Micro-food web interactions involving bacteria, nematodes, and mycorrhiza enhance tree P nutrition in a high P-sorbing soil amended with phytate

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Abstract

Phytate is considered a poorly available plant P source but proved to be useful for particular soil bacteria strains. In soil-free conditions, it has been shown that bacteria locked up the mineralized phosphorus from phytate whereas bacterial grazers like nematodes were able to deliver P to plants. Here, we aimed to determine if the interactions between phytate-mineralizing bacteria, bacterial grazer nematodes, and mycorrhizal fungi could increase plant P acquisition from phytate in high P-adsorbing soils. *Pinus pinaster* was grown in a Cambisol supplemented with phytate. Plants, whether associated or not associated with the ectomycorrhizal fungus *Hebeloma cylindrosporum*, were either inoculated or not inoculated with the phytase-releasing bacteria *Bacillus subtilis* and the bacterial-feeding nematode *Rhabditis* sp. After 100 days, the dual inoculation of bacteria and nematodes significantly increased net plant P accumulation. We observed that, on average, mycorrhizal plants accumulated more P in their shoots than non-mycorrhizal plants. However, the highest plant P acquisition efficiency was found when the three soil organisms were present in the *P. pinaster* rhizosphere. We conclude that, in a highly inorganic P-fixing soil, plant P acquisition from phytate strongly depends on the grazing of phytate-mineralizing bacteria. Our results confirm the importance of the soil microbial loop to improve plant P nutrition from phytate, which should be considered a route to improve the utilization of this source of poorly available P by plants.

Keywords

Phosphorus cycling; microbial loop; *Bacillus subtilis*; *Pinus pinaster*; *Hebeloma cylindrosporum*; *Rhabditis* sp.

1. Introduction

Phosphorus (P) limits plant productivity in many terrestrial ecosystems (Holford, 1997; Vitousek et al., 2010). Depending on the soil, P could be abundant as organic P (Po), with compounds including monoesters, inositol phosphates, diesters and phosphonates. Although depending on the environmental conditions, monoesters and diesters tend to be more labile than the inositol phosphates which accumulate in soil (Darch et al., 2014; George et al., 2018). A direct uptake of Po compounds by roots and mycorrhizal fungi has not been documented excepted one report suggesting that non-mycorrhizal poplar (*Populus x canescens*) and beech (*Fagus sylvatica*) roots might take up ADP and/or AMP by yet unknown nucleotide transporters (Scheerer *et al.*, 2019). Hence, it is generally assumed that plant roots take up P as inorganic P (Pi) in the form of free orthophosphate (HPO_4^{2-} or $H_2PO_4^-$; Bucher, 2007). The mineralization of Po is thus a key step in P cycling to supply available P to plants (Marschner, 1995).

The soil Po pool can be mineralized by microbial enzymes, such as phosphatases and phytases (Rodriguez & Fraga, 1999), into Pi potentially available for plants (Hayes, Simpson & Richardson, 2000; Richardson, Hadobas & Hayes, 2000; Mullaney & Ullah, 2003). However, large amounts of Pi released by microbial enzymes are immobilized in the microbial biomass (Oberson et al., 2001; Pistocchi et al., 2018); they are strongly adsorbed by soil constituents such as minerals (especially clays) and organic matter (Holford, 1997) or combined with cations (Ca, Al, Fe). Therefore, only a small amount of Pi from organic P mineralization, estimated to be in the 1–10 µM range (Hinsinger et al., 2011), will effectively supply the free Pi pool. Consequently, two key ecological functions based on rhizosphere biological interactions have evolved over time to increase the efficiency of plant P absorption: (i) widespread mycorrhizal symbiosis between fungi and plant roots (Smith & Read, 2008) and

(ii) microbial loops involving interactions between bacteria and bacterial-feeders (protists and nematodes) (Clarholm, 1985b).

In forest ecosystems, the mutualistic symbiosis between plants and ectomycorrhizal fungi is one of the most important biological interactions involved in P acquisition by trees (Plassard & Dell, 2010). The extraradical hyphae of ectomycorrhizal fungi are able to prospect a large volume of soil, overcoming the Pi depletion zone created by plant roots (Becquer et al., 2014). The fungal cells can also release carboxylates (Finlay, 2008) and enzymes (Colpaert & van Laere, 1996; Courty et al., 2006; Louche et al., 2010) to increase P acquisition from soil mineral and organic sources (Plassard et al., 2011). After uptake, P is transported along the hyphae towards ectomycorrhizas as polyphosphates accumulated in mobile vacuoles (Nehls & Plassard, 2018).

Among Po forms in soil, phytate (*myo*-inositol hexakisphosphate, $C_6H_{18}O_{24}P_6$) is considered as the most abundant source (Alexander, 1977; Turner, 2007) but also as the poorest P source to plants because most of the plant species are not able to release phytases (enzymes required to release the Pi group from its ester bond) into their rhizosphere (Richardson et al., 2001). In contrast, rhizosphere bacteria are able to produce phytases (Mullaney & Ullah, 2003; Turner, 2007) that could promote the release of Pi from phytate in the vicinity of plant roots. Therefore, the inoculation of phytate-mineralizing bacteria in the rhizosphere of plants is potentially highly beneficial for plant P nutrition (Richardson et al., 2001).

This effect could be strengthened when soil bacterial grazers are present in the rhizosphere of plants as part of the soil microbial loop (Clarholm, 1985b). Bacterial feeders (mostly protists and nematodes) release nutrients stored in the microbial biomass, increasing the activity of decomposers (Kuikman et al., 1991, Jansen & Vanveen, 1991; Clarholm, 2005) and the nutrients become available to plants (Bonkowski, 2004). Trap et al. (2016) showed that soil bacterivores could significantly enhance plant P nutrition, up to 30%, through their grazing

activities. However, the increase in soil P availability induced by the interactions between bacteria and bacterial-feeders has been reported to be slight (Anderson et al., 1978; Darbyshire et al., 1994; Djigal et al., 2004a), high (Cole et al., 1978), or sometimes even null (Griffiths, 1986). It is difficult to explain these discrepancies, mainly because the mechanisms and the controlling factors by which bacterivores increase plant P acquisition have not been identified clearly.

The experiment carried out by Irshad et al. (2012) in an agarose medium showed that the presence of both bacterial-feeding nematodes and phytase-producing bacteria is required to increase the P content in *Pinus pinaster* seedlings, probably by increasing the activity of phytate-mineralizing bacteria. However, we do not know whether similar interactive effects can be obtained in experiments using high P-sorbing soils rather than low-Pi-sorbing agarose. In this study, we aimed to determine if the presence of the above-mentioned potential rhizosphere mutualists in the rhizosphere, alone or together, promotes plant P acquisition from phytate in a highly P-sorbing soil with a very low level of available P. For that purpose, we grew *P. pinaster* seedlings, either associated or not associated with the ectomycorrhizal fungus *Hebeloma cylindrosporum*, in a Cambisol amended with phytate, and either inoculated or not inoculated with phytase-releasing bacteria (*Bacillus subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp.). We formulated four hypotheses. We first assumed that the plant alone, without mycorrhiza nor free-living organisms, does not get P from phytate, as observed in agarose (Irshad et al., 2012). Because mycorrhizal fungi are well-known to take up Pi not available to roots, we expected higher plant P uptake when the ectomycorrhizal fungus is inoculated, irrespective of the presence of free-living organisms (H2). We supposed that the inoculation of free-living organisms will enhance phytate mineralization and plant P availability (H3). Finally, we supposed that the plant P uptake from phytate would be maximal when all potential rhizosphere mutualists are inoculated (H4).

