**Pinus pinaster** seedlings and their fungal symbionts show high plasticity in phosphorus acquisition in acidic soils

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Summary Young seedlings of maritime pine (**Pinus pinaster** Soland in Aıt.) were grown in rhizoboxes using intact spodosol soil samples from the southwest of France, in Landes of Gascogne, presenting a large variation of phosphorus (P) availability. Soils were collected from a 93-year-old unfertilized stand and a 13-year-old **P. pinaster** stand with regular annual fertilization of either only P or P and nitrogen (N). After 6 months of culture in controlled conditions, different morphotypes of ectomycorrhiza (ECM) were used for the measurements of acid phosphatase activity and molecular identification of fungal species using amplification of the ITS region. Total biomass, N and P contents were measured in roots and shoots of plants. Bicarbonate- and NaOH-available inorganic P (Pi), organic P (Po) and ergosterol concentrations were measured in bulk and rhizosphere soil. The results showed that bulk soil from the 93-year-old forest stand presented the highest Po levels, but relatively higher bicarbonate-extractable Pi levels compared to 13-year-old unfertilized stand. Fertilizers significantly increased the concentrations of inorganic P fractions in bulk soil. Ergosterol contents in rhizosphere soil were increased by fertilizer application. The dominant fungal species was *Rhizopogon luteolus* forming 66.6% of analysed ECM tips. Acid phosphatase activity was highly variable and varied inversely with bicarbonate-extractable Pi levels in the rhizosphere soil. Total P or total N in plants was linearly correlated with total plant biomass, but the slope was steep only between total P and biomass in fertilized soil samples. In spite of high phosphatase activity in ECM tips, P availability remained a limiting nutrient in soil samples from unfertilized stands. Nevertheless young **P. pinaster** seedlings showed a high plasticity for biomass production at low P availability in soils.

Keywords: ectomycorrhizal morphotypes, fertilizers application, fungal biomass, P contents, phosphatase activity, plant plasticity.

Introduction

Maritime pine (**Pinus pinaster** Soland in Aıt.) is a tree species cultivated over an area of one million hectares named Landes of Gascogne situated in the southwest of France. Although this species represents only 6.5% of total forest area in France, it holds an important economical value as it produces ca. 20% of French softwood (Bert and Daniçon 2006). These monoculture forests are established in spodosols predominantly characterized by acidic sandy soils with a low cation exchange capacity and high Fe and Al contents in surface layers (Trichet et al. 1999). Due to these high Fe and Al contents, low pH values and low overall total P contents of these soils result in low P availability that is considered as the main limiting factor for tree growth (Bonneau 1995, Trichet et al. 2009).

Therefore, the growth and establishment of **P. pinaster** seedlings under low nutrient soil conditions require fertilizer management practices. Frequently, fertilizers are applied in forest ecosystems once during plantation establishment. However, the productivity of *Picea abies* ([L.] H. Karst) and *Pinus sylvestris* (L.) in Sweden (Axelsson and Axelsson 1986), *Pinus taeda* (L.) in USA (Albaugh et al. 1998), *Pinus radiata* (D. Don) in Australia (Waterworth et al. 2007) and *P. pinaster* (Trichet et al. 2008) in France can be much higher when nutrient availability is optimized continuously or added annually instead of only a single application immediately at plantation. Trichet et al. (2008) showed that annual optimization of nutrients resulted in a significant increase in the aboveground productivity of maritime pine in the first five growing seasons following the application. In the later stages of tree growth, fertilization with P in P deficient soils can further increase the plant growth (Gentle et al. 1965, Neilsen et al. 1984). However, the extent and duration of growth response to P application are dependent on the characteristics of soils, especially P sorption capacity, pH and intrinsic P contents (Pritchett and Comerford 1982). In addition to mineral P pools, among which ortho-phosphate (Pi) is the only available P form for plant uptake, forest soils contain organic P (Po) pools composed of
Plants develop various strategies to fulfill their P requirements, such as modification of root structure, association with ectomycorrhizal (ECM) fungi and release of acid phosphatase. An increase in fine roots has been reported in low fertility soil as compared to high fertility soils in coniferous species such as *P. taeda* (Albaugh et al. 1998, Maier and Kress 2000) and *P. pinaster* (Achat et al. 2008). Woody plants, the gymnosperms and several angiosperms growing in boreal and temperate regions have a symbiotic association with mycorrhizal fungi that form ECM roots (Marmeisse et al. 2004). Mycorrhizal association between plant and fungi is considered as the most prevalent strategy to increase phosphate acquisition by plants (Smith et al. 2000). The ECM fungal species can augment the absorbing surface of mycorrhizal plants compared to non-mycorrhizal plants due to the extended hyphal development in soil (Rousseau et al. 1994). In 13-year-old *P. pinaster* unfertilized plots, Bakker et al. (2009) reported that ECM hyphal length was 25 times higher than that of fine roots. It represents ca. 96% of total length of absorbing structure (fine root + hyphae). In addition to the increased soil exploration, ECM fungi have exhibited a release of phosphatase in culture medium (Tibbet et al. 1998, Quiquampoix and Mousain 2005) that could play a crucial role in mobilizing Pi from organic P pools of soils. This effect could be particularly important in the soils of old forests where organic P pools may represent a large fraction of total P.

