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Positive growth response of *Pinus pinaster* seedlings in soils previously subjected to fertilization and irrigation

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ABSTRACT

Nutrient availability, particularly phosphorus (P), and water are major factors limiting tree growth in plantations of maritime pine (*Pinus pinaster*) in southwestern France. Applied intensively, fertilization and irrigation could have differential effects on the actual nutrient availability to the trees, especially P. These practices could modify the geochemical cycling of P between mineral and organic forms in soil as well as the enzyme activities able to mobilize soil organic P, namely, acid phosphatases. The result is modified growth response and mineral nutrition of the trees. Our research objective was to evaluate the growth and mineral nutrition of *P. pinaster* seedlings, together with soil P cycling, in response to prior fertilization and irrigation practices performed in the field. Seedlings were grown in a growth chamber in rhizoboxes containing soil samples from a 13-year-old maritime pine forest stand, previously fertilized annually (C: no fertilization, P: phosphorus only, F: complete fertilization NPKCaMg) with and without irrigation for 7 years. Plants formed ectomycorrhizal roots (ECM) mainly with the basidiomycete *Rhizopogon luteolus*. Fertilization significantly increased bicarbonate and hydroxide extractable inorganic P (P_i) and organic P (P_o) while it decreased ECM acid phosphatase activity. Plants were hardly able to acquire any P from control soils, despite a high phosphatase activity assayed in ECM. Seedlings grown in soil with previous complete fertilization and irrigation displayed the strongest ability to deplete P_o and to produce biomass. Compared to control soils, P accumulations were significantly greater in seedlings from the fertilized treatments. Diagnostic approaches confirmed deficiency status of P in control soils resulting in increased plant N. In contrast, both P and N were sufficient in soils from the irrigated and complete fertilization treatment, and a steady growth response of seedlings was observed. We suggest that the significant differences observed in P_o mobilization based on prior fertilization and irrigation practices could be due to differential turnover of microbial populations.

1. Introduction

Forest management has recently received special attention throughout the world due to the growing demand for fiber, wood, bio-energy production (Kauter et al., 2003) and CO_2 offsets (Graham et al., 1992). These demands can be satisfied by increasing biomass production of plants, which requires the diagnosis of specific limiting factors at the stand level (Fox, 2000). Nutrients and water are major factors limiting the forest production, and could be alleviated by intensive management practices. High fertility soils increase nutrient concentration, leaf area indices and whole growth of plants (Samuelson et al., 2001), while favorable water availability provides a bulk-flow pathway for nutrient uptake (Marschner, 1986) and maintains turgidity for growth and higher stomatal conductance for photosynthesis (Kozlowski et al., 1991). Field trials including fertilization and irrigation showed a greater increase of annual production, sometimes by four times compared to control plots; however, as these effects were not isolated, either nutrients or water may be the limiting resource (Albaugh et al., 2012). Indeed, forest productivity is generally limited by nutrient availability, but the response to nutrient amendments is also linked with adequate moisture availability (Lockaby et al., 1997; Samuelson, 1998).

Sites responding to both nutrient and water addition displayed either additive or interactive effects. For examples, fertilization had a greater effect on the growth of *Pinus taeda* than irrigation without any interactive effect (Albaugh et al., 2004). Coyle and Coleman (2005) observed strong response to irrigation, nitrogen (N) fertilization and the interaction of both irrigation and fertilization, but the responses were considerably different for *Populus deltoides* and *Platanus occidentalis*. Irrigation and N fertilizers increased the

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growth of *Pinus radiata* but the response to irrigation was stronger in the early than in the later stages of plant growth (Waterworth et al., 2007). Similarly, the growth of *Pinus pinaster* was increased by irrigation alone as well as by the interactive effect of both irrigation and fertilizer treatments i.e. and NPKCaMg and phosphorus (Trichet et al., 2008).

Bakker et al. (2009) showed that irrigation and fertilization increased the amount of ectomycorrhizal (ECM) hyphae and specific root length of *P. pinaster* compared to control treatments. Due to the increase of soil exploration by hyphae associated with the roots (Rousseau et al., 1994), mycorrhizal association is considered as an important strategy to increase phosphate acquisition by plants (Smith et al., 2000; Smith and Read, 2008; Plassard and Dell, 2010; Cairney, 2011). In addition to improving soil exploration, ECM fungi have the ability to release phosphatase into their environment, be it culture medium (Bousquet et al., 1986; Tibbett et al., 1998; Quiquampoix and Mousain, 2005; Plassard et al., 2011) or soil (Antibus et al., 1992; Chen et al., 2002; van Aarle and Plassard, 2010), suggesting that ECM play a crucial role in converting organic P to inorganic P. Maritime pine (*P. pinaster* Soland in Aït.) is a tree species cultivated in the "*des Landes de Gascogne*" region located in southwestern France covering an area of ca 900,000 ha (Augusto et al., 2010). This tree species has a high economic value as it produces ca 20% of French softwood (Bert and Danjon, 2006). This monoculture forest is made up of even-aged tree stands established in different soils according to the site classes of this region (humid moorlands, mesic moorlands, dry moorlands and coastal dunes) (Augusto et al., 2010; Achat et al., 2011). However, most of the soils are acidic sandy podzols, with high Fe and Al contents in surface layers, low cation exchange capacity (CEC) and low total P contents (Trichet et al., 1999). Therefore, these soils are characterized by a low P availability that is the main limiting factor for tree growth (Bonneau, 1995; Trichet et al., 2009). In this region, the availability of water is characterized by the presence of a permanent water table, fluctuating between the soil surface in winter and a depth of 1.8 m in summer. The roots of *P. pinaster* remain at 1 m distance over the water table but the water balance is geographically variable (Loustau et al., 1999a). Management practices in the last three decades of 20th century like drainage, tillage and phosphorus addition has increased the average productivity of this region from 4.8 $m^3\ ha^{-1}\ y^{-1}$ to 11 $m^3\ ha^{-1}\ y^{-1}$. However, the long-term productivity in the next five decades is predicted to decrease due to reduction of soil and atmospheric moisture level (Loustau et al., 2005). Therefore, optimization of management practices could benefit from a more integrated and accurate quantification of the effects of water and nutrient addition on soil nutrient availability and plant productivity.