2. Materials and Methods

2.1. Plant and mycorrhizal synthesis

Maritime pine (*P. pinaster)* seedlings were grown from seeds (Medoc, Landes-Sore-VG, France) as described by Becquer et al. (2017). Briefly, the seeds were first soaked in deionized water at 4 °C for 48 h before being sterilized in a 30% H_2O_2 solution for 30 minutes and rinsed several times with 1 L of sterile deionized water. The seeds were deposited in a solid medium (agar 1.5 g L^{-1} and glucose 2 g L^{-1}) in Petri dishes (90 mm diameter) placed at 25 °C in the dark for 2–3 weeks. Petri dishes showing fungal or/and bacterial development around the seeds were disposed of. Hence, we selected dishes containing actual sterile germinated seeds to prepare plants in glass test tubes (see below and Figure 1A).

A dikaryotic strain of the ectomycorrhizal basidiomycete *H. cylindrosporum* (Debaud & Gay, 1987) was used to get ectomycorrhizal *P. pinaster*. The fungus was grown in the dark at 24 °C in a standard nitrate medium (Louche et al., 2010) for three weeks.

Glass test tubes (15 cm height) containing a filter paper (Whatman 1542–240 Hardened Ashless Grade 542) inserted vertically inside the tube and 10 ml of a glucose solution (10 g L^{-1}) were sterilized twice at an interval of 48 h (Becquer et al., 2017). The glucose solution of each tube was then replaced by a nutritive solution containing 1 mM nitrate and 0.2 mM phosphate (Irshad et al., 2011). Plants, whether inoculated or not with *H. cylindrosporum*, were prepared with sterile germinated seeds having a root more than 2 cm long, but with their external teguments still attached (Becquer et al., 2017). To prepare mycorrhizal (M) plants, three small pieces of solid nitrate medium with *H. cylindrosporum* mycelium were placed on the filter paper near the roots (Figure 1A and supplementary material 1). Non-mycorrhizal plants (NM) were produced by placing germinated seedlings in the tubes without the fungus. The tubes were kept for two months in a growth chamber (16/8 h light/dark cycle at 24/18 °C,

144 400 µmol m^{-2} s^{-1}, 400-700 nm, RH 80%). The level of the nutrient solution (15 ml per tube)
145 was topped up every week in sterile conditions.
146
147 ***2.2. Bacterial strain and nematodes***
148 The bacterial strain used in the experiment was isolated from ectomycorrhizal roots collected
149 in a 15-year-old *P. pinaster* plot in the Landes Region, near Bordeaux (France). The strain
150 was identified as *Bacillus subtilis* (named 111b; Irshad et al., 2011) and was able to use
151 phytate as the sole source of P via the release of an alkaline phytase. For the inoculation
152 experiments, the bacteria were cultivated in a liquid medium, as described in Irshad *et al.*
153 (2011) for 4 d at 26 °C. The culture medium was centrifuged at 3500 g for 10 minutes and the
154 pellet was washed twice before being suspended in a volume of sterile deionized water
155 adjusted to get approximately 2 million bacteria per mL. Monoxenic populations of
156 nematodes (*Rhabditis* sp.) were isolated from the soil collected in the same plot (Landes,
157 France) and were cultured in *B. subtilis* (bacterial strain 111b) following the procedure
158 described previously (Irshad *et al.*, 2011). *Rhabditis* is a common, ubiquitous genus that is
159 classified in the first class along the colonizer-persistent gradient (cp) into the maturity index
160 (Bongers, 1990).
161
162 ***2.3. Soil preparation and phytate addition***
163 The soil used for the co-inoculation experiment corresponded to the A layer of a chromic
164 cambisol collected in the south of France (Cazevieille, Herault). It is a clay soil (48% clay, pH
165 in water = 6.9, total N = 2.5 g kg^{-1}, total P = 0.9 g kg^{-1}) with a very low concentration of
166 bicarbonate-extractable inorganic P (c.a. 3 mg kg^{-1}; Casarin et al., 2003). Before use, the soil
167 was dried at ambient temperature and sieved at 2 mm. Then, the soil was mixed with acid-
168 washed (HCl 1N) glass beads (50/50, w/w) to get homogenous humidification of the soil-bead

169 material during the experiment. The soil and glass bead mixtures were prepared for each
170 replicate by mixing them in a 50-ml polypropylene tube with 15 g of soil, 15 g of glass beads,
171 and 15 ml of a nutrient solution containing 9.3 mM KNO_3, 9.3 mM $MgSO_4$ $7H_2O$, 18.6 mM
172 $CaSO_4$, 200 µg L^{-1} thiamine hydrochloride, 2.3 ml L^{-1} 1% Fe citrate, and 1 ml L^{-1} solution of
173 micronutrients (Morizet & Mingeau, 1976). The soil was then autoclaved twice a week at 115
174 °C for 40 min.
175 A solution of phytate (9.24 g L^{-1}, inositol hexakisphosphate sodium salt from Sigma, ref
176 P0109) was prepared in sterile deionized water. Before sterilization by filtration (0.2 µm), the
177 pH of this solution was adjusted to 7 with HCl. After cooling the soil at room temperature,
178 each tube was opened in sterile conditions to add 46.2 mg of phytate (5 ml of the phytate
179 solution) and 350 µg of cholesterol (70 µl of cholesterol 5 mg L^{-1}, in ethanol). These amounts
180 of phytate and cholesterol were equal to those added to the agarose medium by Irshad et al.
181 (2012). The tubes were then shaken by inversion overnight to get a homogeneous soil
182 suspension.
183
184 ***2.4. Experimental design***
185 The experiment was carried out in sterile conditions in 12 x 12 cm square Petri dishes (Figure
186 1B-E and supplementary material 1). Each dish is first pierced with two holes, one in the lid
187 for watering and the other in a corner in order to allow the growth of the plant shoot outside
188 the dish and filled with the soil-bead-nutrient solution mixture from one polypropylene tube.
189 Two-month-old plants were removed from the test tubes and transferred to the Petri dishes.
190 Six biological treatments, corresponding to two levels of plant mycorrhizal status combined
191 with three inoculation treatments, were set up.
192 Each plant, associated (M) or not associated (NM) with the ectomycorrhizal basidiomycete *H.*
193 *cylindrosporum*, was (i) not inoculated with free-living organisms (None), (ii) inoculated with