The objective of this study was to evaluate the ability of young *P. pinaster* seedlings to cope with a large variation of P availability in intact spodosol soil samples that were collected from the southwest of France in terms of growth responses and mineral nutrition. The soil samples were collected from a 93-year-old unfertilized stand and from a 13-year-old *P. pinaster* stand with annual fertilization of either only P or N and P (Trichet et al. 2008, Bakker et al. 2009). The former soil was chosen based on the presumed high levels of organic P as a major P fraction accumulated over 93 years, and the latter soil due to its younger age and a presumed major fraction of inorganic P. Plants were grown in rhizoboxes (Casarin et al. 2004, Torres Aquino and Plassard 2004) containing these soils, and we assumed that the root system would associate with the indigenous ECM fungal species present in the intact soil. As fertilizers are applied in interline position (Trichet et al. 2008, Bakker et al. 2009), we investigated the variation of soil P pools between line and interline tree positions. It was hypothesized that mycorrhizal association of plants might have affected the mobilization of organic P in the rhizosphere soil compared to the bulk soil. Therefore, we investigated the relationship between mineral P availability, acid phosphatase activity of ECM root tips and bioavailability of P for plants measured as P contents in roots and shoot biomass.

### Materials and methods

#### Forest stand description and soil collection

Soil samples used in this study were collected in two *P. pinaster* stands located in the Gascogne region in the southwest of France. In both stands, soils are sandy spodosol developed on Aeolian sandy deposits of the quaternary era. Mean annual air temperature is 12.5 °C, and average precipitation rate is 950 mm, with frequent prolonged periods of drought in summer. The first stand was a 93-year-old forest of *P. pinaster*, and the soil had never been fertilized since plantation establishment. The second stand was a 13-year-old planted forest divided into plots with different fertilizer regimes: no fertilizer (control, C), phosphorus fertilizer (P) and complete mineral fertilizers (F) application. Each plot measured 60 × 36 m with an exclusion of 10-m border area. In both stands, the trees were planted in lines with a 2 m distance from tree to tree and with a 4 m distance from line to line. Cores of mineral soil (15 cm length and 8 cm diameter) were collected from the tree line (L) and interline (IL) for each stand using a manual auger. A brief description of the soil samples according to their sampling location and stand characteristics is given in Table 1. Samples were collected in April 2006 and were maintained at 4 °C for three months before using them as substrate for young seedlings in rhizoboxes.

As soil samples were used without any further treatment, they served as indigenous soil fungi and bacterial inoculants.

#### Plant preparation and culture in rhizoboxes

Seeds of maritime pine (*P. pinaster* from Medoc, Landes-Sore-VG source, France) were surface disinfected by immersing in H₂O₂ 30% (w/w) for 30 min, then rinsed several times with sterile water. Finally, they were soaked in sterile water and maintained at 4 °C during 48 h for stratification. Germination of stratified seeds was carried out in water-moistened vermiculite previously sterilized twice (121 °C, 15 min) and placed in a growth chamber. After 2 months, germinated pine seedlings were transferred to a mist chamber to develop lateral root system. The mist was produced from distilled water supplied regularly at the bottom of the chamber. The tap root was trimmed repeatedly with sterilized scissors. After 1 month, plants were transferred to rhizoboxes described in Torres Aquino and Plassard (2004). Briefly, the rhizobox consisted of two Perspex plates (20 × 10 cm) separated by 3 mm spacers. The spacers made it possible to establish a 3 mm thick layer of soil, using 70 g of intact fresh soil. Coarse root pieces were removed from the soil. After the installation of soil in the first Perspex plate with spacers, a sterile piece (7 × 10 cm) of glass
Table 1. Brief description of soil samples used to grow young seedlings of *P. pinaster* in rhizoboxes. Soils were sampled in *P. pinaster* stands differing by tree age and fertilization regime. In each plot, paired sampling was carried out in tree line (line) or between two lines of trees (interline).

