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Grazing by nematodes on rhizosphere bacteria enhances nitrate and phosphorus availability to *Pinus pinaster* seedlings

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ABSTRACT

The microbial loop is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. This microbial loop is based on the grazing of bacteria by predators such as bacterial-feeding nematodes. However, little is known about the impact of grazing by nematodes on the mineral nutrition of woody plants. This study was undertaken to quantify the effect of nematode grazing on bacteria in the rhizosphere on the root architecture, growth and mineral nutrition (N and P) of a woody species (*Pinus pinaster*). Young *P. pinaster* seedlings were cultivated for 35 days in a simplified sterile experimental system with bacteria (*Bacillus subtilis*) and bacterivorous nematodes (*Rhabditis* sp.) isolated from soil samples collected from a 15-year old stand of maritime pine. To check the hypothesis that bacteria could be a source of nutrients, especially N, two N sources were supplied in the medium: (i) bacterial N labeled with ^{15}N and (ii) nitrate. Phosphorus was supplied as insoluble inorganic tri-calcium phosphate (TCP). The results showed that the ^{15}N flow from the bacteria to the plant shoots was only significant when nematodes were present, with an average accumulation of 14 ± 5 μg plant^{-1} of ^{15}N. Plants cultivated with nematodes also accumulated significantly more total N in their shoots than sterile ones or inoculated with bacteria, resulting in a net average increase in N of 700 μg plant^{-1}. The same result was observed for the total P accumulation in the shoots, as plants with nematodes accumulated an average of 300 μg plant^{-1} more P than sterile ones or inoculated with bacteria. However, the presence of bacteria, whether alone or with nematodes, did not modify the root architecture. These results demonstrated that the presence of bacterial-feeding nematodes significantly enhanced N and P availability to *P. pinaster* seedlings, probably by improving plant use of nitrate and insoluble P supplied in the medium.

1. Introduction

The microbial loop is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. This is based on the assumption that newly grown bacteria mobilize nutrients that are not easily accessible to plants, in particular N and P, which are then made available to plants by bacterial grazers (Clarholm, 2005; Kuikman et al., 1991). Of these bacterial grazers, protozoa and nematodes play a major role releasing nutrients sequestered in the bacterial biomass in the rhizosphere (Bonkowski et al., 2009; Villenave et al., 2004). These predators themselves are likely to benefit from increased plant growth since bigger plants may allocate more carbon underground and support a greater root biomass resulting in increased prey (bacteria) density (Bonkowski and Brandt, 2002; Bonkowski, 2004; Phillips et al., 2003). The hypothetical mechanism for releasing N via predation is that bacteria have a lower C/N ratio (approximately 5:1) than their predators (e.g. bacterial-feeding nematodes with approximately 10:1) and so the excess mineral N produced during grazing stimulates the plant growth (Anderson et al., 1983; Wood et al., 1982).

Most research has been performed with annual plants and protozoa, highlighting the role of protozoa on N mineralization, root architecture and plant growth (Bonkowski, 2004; Krome et al., 2009). Few studies have been carried out with woody plants. So far as we are aware, only two studies have reported the effect of protozoa on growth and mineral nutrition of *Picea abies* (Bonkowski et al., 2001; Jentschke et al., 1995) and no results are available for the effect of nematodes on woody plants, despite the ecological importance of nematodes in the soil (Ritz and Trudgill,

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1999). Bacterial-feeding nematodes are a major component in the soil. For example, of the whole nematode population extracted from a mixed deciduous forest, 20–50% of nematodes were bacterial-feeders (Bernard, 1992; Liang and Shi, 2000). Furthermore, a high proportion of bacterial-feeding nematodes (90–99%) are found at sites of high microbial activity, such as the rhizosphere (Griffiths, 1994; Li et al., 2001). Studies carried out with various bacterial-feeding nematode species showed that these organisms increased N mineralization and plant growth of herbaceous species (Djigal et al., 2004a, b; Ingham et al., 1985).

In forest ecosystems, N and P nutrition is always a growth limiting factor (Aerts and Chapin, 2000; Comerford et al., 2002). However, according to Joergensen and Wichern (2008), the mean value of the bacterial contribution to the total microbial C in forest soils ($30 \pm 4.3\%$, mean $\pm$ standard deviation, $n = 125$) is close to that calculated in agricultural situations ($25 \pm 2\%$, mean $\pm$ standard deviation, $n = 267$). This bacterial biomass may constitute an important pool of nutrients in forests if mobilized by predation activity (Osler and Sommerkorn, 2007). This mobilization of mineral nutrients sequestered in the microbial biomass may be of particular importance in poor nutrient forest ecosystems such as the Landes forest in south-west France. This forest ecosystem, consisting of stands of maritime pine (*Pinus pinaster* Soland in Ait.), is used for intensive wood production and requires integrated management strategies to maintain optimal soil fertility (Trichet et al., 1999, 2008).

This study was carried out to assess whether grazing by nematodes on the bacteria in the rhizosphere of a woody plant (*Pinus pinaster*) could improve the mineral nutrition (N and P). Young *Pinus pinaster* seedlings were cultivated with bacteria (*Bacillus subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp.) isolated from soil samples collected from a 15-year old stand of maritime pine. The fate of the bacterial N was monitored by inoculating the plants with bacteria that had been labeled with ^{15}N. The effect of grazing by nematodes on the root architecture, plant growth and mineral nutrition (N and P) was also quantified.