194 the phytate-mineralizing bacteria *Bacillus subtilis* (+Bac), or (iii) inoculated with both the
195 bacteria and bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). The bacteria and the
196 nematodes were inoculated at a rate of approximately 10^6 bacteria and 300 nematodes per
197 dish, supplied in 0.5 ml solution each. Six replicates were set up for each biological treatment,
198 giving a total of 36 Petri dishes. The Petri dishes were placed horizontally in the same growth
199 chamber as described above and covered with aluminum foil to limit the light on the roots.
200 The plants were allowed to grow for 100 days. Each Petri dish was watered once a week with
201 sterile water to compensate for water loss during the experiment. At the beginning of the
202 experiment (time 0), 3 NM and 3 M plants were used to determine the initial values of root
203 and shoot dry weight and their P accumulation.
204
205 ***2.5. Plant and soil analysis***
206 At the end of the experiment, the shoots and the roots of each plant were separated. Intact root
207 systems were cleaned, then scanned and digitized using WinRHIZO
208 (http://www.regentinstruments.com/products/rhizo/Rhizo.html) to measure parameters such
209 as length, area, number of tips, number of forks, and number of crossings. The root and shoot
210 dry biomass were determined after oven-drying at 60 °C for 48 h. The total P content in the
211 shoots and roots was determined after crushing and mineralizing plant tissues with 36N
212 H_2SO_4 acid, as described by Aquino & Plassard (2004). The P concentration in the
213 mineralized solution was analyzed using colorimetry with malachite green (Ohno & Zibilske,
214 1991). Absorbance was measured at 630 nm. The P acquisition efficiency of the plants was
215 calculated by dividing the total plant P by the root length.
216 Furthermore, we collected as much soil-bead mixture as we could possibly could without
217 damaging the roots and used it (i) to check for the presence of living nematodes and (ii) to
218 quantify the abundancy of 18S rDNA gene copies. Nematode survival rates were determined

by active filtering through fine cellulose wadding paper for 48 h and counted using a binocular stereoscopic microscope (Southey, 1986). For each sample, microbial DNA was extracted from 0.250 g of soil-bead mixture following the procedure developed by Tournier *et al.* (2015). The quality of the extracted DNA was improved by adding 500 µL of 5.5 M guanidine thiocyanate during the seventh step of DNA extraction. Quantitative real-time PCR was performed on 2.5 ng of extracted DNA to quantify fungal (18S) rDNA gene copies in each sample. The PCR products were amplified with SYBR® Green Master Mix (BIORAD, USA) using the primer pairs FR1 forward (AICCATTCAATCGGTAIT) and FF390 reverse (CGATAACGAACGAGACCT) to estimate the number of 18S rDNA gene copies per gram of soil. The thermocycling pattern used was as follows: 98 °C for 2 min; 39 cycles at 98 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s; and a final elongation step at 55 °C for 5 s and 25 °C for 30 s.

2.6. Statistical analysis

The mean and standard deviation (n = 6) for each measured variable were calculated for each modality. Two-way analysis of variances was conducted to test the “mycorrhiza” and “free-living organisms” factors and their interaction on plant variables. We conducted one-way ANOVA followed by post hoc Tukey HSD test on all modalities as factors. The normality of ANOVA residues was checked using the Shapiro-Wilk test. Using the Student t-test, we also tested whether the final plant biomass and P amount values for each biological treatment were different from the initial value. All statistical analyses were performed using the R software (Team, 2016) with statistical significance at 5%.

3. Results

3.1. Plant biomass and root architecture

244 At the end of the experiment, plant shoot and root dry biomasses were significantly higher
245 than those measured in two-month-old-plants (Figure 2). The two-way ANOVA (Table 1)
246 showed that the shoot and root dry biomasses were significantly affected by the presence of
247 the ectomycorrhizal fungus *H. cylindrosporum,* irrespective of the presence of free-living
248 organisms, with no interactive effect between the two main factors "mycorrhiza" and "free-
249 living organism" (Table 1). Taken as a whole, M treatment decreased shoot and root biomass
250 compared to NM treatment (Figure 2). The inoculation of free-living organisms did not
251 modify shoot biomasses of NM or M plants, nor the root biomasses of NM plants. However,
252 M plants not inoculated (None treatment) had lower root biomass (- 43 %) than NM plants,
253 whether not inoculated (None) or inoculated with bacteria and nematodes (+Bac +Nem).
254 Also, M plants inoculated with bacteria (+Bac) had lower root biomass (- 30 %) than NM
255 plants inoculated with bacteria and nematodes (+Bac +Nem). Only M plants inoculated with
256 nematodes and bacteria (+Bac +Nem) had the same root biomass as NM plants (Figure 2).
257 Regarding the root parameters (Table 2), the two-way ANOVA showed only a significant
258 effect of the M treatment and no significant interaction between the factors "mycorrhiza" and
259 "free-living organisms" for all the root variables (Table 3). The effect of the fungus was
260 confirmed by the one-way ANOVA (Table 2). In average, M treatment decreased all the root
261 parameters, with a stronger effect on root ramification (- 130% for the number of crossings, -
262 93 % for the number of forks, - 70 % for the number of tips) than on root length (- 47 %) and
263 surface areas (- 27 %) (Table 2).
264
265 ***3.2. Plant P accumulation***
266 At harvest, only the shoots of NM plants inoculated with bacteria and nematodes (+Bac
267 +Nem), while those of all M plants, accumulated higher amounts of P than two-month-old
268 plants (Figure 3). In roots, the same trend was observed for NM plants, but only M plants

inoculated either with bacteria (+Bac) or with bacteria and nematodes (+Bac +Nem) accumulated more P than two-month-old plants (Figure 3). The two-way ANOVA showed that shoot P was significantly affected by the two factors "mycorrhiza" and "free-living organism" while root P was affected only by the presence of free-living organisms, with no interactive effect between these two main factors (Table 4). Taken as a group, M plants accumulated more P in their shoots than the NM plants, irrespective of the presence of free-living organisms (Figure 3). In contrast, P accumulation in roots did not differ between NM and M plants. Finally, the calculation of total P accumulation per plant (Supplementary Information 1) confirmed a different effect of free-living organisms in NM and M plants. In NM plants, inoculation of bacteria alone (+Bac) did not modify the P accumulation whereas the presence nematodes (+Bac, +Nem) was able to increase dramatically total P accumulation, either by 88 % when compared to None treatment or by 98 % when compared to +Bac treatment. In M plants, the inoculation of bacteria already increased by 40 % the P amounts compared to None treatment. The addition of the nematodes increased by 66% the P amounts compared to None treatment. However, M plants inoculated either with bacteria (+ Bac) or with bacteria and nematodes (+Bac +Nem) accumulated the same amounts of total P per plant.