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Sampling location</th>
<th>Stand age (years)</th>
<th>Treatment</th>
<th>Fertilization regime</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO-L</td>
<td>Line</td>
<td>93</td>
<td>Control</td>
<td>No fertilizer application</td>
</tr>
<tr>
<td>CO-IL</td>
<td>Interline</td>
<td></td>
<td>Old</td>
<td>No fertilizer application</td>
</tr>
<tr>
<td>C-L</td>
<td>Line</td>
<td>13</td>
<td>Control</td>
<td>Annual P fertilization in interline¹</td>
</tr>
<tr>
<td>C-IL</td>
<td>Interline</td>
<td></td>
<td>P</td>
<td>Annual complete fertilization in interline²</td>
</tr>
<tr>
<td>P-L</td>
<td>Line</td>
<td>13</td>
<td>Complete</td>
<td></td>
</tr>
<tr>
<td>P-IL</td>
<td>Interline</td>
<td></td>
<td>Fertilization</td>
<td></td>
</tr>
<tr>
<td>F-L</td>
<td>Line</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-IL</td>
<td>Interline</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Mean rate in 1998–2005 of 32 kg P ha⁻¹ year⁻¹ (Bakker et al. 2009).
²Mean rate (in kg ha⁻¹ year⁻¹) for 1998–2005 of 84 N, 32 P, 56 K, 22 Ca, 7 Mg, 1.3 B, 2.9 Cu, 2.1 Mn and 0.6 Zn (Bakker et al. 2009).

fibre paper sheet wrapped in a nylon cloth was placed at the bottom of the soil layer. This sheet was in contact with a water reservoir to ensure water supply to the soil and plants. The root system of young seedling was spread on the soil layer in the rhizobox, and the system was closed with a second Perspex plate, clamps and sticky tape. The rhizoboxes were transferred to a container containing distilled water, and the plants were allowed to grow in the growth chamber for 6 months with a regular supply of distilled water. Growth conditions include 16/8 h light/dark cycle at 25/18 °C for 6 months with a regular supply of distilled water. Growth conditions include 16/8 h light/dark cycle at 25/18 °C, 70% rh, CO₂ concentration of c. 350 mm³ l⁻¹ and a PAR of c. 400 µmol m⁻² s⁻¹ (400–700 nm).

**Plant and soil harvest**

Rhizoboxes were dismantled, and the root system was gently pulled out from the soil layer. Soil attached to (rhizosphere soil) and away from the roots (bulk soil) was separated carefully. Each root system was examined under stereomicroscope to pick up ECM root tips, and the ECM tips were morphologically classified. Each ECM morphotype was used for acid phosphatase activity. The subsamples of each morphotype were stored at −20 °C for molecular identification. The weight of fresh and freeze-dried roots and shoot was recorded. The soil separated into bulk and rhizosphere soils was also freeze dried before analysis.

**Phosphatase activity**

Phosphatase activity (Tabatabai 1982) of four ECM tips for each morphotype was estimated separately. The ECM tips were incubated for an hour in 10 mM solution of 1-pNPP prepared in acetate buffer (25 mM, pH 5.4). The reaction was stopped by adding 0.5 M NaOH. A blank sample was prepared for each morphotype by adding NaOH and ECM tips simultaneously before incubation. Optical density of samples was measured at 400 nm, and the enzymatic activity was calculated (nmol of 1-pNP produced min⁻¹ g⁻¹ of fresh ECM weight) by the following equation:

\[
\text{phosphatase activity} = \frac{\Delta OD \times 1.2 \times DF}{t \times FW \times 0.0188},
\]

where ‘ΔOD’ is the difference between optical density of blank and sample, ‘1.2’ is the final reaction volume (ml), ‘DF’ is the dilution factor, ‘t’ is the time (minutes) of incubation, ‘FW’ is the fresh weight of ECM tip and ‘0.0188’ is the coefficient of molar extinction for 1-p-nitrophenolate (ml nmol⁻¹ cm⁻¹).

**Plant and soil analysis**

Bulk and rhizosphere freeze-dried soils were gently sieved (2 mm) to separate soil and ECM fungal hyphae along with dead plant tissues. Freeze drying helped to preserve ergosterol contents and smooth removal of fungal hyphae particularly in sandy soils. The material remaining in the sieve was ground finely. Ergosterol contents were measured in soil and in sieved material as described in Plassard et al. (2000). Briefly, 0.1 g of dried material was incubated for 24 h in a 1 ml solution of methanol and polyedylar (Serva, Heidelberg, Germany) (0.5% w/v). The samples were centrifuged (14,000 g, 15 min), and the supernatants were filtered through 0.45 µm nylon syringe filter (514-0067, VWR International). The concentration of ergosterol in methanol extracts was determined at 270 nm by high-performance liquid chromatography using a C18 column and eluted with methanol flowing at 1 ml min⁻¹.

Sieved soil was used to measure bicarbonate and hydroxide extractable inorganic and organic P fractions. Plant available fraction of P was extracted by shaking 0.3 g of sieved soil for 30 min in 6 ml of NaHCO₃ (0.5 M, pH 8.5) (Olsen et al. 1954). Similarly, 0.5 g of soil was shaken for 16 h in 5 ml of NaOH (0.1 M) to extract less labile fraction of P associated with amorphous Al- and Fe-phosphates (Tiessen et al. 1984, Sharpley et al. 1999). Bicarbonate and hydroxide soil extracts were diluted with distilled water (1/6, v/v), then acidified with HCl 12 N (1/600, v/v) to precipitate humic material before assaying Pi concentrations. The same soil extracts (bicarbonate and hydroxide) were mineralized with 12 N HCl (v/v) at 110 °C for 16 h. As shown in our preliminary experiments, these conditions made it possible to mineralize all organic P contained in the solution (data not shown). Organic P
concentration in soil extracts was calculated by the difference between Pt and Pi for both NaHCO₃ and NaOH extracts.

