Role of phosphatase secreted by ectomycorrhizal fungi associated with maritime pine (Pinus pinaster) for the mobilization of phosphorus from podzols of Landes ecosystem

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(Montpellier SupAgro)

ECOLE DOCTORALE
Systèmes Intégrés en Biologie, Agronomie, Géosciences, Hydrosciences et Environnement
(SIBAGHE)

DOCTORAT

Discipline: Ecosystèmes

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ROLE DES PHOSPHATASES SECRETEES PAR LES CHAMPIGNONS ECTOMYCORHIZIENS ASSOCIES AU PIN MARITIME (*PINUS PINASTER*) DANS LA MOBILISATION DU PHOSPHORE DES PODZOLS DE L'ECOSYSTEME LANDAIS

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Date de soutenance, le 23 octobre 2009

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Rôle des phosphatases sécrétées par les champignons ectomycorhiziens associés au pin maritime (*Pinus pinaster*) dans la mobilisation du phosphore des podzols de l'écosystème landais.

Résumé

Le Pin maritime (*Pinus pinaster*) est largement planté dans le massif des Landes (sud-ouest de la France). Le phosphore (P) limite la croissance dans ces sols et l'apport de fertilisants P est commun pour l'alimentation des arbes en P. L'hypothèse centrale de ce travail est que l'activité phosphatase (Pase) sécrétée par les champignons ectomycorhiziens (ECM) associés à *P. pinaster* pourrait hydrolyser le P organique (Po) de fractions labiles et augmenter la disponibilité de P. Les relations entre les différentes fractions de P du sol, la croissance des arbres et leur nutrition minérale et l'activité Pase des ECM ont été étudiées au champ ou en conditions contrôlées. Les propriétés des arbres et du sol décrivent significativement les variations des fractions de P et de l'activité Pase au champ. Les variations saisonnières des pools de Po suggèrent que P est mobilisé au printemps et immobilisé en automne. Cependant, la croissance de *P. pinaster* est significativement augmentée par la fertilisation P. L'activité Pase dépend de multiples facteurs incluant l'âge des arbres, l'humidité du sol et la disponibilité de P. Des expériences en rhizotrons au laboratoire ont montré que l'activité Pase des ectomycorhizes est forte dans les sols non fertilisés et faible dans les sols fertilisés. Cependant, cette forte activité Pase n'est pas suffisante pour augmenter la biodisponibilité de P dans les sols non fertilisés. Une croissance équilibrée de *P. pinaster* a été obtenue dans des échantillons de sol ayant reçu une fertilisation complète NPK et irrigués au champ. Nous suggérons que la forte activité Pase mesurée dans ces conditions a pu améliorer la nutrition P des jeunes plantes.

***Mots-clés**: Phosphphore organique, Activité phosphatase, Champignons ectomycorhiziens, Disponibilité en P du sol, Rhizosphère, Fertilisation P*

Role of phosphatase secreted by ectomycorrhizal fungi associated with maritime pine (*Pinus pinaster*) for the mobilization of phosphorus from podzols of Landes ecosystem.

Abstract

Pinus pinaster is a tree species planted at large scale in soils of Landes located in southwest of France. Mineral nutrients, particularly phosphorus (P) are major growth limiting factors in these soils. Application of P fertilizers is a common practice to increase P availability. The central hypothesis of this work is that acid phosphatase secreted by ectomycorrhizal (ECM) fungi associated with *P. pinaster* could hydrolyse labile organic P (Po) fractions to increase P availability. Studies aiming at determining the relationships occurring among different P fractions in soils, tree growth, mineral nutrition and phosphatase activity of ECM morphotypes were carried out in the field or in controlled conditions. Results showed that plant as well as soil properties, significantly described the variations of both P fractions and acid phosphatase activity. Seasonal variation of Po fractions suggested mobilization of P in spring and immobilization in autumn. However, P was highly deficient throughout the ecosystem and growth of *P. pinaster* was significantly increased by P fertilization in field. Acid phosphatase activity was linked to multiple factors including trees age, soil water contents and P availability. Rhizobox experiments in laboratory showed high phosphatase activity in control soils and it was drastically decreased in fertilized plots. However, this high phosphatase activity was not enough to increase P availability in control soils. A steady growth response of *P. pinaster* was observed only in soil samples from the field with irrigated NPK treatment. It was suggested due to high phosphatase activity resulting to ameliorate P nutrition of young seedlings.

***Key words**: Organic phosphorus, Phosphatase activity, Ectomycorrhizal fungi, Soil P availability, Rhizosphere, P Fertilisation*

Remerciements

C’est à Mr Benoît JAILLARD, Directeur de l’ex-UMR Rhizosphère & Symbiose de l’INRA Montpellier que s’adressent en premier lieu mes remerciements pour m’avoir accepté au sein de son équipe.

Qu’il me soit permis d’exprimer ma reconnaissance à Mme Claude PLASSARD, Chargée de Recherches à l’INRA-Montpellier (UMR Eco & Sol), pour avoir dirigé mon travail en me faisant profiter de son savoir malgré ses nombreuses occupations. Ses critiques constructives et ses conseils m’ont aidé à progresser dans ce travail. Je la remercie pour son soutien et surtout pour la confiance qu’elle m’a témoignée depuis mon arrivée au laboratoire. Je lui exprime ma gratitude pour l’attention qu’elle m’a accordée tout au long de cette thèse.

Je remercie vivement et sincèrement Mr Jean GARBAYE et Mr Emmanuel FROSSARD pour l’honneur qu’ils me font en acceptant de participer au jury de cette thèse dont ils sont les rapporteurs**.**

J'exprime ma gratitude à Steven CRIQUET d'avoir accepté d'examiner ce travail.

Je n’aurais garde d’oublier Philippe HINSINGER, Directeur de Recherches à l’INRA-Montpellier (UMR Eco&sol), Christian MOREL, Ingénieur de Recherches à l’INRA de Bordeaux, Siobhan STAUNTON Directeur de recherches à INRA Montpellier et Roland MARMEISSE, de l’université de Lyon I, pour l’intérêt qu’ils ont manifesté dans ce travail. Je leur exprime ma gratitude pour la bienveillante attention qu’ils m’ont accordée tout au long de ma thèse. J’ai pu ainsi largement bénéficier de leur gentillesse, de leurs conseils et de leur grande compétence.

Je me dois de rendre hommage à Benoît CLOUTIER-HURTEAU de m’avoir guidé, conseillé et aidé pour l’aboutissement de cette thèse. J’ai pu apprécier non seulement sa gentillesse mais également sa participation et dévouement à une grande partie des analyses statistiques réalisées au cours de cette thèse. Je ne cesserai de le remercier.

Il existe des personnes que je ne saurai jamais assez remercier, ce sont les assistants-ingénieurs, techniciens et ACP de l’UMR Eco&Sol qui m’ont aidé dans la réalisation pratique de cette thèse: Myriam, Elvire, Didier, Lucien, Michael, Laurie, Gabrielle, Gérard, Catherine, et Helène. J’ai trouvé ici, l’occasion de leur exprimer ma gratitude et de leur dire Merci.

Je voudrais aussi remercier les personnes avec qui j’ai partagé des moments de travail acharné à trier des ectomycorhizes à Bordeaux ! Ce sont Antoine, Yves et Odile du LSTM, Jacques, Mark,

Etienne de l'INRA de Bordeaux. Je remercie aussi toutes les personnes qui nous ont abondamment pourvus en échantillons à trier et je pense à Pierre et à Laurent ainsi qu'aux techniciens de l'INRA Bordeaux sans qui nous n'aurions pas pu avoir le matériel.

Ce travail n'aurait pu être mené à son terme sans l'aide directe ou indirecte de nombre d'amis et collègues du laboratoire ou d'ailleurs. Je citerai plus particulièrement mon ami et collègue doctorant Julien LOUCHE avec qui j'ai partagé des moments agréables. Il a su me donner du courage pour affronter les longues journées passées au laboratoire. Qu'il reçoive à travers ces brefs remerciements l'expression de toute mon amitié.

Je citerai aussi Usman, Nicolas, Nora, Siham, George, Arshad, Bilal, Mustafa, Shafiq et tous mes compatriotes pakistanais. Je les remercie beaucoup et les assure de ma sincère amitié. C'est une chance pour moi de les avoir tous connus.

Je remercie également toute personne ayant contribué de prés ou de loin pour l'aboutissement de cette thèse.

Au moment de présenter ce travail, mes pensées vont à toute ma famille dont l'affection est pour moi un constant réconfort. Quoi que je dise, quoi que je fasse je ne saurais jamais assez les remercier.

Enfin, je remercie la « Commission de l'Education Supérieure » (Higher Education Commission, HEC) du gouvernement pakistanais pour le financement qu'il m'a accordé pour mes études ainsi que la Société Française d'Exploitation des Ressources Educatives (SFERE) pour la gestion administrative en France.

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Introduction générale

La forêt des «Landes des Gascogne », située dans le sud-ouest de la France, représente la plus grande surface forestière plantée en Europe. Le massif est en effet planté par une monoculture de pin maritime (*Pinus pinaster*, Ait in Soland), sur environ 1 million d'hectare (Inventaire Forestier National; www.ifn.fr). Les températures et les précipitations moyennes annuelles de la région varient entre 10-15 °C et 750-1250 mm respectivement. Les sols de ce massif sont issus d'une déposition éolienne de sable grossier, au cours du Pléistocène. Ils sont caractérisés par leur acidité, leur faible fertilité et leur richesse en matière organique. Ces caractéristiques les situent dans la catégorie « Spodosols Entique à Albique » (Food and Agriculture Organisation (FAO)/International Union of Soil Science 2006). Selon la profondeur de la nappe phréatique, un horizon spodique cimenté peut se former à une profondeur variant de 40 à 100 cm (Trichet et al. 1999).

La très vaste majorité des surfaces boisées sont plantées, et leur production a doublé au cours des quatre dernières décennies grâce aux pratiques de gestion forestière. Seule 6,5% de la superficie forestière totale de la France est occupée par le pin maritime. Malgré cette faible superficie, elle assure plus de 20% de la production française de bois de résineux (Bert and Danjon 2006). La fabrication de papier, de meubles et d'emballages, ainsi que toute l'industrie de transformation de bois qui sont installés dans la région, assurent plus de 34000 emplois (INSEE Aquitaine n° 160 Novembre 2006).

Dans le cas d'une production intensive il est nécessaire de développer une bonne stratégie de gestion pour maintenir une fertilité optimale de ces sols sableux, tout en ayant une production élevée et durable (Trichet et al. 1999). Dans cette optique de production, des essais ont été menés en appliquant une fertilisation azotée et phosphatée (Bonneau 1995). Toutefois, P reste l'élément nutritif majeur le plus limitant pour les forêts (Attiwill and Adams 1993; Aerts and Chapin 2000; Comerford et al. 2002). Actuellement, plus de 60% de la superficie forestière est traitée avec P minérale. Ce traitement est appliqué en une seule fois ou deux fois durant toute la durée de vie du peuplement. Cependant, l'effet de l'apport d'une fertilisation annuelle sur la productivité des arbres est étudié en parcelles expérimentales (Trichet et al. 2009).

La faible fertilité des sols en P peut avoir différentes causes qui sont la faible teneur en P total, l'association du P avec les argiles, la matière organique et les ions métalliques Al ou Fe sous forme d'oxydes insolubles, très peu disponibles pour les plantes. Environ 70-90% de P apporté par fertilisation peut se trouver indisponible, contribuant à une absence d'effet de la

fertilisation P apportée à la production des cultures (Holford 1997). Enfin, une grande proportion de P (Jusque à 80% du P total) peut se trouver immobilisé sous forme de molécules organiques diverses (Condron and Tiessen 2005; Achat 2009). L'application d'engrais minéraux est une pratique habituelle dans les systèmes agricoles mais il existe très peu de travaux sur l'application de fertilisants minéraux dans les systèmes forestiers. La productivité de *Picea abies* ((L.) H. Karst) et de *Pinus sylvestris* (L.) en Suède (Axelson and Axelson 1986), *Pinus taeda* (L.) aux USA (Albaugh et al. 1998), *Pinus radiata* (D. Don) en Australie (Waterworth et al. 2007) et de *P. pinaster* (Trichet et al. 2008) en France, a été augmentée en optimisant la disponibilité des éléments nutritifs. Trichet et al. (2009) ont récemment suggéré que la fertilisation dans les sols des Landes permet d'augmenter le volume cumulé de bois de 20 à 40% au cours d'un cycle de production ou de réduire de 4 à 5 ans le temps du cycle de production.

Bien que le rôle des fertilisants phosphatés dans l'amélioration de la productivité des sols pauvres en cet élément soit prouvé, leur utilisation suscite toujours des interrogations et ce pour plusieurs raisons: le coût élevé des fertilisants, source naturelle limitée et non renouvelable, leurs dangers potentiels sur la nature par le processus d'eutrophisation. Pour toutes ces raisons, l'être humain doit bien gérer sa fertilisation phosphatée.

En réponse à la déficience en P, les plantes mettent en œuvre différentes stratégies pour répondre à leurs exigences en P. Une première stratégie consiste à modifier le système racinaire en augmentant la surface d'échange. Une augmentation du nombre de racines fines a été observée dans les sols pauvres en P par rapport aux sols riches en cet élément, chez les conifères comme *Pinus taeda* (Albaugh et al. 1998, Maier and Kress 2000) et *P. pinaster* (Achat et al. 2008). Une autre stratégie consiste à développer un système d'acquisition plus efficace du P en sécrétant des acides organiques et des phosphatases dont le rôle est de mobiliser le P complexé du sol. Ces phénomènes sont accrus par les microorganismes de la rhizosphère et la symbiose avec des champignons mycorrhiziens (Casarin et al. 2004). La symbiose mycorhizienne est considérée comme la stratégie la plus répandue pour accroitre l'acquisition de P par les plantes (Smith et al. 2000). Les plantes ligneuses, gymnospermes et certaines angiospermes, qui croissent dans les régions boréales et tempérées forment des associations symbiotiques avec les champignons ectomycorhiziens (Marmeisse et al. 2004). Ces derniers peuvent augmenter la surface d'absorption des plantes mycorhizées, comparée à celle des plantes non mycorhizées, par le développement des hyphes dans le sol (Rousseau et al. 1994). Dans une plantation de Pin non fertilisée, âgée de 13 ans, Bakker et al. (2009) a signalé que la longueur des hyphes des ECM est 25 fois supérieure à celle des racines fines.

Ils représentent 96% de la longueur totale des structures absorbantes (racines fines + hyphe). En plus de l'augmentation de l'exploration du sol, les ECM libèrent des phosphatases dans les milieux de culture (Tibbett et al. 1998, Quiquampoix and Mousain 2005) ce qui pourrait avoir un rôle crucial dans la mobilisation du Pi à partir des pools de P organique des sols.

À ce jour, une étude approfondie a été effectuée pour caractériser la composition des pools de P dans l'écosystème landais (Achat, 2009). Cependant, les relations entre les pools de P dans cet écosystéme et les activités des phosphatases sécrétées par les ectomycorhizes associées à *P. pinaster* n'ont jamais été étudiées. Dans ce travail, nous avons conduit une étude dans différentes parcelles de Pin maritime choisies pour les modes de fertilisation en P variés et l'âge des plantations. Nous avons tout d'abord déterminé la taille des différents pools de P par les méthodes chimiques habituelles. Nous avons aussi comparé la taille du pool de P facilement disponible obtenu par extraction chimique avec les résultats obtenus par des approches plus sensibles et plus avancées tels que la cinétique des échanges isotopiques du P inorganique soluble dans le cadre d'une collaboration avec l'INRA de Bordeaux. Nous avons également déterminé l'activité de la phosphatase acide des ectomycorhizes associées aux racines de *P. pinaster* au champ et en chambre de culture. Nos résultats ont permis (i) d'evaluer la contribution réelle des fractions de Po dans les sols forestiers (ii) de déterminer la variabilité des phosphatases acides des ECMs en relation avec le statut P du sol (iii) de déterminer les fractions de P utilisées par les plantes mycorhizées et leur relation avec l'activité phosphatase.

General introduction

The forest in Landes of Gascogne are situated in southwest of France, and represent the largest planted forest in Europe. It is composed of monoculture of maritime pine (*Pinus pinaster* Ait in Soland), covering a surface area of ca 1 million hectare (Inventaire Forestier National; www.ifn.fr). The average annual temperature and rainfall of the region range between 10-15 °C and 750-1250 mm respectively. Soils are developed from a coarse sandy Aeolian parent material deposited in the Pleistocene and are characterized as acidic, unfertile and rich in organic matter. These can be classified as Entic to Albic spodosols (Food and Agriculture Organization (FAO)/International Union of Soil Science 2006). Depending on the depth of water table, the lenses of a cemented spodic horizon can occur between the depth of 40 and 100 cm in the soil (Trichet et al. 1999).

The whole range of forest is almost planted and in the last four decades, its production has doubled due to forest management practices. Although only 6.5 % of total forest area in France is occupied by *P. pinaster*, it produces over 20 % of total softwood in France (Bert and Danjon 2006). Large number of paper, furniture, packing, construction, transport and other wood processing industries are established in this region, producing over 34000 employments (INSEE Aquitaine n° 160 November 2006).

This intensive woodland production in sandy soils, with low mineral nutrition needs integrated management strategies, to maintain optimal soil fertility (Trichet et al. 1999) for high and durable forest production. In order to obtain high production various fertilizers trials have been conducted including both nitrogen and phosphorus applications (Bonneau 1995). However P remained major limiting nutrient in forest as well as crop lands (Attiwill and Adams, 1993; Aerts and Chapin 2000; Comerford et al. 2002). P fertilizers are applied in about 60 % of forested area of the region. The treatment of P is commonly applied once or twice during the rotation period of stand. However, the effect of annual fertilization on productivity of trees is also studied in an experimental plot (Trichet et al. 2009).

Low P concentration in soils could be due to its low total P contents in soil, high fixation capacity with clay, organic matter and metal ions (Al, Fe) forming insoluble oxides. Once the P fertilizers are applied, ca 70-90 % of P fertilizers is adsorbed and becomes unavailable without giving any immediate contribution to crop production (Holford 1997). Moreover, a major proportion of P is immobilized in the form of organic molecules. The studies have demonstrated that upto 80 % of total P could be found in organic forms (Condron and Tiessen 2005; Achat 2009). Application of mineral fertilizers in cropping systems has

been a common practice but in forest system the number of studies is limited. The productivity of *Picea abies* ((L.) H. Karst) and *Pinus sylvestris* (L.) in Sweeden (Axelson and Axelson 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, was increased by optimizing nutrient availability. Trichet et al. (2009) recently suggested that P application in the soils of Landes could increase 20-40 % cumulative volume at rotation age or reduce 4-5 years rotation age of *P. pinaster* stands.

Although P applications are proven important management practices to increase forest productivity in soils with P limitation, their use is interrogated due to high rising cost of phosphate fertilizers, limited and non-renewable natural resources, and its potential environmental hazard by degrading the surface water through the process of eutrophication. For all these reasons, one should develop better strategies of P fertilization and optimum Pi availability in soils.

Plants develop various strategies to fulfil their P requirements, such as modification of root structure by increasing the exchange surface. An increase in fine roots has been reported in low fertility soil as compared to high fertility soils in coniferous species such as *Pinus taeda* (Albaugh et al. 1998, Maier and Kress 2000) and *P. pinaster* (Achat et al. 2008). Plants also develop efficient P acquisition strategies at the level of their root system, by secreting phosphatase enzymes and organic acids which could mobilize unavailable P in soils. These processes are enhanced by rhizosphere micro-organisms and mycorrhizal symbiosis (Casarin 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). Woody plants, the gymnosperms and several angiosperms growing in boreal and temperate regions, have a symbiotic association with mycorrhizal fungi that form ectomycorrhizal (ECM) roots (Marmeisse et al. 2004). The ECM fungal species can augment the absorbing surface of mycorrhizal plants compared to non mycorrhizal plants due to 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 represented ca 96% of total length of absorbing structure (fine root + hyphae). In addition to increased soil exploration, ECM fungi have exhibited a release of phosphatase in culture medium (Tibbett et al. 1998, Quiquampoix and Mousain 2005) that could play a crucial role to mobilize Pi from organic P pools in soils.

To date, a single comprehensive study has been performed to characterise the composition of P pools in this ecosystem “Landais” (Achat 2009). However, relationships

between P pools in this ecosystem and phosphatase activity secreted by ectomycorrhizal (ECM) fungi symbiotically associated with *P. pinaster* have never been studied. In this work, we aimed at conducting such a comprehensive study, in a range of *Pinus pinaster* plots differing mainly by the management of P fertilization practices and age of plantations. First, we determined the size of different P pools, by routine chemical extraction methods in these soils. The results of plant available P (Olsen P) obtained were compared with those obtained in these soils using more sensitive and advance approach of isotopic exchange kinetics (Pi-isotopic dilution) in the frame of project collaboration with INRA Bordeaux. We also focused on the determination of acid phosphatase activity of ectomycorrhizae associated with *P. pinaster* trees in the field as well as with the young seedlings in growth chamber. Our results would help 1) to evaluate the actual contribution of organic P in total P present in forest soils 2) to determine the variability phosphatase activity of ectomycorrhizae in relation to soil P status 3) to determine the pools of P, from which ectomycorrhizal plants take up P and what is their relation to phosphatase activity.

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CHAPTER 1
Literature review

1. Biological importance of phosphorus

Phosphorus (P) is an element fundamental to life. It plays important role in the structure and functions of all living organisms. It is an integral part of cell components such as phospholipids, and nucleic acids. It is also involved in storing and transferring biochemical useful energy through the phosphate anhydride bond. A few examples of important biological compounds with P are shown in **Figure 1.1**, with their structural formula (Plante 2007). Agronomically, it is second major nutrient after nitrogen and a sufficient amount of P is necessary for plants in order to complete its life cycle.

2. General cycle of phosphorus

Chemically P is a highly active non metal element, which is present on earth as two major forms i.e. inorganic P (Pi) and organic P (Po). According to Walbridge (1991), P cycle in the soil is divided into two subsystems (figure 1.2). These are geochemical subsystem (Pi forms) and biological subsystem (Po forms). Both geochemical and biological subsystems are further characterised respectively into Pi and Po pools. The concentrations of Pi in soil solution depend on the soils properties, biogeochemical processes (mineralization/ immobilization, adsorption/desorption, precipitation/dissolution and ion exchange), soil parent material and environmental factors (biotic and abiotic).

The major input of P in soil is rock derived, which strictly restricts its input rate and makes it exhaustable (Walker and Syers 1976). Especially in highly weathered soils where P is no longer supplied from its parent material and dust deposition is the major input source in these cases (Vitousek 2004). At the same time, the mobility of P in soils is restricted and few losses of P through leaching can occur, where P in dissolved organic matter may lead to its depletion with the passage of time. Other potential losses of P from soils could be harvesting of vegetation and soil erosions. Phosphorus does not show large microbially mediated fluxes as it is not used as a primary energy source for microbial oxidation. Nevertheless, soil organisms are ultimately implicated in the cycling of soil P. They contribute in the solubilisation of inorganic, mineralization of organic and immobilization of available soil P. Collectively, both biological and geochemical cycles are strongly interlinked and significantly affect the rate of various biological or geochemical processes.

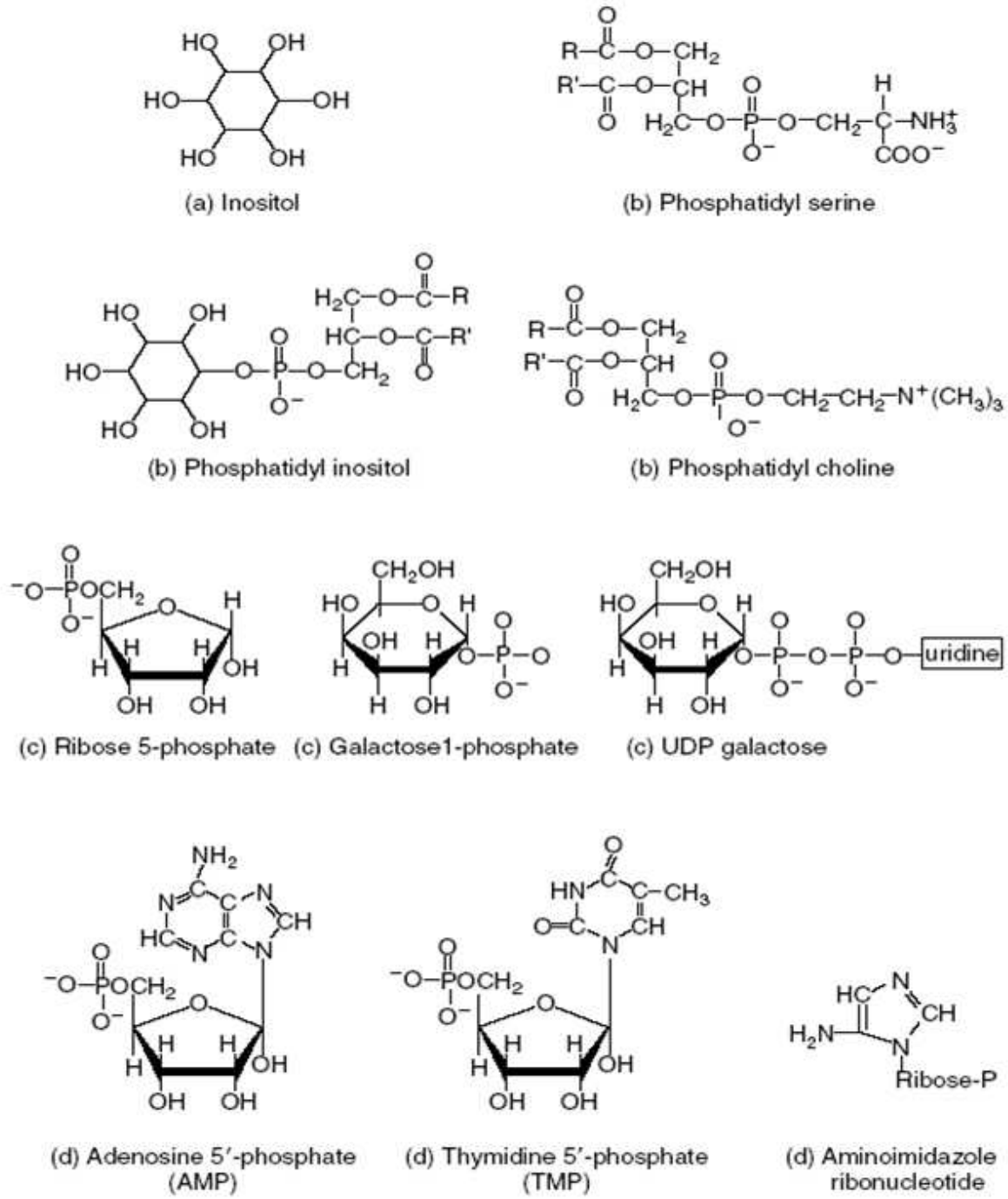


Figure 1.1: Organic phosphorus compounds. (a) Inositol. If given six phosphorus substitutions on a ring (C-P linkages), it becomes inositol hexaphosphate. Fewer substitutions yield 1-, 2-, 3-, 4-, or 5-phosphatidyl inositol phosphates. (b) Phosphoglycerides (C-O-P linkages), shown linked to serine, inositol or choline. (c) Phosphate sugars. (d) Nucleic acid components (adapted from Plante 2007).

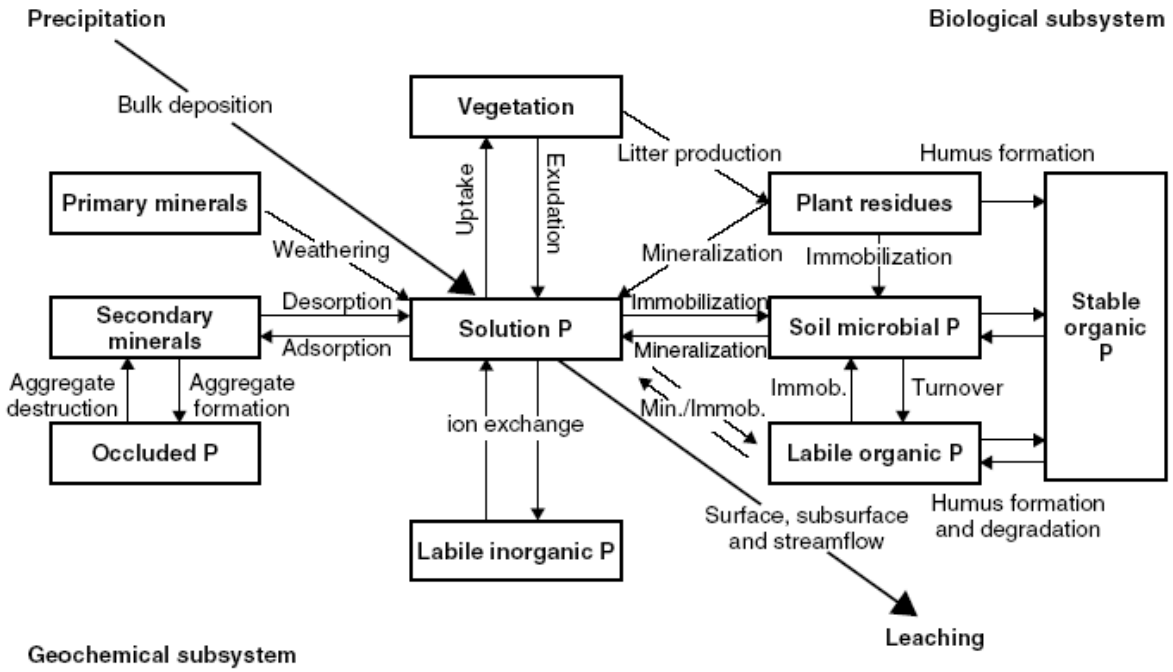


Figure 1.2: Schematic representation of phosphorus cycle consisting of a biological subcycle in the upper right and a geochemical subcycle in the lower left (Walbridge 1991).

3. Nature and forms of phosphorus in soil

Phosphorus in soil is present in the form of inorganic and organic phosphates and their derivatives. The primary mineral form of P is apatite, with the basic formula $Ca_{10}(PO_4)_6X_2$, where Ca can be substituted with Na, Mg and "X" is anion and it could be F^-**,** Cl^-, OH^- and CO_3^{2-} (Paul and Clark 1989, Frossard et al. 1995). The diverse combinations of cations and anions result in over 170 forms of inorganic P (Holford 1997, Plante 2007). Fluorapatite ($Ca_{10}(PO_4)_6F_2$) is the most common apatite mineral found at the earth.

Organic compounds of P are largely derived from plant residues, microbial cells, and metabolic products. The components of soil organic matter are often similar to the source materials (Bonneau 1995). Approximately 1% of the organic phosphorus is in the phospholipids fraction; 5 to 10% is in nucleic acids or degradation products, and up to 60% is in an inositol polyphosphate fraction (Turner et al. 2005a, Quiquampoix and Mousain 2005). A significant portion of the soil organic fraction is unidentified. Total P in soils has been estimated ranging from 200-1500 mg P kg^{-1} (Mengel and Kirkby, 1987) or 100-3000 mg P kg^{-1} (Frossard et al. 1995).

3.1. Inorganic form of phosphorus

The only form of P taken up by plants is orthophosphate (HPO_4^{2-}, $H_2PO_4^-$) (Ullrich-Eberius et al. 1984, Furihata et al. 1992), while P in soil solution rarely exceeds 10 µM (Bieleski, 1973; Marschner, 1995; Schachtman et al. 1998; Hinsinger 2001). The orthophosphate ions are mostly associated with soil minerals like clays, oxides of Fe and Al, and carbonates forming mineral phosphates. Depending upon the availability of mineral phosphates as plant nutrient, it is classified into three pools. The dissolved phosphates in soil solution, the phosphates adsorbed at the surface of mineral easily mobilized (labile pool) in soil solution during plant growth and the phosphates that are strongly adsorbed (non labile pool) on the mineral surfaces (Mengel and Kirkby 1987).

The presence of orthophosphate (HPO_4^{2-}, $H_2PO_4^-$) ions and their relative concentration in solution is controlled by soil pH (Lindsay 1979, Barber 1984, Mengel and Kirkby 1987). **Table 1.1** gives the three reactions of dissociation of phosphoric acid and the values of acid dissociation constant (pKa) indicate that HPO_4^{2-} and $H_2PO_4^-$ ions are the dominant forms between pH 4-7, pH values occurring in most of the soils. pKa is the negative logarithm of acid dissociation constant (Ka). The Ka and pKa of an acid are calculated by the following equation:

$$-\log [Ka] = -\log [H^+] - \log [A^-]/[AH] \longrightarrow pKa = pH - \log [A^-]/[AH]$$

Where, A in the equation represents the anion associated in an acid. Both mono- and di-orthophosphates are present in equilibrium at neutral pH. The pH value of soil above or below neutral indicates the abundance of HPO_4^{2-} or $H_2PO_4^-$ ions species respectively in soils solution.

Table 1.1: Dissociation constants of orthophosphoric acid dissociation reactions (Lindsay 1979).

Dissociation	Equilibrium reaction	pKa
1	$H_3PO_4 \Leftrightarrow H^+ + H_2PO_4^-$	2.15
2	$H_2PO_4^- \Leftrightarrow H^+ + HPO_4^{2-}$	7.20
3	$HPO_4^{2-} \Leftrightarrow H^+ + PO_4^{3-}$	12.35

Orthophosphate ions are capable of making association with other chemicals in the soil to form complex ions called ligands. They have strong ability to make complexes with Ca and Mg in alkaline soils and Fe and Al in acidic soils. In alkaline soils Pi is converted into insoluble calcium phosphates, but in acid soils it is converted into Fe and Al insoluble

phosphates. Hence, in both cases, the concentrations of soil solution Pi remain very low. Nevertheless, the speciation of Pi depends upon the presence of other competitor ligands particularly organic anions (Hinsinger 2001). More then one half of the inorganic P is associated with soil solid phase and at solid-liquid interface numerous physicochemical processes (adsorption/desorption, precipitation/dissociation) play crucial role in the regulation of Pi concentrations in soil solution (Frossard et al 2000, Hinsinger 2001, Morel 2002).

On the other hand, fertilizers are applied to increase the availability of P in soil solution. Well known chemical forms of phosphorus fertilizers are ammonium or calcium phosphates. Here again most of the P applied as fertilizers (ca 80 %) is rendered unavailable for plant uptake due to its fixation with cations (Al and Fe) and minerals at exchange sites (Fontes and Weed 1996). This fixation of applied P with cations is also converted into precipitated mineral phosphates. However, the fixation and availability of P after addition of fertilizers depend on the type of fertilizer added (mono-calcium phosphates, di-calcium phosphates and tri-calcium phosphates etc), humidity, temperature and time of application (Barber 1984).

3.1.1. Factors affecting P availability in soil

3.1.1.1. Adsorption and desorption of P ions

The adsorption corresponds to the passage of phosphate ions from soil solution to solid phase through electrostatic or ligand exchange associations. The amount of P adsorbed at the constituents of soil surface like oxides and hydroxides of Fe and Al is variable. These oxides and hydroxides have different surface polarities, which are subjected to soil pH and are capable of holding phosphoric ions by electrostatic action or the formation of ligands with other chemical identities producing variable charges. The phosphate ions are negatively charged, so they are adsorbed at the positive charged sites including clay minerals and oxides of Fe and Al in case of acid soil (Barrow, 1987; Hinsinger 2001). The P ions are adsorbed, either directly at the broken edges of clay minerals or indirectly through polyvalent cations like Fe and Al. Since the adsorption is surface phenomenon, it often takes place at the interface of solid and aqueous phases.