2. Materials and methods

2.1. Plant production

Maritime pine seedlings (*Pinus pinaster* Soland in Ait. from Medoc, Landes-Sore-VG source, France) were grown from seed in sterile conditions and then placed in test tubes as described by Plassard et al. (1994). Each tube received 10 ml of a sterile nutritive solution (0.2 mM $Ca(NO_3)_2$, 0.6 mM KNO_3, 0.2 mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KCl, 50 μg l^{-1} thiamine hydrochloride, 0.5 ml l^{-1} 1% Fe citrate, 0.2 ml l^{-1} micronutrient solution (Morizet & Mingeau, 1976)) which was renewed once a week in sterile conditions. The plants were placed in a growth chamber under the conditions described by Ali et al. (2009) for two months before inoculation.

2.2. Bacteria

The bacterial strain used in this study was isolated from pine roots collected from a 15-year old stand of *Pinus pinaster* in the Landes Region, near Bordeaux. This strain was chosen because it was able to use either nitrate or ammonium as the sole source of N in pure culture. This isolate was identified as *Bacillus subtilis* from DNA sequencing. To label their N pool with ^{15}N, the bacteria were grown in a synthetic medium containing 5.2 mM $(^{15}NH_4)_2SO_4$ labeled at 77%, macronutrients (11 mM KCl, 2 mM $MgSO_4.7H_2O$, 5 mM $CaCl_2$, 1 mM KH_2PO_4), 5 g l^{-1} glucose and 50 mM MOPS (3-[N-morpholino]propane-sulfonic acid, Sigma ref M-1254) to maintain the pH of the medium close to 7 after the complete assimilation of NH_4^+ by the bacteria. After autoclaving (115 °C, 40 min), micronutrient (4 ml l^{-1}) and vitamin (1 ml l^{-1}) solutions were added to the medium as described by Tatry et al. (2009, Supplementary material). The volume of medium was calculated to provide 2 mg of ^{15}N of bacterial origin per plant, which was roughly the amount of total N contained in the plants at the beginning of the inoculation experiment. The bacteria were grown on a shaker (150 rpm) at 26 °C for 83 h, until the NH_4^+ had completely disappeared from the medium measured using the Berthelot method (Martin et al., 1983). The medium was centrifuged (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^+. The bacteria were then re-suspended in 8 ml of 10 mM $CaCl_2$ solution before use.

2.3. Isolation, multiplication and identification of nematodes

Nematodes were isolated from the soil of the same 15-year old stand of *Pinus pinaster* using the Cobb sieving method (s'Jacob and van Bezooijen, 1986). Bacterial-feeding nematodes were selected by growing them on *Bacillus subtilis* cultures added to solid medium containing 1% agar, 3 g l^{-1} Tryptic Soy Broth (Fluka ref 22092) with added cholesterol (5 μg l^{-1} of media). Monoxenic populations of Rhabditidae were obtained by sterilizing the eggs of a single gravid female with Na0Cl. Rhabditidae were removed from the breeding TSA plates by washing the surface with a sterile NaCl solution (1%). They were washed from most *Bacillus subtilis* by centrifugation (1000 rpm, 5 min) and re-suspended in sterile NaCl solution (1%) to give a density of 80 nematodes ml^{-1} for further use in the inoculation experiment.

Genus identification of the Rhabditidae selected was realized by molecular analysis of 18S rDNA gene. DNA was extracted from single nematodes placed in a volume of lysis buffer (Tris–HCl 200 mM, NaCl 200 mM, β-mercaptoethanol 1% (vol/vol), proteinase K 0.8 g l^{-1}) and ultrapure water (25 μl each). After centrifugation (1 min, 5000 g), the mixture was incubated at 58 °C for 1 h in shaken conditions (900 rpm). After cooling at −20 °C for 1 h, the mixture is incubated again at 58 °C overnight. Three microlitres of the DNA extract were used for PCR amplification with Taq polymerase (GoTaq® Flexi DNA Polymerase, Promega) using the primer pair: F3730 forward (5′-ACGGGGGGAGGGCAAGTCTGGTGCCA-3′) and F3729 reverse (5′-TTTACGGTAGAACTAGG-3′) from Eurogentec SA (Liège, Belgium). The thermo cycling pattern used was 95 °C for 2 min (one cycle); 95 °C for 30 s, 61 °C for 30 s and 68 °C for 30 s (25 cycles) and 68 °C for 10 min (one cycle). After checking the presence of one single band of ca 500 bp on gel electrophorese, PCR products were sequenced and identified to genus level by launching a query through blast of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Experimental design

Three treatments were set up: (1) sterile plants (S), (2) plants inoculated with bacteria (B) and (3) plants inoculated with bacteria plus bacterial-feeding nematodes (BN).

Six to seven replicates were set up for each treatment (S, B and BN) to give a total of 19 plants. The experiment was carried out in square Petri dishes (12 × 12 cm) in sterile conditions with a hole to allow the plant shoots to develop outside the dish. Each Petri dish was filled with 70 ml of solid nutrient medium containing 1.5% agarose (Eurogentec Molecular Biology Grade), 1 mM $MgSO_4.7H_2O$, 50 μg l^{-1} thiamine hydrochloride, 0.5 ml l^{-1} 1% Fe citrate, 0.2 ml l^{-1} micronutrient solution (Morizet and Mingeau, 1976), 1 mM KNO_3 as the sole source of N and 4 g l^{-1} insoluble $Ca_3(PO_4)_2$ (TCP) from Fluka (CAS 7758-87-4) as the sole source of P. The quantity of TCP (4 g l^{-1}) was calculated to give 54 mg of insoluble inorganic phosphorus per plant