Plant roots and shoot materials were finely ground, and 50 mg of roots or shoot dry matter was mineralized in a Pyrex glass tube under a chemical fume hood using 1 ml of H₂SO₄ (36 N) at 330 °C for 30 min (McDonald 1978) in a tube mineralization block. If the solution was not transparent, the tubes were removed and allowed to cool down, and 0.2 ml of H₂O₂ was repeatedly added until the solution became transparent. After the dilution of H₂SO₄ to 0.1 N, ammonium was assayed using phenol colorimetric method of Berthelot (Martin et al. 1983). Orthophosphate P was assayed in 0.1 N H₂SO₄ plant digest and in soil solutions using the malachite green method (Ohno and Zibilske 1991).

Identification of indigenous fungal species

Fungal DNA from frozen individual ECM morphotypes was extracted using the DNaseasy Plant Mini Kit according to the manufacturer’s instructions (QIAGen S.A.). Three microlitres of the DNA extract were used for PCR amplification with Taq polymerase (18038-026, Invitrogen) using the primers ITS1-F and ITS4 (White et al. 1990). The thermocycling pattern used was 94 °C for 5 min (one cycle); 94 °C for 30 s, 53 °C for 1 min and 72 °C for 45 s (35 cycles) and 72 °C for 10 min (one cycle). Samples displaying one single band on gel electrophoresis (90% of DNA extracts) were sequenced from AGOWA GmbH, Berlin, Germany (http://www.agowa.de). All sequences were identified to genus and species level by launching a query through blastn algorithm of UNITE online molecular data base service (Köljalg et al. 2005).

Statistical analyses

The analyses of variance were performed to evaluate significant difference between different soil samples, plant responses, ergosterol contents in bulk and rhizosphere soils, and phosphatase activity. Mean values (n = 6–12 for soil and plant parameters and n = 24–40 for acid phosphatase activity of ECM root tips) were compared using least significant difference of the Fisher model (P < 0.05), and error bars on each mean value denote standard error (SE). Relationships between total N and P and biomass in plants were drawn by simple linear regression. All data were analysed using the Statistica software package (Statistica 8, Statsoft Inc., Tulsa, OK). The first statistical analysis of plant data obtained from the two control treatments (CO and C) showed non-significant differences between lines and interline. Therefore, these data from line and interline were pooled together in CO and C treatments and were compared with other soil treatments.

Results

Soil inorganic P fractions

Concentrations of readily available inorganic P assayed in bulk soil using bicarbonate solution were the lowest (2 mg P kg⁻¹ soil) in control plot with 13-year-old trees, regardless of the sampling position of the soil (Table 2). Forest age (93 years, CO soil) resulted in an increase of bicarbonate-extractable P (4 mg P kg⁻¹ soil) in soils collected from line position. Fertilizer application dramatically increased P concentrations assayed in interline soils, with values of 50 and 62 mg P kg⁻¹ soil in P and F plots, respectively.

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>P-inorganic (mg kg⁻¹ of dry soil)</th>
<th></th>
<th>P-organic (mg kg⁻¹ of dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>NaOH</td>
<td>NaHCO₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO-L</td>
<td>4.4</td>
<td>4.4</td>
<td>8.2</td>
</tr>
<tr>
<td>CO-IL</td>
<td>2.5</td>
<td>3.1</td>
<td>4.2</td>
</tr>
<tr>
<td>P</td>
<td>0.01</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>C-L</td>
<td>1.7</td>
<td>2.1</td>
<td>8.2</td>
</tr>
<tr>
<td>C-IL</td>
<td>1.5</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>P</td>
<td>0.3</td>
<td>0.04</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P-L</td>
<td>4.5</td>
<td>3.7</td>
<td>10.0</td>
</tr>
<tr>
<td>P-IL</td>
<td>50.5</td>
<td>50.6</td>
<td>83.9</td>
</tr>
<tr>
<td>F-L</td>
<td>14.1</td>
<td>13.0</td>
<td>26.4</td>
</tr>
<tr>
<td>F-IL</td>
<td>62.3*</td>
<td>50.8</td>
<td>92.9*</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 2. Concentrations of organic and inorganic P extracted by NaHCO₃ and NaOH from bulk (B) or rhizosphere (R) soil collected in rhizoboxes containing P. pinaster plants grown for 6 months in different soil samples (see Table 1 for details). Soil samples were used intact and during plant growth, the rhizoboxes were watered with water only. Measurements of P concentrations were carried out on freeze-dried soils. Values are mean (n = 6). Probabilities to have significant differences between line and interline are indicated for each soil treatment using the LSD Fisher model. Asterisk denotes a significant difference between B and R soils (LSD Fisher model, P < 0.05).
Despite equal mean annual rate of P supply (32 kg ha\(^{-1}\) year\(^{-1}\)), Table 1) to P and F plots, NaHCO\(_3\)-extractable P concentrations measured in P-L samples (4 mg P kg\(^{-1}\) soil) were much lower than those measured in F-L samples (14 mg P kg\(^{-1}\) soil) (Table 2). Inorganic P concentrations in NaOH extracts from bulk soils were generally twice as high as bicarbonate Pi and followed the same trends of variation between other soil samples (Table 2). Bicarbonate- and NaOH-extractable Pi concentrations from rhizosphere soils were not significantly different compared with those from bulk soils, except in interline soil samples in F plots, where they are significantly decreased. The depletion of NaHCO\(_3\)- and NaOH-extractable Pi was recorded as 20% and 10%, respectively (Table 2).

