterol (final concentration of 5 mg/L) added in cooled, autoclaved medium and already inoculated with 0.4 mL culture of *Bacillus subtilis*. A nematode population able to eat the *B. subtilis* strain 111b and multiply on it was then multiplied individually with the same bacterial culture for further use. This nematode belonged to the *Rhabditis* sp.

Experimental Design and Treatments

Experiment of co-inoculation consisted of 3 treatments that were:

a. Mycorrhizal (M) *P. pinaster* seedlings associated with the ectomycorrizal fungus, *H. cylindrosporum*, strain D2 (n= 8).

b. M *P. pinaster* seedlings+ *Bacillus subtilis*, strain 111b (n=8).

c. M *P. pinaster* seedlings+ *Bacillus subtilis* + Nematodes (n=8).

Co-inoculation experiment was carried out in square petri dishes (12 x 12 cm) and filled with a solid medium containing nitrate as the sole source of N and insoluble P as the sole source of P. 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 *B. subtilis* culture (0.5 mL/plant) were spread over the surface of agar medium, together with 0.5 mL nematode culture (representing approximately 40 nematodes/plate). The root system of 2-months 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 covered with aluminium foil to minimize root system exposure to lighting in the growth chamber.

Root Growth Parameters Measurements

At the end of the experiment, the root systems were scanned using WinRHIZO

(http://www.regentinstruments.com/products/rhizo/Rhizo.html) after opening the dishes to measure the root length. The other root architecture parameters i.e., surface area, number of tips and forks of the roots were also measured.

Nitrogen and Phosphorous Analysis

For chemical analysis, plants were harvested at the end of the co-inoculation period (35 days) and separated into roots and shoots. After weighing different fresh parts of plants were freeze-dried and dry weights of these samples were then 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 remained in nutrient solution after culture of *B. subtilis* was assayed using the phenolic colorimetric of Berthelot (Martin *et al.*, 1983). Total P contents were determined after mineralization with H_2SO_4 36N as described by Torres Aquino and Plassard (2004). Free orthophosphate concentration was assayed in mineralizate according to Ohno and Zibilske (1991) with Malachite green. Concentration of ammonium remained in nutrient solution after culture of *B. subtilis* was assayed using the phenolic colorimetric of Berthelot (Martin *et al*., 1983).

Statistical Analysis

The data obtained were first visualized using Statistica software. Then the data visualized by Kolmogorov-Smiron and Lilliefors test for its distribution suitable to ANOVA. The differences between treatments were then analysed using one way ANOVA followed by multiple comparisons of means (Tukey's test) were performed at $p \leq 0.05$.

Results

Assimilation of $^{15}NH_4$ in Bacterial Biomass

The strain 111b was grown in pure culture and the evolution of ammonium ($[NH_4^+]$) remaining in the culture medium was observed. After 83 h of culture, most of ammonium had disappeared from the medium (Fig. 1). Assuming that all $[NH_4^+]$ 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 37 mg NH_4^+ from the medium. Bacterial cells were grown until the $[NH_4^+]$ remained in the culture medium was very close to zero. This was observed after 83 h of culture. After this time, bacteria accumulated 41 mg of total N corresponding to 32 mg of ^{15}N.

Plant Biomass and Root Parameters

The effect of inoculation on dry biomass accumulation of plants of *P. pinaster* seedlings is shown in Fig. 2. Mycorrhizal plants (m) accumulated significantly lower amounts of biomass than plants grown together with mycorrhiza and bacteria (treatment m+bac). The addition of predator nematodes along with bacteria was found intermediate in total dry biomass accumulation among m (lowest) and m+bac (highest) treatments. Culture of M plants with *B. subtilis* and with nematodes increased the plant biomass by 17% relative to the mycorrhizal plants.

Root lengths of mycorrhizal pine (Table 1) plants after 35 days of growth with bacteria and nematodes showed a significant increase in lengths as we increased the addition of micro-organisms in the rhizosphere. The addition of bacteria (m+bac) and bacteria plus nematodes (m+bac+nem) increased 59% of root length compared to mycorrhizal plants. The other root parameters (surface area, tips and forks) of *P. pinaster* seedlings followed the same trend as for root lengths addition of microbial partners gave significant increase in these parameters (Table 1).

Nitrogen Accumulation in Shoot

Fig. 3a shows that the total amounts of nitrogen accumulated in shoots vary significantly according to the inoculation treatments. Maximum accumulation of total nitrogen (2.54 mg/plant) occurred in mycorrhizal *P. pinaster* seedlings associated with bacteria (m+bac). In contrast, mycorrhizal plants grown alone accumulated the lowest amounts of total N in their shoots. The addition of nematodes plus bacteria to mycorrhizal plants was remained as that of total biomass accumulation.