Soil pH plays an important role in the adsorption and desorption of phosphates. The charge on the surface of Fe and Al oxides vary with the pH (Strauss et al. 1997). The high point of zero charge for metal oxides (generally between pH 7 and 10) makes them positively charged in most of the soil pH ranges. In acid soil (pH 4), the metal oxides possess strong

positive charges which result into strong adsorption of negatively charged (mono-valent or divalent) orthophosphates (Strauss et al. 1997; Hinsinger 2001). Thus adsorption of P ions to the surface of Fe and Al oxides at low pH could result in a stronger retention of P, ultimately reducing the Pi availability and it mobility. In contrast, desorption of Pi occurs by reducing the concentrations of P ions in soil solution and increasing the concentrations of competitor anions. In both the cases, adsorption-desorption equilibrium shifts towards the desorption (Hinsinger 2001).

3.1.1.2. Precipitation and dissolution of P ions

Phosphate ions are able to combine with numerous elements to form mineral phosphates. Principally, the precipitation takes place with Fe, Al and Ca (Lindsay et al. 1989). In calcareous soils with neutral or alkaline pH, the availability of P is reduced by the formation of insoluble calcium phosphate (Sample et al. 1980). The precipitation of ion phosphates is increased either with increasing pH or Ca concentration. But, if soils are strongly acidic (pH less then 4.5), the abundance of Fe and Al induces formation of insoluble or sparingly soluble Fe and Al phosphates. The Fe and Al phosphates are initially amorphous and bear large surface area per gram of soil. The amorphous forms are converted to crystalline forms with the passage of time. The increasing crystalinity reduce surface area, which is associated to decrease Fe and Al phosphates minerals. Similarly, very high pH (pH > 8) on one side increases precipitation but on the other hand it could limit adsorption of phosphates (Mengel and Kirkby 1987) by decreasing surface area through increasing crystalinity at very high pH in alkaline soisl.

3.1.1.3. Organic matter and sorption of P

In addition to phosphorus mineralization and immobilization, it appears that organic matter has indirect, but sometimes inconsistent effects on soil phosphorus reactions. Lopez-Hernandez and Burnham (1974) reported a positive correlation between humification (the process of formation of humus i.e. partially decayed organic matter) and phosphorus-sorption capacity. In contrast, Wild (1950) concluded that the phosphorus-sorption capacity of organic matter is negligible. It is often observed that organic matter hinders phosphorus sorption, thereby enhancing availability. Humic acids and other organic acids often reduce phosphorus adsorption through the formation of complexes (chelates) with Fe, Al, Ca, and other cations that react with phosphorus (Bradely and Sieling 1953; Nagaraja et al. 1970; Holford and Mattingly 1975).

3.1.1.4. Characterization of P pools in general agronomic terms

Phosphorus availability has been often characterized in general terms (a) as solution phosphorus, often known as the intensity factor, (b) as readily available or labile phosphorus, often known as the quantity factor, and (c) as non-labile phosphorus (Sanchez 2007). The labile fraction might include easily mineralize-able organic phosphorus, low-energy sorbed phosphorus, and soluble mineral phosphorus. The non-labile fraction might include resistant organic phosphorus, high-energy sorbed phosphorus, and relatively insoluble phosphate minerals. As plants take up phosphorus from the solution, it is replenished from the labile fraction, which in turn is more slowly replenished by the non-labile fraction. The soil buffering capacity, indicator of the capacity factor, governs the distribution of phosphorus among these pools (Sanchez 2007). In other study (Frossard et al. 2000) the intensity, quality and capacity factors of Pi availability have been defined with reference to Pi estimated by method of ^{32}P-isotopic dilution. The intensity factor is provided by concentration Pi in soil solution, the quantity factor is provided by the amount of isotopically exchangeable Pi and capacity factor is provided by the rate of disappearance of radioactive Pi from soil solution.

3.2. Organic forms of phosphorus

The compounds with hydrocarbon bonds are named as organic compounds, and the presence of P in these compounds linked either with C or H signifies organic phosphorus. The list of organic P compounds is still undefined because a significant number of organic P compounds are uncharacterised (Turner et al. 2005a, Turner et al. 2005b). Organic compounds of P are largely derived from plant residues, microbial cells, and metabolic products. The most common forms of organic P in soil with their structure and origin are summarised in **Figure 1.3**. The phospholipids present in soil as choline result from hydrolysis of lecithin, similarly nucleic acids that enter the soil are degraded rapidly by soil microorganisms (Anderson 1967, Ko and Hora 1970). The constituent parts of nucleic acids are identifiable in hydrolysates of soil extracts. These include cytosine, adenine, guanine, uracil, hypoxanthine, and xanthine. The last two are decomposition products of guanine and adenine. The more stable, and therefore more abundant, constituents of the organic phosphorus fraction are the inositol phosphates. Inositol polyphosphates are usually associated with high-molecular-weight molecules extracted from the soil, suggesting that they are important component of humus (Omotoso and Wild 1970, Steward and Tate 1971). While, the sizes of the organic P pools in soil generally occur in the order inositol phosphate > polymer organic phosphate > nucleic acid P > phospholipid P (Turner et al. 2005a,

Quiquampoix and Mousain 2005), the concentrations of these pools within the soil biota occur in the reverse order. Moreover, the proportion of these compounds may be higher in forest soils (Attiwill and Adams 1993, Turner et al. 2005a). Organic compounds containing P, particularly inositol phosphates, are sparingly available because they are adsorbed by clay minerals and form complexes or precipitates with oxides of Fe and Al in acid soil or Ca and Mg in alkaline soils. Celi et al (1999, 2003) reported the adsorption of *myo*-inositol hexakisphosphate (phytate) on pure iron oxides and phyllosilicates. However, the adsorption of organic phosphorus may take few hours to days (Barrow and Shaw 1975) to attain equilibrium. Desorption of inorganic and organic phosphorus was found to increase with pH (Celi et al. 2003, Martin et al. 2004), with the percentage of phosphorus saturation (He et al. 1994, Martin et al., 2002) and in the presence of competing ligands such as citrate, oxalate or carbonate (Martin et al. 2004).

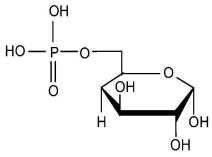
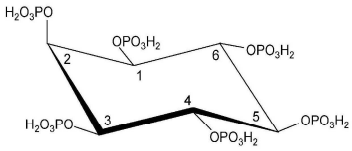
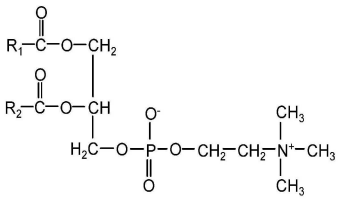
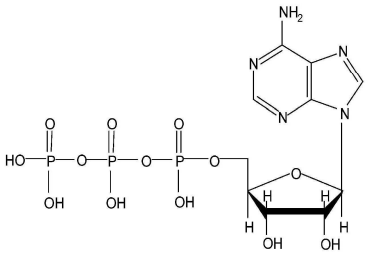
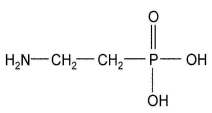
Functional class	Example compound	Structure	Comments
Phosphate monoester	D-Glucose 6-phosphate		Common sugar phosphate. Other sugar phosphates include glucose 1-phosphate and fructose 6-phosphate
Phosphate monoester	*myo*-Inositol hexakisphosphate (phytic acid)		Dominant organic phosphorus compound in plant seeds and most soils, where it is strongly stabilised. Regarded as relatively recalcitrant in the environment
Phosphate diester	L-α-Phosphatidyl choline (lecithin)		Phospholipid commonly found in plants and microorganisms. One of the two common phospholipids in soil
Organic polyphosphate	Adenosine 5′-triphosphate		Involved in biochemical energy transfer. Uridine, cytidine, guanosine and thymine triphosphates are also common in biological systems
Phosphonate	2-Aminoethyl phosphonic acid		Most common naturally occurring phosphonate, found in a variety of organisms and cold, acidic soils

Figure 1.3: Common soil organic phosphorus compounds (adapted from Turner et al. 2005a).

The extent and the rate of adsorption of organic phosphorus in soils, sediments, and onto their minerals depend on the structure of the organic phosphorus compound, notably in terms of the number of phosphorus groups and molecular size. Among the different compounds, inositol phosphates are sorbed to clay minerals to a greater extent than nucleic acids, phospholipids and simple sugar phosphates (McKercher and Anderson 1989, Leytem et al. 2002). In this way, the highly sorbed organic compounds are more stable and their enzymatic hydrolysis is limited (Celi and Barberies 2005, Turner et al. 2005a).

Phosphorus undergoes mineralization and immobilization. The net phosphorus release depends on the phosphorus concentration of the residues undergoing decay and the phosphorus requirements of the active microbial population (Alexander 1977). Studies have shown that organic phosphorus is much more mobile in soils than inorganic sources (Hannapel et al. 1964) and some losses from soil in dissolved organic form have been observed in highly weathered soils (Vitousek 2004).

3.2.1. Microorganisms and transformation of P

Normally, soils contain a wide range of microorganisms capable of releasing inorganic orthophosphate from organic phosphates of plant and microbial origin (Alexander 1977; Cosgrove 1977). Conditions that favour the activities of these organisms, such as warm temperatures and near-neutral pH values also favour mineralization of organic phosphorus in soils (Alexander 1977; Anderson 1975). The enzymes involved in the cleavage of phosphate from organic substrates are collectively called phosphatases. Microorganisms produce a variety of phosphatases that mineralize organic phosphate (Feder 1973). The phosphatase group of enzymes includes phytase enzymes that catalyze the release of phosphate from phytate and the nuclease enzymes that hydrolyse phosphate from nucleic acids. About 70-80 % of enzymes could be released by microbial populations. It includes bacteria such as *Bacillus spp*., *Serratia spp*., *Proteus spp*., *Arthrobacter spp*., and *Streptomyces spp*., and fungi as *Aspergillus spp*., *Penicillium spp*., *Rhizopus spp*., and *Cunninghamella spp* (Plante 2007). Phosphorus released to the soil solution from the mineralization of organic matter might be taken up by the microbial population, by growing plants, transferred to the soil inorganic pool, sorbed to mineral surfaces or less likely lost by leaching and runoff (**Figure 1.2**).

3.2.2. Microbial solubilisation of P

It is fact that phosphorus is highly immobile in the soil, and fertilizers amendments for sustainable plant growth are rendered less effective due to sorption (adsorption/precipitation)

of P and high cost of phosphate fertilizers. Traditionally, P fertilizers are produced by chemical processing of ores of mineral phosphate, which is costly and environment non friendly approach. However, few studies have suggested that the microbial solubilisation of rock phosphate could be a useful alternative (Whitelaw 2000). About 40 million tons of rock phosphate deposits in India could be used as a source of P. The mixing of rock phosphate with plant residues in compost or in some cases the use of P-solubilising bacteria to increase biologically P availability. The development of agricultural inoculants has been difficult and knowledge of the genetics of phosphate solubilisation is still sparse (Rodriguez et al. 2006). Phosphate-solubilising microorganisms are suspected to convert the insoluble rock phosphates into soluble forms through the processes of acidification, chelation, and exchange reactions, but the detailed mechanisms are still unresolved. Carbonic acid and HCO_3^-, derived from respiratory CO_2 are of prime importance in the weathering of soil minerals, but there is poor correlation between CO_2 levels and dissolution of apatite. Illmer and Schinner (1995) showed that *Aspergillus niger* produced citrate, oxalate, and gluconate. They suggested that organic acid production may be an important mechanism for solubilising Al-phosphates, but it was not the only effective mechanism. They found that other organisms, such as *Penicillium aurantiongriseum* and *Pseudomonas* sp. were effective at solubilising Al- or Ca-phosphates without producing organic acids. The release of protons, associated with respiration or NH_4 assimilation is also suggested as a responsible mechanism in this scenario. Organic acids produced in rhizosphere by plant roots and associated microorganisms may act as chelating agents. These organic chelates form complexes with Ca, Fe, or Al, thereby releasing the phosphates to solution. Presently it is impossible to select any given mechanism from the alternatives, and some researchers have questioned whether sufficient acidity or chelating agents can be generated by microbes, to appreciably affect P solubility.

3.2.3. Microbial immobilization of P

Soil microorganisms can cause fixation or immobilization of P in the soil either by promoting the formation of inorganic precipitates or by assimilation into constituent cellular or intracellular polyphosphate granules. In soils immobilization through cells by the fixation of Al, Fe and Ca has been observed. Phosphorite (rock phosphate) precipitation is favoured indirectly by microorganisms, by making available reactive phosphate or calcium, or by creating environmental conditions that help phosphate precipitation. The intensity of immobilization of P is affected by the C: P ratio of the decomposing organic materials and P availability in solution. If insufficient P is available in the substrate for assimilation of the

substrate C, inorganic P from the soil solution will be used and net immobilization occurs. In general, C:P < 200 may result in net mineralization, C:P < 300 results in net immobilization, and C:P ratios between 200 and 300 result in little net change in soluble P concentrations (Plante 2007).

4. Estimation or characterization of soil phosphorus

4.1. Inorganic phosphorus

Crop response and total P contents of soils are often poorly correlated, because P availability could be very low (Hinsinger 2001) even if total P contents are very high and vice versa. This leads to the development of P extraction methods representative of soil type or the pool of P available for plant uptake. These methods are based on the type of reaction taking place to remove P from solid phase. Four basic reactions by which P is removed from solid phase are: 1) dissolving action of acid, 2) anion replacement to enhance desorption, 3) complaxing the cation binding P, and 4) hydrolysis of cations binding P. Brief description of soil P extraction methods have been summarised in **table 1.2.**

4.2. Organic phosphorus

Despite the importance of soil organic phosphorus, its chemical nature and dynamics remain poorly understood. The various organic compounds in soil extracts can be separated by different techniques. These include mass spectroscopy, nuclear magnetic resonance spectroscopy (NMR) (Cade-Menun and Preston 1996), high pressure liquid chromatography (HPLC) and enzymatic hydrolysis like phosphatase and phytase. The last one is useful tool to separate extractable organic phosphorus into functional classes based on susceptibility to enzymatic cleavage (Pant et al. 1996, Turner et al. 2003, Hayes et al. 2000). However, like inorganic phosphorus, the extraction of organic P is complex and is separated into five broad categories depending on the objective of study (Turner et al. 2005a).

4.2.1. Quantitative protocol to extract total organic P from soils.

There are several methods that can be used to assy total organic phosphorus (Po) from the soil. These methods are important because there is no direct method which determines the total organic phosphorus in soil *"in situ"*. It also means that it is impossible to estimate accurately the efficiency of quantitative extraction.

Table 1.2: Common chemical extractants used in various soil P tests

Name of Soil P test	Extractant	Form of P extracted	Reference
AB-DPTA	1M NH_4HCO_3 + 0.005 M DPTA, pH 5	Ca, Fe and Al-bound P	Soltanpour and Schwab 1977
Bray I	0.025 M HCl + 0.03 M NH_4F	Acid soluble P form especially Fe and Al-phosphates	Bray and Kurtz 1945
Bray II	0.1 M HCl + 0.03 M NH_4F	Acid soluble P form especially Fe and Al-phosphates	Bray and Kurtz 1945
Mehlich 1	0.05 M HCl + 0.0125 M H_2SO_4	Acid soluble P Fe and Al-phosphates and P on colloidal surface	Mehlich 1953
Mehlich 3	0.015 M NH_4F + 0.2 M CH_3COOH + 0.025 M NH_4NO_3 + 0.013 M HNO_3	Acid soluble P Fe and Al-phosphates and extraction of micronutrients in the same extract	Mehlich 1984
Morgan	0.54 M HOAc + 7 M NaOAc, pH 4	P from weak complexes with polyvalent metal ions	Morgan 1941
Truog	0.001 M H_2SO_4 + $(NH4)_2SO_4$, pH 3	Sulphate ion dissolve Fe and Al-phosphates and P on colloidal surface	Truog 1930
Olsen	0.5 M $NaHCO_3$, pH 8.5	Remove Ca bound P and P bound with Fe-oxides surface	Olsen et al. 1954
Citric acid	1 % citric acid	P from weak complexes of polyvalent metal ions	Dyer 1894
EDTA	0.02 M Na_2-EDTA	Remove chelated P	Ahmed and Islam 1972
AER	Anion exchange resin (AER)	Anion (Cl^{-1}, HCO_3^{-1}) exchangeable P	Sharpley 2000
IIP	Fe-oxides impregnated paper (IIP)	Displace Pi located at solid phase	Chardon 2000
Sequential extraction	Resin, 0.5 M $NaHCO_3$, 0.1 M NaOH, 1 M HCl, 18 M H_2SO_4 + H_2O_2	Successive remval of different pools of P in the same soil samle	Tiessen et al. 1984
Water	Water	Soil solution P	Forsee 1942
$CaCl_2$	0.01 M $CaCl_2$	Bio-available P	Houba et al. 1990

The alkaline solvent like NaOH is supposed to be the most effective reagent for the extraction of organic phosphorus (Anderson 1967). Similarly calcination (550°C) and extraction of phosphorus with dilute sulphuric acid before and after calcinations is used to estimate total organic P (Saunders and Williams 1955). However, Po is overestimated with ignition methods by increasing the solubility of inorganic phosphates (Williams et al. 1970) following ignition. Similarly, acid extraction of non-ignited samples rarely extracts all inorganic P, which can result into overestimation of organic phosphorus.

4.2.2. Sequential extraction for separating Po into discrete pools based on relative solubility

Sequential extraction protocols are developed to obtain supplementary information on the nature of soil phosphorus. A single sample of soil is subjected to increasingly stronger solvents. In this way P is separated into fractions based on chemical solubility (Bowman and Cole 1978, Hedley et al. 1982). The Po estimated in various soil extracts used in sequential procedure can be characterised into pools of P with agronomic relevance (Labile, moderately labile and non labile etc) (Hedley et al. 1982). The individual Po pools are also added to get total Po concentrations in soil. However, the information obtained by sequential extraction should be treated carefully when describing the pools of phosphorus (Hedley et al. 1982). The chemical nature of the organic phosphorus within the operationally defined factions is understood poorly, and specific groups of compounds are probably present in more than one fraction. Fractions (inorganic or organic) that are bio-available in one soil may not be bio-available in the other soil (Frossard et al. 2000).

4.2.3. Single-step method for the extraction of Po used afterward for suitable speciation

The extractant, which causes minimum chemical and structural alteration and maximum recovery, is believed ideal extractant. In true sense, most of the protocols make use of strong acid or base for the extraction of Po from soil, which unavoidably change the chemical nature of at least some Po compounds (Turner et al. 2005a). The choice of the post speciation procedure may change the choice of the extractant. The speciation of Po by NMR is commonly conducted at high pH for optimum spectral resolution, so alkaline extraction (NaOH) is very often used to extract Po from soils (Newman and Tate 1980). In this way the choice of extractant could influence the recovery of Po as well as the composition of extracted compounds (Cade-Menun and Preston 1996).

4.2.4. Compound specific extractions of soil organic P

Various methods are practiced to estimate specific Po compounds in soil. These make use of specific reagent which extracts particular class of compounds (Anderson et al. 1980) like phospholipids (Kowalenko and McKercher 1970), sugar phosphates (Mueller-Harvey and Wild 1987), inositol phosphates (McKercher and Anderson 1968), adenosine tri-phosphates (Brookes et al. 1987), nucleic acids and nucleotides (Anderson 1967). These procedures are mostly laborious and are often limited by incomplete extraction or post extraction difficulties (Turner et al. 2002).

4.2.5. Extraction of organic P pools with biological or environmental relevance

P estimated with bicarbonate solution and with isotopic dilution is used to estimate potentially plant available phosphorus. Similarly water soluble organic P estimated the mobility of P in environment (Turner and Haygarth 2001). Although, a very little knowledge is available about the compounds dissolved in water, they can be characterised by phosphatase hydrolysis (Shand and Smith 1997, Turner et al. 2002). The knowledge is lacking about the bioavailability of bicarbonate extractable Po, however the hydrolysis of bicarbonate extractable Po by phosphatase has shown both large (Turner et al. 2003) and small (Hayes et al. 2000) proportions of hydrolysable compounds. Thien and Myers (1992) reported an increase in microbial biomass by the addition of labile substrate carbon. This increase in microbial biomass was related to increase bicarbonate extractable Po. Hayes et al (2000) used 50 mM citric acid at pH 2.3 to estimate plant available Po in Australian soils, with speciation of extracted compound by phosphatase hydrolysis. A large proportion of Po was hydrolysed by phosphomonoesterase and phytase. However, only a small amount of Po extracted by water and bicarbonate was hydrolysed by these enzymes. The secretion of organic anions like oxalate citrate and malate etc, may chelate the metals like Al and solubilise associated organic phosphorus in soil (Hocking 2001).

5. Phosphatase activity

Phosphatase activity plays a vital role to increase the mobility and bioavailability of P in soils. Their role is more important in rhizosphere than in bulk soils (Williamson and Alexander 1975). Organic phosphorus in the soil is hydrolysed by the secretion of phosphatases into the soil (Abdalla 1994, Turner and Haygarth 2005). The secretion of phosphatase depends upon the availability of phosphorus and the demand of P either by plant or microorganisms (Abdalla 1994, Chen et al. 2003a, b) Under deficient P condition the phosphatase activity is higher compared to P sufficient conditions, both in culture medium (Bousquet et al. 1986) as well as in soils (Kroehler and Linkins. 1988, Antibus et al. 1992, Chen et al. 2002). These enzymes are commonly subjected to inhibition, biodegradation and stabilization processes in soil. As soil contains clay, heavy metals and organic carbon, the fixation and humification of these enzymes make it difficult to predict their fate and regulation in soils (Sinsabaugh 1994, Dick et al. 2000, Kelleher et al. 2004, George et al. 2005). Chen et al. (2000) reported higher phosphomonoestrase and phosphodiesterase activities in grassland ecosystem than in forest ecosystem. They related these low activities with low organic, microbial biomass and soil pH in forest soils as compared to grassland soils.

The ability of phosphatase activity to hydrolyse organic P is reported different under different conditions. Hence the relation between phosphatase activity and the mineralization of organic P is not well established (Condron and Tiessen 2005). However, a paired site study conducted by Chen et al. (2002) in glasshouse showed that alkaline phosphatase and phosphodiesterase activity was correlated with depletion of NaOH extractable organic P in rhizosphere of *Pinus radiata* compared with ryegrass. Liu et al. (2004, 2005) also observed that the depletion of NaOH extractable organic P was related to acid phosphatase activities in the rhizosphere of *Pinus radiata* seedlings. Furthermore, the phosphatase activity of mycorrhizal roots is reported higher than the activity of roots without mycorrhizal associations (Perez-Moreno and Read 2000). These mycorrhizal associations result into hyphal extension which may extend from few mm to cm, and the phosphatase activity is positively correlated with the hypal length (Haussling and Marschner 1989)

The increased phosphatase activity in the rhizosphere of radiata pine has been associated with the high microbial and root activities (Chen et al. 2002, Liu et al. 2004; 2005, Chen et al. 2006). However, the relation could be variable from one ecosystem to other ecosystem. The variations of activities (microbial and root) were less consistent in field experiments (Chen et al. 2000, 2003a). The reduced enzyme activity in bulk soil could be due to dilution effect i.e. low root activities in bulk soil compared to rhizosphere soils. In a glasshouse experiment, Chen et al. (2003b) reported that root surface phosphatase activities were up to 13 times higher in *Pinus radiata* than in ryegrass. Together with the nature of low mobility of P in soil (Bar-Yosef 1996) and low microbial and root activity in bulk soil, it is supposed that root phosphatase (rhizosphere soil) rather than soil phosphatase enzyme (bulk soil) might play a more important role in the mineralization of soil organic P and consequently P uptake by plants (Badalucco and Kuikman 2001).

6. Low molecular weight organic acids

Most of the soil organic P is not in soil solution and ultimately it is unavailable for plant as well as for enzymatic hydrolysis. The low molecular organic acids secreted by microorganisms and plant roots could solubilise organic P and increase the availability of Po for enzymatic hydrolysis. Indeed, it has been suggested that it is not enzymatic activity that determines the mineralization of organic P but this is the solubility of organic P that determines its mineralization in soils (Adams and Pate 1992). The low molecular organic acids such as oxalic, malic and citric acids are largely secreted into rhizosphere (Jones 1998, Ström et al. 2005). These organic acids have been shown to play a significant role in

increasing the availability of sparingly soluble inorganic P compounds. The increased availability of inorganic P is due to decrease in pH (Gahoonia and Nielsen 1992, Zou et al. 1995) and chelation of metal cations (Fox and Comerford 1990, Grierson 1992, Jones and Darrah 1994). The chelation of metal cations (Fe and Al) releases P in the soil solution (Fox and Comerford 1990). However, the role of organic acids to solubilise organic P has not been studied frequently (Bar-Yosef 1996, Jones 1998, Ström et al. 2002). Fox and Comerford (1990) have shown oxalic acid might enhance the solubility of organic P in soil, which ultimately increase accessibility of organic P for enzyme hydrolysis (Adams and Pate 1992). Recently, Tang et al (2006) reported that malate and oxalate could enhance the solubility of phytate salts for enzyme hydrolysis. Similarly, Hayes et al (2000) proposed that organic acids play an important role to increase the accessibility of organic P.

In most of the forest studies, organic acid concentrations in the soil solution are not assayed directly because of measurement difficulties. However, organic acids represent 10 % of water soluble organic carbon, hence the quantification of water soluble organic carbon concentrations has been used (Scott and Condron 2004) to estimate that of organic acids. It is also reported that the latter is mostly derived through root exudates (Huang and Schoenau 1998). In short term rhizosphere study Chen et al (2002) found that water soluble organic carbon was higher in the rhizosphere of *Pinus radiata* compared to ryegrass. In another study, Chen et al (2004) reported a negative relation between water soluble organic carbon and NaOH extractable organic P. It suggests that the decrease in NaOH extractable Po may be due to secretion of organic acids which is positively correlated with water soluble organic carbon. The forest ecosystem may hold higher organic acid release compared to grassland. This could be due the release of organic acids by the fungi that are known to produce principally citrate and oxalate (Dutton and Evans 1996, Gadd 1999). Most of the data reporting this capacity of anion secretion are dealing with saprophytic and pathogenic fungi. However, fungal species associated with tree roots via the formation of ectomycorrhizae are also reported to possess these abilities. Oxalate crystals are observed in the hyphal sheath of ectomycorrhizae of *Pinus radiata* (Malajczuk and Cromack 1982). The secretion of oxalate has been noted in non mycorrhizal and mycorrhizal roots of *Pinus pinaster*. However the secretion of oxalate was higher in mycorrhizal roots compared to non mycorrhizal roots (Casarin et al. 2003). The secretion of oxalate by fungi is favoured by nitrate compared to ammonium in medium culture (Lapeyrie et al. 1987). Similarly, the presence of bicarbonate and calcium carbonate favoured the secretion of oxalate whereas the deficiency or low availability of inorganic P did not affect the secretion of oxalate in culture medium (Arvieu et al. 2003). A high variability in

the capacity to produce oxalate was found among ectomycorrhizal species grown *in vitro*, with species not able to release oxalate (e.g. *Hebeloma cylindrosporum*) or releasing huge amounts of oxalate (e.g. *Rhizopogon roseolus*) (Arvieu et al. 2003). These contrasting capacities to release oxalate were maintained in the rhizosphere of ectomycorrhizal roots of *P. pinaster* formed by the two fungal species mentioned above (Casarin et al. 2003, 2004). In addition, *R. roseolus* increased significantly the growth and the concentration of P in the host plant compared to non mycorrhizal plants (Casarin et al. 2004), indicated that the release of oxalate improved mineral P acquistion by the host plant. In forest ecosystems, such a variability to produce oxalate among ectomycorrhizal fungal species may exist. Interestingly, it should be possible to check this hypothesis by using the micro-plate method recently developed by Rineau et al. (2008) enabling to measure production rate of oxalate by individual ectomycorrhizal tips at a large scale.

7. Mycorrhizae

The term mycorrhiza is derived from a Greek word “mycorrhiza” which means “fungus root”. In a study of plant-microbe relationship, Frank (1885) used this term for the first time. Fungi make variable associations with roots, depending on the type of association mycorrhizae are classified into seven groups. These include ectomycorrhizae, endomycorrhizae, ectendo-mycorrhizae, arbuscular mycorrhizae, arbutoid mycorrhizae, monotropoid mycorrhizae and orchidaceous mycorrhizae. The brief characteristics of these mycorrhizal types are given in **table 1.3.**

Arbuscular and ectomycorrhizal types of symbiotic associations are most widely and abundantly found on earth (Smith and Read 1997, Allen et al. 2003). Brundrett (2002) reported that arbuscular mycorrhizae develop the most common mutual relationship with more than 80 % of vascular plants. According to Redecker et al (2000) arbuscular mycorrhizae form obligate mutual association and they belong to the phylum Glomeromycota, omnipresent in global ecosystems. The main characteristic of this type of mycorrhizal symbiosis is the development of “hyphae” in the root cortex, forming arbuscules that are looked as intracellular colonisation of healthy plant cells. However, both plasma membranes, from fungal and root cells, remained intact but without any direct connection between them (e.g., through the formation of plasmodesma). This symbiosis can form vesicles in the cortex and always produces extraradical hyphae colonising the soil and bearing spores.

Ectomycorrhizae (ECM) fungi are also widely distributed and make associations with only 3 % of the vascular plant families (Smith and Read 1997). However, ECM fungi possess

a huge diversity which arises from fungal partners including about 5, 000 to 6,000 species, mostly belong Basidiomycota with few examples of Ascomycota and Zygomycota (Molina et al. 1992, Castellano and Bougher 1994). The roots of ECM trees and shrubs including Pinaceae, Cupressaceae, Fagaceae, Betuaceae, Salicaceae, Dipterocarpaceae, and Myrtaceae host most of the fungal species richness. The development of mutual relationship in ECM is believed to have been independently evolved through multiple mutual and non mutual stages, via lineages (Hibbett et al. 2000). This type of symbiosis develops outside root surface through the formation of a fungal sheath and inside the root cortex through the formation of Hartig net. In contrast to arbuscular mycorrhizae, the fungal hyphae develop only between cortical root cells, in the middle lamella of host cell walls, to form the Hartig net. Like arbuscular mycorrhizae, the fungus produces extraradical hyphae that explore the soil.

Ectendomycorrhizae bear characteristics of both ECM and arbuscular mycorrhizal fungi like the formation of Hartig net with intracellular colonization and some sheath structure. The fungal sheath structure is reduced in ectendomycorrhizae compared with ECM. The Hartig net is named as an inward growth of hyphae which penetrates into the root structure. Intracellular penetration of healthy plant cells by these fungi also does occur, a characteristic unlike that of ECM but consistent with AM. Ectendomycorrhization develops with the roots of many angiosperm and gymnosperm plant species. The fungal symbionts belong to the members of Basidiomycota, Ascomycota, and Zygomycota. Depending upon the plant species same fungal species can develop ECM or ectendomycorrhizal association.

Arbutoid mycorrhizae possess characteristics of both ECM and AM fungi. It means that they have well developed mantle, a Hartig net and prolific extrametrical mycelium. Additionally, intracellular penetration occurs and hyphal coils are produced in autotrophic cells. The species which are associated with these mycorrhizae belong to members of Ericales which are also called *Arbutus* and *Arctostaphylos* species. The fungal symbionts are exclusively Basidiomycete species, which may form ECM with other autotrophic hosts.

Monotropoid and orchid mycorrhizal associations are developed between Basidiomycete fungi and achlorophyllous plant species. Monotropoid mycorrhizae are formed between plants of the Monotropaceae family and a specific subset of fungi in the Russulaceae or the Boletaceae families. Orchid mycorrhizae are present only in association with Basidiomycete species. Higher specificity for plant species is a characteristic of monotropoid and orchid mychorrhizal associations.

Table 1.3: Nomenclature of mycorrhizae and their attributes (adapted from Smith and Read, 1997)

Mycorrhizal type	**Arbuscular**	**Ectomycorrhizae**	**Ectendomycorrhizae**	**Arbutoid**	**Monotropoid**	**Ericoid**	**Orchidaceous**
Fungi	Glomeromycetes	Basideomycetes Ascomycetes Zygomycetes	Basideomycetes Ascomycetes	Basideomycetes	Basideomycetes	Ascomycetes	Basideomycetes
Plant	Bryophytes Pteridophytes Gymnosperms Angiosperms	Gymnosperms Angiosperms	Gymnosperms Angiosperms	Ericales	Monotropoideae	Ericales Bryophytes	Orchidaceae
Intracellular colonization	+	-	+	+	+	+	+
Fungal sheath	-	+	+/-	+/-	+	-	-
Hartig net	-	+	+	+	+	-	-
Vesicles	+/-	-	-	-	-	-	-
Achlorophylly	-	-	-	-	+	-	+

Symbol + denotes the presence and symbol – denotes the absence of attribute.

It had been thought that these mycorrhizal associations were formed exclusively with Basidiomycete fungal species; however, it has recently been discovered that several species of tropical achlorophyllous epiphytes form mycorrhizal associations with AM fungal species in the Glomeromycota (Bidartondo et al. 2002). Ericoid mycorrhizae are known to form between autotrophs in the Ericaceae and fungi in the Ascomycota. Intracellular penetration of root cells occurs and there is no mantle or Hartig net development.

8. Ectomycorrhizal diversity

Ectomycorrhizae present a significant level of diversity within the ecosystem as well as across the ecosystems. In a number of field and glasshouse studies, different surveys have been conducted to evaluate the diversity of ECM. Jonsson (1998) has surveyed a Swedish boreal forest showing between 60,000 and 1.2 million ectomycorrhizae in one square meter of forest soil. Furthermore over 95 % of root tips examined were associated with ectomycorrhizal fungi. In another study Bruns (1995) found 13 to 35 fungal species associated in 0.1 ha area, presenting large diversity of ECM. The characterization of individual ECM species has been reported to possess different physiological features (Abuzinadah and Read 1986, Samson and Fortin 1986) and functional role to their host tree (Cairney 1999, Koide et al. 2007). The high diversity of ECM species suggests that there is a potential for significant community level effect on host plant performance.

In low fertility conditions, the inoculation of birch seedlings produced more biomass when inoculated with several fungal species compared with the inoculation of single species (Jonsson et al. 2001). However, this effect was not observed under high fertility conditions. Similarly, Baxter and Dighton (2001) showed that the diversity of ECM per seedling was a better indicator to improve nutrient status of birch (*Betula populifolia*) than species composition and colonization rate. Recently, Baxter and Dighton (2005) have observed that productivity and nutrient uptake of *Pinus rigida* were increased after one year growth by increasing the diversity of ECM on root system. Moreover, the effect was considered due to fungal species composition.

8.1. Formation of ectomycorrhizal symbiosis

The formation of ectomycorrhizae does not take place haphazardly but there exists a cellular system of recognition between plant roots and their fungal partners. The host plant and their fungal symbionts choose their specific partner (Guillot 1997). The plants with and without mycorrhization are characterised by morphological modifications which take place

during the process of colonization of plant roots with fungal hyphae (Tagu et al. 2002). The process of mycorrhization can be divided into four steps (Matin et al. 1997), pre-infection, initiation, and differentiation and functioning.

8.1.1. Pre-infection

The germinating fungal spores develop in soil with their own nutrient resources that are limited. These germinated spores exchange signals with the roots of host plant through the colonising hyphae. The colonising hyphae may also belong to the fungi, which are already in mycorrhizal association. The communication process from one cell to the other may be different in mycorrhizal hyphae and hyphae germinating from spores because of having different nature and concentration of signal molecules (Tagu et al. 2002). However, the exchange of signals is controlled by a variety of morphological, biochemical and molecular processes which play a crucial role in the functioning of mycorrhizae (Martin et al. 2001, Sundaram et al. 2001). This colonization of hyphae takes place before the emergence of new root (Tranvan et al. 2000).