**Soil organic P fractions**

Organic P (Po) concentrations in bulk soils extracted with bicarbonate varied little between treatments (Table 2). In control plots, soils from lines contained higher Po concentrations than soils from interlines, contrary to concentrations observed in P or F plots, where Po was higher in interline. The concentrations of NaOH-extractable Po in bulk soils were increased 3- to 5-fold in both line and interline positions. However, the pattern of accumulations was similar to that observed in NaHCO\(_3\)-extractable Po. When assayed in rhizosphere soils, NaHCO\(_3\)-Po concentrations displayed the same trends as those observed for bulk soil and were either equal to (control old and control) or higher than (P and F plots) the values assayed in bulk soils. The concentrations of NaOH-Po in rhizosphere soils were equal to the values assayed in bulk soils, except in soils from the lines position of CO treatment and from the interlines position of F treatment, where they are respectively higher and lower than the values assayed in corresponding bulk soils (Table 2).

**Fungal growth and ectomycorrhizae formation**

Fungal development was estimated by assaying ergosterol, a sterol characterizing living fungal cells as it is only found in the plasma membrane. The sieved soil contained extremely low ergosterol contents compared to the material remaining in the sieve (data not shown), indicating that sieving was efficient to isolate the hyphae from freeze-dried soil samples. As indicated in Figure 1, ergosterol contents of material sampled in rhizosphere were higher than those assayed in bulk soil, whatever the treatments. The increase was highly significant in fertilized treatments as compared to control (CO and C) treatments. Ergosterol contents measured in fertilized treatments were relatively higher than the values measured in the controls (Figure 1). The identification of fungal species forming ECM tips showed that the basidiomycete *Rhizopogon luteolus* was the dominant species regardless of the treatments, as it presented 66.6% of the ECM tips analysed using molecular tools. We also found *Sphaerospora brunea* (28%) and the rare presence of *Laccaria bicolor*, (4.7%) only in F treatment. Therefore, phosphatase activity of ECM corresponded mainly to those of *R. luteolus*. As shown in Figure 2A, phosphatase activity was the highest (n = 25–40) in ECM collected in soil from the 93-year-old forest, regardless of the soil position (L or IL). Activities measured in ECM collected in soil from the control plot of 13-year-old forest were not significantly different from those measured in the soil sample collected in the line of P-fertilized plot. However, they were significantly different from those measured in ECM found in F soil (L and IL samples) and Po soil (IL) (Figure 2A). As shown in Figure 2B, the activities decreased when rhizosphere NaHCO\(_3\)-extractable Pi concentrations increased and vice-versa. Nevertheless, the activities measured in ECM collected from soils were highly variable; NaHCO\(_3\)-extractable Pi was lower than 5 mg kg\(^{-1}\) soil (CO, C and P-IL), contrasted with the activities measured in soils from CO which were significantly higher. Moreover, phosphatase activity did not show any relation with Po fractions in soils.