To address this issue, we quantified the growth and mineral nutrition, particularly P, of newly planted *P. pinaster* seedlings in soil samples collected from plots of a 13 year-old *P. pinaster* stand, which had been subjected to varying fertilization and irrigation regimes annually for the previous 7 years. Plants were grown in rhizoboxes, under controlled conditions in a growth chamber. Our hypothesis was that fertilization and irrigation could interact to modify the availability of nutrients to plants, particularly that of P, compared to either treatment alone through modifications to the soil P cycle. Therefore, we quantified the pools of soil mineral and organic P and the enzyme activities able to mobilize soil organic P, namely acid phosphatases, produced by ectomycorrhizal roots.

2. Material and methods

2.1. Soil sampling and site description

The soil samples were collected from a maritime pine forest stand located in the region "*Les Landes de Gascogne*" situated in southwest of France. Climatic conditions are a mean annual air temperature of 12.5 °C and an average precipitation rate of 950 mm, with frequent prolonged period of drought in summer. Soils are characterized as the sandy spodosols developed on Aeolian sandy deposits of quaternary era. The forest stand consisted of 13-year-old *P. pinaster* trees maintained regularly by various agronomic practices over the past 7 years. Included are blocks with different fertilizer regimes: no fertilizer (control, C), phosphorus fertilizer (P) and complete mineral fertilizer (F) application without irrigation (C, P and F respectively) and with irrigation (CI, PI and FI respectively) (Table 1). Each block measured 60 × 36 m with an exclusion of a 10 m border area. Trees were planted in lines with a 2 m tree-to-tree distance and a 4 m line-to-line distance. In each block, soil cores (15 cm deep × 8 cm diameter) were collected with a manual auger from interline position located roughly at equidistance between two lines of trees in November 2006. Basic chemical properties of the soil samples (total N, total C, CEC, pH in water and aluminum content) were measured by the Soil Analysis Laboratory of INRA, Arras France (http://www5.lille.inra.fr/las) and are presented in Table 2. Soils were kept for three months at 4 °C before serving as a substrate in rhizoboxes without any further treatment. The indigenous soil fungi and bacteria served as inoculants and soil mineral nutrients were supplied as the sole source of nutrition for the young seedlings.

2.2. Seedling development and plant growth in rhizobox

Seeds of maritime pine (*P. pinaster* Soland. In Ait. from Medoc, Landes-Sore-VG source, France) were disinfected and stratified as previously described (Ali et al., 2009), before sowing in vermiculite, which was twice autoclaved (121 °C, 15 min) and soaked in distilled water. After 2.5 months in a growth chamber (16/8 h light/dark cycle at 25/18 °C, 70% rh, CO_2 concentration of *c.* 350 $mm^3\ l^{-1}$

Table 1
Description of soil samples used to grow young seedlings of *P. pinaster* in rhizoboxes. Soils were sampled from the field of 13-year-old *P. pinaster* stands, which had received annual treatments for the previous 7 years.

Soil sample	Description
C	No irrigation and no fertilizer application
P	No irrigation and annual phosphorus application in interline[a]
F	No irrigation and annual complete fertilizers application in interline[b]
CI	Regular irrigation and no fertilizer application
PI	Regular irrigation and annual phosphorus application in interline[a]
FI	Regular irrigation and annual complete fertilizers application in interline[b]

[a] Mean rate in 1998–2005 of 32 P kg ha^{-1} $year^{-1}$ (Bakker et al., 2009).
[b] Mean 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).

Table 2
Chemical properties of the soil samples used for plant growth.

Soil	Total N (g kg^{-1})	Total C (g kg^{-1})	C/N	CEC[a] (cmol kg^{-1})	pH (H_2O)	Aluminum[b] (g/100 g)
C	0.735	21.1	28.7	1.34	4.08	0.0416
P	0.943	26.9	28.5	2.17	4.05	0.0434
F	0.731	18.6	25.5	1.19	4.30	0.0645
CI	0.743	19.4	26.1	1.51	4.38	0.0444
PI	0.77	21.2	27.5	1.69	4.23	0.0348
FI	1.15	26.3	22.9	2.42	4.38	0.0720

[a] Cation Exchange Capacity was determined using the cobaltihexamine method.
[b] Al was determined using the TAM method.

and a PAR of *c.* 400 μmol m^{-2} s^{-1} (400–700 nm)), the young seedlings were transferred in rhizoboxes as described in Ali et al. (2009). Each rhizobox contained 70 g of unsieved soil from which coarse root fragments had been removed. For each soil treatment (C, P, F, CI, PI and FI) ten rhizoboxes were prepared simultaneously, eight with a young seedling of *P. pinaster* and two without seedlings (control rhizoboxes). Rhizoboxes with and without plants were placed in containers with distilled water to ensure water supply to the seedlings through a sterile glass fiber sheet in contact with soil (Torres Aquino and Plassard, 2004). All the rhizoboxes were maintained under similar conditions for eight months, in the growth chamber at aforementioned conditions, with regular supply of distilled water. In addition, six young plants were weighed and kept at −80 °C, to determine initial plant N and P contents.