We estimated the root P acquisition efficiency by dividing the total plant P amount by the root length (Figure 4). The two-way ANOVA (Table 5) showed no significant interaction between the factors "mycorrhiza" and "free-living organisms" on this parameter. In contrast, P acquisition efficiency was highly affected by both the presence of the mycorrhizal fungus *H. cylindrosporum* and the presence of free-living organisms. Within the same inoculation treatment, M plants had always higher values of P acquisition efficiency than NM plants, with increases of 78 % (None), 117 % (+Bac) and 47 % (+Bac +Nem). In NM plants, the presence of *Rhabditis* sp. strongly increased P acquisition efficiency compared to the two other

294 treatments, by 90 % (None) and 70 % (+ Bac). In M plants, the presence of *Rhabditis* sp.
295 increased P acquisition efficiency only compared to plants not inoculated (None), by 60%.
296
297 ***3.3. Abundance of fungal DNA and nematodes***
298 To assess the soil colonization by *H. cylindrosporum* hyphae, we measured the number of 18S
299 rDNA gene copies of the fungus in soil DNA. As expected, the abundance of 18S rDNA
300 copies was significantly higher in soil with M plants than in soil with NM plants (Figure 5).
301 However, according to the two-way ANOVA (Table 6), the fungal rDNA gene copies number
302 was significantly affected by the interaction between the factors “mycorrhiza” and “free-
303 living organism. We did not find nematodes in the "None" and "+Bac" treatments. There were
304 ca 2000 nematodes per Petri dish in the soil in the "+Bac +Nem" treatments, irrespective of
305 the presence of the mycorrhizal fungus.
306
307 **4. Discussion**
308 ***4.1. Effects of rhizosphere organisms on plant growth***
309 We showed that (i) the presence of the mycorrhizal fungus had a slight negative impact on
310 shoot biomass and (ii) the presence of free-living organisms did not have any significant
311 effect on the accumulation of shoot biomass. At first glance, our results are opposed to those
312 found by Gyaneshwar et al. (2002), Djigal et al. (2004a), and Smith and Read (2008), who
313 showed that rhizosphere bacteria, bacterial-feeding nematodes, and mycorrhizal fungi
314 increased shoot biomass, respectively. However, this discrepancy can be explained by the
315 protocol used among these different studies. During the two-month pre-experiment required
316 for the mycorrhizal synthesis, plants were grown in a nutrient solution and accumulated P in
317 their tissues. Once placed in Petri dishes, they recycled their internal P despite the low P soil
318 availability in order to sustain shoot biomass production. Plants are able to vary their

stoichiometry to sustain shoot biomass production despite low soil mineral fertility (Gusewell, 2004) and this was shown for maritime pine (Ali et al., 2009; 2014). The negative effects of the mycorrhizal fungus on shoot biomass can also be explained by the short duration of the experiment. It has been shown that the effect of *H. cylindrosporum* on the rate of increase of *P. pinaster* shoot biomass depends on the duration of the experiment (Aquino & Plassard, 2004). A period of 100 days does not appear to be long enough to detect significant effects of the mycorrhizal fungus on shoot growth, especially with slow-growing plant species such as *P. pinaster*. Despite these considerations, it was decided to stop the experiment after 100 days because of the strong root development in the Petri dishes. It would be interesting to continue the inoculation experiment for a longer period using larger containers in order to allow sufficient space for root development.

4.2. Effect of organisms on plant P acquisition

The analysis of the P content in the shoots and roots and the total P amount in the plants at the end of the experiment showed that the presence of bacteria with bacterial-feeding nematodes strongly increased the plants' P acquisition. The presence of *Bacillus subtilis* (alone or not) is central for the mineralization of phytate by the extra-cellular enzyme (Jorquera et al., 2011; Becquer et al., 2014), but the inoculation of bacteria alone in the non-mycorrhizal plants did not significantly affect plant P nutrition. In other words, without the nematodes, the P mineralization did not result in higher P availability. The inoculation of both bacteria and nematodes is thus decisive for the net flow of P from phytate to the plant. This agrees with the previous study carried out by Irshad et al. (2012) on agarose medium. Our results suggest that the interaction between bacteria and bacterial-feeding nematodes improved soil P availability for plants by stimulating the phytate-mineralizing bacterial activity and releasing microbial biomass P (Trap et al., 2016). Similar patterns were found by Ranoarisoa et al. (2018) with

rice growing in Ferralsol from Madagascar with or without *Acrobeloides* sp., but only when the soil pH was corrected with dolomite input. The promotion of the bacterial Po-mineralizing activity by nematode inoculation has also been found by Djigal et al. (2004a; 2004b), who showed that bacterial grazers increased soil phosphatase activity in the rhizosphere of maize at all plant growth stages. From our data, we cannot decipher whether the nematodes increased plant-available P through (i) higher microbial activity (excess of mineralized P not immobilized in the microbial biomass) and/or (ii) excretion (P immobilized in the microbial biomass and then released by the nematodes). The use of isotopic labeling (^{33}P) should constitute the best way to achieve this goal (Ranoarisoa et al., 2018).

In contrast, the sole presence of the fungus did not increase plant P acquisition in "None" treatment. The absence of plant response towards fungal inoculation is also probably due to the duration of the experiment. Aquino and Plassard (2004) found that the same strain of *H. cylindrosporum* had a beneficial effect on *P. pinaster* P nutrition after 6 months of growth, but no effect after 4 months. However, it is important to note that the inoculation of bacteria in ectomycorrhizal plants led to a significant effect on plant P amount (Supplementary Information 1). This effect was clearly discernible when we calculated the plant P acquisition efficiency, supposing that the Pi uptake rate of *P. pinaster* increased when both *B. subtilis* and *H. cylindrosporum* were inoculated. As the fungus alone was shown to be poorly able to use phytate in pure culture (Irshad et al., 2012), it could have been more efficient to use Pi resulting from bacterial phytate mineralization. In this respect, the first hypothesis is that the intense exploration of the soil by the hyphae would allow a higher ability of the mycorrhizal roots to take up Pi released by the bacterial enzymes. Furthermore, we must not exclude the possibility that, during the growth of the fungus, bacteria became attached to the hyphae and move in the soil (Kohlmeier et al., 2005) promoting (i) the contact between the bacteria and the substrate (phytate) and (ii) phytate mineralization. Finally, it is also possible that the high

369 expansion of fungal hyphae promotes carbon input into the soil away from the roots,
370 increasing bacterial activity in larger soil volumes. For instance, by manipulating the
371 endomycorrhizal fungus *Glomus intraradices* and protozoa in the rhizosphere of *Plantago*
372 *lanceolata*, Koller et al. (2013) showed that the presence of the bacterial grazers stimulated
373 the translocation of carbon from the plant via the fungus into the soil, fueling the microbial
374 loop (Clarholm, 1985a) and resulting in greater plant nutrition in comparison to plants without
375 protozoa. No such mechanism has been observed for ectomycorrhizal fungi, but our results
376 greatly encourage further studies focusing on this feature.
377
378 ***4.3. Effects of microorganisms on root growth and architecture***
379 The most notable effect was that *H. cylindrosporum* decreased the root biomass, irrespective
380 of the presence of free-living organisms. This agrees with a previous study carried out using
381 the same fungal isolate associated with *P. pinaster* (Aquino & Plassard, 2004). The reduced
382 root development, which was associated with ectomycorrhizal fungi, was attributed to the
383 high ability of the extraradical hyphae of the fungus to take up P from soil (Aquino &
384 Plassard, 2004; Plassard & Dell, 2010).
385 The analysis of the root architecture underlined the strong significant effect of the fungus on
386 the root compartment. In agreement with the findings of Jentschke et al. (1995) and
387 Bonkowski et al. (2001), the presence of the mycorrhizal fungus resulted in a less complex
388 root system. All root parameters were significantly reduced by the presence of the
389 ectomycorrhizal fungus. As discussed above, this pattern could be due to the high ability of
390 the ectomycorrhizal fungus to acquire P from the soil, thus shifting the plant carbon allocation
391 from the roots to the fungus (Brown *et al.*, 2013). In contrast to the ectomycorrhizal fungus,
392 bacteria and nematodes did not modify significantly the root architecture. Changes in the root
393 architecture in the presence of bacterial feeders (especially protozoa) have been interpreted as

hormonal non-nutritional effects on the lateral root production (Bonkowski, 2004; Bonkowski & Clarholm, 2012). Here, the soil was inoculated with a population of *B. subtilis* (not with a microbial community), which probably limited the ability of nematodes to induce non-nutritional effects (Ranoarisoa et al., 2018).