**Plant growth and mineral nutrition**

Table 3 indicates that dry biomass in roots and shoots did not vary largely between treatments. However, shoot biomass was the highest in F-L and P-IL treatments, and root biomass was the highest only in P-IL treatment. Soil receiving complete fertilization (F-IL) produced plants with a similar root and shoot biomass as in control soil (CO and C). The concentrations of total N in roots and shoots of plants grown in soil from the fertilized plots did not vary whatever the treatment and were lower than those measured in shoots from the two control treatments (CO and C) and in roots from the CO treatment only (Table 3). In contrast to N, total P concentrations in roots and shoots were dramatically increased by 10- and 6-fold in plants.
grown in fertilized soils (P-IL and F-IL, respectively) compared to plants grown in soil from CO treatment. However, P concentrations measured in roots and shoots of plants grown in soils from control or P-L treatments were as low as those measured in CO treatment. The culture of plants in soil from F-L treatment induced a significant increase in P concentrations measured in plants in comparison with the previous treatments, but this increase was lower than that observed in P-IL and F-IL treatments. Root length was the highest in C and P-IL treatments, while complete fertilization (F-IL) and CO soils significantly decreased this parameter (Table 3). Plotting total N contents and total biomass accumulated in each plant showed that these two variables are linearly correlated for all treatments ($R^2 = 0.55$, $P < 0.01$). However, linear regression calculated for each soil treatment showed stronger relationships than that found with the whole data set, except in P-IL plants (Figure 3A). The highest $R^2$ value was observed in C treatment ($R^2 = 0.95$) and the lowest one in CO treatment ($R^2 = 0.61$) (Figure 3A). Contrary to the total N contents, the total P contents varied widely among the soil treatments (Figure 3B). Total P contents in plants from CO, C and P-L treatments were low and varied linearly with biomass. The plants grown in F-L soil samples showed higher P contents than in CO, C and P-L, and P contents were still highly correlated with total biomass. However, the plants grown in F-IL and P-IL soil samples displayed the highest levels of P contents that were not linearly correlated ($P > 0.05$) with the plant biomass (Figure 3B). Plotting the total amounts of N per plant against the total amounts of P per plant showed that these two variables were positively correlated in all treatments, except in P-IL (Figure 4). These data show that the plants were able to uptake N despite having low P availability in soil (Figure 4). Overall, these results indicate that the plants were able to produce variable amounts of biomass per unit of P taken up from soil. Plotting ratio between biomass plant$^{-1}$ and total P plant$^{-1}$ against the concentrations of

Table 3. Accumulation of biomass, total N and total P in shoots and roots and root length per plant measured in *P. pinaster* plants grown for 6 months in rhizoboxes containing soil samples from different provenances (see Table 1 for details). Values are mean ($n = 6$ for all treatments but $10 < n < 12$ for CO and C treatments) with standard error between brackets. Different letters show significant difference between treatments according to the LSD Fisher model ($P < 0.05$).

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Biomass (g dwt plant$^{-1}$)</th>
<th>Total N (mg g$^{-1}$ dwt)</th>
<th>Total P (mg g$^{-1}$ dwt)</th>
<th>Root length (cm plant$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td>CO-L + CO-IL</td>
<td>0.25b (0.02)</td>
<td>0.16d (0.01)</td>
<td>12.17a (0.68)</td>
<td>10.19a (0.49)</td>
</tr>
<tr>
<td>C-L + C-IL</td>
<td>0.25b (0.02)</td>
<td>0.17d (0.01)</td>
<td>10.12b (0.23)</td>
<td>7.35b (0.24)</td>
</tr>
<tr>
<td>P-L</td>
<td>0.31b (0.06)</td>
<td>0.22c (0.05)</td>
<td>6.61c (0.56)</td>
<td>6.65b (0.23)</td>
</tr>
<tr>
<td>F-L</td>
<td>0.33a (0.05)</td>
<td>0.24b (0.03)</td>
<td>7.72c (0.26)</td>
<td>6.61b (0.25)</td>
</tr>
<tr>
<td>P-IL</td>
<td>0.35a (0.02)</td>
<td>0.29a (0.01)</td>
<td>7.14c (0.36)</td>
<td>6.68b (0.25)</td>
</tr>
<tr>
<td>F-IL</td>
<td>0.26b (0.02)</td>
<td>0.17d (0.02)</td>
<td>8.05c (0.89)</td>
<td>6.98b (0.15)</td>
</tr>
</tbody>
</table>

Figure 2. Acid phosphatase activity from ECM root tips estimated by the hydrolysis of pNPP as a function of the soil samples (A) or the concentrations of NaHCO$_3$-extractable Pi in the rhizosphere soil of each soil sample (B). Mean values ($n = 24–40$) ± 1 SE are shown. Different letters indicate the significant differences between soil samples (LSD Fisher model, $P < 0.05$).
NaHCO$_3$-extractable Pi in rhizosphere soil demonstrated the inverse relationship between these two parameters and the extreme plasticity of *P. pinaster* to produce biomass as a function of P availability in the soil (Figure 5).

**Discussion**

Measurements of bicarbonate-extractable P concentrations in bulk soils from unfertilized plots confirmed the low P availability of these spodosol soils. Nevertheless, soils taken from the vicinity of old trees (CO-L) contained more bicarbonate-extractable Pi than soils taken near to young trees (C-L). This could be due to the recycling of Pi from litter fall, together with a low rate of Pi uptake by the roots of these 93-year-old trees. Low availability of P in soil could be due to the multiple factors such as immobilization of available P by adsorption of phosphates with Al and Fe, rendering Pi as a less labile phosphate source of P (Fontes and Weed 1996, Barroso and Nahas 2005) or immobilization in the form of organic P (Holford 1997). The P fertilizers that were applied to enhance the sustainability of the stand dramatically increased NaHCO$_3$-$P_i$, especially NaOH-extractable Pi concentrations in the bulk soil from P-IL and F-IL samples compared to the values of C (L and IL), P-L and F-L. In addition, only NaOH-extractable Po concentrations in the bulk soil were significantly higher in P-IL and F-IL than in P-L or F-L samples. Thus, our data suggest that most of the P applied in the stand was fixed with Al and Fe, and was also partly immobilized into organic forms in situ as NaOH is thought to extract phosphate associated with Al and Fe (Fontes and Weed 1996).