2.3. Harvesting of experiment and chemical analyses

In order to isolate ectomycorrhizal root tips (ECM), the root system of each plant was gently pulled out of the rhizoboxes, after disassembly. Ectomycorrhizal root tips were picked up from each root system under a stereomicroscope and were classified into different morphotypes to assay phosphatase activity. Subsamples of each morphotype were stored at −20 °C for molecular identification. Fresh and dry weights of roots and shoots were recorded before and after freeze-drying, respectively. Total N and total P contents were measured as described in Ali et al. (2009). Briefly, 1 ml of 36N H_2SO_4 was used to mineralize 50 mg of finely grinded plant material at 330 °C for 30 min (McDonald, 1978) in a tube mineralization block. If the solution remained unclear, 0.2 ml of pure hydrogen peroxide (110 vol, not stabilized with phosphate) was repeatedly added until the color of solution became transparent. Free ammonium and orthophosphate P concentration was then assayed in H_2SO_4 diluted to 0.1N using phenol colorimetric method of Berthelot (Martin et al., 1983) and malachite green method (Ohno and Zibilske, 1991), respectively.

2.4. Soil analyses

All soil contained in each rhizobox was collected and air-dried prior to analysis. Phosphorus fractions were determined in sieved soil (2 mm) as described by Ali et al. (2009). Plant available P was extracted with 0.5M, $NaHCO_3$, pH 8.5 (1/20, g/v) by shaking for 30 min at room temperature (Olsen et al., 1954). We choose this method of extraction because it has been determined as the best one to estimate "available" P not only in calcareous but also in acid soils (Fardeau et al., 1988). Less labile P, associated with amorphous Al- and Fe-phosphates (Tiessen et al., 1984; Sharpley et al., 1999) was extracted by shaking soil for 16 h in 0.1M NaOH (1/10, g/v) at room temperature. After dilution with distilled water (1/6, v/v), soil extracts were acidified with 12 N, HCl (1/600, v/v) to precipitate humic material by centrifugation (4600 g, 25 min) before directly assaying P_i concentrations using malachite green method. The same soil extracts were mineralized with 12 N HCl (v/v) at 110 °C for 16 h (Ali et al., 2009). The excess of protons was eliminated by mixing the resulting HCl solution (6N) with CH_3COONa (1.25 M) (1/6, v/v) before assaying P with the malachite green method. Organic P concentration was calculated as the difference between Pt and P_i for both $NaHCO_3$ and NaOH extracts.

2.5. Phosphatase activity of ECM tips

Five independent ECM tips of each morphotype were used to estimate acid phosphatase activity. ECM tips were incubated individually for an hour in acetate buffer (25 mM, pH 5.4) with 10 mM *para*-nitrophenol phosphate (*p*NPP, Sigma Ref. N4645). The reaction was terminated by adding 0.5 M NaOH into the incubated tubes. For each morphotype, a blank sample was also prepared by adding NaOH to the *p*NPP solution and ECM tips, simultaneously, before incubation. The yellow color resulting from the production of *p*NP (*para*-nitrophenolate) was measured at 400 nm and enzyme activity was calculated (nmol of *p*NP produced min^{-1} g^{-1} of fresh ECM weight) from the equation:

$$\text{Phosphatase activity} = (\Delta OD \times V \times DF)/(t \times 0.0188 \times FW)$$

In the equation, "ΔOD" indicates the difference between optical density of blank and the samples, "V" is the final reaction volume (ml), "DF" stands for dilution factor, "t" denotes the time of incubation (minutes), "0.0188" is coefficient of molar extinction for *p*-nitrophenolate (ml $nmol^{-1}$ cm^{-1}), "FW" describes the fresh weight (g) of ECM tips calculated using their volume and a density of 1 kg l^{-1}. The volume of ECM tips was calculated using the average diameter and length of ECM root tips taken by automated image analysis software, WinRhizo 2005b (Regent Instruments. Inc., Canada).

2.6. Molecular identification of indigenous fungal species

Fungal DNA was extracted from frozen individual ECM morphotypes using the DNeasy Plant Mini Kit according to the manufacturer's instructions (QIAgen S.A.). DNA amplification was carried out using the primers ITS1-F and ITS4 (White et al., 1990) as described previously (Ali et al., 2009). Gel electrophoresis (agarose 1.5%) was performed to verify DNA amplification. The amplicons were sequenced from Cogenics (https://www.cogenicsonline.com). Identification of the fungal ECM genus and species was performed by launching the sequences and running the blastn program on UNITE online molecular data base service (Kõljalg et al., 2005).

2.7. Statistical analyses

Analyses of variance were performed to evaluate significant difference between different soil samples, plant responses and phosphatase activities. Means of each treatment were compared using Fisher's least significant difference ($P < 0.05$). Relations between biomass, total N and P in plants were performed by simple linear regression. All data were analyzed using Statistica software package (Statistica 8, Statsoft Inc. Tulsa OK, USA.). Diagnostic tool of vector analysis was performed to evaluate plant response to N and P in different soil treatments (Imo and Timmer, 1997; Salifu and Timmer, 2001, 2003). Soil treatment with phosphorus application (P) was taken as reference, since this treatment added P annually according to the foliar requirements of *P. pinaster* in the field to overcome P deficiency in the forest stand. The relative biomass, N and P contents, and N and P concentrations were expressed in vector nomogram. The arrowhead lines in Fig. 4 indicate the direction of dispersion of each vector from the reference soil sample (P) and length of the lines indicates the magnitude of dispersion. The arrows in vector nomograms (Fig. 4) are represented only for significant effects; plant responses to N and P were interpreted (Imo and Timmer, 1997; Salifu and Timmer, 2001, 2003).