P Accumulation in Shoot

Fig. 3b shows that the total amounts of phosphorous accumulated in shoots vary significantly according to the inoculation treatments. Maximum accumulation of total phosphorous occurred in mycorrhizal *P. pinaster* seedlings inoculated with bacteria (m+bac). Inoculation of nematodes plus bacteria to mycorrhizal plants increased significantly the P accumulation in shoots compared to mycorrhizal plants.

^{15}N Accumulation in Shoot

Fig 4 shows the average values of ^{15}N accumulated in shoots from ^{15}N contained in bacterial biomass. Differences between treatments were highly significant with a greatest accumulation occurring in mycorrhizal plants inoculated with bacteria and nematodes (m+bac+nem) with a value 4 µg/plant. In contrast, plants grown with bacteria, inoculated with *H. cylindrosporum* (m+bac), accumulated very low amounts of ^{15}N.

Discussion

The effect of presence of bacteria, whether or not accompanied by nematodes that are able to feed on them,

Table 1: Root growth parameters (length, surface area, number of tips and forks) measured in 35 days old mycorrhizal *P. pinaster* plants of different status, either alone or with *B. subtilis* (m+bac) or of the bacteria with the bacteria-feeding nematodes, *Rhabditis* sp. (m+bac+nem)

	Root growth parameters (plant^{-1})			
Treatments	Lengths (cm)	Surface area (cm^2)	Number of Tips	Number of Forks
M	145b	40b	306b	444b
m+bac	227a	64a	477a	571ab
m+bac+nem	233a	66a	446ab	618a
P-Values	0.00	0.00	0.03	0.02

Table 2: Percent increase in plant biomass, root growth and nutrient status of pine seedlings as a result of addition of microbial biodiversity in the rhizosphere. Data is obtained in comparison with mycorrhizal plants only and by putting together bacteria and bacteria plus nematodes inoculated plants

	Plant dry biomass	Root growth parameters				Nutrient shoot accumulation		
Treatment		lengths	surface area	tips	forks	Phosphorus	Nitrogen	^{15}N
m+bac+nem	16	59	64	50	34	51	39	487

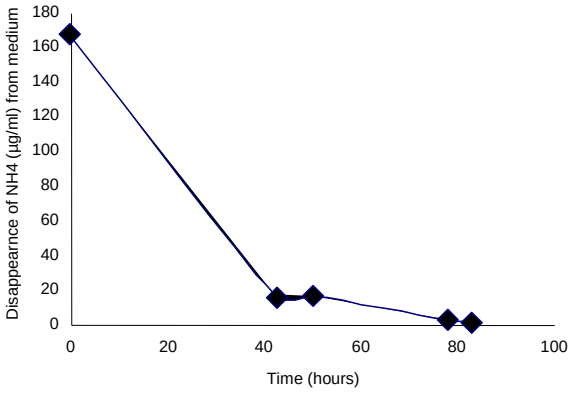

Fig. 1: Depletion of ammonium concentration during the culture of *Bacillus subtillus*, strain 111b. Ammonium was supplied as $(^{15}NH_4)_2SO_4$ labelled at 77%

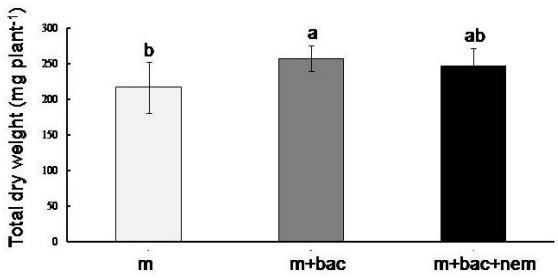

Fig. 2: Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis sp.*) on dry shoot biomass of *mycorrhizal P. pinaster* seedlings 35 days of the inoculation experiment. Bars with different letters shows significant differences (Tukey's minimum significant difference test, $P \leq 0.05$). Each bar is the mean of 8 replicates per treatment (m: mycorrhizal, m+bac: mycorrhiza plus bacteria, m+bac+nem: mycorrhiza, bacteria plus nematodes)