Two treatments with fertilizer application presented much lower bicarbonate- and NaOH-extractable Pi concentrations that were applied to enhance the sustainability of the stand dramatically increased NaHCO$_3$-$P_i$, especially NaOH-extractable Pi concentrations in the bulk soil from P-IL and F-IL samples compared to the values of C (L and IL), P-L and F-L. In addition, only NaOH-extractable Po concentrations in the bulk soil were significantly higher in P-IL and F-IL than in P-L or F-L samples. Thus, our data suggest that most of the P applied in the stand was fixed with Al and Fe, and was also partly immobilized into organic forms in situ as NaOH is thought to extract phosphate associated with Al and Fe (Fontes and Weed 1996).

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in soil collected from the lines than in interlines positions. It confirms that the P in soil was not or slightly mobile as claimed by several authors (see for e.g., the reviews from Schachtman et al. 1998, Hinsinger 2001, Vance et al. 2003), and the fertilizers applied in the interlines position did not show significant mobility, and bicarbonate- and hydroxide-extractable Pi pools were as low as in control treatment. However, the soil collected in lines from the F plot contained greater bicarbonate- and NaOH-extractable Pi concentrations than the soil collected from the P plots despite the same annual rate of P supply in P and F treatments (32 kg ha\(^{-1}\) year\(^{-1}\), Trichet et al. 2008, Bakker et al. 2009). This enhanced Pi availability could be due to the intensive development of forest floor annual vegetation (Phytolacca spp) only observed in F plots (personal observation), leading to an accelerated recycling rate of Pi accumulated in the aerial parts of annual species during the growing season returning to the soil in winter. In addition, the root compartment of these plant species may also play a role in Pi recycling, as Achat et al. (2008) reported that 90% of total fine roots are composed of forest floor annual vegetation species in the upper soil layer in this forest ecosystem.

The comparison of NaHCO\(_3\)- and NaOH-extractable Pi measured in bulk or rhizosphere soils showed non-significant changes, except in the soil samples collected from interline position of F plots where the rhizosphere pools are significantly decreased compared to bulk ones. The depletion of Pi in rhizosphere could be due to the P uptake by the plants and the microorganisms developing in association with the roots such as ECM fungi. Significant high concentrations of total ergosterol in F-IL soil (rhizosphere and bulk) are in good agreement with the depletion of Pi in rhizosphere. These results are in agreement with Bakker et al. (2009), who reported significantly more fungal hyphae in phosphorus-fertilized treatments than in control in P. taeda forest stand and with Parrent and Vilgalys (2007), who reported the stimulation of extramatrical mycelia with N fertilization in P. taeda forest. In addition, these soil conditions may also have favoured the development of bacterial populations associated with ECM fungi, as demonstrated for L. bicolor (Duponnois and Garbaye 1991a, 1991b). Consequently, this enhanced growth of fungal populations and possibly of bacterial populations may be responsible for the depletion of rhizosphere bicarbonate- and NaOH-extractable Pi from rhizosphere of F-IL soils.

On the other hand, the comparison of NaHCO\(_3\)-extractable Po measured in bulk or rhizosphere showed increased Po pools in rhizosphere soil, especially in fertilized plots. This could be due to the better development of fungal populations observed in these conditions and/or their associated bacterial populations. The use of Pi and the assimilation of P as organic forms by the microbial cells could lead to the enrichment of Po pools observed in these conditions. However, these trends are less obvious for NaOH-extractable Po, suggesting two different pools of Po extracted by NaHCO\(_3\) and NaOH.

Molecular studies of ECM morphotypes revealed that R. luteolus formed most of the ECM tips whatever the treatment. The high capacity of R. luteolus to develop an association in these conditions could be due to a high survival capacity of the spores present in the soil (Massicotte et al. 1994, Colgan Iii and Claridge 2002, Bruns et al. 2009). The R. luteolus was frequently identified in ECM tips in field surveys of the same plots (unpublished data). The second species was Sphaerosporea brunnea. However, it could be considered as an opportunist species, as it is a common contaminant in nurseries producing mycorrhizal plants (Garcia-Montero et al. 2008). Acid phosphatase activity measured by incubating ECM in the artificial substrate \(pN\)PP differed widely according to the soil samples. Plotting this phosphatase activity against bicarbonate-extractable Pi from the rhizosphere soil showed an inverse relationship between these two variables, indicating that the enzyme activity is enhanced by low P availability. Our data are in agreement with the previous studies carried out with ECM grown in soil (Kroehler and Linkins 1988, Antibus et al. 1992, Chen et al. 2002) or with ECM fungus grown in pure culture (Bousquet et al. 1986). However, despite comparable levels of bicarbonate-extractable Pi in CO, C and P-IL soil samples, ECM collected in CO soil displayed the highest phosphatase activity. This could be due to either an overestimation of the actual Pi concentration in the soil solution by bicarbonate extraction or the selection of fungal strains that are able to release more phosphatases in this old, undisturbed forest than the fungal strains present in the young forest.