3. Results

3.1. Soil P availability and its mobilization

In rhizoboxes without trees, the concentrations of $NaHCO_3$ and NaOH extractable inorganic P (P_i) were significantly higher in the fertilized soil samples than in the control ones (Fig. 1a and c). However, the comparison of fertilized soil samples revealed, significantly ($P < 0.05$) higher values of NaOH extractable P_i

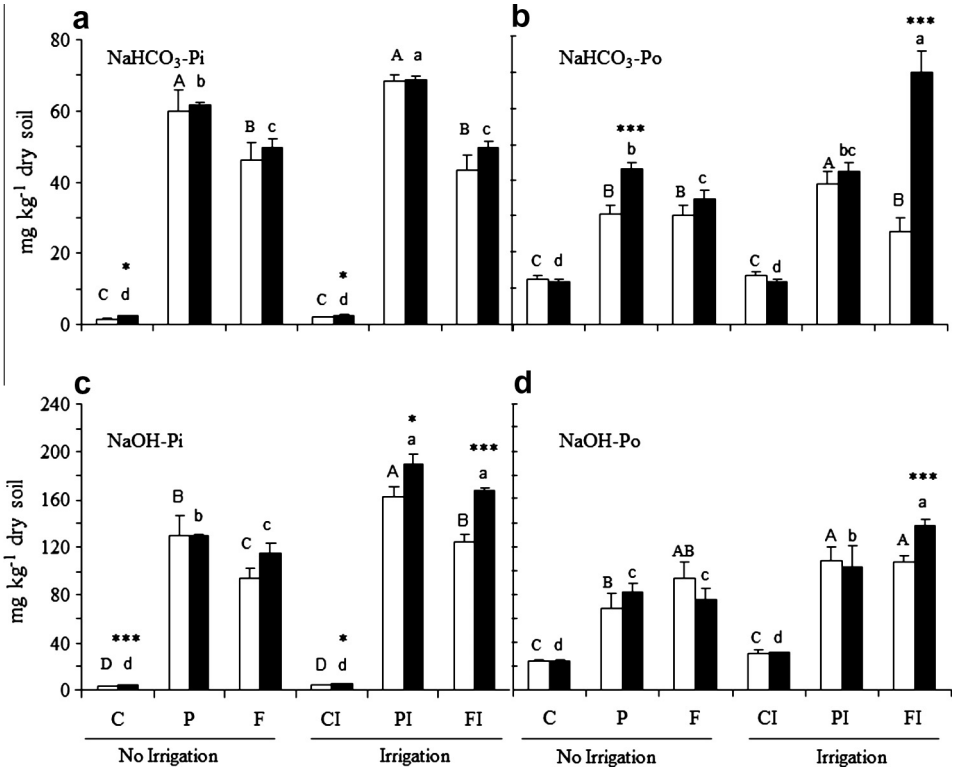

Fig. 1. Concentrations of bicarbonate (a and b) and NaOH (c and d) extractable P_i and P_o in soils with and without growth of maritime pine seedlings. Soils from a 13-year old *Pinus pinaster* stand were treated annually with fertilizers for the previous 7 years (C: no fertilization, P: phosphorus only, F: complete fertilization NPKCaMg) without or with irrigation (I). Unsieved soil samples were placed in a rhizobox, either with one plant (white bars) or without any plants (black bars) in controlled conditions for eight months. Different capital letters on white bars and small letters on black bars denote significance of means comparison using Fisher's LSD at $P < 0.05$ ($n = 8$). A Student's *t*-test was performed to compare the concentration of P with and without plants. Significance levels are given as: *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$; no symbol, $P > 0.05$.

concentrations in soil with P and PI treatments, compared to F and FI treatments, despite having received the same levels of P supply. As shown in Table 2, these treatments modified other soil chemical properties than P contents. Without irrigation, P treatment increased total N and C concentrations and the cation exchange capacity (CEC) without clearly affecting pH and exchangeable Al contents. Remarkably, the F treatment did not modify soil properties except for the pH and exchangeable Al content when compared to the C treatment. When irrigation was also applied, the most obvious changes were observed in FI soil, with a strong increase of N and C contents, CEC and exchangeable Al contents compared to CI and PI treatments (Table 2). The depletion of P_i was generally observed in rhizoboxes bearing young plants of *P. pinaster* as compared to rhizoboxes without seedlings. It was significant (*t*-test, $P < 0.05$) in control (C, CI) soil samples for both bicarbonate and hydroxide extracts (Fig. 1a and c). Additionally, in fertilized treatments, the depletion of P_i by plants was also observed, but only NaOH extractable P_i was significantly different in PI and FI soils (Fig. 1c).

Organic P (P_o) measured in soil from rhizoboxes without plants represented the dominant form (>80%) of extractable P in control (C and CI) treatments, regardless of the extraction type, while P_i was the dominant fraction (>50%) in all fertilized soil samples excepted for bicarbonate-extractable P_o in soil from FI treatment (Fig. 1b and d). Plants showed a variable ability to deplete the P_o pool from the soil samples (Fig. 1b and d) as we observed a significant decrease of sodium bicarbonate extractable P_o (*t*-test, $P < 0.05$) only in phosphorus (P) and irrigated and complete fertilizer (FI) treatments. This depletion was much stronger in FI (71%) than in P (25%) soil samples (Fig. 1b). The depletion of NaOH extractable P_o pool was significant only in FI soil samples (Fig. 1d).

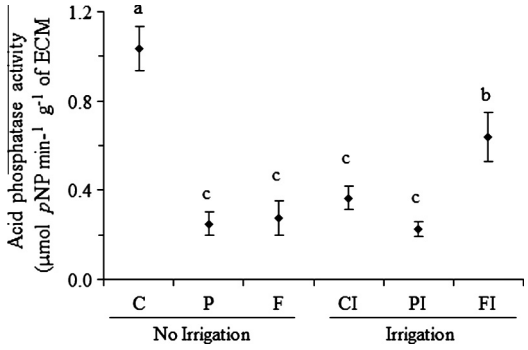

Fig. 2. Acid phosphatase activity secreted by ECM root tips ($n = 8$–25) of *P. pinaster* grown in soils that were previously managed annually with different fertilizers with and without irrigation. Different letters represent least significant differences of Fisher ($P < 0.05$) and bars are standard errors. Soils and plant growth conditions are the same as in Fig. 1.