8.1.2. Initiation

It is the establishment of physical contact between two partners after their mutual recognition. Once the fungi have colonised at the surface of the roots, large morphological modifications occur in fungal cells. The fungal cells are adhered firmly with the cell wall of host plant, which protrudes out. It is followed by the ramification of hyphae (Tagu et al. 2002). Mycorrhizae are reported to respond to root exudates (amino acids, flavonoids, sugars and volatile compounds), and their particular role is well established (Béguiristain and Lapeyrie 1997). Horan and Chilvers (1990) tested the compatibility of *Pisolithus tinctorius* and *Paxillus involutus* by adjusting a permeable membrane between plant and fungi. The hyphae of compatible fungi were attracted towards the membrane while the hyphae of non compatible fungal strains were not attracted by membrane. This suggests the specificity of root exudates to attract compatible strains for mycorrhization. Similarly, fungal hormones are also important for the ramification and growth of hyphae (Gogala 1991). Studies have confirmed that fungal indol acetic acid (IAA) control the principal anatomical characteristic and the expression of genes in the ectomycorrhizae of Pine (Gay et al. 1994).

8.1.3. Differentiation

Ectomycorrhizal fungi form a symbiotic relationship with a plant by forming a sheath around its root tips. The fungus then penetrates into the root along the middle lamellae between cell walls by inward growth of hyphae, thereby form a Hartig net, a complex network of fungal hyphae that is the site of nutrient exchange between the fungus and the host plant. The fungi and the plant essentially fuse their walls, and nutrient exchange appears to take place across these walls. The cell wall becomes wider and less compact after the penetration of hyphae between the cortex cells (Martin et al. 1999). Generally, mycorrhizal roots are largely transformed and they represent the morphology of short roots (Dexheimer 1997).

8.2. Functioning of ectomycorrhizae in forest stands

Forest trees are naturally dependent on symbiotic associations with ectomycorrhizal fungi. The fungi take up minerals (phosphorus, nitrogen, sulphur and zinc etc.) from soil and transfer them to the plant through extended functional root system (Allen 1991) and in response plants deliver carbon to the fungi. ECM fungi possess very limited ability to degrade or hydrolyse the complex carbohydrates, which are derived through plant debris. Instead, these fungi depend upon their hosts for the energy. The ectomycorrhizal fungi are able to link root systems of several trees through hyphal extension. A large proportion of ECM fungi belong to basidiomycetes, which includes well known genera of *Amanita, Cortinarius, Lactarius, Russula and Suillus* (Hacskaylo 1972). Ectomycorrhizal associations are widely present, particularly in temperate regions with plants belonging to the following genera: *Pseudotsuga*, *Picea*, *Pinus*, *Abies*, *Salix*, *Quercus*, *Betula* and *Fagus*. In this association the fungus gets C and other essential organic substances from the tree and in return supports the tree in taking up water, mineral salts and metabolites. The fungus also protects trees from parasites, nematodes, and soil pathogens. Indeed, most forest trees are highly dependent on their fungal partners and the development of plants in soil with poor fertility in absence of mycorrhizal symbiosis could be very low. This effect has been observed in exotic pine transplantation in different parts of the world. In Western Australia, *Pinus radiata* and *P. pinaster* seedlings failed to grow in nursery beds without ECM fungi (Lakhanpal 2000). High ectomycorrhizal diversity is important in the healthy functioning of woodlands. Different fungi appear to occupy different niches. Some may be more proficient at supporting the tree in taking up particular nutrients, others may be specialized at protecting against pathogens, and others may assist in enzyme production (Akema and Futai 2005).

8.2.1. Rhizosphere and Ectomycorrhizae

The rhizosphere is characterised as an area in the vicinity of root having stimulated microbial activities due to exudation of organic substances (Grayston et al. 1998). These root exudates are considered as the indicators that communicate and initiate biological and physiological interaction between roots and soil born organisms (Walker et al. 2003) and root itself is considered as an indicator which facilitates communication (Bais et al. 2006). Roots are reported to secrete small molecules such as amino acids, organic acids, sugars, phenolic and other secondary metabolites, presenting large diversity, whereas compounds with high molecular weight such as polysaccharides and protein are less diverse but consist of major proportion of root exudates (Stotz et al. 2000, Bais et al. 2006). Plant roots are known to communicate with soil borne organisms, although some can be positive (symbiotic) and others can be negative (parasitic or pathogenic) for the plant. The growth rate of ectomycorrhizal fungi is promoted by pine root exudates, though different fungal species often react differently (Melin 1963). In negative plant-soil borne organism associations root exudates may function to defend plant roots.

8.2.2. Water stress and supply

It is generally hypothesised that mycorrhizal associations increase hydraulic conductivity and water use efficiency of plants. Coleman et al. (1990) studied the hydraulic conductivity of Douglas fir (*Pseudotsuga menziesii*) seedlings inoculated with *Laccaria bicolor* or *Hebeloma crustuliniforme*. The seedlings were grown under different levels of fertilization (1, 10 and 100 µM, P). The increase in tissue P and decrease in root to shoot ratio was correlated with high hydraulic conductivity in each of the mycorrhizal treatments. Nardini et al. (2000) determined the physiological response of ectomycorrhization between *Tuber melanosporum* and *Quercus ilex.* They observed that inoculated seedlings showed higher net assimilation and stomatal conductance than non-inoculated seedlings. Root hydraulic conductance per unit root surface area of inoculated seedlings was reduced to 0.44% that of non-inoculated seedlings but had 2.5 times more fine root surface area than non-inoculated seedlings. However, when root conductance was related with leaf area, the inoculated seedlings showed 1.27 times more root conductance per unit leaf area compared to non-inoculated seedlings.

The *Pinus pinaster* seedlings were inoculated with *Hebeloma cylindrosporum* and seedlings, whether inoculated or not, were allowed to grow in sandy dune soil (Bogeat-Triboulot et al. 2004). A drought stress of three weeks was given after six months of culture.

Inoculated seedlings were 75 % mycorrhized with *Hebeloma cylindrosporum* while non inoculated seedlings showed presence of exotic species like *Thelephora terrestris* (50%) and *Laccaria bicolor* (30%) and to a lesser extent by *H. cylindrosporum* (20%). The hydraulic conductivity was reported higher in plants associated with *H. cylindrosporum* compared to other species. Inoculation of *P. pinaster* trees with *Pisolithus* sp increased tree growth in drought conditions (Lamhamedi et al. 1992). The results suggest that fungal inoculation in arid and semi arid regions may help forest management.

8.2.3. Nutrient uptake and ectomycorrhizae

Plant nutrients, except nitrogen are derived from weathering of primary minerals and the access of plant towards these nutrients (P and K) is often limited. The, ectomycorrhizal association is able to improve mineral nutrition (N, P and K) One hypothesis is that ectomycorrhizal fungi mobilize essential plant nutrients directly from minerals through excretion of organic acids. This enables ectomycorrhizal plants to utilize essential nutrients from insoluble mineral by altering nutrient cycles in forest systems (Landeweert et al. 2001). Wallander et al. (2004) inoculated pine (*Pinus sylvestris*) seedlings with indigenous ectomycorrhizal fungi using forest soil with four levels of wood ash addition (0, 1, 3 and 6 t ha^{-1}), and estimated the demand for P and K by seedlings grown in the different soils by measuring the uptake of isotopically marked ^{32}P and ^{86}Rb (homologe of K) in the root. They also assayed the utilization of P from apatite. The comparison of uptake by ectomycorrhizal mycelium and uptake by roots showed better uptake by mycellium. Uptake of P from apatite was on average 23% of total seedling P which illustrated the role of ectomycorrhizal to utilize the P from apatite. Many other studies **(Table 1.4)** also highlight the role of ectomycorrhizae associated with pine species to increase the uptake of N, P or K nutrition.

9. Phosphorus acquisition from soil and plant nutrition

Plants take up phosphorus in the form of phosphate ion which is very scarce in soil solution. That is why, phosphorus deficiency is considered as one of the major nutritional limitations for plant growth and production (Barber et al. 1963). The volume of the soil explored by roots determines the amount of P that can be potentially taken up by plant. However, the volume of the soil occupied by roots is very low (1%) compared to volume of the soil. In this way, very low amount of P can be taken up directly by root interception (Barber 1995). Other processes like convection and diffusion of P play an important role to increase nutrient uptake.

Table 1.4: Examples of positive effects of ectomycorrhizal associations on mineral nutrition of pine trees.

Host plant	Ectomycorrhizal fungal species	Nutrient improved	References
Pinus sylvestris	*Rhizopogon roseolus, Suillus bovinus, Pisolithus tinctorius, Paxillus involutus*	N	Finlay et al. 1988
P. sylvestris	*Pisolithus arhizus*	N	Högberg 1989
P. contorta	*Thelephora terrestris*	N	Finlay and Söderström 1992
P. taeda	*Cenococcum geophilum, Pisolithus tinctorius*	P	Rousseau et al. 1994
P. rigida	*Laccaria bicolor, Pisolithus tinctorius, Paxillus involutus*	P	Cumming 1996
P. sylvestris	*Paxillus involutus, Suillus luteus, Suillus bovinus, Thelephora terrestris*	P	Colpaert et al. 1999
P. sylvestris	*Suillus variegates*	P	Wallander, 2000
P. resinosa	*Pisolithus tinctorius*	N	Wu et al. 2003
P. sylvestris	*Amanita rubescens, Laccaria deterrimus*	N	Taylor et al. 2004
P. sylvestris	*Indigenous ectomycorrhizal fungi*	P, K	Wallander et al. 2005

The process of convection depends upon the availability of water, concentration of P in solution and the water requirement of plants. The processes of root interception and convection generate a concentration gradient of P in rhizosphere soils and bulk soils. This concentration gradient result into the diffusion, from the area of higher Pi concentration (bulk soil) to the area of lower concentration (Hinsinger 2001). Diffusion of P is linked with the process of adsorption and desorption. The decrease in Pi concentration in solution induces desorption of Pi in soils. According to Morel (2002) P concentration gradient not only affects soil solution P but also the P present at solid-solution interface. The amount of P in soil solution is also maintained by mineralization of organic P including microbial organic P by enzymatic hydrolysis (Oberson and Joner 2005). Despite all these processes of replenishment of the soil solution, the concentration of P rarely exceeds 10 µM in the vast majority of soils (Bieleski 1973, Hinsinger 2001). This low Pi availability results into high differences between soil solution Pi (µM) and plant cells (mM) concentrations (Raghothama and Karthikeyan 2005). In order to maintain sufficient level of cellular Pi, plants require efficient uptake and translocation Pi for normal functioning. In this way plants need to acquire Pi against high

concentration gradient across the plasma membrane. A dual model of P uptake dealing with high and low affinity uptake mechanisms is widely accepted to explain the concentration dependent acquisition of phosphate ions (Ullrich-Eberius et al. 1984, Amijee et al. 1991). Regardless of the concentration of Pi in soil solution, P concentration in cytoplasm of plant is kept constant (5-10 mM) except under acute deficiency conditions. On the other hand concentrations of Pi in vacuoles vary largely depending on the P status. Under condition of acute deficiency it may be undetectable, and may increase up to 25 mM with higher P status of soils (Mimura 1995). Plants make adjustments according to P availability, as under P deficiency, plants grow more roots, increase the rate of uptake by roots from the soil, re-translocate Pi from older leaves, and deplete the vacuolar stores of Pi. Additionally, mycorrhizal fungi may colonize more extensively the roots. Conversely, under sufficient P supply when absorption exceeds the demand, a variety of processes play their role to prevent the accumulation of Pi to toxic concentrations (Lambers et al. 2008). It could be conversion of Pi into organic storage compounds (e.g. phytic acid), down regulation of Pi uptake rate from the outside solution (Lee et al. 1990), and the loss of Pi by efflux, ranging between 8 to 70% of the influx (Bieleski and Ferguson 1983). It is clear from both kinetic and molecular studies that the capacity to transport Pi across cellular membranes involves several different transporters and is in some way regulated by the external supply of Pi.

10. Objectives and strategy of research

10.1. Basic hypothesis

The review of literature shows that P is an important mineral nutrient element for plant growth, and its availability is often limited in the soils, throughout the world. However, the proportions of unavailable P fractions, particularly organic P fractions could be high particularly in forest ecosystems. High Po and low Pi availability in forest soils has often resulted to decrease forest production. In order to increase Pi availability for plants, P fertilizers are applied in soils to increase plant growth. At the same time ectomycorrhizal fungi and their bacteria associated with these fungi could be beneficial for plants to increase P availability and its uptake. This could be through the extension of fungal hyphae, secretion of acid phosphatases into the soil environment which could hydrolyse Po compounds in soil solution. These enzymes are well documented for their capacity to hydrolyse Po compounds in culture medium. However, very few studies describe the actual role of these enzymes under soil condition and their capacity to hydrolyse Po in soils in relation to P status of the soils.

With reference to above discussed avenue of the research the principal hypothesis of my theis was the following:

"The acid phosphatase secreted by ectomycorrhizal fungi could change the availability of Pi in soil"

10.2. Objectives

The general objectives of my thesis are to determine the relation between different P fractions in soils and phosphatase activity of ectomycorrhizal morphotypes, which are both influenced by soil as well as plant properties. It would further help to determine the role of Pi availability and phosphatase activity on the *P. pinaster* growth. In order to realise these objectives, field as well as laboratory experiments were conducted to address following questions:

1) What is the actual contribution of organic P in total P present in soils of Landes?
2) What is the variability of ectomycorrhizal phosphatase activity in relation to P status in soils?
3) Which are the pools of P, from which ectomycorrhizal plants take up P and what is their relation with phosphatase activity?

10.3. Experimental strategy

Two sampling campaigns (April and November, 2006) of Landes des Gascogne, have been carried out to collect bulk soil samples from a range of *P. pinaster* plots differing by the management of P fertilization practices and age of plantations. These soil samples were used to sort out ectomycorrhizae root tips as well as to prepare composite soil samples for soil chemical analysis. Fresh soil samples of an experimental site called "Parcelle L" were brought intact (with out sorting ECM root tips), and stored 4°C in dark prior to conduct rhizobox experiments for both sampling campaigns.

The results of the field soil samples and measurement of phosphatase activity of ECM root tips will be presented in the form of following two articles:

1) Effect of stand age and season on the different phosphorus fractions characterised in spodosols of Landes of Gascogne.
2) Effect of P fertilizers treatments and stand age on phosphatase activities of ectomycorrhizal fungal species associated with *Pinus pinaster* trees sampled in spodosol soils of Landes of Gascogne.

Similarly, two rhizobox experiments in growth chamber were used to grow young seedlings of *P. pinaster*. The results obtained will also be presented in the form of following two articles:

3) *Pinus pinaster* seedlings and their fungal symbionts show high plasticity in phosphorus acquisition in acidic soils.
4) Organic P mobilisation by young ectomycorrhizal *Pinus pinaster* in controlled conditions from native spodosol soil samples is highly dependent upon fertilisation and irrigation story of soil.

The thesis manuscript will be composed of five chapters a chapter of review literature followed by four chapters of above listed scientific articles. The general conclusions and perspectives will be given in the last section of manuscript.

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CHAPTER 2

Effect of forest stand age and season on different phosphorus fractions characterised in spodosols of Landes of Gascogne, France

Phosphorus being the second most important mineral nutrient for plant growth after nitrogen is often limited in the soils. Although the total amount of P in most of the soils could be significantly high but the amount of P available to plants is commonly very low and it rarely exceeds to 10 µM (Hinsinger 2001). The processes like adsorption and immobilization of available Pi (Frossard et al 1995) could be responsible for the low Pi availability in soils. The rate of these processes could be controlled by soil physicochemical properties and soil management practices like fertilization and soil cultural practices. The soils of ecosystem Landais are known to be very limited by P nutrition (Trichet et al. 1999). The plantation of *Pinus pinaster* having different age of trees and forest management practices, particularly fertilization with mineral P fertilizers could affect soil physicochemical properties like the accumulation of organic matter. Both the soil as well as the plant characteristics could significantly affect different fractions of inorganic P (Pi) and organic P (Po). In addition to soil and plant characteristics, soil microorganisms (fungi and bacteria) and environmental factors (seasonal variations) could also play an important in the variability of P fractions. The plant roots and soil microorganisms could have maximum influence in the first 15 cm of mineral soil horizon, as the density of fine roots associated with ectomycorrhizae could be highest (90 %) in this area (Taylor and Bruns 1999).

The objectives of this chapter (Chapter 2) were the following:

1) To characterise various fractions of Pi and Po in forest ecosystem of Landes of Gascogne and to evaluate the contribution of Po fractions in total P of these soils.
2) To determine the variation of Pi and Po fraction in forest ecosystem and to identify the factors (plant soil and environmental) which can explain these variation.

In order to realise the first objective, soil samples from lines of trees and interlines of trees were taken from 18 plots covering a whole range (1 million hectare) of forest in spring 2006 and autumn 2006. Pi and Pt by various methods (Olsen, NaOH and H_2SO_4, Pi-isotopic dilution) were determined and the contribution of Po in each fraction was calculated.

Soil physicochemical properties (pH, water contents, Alox, total N, total C,) as well the various Pi and Po fractions (Olsen, NaOH, H_2SO_4, Pi-isotopic dilution) were estimated in

spring. For autumn the Pi and Po fraction (Olsen, NaOH and H_2SO_4) and pH were determined in the soil samples, however the values of total N and total C were deduced by the relationships between heat weight loss (550°C) and total N and total C in spring. The data of Pi and Po fractions thus obtained were used to evaluate seasonal variations and soil physicochemical properties were used to identify the indicators which control the variance of Pi and Po fraction in the studied soils.

Additionally, the concentrations of Pi measured by Olsen method and Pi measured by isotopic dilution technique (30 minutes of kinetics) were used to compare the pertinence of these two methods The physicochemical properties were also used to know, how they affect the parameters (physical-chemical reactions) of kinetics of isotopic dilution.

Subsequently, the results obtained are compiled for a scientific publication in pre-reviewed journal "*Biogeochemistry*" titled as: "Effect of forest stand age and season on different phosphorus fractions characterised in spodosols of Landes of Gascogne, France"

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CHAPTER 2

Effect of forest stand age and season on different phosphorus fractions characterised in spodosols of Landes of Gascogne, France

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Abstract

Phosphorus presents major limitation for plant growth in most of the forest ecosystems and the knowledge of P cycle in this system is insufficient. The forest range of Landes of Gascogne is also characterized as P deficient affecting plant growth in this region. The current study was performed to identify the factors controlling the variation of P in stands of *Pinus pinaster* forest, with different age of trees and fertilizer treatments. The fractions of P (bicarbonate, hydroxide and H_2SO_4) and soil physicochemical properties were measured to evaluate the effects of treatments, seasons and soil properties on P fractions. Pi-isotopic dilution was measured and compare with labile bicarbonate fraction. The results showed comparable values for bicarbonate and Pi-isotopic dilution, which were very low ranging between 2-10 mg g^{-1} of soil. Over 50 % of the variance of P fractions (bicarbonate, hydroxide and H_2SO_4) was explained by soil treatments and total C, total N, organic matter and Al_{ox} in both spring and autumn. The significant difference between control and fertilized soils was observed only in bicarbonate and hydroxide extractable Pi fractions. These two fractions were highly correlated to each other and were controlled negatively by Al_{ox} and positively by H^+, P fertilizers in soils. The Pi concentrations were higher for bicarbonate and hydroxide and lower for H_2SO_4 in spring compared to autumn. While Po concentrations were lower for bicarbonate and hydroxide and higher for H_2SO_4 in spring compared to autumn, showing immobilization of P in autumn and mineralization in spring. Soil physicochemical properties were not only controlling variance of P fraction but also presented high correlation with parameter Pr1min of isotopic dilution, controlling the physical-chemical reactions during the first minute of isotopic dilution.

Key words: *P. pinaster, P avaiability, isotopic dilution, soil properties, seasonal P variation, fertilization.*

1. Introduction

Phosphorus (P) is an essential nutrient element and is identified as first or second limiting factor for plant growth in forest, grassland and cultivated systems (Aerts and Chapin 2000; Comerford et al. 2002). The preferred form of P for plant uptake is orthophosphate (HPO_4^{2-}, $H_2PO_4^{1-}$), but the knowledge of forms of P (inorganic and organic) in a particular system is pivotal to understand the P availability and sustainability of management practices (Frossard et al. 2000). In an undisturbed ecosystem total P and its inorganic and organic components remain principally closed, with little loss through leaching or biomass harvesting. In such type of system, the cycling of P and its pools is determined by topography of the region, soil parent material, climatic conditions and plant biomass (Solomon et al. 2002). Conversely, in anthropogenic soils the balance in the forms of P is largely changed due to variable plant biomass production and organic matter inputs resulting through management and fertilization treatments (Bowman et al. 1990; Magid et al. 1996). In this regard, little is known about the seasonal variation in soil P and its fractions. In addition, the published data give diverging and conflicting seasonal pattern of soil solution and bicarbonate extractable P in soils studied previously (Tate et al. 1991; Sharpley et al. 1995; Pote et al. 1999; McDowell and Trudgill 2000; Chen et al. 2003). These differences may be due to variable climates, soil types, soil cultural practices and analytical procedures particularly soil storage conditions (Turner et al. 2003; Styles et al. 2006) and soil phosphorus testing method.

Other factors (physicochemical) controlling P fractions include additions of phosphate fertilizers, P solubilisation (dissolution or desorption), removal of solution P (adsorption/precipitation), mineralization and immobilization of P through organic pools (Frossard et al. 2000; Hinsinger 2001). Studies have shown that processes controlling different fractions of P in soil are strongly mediated by edaphic factors, like seasonal variation in temperature may reduce P sorption, following the formation of crystals of Al and Fe oxides and hydroxides with low surface area (Pote et al. 1999). The variation in soil temperature, moisture and redox potential are also supposed to affect interaction between P, organic acids and components of organic matter. These and other factors such as exchangeable cations and pH also contribute to determine the availability of P in soil solution (Brennan et al. 1994).

In order to better understand dynamics of P and its fractions, P is characterized into different pools using chemical extractants (Hedley et al. 1982; Tiessen and Moir 1993). According to Hedley procedures P extracted with an anion exchange resin and 0.5 M $NaHCO_3$ (pH 8.5) is named as labile pool and P extracted with 0.1 M NaOH is named as moderately labile for plant uptake. Although these single chemical and sequential extraction

methods are commonly used analytical procedures (Bonneau et al. 2003; Chen et al. 2000; Comerford et al. 2002), these methods do not explain the kinetics of ionic P species between soil solid and soil solution phases, which are controlled by physicochemical processes (Frossard et al. 2000; Hinsinger 2001). Indeed, the use of isotopic dilution method has shown its importance to determine the movement of P ions between solid and liquid phases of soil as the function of time (Frossard et al. 1994; Fardeau 1996; Bühler et al. 2003; Achat et al. 2009). Apart from characterization of different pools of P in soils of temperate forests very little is known about the factors controlling the dynamics of different P fractions and their variation among the seasons. Moreover, data is lacking to compare isotopic dilution technique and the most routine soil test methods. Published data do not show any study which relate soil or plant properties with the parameters which belong to the physical-chemical reactions in the kinetics of isotopic dilution technique.

The forest range of the Landes of Gascogne in southwest of France cover ca 1 million ha, planted with maritime pine (*Pinus pinaster* Ait in Soland). The soils are sandy spodosols commonly fertilized with P fertilizers because the soils are characterized as P-deficient (Trichet et al. 2000). The development of soil and understory annual vegetation has been changed over the time resulting into variable accumulation of organic material due to the differences in topology and water contents (Augusto et al. 2006). A comprehensive study was conducted in to evaluate Pi availability and its variation with respect to soil properties.

Objectives of the present study were to 1) characterize different pools of P in forest soils and 2) to evaluate the effects of soil properties, season and fertilizer management practices on P pools. We hypothesized that plants of *Pinus pinaster* as well as soil properties could affect the different fractions of P in forest soils. We also aimed at linking the results of most routine soil Pi test (Olsen Pi) and Pi estimated by isotopic dilution method, with comparable time of contact between soil and extraction solution. We further, used short and long time, physical-chemical reactions parameters to compare with soil as well as plant properties.

2. Material and methods

2.1. Description of experimental station

The station of study, known as Landes of Gascogne is located in southwest of France. The mean annual temperature ranges from 10 to 15°C (from west to east) and precipitation ranges from 750 to 1,250 mm yr^{-1} (from south to north) and is irregularly distributed over the

year. Soils are developed from a coarse sandy Aeolian parent material deposited in the Pleistocene and are characterized as acidic, less fertile and rich in organic matter. These can be classified as Entic to Albic spodosols (Food and Agriculture Organization (FAO)/International Union of Soil Science 2006). Depending on the depth of water table, the lenses of a cemented spodic horizon can occur between the depth of 40 and 100 cm in the soil (Trichet et al. 1999). The soils are planted with *P. pinaster* consisting of nearly one million hectare. Most of the stands are intensively managed, and composed of different age class stands ranging from 6 to 93 years. The trees of *P. pinaster* are grown evenly in lines with 2 m tree to tree distance and 4 m interline tree distance. Fertilizers trials especially of phosphate fertilizers are conducted over a whole forest range.

2.2. Soil sampling

Paired soil samples from line and interline tree positions were taken during two sampling campaigns in April 2006 (spring) and November 2006 (autumn) from 19 plots located at 11 sites, covering a whole range of forest. Seven sets of soil cores (15 cm length x 8 cm diameter) from mineral soil layer were taken after removing litter layer, from each sampling position. Seven out of 11 sites contained both P treatment (+P) and no fertilizer treatment (control) plots, whereas other 4 sites contained plots with either only +P treatment or control treatment. Collectively, 37 sets of samples were taken from both line and interline tree positions (15 +P and 22 controls). Fertilizers (+P) were commonly applied once at the time of plantation or in some plots at the time of plantation as well as during the plant rotation period. The details of sampling plots are mentioned in **table 1**. A composite sample of each set of seven samples was prepared by sieving the soil through 2 mm mesh size sieve. These samples were stored at 4°C and sub samples were air dried for chemical analyses that were carried out under similar conditions for all samples in order to overcome storage induced differences in P concentrations (Turner 2005).

2.3. Estimation of P fractions

Total P (Pt) in the soils was determined by igniting air dried soil samples at 550°C for 4 h in muffle furnace (Saunder and Williams 1955). The ignited and air dried soil was shaken with 1 N H_2SO_4 (1:50 soil to solution ratio) for 16 h using end-to-end shaker. The samples were centrifuged (14000 g, 15 min) and the supernatants were used for P assay. The phosphorus estimated in ignited and air dried soil represented Pt and Pi respectively. Total Po (H_2SO_4) was calculated by the difference between Pt and Pi. Sieved and air dried soil samples

were also used to measure bicarbonate and hydroxide extractable inorganic and organic P fractions. Bicarbonate extractable P known as, plant available P was extracted by shaking 0.3 g of sieved soil for 30 minutes in 6 ml of $NaHCO_3$ (0.5 M, pH 8.5) (Olsen et al, 1954).

Table 1: A brief description of sites characteristics used in this study

Sites name	Sites code	Soil treatment	P_2O_5 input [a] kg ha^{-1}	Sampling position	Age of tree population Years
Blagon	1	Control	0	Line	8
Blagon	2	Control	0	Interline	8
Blagon	3	+P	120	Line	8
Blagon	4	+P	120	Interline	8
Le Bray	5	+P	120	Line	36
Le Bray	6	+P	120	Interline	36
Baudes	7	Control	0	Line	93
Baudes	8	Control	0	Interline	93
L plot	9	Control	0	Line	13
L plot	10	Control	0	Interline	13
L plot [b]	11	+P	90	Line	13
Vieille 1	12	Control	0	Line	29
Vieille 1	13	Control	0	Interline	29
Vieille 2	14	Control	0	Line	26
Vieille 2	15	Control	0	Interline	26
Caudos	16	Control	0	Line	8
Caudos	17	Control	0	Interline	8
Caudos	18	+P	120	Line	8
Caudos	19	+P	120	Interline	8
Retjons	20	Control	0	Line	8
Retjons	21	Control	0	Interline	8
Retjons	22	+P	120	Line	8
Retjons	23	+P	120	Interline	8
Saumejan	24	Control	0	Line	6
Saumejan	25	Control	0	Interline	6
Saumejan	26	+P	120	Line	6
Saumejan	27	+P	120	Interline	6
Lue	28	Control	0	Line	9
Lue	29	Control	0	Interline	9
Lue	30	+P	120	Line	9
Lue	31	+P	120	Interline	9
Mimizan	32	Control	0	Line	49
Mimizan	33	Control	0	Interline	49
Mimizan	34	+P	120+120	Line	49
Mimizan	35	+P	120+120	Interline	49
Marcheprime	36	Control	0	Line	32
Marcheprime	37	Control	0	Interline	32

a P was applied in most of the sites at the time of pine plantation except site L where fertilizers are applied annually (in June or July) and site Mimizan where P was applied at plantation of pine trees and after 41 years of trees plantation

b The interline of the fertilized L plot was excluded from analysis of database to limit the statistical artifacts.

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 to Al and Fe-oxides (Tiessen et al 1984; Sharpley 1999). Soil extracts were diluted with distilled water (1/6, v/v), then acidified with 12 N HCl (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 by our preliminary experiments, these conditions made it possible to mineralize all organic P contained in the solution (data not shown). The organic P concentration in soil extracts was calculated by the difference between Pt and Pi for both $NaHCO_3$ and NaOH extracts. The concentration of Pi in all solutions was measured using malachite green method (Ohno and Zibilske 1991).

2.4. Determination of diffusive P by isotopic dilution

In addition to the chemical P fractionation scheme, a sound mechanistic approach was used to quantify soil P status. It consists of analysing both the soil P ions concentration (Cp, mg l^{-1} solution), i.e. plant available $H_2PO_4^-$ and HPO_4^{2-}, and the corresponding capacity of the soil solid phase to equilibrate P ions in solutions under a gradient of P ions concentration between liquid and solid phases of soil. The gross amount (Pr, mg kg^{-1} soil) of diffusive P ions between liquid and solid phases of soil suspensions at steady state was determined using the isotopic labelling and dilution technique proposed by Fardeau (1993, 1996). The procedure consists, first to obtain a dataset of Cp and of associated kinetics of Pr for short periods of isotopic dilution, then to use this dataset for the calibration of mathematical equations, and finally to use these equations for simulation of Pr values over the more longer periods. The Cp and Pr determinations were carried out only in soils of spring campaign according to the procedure used by Achat et al. (2009).

Briefly, five soil suspensions (10 g soil in 14.9 ml of distilled water and 0.1 ml of biocide (Micro-O-protect, containing bromo-nitro-dioxane 2-methylisothiazolone and ethanol, Product 1585 720, Boehringer Mannheim Corporation, Indianapolis, IN, USA)) were prepared for each sample, corresponding to 10, 40, 100, 400 and 1000 min dilution period. Biocide was used to avoid microbial activity. The soil suspensions were allowed to equilibrate for 16 h at 20 ± 2°C, by gently stirring on a roller (40 cycle min^{-1}). The 0.1 ml of carrier free $^{32}PO_4$ ions (R) solution was introduced into soil suspension at time zero to label the amount of P ions in solution (Pw, mg kg^{-1} soil). Introduction of R does not affect the steady-state of soil suspensions since R is around 10^{-6} fold lower than Pw, i.e. soil solution P concentration (Cp, mg l^{-1} solution) and Pw does not change significantly during the isotopic dilution kinetics.

About 2.5 ml of soil suspension was taken with disposable syringe after 10, 40, 100, 400 and 1000 min from each sample and immediately filtered through 0.2 µm membrane filters. These aliquots were used to determine the radioactivity remaining (r) after these periods of isotopic dilution. Additionally, the same procedure was carried out in distilled water containing only biocide (no soil) to determine R at any given time. The r and R values were enumerated simultaneously in the counter (Packard TR 1100) using a liquid scintillation cocktail and isotopic dilution ratio (r/R, dimensionless) was calculated. The Cp values was also determined (Cp, mg l^{-1}) in all aliquots using malachite green colorimetric method.

From this Cp and r/R dataset, the amount (Pw, mg P kg^{-1}) of P ions in solution in which R is diluted, the Pr values and the amount of isotopic exchangeable P ion (E, mg kg^{-1} soil) in which R is diluted were calculated using the following equations (Fardeau et al. 1991; Fardeau, 1993; 1996) obtained by applying the isotopic dilution principle:

$$Pw\ (mg\ \ kg^{-1}) = \frac{Cp \times V}{M} \text{ ----------1}$$

Where V is volume (15 ml) and M is weight of soil (10 g) used for soil suspensions

$$Pr\ (mg\ \ P\ kg^{-1}) = \frac{Pw\ (R\ -\ r)}{r} = Pw\left(\frac{1}{(r/R)} - 1\right) \text{ ----------2}$$

$$E\ (mg\ \ P\ kg^{-1}) = \frac{Pw}{(r/R)} = Pr + Pw\ = \text{(Pi-isotopic dilution) ----------3}$$

For a given soil, the parameters estimates of kinetics of the 5 Pr values per soil was fitted to the following equation using the procedure PROC NLIN of Statistical Analysis Software (SAS, 200) that minimizes residual variability:

$$Pr = Pr1min \times t^{n}\text{, with Pr < limit value ----------4}$$

where Pr1min is the Pr value after 1 min. The parameter n accounts for slow physical-chemical reactions with time. Because slow reactions of orthophosphate ions in solution with soil solid phase can occur for several months, the limit value of Pr, obtained when the isotopic equilibrium is reached, can not be determined experimentally. The limit value of Pr is generally calculated by excess considering that inorganic P in soil can take part in isotopic dilution (Fardeau, 1993; Frossard et Sinaj, 1997).

The Eq. (4) can be used to calculate Pr values for any durations of time up to the limit value. As an example, we used Eq. 4 to calculate Pr values after 30 minutes of isotopic dilution and then Eq. (3) to calculate the E values after 30 minutes (Pi-isotopic dilution after

30 minutes of kinetics) in order to compare them with the bicarbonate (Pi-Olsen) extractable P in soils.

2.5. Other soil chemical and plant analyses

Fresh soil was used to determine percentage of water content (WC) and pH. Soil was heated overnight at 105°C in tarred aluminium dish and WC was calculated by the loss of weight before and after heating. Soil pH was measured in 10 mM solution of $CaCl_2$ using a 1:5 soil to solution ratio. Air-dried soil samples from spring campaign were used to determine total organic carbon (C), total organic nitrogen (N) and total oxalate extractable aluminium (Al_{ox}) by *Laboratoire d'Analyses des Sols* belonging to National Institute for Agronomic Research (INRA), Arras, France, using standard French analytical norms (AFNOR, 1999). Briefly, total organic C content was estimated by oxidation with potassium dichromate and sulphuric acid (NF ISO 14235); total organic N content was estimated by the Kjeldahl method (NF ISO 11261) and the Al_{ox} was determined by McKeague and Day (1966) method. We observed that the values of total C and total N concentrations were highly correlated with soil heat loss weight at 550°C (HLW) when ignited for determination of total P (see supplementary data, Fig. 1). Therefore, we estimated the values of total C and N concentrations in soil sampled in autumn by using the linear regressions fitted on the variation of HLW (given by the difference in weight of soils dried at 105°C overnight and soils ignited at 550°C for 4 h) with total C and N in spring. The equations used were: C total = 5.7 x HLW – 0.79 and N tot = 0.22 x HLW – 0.14 (see supplementary data, Fig. 1). The growth of the trees was estimated by measuring the circumference at breast height. The ratio of circumference at breast height to age of tree (Circ:Age in mm $year^{-1}$) was calculated to take into account the increase of diameter with tree age.