4.4. Relevance of our simplified conditions to study micro-food web interactions

In this study, we used an experimental system with simplified and artificial conditions to study the effects of the micro-food web interactions on phytate mineralization as we worked with sterilized soil-bead mixture and addition of selected free-living organisms. Compared to *in situ* ecosystems presenting huge variations of temperatures and soil humidity, these two environmental factors varied little during all the experiment, thus favoring enzyme (Rinkes et al., 2013) and root activities for mineral uptake (Gessler et al., 1998). Although sterilized, our soil-bead mixture enabled us to take into account the exchanges between mineral nutrients and the soil as well as the rhizosphere changes able to modify greatly mineral nutrients bioavailability for plants (Bravin et al., 2010). The control of biodiversity, although low in comparison to field conditions, enabled us to quantify the potential contribution of each actor, alone or in combination, to the mineralization of phytate and finally, to the bioavailability of P released for the plant. Thus, as underlined by Mezeli et al. (2020), our experimental conditions were suitable to reveal the effects of the soil microbial loop on phytate mineralization and plant P bioavailability. Hence, the next step of this work will be to use different richness levels of free-living organisms added in non-sterilized soil in order to take into account better their interactions with soil biodiversity which might modify the final plant P bioavailability.

5. Conclusion

We formulated four hypotheses. First, we supposed that the plant alone, without mycorrhiza nor free-living organisms, is not able to uptake P from phytate (H1). This hypothesis was validated since no P was acquired from phytate when no rhizospheric organisms were inoculated. Second, we expected higher plant P uptake in the presence of *H. cylindrosporum* in comparison to the plant alone (H2). This hypothesis was partially validated since the presence of the fungus did not result in higher plant P nutrition in the "None" treatments, but the inoculation of bacteria in ectomycorrhizal plants led to a significant effect on plant P amount. The third hypothesis suggested that the presence of free-living organisms, bacteria, and nematodes, would increase the plants' P uptake. This hypothesis was fully validated and constitutes the most remarkable result of the present study. The strong positive effect of the presence of both phytate-mineralizing bacteria and bacterial-feeding nematodes on P acquisition by plants growing in soil amended with phytate is central and suggests that interactions between bacteria and bacterial-feeding nematodes are essential to increase soil Pi availability for plants. Finally, the last hypothesis, supposing that plant P uptake from phytate will be maximal when all potential rhizosphere mutualists are inoculated, was also validated. The findings are particularly noteworthy because they show the importance of the rhizosphere micro-food web role on plants' P nutrition from a recalcitrant organic P source despite high Pi adsorption onto soil components. Further investigations are required to assess better the involved mechanisms. They could consist of the quantification of phosphatases and phytases activities together with available Po and Pi in rhizosphere soil to determine the possible relationships between soil micro-food web interactions and these parameters. Exploring the benefits of rhizosphere interactions and identifying the potential drivers of these interactions appear as pertinent goals to develop agricultural management practices that could take advantage of the beneficial interactions of these organisms.

Acknowledgments

We would like to thank the three anonymous reviewers for their helpful suggestions that helped us improve the manuscript. This project was part of the ANR project “UNLOCKP” (ANR 11 BSV7 015 01; France) awarded to C. Plassard. The work by M.P. Ranoarisoa was also funded by the UNLOCKP project. The work was also supported by the program ‘Investments for the future’ (ANR-10- LABX-04-01) through the use of the Ecotrop platform from CeMEB labEx. We are grateful to Pierre Trichet (ISPA, INRA Bordeaux) for providing the soil samples for nematode isolation. We also thank Anne Jimenez and Cécile Villenave (Elisol Environment) for their help and advice on the production of nematodes. We thank Valentin Lauret, Guillaume Pugliese, Sebastian Mira, and Olivier Pacholick for their help during the experiments.

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Captions to figures

Figure 1. Schematic procedures to obtain the plants for inoculation experiments.

A: ectomycorrhizal synthesis in glass tubes with *Pinus pinaster* and *Hebeloma cylindrosporum*. Non mycorrhizal plants are obtained without fungal plugs added. The inset diagram shows the home-made closing system of each Teflon tube. B-E: preparation of square Petri dishes with soil-bead mixture. B: two holes for plant passage and watering are first made with a hot tool. C: the sterile soil-bead-nutrient solution mixture is poured into the dish. D: a two-month-old pine seedling is taken of the glass tube and placed on the soil-bead mixture before addition of free-living organisms (*B. subtilis* and/or *Rhabditis* sp.). E: the hole for watering and the dish are closed with pieces of sticky tape.

Figure 2. Biomass accumulation in shoots and roots of *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M) and different inoculation treatments.

Two-month old plants were grown for 100 d in a cambisol supplemented with phytate with different inoculation treatments that were: no addition of living-free organisms (None), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test). Asterisk indicates a mean significantly different from the initial value measured in two-month old plants at $p<0.05$ (Student's t-test of mean comparison)

Figure 3. Total P amount in shoots and roots of *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M).

Two-month old plants were grown for 100 d in a cambisol supplemented with phytate with different inoculation treatments that were: no addition of living-free organisms (None), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test). Asterisk indicates a mean significantly different from the initial value measured in two-month old plants at $p<0.05$ (Student's t-test of mean comparison).

Figure 4. P acquisition efficiency of *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M).

Two-month old plants were grown for 100 d in a cambisol supplemented with phytate with different inoculation treatments that were: no addition of living-free organisms (none), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c, d) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test).

Figure 5. Abundance of fungal (18S) rDNA gene copies measured by qPCR in soil with *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M) after 100 d of growth in a cambisol supplemented with phytate and with different inoculation treatments.

Inoculation treatments were: no addition of living-free organisms (None), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test).

Supplementary information 1. Pictures of the devices used to produce plants for inoculation experiments

Supplementary information 2. Plant total P amounts according to biological treatments.