induced contrasted effects on plant biomass of *P. pinaster* seedlings after only 35 d of contact. Amounts of biomass accumulated in mycorrihzal plants were the lowest ones (Fig. 2), suggesting that these plants grew very slowly. The addition of bacteria significantly improved shoot growth of mycorrhizal plants, suggesting that the addition of another microbe to plant rhizosphere gave a synergistic effect. The presence *of B. subtilis* with mycorrhiza tended to reinforce the positive effect on all parameters measured (Fig. 1; Table 1). This positive effect could be due to specific relationship occurring between the hyphae of *H. cylindrosporum* and this bacterial strain, which was isolated from ectomycorrhizal roots collected in the field as reported previously (Bonfante and Anca, 2009; Ngakou *et al*., 2012). Vosatka (1994) 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 to mycorrhizal plants had same effect on root 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 bacteria, through provision of channels to resources in medium as suggested by several researchers (Bonkowski *et al*., 2004, Ferris *et al*., 2010; Irshad *et al*., 2011, 2012). Our results are in the same line with 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.

Pine seedlings grown in presence of fungi, bacteria and nematodes as a bacterial grazers accumulated strikingly different amounts of total N (Fig. 3a) and nitrogen marked as ^{15}N (Fig. 4) in their shoots. Amounts of total N accumulation in shoots of mycorrhizal plants were the lowest ones compared to other treatments. This low accumulation could be due to the inability of plants to take up N supplied as KNO_3 1 mM in the agar medium. This could be due, in turn, to the absence of soluble orthophosphate (Pi) available for uptake as all phosphorus

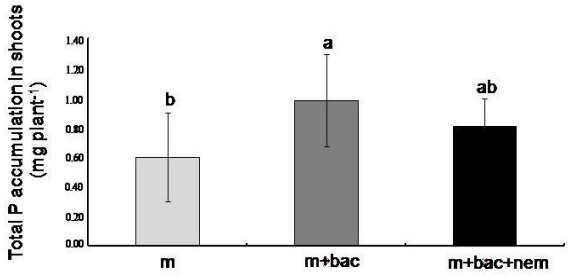

Fig. 3a: Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis sp*.) on the accumulation of N and P in shoots of mycorrhizal *P. pinaster* seedlings after 35 days. Values are the means of 8 replicates per treatment and differences between treatments for a given parameter were analyzed with ANOVA. The different letters are significantly different at P≤0.05

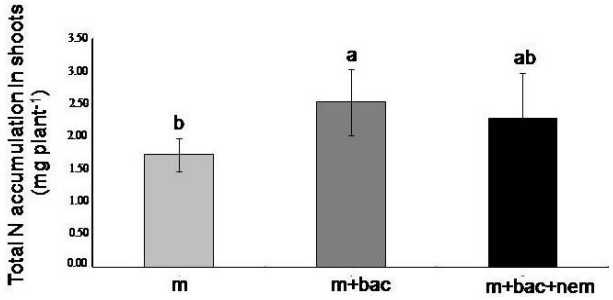

Fig. 3b: Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis sp*.) on theaccumulation of N and P in shoots of mycorrhizal *P. pinaster* seedlings after 35 days. Values are the means of 8 replicates per treatment and differences between treatments for a given parameter were analyzed with ANOVA. Different letters are significantly different at P ≤ 0.05

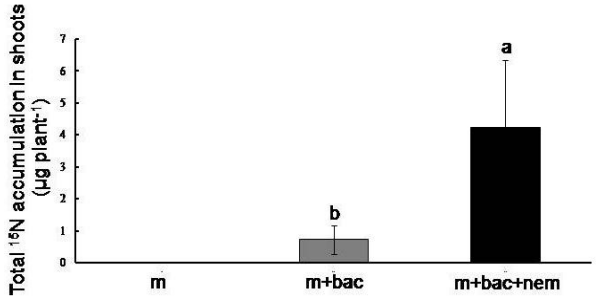

Fig. 4: Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp.) on ^{15}N accumulation in shoots of *P. pinaster* seedlings after 35 days of co-inoculation treatments (m: mycorrhizal, m+bac: mycorrhizal plus bacteria, m+bac+nem: mycorrhizae plus bacteria plus nematodes). Bacteria were labeled with ^{15}N and supplied to the plants at a rate of 2 mg ^{15}N per plant in +bac and +bac+nem treatments. Each bar is the mean of 8 replicates per treatment and error bars are the standard deviations. Bars with different letters are signifcantly different (Tukey's minimum significant difference test, P≤0.05)