3.2. Ectomycorrhizae and phosphatase activity

Acid phosphatase activities of ectomycorrhizal root tips (Fig. 2) were significantly higher (1.03 µmol *p*NP min^{-1} g^{-1}) when plants were grown in non-irrigated control soil samples compared to all other soil samples. The fertilization treatments without irrigation (P and F) as well as P + irrigation (PI) dramatically decreased phosphatase activities from ECM tips by factors ranging from 2.7 to 3.5. The decrease of phosphatase activity was also strong (factor of 1.8) when plants were grown in CI soil samples. Finally, the decrease of phosphatase activities was the lowest (factor of 0.6) in ECM tips developed in soils sampled from FI plot (Fig. 2). Molecular

Table 3
Accumulation of biomass and concentrations of N and P in *P. pinaster* plants grown for 8 months in rhizoboxes containing soils from different provenances (see Table 1 for details). Analysis of variance was performed and means are compared using Fisher's LSD. Different letters in the same column indicate significant difference ($P < 0.05$) between soils ($n = 8$).

Soil Samples	Dry biomass (g plant^{-1})			Concentrations of N (mg g^{-1} dry weight)		Concentrations of P (mg g^{-1} dry weight)	
	Shoot	Roots	Plant	Shoot	Roots	Shoot	Roots
C	0.41b	0.29d	0.70c	6.80a	4.68a	0.32c	0.40c
P	0.57b	0.40cd	0.96bc	3.71b	4.05b	1.47b	2.06b
F	0.51b	0.64b	1.15b	3.94b	3.58b	1.15b	1.96b
CI	0.47b	0.37cd	0.84bc	6.12a	4.70a	0.28c	0.43c
PI	0.50b	0.48bc	0.98bc	3.60b	3.67b	1.86a	2.71a
FI	0.91a	0.82a	1.73a	3.49b	3.65b	1.39b	1.80b

identification of ECM tips indicated that *Rhizopogon luteolus* was the dominant fungal species, as it represented 80% of the total analyzed morphotypes, whatever the treatment. The remaining 20% of ECM tips were identified as *Sphaerosporella brunnea.*

3.3. Plant growth and mineral nutrition

Plant analysis (Table 3) indicated a significant ($P < 0.05$) increase in shoot biomass of plants only in the FI (+145%) treatment compared to C treatment. In contrast, root biomass was significantly increased in F (120%), PI (65%) and FI (182%) rhizoboxes compared to control (C) rhizoboxes. Finally, only plants grown in FI soil samples displayed a significant increase in shoot and root biomass. Considering total biomass of plants, complete fertilizer treatments (F and FI) only gave significant increase compared to control conditions (C).

Concentrations of N ranged within a factor of 2 across all treatments for both roots and shoots, and were the highest in the controls, irrigated and non-irrigated (C and CI) (Table 3). In contrast to N concentrations, plant P concentrations varied over a factor of 6 across all treatments and were lowest in the control treatments (C and CI). The analysis of variance ($P < 0.05$) also showed that the concentrations of P measured in shoots and roots were highest in the PI treatment but were also significantly higher in soils from P, F and FI treatments than in control (C and CI) treatments (Table 3).

Values calculated for net accumulation of N and P are given in Table 4. The shoots systematically accumulated less N compared to the roots in fertilized treatments, whereas the accumulation was higher in the shoots of control plants both with and without irrigation. Data indicated significantly higher net accumulation of N in the shoots of C, CI and FI treatment ($P < 0.05$), whereas N accumulation in the roots was only significantly higher in FI plants followed by F treatment. Finally, total net accumulation of N only increased significantly in plants of FI soil samples (Table 4).

Values of net P accumulation calculated from the shoots of plants grown in the fertilized, irrigated (PI and FI) soil were significantly higher than those calculated from plant shoots grown in nonirrigated F and control treatments. Remarkably, there was a net decrease in P accumulation in the shoots of plants grown in C and CI soils, while in roots, a very low average net accumulation of P was calculated. Similarly, values of net P accumulation in the roots of plants grown in control soils (C and CI) were much lower than those measured in plants from fertilized soils, whether or not irrigated. Total net accumulation of P per plant was significantly higher in plants from fertilized soils than from controls, where it was slightly negative. Interestingly, net P accumulation in plants grown from irrigated, fertilized soils was increased by 56% (PI) and 60% (FI) relative to plants grown in P and F soils, respectively (Table 4).

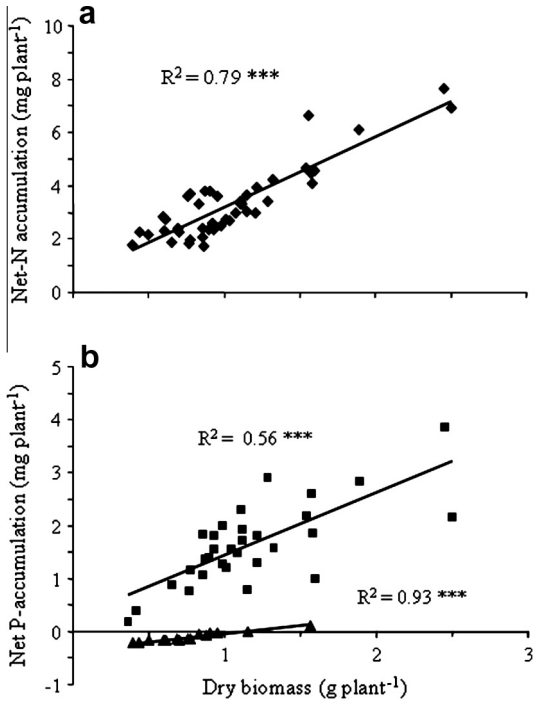

Fig. 3. Relation of total net accumulation of N (a) and P (b) per plant with total dry biomass of *P. pinaster* plant. In (a) the regression was calculated using all data. In (b), regression line plotted over triangles denotes plants grown in control (C and CI) soil samples and regression line over squares denotes plants grown in fertilized (P, PI, F and FI) soil samples. Values in brackets represent the coefficient of linear regression (R^2) and asterisks denote significance of relationships (*** = $P < 0.001$). Soils and plant growth conditions are the same as in Fig. 1.