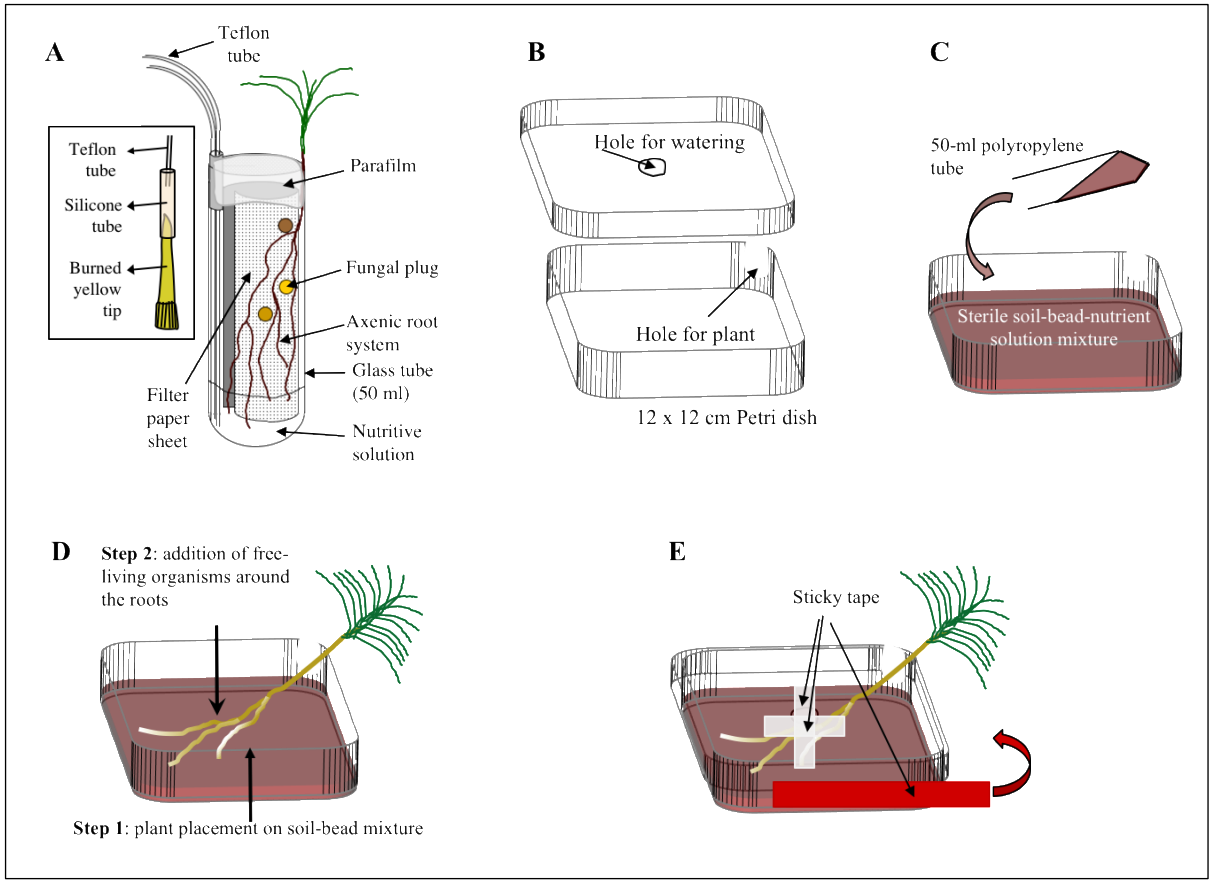

Figure 1. Schematic procedures to obtain the plants for inoculation experiments.

A: ectomycorrhizal synthesis in glass test-tubes with *Pinus pinaster* and *Hebeloma cylindrosporum*. Non mycorrhizal plants are obtained without fungal plugs added. The inset diagram shows the home-made closing system of each Teflon tube. B-E: preparation of square Petri dishes with soil-bead mixture. B: two holes for plant passage and watering are first made with a hot tool. C: the sterile soil-bead-nutrient solution mixture is poured into the dish. D: a two-month-old pine seedling is taken of the glass tube and placed on the soil-bead mixture before addition of free-living organisms (*B. subtilis* and/or *Rhabditis* sp.). E: the hole for watering and the dish are closed with pieces of sticky tape.

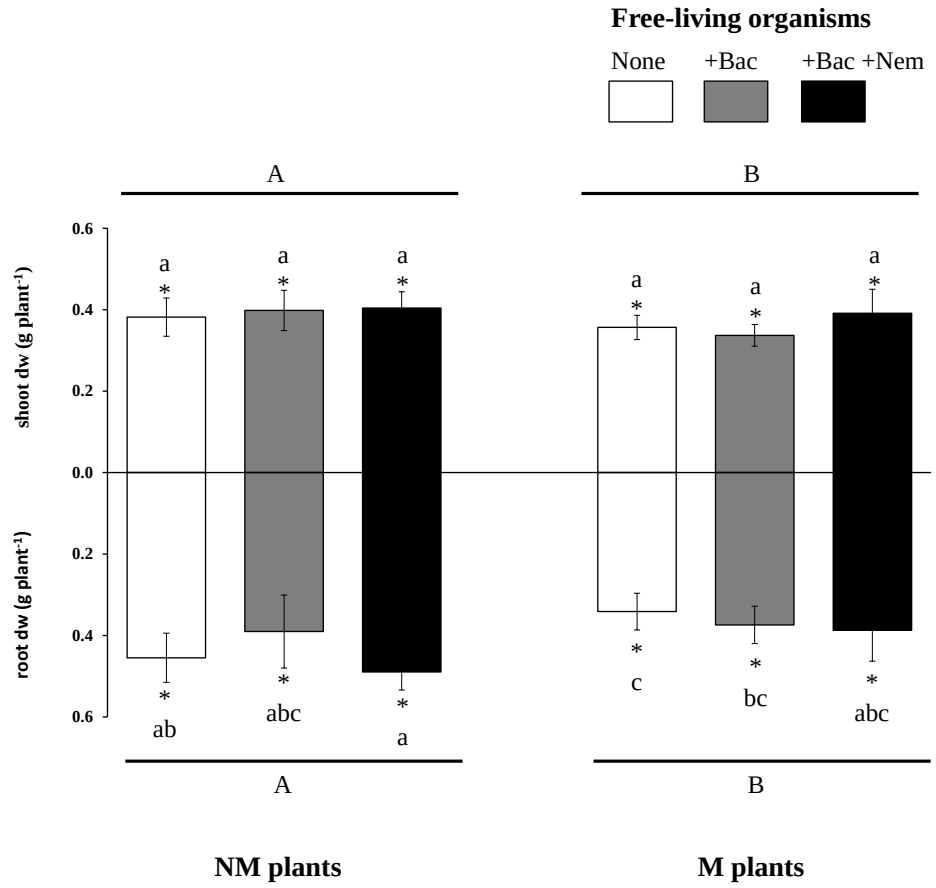

Figure 2. Biomass accumulation in shoots and roots of *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M) and different inoculation treatments.