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 the nitrate uptake by mycorrhizal plants. The presence of bacteria dramatically changed this situation by showing maximum accumulation of nitrogen compared to other treatments. The addition of nematodes also increased total N accumulation in the shoots of pine seedlings by around 0.54 mg plant^{-1} that could come from nitrate in agar medium or from organic ^{15}N supplied with bacteria. The magnitude of ^{15}N accumulation in shoots (treatment m+bac+nem) amounting to 4 µg of ^{15}N (Fig. 4) clearly indicated that most of N is coming from NO_3^-. Thus, our results confirmed the functional role of bacteria and their grazer nematodes in N mineralization as previously shown in other situations (e.g., Bonkowski, 2001, 2004; Kamra *et al*., 200; Irshad *et al*., 2011, 2012). More specifically, Mao *et al.* (2006) found the same positive impact of bacteria grazing nematodes on tomato seedlings. However, in our experimental conditions, the positive effect of bacteria and their grazer nematodes on shoot growth and N nutrition of NM plants seemed to be due to an indirect effect via improved P nutrition and improved nitrate uptake rather than to a direct uptake of ^{15}N. Similarly, findings of Buchan *et al.* (2013) showed contribution of free-living nematodes on total nitrogen mineralization, with clear evidence of activity of nitrifying organisms stimulated by nematode grazing.

However, in any case our results strongly suggest that the ectomycorrhizal association plus bacteria lead to a positive effect on N accumulation in the shoots of the host plant. As observed in m+bac plants, the addition of labelled N (^{15}N) in bacteria did not result in a significant accumulation of ^{15}N in shoots of mycorrhizal plants. The presence of nematodes resulted in a ^{15}N accumulation in shoots of plants but the amount was very low compared to total N accumulation (Fig. 4). Similar results are reported with non mycorrhizal plants inoculated with ^{15}N marked bacteria and their grazer nematodes (Irshad *et al*., 2011). This is might be due to competition that could have occurred between the hyphae and the roots to take up the mineralized ^{15}N from the bacteria and it remained in the roots rather than become visible in shoots.

According to P accumulation, mycorrhizal plants associated with the ectomycorrhizal fungus *H. cylindrosporum* and *B. subtilis* accumulated the highest amount of total P in their shoots (Fig. 3a). And the treatment (m+bac+nem) accumulated significant amount of total P as compared to mycorrihzal plants alone, suggesting that there is a competition between plant and fungi for P uptake. Torres Aquino and Claude Plassard (2004) also provided evidence which gave the evidence that P accumulation is strongly dependent on the growth stage of seedlings.

In this study, we demonstrated that mycorrhiza and bacteria together were able to exert a positive effect for the uptake of insoluble inorganic P from the medium. Similar effect of

AM fungi and related bacteria was reported by Puppi *et al*. (1994). However, our results suggest that this positive effect is not simply due to a better exploitation of nitrate from the medium but also by the action of bacteria on P solubilization activity (Richardson *et al*., 2001).

The microbial inoculation of plants for enhancement of growth has been reported for more than 100 years. Until now individual microbes inoculation studies got much attention then combined effect of microbial food web on plant growth. We tried to study together three microbial trophic level groups including plant (first trophic group), fungi and bacteria (second trophic group) and their predator nematodes (third trophic group). Specifically, we looked upon plant growth, nutrient status and exact N flow through microbes to plant shoots. The results we obtained suggest that presence of microbial food web in roots is as important that if we delete only bacteria and their grazer nematodes. From it we can reduce root growth up to (50%) and nutrient flow up to (45%) (Table 2). This increase in growth and nutrients are in agreement with the data obtained by Irshad *et al*. (2011) from non mycorrhizal plants. Same effect was observed by Koller *et al*. (2013) who found that plant N uptake *via* mycorrhizal symbiosis strongly benefits from the presence of protozoa. However, the results are not in agreement with the studies of Jentschke *et al.* (1995) and Bonkowski *et al.* (2001) who reported the persistence of the negative effect of ectomycorrhizal symbiosis on root development of Norway spruce seedlings (*Picea abies*) after plant inoculation with either bacteria or bacteria plus protozoa. But the organisms and experimental conditions used in above results were different.

In conclusion, the natural roles of rhizosphere organisms may have been reduced due to global warming, high inputs of inorganic fertilizers, herbicides, and pesticides. Microbial diversity in these systems has been reduced and the functional consequences of this loss of diversity are still unknown. The data obtained indicates that the complementary effect of microbes have positive influence on plant growth and nutrient uptake. This will help compare the situations of disturbed ecosystems, and a better understanding of the interactions of soil microorganisms with each other and with plants for the development of sustainable management of soil fertility and crop production in changing climate.

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