Growth parameters and mineral nutrition indicate a high plasticity of P. pinaster to cope with low concentrations of available P in soils. However, N availability was not as limiting as P availability. Indeed, N concentrations and N contents per plant were not as variable as for P, especially when plants were grown in CO soil samples. In these conditions, the concentrations assayed in roots and shoots were remarkably close to those measured in 6-month-old P. pinaster associated with the ECM basidiomycete Hebeloma cylindrosporum, and N was supplied in nutritive solution containing ammonium and nitrate (2 mM each) (Conjeaud et al. 1996). This high N availability was in agreement with the high total N concentrations measured in the soil sampled in CO forest (2.6 mg N kg\(^{-1}\) of soil) that was three times higher than other treatments (0.7–1.0 mg N kg\(^{-1}\) of dry soil). This suggests that N accumulated in this soil was easily used up by the mycorrhizal plants. By contrast, soil samples that received a complete fertilization did not result in an increase of biomass nor total N contents. Such negative effects of additional fertilization, particularly N, have been shown to reduce net primary production, foliar and fine root biomass in field plots (Aber et al. 1989, Albaugh et al. 1998). Alternatively, the growth of P. pinaster plants could have reached a plateau with increasing doses of either N or P fertilizers, as observed for Eucalyptus grandis (Conroy et al. 1992).
In contrast to N, the relationships between biomass and total P per plant were highly different in different soil treatments. The results showed that the linear regressions of these two variables were highly significant in all treatments except in soils with fertilizer application (P-IL and F-IL). Plants grown in soil samples with NaHCO₃-extractable Pi ranging from 1.5 (C) to 4.5 mg kg⁻¹ (CO and P-L) presented comparable P contents in the whole plants; however, the values of P contents were very low. It indicates that P was the limiting nutrient factor. Moreover, the plants grown in CO soil presented the highest values of acid phosphatase activity in ECM. This suggests that phosphatase activity was not sufficient per se to overcome the P limitation in CO, C and P-L soils. This could be due to several factors related to the environmental conditions for hydrolysis of P occurring in the soil solution that may be very different from the conditions used to measure pNPPase activity in vitro. Indeed, CO soils are very dark, and the measurement of fluorescence on water soil extracts showed that CO soils contained more fluorescent compounds than the other soils (personal observation). These observations were based on the wavelength in the peak values of acid and fulvic compounds. These soluble molecules could inhibit the phosphatase activity in soil conditions (Allison et al. 2007). On the other hand, soil organic P molecules might not be highly degradable with the enzymes produced by the ECM fungi.

A slight increase in soil P availability up to 14 mg NaHCO₃-extractable Pi kg⁻¹ dry soil (F-L) strongly increased the P contents that remained highly correlated with biomass production and N contents per plant. In contrast, the highest bicarbonate P concentrations still increased the total P contents of plants (interline position of P and F treatment) that was not used to produce biomass, indicating a luxury consumption of P, as reported for other plant species (Verhoeven and Schmitz 1991, Aerts and Chapin 2000). Conversely, we observed a decreased biomass production in F-IL treatment. This could result from the down-regulation of root length observed in these two variables were highly significant in all treatments except in soils with fertilizer application (P-IL and F-IL). Plants grown in soil samples with NaHCO₃-extractable Pi ranging from 1.5 (C) to 4.5 mg kg⁻¹ (CO and P-L) presented comparable P contents in the whole plants; however, the values of P contents were very low. It indicates that P was the limiting nutrient factor. Moreover, the plants grown in CO soil presented the highest values of acid phosphatase activity in ECM. This suggests that phosphatase activity was not sufficient per se to overcome the P limitation in CO, C and P-L soils. This could be due to several factors related to the environmental conditions for hydrolysis of P occurring in the soil solution that may be very different from the conditions used to measure pNPPase activity in vitro. Indeed, CO soils are very dark, and the measurement of fluorescence on water soil extracts showed that CO soils contained more fluorescent compounds than the other soils (personal observation). These observations were based on the wavelength in the peak values of acid and fulvic compounds. These soluble molecules could inhibit the phosphatase activity in soil conditions (Allison et al. 2007). On the other hand, soil organic P molecules might not be highly degradable with the enzymes produced by the ECM fungi.

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In conclusion, these results showed the high capacity of P. pinaster to produce plant biomass when grown with low P availability. The P applied in the form of mineral fertilizers remained highly immobile, and the high phosphatase activity was not sufficient to overcome P limitation of P. pinaster in soil with a low phosphorus availability.

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