Two-month old plants were grown for 100 d in a cambisol supplemented with phytate with different inoculation treatments that were: no addition of supplementary organisms (None), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test). Asterisk indicates a mean significantly different from the initial value measured in two-month old plants at $p<0.05$ (Student's t-test of mean comparison)

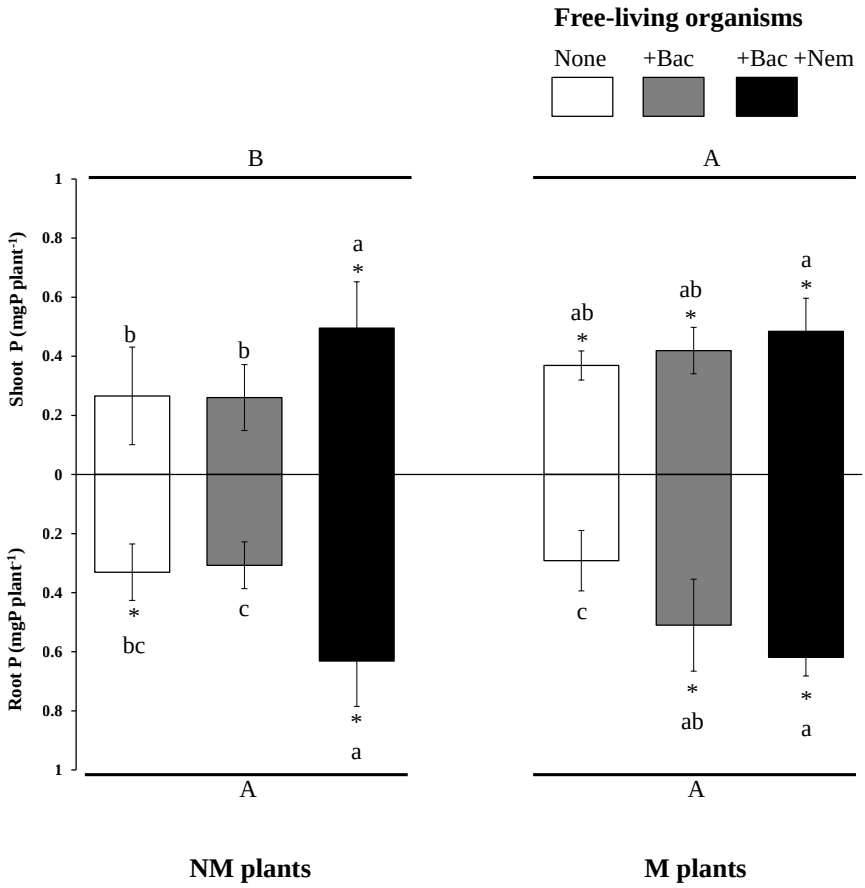

Figure 3. Total P amount in shoots and roots of *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M).

Two-month old plants were grown for 100 d in a cambisol supplemented with phytate with different inoculation treatments that were: no addition of supplementary organisms (None), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test). Asterisk indicates a mean significantly different from the initial value measured in two-month old plants at $p<0.05$ (Student's t-test of mean comparison).

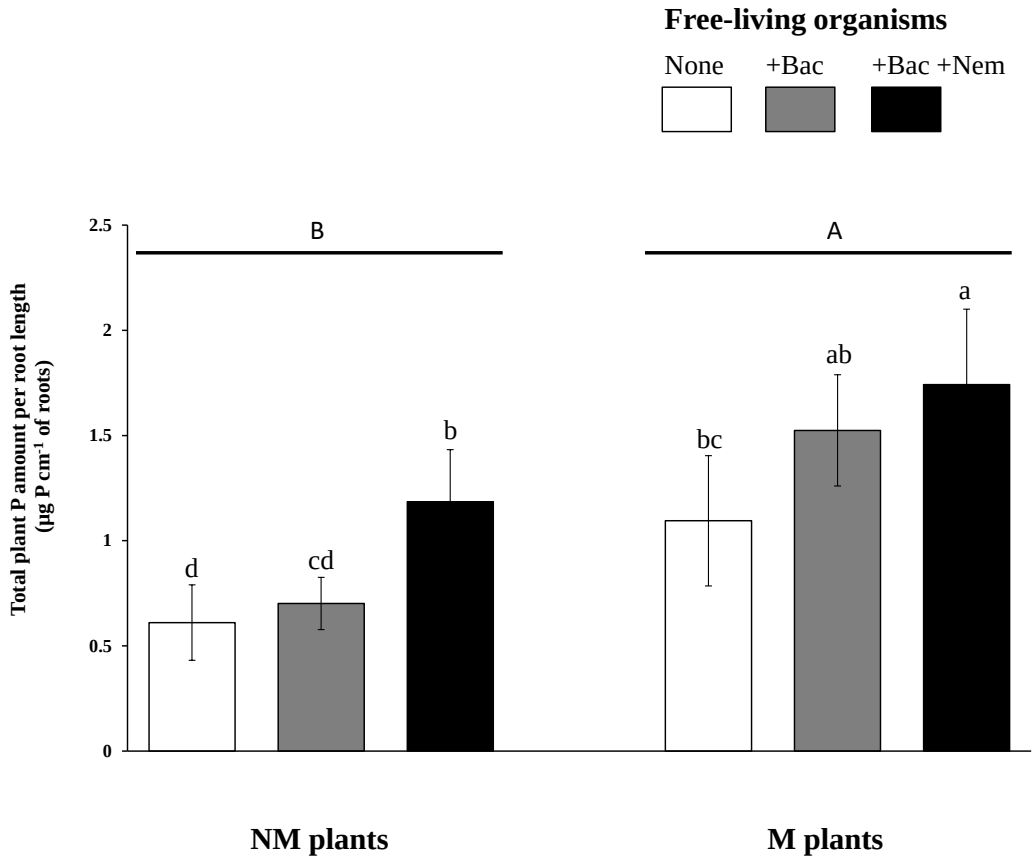

Figure 4. P acquisition efficiency of *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M).

Two-month old plants were grown for 100 d in a cambisol supplemented with phytate with different inoculation treatments that were: no addition of supplementary organisms (none), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c, d) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test).