2.6. Data arrangement and statistical analyses

The data was arranged into P fertilized plots (+P, n = 15) and unfertilized (control, n = 22) plots to evaluate the effect of fertilizer treatment on Pi and Po fractions for both spring and autumn. The effect of plants age on different fractions of Pi and Po in soils was evaluated by grouping only control plots into 6-13 years, 26-49 years and 93 years old plants groups, for both spring and autumn. All statistical analyses were performed in R open source software (R Development Core Team, 2009). The *stats* library of R software was used for the tests of normality and comparison of means as well as Pearson correlations (functions *ks.test*, *wilcox.test* and *cor.test*, respectively), whereas with the *vegan* library (Oksanen et al., 2009),

redundancy analysis (RDA) and partition of variation were performed (functions *rda* and *varpart*, respectively). The significance level used in this study was set to $\alpha = 0.05$.

For each variable, normality of the datasets was tested using the Kolmogorov-Smirnov test. If necessary, logarithmic transformations were applied to the variables, to achieve a normal distribution. The influence of sampling season and tree age for Pi and Po fractions as well as the influence of soil treatment for physical-chemical parameters Pr1min and n determining the rate of solid-liquid exchange reactions were evaluated using mean comparisons. These comparisons were made only on paired samples by using the non-parametric Wilcoxon signed-rank test. The effect of tree age on soil Pi and Po concentrations was evaluated only on control sites, in order to exclude the influence of fertilization on P fractions. Three groups of tree ages were created, which corresponded to young (6-13 years old; n = 23), middle age (26-49 years old; n = 12) and old trees (93 years old; n = 2). The mean comparisons were only assessed between young and middle age groups using the non-parametric Mann-Whitney test. Due to small sample numbers in the old trees group, it was impossible to compare the results of this group with other two groups.

At whole dataset level, heterogeneity of soil P fractions as well as Pr1min and n parameters occur between sampled plots. Factors controlling the variability of these response variables were assessed using RDA describing, on one hand, soil P fractions and, on the other hand, Pr1min and n parameters. The explanatory variables tested by RDA were the sample position (line/interline) identified by a binary variable, the soil treatments (control/+P) identified by a binary variable, the age of each soil plot since last fertilization identified by a set of dummy binary variables and significant soil properties identified using a forward selection procedure. These RDA were made on standardized variables and the significance of the canonical relations as well as the canonical axes were tested using unrestricted Monte-Carlo permutation test (10,000 simulations) performed under reduced model. For Pi and Po concentrations, two RDA ordination biplots, preserving correlations among variables (type-2 scaling), were used to document the effects associated with soil treatment and soil properties, respectively. The discrimination between soil treatment and soil property effects were also assessed using partition of variation of the measured soil Pi and Po concentrations on spring and autumn sampling campaign. Furthermore, for the functional parameters Pr1min and n, Pearson correlations were computed and used to identify the key soil and tree properties active on these parameters.

3. Results

3.1. Soil physicochemical properties

The descriptive statistics of soil physicochemical properties are presented in Table 2. The data indicate that total C, total N, cation exchange capacity (CEC) and Al_{ox} concentration were highly variable in studied forest soils, while pH was relatively stable over the various plots. The comparison of soil treatment showed that mean values of almost all physicochemical properties were slightly higher in control soil plots than in P fertilized plots except soil pH which was slightly lower in control soils. The seasonal variations show that the mean values of total C and total N in autumn were slightly lower in autumn as compared to spring.

Table 2: Descriptive statistics of the soil properties expressed according to sampling campaign (spring / autumn) and soil treatments (Control / +P).

Soil treatment	Descriptive statistics	Total C [a]	Total N [a]	CEC	Water content	pH in $CaCl_2$ [b]	Al_{ox} [c]
		g kg^{-1}	g kg^{-1}	cmol(+) kg^{-1}	%		g $100g^{-1}$ DWS
				Spring			
Control	Mean	28.6	0.99	2.17	4.68	3.25	0.03
	SD [d]	15.0	0.62	0.79	4.93	0.11	0.03
	Range	13.7 – 76.7	0.39 – 2.93	1.05 – 3.87	0.43 – 20.0	3.07 – 3.72	0.01 – 0.12
	n	21	21	21	21	21	21
+P	Mean	27.8	0.94	2.06	4.43	3.30	0.03
	SD	9.63	0.31	0.57	3.20	0.15	0.02
	Range	14.1 – 51.0	0.46 – 1.70	1.18 – 3.04	0.74 – 9.98	3.01 – 3.89	0.01 – 0.06
	n	15	15	15	15	15	15
				Autumn			
Control	Mean	25.4	0.87	- [e]	5.62	3.27	-
	SD	9.58	0.37	-	2.35	0.14	-
	Range	11.3 – 42.3	0.33 – 1.53	-	1.62 – 10.9	3.09 – 3.94	-
	n	22	22	-	22	22	-
+P	Mean	25.1	0.87	-	5.81	3.31	-
	SD	10.7	0.41	-	2.63	0.14	-
	Range	13.8 – 49.4	0.43 – 1.80	-	1.07 – 9.70	3.12 – 3.90	-
	n	15	15	-	15	15	-

a The data of total C and N for autumn campaign were estimated. The estimation was based on relationships derived from the spring database between heat loss weight of sample at 550 °C and total C and N content (please consult material and method for details).
b pH was measured in 10 mM $CaCl_2$ solution and with 1:5 soil-to-solution ratio.
c Al_{ox} = extraction of Al by ammonium oxalate.
d SD = standard deviation.
e No data available.

However, percentages of WC in autumn were higher compared to spring both in control and P fertilized soils. Looking at the minimum and maximum values of total C and total N, the minimum values did not differ largely between two seasons, while the maximum values considerably decreased in autumn when compared with spring soil samples. As against, the minimum range values of WC, both in control plots as well as in P fertilized plots are higher in autumn compared to soils collected in spring.

3.2. Characterization of soil P fractions

The P concentrations belonging to different P pools (Pi-isotopic dilution and $NaHCO_3$, NaOH and H_2SO_4 extractable Pi and Po) are given in figure 1. The comparison between the two methods used to estimate the plant available P in soils of spring campaign, estimated either by a kinetic approach (Pi - isotopic dilution) or a static one (Pi-Olsen), indicated that both methods gave similar results in control as well as in P amended soils (Fig. 1a). Moreover, P fractions were systematically higher but statistically non-significant in P fertilized plots compared to non-fertilized plots. These differences were more observable in Pi fraction than in Po fraction regardless the sampling season (Fig. 1). The bicarbonate and hydroxide extractable Pi and total Po (H_2SO_4) fraction were significantly higher (Wilkoxon signed-rank test, $P < 0.05$) in spring compared to autumn for each treatment (control or +P). Both bicarbonate and hydroxide extractable Po fractions and H_2SO_4 extractable Pi were higher in autumn rather then spring (Fig. 1). The effects were statistically significant only for bicarbonate extractable Po and hydroxide extractable Po in P treated plots. Organic P fraction indicated that total Po (ignition at 550°C) was significantly higher in spring than in autumn while bicarbonate and hydroxide extractable Po represented opposite pattern of variation (Fig. 1).

3.3. Parameters controlling Pi-isotopic dilution

Parameters determining the quantity of diffusive phosphate ions due to physical-chemical reactions at solid-liquid interface in the first minute (Pr1min) and slow reaction over the time (n) were analyzed for control and P fertilized soils (Table. 3). The average values of Pr1min parameter was significantly (Wilcoxon signed-rank test, $P = 0.03$) higher in P fertilized (+P) plots compared to unfertilized (control) plots, whereas, parameter n was not different between +P and control soil plots. However, the values of parameters Pr1min and n were highly variable for both control and +P soil plots (Table 3). RDA results showed that the variance of Pr1min parameter was significantly (Monte-Carlo permutation test, $P = 0.01$)

affected by the age since last fertilization as well as soil total N contents, while variance of the n parameter was only affected by soil properties, particularly Alox concentration and CEC (Table 4). In order to extend our understanding of soil processes involved in the isotopic dilution measurement, correlations were determined between each soil property and parameters Pr1min and n (Table 5). These correlations indicated that Pr1min was significantly and positively correlated with total C, total N, and Al contents as well as CEC and WC of soils. Similarly, n parameter was significantly and positively correlated with Al contents and negatively correlated with H^+ concentration and CEC of soils.

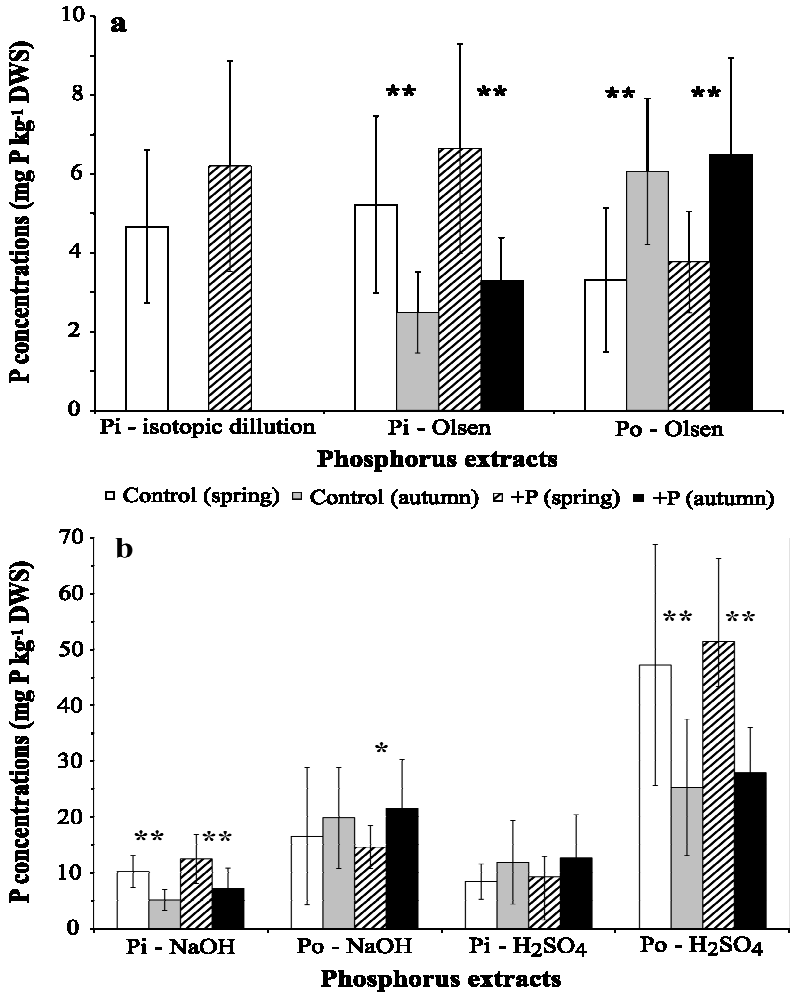

Figure 1: Mean and standard deviation of P measured by isotopic dilution after 30 minutes isotopic dilution experiment (Pi-isotopic dilution) as well as inorganic (Pi) and organic (Po) P measured by Olsen (a), NaOH and H_2SO_4 (b) extracts in both control and P-fertilized plots. For each Pi and Po fraction in soil extracts and for each soil treatment, the seasonal effect between spring and autumn sampling campaign were evaluated by the Wilcoxon signed-rank test on 22 and 15 paired samples for control and P fertilized plots respectively (significant at * $\alpha = 0.05$ and ** $\alpha = 0.01$).

Table 3: Descriptive statistics of physical-chemical reaction (Pr = Pr1min x t^n) used to calculate soil P concentration by isotopic dilution (spring database).

Isotopic dilution parameters [a]	Descriptive statistics [b]	Soils studied		Statistical significance [c]
		Control	+P	p value
Pr1min	Mean	0.37	0.46	0.03
	SD [d]	0.32	0.35	
	Range	0.02 – 0.97	0.08 – 1.26	
N	Mean	0.33	0.32	0.62
	SD	0.10	0.06	
	Range	0.10 – 0.51	0.24 – 0.44	

a For each sample, the values of these parameters were estimated during a 1000 minutes isotopic dilution kinetic. For more details on the isotopic dilution technique, read the material and methods section.
b Descriptive statistics were calculated on all the samples of a given soil treatment (Control: n = 21; +P = 15).
c Mean comparisons of isotopic dilution parameters were assessed using the Wilcoxon signed-rank test on sites having both control and P fertilized plots (12 pairs).
d SD = standard deviation.

Table 4: Summary of RDA describing the relationships between physical-chemical parameters (Pr = Pr1min x t^n) used to calculate soil P concentration by isotopic dilution and environmental factors.

Environmental factors [a]	Pr1min			n		
	Canonical eigenvalues	Adjusted R^2 (%)	Statistics (F) [b]	Canonical eigenvalues	Adjusted R^2 (%)	Statistics (F)
Sample position	0.0014	0.0	0.47	< 0.001	0.0	0.02
Soil treatment	0.0023	0.0	0.73	< 0.001	0.0	0.08
Time since the last fertilization	0.078	59.8	6.20**	0.0024	5.1	1.19
Soil properties	0.047	40.8	25.2**	0.0028	34.8	10.3**

a The effect of each environmental factor were tested using RDA. The sample position (line/interline) and soil treatments (control/+P) were described by one binary variable for each case, whereas time since last fertilization was described by dummy binary variables. As indicated by the forward selection procedure, total N content as well as Al content extracted by ammonium oxalate and CEC were the significant soil properties integrated in the RDA models, describing respectively the Pr1min and n parameters.
b The statistical significance of the RDA was tested using Monte-Carlo permutation test performed under reduced model after 10,000 simulations (** significant at $\alpha = 0.01$).

Table 5: Pearson correlations coefficients between physical-chemical parameters (Pr = Pr1min x t^n) used to calculate soil P concentration by isotopic dilution and, measured soil and tree properties.

Soil properties	Parameters	
	Pr1min	**N**
	r [a]	R
Ctot	0.57**	-0.11
Ntot	0.65**	0.06
CEC	0.42*	-0.34*
WC	0.57**	0.08
H^+	0.08	-0.41*
Al	0.52**	0.45**
Circ : Tree Age	- 0.07	0.21

a Correlations are considered significant at $\alpha = 0.05$ (*) and at $\alpha = 0.01$ (**).

3.4. Factors controlling the variance of soil P fractions

The partition of the variance of Pi and Po fractions ($NaHCO_3$, NaOH, and H_2SO_4 extracts) between soil properties and soil treatments along with age since last fertilization is illustrated in Fig. 2.for both sampling campaigns. In spring and autumn sampling campaignes the exclusive effect associated to soil treatment and age since last fertilization explained 21.5 % and 35 % of adjusted variance of soil P fractions respectively. These fractions were highly significant at $\alpha = 0.01$. Similarly, 30.5 and 23 % of adjusted variance ($P < 0.01$) of soil P fractions were explained exclusively by soil physicochemical properties in spring and autumn sampling campaigns, respectively. The significant soil properties integrated in the statistical models were total N, Al_{ox} and H^+ concentrations for the spring period and total C and H^+ concentrations for the autumn dataset. A small amount of variance of soil P fractions (2 %) was shared between the two groups of explanatory variables in autumn sampling campaign, whereas no similar observation of shared variance occurred for the spring dataset.

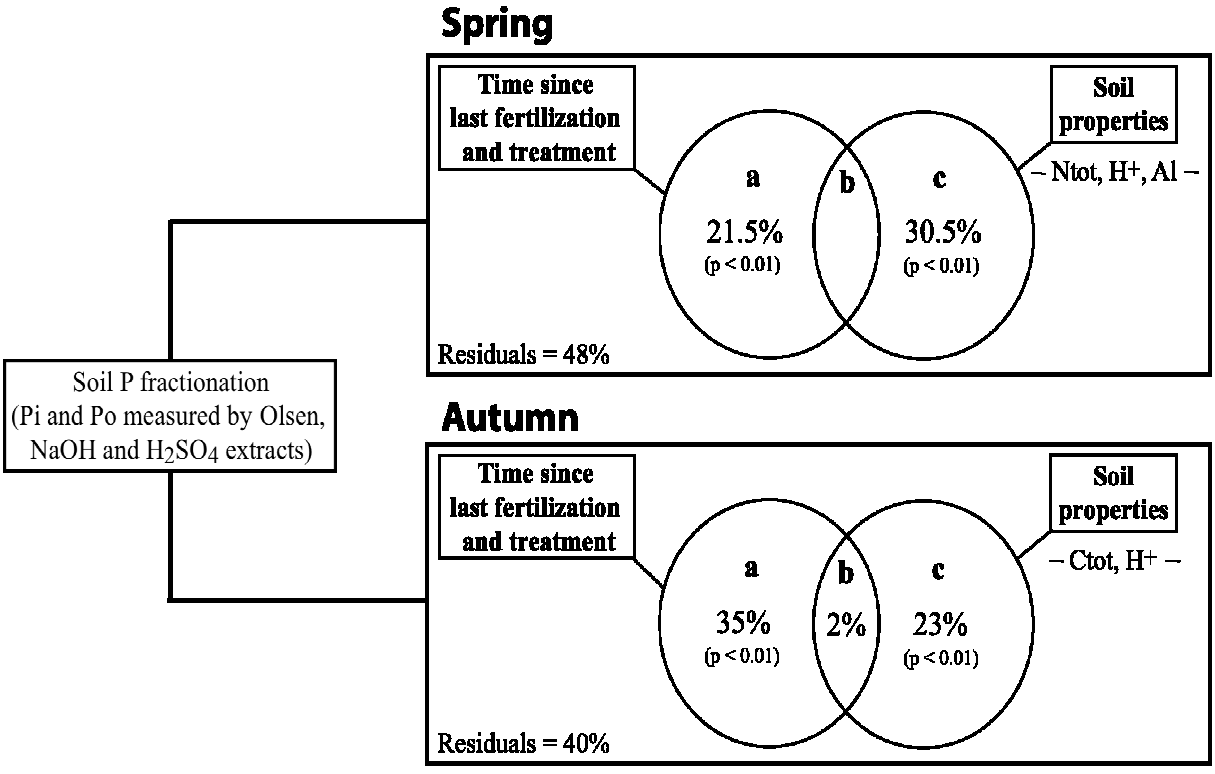

Figure 2: Venn diagrams of partition of the variation of inorganic (Pi) and organic (Po) P concentrations from various soil extracts (Olsen, NaOH and H_2SO_4) for the two sampling seasons. The rectangles represent the total variance of Pi and Po concentrations in various soil extracts while each circle is associated to a given group of significant explanatory variables (soil treatment and age since last fertilization as well as soil properties). The exclusive effect of the Pi and Po concentrations by each group of explanatory variables (surfaces a and c), the co-variation attributed to the two groups of explanatory variables (surface b) and the unexplained variations (residuals) are shown in these diagrams. The adjusted R^2 (expressed in %) of the variance of Pi and Po concentrations for each surface of the Venn diagrams are presented and were obtained from equations described in Legendre and Legendre (1998). Significance of a and c surfaces of the Venn diagrams were evaluated using Monte-Carlo permutation test performed under reduced model after 10,000 simulations.

These results suggested that in this study, slight but statistically non-significant interactions occurred between these two groups of explanatory variables. Finally, the amount of unexplained variance reached 48 % in the spring dataset and 40% in the autumn sampling campaign.

In order to improve our knowledge on how soil properties as well as soil treatment and age since last fertilization controlled the different fractions of P in soil, partial RDA were performed. The partial RDA illustrating the exclusive effect of soil physicochemical properties on soil P fractions of the spring dataset is presented in Fig. 3a. Along the first canonical axis, bicarbonate and hydroxide extractable Pi were strongly correlated to each other and were strongly and positively linked together and were clearly related to H^+ concentration and to a lesser extent to total N contents. Similarly, bicarbonate and H_2SO_4 extractable Po were correlated to each other and were positively related to total N and H^+ concentrations. The second axis of the biplot seemed to better describe the relationships of hydroxide extractable Po and H_2SO_4 extractable Pi with soil properties. Indeed, along this axis, these two P fractions were positively related to soil Al_{ox} concentration.

The results of the partial RDA, illustrating the exclusive effect of soil treatments and age since last fertilization on soil P fractions of the spring sampling campaign, revealed that most of the P fractions were explained by the first canonical axis, which explained a significant fraction of variance set to 14.7 % (Fig 3b). Contrary to the first canonical axis, the second canonical axis explained a non-significant fraction of soil P variance and will not be discussed in this work. Bicarbonate and hydroxide extractable Pi concentrations were highly positively correlated to each other and were positively related to the application of P fertilizers and inversely related to time since last fertilization. The Po fractions did not exhibit high variations, except for bicarbonate extractable Po that showed comparable behavior to bicarbonate and hydroxide extractable Pi (Fig. 3b). The H_2SO_4 extractable Po was also positively related to P fertilizers and inversely linked to time since last fertilization, while hydroxide extractable Po showed completely opposite patterns.

Partial RDA produced on the autumn sampling campaign gave similar results as obtained on the spring dataset. Therefore, the results will not be exhaustively presented and discussed here, but partial RDA biplots are available as supplementary information in Fig 2. The main differences between sampling campaigns were observed in the partial RDA describing the exclusive effect of soil physicochemical properties.

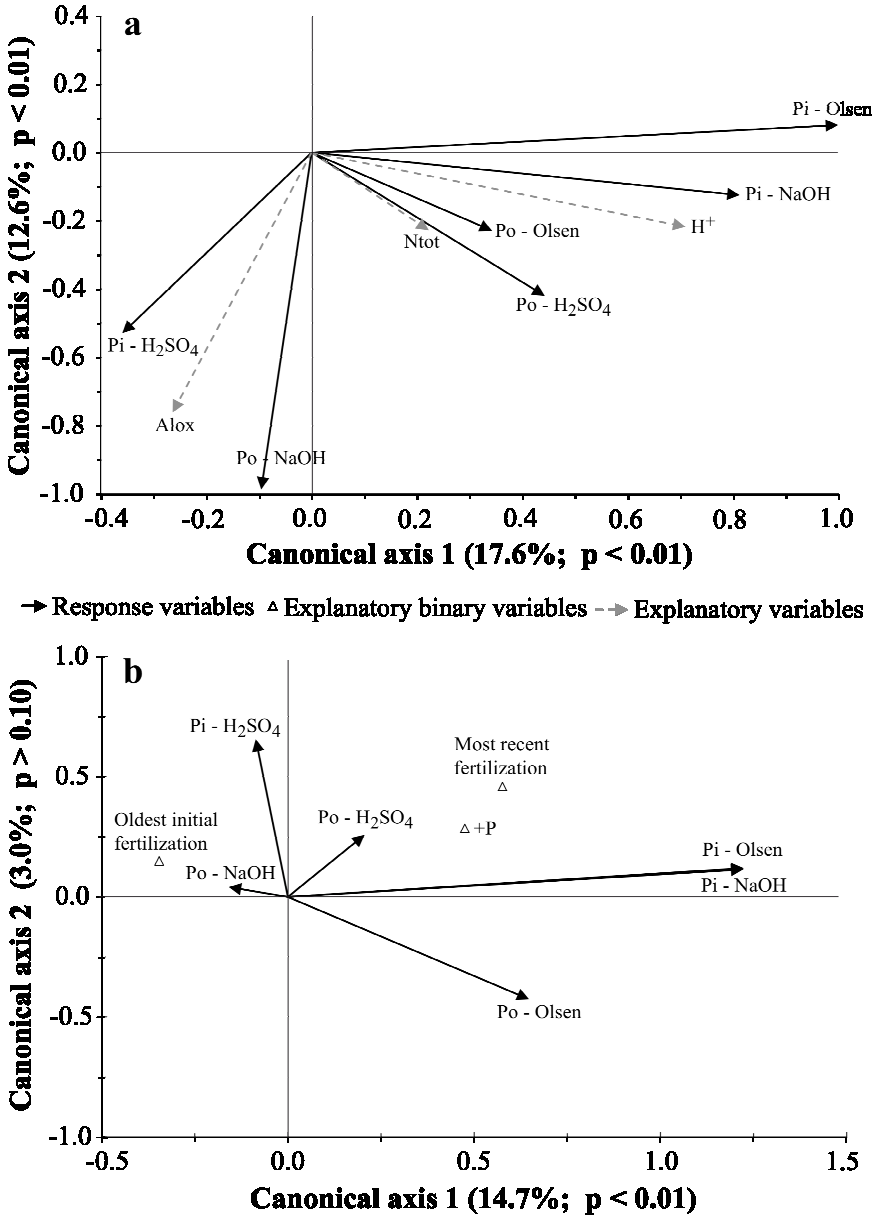

Figure 3: Redundancy analysis ordination biplot (scaling type 2) of forest soil plots representing, either the exclusive links of soil treatment and time since last fertilization (a) or, the exclusive links of soil properties (b) with inorganic and organic P concentrations (Pi and Po respectively) in various soil extracts (Olsen, NaOH and H_2SO_4) of spring sampling campaign. For each biplot, either the influence of soil treatment and time since last fertilization (a) or the soil properties (b) were controlled in order to isolate the influence of the other group of variables. The combination of all canonical axes explained 21.5% (a) and 30.5% (b) of the adjusted significant ($\alpha = 0.01$) variance of Pi and Po fractions. Soil treatment is described by a binary variable showing P fertilized plots (+P) and time since last fertilization by two binary variables expressing the two periods of soil fertilization observed across the whole range of plots (most recent and oldest initial fertilization). Significant soil properties integrated in the model expressed the total content of nitrogen (total N), Al content extracted by ammonium oxalate (Al_{ox}) and proton concentration (H^+).

Indeed, the comparison of the partial RDA from both sampling campaigns revealed that the organic matter behavior, as suggested by total C or N measurements, was more strongly related to soil P fractions than H^+ concentrations in autumn compared to spring. Finally, the effect of stand age on soil P fractions was also evaluated in both spring and autumn sampling campaigns. To isolate this effect, the concentration of soil P fractions of

only the control plots were considered and were grouped according to stand age (young, middle age and old trees) (Fig. 4). The concentrations of Po fractions were increased between 10-60 % in plots with high age forest stand (middle and old) compared to plots with young trees both in spring and autumn.

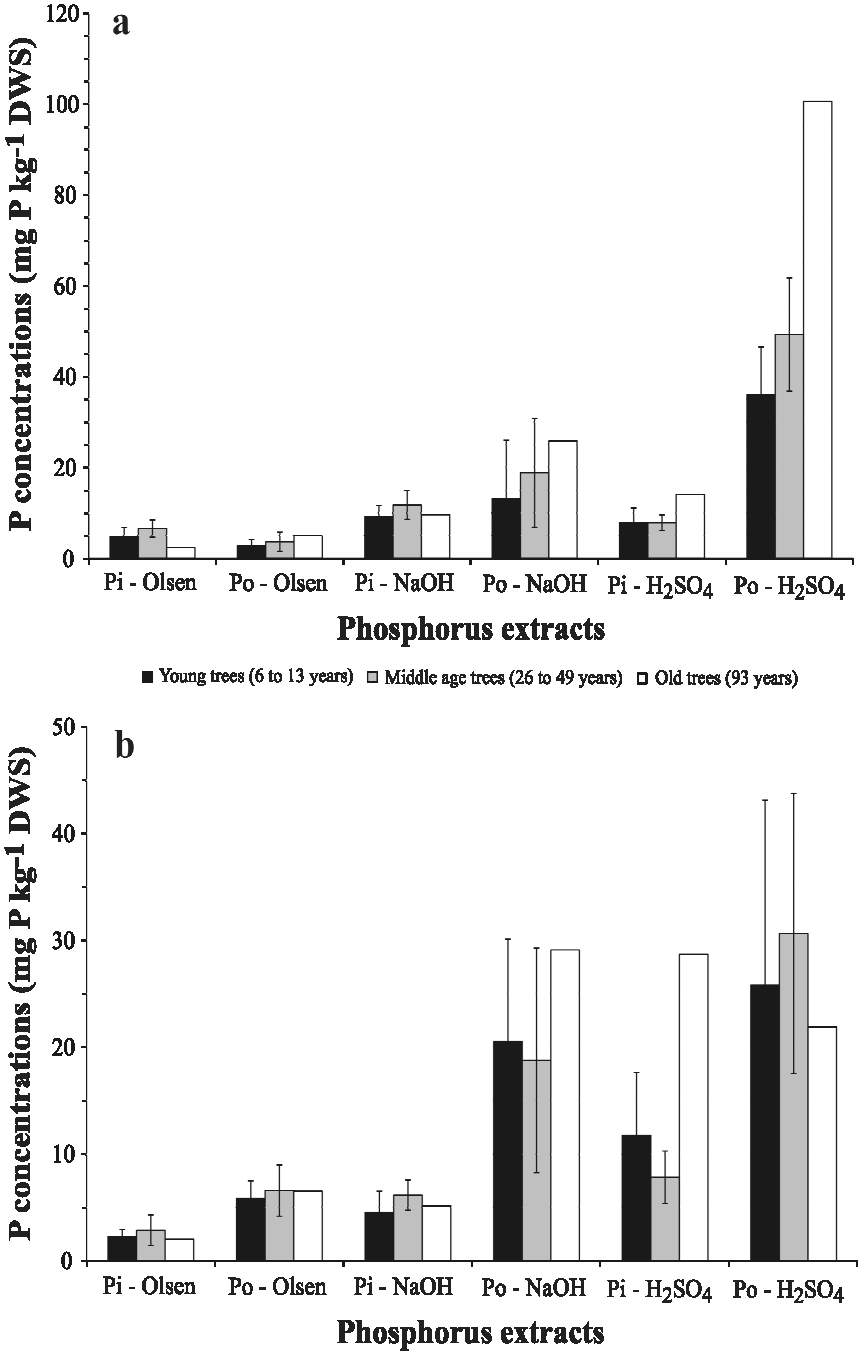

Figure 4: Mean and standard deviation of inorganic (Pi) and organic (Po) P concentrations in various soil extracts sampled in spring (a) and autumn (b) from control treatment plots of *P. pinaster* stands with different ages. The trees age effects on soil P fractions were only investigated on the control plots and the mean comparisons were only assessed between young and middle age trees, because of very few samples classified as old trees.

The increase of Po fractions with trees age were higher (2-3 folds) in spring compared to autumn. However, Po-H_2SO_4 in autumn was strongly decreased in 93 years old tree stands compared to younger tree stands as well as Po-H_2SO_4 concentrations in spring (Fig. 4b). Bicarbonate and hydroxide extractable Pi concentration was higher in plots with middle age stands compared to plots with young and old stands, while Pi-H_2SO_4 was systematically higher in plots with old trees compared to other two (young and middle) age group stands. Bicarbonate and hydroxide extractable Pi was 15 % -55 % higher in spring as compared to autumn depending upon the trees age and soil extract, while Po was lower in the same range of magnitude.

4. Discussion

4.1. Soil physicochemical variables

Soil physicochemical properties, soil treatments, plant age and soil reactivity regarding P availability is important to interpret the results of this study. The brief description of these variables is mandatory to elaborate the objectives of the study. Results mentioned that the physicochemical properties of soils presented high variation among different soils. These variations of physicochemical properties, like $Al_{ox,}$ Fe_{ox}, CEC, OM (soil heat loss), total C and total N were linked with soil water contents as well as the development of soil horizons (Achat 2009). Generally, humid soils contained more OM, CEC, and Al_{ox} as compared to dry soils. In addition, these properties were also affected by fertilizer treatments (+P) and seasons (Table 2). The higher water contents in autumn could be explained by the high precipitation and number of wet days and low temperature in autumn as compared to spring, reported in the region. The pH ($CaCl_2$) also showed large variations between different soil plots as well as seasons. However, the low absolute pH values compared to that of Achat (2009) in these soils could be due to $CaCl_2$ soil solution. According to Page et al. (1982) the lower pH in $CaCl_2$ (0.6 ± 0.2) soil solution is due to easily dissociable and exchangeable protons, while water solution contains only easily dissociable protons.

4.2. P fractions

The hydroxide extractable Po presented the major fraction of total organic P in these soils and the values (3.7-49.8 mg kg^{-1}) were comparable with the results of Nwoke et al. (2003). The high organic fractions of P (upto 80 %) in soil (Condron and Tiessen 2005; Achat 2009) have been investigated to be an important source of P availability in forest ecosystem,

particularly in the systems of unfertilized topsoil (Beck and Sanchez 1994). Application of mineral P fertilizer increased bicarbonate, hydroxide and H_2SO_4 extractable Pi (Börling et al. 2004) as well as Po concentrations but the effect was not statistically significant (Fig. 1). Similarly, the Pi-isotopic dilution was also increased in fertilized plots. This shows that a part of P applied increased inorganic fraction and the rest was immobilized, adsorbed or precipitated, hence reducing the differences of Pi in soil solution (Frossard et al., 2000; Hinsinger 2001) of fertilized treatments. Moreover, a significant amount of Pi could be taken up by plants resulting into high plant growth ($P < 0.05$) in fertilized plots compared to unfertilized plots (Payn et al. 2000; Fox et al. 2006; Trichet et al., 2009).

The Pi isotopic dilution (30 minutes of kinetics) and bicarbonate extractable Pi fractions indicated comparable values, suggesting that both methods were probably characterizing same pool of P in these soils. Both, bicarbonate (Olsen et al 1954) and Pi by isotopic dilution (Bühler et al., 2003) provide information about the P availability for plant uptake. However, the Pi-isotopic dilution provides supplementary information like intensity, quantity and capacity of Pi availabilityity (Fardeau 1996; Frossard and Sinaj 1997; Frossard et al., 2000; Bühler et al., 2003). We used Pr1min and n parameters, to describe availability of P, the effect of environmental factor on these parameters and their relation with soil and tree properties. The significant effect of fertilizers on Pr1min parameter suggested that application of fertilizers increased availability of instant P in these soils while fertilizers did not affect significantly n parameter. Similar type of information could be drawn from scores of redundancy analysis, (Table 4) showing positive relation with time of fertilization. Nevertheless, soil properties significantly controlled both Pr1min and n parameters as explained by redundancy analysis. Pearson correlation with individual parameters of soil properties further explained that both Pr1min and n parameters showed better correlation with Al_{ox}. It could suggest that Al_{ox} is the variable which explained the capacity of sorption of P in acid soils (Daly et al., 2001; Maguire et al. 2001; Villapando and Graetz 2001; Börling et al. 2004; Horta and Torrent 2007). The parameters related to organic matter (total C, total N, water contents and CEC) were positively correlated with Pr1min parameter. It suggests that organic matter can reduce fixation of P or it inhibits adsorption of P on inorganic surfaces (Iyamuremye and Dick 1996; Brady and Weil 2002). Mechanism responsible for inhibiting adsorption could be large humic molecules, organic acids produced during organic matter decomposition adhered at the surface of metal hydrous oxides and chelation of low molecular weight organic acids with Al and Fe, preventing the fixation of P at adsorption sites (Iyamuremye and Dick 1996; Brady and Weil 2002; Santruckova et al. 2004).

4.3. Seasonal variation of P fraction

The seasonal variation in phosphorus pools in forest soil has not been studied extensively. In the limited numbers of studies, the difference in seasonal pattern of organic and inorganic P fractions could be explained by the variation in soil temperature, moisture and plant uptake over the seasons and soil types (Mcgrath et al. 2000; Chen et al. 2003). The amount of inorganic P in soils is also controlled by the factors, like adsorption-precipitation of inorganic P, microbial immobilization and mineralization (Perrott et al., 1990; Frossard et al. 2000; Hinsinger et al. 2001). In this study the concentration of bicarbonate and hydroxide extractable Pi was significantly higher in spring as compared to autumn. However, the bicarbonate and hydroxide extractable Po concentration were significantly higher in autumn compared to spring (Fig. 1). The results are in accordance with the findings of Magid and Nielsen (1992), in pasture grassland and arable soils of Denmark and Fabre et al. (1996) in temporarily flooded riparian forest soils of France. This could be interpreted by an increased rate of mineralization of labile Po by the microbial activity combined with a low Pi uptake by plant during winter, resulting in the decrease of Po and increase of Pi pools in spring. In contrast, active plant growth and increased organic inputs during the growing season (late spring and summer) could decrease and increase Pi and Po concentrations, respectively. The decrease of total Po (H_2SO_4) in autumns also suggest that a significant amount of P has been immobilised and taken up by plant (both pine and understory annual vegetations) during high growth period (spring and summer). The P taken up by plants would be returned into soil reserves by degradation of litter fall and annual vegetations during the coming winter and autumn. Nevertheless, high abrupt decrease of total Po in some sites (Baudes Vielle and Mimizan) could also be suspected due to spatial variation along with seasonal variations.