Table 4
Net N and P accumulation in shoots, roots and total plant, and [N:P] ratios of shoot and root concentration of *P. pinaster* grown in rhizoboxes for 8 months (see Table 1 for details). Values are means ($n = 8$) and different letters denote significant differences within a column (one-way ANOVA, mean comparison test using Fisher's LSD at $P < 0.05$).

Soil samples	Net N-accumulation (mg plant^{-1}) in:			Net P-accumulation (mg plant^{-1}) in:			[N:P] ratios in:	
	Shoot	Roots	Total	Shoot	Roots	Total	Shoot	Roots
C	1.74a	1.16c	2.90b	−0.19c	0.06c	−0.13d	21.1a	11.80a
P	1.21b	1.45c	2.66b	0.47ab	0.73b	1.20c	2.74b	2.14b
F	1.13b	2.12b	3.25b	0.23b	1.22a	1.45bc	3.71b	1.84b
CI	1.92a	1.54c	3.46b	−0.20c	0.11c	−0.09d	22.4a	11.10a
PI	0.97b	1.58c	2.55b	0.63a	1.25a	1.88ab	2.08b	1.36b
FI	2.28b	2.82a	5.10a	0.92a	1.40a	2.32a	2.67b	2.05b

Plotting the total net N accumulation as a function of total plant dry biomass showed that these two variables were highly correlated ($R^2 = 0.79$, $P < 0.001$) (Fig. 3a). This relationship explained the high net accumulation of N with increasing plant growth and vice versa (Fig. 3a). Compared to N, the value of the regression coefficient between net P accumulation and total biomass per plant for all soils was smaller but remained significant (51%) (not shown). However, when fertilized (P, F, PI, FI) and control (C, CI) treatments were analyzed separately, the coefficient of linear regression for net P accumulations as a function of total plant biomass was increased to 56% for fertilized (P, F, PI, FI) treatments and 93% for the unfertilized control (C and CI) treatments (Fig. 3b).

3.4. Diagnostic analysis

N:P ratio (Table 4) indicated higher absolute values in control treatments (~20 and ~12) for shoot and roots respectively, than in other treatments. These high values of N:P ratio in plants from control soil samples confirmed that availability of N and P are very unbalanced, with non-limiting N and highly limiting P. Due to P accumulation in plants grown in fertilized soil samples, values of N:P ratio fell by a factor ranging from 5 to 10 compared to control plants. Irrigation did not affect the N:P ratio significantly in either fertilized or control plants. Furthermore, no significant shift in N:P ratio was observed in P and F soil samples.

The diagnostic approach of vector analysis was also used to evaluate the effect of N and P nutrient on biomass production. The treatment P was used as a reference (100%) since soils at study site were limited with phosphorus nutrition. As shown in Fig. 4a, plants from C and CI treatments showed luxury consumption of N, with relatively high accumulation of N in shoot. Plants from PI and F treatments showed no vector because N accumulation was not significantly different from the reference P treatment. Plants from FI treatment showed a sufficiency response, as relative biomass and N contents were increased significantly without increasing relative N concentration in shoots. The vector nomogram for P nutrient (Fig. 4b) showed a significant depletion of relative P contents and a reduction in P concentration without a significant increase in the shoot biomass of control plants. These conditions indicate the re-translocation of P in control plants. The plants in PI treatment indicate a low level of luxury consumptions resulting into accumulation of P without a significant change in biomass. In FI treatment, P contents and plant biomass were increased significantly without significant change of P concentration as compared to reference treatment. Like N, these conditions also showed the sufficiency response, with steady-plant growth in FI treatment.

4. Discussion

4.1. Soil P fractions

Our study featured soil that had been subjected to varying histories including repeated application of phosphate mineral fertilizers, supplied either alone or together with other macronutrients. While these practices are becoming more common, our results suggest they can lead to major changes to soil P cycling. The history of P applications to the soil, applied annually in March (Trichet et al., 2008), 7 months before our experiments began, not only significantly augmented the soil pools of P_i but also P_o in both bicarbonate and hydroxide extracts. The dominant pools of the soil P (NaOH extractable P_i and P_o) showed that most of the applied P was found associated with Al and Fe, representing a less labile source of phosphate (Fontes and Weed, 1996; Barroso and Nahas, 2005) together with a significant amount of P also immobilized into organic P forms. Despite this, a sufficient amount of P_i was readily available (bicarbonate P_i) for plant uptake in fertilized soils. In contrast, unfertilized soils displayed a very low P availability, in agreement with the studies of Bonneau (1995), Trichet et al. (2009). Whereas, irrigation had almost no effect on $NaHCO_3$-P_i, it significantly increased NaOH-P_i in fertilized treatments. This could be due to the enhancement of several known mechanisms by increased water availability such as the dissolution of amorphous phosphorus compounds (Al and Fe phosphates) (Holford and Patrick, 1979) and/or an improved P diffusion (Barber, 1980). Interestingly, the combination of irrigation and complete fertilization resulted in the highest concentrations of P_o, especially after extraction with bicarbonate that could be due to increased microbial biomass (Ruppel and Makswitat, 1999). This hypothesis is consistent with our findings of FI soil featuring the highest levels of total N and C contents potentially reflecting an increase in microbial biomass. Also, Achat et al. (2012) found a positive correlation between microbial P and the gravimetric water content of mineral soil in the region "*des Landes de Gascogne*". Conversely, the lower concentrations of P_i (bicarbonate and hydroxide) in F soil could be due to the intensive development of forest floor annual vegetation (*Phytolacca* spp.) only observed in F plots (field observation). This understory, whose roots can contribute 90% of the total fine roots in these forest soils (Achat et al., 2008; Gonzalez et al., 2013), could significantly deplete the Pi pools from these soils, leading to lower P_i concentrations in F than in the P treatment.