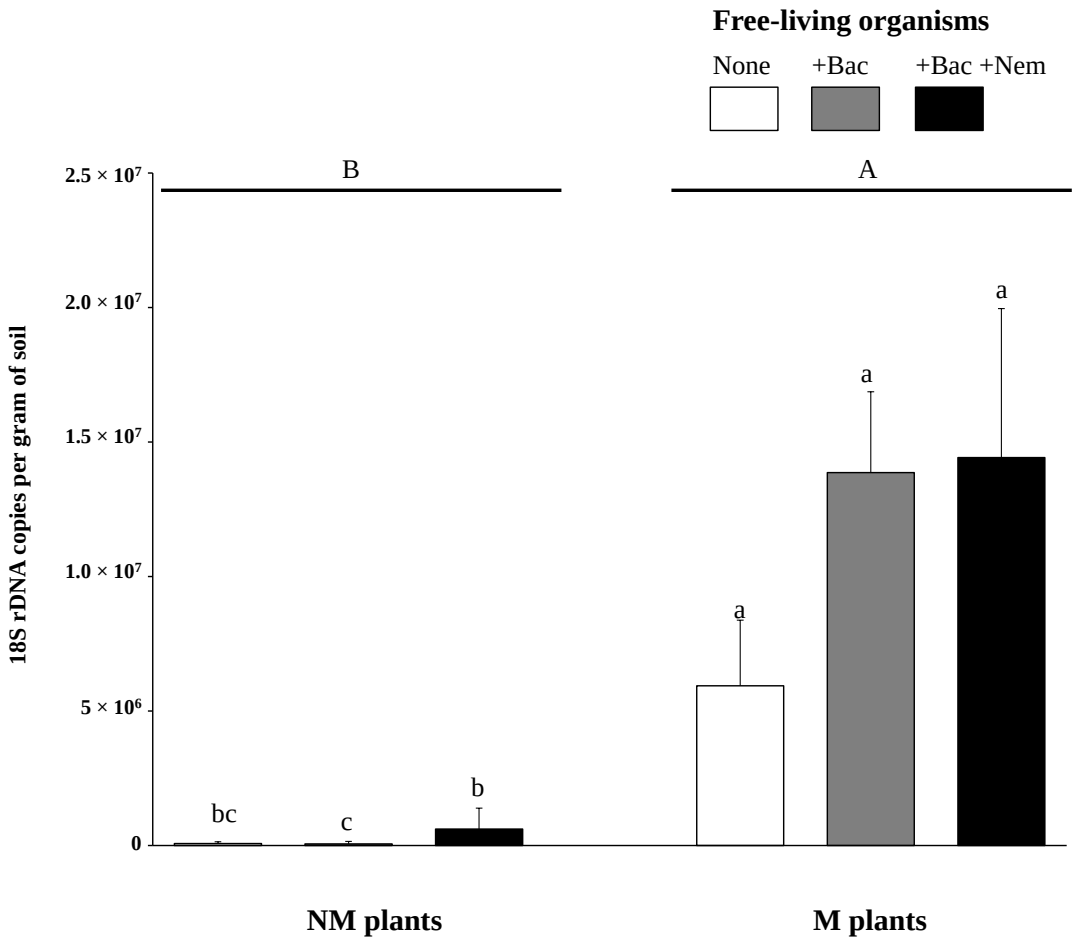

Figure 5. Abundance of fungal (18S) rDNA gene copies measured by qPCR in soil with *P. pinaster*, whether not associated (NM) or associated with the ectomycorrhizal basidiomycete *H. cylindrosporum* (M) after 100 d of growth in a cambisol supplemented with phytate and with different inoculation treatments.

Inoculation treatments were: no addition of supplementary organisms (None), addition of a phytate-mineralizing bacteria *Bacillus subtilis* (+Bac) or addition of bacteria together with bacteria-feeding nematodes *Rhabditis* sp. (+Bac +Nem). Bars are the means (n=6) with standard deviation. Different capital letters (A, B) indicate significant differences among mycorrhizal fungal status at $p<0.05$ (two-way ANOVA). Different lowercase letters (a, b, c) indicate significant differences among biological treatments at $p<0.05$ (one-way ANOVA followed by post hoc Tukey HSD test**).**

Table 1. Statistics (F and p-values) of two-way ANOVA performed on biomass accumulation in shoots and roots of *P. pinaster.*

Plants were grown for 100 days in a Cambisol supplemented with phytate, whether or not associated with the ectomycorrhizal fungus *H. cylindrosporum*, referred as "Mycorrhiza" (M) and whether or not inoculated with free-living microorganisms (bacteria and bacterial-feeding nematodes) referred as "Free-living organisms" (FO) as factors.

Factors	Dry biomass (g plant^{-1}) in:	
	Shoot	Root
Mycorrhiza (M)	5.25 *	13.71 ***
Free-living organisms (FO)	1.81	2.56
M × FO	1.01	2.16

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 2. Root parameters measured in *P. pinaster* grown for 100 days in a Cambisol supplemented with phytate with different biological treatments.

Values are the means (n=6) with standard deviation between brackets. NM: non-mycorrhizal, M: mycorrhizal with *H. cylindrosporum*, None: no inoculation, +Bac: inoculation with *B. subtilis*, +Bac +Nem: inoculation with *B. subtilis* and *Rhabditis* sp.

		Root parameters (plant^{-1})				
Biological treatment		Length (cm)	Area (cm^2)	Tips (Number)	Forks (Number)	Crossings (Number)
NM	None	989 a (159)	150 a (19)	1851 a (387)	2726 ab (701)	428 a (143)
NM	+Bac	828 a (224)	133 a (30)	1745 ab (473)	2554 ab (930)	312 ab (125)
NM	+Bac +Nem	976 a (146)	154 a (23)	1768 ab (318)	3404 a (940)	472 a (161)
M	None	608 a (321)	107 a (50)	1200 bc (494)	1558 b (1267)	194 b (185)
M	+Bac	610 a (15)	112 a (5)	942 c (72)	1290 b (114)	142 b (20)
M	+Bac +Nem	647 a (90)	123 a (22)	1019 c (277)	1641 ab (407)	177 b (33)
NM vs M	one-way ANOVA p-value	***	**	***	***	***

Different letters (a, b and c) indicate significant differences between biological treatments and stars (*p<0.05, **p<0.01, ***p<0.001) indicate significant differences between presence and absence of mycorrhiza according to one-way ANOVA followed by post hoc Tukey HSD test.

Table 3. Statistics (F and p-values) of two-way ANOVA performed on root parameters of *P. pinaster.*

Plants were grown for 100 days in a Cambisol supplemented with phytate, whether or not associated with the ectomycorrhizal fungus *H. cylindrosporum*, referred as "Mycorrhiza" (M) and whether or not inoculated with free-living microorganisms (bacteria and bacterial-feeding nematodes) referred as "Free-living organisms" (FO) as factors.

Factors	Length (cm)	Area (cm^2)	Tips (Number)	Forks (Number)	Crossings (Number)
Mycorrhiza (M)	24.86 ***	12.59 *	36.34 ***	32.03 ***	30.01 ***
Free-living organisms (FO)	0.87	1.12	0.79	1.90	2.04
M × FO	0.60	1.02	0.13	0.10	0.73

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 4. Statistics (F and p-values) of two-way ANOVA performed on P amount in shoots and roots of *P. pinaster*.

Plants were grown for 100 days in a Cambisol supplemented with phytate, whether or not associated with the ectomycorrhizal fungus *H. cylindrosporum*, referred as "Mycorrhiza" (M) and whether or not inoculated with free-living microorganisms (bacteria and bacterial-feeding nematodes) referred as "Free-living organisms" (FO) as factors.

Factors	Plant P (mg P plant^{-1}) in:	
	Shoot	Root
Mycorrhiza (M)	4.42 *	1.76
Free-living organisms (FO)	7.42 **	23.94 ***
M × FO	1.58	4.08

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 5. Statistics (F and p-values) of two-way ANOVA performed on P acquisition efficiency of *P. pinaster*.

Plants were grown for 100 days in a Cambisol supplemented with phytate, whether or not associated with the ectomycorrhizal fungus *H. cylindrosporum*, referred as "Mycorrhiza" (M) and whether or not inoculated with free-living microorganisms (bacteria and bacterial-feeding nematodes) referred as "Free-living organisms" (FO) as factors.

Factors	**P acquisition efficiency** ($\mu g\ P\ cm^{-1}$ of roots)
Mycorrhiza (M)	51.61 ***
Free-living organisms (FO)	17.46 ***
M × FO	1.4

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 6. Statistics (F and p-values) of two-way ANOVA performed on fungal (18S) rDNA gene copies number measured by qPCR in soil with *P. pinaster.*

Plants were grown for 100 days with the inoculation of ectomycorrhizal fungus referred as "Mycorrhiza" (M) and inoculation of free-living microorganisms (bacteria and bacterial-feeding nematodes) referred as "Free-living organisms" (FO) as factors.

Factors	18S rDNA gene copies number per gram of soil
Mycorrhiza (M)	194.71 ***
Free-living organisms (FO)	3.66 *
M × FO	3.36 *

* $p<0.05$, ** $p<0.01$, *** $p<0.001$