4.4. Factors controlling variance of soil P fractions

The partition of the variance of soils inorganic and organic P fractions was explained in terms of soil properties and treatment with age since last fertilization for both spring and autumn (Fig. 2). These two sets of studied variables explained significantly over 50 % of variance, showing some seasonal shifts between spring and autumn. In spring the high proportion of variance controlled by soil properties could be due to biological processes (Chen et al. 2003) or change in solubility (Magid and Nielsen 1992). These processes are influenced by soil moisture and temperature (Tate et al. 1991; Fabre et al. 1996). The high rate of mineralization of organic matter in spring (Perrott et al, 1990; Tate et al. 1991; Chen et al. 2003), which is highly related to total C, total N and Po fraction (Achat 2009), could

highlight the importance of soil properties in explaining high proportion of variance. However, soil treatments and age since last fertilization is considered to affect plant growth (Trichet et al. 2009), altering canopy size and litter turnover in the soils. In this way soil treatments and age of fertilization could be suggested to control larger proportion of variance (35 %) in autumn through immobilization of Po as well as organic matter (Harrison 1987; Perrott et al. 1990; Tate et al. 1991; Chen et al. 2003). The proportion of residual variance in these spodosols might be explained by many factors, like podzolization (Kitayama et al., 2004), depth of water table (Achat et al. 2009), soil formation and type of understory vegetations (Trichet et al 1999, Augusto et al. 2006), microbial and soil enzyme activities (Wang et al., 2008), other nutrients like N and K (Trichet et al. 2009) and other processes controlling P availability in soils (Frossard et al. 2000).

For strengthening our understanding about the explanatory and response variable in partition analysis, redundancy analysis were performed on data sets of both spring and autumn. Results indicate that labile fractions of Pi (bicarbonate and hydroxide) were related to soil pH (Fig. 3a) controlling the processes of sorption and desorption (Hinsinger 2001) and the addition of fertilizers and age of fertilizer since last application. The effect of age of fertilizer or age of forest stand (Fig. 4) suggested that as soil developed, the part of labile Pi became unavailable by immobilization into organic or occluded form (Walker and Syers 1976; Frizano et al. 2002; Frizano et al. 2003). Valdespino et al (2009) suggested that available pools of Pi decreased with forest age while total phosphorus did not change with forest age. Moreover, the organic fractions of P (bicarbonate and H_2SO_4) were strongly associated with the organic matter while hydroxide extractable Po was associated with Al_{ox} present in these acid soils, which is in agreement with the idea of P sorption with Al_{ox} and organic matter (Barrow 1987; Frossard et al. 1995; Hinsinger 2001; Celi and Barberis 2005). Bicarbonate extractable Pi in the acid soils was in accordance with the hypothesis of Kuo (1996) who suggested that Olsen method could be used for both acid and alkaline soils. In this study bicarbonate extractable Pi presented comparable values with Pi-isotopic dilution (30 minutes) suggesting the pertinence of Olsen method in acid soils.

5. Conclusion

Along with both labile (bicarbonate) and moderately labile (hydroxide) fractions of P, the total contents of P were very low in these soils. Organic fractions represented major proportion of total P (up to 80%) in these soils. Application of P fertilizer increased all fractions of P as compared to control plots but the effect was not statistically significant.

However fertilization increased significantly parameter Pr1min compared to control, suggesting that fertilization increased readily available P for plant uptake. The remarkable significant seasonal variation was observed in both bicarbonate and hydroxide extracts. Pi fractions were higher in spring compared to autumn but Po fractions were dominant in autumn for both bicarbonate and hydroxide extractions. As against, the results for H_2SO_4 extractable Pi and Po were opposite. Over 50 % of the variance in P pools was explained by soil treatments and its properties. The fertilization and H^+ were positively controlling labile and moderately labile Pi fractions, however concentrations of H^+ was more important for moderately labile (NaOH) Pi fraction. Moreover, bicarbonate and Pi-isotopic dilution represented comparable value for both control and fertilized soil. The total C, total N and organic matter were highly correlated to each other and were principally controlling Po fractions and partly Pi fractions except hydroxide extractable Po and dilute acid extractable Pi which was positively controlled by Al_{ox}. Similarly, soil properties (physicochemical) were also playing their role in regulating concentration of Pi-isotopic dilution. Further research at large scale, with more explanatory variables (biotic and abiotic) would help to increase our understanding about the variation of P in forest ecosystem.

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7. Supplementary data

Table 1: Soil properties and P concentrations in various soil extracts sampled from fields of *Pinus pinaster* in spring 2006.

Sites code[a]	**pH**[b]	**Water content**	**Total C**	**Total N**	**Olsen extract**		**NaOH extract**		**H_2SO_4 extract**	
					Pi[c]	Po	Pi	Po	Pi	Po
		% (w/w)	------- g kg^{-1} -------		---------- mg P kg^{-1} ----------					
1	3.42	1.04	14.9	0.46	2.85	1.36	6.18	7.92	6.03	23.6
2	3.40	0.88	21.0	0.64	3.69	1.14	6.69	3.74	4.42	28.2
3	3.40	0.87	22.6	0.75	4.24	2.18	10.6	14.9	4.38	30.4
4	3.38	1.26	21.2	0.72	5.86	3.03	9.38	8.08	6.34	36.3
5	3.20	9.98	51.0	1.70	7.10	4.36	13.3	18.6	5.46	59.1
6	3.34	8.76	36.8	1.21	4.69	3.31	9.55	9.14	13.8	42.2
7	3.30	20.0	76.7	2.93	2.41	2.98	9.04	21.9	15.6	101
8	3.30	15.6	60.8	2.33	2.32	7.06	10.1	29.7	12.4	99.8
9	3.27	2.24	21.9	0.72	4.97	3.25	10.1	11.4	5.67	34.9
10	3.39	5.31	21.1	0.74	5.53	5.04	11.2	17.9	9.57	29.1
11	3.42	4.96	26.5	0.97	11.1	4.74	21.8	18.6	14.9	54.7
12	3.26	4.46	24.3	0.72	5.37	2.05	8.88	9.81	11.8	43.0
13	3.24	3.76	33.2	0.96	8.90	2.60	13.9	10.6	6.71	49.2
14	3.09	5.28	20.6	0.54	5.42	2.37	9.72	41.4	8.37	49.0
15	3.07	2.71	19.7	0.52	4.15	2.43	9.04	7.81	7.89	42.9
16	3.22	9.41	24.0	1.22	1.88	2.84	8.37	49.8	13.0	56.2
18	3.89	5.12	22.3	0.97	3.87	4.30	9.72	20.0	12.6	55.6
19	3.64	5.23	19.8	0.87	3.14	3.26	8.88	14.4	9.83	43.5
20	3.72	0.82	27.1	0.75	4.69	4.07	10.7	11.0	7.21	44.9
21	3.11	1.66	25.3	0.76	8.12	4.60	13.9	11.8	6.34	44.7
22	3.20	2.82	37.3	1.01	7.70	3.65	12.9	15.3	10.0	61.6
23	3.10	6.83	30.6	0.91	10.5	4.32	18.8	14.6	13.5	45.2
24	3.19	0.54	15.2	0.42	3.49	1.86	7.36	6.74	4.55	31.9
25	3.26	0.43	13.7	0.39	2.83	2.70	6.69	3.13	13.0	21.8
26	3.25	1.02	20.4	0.57	5.79	2.20	9.72	16.0	6.92	37.2
27	3.29	0.74	27.7	0.84	7.31	4.37	10.6	12.1	7.01	44.7
28	3.28	1.55	25.8	0.87	6.56	1.75	9.04	12.1	10.8	36.2
29	3.22	4.17	30.1	1.02	7.74	1.95	10.4	9.21	5.53	43.7
30	3.31	0.85	18.0	0.63	5.88	2.73	10.9	12.4	14.0	44.0
31	3.46	8.15	14.1	0.46	3.09	2.14	6.35	9.28	6.45	55.1
32	3.32	4.69	24.8	1.00	5.61	3.44	11.4	29.3	7.21	32.7
33	3.20	4.36	25.0	1.04	5.61	2.37	9.89	8.80	6.71	41.9
34	3.01	2.81	34.2	1.17	9.00	6.98	18.1	19.8	5.00	86.7
35	3.27	7.06	33.8	1.25	10.3	4.79	16.1	15.4	8.51	73.6
36	3.19	4.38	36.6	1.38	8.98	6.04	18.3	21.2	6.65	68.9
37	3.13	5.00	38.1	1.40	8.42	7.73	12.9	21.7	7.03	66.3

a The plot No. 17 was excluded from database of April 2006 due to analytical problem during the measurement of Pi and Po concentrations extracted with H_2SO_4.

b Soil pH was measured in 10 mM $CaCl_2$ solution using 1:5 soil-to-solution ratio.

c Pi = inorganic P concentration; Po = organic P concentration obtained by the difference between total P and Pi concentrations.

Table 2: Soil properties and P concentrations in various soil extracts sampled from fields of *Pinus pinaster* in autumn 2006.

Sites code	pH[a]	Water content	Total C[b]	Total N[b]	Olsen extract		NaOH extract		H_2SO_4 extract	
					Pi[c]	Po	Pi	Po	Pi	Po
		% (w/w)	g kg^{-1}		mg P kg^{-1}					
1	3.35	4.71	15.0	0.47	1.50	3.11	1.79	12.0	17.9	14.7[d]
2	3.34	4.78	15.0	0.47	1.73	3.44	1.80	9.92	16.9	13.4[d]
3	3.28	6.45	20.1	0.67	2.13	4.35	2.79	16.4	17.6	18.9
4	3.32	4.56	16.9	0.55	2.42	5.97	3.62	14.0	16.9	17.3
5	3.12	9.41	49.4	1.80	3.13	7.43	6.30	29.6	20.7	39.6
6	3.20	8.90	48.5	1.77	3.83	7.59	8.41	27.6	33.1	30.9
7	3.11	8.37	39.5	1.42	2.13	6.07	5.47	28.6	29.6	25.1
8	3.18	6.96	38.0	1.36	1.88	6.94	4.79	29.5	27.7	18.7
9	3.48	4.72	22.2	0.75	2.21	6.97	6.65	20.8	22.0	7.26
10	3.53	5.00	22.4	0.76	2.44	5.74	5.82	24.7	19.0	22.4
11	3.43	6.18	25.9	0.89	5.43	11.4	14.6	36.0	21.2	21.3
12	3.15	4.89	17.2	0.56	2.72	4.24	8.39	6.07	12.5	16.5
13	3.28	4.92	12.5	0.38	1.51	3.55	8.12	5.92	8.44	12.0
14	3.12	9.56	28.3	0.99	6.26	10.8	5.36	12.5	9.74	26.0
15	3.12	5.28	22.5	0.76	2.67	4.50	5.99	14.8	8.47	23.1
16	3.94	8.10	28.2	0.98	1.58	7.30	4.33	33.7	12.5	43.3
17	3.86	10.9	36.0	1.28	1.66	8.23	6.33	38.3	11.3	67.6
18	3.90	5.24	16.5	0.53	2.29	6.18	15.1	15.6	9.47	29.6
19	3.87	8.79	23.5	0.80	2.74	5.84	8.66	32.8	10.4	35.6
20	3.12	8.53	31.9	1.12	3.85	7.19	7.64	21.3	8.03	35.6
21	3.09	5.58	30.3	1.06	2.92	7.14	5.64	27.3	8.98	36.3
22	3.15	9.70	30.2	1.06	2.99	8.34	5.85	26.4	9.05	29.1
23	3.21	6.00	26.2	0.91	3.38	6.81	8.06	27.0	7.92	30.4
24	3.30	4.36	11.3	0.33	2.29	4.62	2.14	7.43	4.50	9.97
25	3.24	4.10	15.8	0.51	2.21	4.40	3.66	21.2	5.68	14.8
26	3.23	4.93	27.7	0.96	3.46	7.30	5.82	16.2	6.72	32.2
27	3.26	5.11	20.4	0.68	2.05	5.26	4.68	10.7	6.32	20.2
28	3.31	6.62	20.6	0.69	2.76	5.58	4.98	18.9	7.28	24.3
29	3.26	6.11	20.5	0.68	1.74	6.16	3.33	10.6	6.61	19.7
30	3.36	5.12	15.4	0.49	3.92	2.83	6.52	10.7	7.58	16.7
31	3.43	4.61	13.8	0.43	5.47	1.27	5.16	8.02	7.69	21.1
32	3.26	1.62	23.1	0.79	2.29	6.62	5.01	26.3	5.71	42.0
33	3.38	3.14	23.7	0.81	2.68	7.65	5.17	26.4	6.10	47.6
34	3.26	1.07	21.1	0.71	2.20	8.67	4.35	24.9	6.38	35.7
35	3.32	1.12	21.6	0.73	3.93	7.98	8.58	25.9	8.20	39.5
36	3.35	2.47	41.7	1.50	2.21	8.27	6.37	34.8	6.03	42.4
37	3.10	2.87	42.3	1.53	2.44	6.89	4.68	23.0	5.47	35.3

a Soil pH was measured in 10 mM $CaCl_2$ solution using 1:5 soil-to-solution ratio.

b The data of total C and N for November 2006 were estimated. The estimations were based on relationships derived between heat loss weight of soil samples at 550 °C and total C and N content from database of April 2006 (please consult material and method for details).

c Pi = inorganic P concentration; Po = organic P concentration obtained by the difference between total P and Pi concentrations.

d Corrections were applied to these data to minimize analytical problems occurring during the measurement.

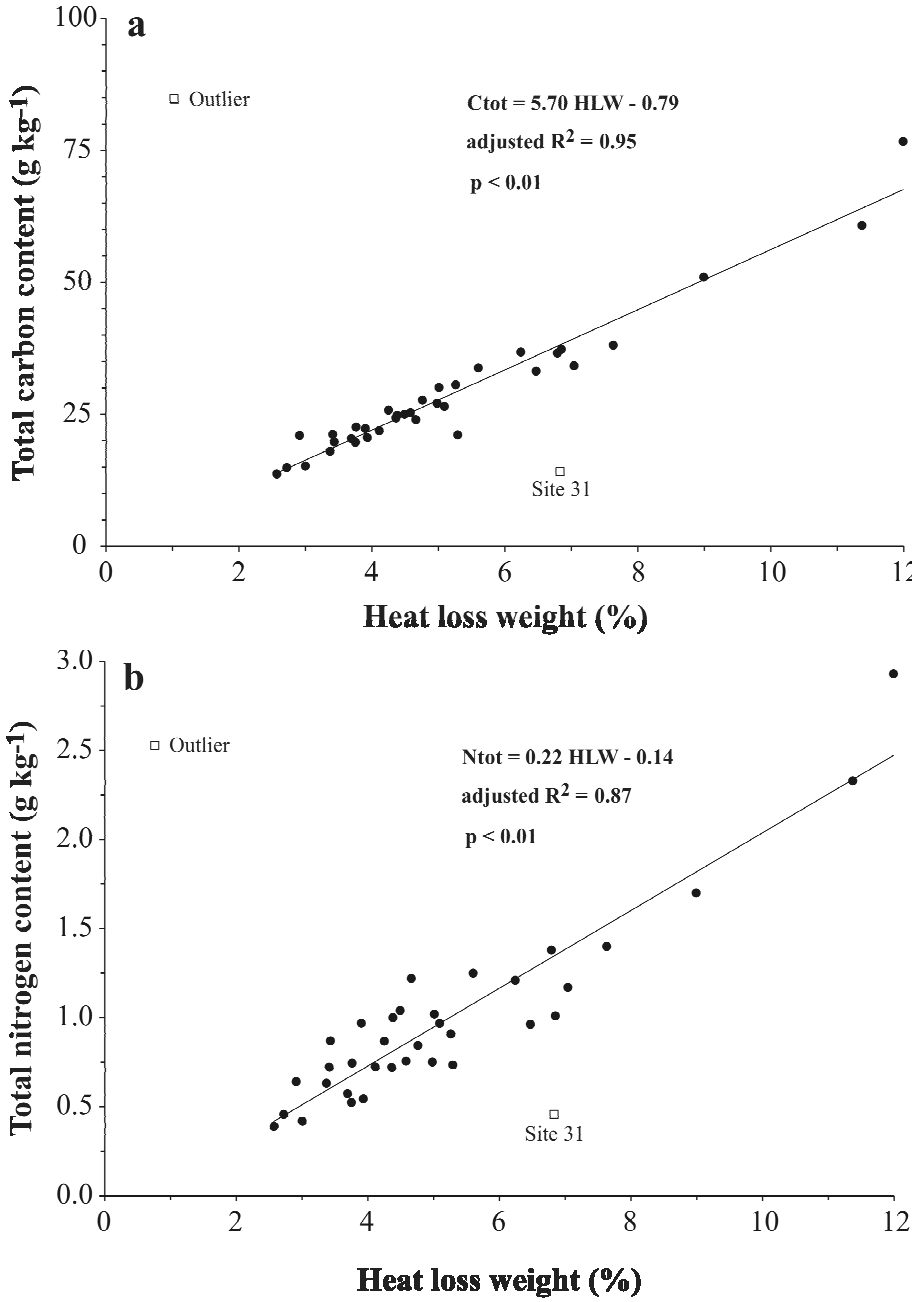

Figure 1: Relationships between heat loss weight (HLM) of soil samples at 550°C with total C (a) and N (b) contents. These relations were derived from the spring sampling campaign and were used to estimate the total C and N contents of soil sampled in autumn. An outlier was excluded from linear regression model when drawing these relationships.

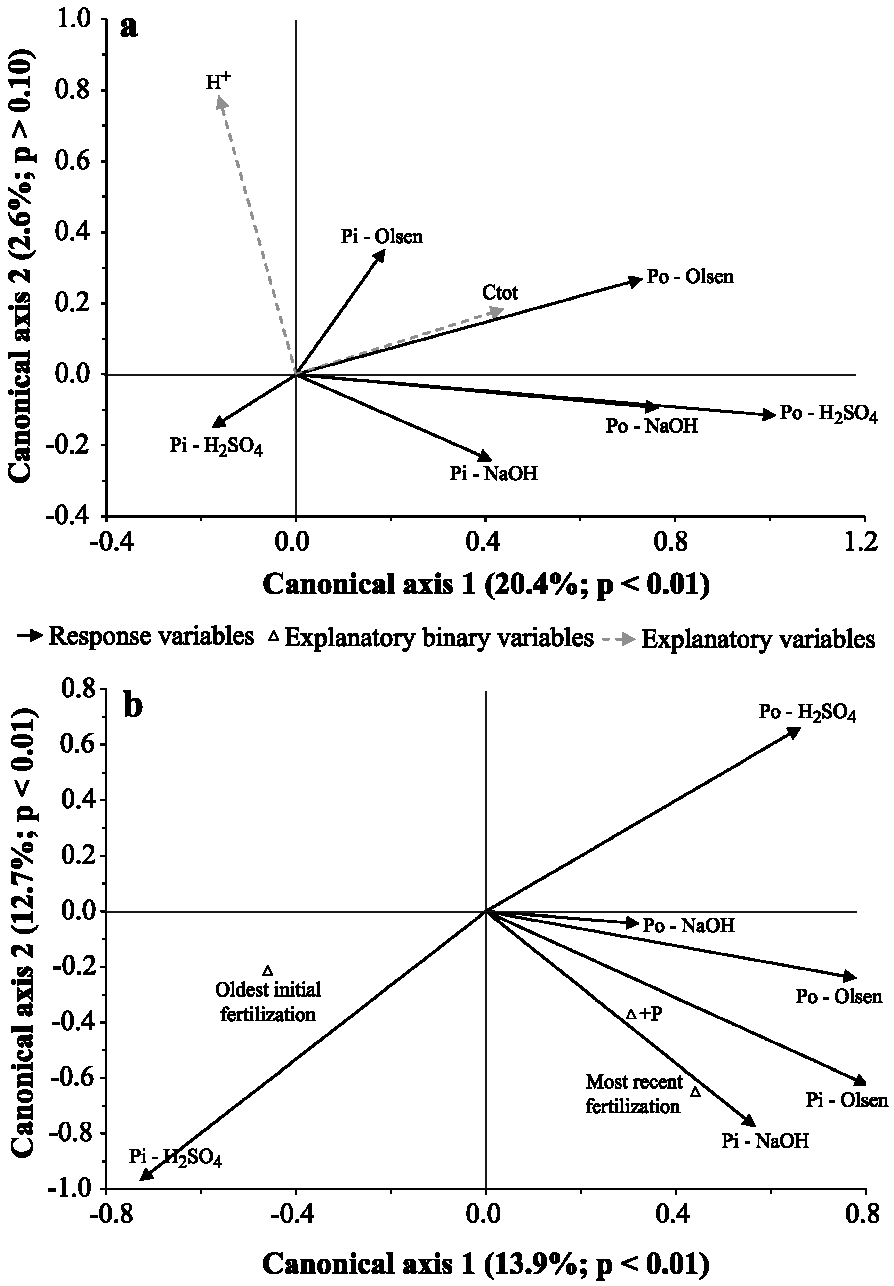

Figure 2: Redundancy analysis ordination biplot (scaling type 2) of forested soil plots representing, either the exclusive links of soil properties (a) or, the exclusive links of soil treatment and time since last fertilization (b) with inorganic and organic P concentrations (Pi and Po respectively) in various soil extracts (Olsen, NaOH and H_2SO_4) of the autumn sampling campaign. For each biplot, either the influence of soil treatment and time since last fertilization (a) or the soil properties (b) were controlled in order to isolate the influence of the other group of variables. The combination of all canonical axes explained 35% (a) and 23% (b) of the adjusted variance of Pi and Po fractions and were both statistically significant at $\alpha = 0.01$. Soil treatment is described by a binary variable showing P fertilized plots (+P) and time since last fertilization by two binary variables expressing the two extremes times of soil fertilization observed across the whole plots studied (most recent and oldest initial fertilization). Significant soil properties integrated in the model expressed the total content of carbon (total C) and proton concentration (H^+).

CHAPTER 3

Acid phosphatase activity of ectomycorrhizal fungi and their abundance as affected by soil and plant properties, in *Pinus pinaster* forest

In the previous chapter it is observed that in 37 sampling positions the Pi and Po concentrations of different fraction showed large variation, which is partly controlled by soil properties and partly by the soil treatments. In holistic terms over 50 % of variance was explained by soil and environmental variables used in the study and up to 80 % of total P was present in the form of organic P fractions. This large fraction of total P in the form of Po is often not accessible for plant uptake, unless it is not hydrolysed into Pi fractions (orthophosphates) in soil solution. Roots of *Pinus pinater* trees are associated symbiotically with ectomycorrhizal fungi. These ectomycorrhizal fungi are known to secrete phosphatases enzymes, which can hydrolyse Po compounds in soil.

The concentrations of Pi and Po fractions were assayed in the first 15 cm of mineral soil horizon after removing superficial litter layer. The objective to use 15 cm mineral soil horizon was to use soil layer which is potentially bio-influenced (Fungi, bacteria and roots).Taylor and Bruns (1999) suggested that around 90 % of fine roots are located in the first 15 cm of soil and an equal proportion of ectomycorrhizae could be associated with the fine roots.

In this chapter (Chapter 3) we focused to determine:

1) What is the morphological diversity of ectomycorrhizae associated with fine roots of *Pinus pinaster*?
2) What are the indicators which could influence the activity phosphatase of ectomycorrhizae associated with fine roots of *Pinus pinaster*?

Seven soil cores (15 cm length x 8 cm diameter) were taken from top 15 cm of mineral soil horizon after removing the surface litter layer. These soil samples were successively sieved through 5 mm and 2 mm sieves in order to sort out coarse and fine roots respectively. Only fine roots showed maximum mycorrhization and almost all fine roots were mycorrhized. The mycorrhizal root apex were detached from roots and then characterized into myrphotypes on the bases of morphological characteristics (Agerer 1987-1999). Each morphological unit was given an alphabetic name called ectomycorrhizae (ECM).

Four replicates of each of the ECM morphotypes with sufficient number of apex were then used to assay their phosphatase activity. The ECM(s) were incubated in the *para*-nitrophenyl phosphate (*p*NPP), which is a synthetic monoester.phosphate. The extracellular phosphatase activity of each ECM was assayed immidiatly after removing from soil samples in order to observe phosphatase activity. These phosphatase activities were used to evaluate their functional diversity by using appropriate statistical analysis. The morphotypes showing more then 90 % of the total specific phosphatase activity of entire data base were used to identify the new indicators, which could potentially control the regulation of phosphatase activity of ECM morphotypes. The explanatory variables used to evaluate the response variable (phosphatase activity) were the different fraction of Pi and Po (Olsen, NaOH and H_2SO_4), the environmental factors (water contents of the soil at the time of sampling) and plant paramenters (fine root biomass, fine root density, specific root length, age of the plantation etc).

The data obtained is used to compose a scientific article for a pre-reviewed "*FEMS microbiology ecology"* titled as "Acid phosphatase activity of ectomycorrhizal fungi and their abundance as affected by soil and plant properties, in *Pinus pinaster* forest"

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CHAPTER 3

Acid phosphatase activity of ectomycorrhizal fungi and their abundance as affected by soil and plant properties, in *Pinus pinaster* forest

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Ready for submission in FEMS microbiology ecology

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Abstract

The diversity and phosphatase activity of ectomycorrhizal (ECM) fungi on fine root tips was studied in mono-specific *Pinus pinaster* forest. The ECM fungi were isolated from bulk soil samples of representative plots in a range of over 1 million ha forest. The soil P fraction and fine roots biomass, density and specific root length were estimated. Using correspondence and canonical correspondence analyses, the diversity of structure and phosphatase activity of different ECM morphotypes was determined. Similarly, the relations between soil and plant properties with diversity of ECM and phosphatase activity were developed. Results showed the presence of 19 ECM morphotypes randomly distributed with respect to presence/absence as well as abundance. The acid phosphatase activity was also highly variable within the same morphotype and plot as well as between morphotypes and plots. The correspondence analysis with soil and plant properties showed that phosphatase activity of ECM was low in plots with high Pi-Olsen or P fertilizers treatments. Moreover, important number of ECM fungi was linked with fine root parameters, both in terms of abundance and phosphatase activity. Significant relationships of soil treatments and soil properties (Pi-Olsen, water contents and Po-H_2SO_4) were observed with the abundance of ECM, whereas for acid phosphatase activity, it was sampling position and soil properties (Pi-Olsen, total N and water contents). Further research should focus on relating edaphic and molecular characterization to attain better understanding of this complex soil-plant-fungal interface.

Key words: *enzyme activity, fungal diversity, fine roots, P fraction, environmental factors, water contents,*

1. Introduction

The fine roots of forest stands in temperate region are associated symbiotically with large diversity of fungal species which develop a structure called ectomycorrhizae (Horton and Bruns 1998; Dahlberg 2001; Landeweert et al. 2003). These ectomycorrhizal (ECM) fungi may contribute to one third of microbial biomass in forest soils (Högberg and Högberg 2002). The various studies have examined the communities of these ECM fungi on relatively small area (Bruns 1995; Horton and Bruns 2001). However, results obtained at a large scale may change our comprehension of community structure and function. These ECM communities have been determined either by taking bulk soil samples and their subsequent analysis to isolate ECM present on root tips, or by making survey of fruiting bodies. However, surveying fruiting bodies hardly represent ca 20-30 % of mycorrhizal species (Gardes and Bruns 1996; Jonsson et al. 1999). Thus, it is inevitable to take bulk soil samples of forest soils, and appropriate methods to sort out ECM fungal community associated with fine roots. Characterization of ECM fungi may be done using morphological characteristics (Agerer 1987-1999) or molecular techniques or now more commonly a combination of both (Koljalg et al. 2000; Horton and Bruns 2001). Community diversity is usually documented as two components, species richness i.e. the number of species in the community and community evenness i.e. a measure of the abundance of each species in the community (Magurran, 1988; Nantel and Neumann. 1992).

The community structure of ECM on root system may be related to nutrient uptake efficiency from the soil solution of different mycorrhizal species (Dighton 1995). It could also be affected by the quality of litter (Conn and Dighton 2000), competitive interaction between colonizing mycorrhizal fungi (Shaw et al. 1995), forest ageing (Last et al. 1987), forest disturbances like forest fire, clear-cutting and thinning (Jones et al. 2003; De Roman and De Miguel 2005; Buée et al. 2005), nitrogen deposition (Lilleskov et al. 2002), irrigation and fertilization (Jones et al. 1990; Fransson et al. 2000; Bakker et al 2009).

These ECM fungi are reported to play a crucial role in determining ecosystem health through their functions like the secretion of enzymes, particularly acid phosphatase (Antibus et al. 1992; Chen et al. 2002; Courty et al. 2005; Mosca et al. 2007) in low P status soils. Acid phosphatases are hydrolytic exo-enzymes which are known to release inorganic P from the soil organic P sources (Tibbett et al. 1998). The acid phosphatase activity of mycorrhizae is up or down regulated by P availability in soil (Chen et al. 2003a, b; Criquet et al. 2007), mineral or organic amendments (Criquet et al. 2008) and P demand by fungi or plant host.

A mono-specific *Pinus pinaster* forest has been established on nearly one million hectare in Landes of Gascogne situated in south west of France. The soils are sandy acidic podzols with 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 results in low P availability that is considered as the main limiting factor for tree growth (Bonneau 1995, Trichet et al 2009). On the other hand, these sandy podzols present organic P up to 80% of total P (Achat et al., 2009), suggesting that phosphatase activity released by ECM fungi may enhance Pi availability by the hydrolysis the Po pools. In this forest, large scale field trials aiming at measuring the effects of P fertilization on tree growth have been established and plots with different stand age, understory vegetation and organic matter accumulation as well as water contents (Augusto et al. 2006) are available. To date, no comprehensive study has been conducted at this large forest ecosystem to evaluate ECM diversity and functions, especially at the level of phosphatase activity. Objective of the present study was to assess the functional diversity of ECM fungi associated with *P. pinaster* roots in the field along a gradient of soil and plant properties. It was hypothesized that different soil P availability, modified by P fertilization practices as well as age of plantations, may influence ECM community structure and phosphatase activity. The soil samples were collected from 18 plots of *P. pinaster* in which age and growth of trees was known. Living ECM tips associated with fine roots were extracted and grouped according to morphological characteristics (colour, form etc) before measuring phosphatase activity in individual morphotype. We also measured fine root and soil physicochemical characteristics. Finally, we used statistical approaches to assess the effect of soil and plant properties on the ECM community structure and the regulation of acid phosphatase activity.

2. Material and Methods

2.1. Description of experimental station

The experimental station known as Landes of Gascogne is located in southwest of France. Soils have developed from a coarse sandy Aeolian parent material deposited in the Pleistocene and are characterized as acidic, less fertile with highly organic matter. These can be classified as Entic to Albic spodosols (Food and Agriculture Organization (FAO)/International Union of Soil Science 2006). Depending on the depth of water table, the lenses of a cemented spodic horizon can occur between the depth of 40 and 100 cm in the soil (Trichet et al. 1999). The plots used in this study had *P. pinaster* plantations ranging from 6 to

93 years. The trees of *P. pinaster* are grown evenly into lines with 2 m tree-to-tree distance and 4 m interline tree distance. Fertilizers trials especially of phosphate fertilizers are conducted over a whole forest range because soils are characterized as P deficient. The mean annual temperature ranges from 10 to 15°C (from west to east) and precipitation ranges from 750 to 1,250 mm yr^{-1} (from south to north) that is irregularly distributed over the year.

2.2. Soil sampling and treatment

Paired soil samples from line and interline tree positions were taken in November 2006 (autumn) from 18 plots and 11 sites, covering a whole range of forest. Seven sets of soil cores (15 x 8 cm, length x diameter) from mineral topsoil layer were taken from each sampling position. Most of the sites (7 sites) contained plots with P fertilizer amendment (+P) and no fertilizer amendment (control). Other 4 sites contained plots with either only +P treatment or control treatment (Table 1). Collectively, 37 sets of samples were taken from both line and interline tree positions. The details of sampling plots are supplied in table 1, denoting age groups of plantation, fertilizer treatments, codes given to each set of samples, amount, form and frequency of P application and sampling position. Each soil core was gently sieved successively through 4 mm and 2 mm mesh size sieves to collect coarse and fine roots respectively. Sieved soils were mixed thoroughly to prepare a composite sample. These samples were stored at 4°C and sub samples were air dried for subsequent chemical analysis.

2.3. Treatment of fine roots and ectomycorrhizal morphotyping

Maximum of living fine roots (diameter < 2 mm) belonging to *P. pinaster* were sorted out of the sieved material using forceps, according to morphological characteristics and root smell (Bakker et al. 2006). Ectomycorrhizal roots are formed on lateral roots forming ramification. The ramification may consist of single dichotomous or cluster structures. In this study each ramification simple or cluster was counted as one ectomycorrhizal apex. A sub-sample of fine roots with a maximum of 300 individual apexes was transferred to a cuvette containing tap water. This sub sample was used to isolate living ectomycorrhizal tips that were grouped into small sub units presenting the same form, colour, turbidity, structure and other distinguishing characteristics (Harvey et al. 1976; Agerer 1987-1999). Each small sub unit was given a name called ectomycorrhizal morphotype.

Table 1: Characteristics of soil plots sampled in *Pinus pinaster* forest.

Plots name	Plots code	Soil treatment	P_2O_5 input [a] kg ha^{-1}	Sampling position	Age class of plantation [b]
Blagon	1	Control	0	Line	1
Blagon	2	Control	0	Interline	1
Blagon	3	+P	120	Line	1
Blagon	4	+P	120	Interline	1
Le Bray	5	+P	120	Line	2
Le Bray	6	+P	120	Interline	2
Baudes	7	Control	0	Line	3
Baudes	8	Control	0	Interline	3
L plot	9	Control	0	Line	1
L plot	10	Control	0	Interline	1
Vieille 1	11	Control	0	Line	2
Vieille 1	12	Control	0	Interline	2
Vieille 2	13	Control	0	Line	2
Vieille 2	14	Control	0	Interline	2
Caudos	15	Control	0	Line	1
Caudos	16	Control	0	Interline	1
Caudos	17	+P	120	Line	1
Caudos	18	+P	120	Interline	1
Retjons	19	Control	0	Line	1
Retjons	20	Control	0	Interline	1
Retjons	21	+P	120	Line	1
Retjons	22	+P	120	Interline	1
Saumejan	23	Control	0	Line	1
Saumejan	24	Control	0	Interline	1
Saumejan [c]	25	+P	120	Line	1
Lue	26	Control	0	Line	1
Lue	27	Control	0	Interline	1
Lue	28	+P	120	Line	1
Lue	29	+P	120	Interline	1
Mimizan	30	Control	0	Line	2
Mimizan	31	Control	0	Interline	2
Mimizan	32	+P	120+120	Line	2
Mimizan	33	+P	120+120	Interline	2
Marcheprime	34	Control	0	Line	2
Marcheprime	35	Control	0	Interline	2

a Additions of P to fertilized plots were made once when the pine trees were planted with the only exception of Mimizan plots, where P additions were made at the beginning of the experiment and eight years before sampling.

b Semi-quantitative variables were used to define tree stands age classes (1 = 13 years and less; 2 = between 26 and 49 years; 3 = 93 years).

c The interline of the fertilized Saumejan plots were removed from the database because no specific phosphatase activity of ECM morphotype was measured at this site.