The depletion of P_i in rhizoboxes with plants indicated that the roots and/or the rhizosphere microbial populations depending on plant C supply were able to take up P from soil. However, the

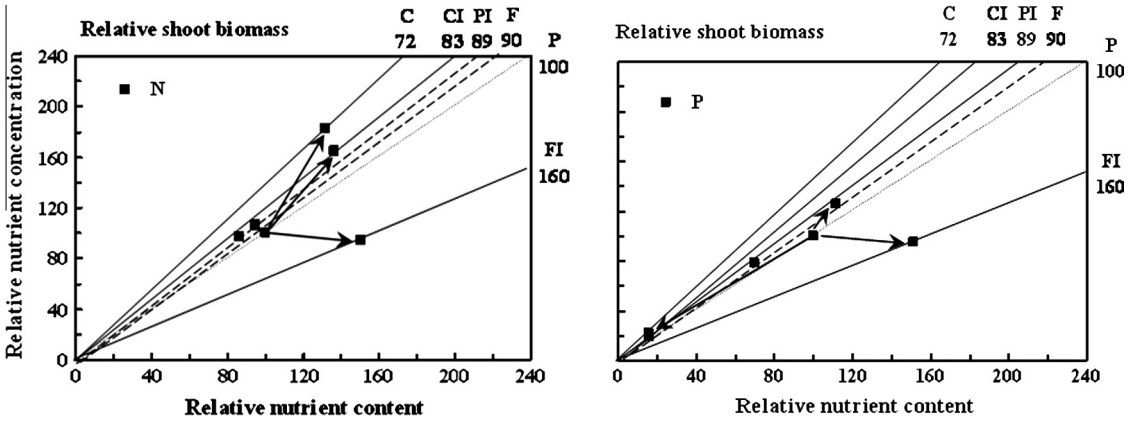

Fig. 4. Vector nomograms of changes in dry biomass, N (a) and P (b) contents as well as concentrations in the shoot of eight months old *P. pinaster* seedlings grown in soil samples, previously annually treated with fertilizers, with and without irrigation. The mean ($n = 8$) values of shoot dry biomass, N and P contents and concentration of P treatment was taken 100%. The mean values of other treatments relative to P treatment are plotted in vector nomogram for diagnosis. The arrowhead lines and complete lines represent significant effects whereas the dashed lines represent non-significant effect compared to the reference P treatment.

significant depletion of hydroxide extractable P_i in fertilized and irrigated treatments compared to fertilized and non-irrigated soils might be due to improved soil biogeochemical properties due to fine roots of understory (Achat et al., 2008) and their associated microbial populations, especially those able to reduce Fe(III)–O–PO_4 complexes, resulting into accelerated dissolution of Fe-associated P in soils (Chacon et al., 2006). In control treatments, the depletion of P_i was significant (*t*-test, $P < 0.05$) but in relative terms, it was very low when compared with fertilized treatments. As plants were not able to accumulate P, principally soil microorganisms might have taken up this small fraction of P_i in these P-deficient soils (Plante, 2007).

The depletion of $NaHCO_3$ extractable P_o in fertilized treatments with young seedlings could be the result of net microbial mineralization of P_o compounds due to low C/P ratio (C/P < 200) (Plante, 2007) and high microbial biomass. Remarkably, the depletion of bicarbonate and hydroxide extractable P_o in FI treatment was highly significant (*t*-test, $P > 0.001$). This effect could be attributed to the observed high phosphatase activity of ECM roots combined to a high fungal biomass. Indeed, greater fungal development was observed in the rhizosphere of *P. pinaster* grown in rhizoboxes with soils from the F treatment (Ali et al., 2009), and from the rhizosphere of *P. pinaster* subjected to the P treatment in the field (Bakker et al., 2009) than in the C treatment. The same effect was observed in *P. taeda* stands after N application (Parrent and Vilgalys, 2007). Conversely, in control treatments the high C/P ratio (C/P > 300) could have induced a net immobilization, as indicated by higher P_o concentration in rhizoboxes with plants. However, these trends are less obvious for NaOH extractable P_o, suggesting two different pools of P_o extracted by $NaHCO_3$ and NaOH as well as their microbial and plant availability.