The length of the core (15 cm) was representative to collect ca 90 % of the ectomycorrhizal root tips which are found in the mineral soils (Taylor and Bruns 1999; Dahlberg 2001; Baier et al. 2006). The length of the fine roots was determined by method of Tennant (1975) based on a modified line intersects method and the biomass was determined after drying sub-samples at 105°C for 24h. Based on similar fine root studies carried out previously, correction factors were applied on the rough values to account for different losses through sieving, washing steps, overestimates due to adhering soil particles and misjudgements with regards to vitality of the roots (living or dead). The final correction factors were +20% for fine root weight and +25% for fine root length (Bakker et al. 2006). Fine root length was expressed as fine root length density (FRLD in cm cm^{-3} of soil) and specific root length (SRL in m g^{-1} of root dry weight). Similarly, root weight was expressed as biomass (FRB g m^{-2} of soil). The growth of the trees was estimated by measuring the circumference at breast height. The ratio of circumference at breast height to age of tree (Circ:Age in mm $year^{-1}$) was calculated to take into account the increase of diameter with tree age.

2.4. Phosphatase activity

Phosphatase activity (Tabatabai 1982) of four ECM tips for each morphotype was estimated immediately after the counting of morphotypes. Each ECM tip was incubated at 30 °C for an hour in 10 mM solution (0.2 ml) of *p*NPP prepared in acetate buffer (25 mM, pH 5.4). The reaction was stopped by adding 1 ml of 0.5 M NaOH after incubation. A blank sample was also prepared for each morphotype by adding NaOH and *p*NPP with ECM tips simultaneously before incubation. Optical density of samples was measured at 400 nm and enzymatic activity was calculated (nmol of *p*NP produced min^{-1} g^{-1} of fresh ECM weight) by the equation:

$$\text{Phosphatase activity} = \left(\Delta\,\text{OD} \times 1.2 \times \text{DF} \Big/ \text{t} \times \text{FW} \times 0.0188\right)$$

Where "ΔOD" is the difference between optical density of blank and sample, "1.2" is final reaction volume (ml), "DF" is dilution factor, "t" is the time (minutes) of incubation, "FW" is fresh weight of ECM tip, "0.0188" is coefficient of molar extinction for *p*-nitrophenolat ($ml.nmol^{-1}$ cm^{-1}). Finally the activity phosphatase was transformed into µmol *p*NP $m^{-1}g^{-1}$ FW for data interpretation.

2.5. Estimation of P fractions in soils

Total soil P content (Pt) was determined by the ignition of soil samples at 550°C for 4 h in a muffle furnace (Saunder and Williams 1955). The ignited and air dried soil was shaken with 1 N H_2SO_4 (1:50 soil to solution ratio) for 16 h using end to end shaker. The samples were centrifuged (14000 g, 15 min) and the supernatants were used for P assay using malachite green method (Ohno and Zibilske 1991). The phosphorus estimated in ignited and air dried soil represented Pt and Pi respectively. Total Po was calculated by the difference between Pt and Pi. Sieved and air dried soil was also used to measure bicarbonate and hydroxide extractable inorganic and organic P fractions. Bicarbonate extractable P known as plant available P (labile P) was extracted by shaking 0.3 g of sieved soil for 30 minutes in 6 ml of $NaHCO_3$ (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 moderately available (less labile) fraction of P associated Al and Fe-oxides (Tiessen et al 1984, Sharpley 1999). Soil extracts were diluted with distilled water (1/6, v/v), then acidified with 12 N HCl (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 by 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 $NaHCO_3$ and NaOH extracts. The P in supernatant of each soil extract before and after mineralization was assayed using malachite green method (Ohno and Zibilske 1991).

2.6. Other soil analyses

Fresh soil was heated overnight at 105°C in tarred aluminium dish and percent water content (WC) was calculated by the loss of weight before and after heating. Soil pH was estimated in 10 mM solution of $CaCl_2$ using a 1:5 soil to solution ratio. Soil physicochemical characteristics like total organic carbon (C) and total organic nitrogen (N) were determined by *Laboratoire d'Analyses des Sols* belonging to National Institute for Agronomic Research (INRA), Arras, France, using standard French analytical norms (AFNOR, 1999). Total organic C content was estimated by oxidation with potassium dichromate and sulphuric acid (NF ISO 14235); total organic N content was estimated by the Kjeldahl method (NF ISO 11261).

We observed that the values of total C and total N concentrations were highly correlated with soil heat loss weight at 550 °C (HLW) when ignited for determination of total

P (see supplementary data, Figure 1). Therefore, we estimated the values of total C and N concentrations in soil samples, by using the linear regressions fitted on the variation of HLW (given by the difference in weight of soils dried at 105°C overnight and soils ignited at 550°C for 4 h) with total C and N in spring 2006. The equations used were: C total = 5.7 x HLW – 0.79 and N tot = 0.22 x HLW – 0.14 (see supplementary data, Figure 1, Chapter 2).

2.7. Characteristics of data set and statistical analyses

The database is composed of response variables integrated in three data matrixes describing the presence/absence of ECM morphotypes, their abundance and specific phosphatase activity at a given plot (Supp. data Table 1, 2 and 3 respectively). It also contains 19 explanatory variables describing the effects associated to the soil treatments (Control/+P), age of forest stands (Table 1), root parameters, tree characteristics (Supp. data, Table 4) and soil properties (Supp. data Table 5). Soil treatment effect was described by one binary variable, tree age populations were divided into three classes of age and described by a set of three binary variables (Table 1), whereas tree parameters and soil properties were described as quantitative variables.

Prior to multivariate statistical analyses, the normality of statistical distribution of variables describing root, tree and soil properties were evaluated using the Kolmogorov-Smirnov test. If necessary, logarithmic transformations were applied to these variables to achieve normal distribution. This test was run in the R open source software by using the function *ks.test* available in the *stats* library (R Development Core Team, 2009). Thereafter, to answer our research objectives, correspondence analysis (CA) was used to perform ordination of sampling plots according to ECM morphotypes data, whereas canonical correspondence analyses (CCA) were used to evaluate the influence of environmental factors on ECM morphotypes. These statistical analyses were both performed in CANOCO 4.5 software for Windows® (ter Braak and Smilauer, 1998). Given the relatively low number of samples, the nature of ECM morphotypes data showing in general strong unimodal distributions and the difficulty to extract relationships from these types of data, we set the level of significance to $\alpha = 0.10$.

The CA and CCA are well adapted to the analysis of ecological data that showed unimodal distribution, mostly because the measure of correspondence is based on χ^2 distance which exclude the double absence of organism in its calculation (Legendre and Legendre, 1998). However, these multivariate statistics are also known to overestimate the influence of rare organisms (Legendre and Gallagher, 2001; Legendre and Legendre, 1998). As mentioned

by Legendre and Gallagher (2001), the high weights given to the rare organisms is not a problem when the aim of the analysis is to produce a reduced-space ordination diagram, since the contribution of rare organisms to first ordination axes are low. However, it does matter when relationships between organisms and environmental factors need to be tested. Therefore, to answer our objectives, this statistical artefact might not pose problem for the ordination of plots, but might be undesirable when documenting relationships between the activity of ECM morphotypes and environmental factors. In order to limit this statistical artefact, we first remove in all statistical treatment the less important ECM morphotypes. For matrixes describing presence/absence and abundance of ECM morphotypes that were principally used to produce ordination of plots, only the two rarest species were removed. On the other hand, for the specific phosphatase activity matrix, we kept ECM morphotypes that account for more than 4% of the total specific phosphatase activity of the dataset (Supp data Figure 1). This procedure allowed us to remove nine ECM morphotypes and to keep more than 94% of the total specific phosphatase activity of the dataset. Combined with this approach, to limit the sensitivity of CA and CCA to rare organisms, the *down-weighting of rare species* function available in the CANOCO software was used for the analyses (ter Braak and Smilauer, 1998).

CA was performed separately on the three response matrixes. By focusing on sample distance (scaling type 1) for these matrixes, ordinations of sampling plots were produced. This might be helpful to characterize plots heterogeneity and to identify sampling plots clusters based on, either presence or abundance of ECM morphotype or their specific phosphatase activity.

Contrary to CA, the influence of environmental factors on ECM morphotypes was only assessed on abundance and specific phosphatase activity matrixes. In both case, only the most active ECM morphotypes in term of phosphatase activity production were kept in the analyses (Supp. Data Table 3). The influence of soil treatment, root and tree properties as well as soil properties were assessed on the entire dataset, whereas the influence associated with the tree age populations was documented only on non-fertilized plots. With this approach, the complex and unclear interactions that might have occurred between the trees age and fertilization treatments in fertilized plots were discarded. Previously, CCA conducted on the entire dataset revealed that sample position (sampling on line or interline) statistically influence the variation of the specific phosphatase activity matrix, whereas no significant effects were observed for abundance matrix and for specific phosphatase activity matrix of non-fertilized soils. The effect of this sampling artefact was removed from the CCA analyses by producing partial CCA, where a binary variable describing sample position was introduced

as co-variable. All the CCA were made on standardized explanatory variables, the significant explanatory variables attributed to each environmental factor were evaluated by using forward selection procedure and the significance of the canonical relations were tested using unrestricted Monte-Carlo permutation test (10,000 simulations), performed under full model. However, due to the sampling artefact already mentioned, restricted permutation tests were used for the CCA conducted on the entire dataset and explaining the variation of specific phosphatase activity matrix. In this case, the permutation of objects was constrained within a sample position. Finally, the identification of relations between response variables and environmental factors were achieved by producing CCA ordination biplots preserving the distance between response variables (scaling type 2).

3. Results

3.1. Distribution of ECM and their phosphatase activity

A total of 17 different morphotypes were observed in monoculture of *P. pinaster* with an average number of 4-5 ECM morphotypes identified per plot, but it ranged between 1 and 8 in different plots of *P. pinaster* (Table 2). Moreover, the abundance of a single morphotype in different *P. pinaster* plots (Supp. data Table 2) was highly variable (0-180 ECM). The average specific phosphatase activity of these ECM morphotypes ranged between 0.41-0.69 µmol *p*NP min^{-1} g^{-1} of FW in all plots, but it varied largely within single site (0.15-1.34 µmol *p*NP min^{-1} g^{-1}), either due to sampling position, soil treatments and stand age groups. However, phosphatase activity was slightly higher in interline tree positions than line position, in +P soil plots compared to control ones and in plots with less than 13 year old stands of *P. pinaster* (Table 1) than in older stands.

The ECM community structure, in terms of presence absence, abundance and specific phosphatase activity was characterized by CA. The data for presence/absence of ECM are expressed in biplots, where first two ordination axes explain 37 % of variance (Figure 1). On these statistics, the focus was made on the inter-plots distances in order to know if variation of community structure occurred in the datasets. Figure 1a showed that the plots used in this study were highly dispersed in a biplot, suggesting highly variable composition of ECM between plots (Fig 1b). The two biplots (Figure 1) further explain that the presence of morphotypes A-B-H and D is similar in plots 1-26-15-22-7, because of similar location of morphotypes and plots in biplot. Conversely, the dispersion of morphotypes and plots in the biplots indicate their differences of presence absence of morphotypes.

Table 2: Descriptive statistics[a] of the community composition and phosphatase activity of ectomycorrhizae (ECM) sampled in *Pinus pinaster* forest.

ECM properties [b]	Descriptive statistics	Sampling position		Soil treatment		Tree age [c]		
		Line	Interline	Control	+P	1	2	3
Number of morphotype per site	Mean	4.72	4.12	4.23	4.77	4.50	4.50	4.00
	SD [d]	1.56	1.62	1.54	1.69	1.98	0.90	-
	Range	2 - 8	1 – 7	1 – 7	2 – 8	1 – 8	3 – 6	2 – 6
	n	18	17	22	13	21	12	2
Specific phosphatase activity per site [e]	Mean	0.53	0.65	0.56	0.63	0.69	0.41	0.53
	SD	0.19	0.37	0.24	0.38	0.33	0.14	-
	Range	0.15 – 0.86	0.24 – 1.34	0.15 – 1.17	0.24 – 1.34	0.15 – 1.34	0.24 – 0.69	0.46 – 0.59
	n	18	17	22	13	21	12	2

a The descriptive statistic was expressed in three different ways representing the main axes of variation anticipated for the database.

b The ECM properties are expressed by site and for the specific phosphatase activity, the activity value of a given site may represent activity of more than one ECM morphotypes.

c Tree age classes correspond to the ones defined in table 1.

d SD = standard deviation.

e The specific phosphatase activity is expressed in µmol *p*NP min^{-1} g^{-1} fresh matter.

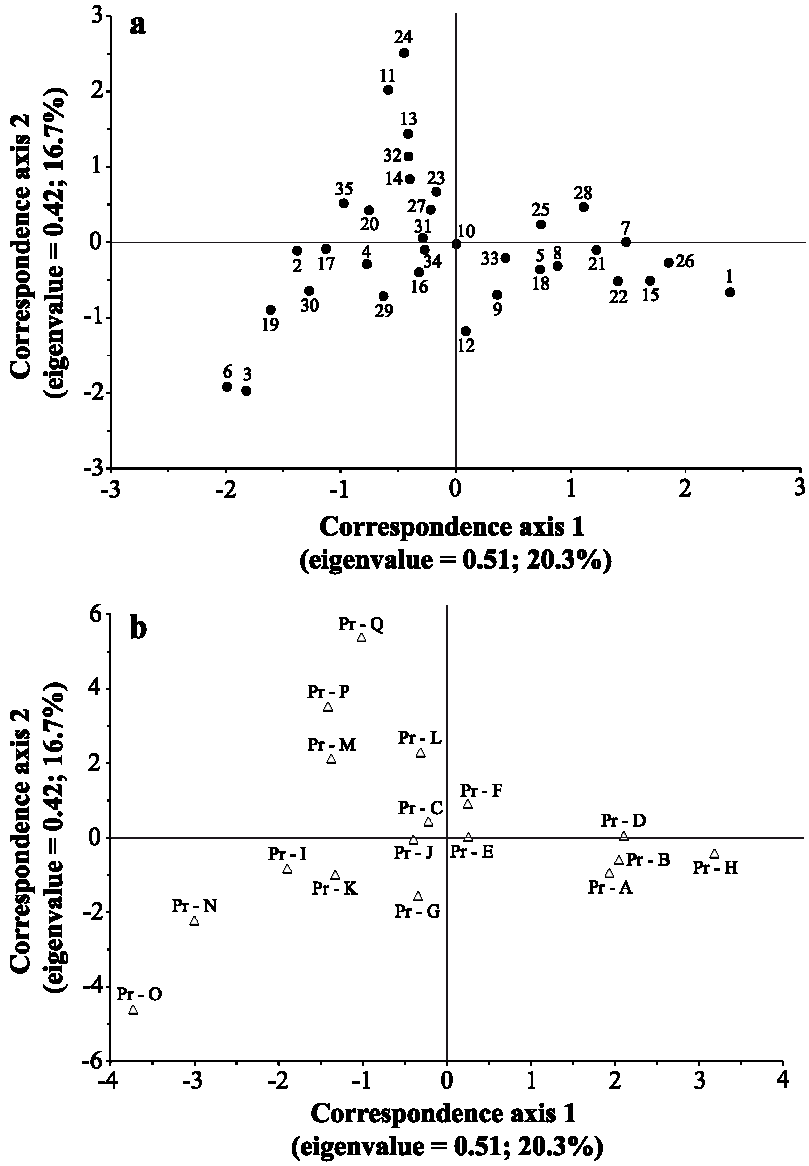

Figure 1: Correspondence analyses ordination biplots (scaling type 1) of all forest soil plots distributed according to matrixes describing the presence/absence of ectomycorrhizae (ECM) morphotypes. The sites (a) and the ECM morphotypes (b) were presented in this figure. Eigenvalue as well as percentage of explained variance of response matrixes are given for each correspondence axis.

Similarly, the first two ordination axes of CA biplot drawn for the abundance of ECM morphotypes explained 31.7 % of variance (Figure 2). The biplots showed the difference among studied plots explained by the abundance of ECM morphotypes. It showed large variations among the plots as shown by the biplot (Figure 2a). The group of plots in the top left quadrant of biplot in Figure 2a showed the abundance of morphotypes present in top left quadrant of biplot in figure 2b. Conversely, the morphotypes present in bottom right quadrant of biplot in Figure 2b were not abundant in top left quadrant of biplot in figure 2a but these were abundant in plots present in the right bottom quadrant of figure 5a. The plot 6 and 3 showed the abundance of rare morphotypes in this biplot.

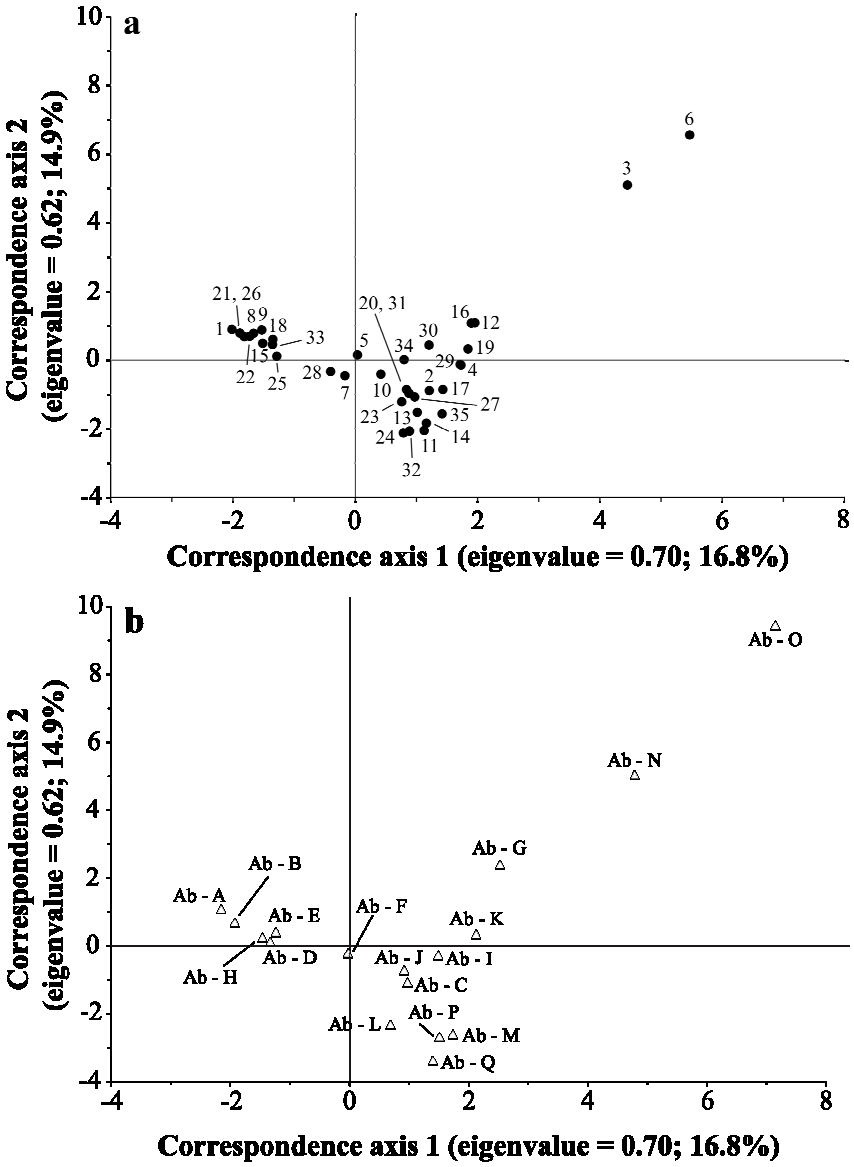

Figure 2: Correspondence analyses ordination biplots (scaling type 1) of all forest soil plots distributed according to matrixes describing the abundance of ectomycorrhizae (ECM) morphotypes. The sites (a) and the ECM morphotypes (b) were presented in this figure. Eigenvalue as well as percentage of explained variance of response matrixes are given for each correspondence axis.

Additionally, the ECM only the morphotypes representing around 95 % of the total phosphatase activity (Supp. data Figure 1), were used to represent their specific phosphatase in CA biplot (Figure 3). As indicated in biplots, 35.8 % of variance was explained by two ordination axes of CA biplot. The distribution of the soil plots (Figure 3a) is explained by the superimposing the distribution (Figure 3b) of specific activity of ECM. However, the activity of morphotypes close to centre of biplot was difficult to associate specific plot as it shows multi-modal response. Finally, the CA for the abundance and phosphatase activity showed large variation with respect to different plots, as it is also shown in descriptive statistic in table 2.

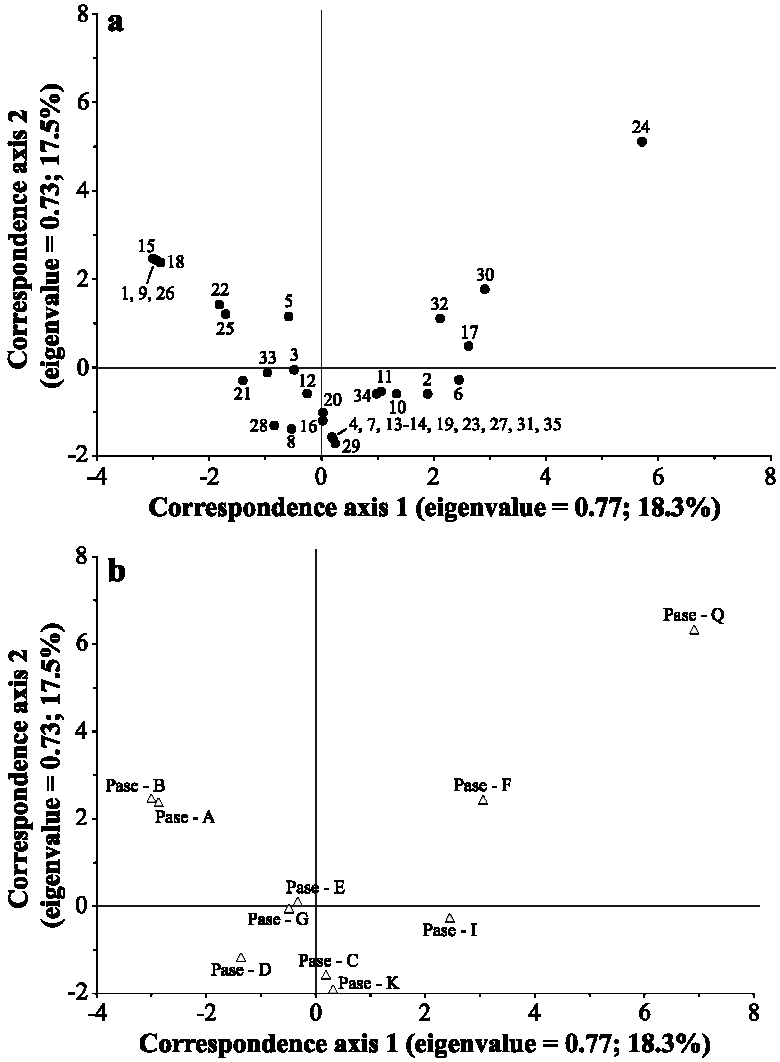

Figure 3: Correspondence analyses ordination biplots (scaling type 1) of all forest soil plots distributed according to matrixes describing the specific phosphatase activity of ectomycorrhizae (ECM) morphotypes. The sites (a) and the ECM morphotypes (b) were presented in this figure. Eigenvalue as well as percentage of explained variance of response matrixes are given for each correspondence axis.

3.2. ECM morphotypes in relation to plant and soil characteristics

The data of descriptive statistic for tree properties (root and shoot) and soil properties was presented with respect to fertilized (+P) plots and control plots. For each parameter of plant and soil properties a large variation was observed for both +P and control plots (Tables 3 and 4). The average values of FRLD, SRL, Circ. and Circ:Age as well as Olsen and NaOH extractable Pi were significantly higher ($P < 0.05$) in +P compared to control soil plots (Table 3 and 4). In addition, the values of pH, WC, total C, total N as well as P fractions were also higher but statistically non-significant in +P plots as compared to control plots.

In order to evaluate the influence of environmental factor on both ECM abundance and phosphatase activity, canonical correspondence analysis (CCA) were performed separately for every environmental factor (Table 5). Therefore, significant influence of soil treatment (+P

and control) for ECM abundance, and sampling position (line and interline) for specific phosphatase activity was observed in these soils (Table 5). In addition, soil properties significantly influence the ECM morphotype abundance as well as specific phosphatase activity.

Table 3: Descriptive statistics, expressed according to soil treatments (Control / +P), and the tree and root properties of samples taken in *Pinus pinaster* forest.

Soil treatment	Descriptive statistics	FRB [a]	FRLD	SRL	Circ	Circ:Age
		g m^{-2} soil	cm cm^{-3} soil	m g^{-1} dwr [c]	mm	mm $year^{-1}$
Control	Mean	121	0.83	10.8	629	29.8
	SD [d]	77.9	0.50	2.85	449	8.55
	Range	28.8 – 311	0.20 – 1.87	6.89 – 17.5	188 – 1668	17.9 – 45.7
	n	22	22	22	22	22
+P	Mean	121	1.11	14.2	585	38.2
	SD	52.5	0.43	2.91	383	9.82
	Range	41.3 – 230	0.38 – 1.91	8.83 – 17.5	229 - 1232	25.1 – 52.1
	n	13	13	13	13	13

a FRB = fine root biomass; FRLD = fine root length density; SRL = specific root length; Circ = tree circumference measured at breast height; Circ:Age = tree circumference-to-tree age ratio.
c dwr = dry weight root.
d SD = standard deviation.

Ordination diagram of CCA grouping all significant explanatory variables to explain response variables i.e. specific phosphates activity (Figure 4a) and abundance (Figure 4b) of ECM morphotypes could increase the understanding of the links between environmental factors and ECM phosphatase activity and their abundance. CCA showed that water contents predominantly controlled the variability of first canonical axes and, the phosphatase activity of morphotypes G, D, A and B was higher in plots with higher water content. In contrast, the activity of morphotypes F, E, L and Q was higher is soil plots with lower water contents (Figure 4a). However, the position of each morphotype on eigenvector of water contents determines their relative response with respect to each other. The second axes of CCA is principally controlled by Pi-Olsen and to lesser extent by total N, In this way, morphotype K showed the phosphatase activity in plots with high Pi-Olsen concentrations while morphotype G followed by Q and I showed phosphatase activity under relatively low Pi-Olsen concentrations. Similarly, the response of specific phosphatase activity of ECM morphotype to passive variables (not significant but included in biplot for interpretation of results) revealed positive relations of morphotypes I and V with high FRB, Pi-NaOH, and FRLD while, inverse relations were observed with morphotypes D, A and C. Furthermore, passive

variable; Pi- H_2SO_4, was positively correlated with water contents and FRB, FRLD and Po-NaOH were positively linked to each other and negatively related to Pi-Olsen in these soils.

Figure 4b explains the abundance of morphotypes with respect to explanatory variables (Pi-olsen, log WC, +P soil treatment, Po-H_2SO_4). The results from CCA showed that most of the ECM morphotypes less abundant in fertilized plots (+P) and in soil with high Pi-Olsen contents, except morphotypes B and D which were abundant in fertilized plots and plots with high Pi-Olsen contents. Similarly, the relative position of the ECM morphotypes on the eigenvector of WC represented their relative abundance. Therefore, morphotype G and B were abundant in soil with high water contents whereas morphotypes Q, K, C and I were abundant in soil with less water contents. Moreover, explanatory variables Po- H_2SO_4 and Pi-Olsen were positively correlated with WC and +P respectively. FRLD and FRB were inversely and Circ:Age was positively linked with +P treatment in soils. However, the proportions of explanation for both phosphatase activity (R^2 = 5.24 %) as well as abundance of morphotypes (R^2 = 5.59 %) were low but statistically significant at α = 0.05 and 0.10 respectively.

In order to evaluate the influence of trees age on specific phosphatase activity of ECM morphotypes as well as its relation with soil and root properties, a CCA was performed only on control plots. The fertilized plots were not included in the CCA to avoid any complex effect that might occur between tree age and P-fertilization. Only significant explanatory variables, obtained by forward selection, were integrated in CCA. The analysis explained 11.9 % of the adjusted variance of phosphatase activity, and were significant at α = 0.01 (Figure 5). The biplots showed that activity of morphotype A, B, I and E was high in young tree plots (< 13 years old), while morphotypes F and K showed high activity in middle-age plots (26 to 49 years old) and high SRL. Both middle-age tree plots and SRL are positively linked to each other and negatively linked to Pi- H_2SO_4 and WC of the soils.

4. Discussion

4.1. Diversity of ectomycorrhizal fungi

This is one of the first studies exploring the morphological diversity of ectomycorrhizal fungi associated with mono specific *P. pinater* forest stand. In agreement with Smith and Read (1997) as well as Taylor et al. (2000), all the fine roots collected in our soil samples were associated with mycorrhizal fungi.

Table 4: Descriptive statistics, expressed according to soil treatment (Control / +P), of the soil properties sampled in *Pinus pinaster* plantations.

Soil treatment	Descriptive statistics	pH in $CaCl_2$ [a]	Water content	Total C [b]	Total N [b]	Olsen extract		NaOH extract		H_2SO_4 extract	
						Pi [c]	Po	Pi	Po	Pi	Po
			% (w/w)	g kg^{-1}	g kg^{-1}	mg P kg^{-1}					
Control	Mean	3.27	5.62	25.4	0.87	2.44	6.15	5.16	20.6	11.8	27.2
	SD [d]	0.14	2.35	9.58	0.37	1.02	1.87	1.83	9.65	7.31	15.0
	Range	3.09 – 3.94	1.62 – 10.9	11.3 – 42.3	0.33 – 1.53	1.50 - 6.56	3.11 – 10.8	1.79 – 8.39	5.92 – 38.3	4.50 – 29.6	7.26 – 67.6
	n	22	22	22	22	22	22	22	22	22	22
+P	Mean	3.31	5.84	25.4	0.88	3.22	6.20	6.87	21.2	12.4	29.0
	SD	0.14	2.83	11.5	0.44	0.94	2.20	3.13	7.95	7.75	8.09
	Range	3.12 – 3.90	1.07 – 9.70	13.8 – 49.4	0.43 – 1.80	2.13 – 5.47	1.27 – 8.67	2.79 – 15.1	8.02 – 32.8	6.38 – 33.1	16.7 – 39.6
	n	13	13	13	13	13	13	13	13	13	13

a pH was measured in 10 mM $CaCl_2$ solution and with 1:5 soil-to-solution ratio.
b The data of total C and N was estimated. The estimation was based on relationships between heat loss weight of sample at 550 °C and total C and N contents derived from a previous sampling campaign (Supplementary data Fig. 1, Chapter 2).
c Pi = inorganic P concentration; Po = organic P concentration obtained by difference between total P and Pi concentrations.
d SD = standard deviation.

Table 5: Summary of canonical correspondence analyses (CCA) describing the relationships of abundance and specific phosphatase activity of ECM morphotypes with environmental and methodological factors.

Environmental factors [a]	Abundance				Specific phosphatase activity			
	Canonical eigenvalues	Adjusted R^2 (%)	F Statistic [b]	Significant variables [c]	Canonical eigenvalues	Adjusted R^2 (%)	F Statistic [b]	Significant variables
Sample position	0.10	0.50	1.17 ^{NS}	Interline	0.31	4.39	2.57 ***	Interline
Soil treatment	0.17	2.84	2.01 **	+P	0.08	0	0.68 ^{NS}	+P
Tree and root properties	-	-	-	No significant variable	0.05	0	0.40 ^{NS}	FRB
Soil properties	0.36	3.92	1.46 *	Pi – Olsen, Po - H_2SO_4, log.WC	0.55	5.24	1.58 *	Pi - Olsen, Ntot, log.WC

a The sample position (line/interline) and soil treatment (control/+P) were described by one binary variable for each case.
b The statistical significance of the canonical relation described by CCA were tested using Monte-Carlo permutation test performed under full model after 10,000 simulations (NS = not significant; * significant at $\alpha = 0.10$; ** significant at $\alpha = 0.05$; *** significant at $\alpha = 0.01$). Further details on CCA are available in *Materials and methods* section.
c The significance of tree and root as well as soil variables was evaluated by the forward selection procedure before producing the CCA.

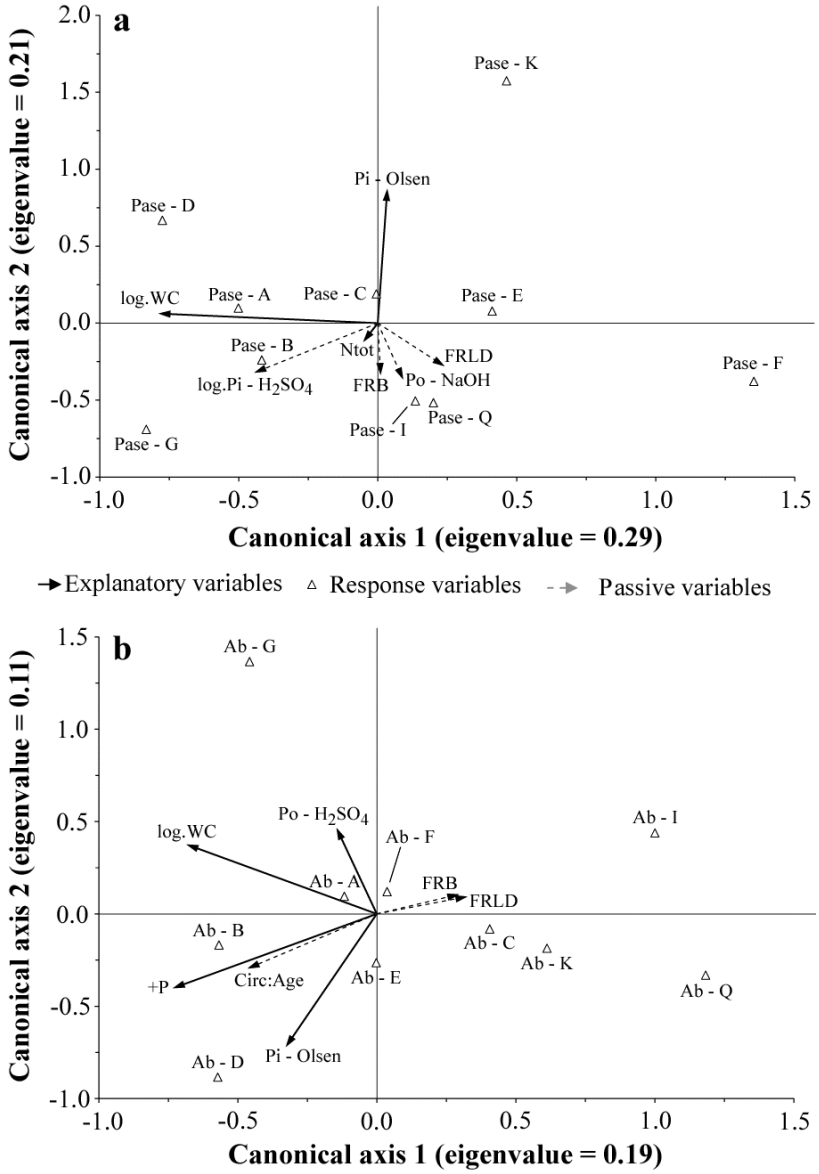

Figure 4: Canonical correspondence analyses ordination biplots (scaling type 2) of all forest soil plots representing the overall influence of soil treatment, soil, root and tree properties on matrixes describing specific phosphatase activity (a) and abundance (b) of ectomycorrhizae morphotypes. For the specific phosphatase activity biplot (a), the influence of sample position was controlled in order to isolate the influence of other environmental factors. The combination of all canonical axes explained 5.24% (a) and 5.59% (b) of the adjusted variance of the respective response matrixes and were statistically significant at $\alpha = 0.10$ (a) and $\alpha = 0.05$ (b), respectively. The significant explanatory variables integrated in the model included soil treatment that is described by a binary variable showing P fertilized plots (+P), the soil water content (log.WC), the total soil nitrogen content (Ntot) as well as inorganic and organic P concentrations measured respectively, in Olsen and H_2SO_4 extractions. Supplementary environmental variables were not compiled in the CCA analyses but were integrated in the biplots to improve the understanding of the system.