4.2. Phosphatase activity of ectomycorrhizae and their molecular identification

Molecular identification of ectomycorrhizal morphotypes revealed that *R. luteolus* formed most of the ECM tips whatever the treatment. The results are in accordance with the findings of Ali et al. (2009). 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 soil (Massicotte et al., 1994; Colgan Iii and Claridge, 2002; Bruns et al., 2009). *R. luteolus* was also frequently found in ECM tips in field surveys of the same plots (unpublished data). The second species was identified as *S. brunnea* considered as a common contaminant in nurseries of mycorrhizal plants (Garcia-Montero et al., 2008); however its abundance was proportionally low. Therefore, it can be suggested that the phosphatase activity assayed in ECM morphotypes belonged to *R. luteolus*. The acid phosphatase activity of ECM assayed using *p*NPP as a substrate was different according to the soil treatments. Acid phosphatase activities were higher in ECM from the C treatment than those measured in ECM from fertilized treatments (P, F, PI, FI). These results are in agreement with the decrease of phosphatase activity systematically observed when high P availability is provided to ECM morphotypes grown in soil (Kroehler and Linkins, 1988; Antibus et al., 1992; Chen et al., 2002; Ali et al., 2009) or to ectomycorrhizal fungi grown in pure culture (Bousquet et al., 1986; Plassard et al., 2011). However, irrigation in CI soil also strongly decreased enzyme activity compared to the C soil and this decline could not be explained by high P availability. In contrast, additional application of nitrogen fertilizers coupled with irrigation (FI treatment) increased the phosphatase activity measured in ECM tips. These results are in agreement with prior studies investigating the ECM tips of *Pinus sylvestris* (Kieliszewskarokicka, 1992; Baar et al., 1997) and those of *Pinus thunbergii* (Taniguchi et al., 2008) displaying enhanced phosphatase activity when nitrogen was applied as fertilizers. Similarly, Ruppel and Makswitat (1999) and Wang et al. (2008) reported an increase in urease and phosphatase activity when nitrogen fertilizer application was coupled with irrigation. These results suggest that the levels of phosphatase activities are not only regulated by P availability.

4.3. Plant growth and nutrient uptake

Soils with complete fertilizer addition (F) had significantly higher plant biomass (+64%) when compared to control soil (C), and irrigation application to these complete fertilized soils (FI) further increased biomass production (+50%) compared to F alone treatment. Both irrigation and fertilization increased maximum total plant biomass up to 145% as compared to control soil samples. Our results are in agreement with those of Trichet et al. (2008), who reported a maximal increase in aboveground biomass of *P. pinaster* in the field in FI plots.

In non-irrigated control soils, P_i availability in soil (Fig. 1) was highly limited and was clearly observable in plant P analyses in tissues. Although, the highest phosphatase activity was in the C treatment it was not enough to increase net P accumulation. In this study, the concentrations of P in tissues were lower than the suggested critical limit (0.6–0.7 mg g^{-1}) at which photosynthetic limitation might have occurred in *P. pinaster* seedlings (Loustau et al., 1999a,b; Delzon et al., 2005). These limitations could have resulted in significantly lower biomass and re-translocation of P from shoots to roots in the seedlings of both C and CI treatments. In P fertilized treatments (P and PI), the accumulation of P in shoots as well as in roots was significantly higher than in C treatments, but the increase in the biomass of roots and shoots was not significant compared to control treatments. Indeed, application of P as well as irrigation (PI) spurred even higher accumulation of P in seedlings, with a significant increase in root biomass only. This suggested that plants showed excess of P accumulation without additional biomass production in both P and PI treatments. In the complete fertilization and irrigation (FI) treatment, plants showed greatest accumulation of N and P as well as biomass, even relative to the F treatment. Thus, the FI treatment likely increased nutrient availability, which resulted in high shoot growth as seen previously in the field (Trichet et al., 2008).

Interestingly, in the FI and F treatments the apparent significant differences of biomass and nutrient accumulation were due to irrigation even though water supply during our experiment was similar and uniform for all treatments. The suggestion then is that the observed effects were not due to the water availability itself during the growth period, but to some other factors occurring in the field, which were different only in FI treatment. This could be due to the difference in microbial population or their functioning and higher total N, total carbon and cation exchange capacity (Table 2) in the FI treatment. In this study, the observed significant increase in ECM phosphatase activity (Fig. 2) and significant mobilization of hydroxide and bicarbonate extractable P_o in FI treatment (Fig. 1) compared to other fertilized treatments could suggest evidence of high microbial activity in the FI treatment. In addition to microbial influences, high nutrient availability and irrigation could increase *P. pinaster* growth by decreasing stomatal limitations and increasing the photosynthetic capacity (Loustau et al., 1999b; Delzon et al., 2005).

N:P ratio and vector analysis was used as an other way to assess the effects of N and P nutrition on plant growth. The high N:P ratio of shoots and roots of *P. pinaster* seedling diagnosed deficiency of P in control treatments compared to fertilized treatments. The high values of N:P ratio both in shoot and roots are in agreement with previous studies reporting the deficiency of P at N:P ratio > 10 in foliage of wetland (Lockaby and Conner, 1999) and Scot pine (Prietzel and Stetter, 2010) forests. However N:P ratio could vary

strongly across plant species and age of seedlings. The more integrated approach of vector analysis, using shoot dry mass, nutrient concentration and content elaborated the diagnosis of both N and P (Timmer and Armstrong, 1989; Salifu and Timmer, 2003). Like N:P ratio, vector analysis also suggested high deficiency of P in control treatments, limiting plant growth. While in FI treatment the diagnosis of both N and P nutrients indicated the steady growth of seedlings, suggesting the beneficial role of irrigation coupled with complete fertilization.

5. Conclusion

The limiting P availability in control soils was confirmed by both analytical as well as diagnostic approaches. Similarly, the sufficiency and steady growth with significantly high biomass production in FI treatment was also confirmed. However, our results also showed that the P_o pool (bicarbonate and hydroxide extractable) in soil receiving irrigation and complete fertilization was the highest before plant growth and the more depleted after plant growth, compared to the treatments applied separately. These results suggest therefore that there is a strong positive interaction between irrigation and complete fertilization in these spodosols, resulting in a highly reactive organic P pool that might be used by *P. pinaster* and its associated ECM, which displayed significantly higher phosphatase activity than in other irrigated or fertilized treatments. In the future, studies determining the soil microbial composition and their activities in soil receiving different regimes of irrigation and fertilization should be helpful to establish the true roles of fertilizers, irrigation or soil microbial communities on plot fertility and plant productivity.

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