However, the total number of morphotypes (19 morphotypes) observed in our study was not as high as observed in other forests, such as pine oak forest (100-135 ectomycorrhizal morphotypes) (Palmer et al. 1994; Tuininga 2000). The apparent low number of mophotypes could be due to several factors. The major ones include monoculture stand which itself might have caused selection of host specific fungal communities and anthropogenic activities (Smith

1990). The occurrence of few ECM morphotypes frequently and abundantly and of others rarely in this study was in accordance with the previous studies in different forests (Taylor et al. 2002, Buée et al. 2005; Koide et al. 2005).

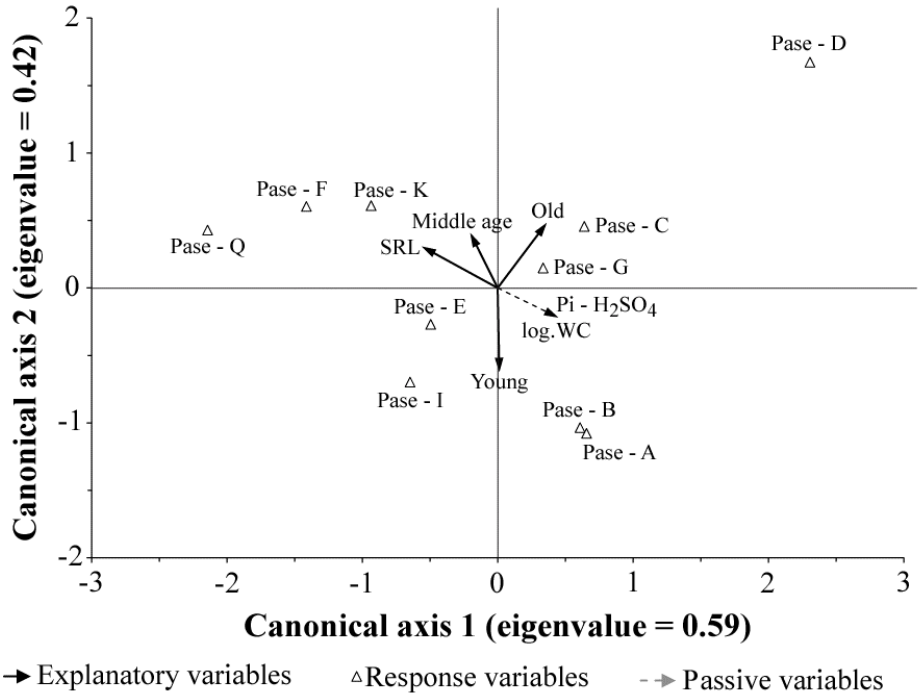

Figure 5: Canonical correspondence analysis ordination biplot (scaling type 2) of non-fertilized forest soil plots representing the overall influence of soil treatments, trees age and soil as well as root and tree properties on matrix describing specific phosphatase activity of ectomycorrhizae morphotypes. The combination of all canonical axes explained 11.9% of the adjusted variance of the response matrix and were statistically significant at $\alpha = 0.01$. The significant explanatory variables integrated in the model included the three binary variables describing the tree ages (Young, Middle age and Old), the specific root length (SRL) and inorganic P concentration measured in H_2SO_4 extraction. Supplementary environmental variable was not compiled in the CCA analysis but was integrated in the biplot to improve the understanding of the system.

The results of canonical correspondence analyses (Tab. 5) showed that the soil fertilization history and variables describing soil properties (Pi-Olsen, Po-H_2SO_4 and WC) influenced significantly the variability of ECM abundance. We did not find the effect of soil pH on ECM abundance despite almost one unit of variation (from 1000 to 100µM of H^+) between soil samples, especially in control plots (Tab. 4), whereas Tyler et al. (1987) observed the decrease in ECM abundance with decreasing pH of soil. No significant effect of trees age on the abundance of ECM was observed. This is in agreement with Jones et al. (2003), who suggested that trees age may not play major role in fungal communities between mature and re-generating forests.

However, when used in a biplot, the combination of all canonical axes integrating the significant variables, they explained only 5.59% of the adjusted variance of the abundance

matrix, indicating that other factors should explain the variability of ECM abundance. Indeed, in addition to the soil properties, other factors such as soil temperature, soil disturbance (Jones et al. 2003) could have affected fungal colonization. On the other hand, physiological responses of host plant to different soil conditions may have changed the relative colonization levels of individual ECM morphotypes on the root system (Baxter and Dighton 2005). Among the possible physiological responses, the variation of carbon allocation to roots and fungal cells as a function of soil conditions could be an important determinant, since the C demand of various fungal species could be more or less (Leake et al. 2001, Culling et al. 2001). The maximum allocation of carbon by plant takes place in the most active part of growing roots (Quick and Schaffer 1996) and the demand on host for carbon varies among ECM fungi. Thus, the preferential allocation of C by plants could favour ECM fungi associated with high compared to low resource allocation area of roots.

4.2. Variability of ECM phosphatase activity and effect of soil and plant properties

The main characteristic of phosphatase enzyme activity of the ECM morphotypes was its high variability (that can reach a factor 10) occurring within the same morphotype as well as between the morphotypes (Table 3, sup. data). Some large variation of enzyme activities and phosphatase activity was also observed among ECM morphotypes collected in the field (Buée et al. 2005; Courty et al. 2006). Indeed, a large variation of acid phosphatase was also reported in laboratory experiments using isolates of *Amanita muscaria* (4 isolates) *Cenococcum geophilum* (3 isolates), *Scleroderma citrinum Pers.* (5 isolates) *and Paxillus involutus* (8 isolates) (Ho 1987; Ho 1989; Antibus et al. 1992; Cairney 1999). Therefore, the high variability of phosphatase activity measured in field samples could be due to intra-specific variability of ECM isolates forming identical morphotypes. However, the higher range of phosphatase activity variation observed in this study compared to the studies carried out in laboratory conditions suggest that factors other than intra-specific variation could explain our results. Notably, the contribution of mycorrhizal associated bacterial populations could not be ignored, as they have some functional relevancies (Garbaye 1994).Otherwise, environmental factors may also play a significant role to regulate the amounts of phosphatase activity produced by the ECM.

In order to further investigate the possible contribution of environmental factors to the variation of the phosphatase activity of different ECM morphotypes, the CCA showed that factors like Pi-Olsen, WC, fertilizers application as well as some plant factors significantly explained the secretion of phosphatase activity. Phosphatase activity of most of the

morphotypes was high with low concentration of labile P concentrations (Figure 4a). The results are in agreement with the hypothesis that phosphatase activity increases under low Pi concentration and decreases with increasing Pi concentration or P fertilizer application. A number of studies are in accordance with our finding that phosphatase activity of ECM was decreased with increasing availability of Pi both in culture medium (Bousquet et al. 1986; Tibbett et al. 1998; Quiquampoix and Mousain 2005) as well as in soils (Kroehler et al. 1988; Antibus et al. 1992; Chen et al. 2002; Ali et al. 2009). Similarly, the antagonistic effects of fertilizers application has been reported on acid phosphatase activity in soils subjected to different organic and inorganic amendments (Criquet et al. 2007; Nèble et al. 2007; Criquet and Braud 2008; Floch et al. 2009).

The fertilizers treatment and phosphorus availability also appeared to decrease FRLD, FRB of *P. pinaster* trees and the abundance (Wright et al. 2009) and phosphatase activity of not all but most of ECM morphotypes was associated with FRB and FRLD in the CCA biplot (Figure 4a, b). It suggested that under low P availability, plant modified its resource allocation resulting into production of more fine roots to cope with P deficiency (Albaugh et al. 1998; Mair and Kress 2000; Achat et al. 2008). Once the fine roots are developed, they are rapidly colonized by mycorrhizal fungi (Smith and Read 1997; Taylor et al. 2000). However, the pattern of ECM abundance did not correspond to the pattern of phosphatase activity variation (compare Figure 2 and 3). Therefore, it remained difficult to assess what was the pattern to express acid phosphatase activity and ECM abundance because the ECM with high phosphatase activity did not mean the ECM with high rate of colonization with fine roots of *P. pinaster*.

Interestingly, the CCA analysis carried out in samples from control plots showed that phosphatase activity of morphotypes was largely associated with eigenvectors of young and middle age tree stands of *P. pinaster* and very few morphotypes strongly expressed their phosphatase activity in old age forest stands. The high phosphatase activity of ECM morphotypes in relatively young tree stands could be due to high growth rate of these plants as compared to older trees. This high growth rate may subsequently induce a high P demand in these plants. Consequently, the ECM morphtypes associated with fast growing and high P demanding roots would have to secrete high phosphatase activity as suggested by other authors (Abdalla 1994, Chen et al. 2003a, b). Hence, high P demand of both fast growing plants and their symbiotic partner may induce high phosphatase activity.

In conclusion, CA presented reliable interpretations to explain the complex system of fungal community structures and their phosphatase activity at this large scale study.

Additionally, the interpretations of CCA helped to link the soil and plant properties with acid phosphatase activity and ECM community. However, the proportion of explanation thus established remained low but statistically significant (Table 5). The important factors controlling the acid phosphate activity and ECM abundance were soil treatment, water contents, Pi-Olsen concentrations and age of tree stands. An extended research program focusing on these important factors as well as the new factors related to soil, plant and ectomycorrhizae could be helpful for developing better relations for ECM diversity and functioning.

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6. Supplementary data

Table 1: Community composition of ectomycorrhizae (ECM) sampled in *Pinus pinaster* forest.

Plots code	ECM morphotype																
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	1 [a]	1						1									
2			1						1	1			1	1			
3							1				1			1	1		
4			1								1						
5	1	1	1			1	1										
6							1		1					1	1		
7			1					1									
8	1	1	1	1		1	1										
9	1	1			1		1			1	1						
10		1	1		1	1			1	1							
11			1			1						1	1			1	1
12	1						1				1						
13			1			1						1	1				
14			1			1	1					1	1				
15	1	1						1		1							
16			1				1			1							
17			1			1			1	1	1			1		1	
18	1	1	1			1	1										
19			1											1			
20			1		1				1	1	1	1	1				
21	1	1	1	1		1		1		1							
22	1	1			1												
23			1		1					1		1					
24						1						1				1	1
25	1	1	1		1							1					
26	1	1				1		1									
27			1														
28		1	1	1		1		1				1					
29			1				1				1						
30						1			1	1	1			1			
31			1		1	1				1	1						
32			1			1				1		1	1				
33		1	1			1	1										
34	1				1				1		1	1					
35			1								1		1				

a Empty cell indicates absence of a given ECM morphotype, while value of 1 in cells indicate the presence of an ECM morphotype for a given site.

Table 2: Abundance of ectomycorrhizae (ECM) morphotypes sampled in *Pinus pinaster* forest.

Plots code	ECM morphotype																
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	42 [a]	15						8									
2			110						20	15			14	5			
3							24				3			12	23		
4			34								65						
5	14	43	60			53	25										
6							4		3					12	24		
7			17					15									
8	29	10	2	3		4	1										
9	54	38			5		3			1	9						
10		17	30		5	2			21	20							
11			21			1						8	6			4	14
12	8						38				67						
13			34			1						10	8				
14			5			6	7					40	31				
15	4	65						4		12							
16			20				53			15							
17			4			5			28	1	5			1		19	
18	38	119	21			12	9										
19			27											8			
20			86		11				3	5	8	8	6				
21	48	180	6	4		7		1		2							
22	68	93			97												
23			39		5					21		22					
24						21						71				29	12
25	34	140	14		31							44					
26	69	82				11		3									
27			47														
28		109	115	11		1		1				16					
29			16				1				27						
30						32			13	1	12			7			
31			125		8	11				19	6						
32			9			4				3		49	15				
33		100	7			25	2										
34	10				32				71		35	6					
35			51								12		46				

a Empty cell indicates absence of a given ECM morphotype, while value in cells indicate number of ECM morphotypes observed for a given site.

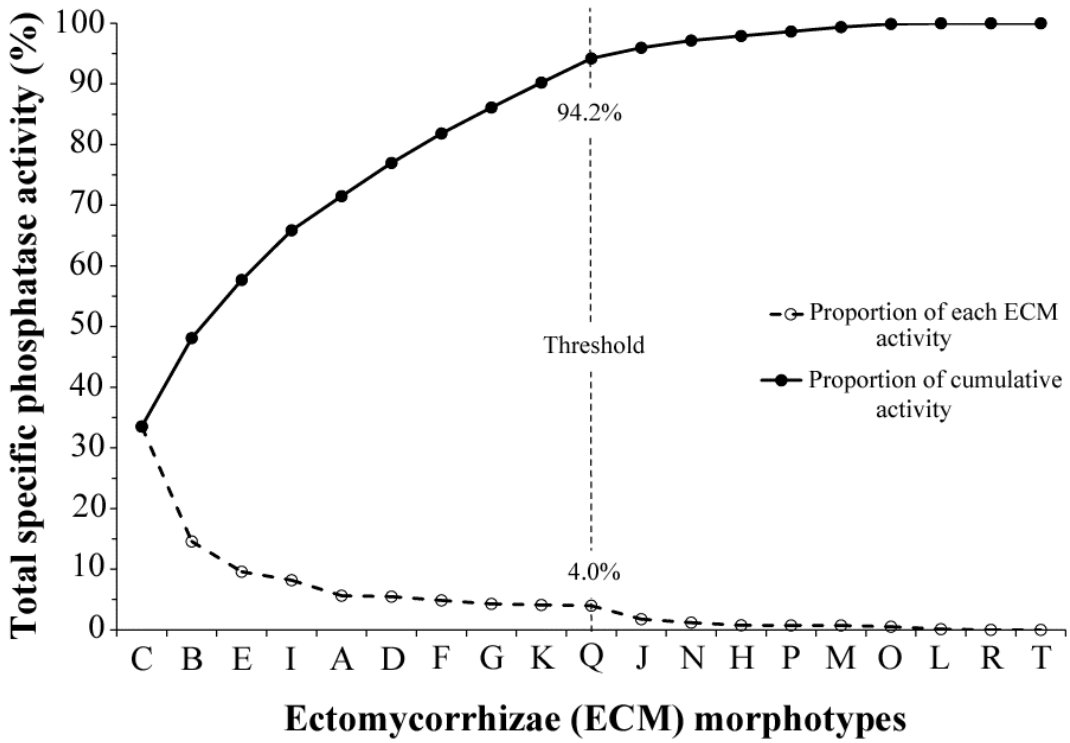

Figure 1: Percentage contribution of each ECM morphotype to the total specific phosphatase activity of dataset. The criteria threshold used to identify the most active ECM morphotypes was set at ca 94.2 % of total cumulative phosphatase activity of all the morphotypes from all plots. The rare and less active morphotypes with their individual activity less then 4% of the total activity of all ECM in all plots were not used in statistical analysis.

Table 3: Specific phosphatase activity (µmol *p*NP min^{-1} g^{-1} fresh ECM tips) of ECM morphotypes sampled in *Pinus pinaster* forest.

Plots code	**ECM morphotype** [a]									
	A	B	C	D	E	F	G	I	K	Q
1	0.22 [b]	0.98								
2			0.30					0.93		
3							0.80			
4			2.39						0.29	
5	0.43	0.60	0.46			0.55	0.63			
6								0.29		
7			0.59							
8			0.48	0.44						
9	0.59	0.68								
10			0.83		0.47			1.56		
11			0.53							0.18
12							0.42		0.17	
13			0.42							
14			0.37							
15		0.58								
16			1.37				0.44			
17						0.41		1.05		
18	0.33									
19			0.45							
20			0.41		0.46				0.33	
21	0.29	0.44	0.68	1,33						
22	0.20	0.30			0.38					
23			0.15							
24						0.38				1.95
25	0.42	0.26	0.12		0.43					
26	0.52	0.71								
27			0.73							
28			0.56	1.16						
29			1.49						1.17	
30						0.64		0.21		
31			0.58							
32			0.30			0.61				
33		0.17	0.31							
34					0.28			0.35	0.23	
35			0.69							

a To avoid statistical artefact associated to rare ECM morphotype, only the morphotype that have contributed ca 94.2 % of cumulative phosphatase activity of dataset were kept for statistical analyses. This procedure has lead to remove from this table the contribution of ECM morphotypes H, J, L-P, Q, and T.

b Empty cell indicate absence of phosphatase activity measurement for a given ECM morphotype and site.

Table 4: Tree and root properties of the plots sampled in *Pinus pinaster* forest.

Plots code	FRB [a] g m^{-2} soil	FRLD Cm cm^{-3} soil	SRL m g^{-1} dwr [c]	Circ mm	Circ:Age mm $year^{-1}$
1	103	0.63	9.12	249	31.1
2	106	0.89	12.6	249	31.1
3	173	1.91	16.7	330	41.2
4	142	1.43	15.2	330	41.2
5	230	1.35	8.83	1018	28.3
6	95.5	0.75	11.7	1018	28.3
7	28.8	0.26	13.8	1668	17.9
8	114	0.52	6.89	1668	17.9
9	235	1.56	9.93	594	45.7
10	311	1.87	8.99	594	45.7
11	218	1.20	8.23	835	28.8
12	102	0.78	11.4	835	28.8
13	101	0.61	9.07	691	26.6
14	71.0	0.59	12.6	691	26.6
15	71.7	0.49	10.3	250	31.3
16	85.7	0.43	7.53	250	31.3
17	99.8	1.16	17.4	417	52.1
18	119	1.38	17.5	417	52.1
19	37.8	0.20	7.99	341	42.7
20	66.2	0.48	10.9	341	42.7
21	57.5	0.52	13.6	384	47.9
22	109	0.80	11.0	384	47.9
23	250	1.70	10.2	213	35.6
24	180	1.75	14.6	213	35.6
25	72.9	0.82	16.9	229	38.1
26	63.3	0.38	8.94	188	20.9
27	43.0	0.42	14.7	188	20.9
28	183	1.27	10.4	311	34.6
29	134	1.48	16.6	311	34.6
30	89.1	1.04	17.5	1104	22.5
31	92.9	0.64	10.4	1104	22.5
32	112	1.12	15.0	1232	25.1
33	41.3	0.38	13.9	1232	25.1
34	76.9	0.77	14.9	781	24.4
35	219	1.15	7.86	781	24.4

a FRB = fine root biomass; FRLD = fine root length density; SRL = specific root length; Circ = tree circumference measured at breast height; Circ:Age = tree-circumference-to-tree-age ratio.

c dwr = dry weight root.

Table 5: Soil properties of plots sampled in *Pinus pinaster* forest.

Plots code	pH[a]	Water content	Total C[b]	Total N[b]	Olsen extract		NaOH extract		H_2SO_4 extract	
					Pi[c]	Po	Pi	Po	Pi	Po
		% (w/w)	g kg^{-1}		mg P kg^{-1}					
1	3.35	4.71	15.0	0.47	1.50	3.11	1.79	12.0	17.9	14.7[d]
2	3.34	4.78	15.0	0.47	1.73	3.44	1.80	9.92	16.9	13.4[d]
3	3.28	6.45	20.1	0.67	2.13	4.35	2.79	16.4	17.6	18.9
4	3.32	4.56	16.9	0.55	2.42	5.97	3.62	14.0	16.9	17.3
5	3.12	9.41	49.4	1.80	3.13	7.43	6.30	29.6	20.7	39.6
6	3.20	8.90	48.5	1.77	3.83	7.59	8.41	27.6	33.1	30.9
7	3.11	8.37	39.5	1.42	2.13	6.07	5.47	28.6	29.6	25.1
8	3.18	6.96	38.0	1.36	1.88	6.94	4.79	29.5	27.7	18.7
9	3.48	4.72	22.2	0.75	2.21	6.97	6.65	20.8	22.0	7.26
10	3.53	5.00	22.4	0.76	2.44	5.74	5.82	24.7	19.0	22.4
11	3.15	4.89	17.2	0.56	2.72	4.24	8.39	6.07	12.5	16.5
12	3.28	4.92	12.5	0.38	1.51	3.55	8.12	5.92	8.44	12.0
13	3.12	9.56	28.3	0.99	6.26	10.8	5.36	12.5	9.74	26.0
14	3.12	5.28	22.5	0.76	2.67	4.50	5.99	14.8	8.47	23.1
15	3.94	8.10	28.2	0.98	1.58	7.30	4.33	33.7	12.5	43.3
16	3.86	10.9	36.0	1.28	1.66	8.23	6.33	38.3	11.3	67.6
17	3.90	5.24	16.5	0.53	2.29	6.18	15.1	15.6	9.47	29.6
18	3.87	8.79	23.5	0.80	2.74	5.84	8.66	32.8	10.4	35.6
19	3.12	8.53	31.9	1.12	3.85	7.19	7.64	21.3	8.03	35.6
20	3.09	5.58	30.3	1.06	2.92	7.14	5.64	27.3	8.98	36.3
21	3.15	9.70	30.2	1.06	2.99	8.34	5.85	26.4	9.05	29.1
22	3.21	6.00	26.2	0.91	3.38	6.81	8.06	27.0	7.92	30.4
23	3.30	4.36	11.3	0.33	2.29	4.62	2.14	7.43	4.50	9.97
24	3.24	4.10	15.8	0.51	2.21	4.40	3.66	21.2	5.68	14.8
25	3.23	4.93	27.7	0.96	3.46	7.30	5.82	16.2	6.72	32.2
26	3.31	6.62	20.6	0.69	2.76	5.58	4.98	18.9	7.28	24.3
27	3.26	6.11	20.5	0.68	1.74	6.16	3.33	10.6	6.61	19.7
28	3.36	5.12	15.4	0.49	3.92	2.83	6.52	10.7	7.58	16.7
29	3.43	4.61	13.8	0.43	5.47	1.27	5.16	8.02	7.69	21.1
30	3.26	1.62	23.1	0.79	2.29	6.62	5.01	26.3	5.71	42.0
31	3.38	3.14	23.7	0.81	2.68	7.65	5.17	26.4	6.10	47.6
32	3.26	1.07	21.1	0.71	2.20	8.67	4.35	24.9	6.38	35.7
33	3.32	1.12	21.6	0.73	3.93	7.98	8.58	25.9	8.20	39.5
34	3.35	2.47	41.7	1.50	2.21	8.27	6.37	34.8	6.03	42.4
35	3.10	2.87	42.3	1.53	2.44	6.89	4.68	23.0	5.47	35.3

a Soil pH were measured in 10 mM $CaCl_2$ solution using a 1:5 soil-to-solution ratio.

b The data of total C and N were estimated. The estimation was based on relationships between heat loss weight of sample at 550 °C and total C and N contents derived from a previous sampling campaign (Supplimentry data, Fig. 1. Chapter 2).

c Pi = inorganic P concentration; Po = organic P concentration obtained by difference between total P and Pi concentrations.

d Corrections were applied to these data to minimize analytical problems occurring during the measurement.

CHAPTER 4

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

It is observed (chapter 2) that Pi and Po fraction in the soil of Landes at the scale of ecosystem are largely variables. The results suggest that fertilizers amendments showed some relevance to control significantly, the total variance of various P fractions measured in 37 soil sampling positions. Similarly, mineral P fertilization also significantly increased the growth of *Pinus pinaster* (Brest height circumference) in field conditions. Chapter 3 revealed 19 different ECM morphotypes and the phosphatase activity of these ECM was seamed to be regulated by soil water contents and Olsen Pi availability.

In order to better understand soil-plant-ectomycorrhizae interface, intact soil samples with contrasting Pi availability were used to grow young seedlings of *Pinus pinaster* in control conditions using rhizoboxes (Torres Aquino and Plassard 2004). Intact soils samples were taken from field and preserved at 4°C in dark prior to use in rhizobox. These samples were supposed to serve as a soil born indigenous inoculum of ectomycorrhizae for the young seedlings.

The objectives of this chapter were to evaluate:

1) The phosphatase activity of ECM associated with young seedlings grown in soils with contrasting P status.
2) The Pi availability in soils taken from line of trees and interlines of trees to evaluate effect of line and interline.
3) The growth response of young seedlings of pine in rhizobox prepared with soils of contrasting soil P status.

The young seedlings of *Pinus pinaster* were grown in soils of two sites. The site “L” with treatments of fertilizers amendments (control, fertilization with P mineral fertilizers and fertilization with NPKCaMg, fertilization was applied in interline position) and site Baudes with out fertilizers amendment were used in rhizoboxes. Both sites were not irrigated and samples were taken from lines of trees and interlines of trees. The site “L” has plantation age 13 years, in contrast site “Baudes” has plantation age 93 years.

The results obtained after 5 months of growth experiment have been published in scientific review “*Tree physiology*” with the following title:

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

References

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CHAPTER 4

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

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Abstract

Young seedlings of maritime pine (*Pinus pinaster*) were grown in rhizoboxes using intact spodosol soil samples from south west 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 ectomycorrhizal were used for measurements of acid phosphatase activity and molecular identification of fungal species using amplification of ITS region. Total biomass, N and P contents were measured in roots and shoot 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 ectomycorrhizal 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 were 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 ectomycorrhizal tips, P availability remained a limiting nutrient in soil samples from unfertilized stands. Nevertheless young *P. pinaster* seedlings showed high plasticity for biomass production at low P availability in soils.

Key words: *Plant plasticity, P contents, fertilizers application, fungal biomass, ectomycorrhizal morphotypes, phosphatase activity.*

1. Introduction

Maritime pine (*Pinus pinaster* Soland *in* Aït.) is a tree species cultivated over an area of one million hectares named Landes of Gascogne situated in south west of France. Although this species represents only 6.5% of total forest area in France, it holds an important economical value as it produce ca 20% of French softwood (Bert and Danjon 2006). These monoculture forests are established in spodosols predominantly characterized by acidic sandy soils with 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 results 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, productivity of *Picea abies* ((L.) H. Karst) and *Pinus sylvestris* (L.) in Sweeden (Axelson and Axelson 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 aboveground productivity of maritime pine in the first five growing seasons following application. In the later stages of tree growth, fertilization with P in P deficient soils can further increase plant growth (Gentle et al. 1965, Neilsen et al. 1984). However the extents and duration of growth response to P application is dependent on characteristics of soils, especially P sorption capacity, pH and intrinsic P contents (Pritchett and Comerford 1982). In addition to mineral P pools, among which orthosphosphate (Pi) is the only available P form for plant uptake, forest soils contain organic P (Po) pools composed of mono and diesters released from plant biomass. Together with microbial and litter fall plant derived inputs, Po pools increase over time and represent a major fraction of total P (up to 80%) in soils (Condron and Tiessen 2005). However, prior to being used by plants or microorganisms, the ester link must be hydrolyzed by phosphatase enzymes to release free orthophosphate ion, the only form of P taken up by plants or microorganisms.

Plants develop various strategies to fulfil their P requirements, such as modification of root structure, association with ectomycorrhizal 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 *Pinus 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 ectomycorrhizal (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 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 increased soil exploration, ECM fungi have exhibited a release of phosphatase in culture medium (Tibbett et al. 1998, Quiquampoix and Mousain 2005) that could play a crucial role to mobilize Pi from organic P pools of soils. This effect could be particularly important in 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 collected from south west of France in terms of growth responses and mineral nutrition. Soil samples were collected from a 93-year-old unfertilized stand, and a 13-year-old *P. pinaster* stand with annual fertilization of either only P or P and N (Trichet et al. 2008, Bakker et al. 2009). The former soil was chosen based on 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 position. It was hypothesized that mycorrhizal association of plant might have affected 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.

2. Material and methods

2.1. Forest stands description and soil collection

Soil samples used in this study were collected in two *P. pinaster* stands located in Gascogne region in south west of France. In both stands, soils are sandy spodosol developed on Aeolian sandy deposits of quaternary era. Mean annual air temperature is 12.5 °C and average precipitation rate is 950 mm, with frequent prolonged period of drought in summer. The first stand was a 93-year old forest of *P. pinaster* and the soil was never fertilized since plantation establishment. The second stand was a 13-year-old planted forest divided in plots with different fertilizer regimes: no fertilizer (control, C), phosphorus fertilizer (P) and complete mineral fertilizers (F) application. Each plot measured 60 x 36 m with an exclusion of 10 m border area. In both stands, the trees were planted in lines with a 2m-distance from tree to tree and a 4m-distance from line to line. Cores of mineral soil (15 cm length, 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 kept at 4°C for three months prior to use 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.

2.2. Plant preparation and culture in rhizoboxes

Seeds of maritime pine (*P. pinaster* Soland. In Ait. from Medoc, Landes-Sore-VG source, France) were surface disinfected by immersing into H_2O_2 30% (w/w) for 30 minutes then rinsed several times with sterile water. Finally they were soaked in sterile water and let at 4°C during 48 hrs for stratification. Germination of stratified seeds was carried out in water-moistened vermiculite previously sterilised twice (121°C, 15 minutes) and placed in a growth chamber. After 2 months, germinated pine seedlings were transferred into a mist chambers in order 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 scissor. After one month, plants were transferred in rhizoboxes described in Torres Aquino & Plassard (2004). Briefly, the rhizobox consisted of two Perspex plates (20 x 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.

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).

Soil sample	Sampling location	Stand Age (years)	Treatment	Fertilization regime
CO-L	Line	93	Control Old	No fertilizer application
CO-IL	Interline			
C-L	Line	13	Control	No fertilizer application
C-IL	Interline			
P-L	Line	13	P	Annual P fertilization in interline[a]
P-IL	Interline			
F-L	Line	13	Complete fertilization	Annual complete fertilization in interline[b]
F-IL	Interline			

[a] Mean rate in 1998-2005 of 32 kg P 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)

After the installation of soil in the first Perspex plate with spacers, a sterile piece (7x10 cm) of glass 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 plant. The root system of young seedling was spread on the soil layer in the rhizobox and the system was closed with second Perpex plate, clamps and sticky tape. The rhizoboxes were transferred in a container containing distilled water and plants were allowed to grow in the growth chamber for six months with regular supply of distilled water. Growth conditions were a 16/8 h light/dark cycle at 25/18°C, 70% rh, CO_2 concentration of *c.* 350 mm^3 l^{-1} and a PAR of *c.* 400 µmol m^{-2} s^{-1} (400-700 nm).

2.3. 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 roots (bulk soil) was separated carefully. Each root system was examined under stereo microscope to pick up ectomycorrhizal root tips (ECM) and ECM tips were morphologically classified. Each ECM

morphotype was used for acid phosphatase activity. The sub samples of each morphotype were stored at –20°C for molecular identification. The weight of fresh and freeze-dried roots and shoot was recorded. Soil separated into bulk and rhizosphere soils were also freeze-dried prior to analysis.

2.4. Phosphatase activity

Phosphatase activity (Tabatabai 1982) of four ECM tips for each morphotype was estimated separately. ECM tips were incubated at 30°C for an hour in 10 mM solution of *p*NPP 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 measures at 400 nm and enzymatic activity was calculated (nmol of *p*NP produced min^{-1} g^{-1} of fresh ECM weight) by the equation:

$$\text{Phosphatase activity} = \left(\Delta\,\text{OD} \times 1.2 \times \text{DF} \Big/ \text{t} \times \text{FW} \times 0.0188\right)$$

Where "ΔOD" is the difference between optical density of blank and sample, "1.2" is final reaction volume (ml), "DF" is dilution factor, "t" is the time (minutes) of incubation, "FW" is fresh weight of ECM tip, "0.0188" is coefficient of molar extinction for *p*-nitrophenolate ($ml.nmol^{-1}$ cm^{-1}).

2.5. Plant and soil analysis

Bulk and rhizophere 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 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 hours in 1 ml solution of methanol and polyclar (Serva, Heidelberg, Germany) (0.5 % W/V). The samples were centrifuged (14000 g, 15 min), and the supernatants were filtered through 0.45 µm nylon syringe filter (514-0067, VWR™ International USA). 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 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 minutes in 6 ml of $NaHCO_3$ (0.5 M, pH 8.5) (Olsen *et al*, 1954). Similarly, 0.5 g of soil

was shaken for 16 hr in 5 ml of NaOH (0.1 M) to extract less labile fraction of P associated with Al and Fe-oxides (Tiessen et al 1984, Sharpley 1999). Bicarbonate and hydroxide soil extracts were diluted with distilled water (1/6, v/v), then acidified with HCl 12N (1/600, v/v) to precipitate humic material before assaying Pi concentrations. The same soil extracts (bicarbonate and hydroxide) were mineralised with HCl 12N (v/v) at 110°C for 16 hr. As shown by our preliminary experiments, these conditions made it possible to mineralise 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 $NaHCO_3$ and NaOH extracts.

Plant roots and shoot material was finely ground and 50 mg of roots or shoot dry matter was mineralised in Pyrex glass tube under chemical fume hood using 1 ml of H_2SO_4 (36 N) at 330°C for 30 minutes (McDonald 1978) in a tube mineralization block. If solution was not transparent, tubes were removed and allowed to cool down and 0.2 ml of H_2O_2 was repeatedly added till the solution became transparent. After dilution of H_2SO_4 to 0.1N, ammonium was assayed using phenol colorimetric method of Berthelot (Martin et al, 1983). Orthophosphate P was assayed in 0.1N H_2SO_4 plant digest and in soil solutions using malachite green method (Ohno and Zibilske, 1991).

2.6. Identification of indigenous fungal species

Fungal DNA from frozen individual ECM morphotypes was extracted using the DNeasy Plant Mini Kit according to the manufacturer's instructions (QIAgen S.A.). Three µl 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 (Kõljalg et al. 2005).

2.7. Statistical analyses

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. Means (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 Fisher model ($P < 0.05$) and error bars on each mean value denote standard error (SE). Relations between total N and P and biomass in plants were drawn by simple linear regression. All data were analysed using Statistica software package (Statistica 8, Statsoft Inc. Tulsa OK, USA.). 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, data from line and interline were pooled together in CO and C treatments and were compared with other soil treatments.

3. Results

3.1. 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 soil (Table 2). Forest age (93 years, CO soil) resulted in an increase of bicarbonate extractable P (4 mg P kg^{-1} soil) in soil 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. Despite of 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 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 22% and 12% respectively (Table 2).

Table 2: Concentrations of organic and inorganic P extracted by $NaHCO_3$ 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 means (n = 6). Probabilities to have significant differences between lines and interline are indicated for each soil treatment using LSD Fisher model. An asterisk denotes a significant difference between B and R soils (LSD Fisher model, $p< 0.05$).

Soil sample	P-inorganic (mg kg^{-1} of dry soil)				P-organic (mg kg^{-1} of dry soil)			
	$NaHCO_3$		NaOH		$NaHCO_3$		NaOH	
	B	R	B	R	B	R	B	R
CO-L	4.4	4.4	8.2	7.8	10.3	12.8	55.4	68.4*
CO-IL	2.5	3.1	4.2	5.1	6.9	7.7	39.7	42.5
p	0.01	0.02	0.06	0.002	0.04	0.02	0.1	0.002
C-L	1.7	2.1	8.2	7.2	7.7	8.1	43.9	46.3
C-IL	1.5	1.6	2.4	3.3	3.9	4.0	17.7	17.3
p	0.3	0.04	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
P-L	4.5	3.7	10.0	5.9	5.3	9.1*	33.8	37.1
P-IL	50.5	50.6	83.9	80.2	7.4	15.0*	50.7	45.3
p	< 0.001	< 0.001	< 0.001	< 0.001	0.04	0.007	0.03	0.06
F-L	14.1	13.0	26.4	25.5	5.4	8.3*	32.8	31.0
F-IL	62.3*	50.8	92.9*	82.4	5.3	14.7*	53.7*	41.6
p	< 0.001	< 0.001	< 0.001	< 0.001	0.9	< 0.001	< 0.001	0.03

3.2. Soil organic P fractions

Organic P (Po) concentrations in bulk soils extracted with bicarbonate varied little between treatments (Table 2). In control plots, soil 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 folds in both line and interline positions. However the pattern of accumulations was as that was 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 soil from the lines position of CO treatment and from interlines position of F treatment, where they are respectively higher and lower than the values assayed in corresponding bulk soils (Table 2).

3.3. 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 that 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 fertilised treatments were relatively higher than the values measured in controls (Figure 1).

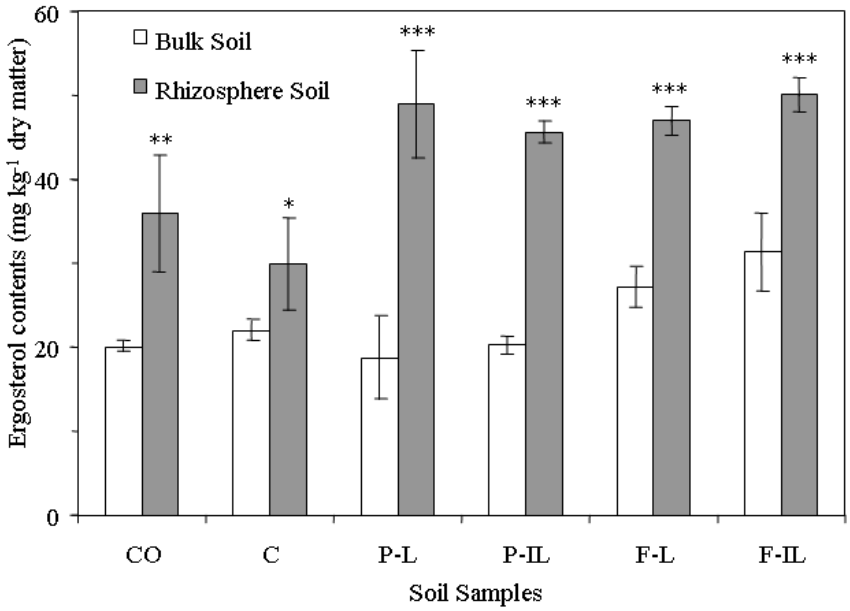

Figure 1: Ergosterol contents estimated in the hyphal plus plant debris material collected from bulk and rhizosphere soils after sieving through 2 mm sieve. Bars of histogram are means (n = 6-12) with standard errors. Significant differences between contents in bulk and rhizosphere soils are signalled by asterisks (* $P< 0.05$, ** $P< 0.01$, *** $P<0.001$, LSD Fisher model).

Identification of fungal species forming ectomycorrhizal tips showed that the basidiomycete *Rhizopogon luteolus* was dominant species regardless the treatments, as it presented 66.6 % of the ECM tips analysed using molecular tools. We also found *Sphaerosporella brunea* (28 %) and 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 were the highest (n = 25-40) in ECM collected in soil from the 93 year-old forest, regardless 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 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 P soil (IL) (Figure 2A). As shown in Figure 2B, the activities decreased when rhizosphere $NaHCO_3$-extractable Pi concentrations increased and vice-versa. Nevertheless, 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.

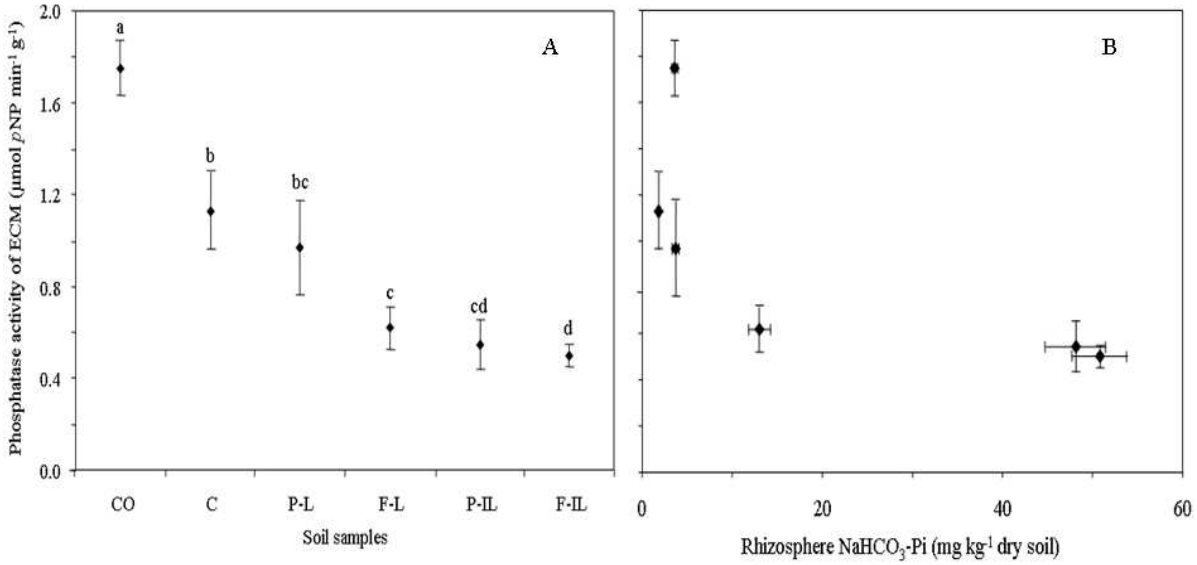

Figure 2: Acid phosphatase activity from ECM root tips estimated by the hydrolysis of pNPP as a function of the soil samples (A) or the concentrations of $NaHCO_3$-extractable Pi in the rhizosphere soil of each soil sample (B). Mean values (n = 24-40) ± 1 SE are shown. Different letters indicate significant differences between soil samples (LSD Fisher model, $P< 0.05$).

3.4. Plant growth and mineral nutrition

Table 3 indicates that dry biomass in roots and shoot 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. Surprisingly, soil receiving complete fertilisation (F-IL) produced plants with similar root and shoot biomass as in control soil (CO and C). Concentrations of total N in roots and shoot of plants grown in soil from fertilized plots did not vary whatever the treatment and were lower than those measured in shoot from the two control treatments (CO and C) and roots from the CO treatment only (Table 3). In contrast to N, total P concentrations in roots and shoot 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 shoot 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 treatment while complete fertilization (F-IL) and CO soils significantly decreased this parameter (Table 3).

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 means (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 LSD Fisher model ($p < 0.05$).

Soil sample	**Biomass (g dwt plant^{-1})**		**Total N (mg g^{-1} dwt)**		**Total P (mg g^{-1} dwt)**		**Root length (cm plant^{-1})**
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	
CO-L+	0.25b	0.16d	12.17a	10.19a	0.44d	0.55d	228.7b
CO-IL	(0.02)	(0.01)	(0.68)	(0.49)	(0.02)	(0.04)	(13.3)
C-L+C-IL	0.25b	0.17d	10.12b	7.35b	0.60d	0.55d	302.2a
	(0.02)	(0.01)	(0.23)	(0.24)	(0.02)	(0.02)	(25.3)
P-L	0.31b	0.22c	6.61c	6.65b	0.67d	0.64d	250.9b
	(0.06)	(0.05)	(0.56)	(0.23)	(0.08)	(0.05)	(33.9)
F-L	0.33a	0.24b	7.72c	6.61b	1.64c	1.73c	282.6b
	(0.05)	(0.03)	(0.26)	(0.25)	(0.11)	(0.13)	(36.5)
P-IL	0.35a	0.29a	7.14c	6.68b	4.69a	3.11b	305.9a
	(0.02)	(0.01)	(0.36)	(0.25)	(0.27)	(0.11)	(18.3)
F-IL	0.26b	0.17d	8.05c	6.98b	3.98b	3.41a	254.3b
	(0.02)	(0.02)	(0.89)	(0.15)	(0.45)	(0.14)	(33.4)

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 total N contents, total P contents varied widely among soil treatments (Figure 3B). Total P contents in plants from CO, C and P-L treatments were low and varied linearly with biomass. 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, plants grown in F-IL and P-IL soil samples displayed the highest levels of P contents that was not linearly correlated (*P > 0.05*) with plant biomass (Figure 3B).

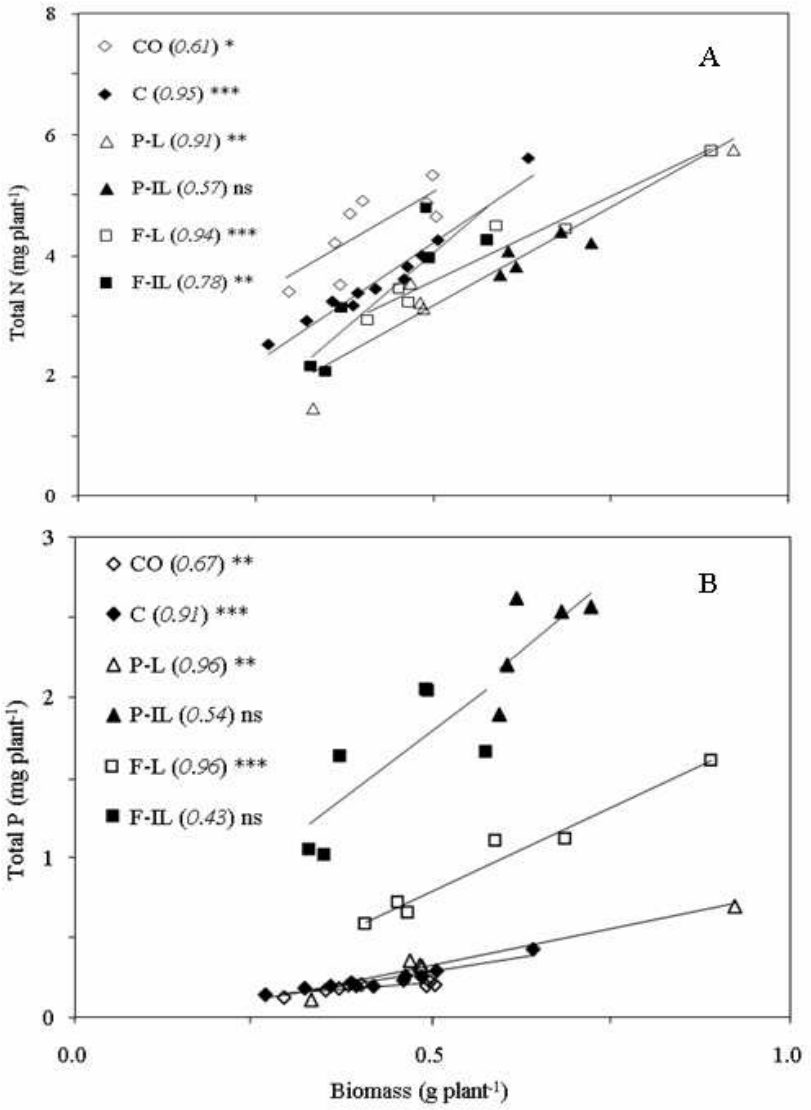

Figure 3: Relation of total N (A) and total P (B) per plant with total plant biomass for different soil treatments (n = 6-12). Plants grown in soil samples collected from line and interline in CO or C treatments are grouped together. Values in brackets represent the coefficient of linear regression (R^2) and asterisks denote significance of relationships for each soil sample type (* = P< 0.05, ** = P< 0.01, *** = P< 0.001).

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. These data show that plants were able to uptake N despite of having low P availability in soil

(Figure 4). Overall, these results indicate that 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 $NaHCO_3$ 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).

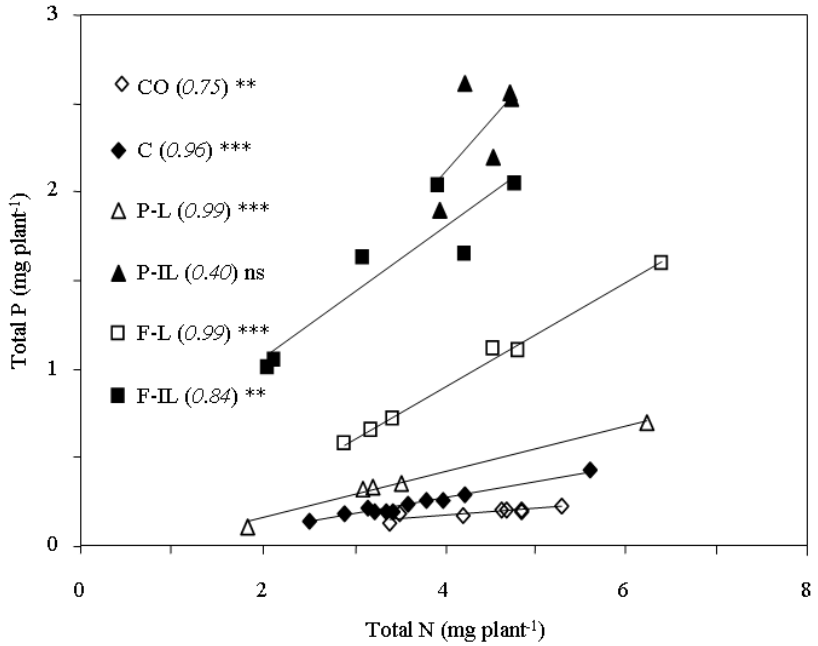

Figure 4: Relationships between total P per plant with total N per plant for different soil treatments (n = 6-12). Plants grown in soil samples collected from line and interline in CO or C treatments are grouped together. Values in brackets represent coefficient of linear regression (R^2) and asterisks denote significance of relationships for each soil sample type (* = P< 0.05, ** = P< 0.01, *** = P< 0.001).

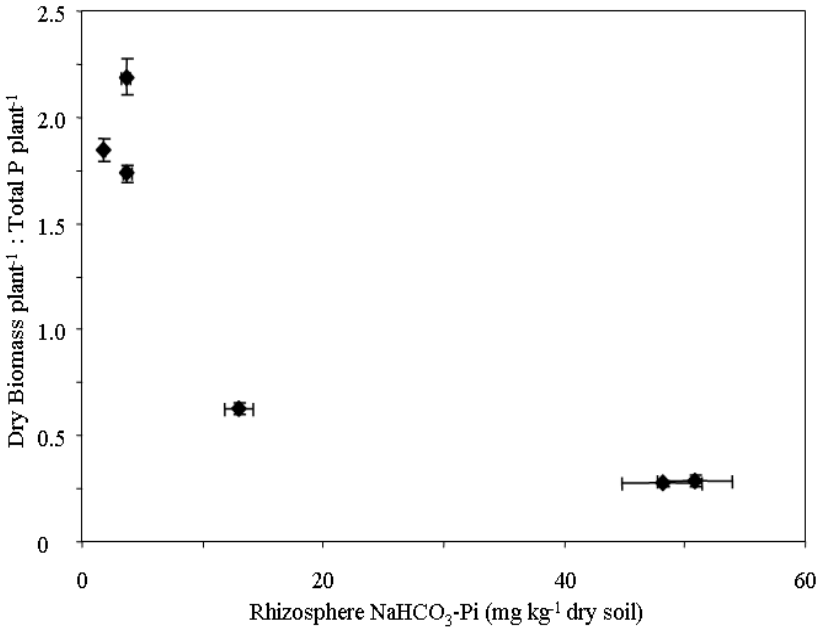

Figure 5: Variation in the ratio of dry biomass to total plant P with concentrations of $NaHCO_3$-Pi in the rhizosphere of *P. pinaster* seedlings grown in the soil samples with different fertilizer regimes. Bars represent standard errors of means (n = 6-12).

4. 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 multiple factors such as immobilization of available P by adsorption of phosphates with Al and Fe, rendering Pi as less labile phosphate source of P (Fontes and Weed 1996, Barroso and Nahas 2005) or immobilization in the form organic P (Holford 1997). P fertilizers applied to enhance the sustainability of the stand dramatically increased $NaHCO_3$ and 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 immobilised 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 in soil collected from lines than in interlines positions. It confirms that P in soil was not or slightly mobile as claimed by several authors (see for ex. the reviews from Schachtmann et al. 1998, Hinsinger et al. 2001, Vance et al. 2003) and the fertilizers applied in 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 F plot contained greater bicarbonate and NaOH extractable Pi concentrations than soil from 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 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 soil samples from interlines 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 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 *Pinus pinaster* forest stand and Parrent and Vilgalys (2007), who reported stimulation of extramatrical mycelia with N fertilization in *Pinus taeda* forest. In addition, these soil conditions may have also favoured the development of bacterial populations associated with ECM fungi, as demonstrated for *Laccaria bicolor* (Duponnois and Garbaye 1991a, b). 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 utilisation 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 ectomycorrhizal morphotypes revealed that *Rhizopogon luteolus* formed most of the ECM tips whatever the treatment. The high capacity *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 and Claridge 2002, Bruns et al. 2009). *R. luteolus* was frequently identified in ECM tips in field surveys of the same plots (unpublished data). The second species was *Sphaerosporella brunnea.* However, it could be considered as an opportunist species, as it is a common contaminant in nurseries producing mycorrhizal plants (García-Montero et al. 2008). Acid phosphatase activity measured by incubating the ECM in the artificial substrate *p*NPP 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 previous studies carried out

with ECM grown in soil (Kroehler et al. 1988, Antibus et al. 1992 Chen et al. 2002) or with the ectomycorrhizal 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 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 concentration 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 much variable as for P, especially when plants were grown in CO soil samples. In these conditions, the concentrations assayed in roots and shoot 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 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 mg N kg-1 of dry soil). This suggests that N accumulated in this soil was easily used 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 linear regressions of these two variables were highly significant in all treatments except in soils with fertilizers application (P-IL and F-IL). Plants grown in soil samples with $NaHCO_3$ 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, plants grown in CO soil presented the highest values of acid phosphatase activity in ECM. This suggests that phosphatase activity was not sufficient enough 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 Po occurring in the soil solution that may be

very different from the conditions used to measure *p*NPPase activity *in vitro*. Indeed, CO soils are very dark and 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 humic and fulvic compounds. These soluble molecules could inhibit phosphatase activity in soil conditions (Allison 2007). On the other hand, soil organic P molecules might not be highly degradable with the enzymes produced by the ECM fungi.

Slight increase in soil P availability up to 14 mg $NaHCO_3$ extractable Pi kg^{-1} dry soil (F-L) strongly increased P contents that remained highly correlated with biomass production and N contents per plant. In contrast, the highest bicarbonate P concentrations still increased total P contents of plant (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 & Schmitz 1991, Aerts & Chapin 2000). Conversely, we observed a decreased biomass production in F-IL treatment. This could result by the down-regulation of root length observed in these conditions (Coyle & Coleman 2005), as shown in several plant species together with the down-regulation of P transport capacities by the roots.

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 immobile spacialement and high phosphatase activity was not sufficient to overcome P limitation of *P. pinaster* in soil with low phosphorus availability.

5. Acknowledgements

We thank Pierre Trichet (EPHYSE, INRA Bordeaux) and Laurent Augusto (TCEM, INRA Bordeaux) for their help in collecting the field samples and Marney Isaac (University of Toronto, Canada) for helpful comments on text. Muhammad A. Ali was supported by a grant from the Higher Education Commission of Pakistan, Julien Louche by a grant from the French Minister of Research and Technology and Myriam Duchemin by the ANR (Agence Nationale de la Recherche) through the FUNDIV project (ANR-06-BDIV-06). The work was funded by the PNBC-ACI ECCO project *"Déterminants naturels et anthropiques de la biodiversité rhizosphérique et de la biodisponibilité du phosphore en forêt atlantique"*. Molecular identification of fungal species was funded by the FUNDIV project.

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CHAPTER 5

Growth response of *Pinus pinaster* seedlings in soils regularly managed by amendments of irrigation and fertilization

The results of chapter 4 showed that application of fertilizers increased significantly, the fractions of Pi and Po in interline position and there was no or very low spatial movement of P towards lines of trees. It was also observed that the phosphatase activity was negatively related to Pi availability (Olsen Pi). However in chapter 3, it was deduced that the phosphatase activity was not only regulated by Pi availability (Olsen Pi) but also it was regulated by water contents. In the other study it has been observed that the phosphatase activity of soils could be increased by the application of nitrogen as fertilizer (Wang et al., 2008). We made an inference that irrigation, NPK fertilization or both irrigation and NPK fertilization could have some relevance with phosphatase activity as well as the availability of Pi for the young seedlings of *Pinus pinaster*. In this chapter, the focus was to evaluate the effect of Pi availability on the growth of *Pinus pinaster* and phosphatase activity of ECM in the soils which were taken from irrigated and non-irrigated plots.

In previous chapter (chapter 4) the significant differences were observed between rhizosphere soils and bulk soils (fungal biomass, Po mobilization). However, it remained questionable, how to separate rhizosphere and bulk soil in a system with extension of fungal hyphae? In order to address this question, rhizobox without *Pinus pinaster* seedlings were prepared for each treatment to evaluate the depletion of Pi or hydrolysis of Po in soils by young seedlings (rhizosphere soil) compared to without seedlings (bulk soil).

The objectives of this chapter were to evaluate:

1) The phosphatase activity of ECM associated with young seedlings grown in soils with contrasting P status.
2) The effect of irrigation on phosphatase activity of ECM and P nutrition of *Pinus pinaster* seedlings.

In order to achieve these objectives young seedlings of *Pinus pinaster* were grown in rhizoboxes for 8 months. The soil samples from site “L” with treatments of fertilizers amendments (control, fertilization with P mineral fertilizers and fertilization with NPKCaMg) with and without irrigation were used in rhizoboxes.

The results obtained after 8 months of growth experiment has been used for a scientific manuscript, which will be submitted in "*Forest Ecology and Management*" titled as "Growth response of *Pinus pinaster* seedlings in soils regularly managed by amendments of irrigation and fertilization".

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CHAPTER 5

Growth response of *Pinus pinaster* seedlings in soils regularly managed by amendments of irrigation and fertilization

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Abstract

Soil samples from 13 years-old *Pinus pinaster* forest stand, managed annually by treatments of fertilizers (control, P and NPKCaMg) with and without irrigation were used to grow young seedlings of *P. pinaster* in rhizoboxes under controlled conditions. Eight replicates of young seedlings for each treatment and control rhizoboxes (without seedlings) were harvested after 8 months and ectomycorrhizal morphotypes were isolated from roots. Bicarbonate and hydroxide extractable Pi and Po in soils and total P and N contents of plants were assayed. Moreover, determination of acid phosphatase activity of ectomycorrhizal morphotypes developed through indigenous soil spores and their molecular identification was performed. The data obtained was used to evaluate the P status of soils and the effect of soil treatments on plant growth as well as acid phosphatase activity of indigenous ectomycorrhizae developed in these soils. Results showed that fertilization significantly increased bicarbonate and hydroxide extractable Pi and Po while the availability of P in soils decreased phosphatase activity. Plants were able to deplete Pi and Po, but the depletion was highly significant in FI treatment. Combination of fertilizers and irrigation (FI) increased maximum biomass +147 % and +50 % in NPKCaMg treatment compared to C and F treatments respectively. The P in control treatments was strongly limiting and plants were hardly able to acquire P from these soils. However, concentrations and uptake of P was significantly higher in seedlings of fertilized treatments. Diagnostic approaches also confirmed deficiency status of P resulting into, up-regulation of N in plants of control soils. In contrast, both P and N were sufficient in soils from irrigated and complete fertilization treatment, and a steady growth response of seedlings was observed. The significant differences with complete fertilization with irrigation were suggested due to microbial population as well as irrigation. More research would be helpful to evaluate the effect of fertilization with irrigation, on microbial populations.

Key words: *acid phosphatase activity, rhizobox, mineral nutrition, microorganisms, vector analysis, P deficiency, plant biomass, P availability*

1. Introduction

Recently, forest management has received special attention throughout the world due to growing demands for fibre, wood, bio-energy production (Kauter et al., 2003) and reduction of CO_2 emission (Graham, 1992). These demands can only be satisfied by increasing plants production which requires the diagnosis of specific limiting factors at stand level (Fox, 2000). Nutrients and water are major limiting factors for the forest production, and could be alleviated by intensive management practices. It has been well accepted that the availability of water and nutrients largely control forest productivity, but few studies quantifying the role of water and nutrients have been conducted (Benson et al., 1992; Jokela et al., 2004). High fertility increases nutrient concentration of tissues, leaf area and plant growth as a whole (Samuelson et al., 2001), and water availability provides a bulk-flow pathway for nutrient uptake and maintains turgidity for growth and higher stomatal conductance for photosynthesis (Kozlowski et al., 1991). Studies conducted while using both fertilization and irrigation as treatments showed that forest production was generally limited by nutrient availability, but the response to nutrient amendments was also linked with adequate moisture availability (Lockaby et al., 1997; Samuelson, 1998).

Sites that responded to both nutrient and water addition showed either additive or interactive effects. For examples, fertilization had more effect on the growth of *Pinus taeda*, than irrigation and the interactive effect of fertilization and irrigation was not significant (Albaugh et al., 2004). Coyle and Coleman (2005) observed strong response with irrigation, nitrogen (N) fertilization and the interaction of both irrigation and fertilization, but the responses were considerably different for *Populus deltoides* and *Platanus occidentalis* species. Irrigation and N fertilizers resulted to increase growth of *Pinus radiata* showing enhanced response to irrigation in the early stages of growth compared to later growth stages (Waterworth et al., 2007). Similarly, the growth of *P. pinater* was increased by irrigation alone as well as by the interactive effect of both irrigation and fertilizers. Fertilizers amendments were of either phosphorus (P) or complete fertilizers (NPKCaMg) forms (Trichet et al., 2008).

Bakker et al. (2009) showed that irrigation and fertilization increased the amount of hyphae from ectomycorrhizal (ECM) fungi 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 for plants to increase phosphate acquisition by plants (Smith et al. 2003). In addition to increased soil exploration, ECM fungi have exhibited a release of phosphatase in culture

medium (Bousquet et al., 1986; Tibbett et al., 1998; Quiquampoix and Mousain, 2005) as well as in soils (Antibus et al., 1992; Chen et al., 2002) that could play a crucial role to mobilize Pi from organic P pools of soils.

Maritime pine (*P. pinaster* Soland in Aït.) is a tree species cultivated over an area of one million hectares in Landes of Gascogne situated in southwest of France. Although this species represents only 6.5% of total forest area in France, it holds an important economical value as it produce ca 20% of French softwood (Bert and Danjon, 2006). These monoculture forests are established in spodosols, predominantly characterized by acidic sandy soils with 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, these soils present low P availability that is considered as the main limiting factor for tree growth (Bonneau, 1995; Trichet et al., 2009). 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). The 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 decrease of soil and atmospheric moisture level (Loustau et al., 2005). Therefore, optimisation of management practices could benefit from a more integrated and accurate quantification of the effects of water and nutrients addition on soil nutrient availability and plant productivity.

We present here the results of a growth chamber experiment conducted in rhizobox on soils samples collected from plots of 13 year-old *P. pinaster* stand which were managed or not by the treatments of irrigation and fertilizers. The objective of the experiment was to evaluate the effect of previously irrigated and fertilized soil, planted with *P. pinaster*, on growth and mineral nutrition, particularly P, of newly planted *P. pinaster* seedlings. We also identified indigenous ectomycorrhizal (ECM) fungi associated with seedlings and quantified the phosphatase activity secreted by these ECM root tips. The effects of plant with its fungal partner (ECM) on different fractions of P in soil samples were evaluated by comparing the concentration of P fractions in soil, with and without seedlings of *P. pinaster*. We also aimed at linking P uptake by plant, P availability in soils and phosphatase activity of ECM in relation to irrigation and fertilization treatments.

2. Material and methods

2.1. Soil sampling and site discription

The soil samples were collected from the field of 13 year-old *P. pinaster* stands located in Gascogne region in southwest of France. Soil composition was characterized by sandy spodosols developed on Aeolian sandy deposits of quaternary era. Mean annual air temperature is 12.5 °C and average precipitation is 950 mm, with frequent prolonged period of drought in summer. The forest stand consisted of 13 year-old *P. pinaster* trees maintained regularly by various agronomic practices since 7 years. It included blocks with different fertilization regimes: no fertilizer (control, C), phosphorus fertilizer (P) and complete mineral fertilizers (F) application without irrigation and with irrigation (CI, PI and FI respectively) (Table 1). Each block measured 60 x 36 m with an exclusion of 10 m border area. Trees were planted in lines with a 2 m tree to tree distance and 4m line to line distance. Soil cores (15 cm length x 8 cm diameter) were collected from interlines positions of each block. Soil samples were collected in November 2006 and were kept at 4°C for three months prior to use them as substrate for young seedlings in rhizoboxes. As they were used without any further treatments, these soil samples served as indigenous soil fungi and bacterial inoculants and inherent mineral nutrients served as a source of nutrition for young seedlings.

Table 1: Brief description of soil samples used to grow young seedlings of *P. pinaster* in rhizoboxes. Soils were sampled from the field of 13 years old *P. pinaster* stands.

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 in1998-2005 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)

2.2. Seedling development and rhizobox culture

Seeds of maritime pine (*P. pinaster* Soland. In Ait. from Medoc, Landes-Sore-VG source, France) were surface disinfected by immersing them into H_2O_2 30% (w/w) for 30 minutes (Mason et al., 1983) then rinsed several times with sterile water. Finally, they were soaked in sterile water and incubated at 4°C during 48 hrs for stratification. Germination of stratified seeds was carried out on sterile (121°C, 15 minutes) water-soaked vermiculite and placed in a growth chamber. After two and half months, the plants were transferred in rhizoboxes described in Torres Aquino and Plassard (2004). Briefly, the rhizobox consisted of two Perspex plates (20 x 10 cm) separated by 3 mm spacers. The spacers made it possible to establish a 3 mm-wide layer of soil, corresponding to about 70 g of fresh soil. After soil installation in the first Perspex plate with spacers, a sterile piece (10 cm x 7 cm) of glass fibre paper sheet wrapped in a nylon cloth was placed at the bottom of the soil layer. This sheet will be in contact with a water reservoir to ensure water supply to the soil. After laying out the root system on soil surface, the system was closed with the second Perplex plate, clamps and sticky tape. The rhizoboxes were transferred in a container containing distilled water and plants were allowed to grow in the growth chamber for eight months with regular supply of distilled water. Growth conditions were maintained as 16/8 h light/dark cycle at 25/18°C, 70% rh, CO_2 concentration of *c.* 350 $mm^3\ l^{-1}$ and a PAR of *c.* 400 µmol $m^{-2}\ s^{-1}$ (400-700 nm). For each soil sample (C, P, F, CI, PI and FI) eight rhizoboxes with a young seedling of *P. pinaster* and two control rhizoboxes without seedlings were prepared simultaneously. Rhizoboxes with and without plants were maintained under similar growth conditions during eight months. Six young plants were weighed and kept at -80 °C, as a reference for the calculation of initial plant N and P amounts.

2.3. Plant and soil harvest

Rhizoboxes were dismantled and the root system was gently pulled out from the soil layer. Each root system was examined under stereo microscope to pick up ectomycorrhizal root tips (ECM) that were classified into different morphotypes to assay phosphatase activity. Sub samples of each morphotype were stored at -20°C for molecular identification. The weight of fresh and freeze-dried roots and shoot was determined. All the soils collected from rhizoboxes were air dried prior to analysis.

2.4. Phosphatase activity

Phosphatase activity (Tabatabai, 1982) of five ECM tips for each morphotype was estimated separately. ECM tips were incubated at 30°C for an hour in 0.2 ml of 10 mM *p*NPP solution prepared in acetate buffer (25 mM, pH 5.4). The reaction was stopped by adding 1ml of 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 enzymatic activity was calculated (nmol of *p*NP produced min^{-1} g^{-1} of fresh ECM weight) from the equation:

$$\text{Phosphatase activity} = \left(\Delta\,\text{OD} \times 1.2 \times \text{DF} \Big/ \text{t} \times \text{FW} \times 0.0188\right)$$

Where "ΔOD" is the difference between optical density of blank and sample, "1.2" is final reaction volume (ml), "DF" is dilution factor, "t" is the time (minutes) of incubation, "FW" is fresh weight (g) of ECM tip calculated using its volume and a density of 1 kg l^{-1}. Volume of ECM tip was calculated using the average diameter and length of ECM root tips taken by automated image analysis software WinRhizo 2005b (Regent Instruments. Inc., Canada), "0.0188" is coefficient of molar extinction for *p*-nitrophenolate ($ml.nmol^{-1}$ cm^{-1}).

2.5. Plant and soil analysis

Sieved soil (2mm) was used to measure P fractions. Plant available fraction of P was extracted by shaking 0.3 g of sieved soil for 30 minutes in 6 ml of $NaHCO_3$ (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, 1999). Soil extracts were diluted with distilled water (1/6, v/v), then acidified with 12 N HCl (1/600, v/v) to precipitate humic material before assaying Pi concentrations. The same soil extracts were mineralised with 12 N HCl (v/v) at 110°C for 16 h. As shown by our preliminary experiments, these conditions made it possible to mineralise all organic P contained in the solution (data not shown). Organic P concentration in soil extracts was calculated as the difference between Pt and Pi for both $NaHCO_3$ and NaOH extracts.

Plant roots and shoot material was finely grinded and 50 mg of roots or shoot dry matter was mineralised with 1 ml of H_2SO_4 (36 N) at 330°C for 30 minutes (McDonald, 1978). If solution was not transparent 0.2 ml of pure H_2O_2 (110 vol, not stabilized with phosphate) was repeatedly added till the colour of the solution was transparent. After dilution of H_2SO_4 to 0.1N, ammonium was assayed using phenol colorimetric method of Berthelot

(Martin et al., 1983). Orthophosphate was assayed in 0.1N H_2SO_4 and in soil solutions using malachite green method (Ohno and Zibilske, 1991).

2.6. Identification of indigenous fungal species

Fungal DNA from frozen individual ECM morphotypes was extracted using the DNeasy Plant Mini Kit according to the manufacturer's instructions (QIAgen S.A.). Three µl of the DNA extracts were used for PCR amplification with *Taq* polymerase (18038-026, Invitrogen) using the primers ITS1-F and ITS4 (White et al., 1990). The thermo-cycling 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). Gel electrophoresis (agarose 1.5%) was performed to verify DNA amplification. Samples with single band on gel were sequenced from COGENICS™ Meylan, France (http://www.cogenics.com). All sequences were identified to genus and species level by launching a query through blastn algorithm of 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 (n = 8 for soil and plant parameters and n = 8-25 for acid phosphatase activities of ECM root tips) were compared using least significant difference of Fisher ($P < 0.05$) and error bars presented for each mean value denote standard error (SE). Relations between biomass, total N and P in plants were calculated by simple linear regression. All data were analysed using Statistica software package (Statistica 8, Statsoft Inc. Tulsa OK, USA.). Vector analysis was performed for diagnosis of plant response to N and P in different soil treatments (Imo and Timmer, 1997; Saifu and Timmer, 2001; Saifu and Timmer, 2003). Soil treated with phosphorus application (P) was taken as reference because soil of experimental location was limited with phosphorus availability. The relative biomass, N and P contents as well as concentrations were expressed in vector nomogram. The arrow head lines in Figure. 5 indicate the dispersion of vector from reference soil sample (P) and length of the lines indicate the magnitude of dispersion. The arrows in vector nomograms (Fig. 5) are represented only for significant effects and plant responses to N as well as P were interpreted (Imo and Timmer, 1997; Saifu and Timmer, 2001; Saifu and Timmer, 2003).

3. Results

3.1. Soil P availability and its mobilization

The concentrations of inorganic P extracted through $NaHCO_3$ and NaOH were significantly higher in the fertilized soil samples compared to control ones (Fig. 1, a and c). However, the comparison of fertilized soil samples revealed, significantly ($P< 0.05$) higher values of Pi concentrations in soil with P and PI treatments, compared to F and FI treatments, despite of receiving same levels of P supply. The depletion of Pi was 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 hydroxyde extracts (Fig. 1a and c). Additionally, in fertilized treatments, the depletion of Pi by plants was also observed, but only NaOH extractable Pi was significantly different in PI and FI soils (Fig. 1c).

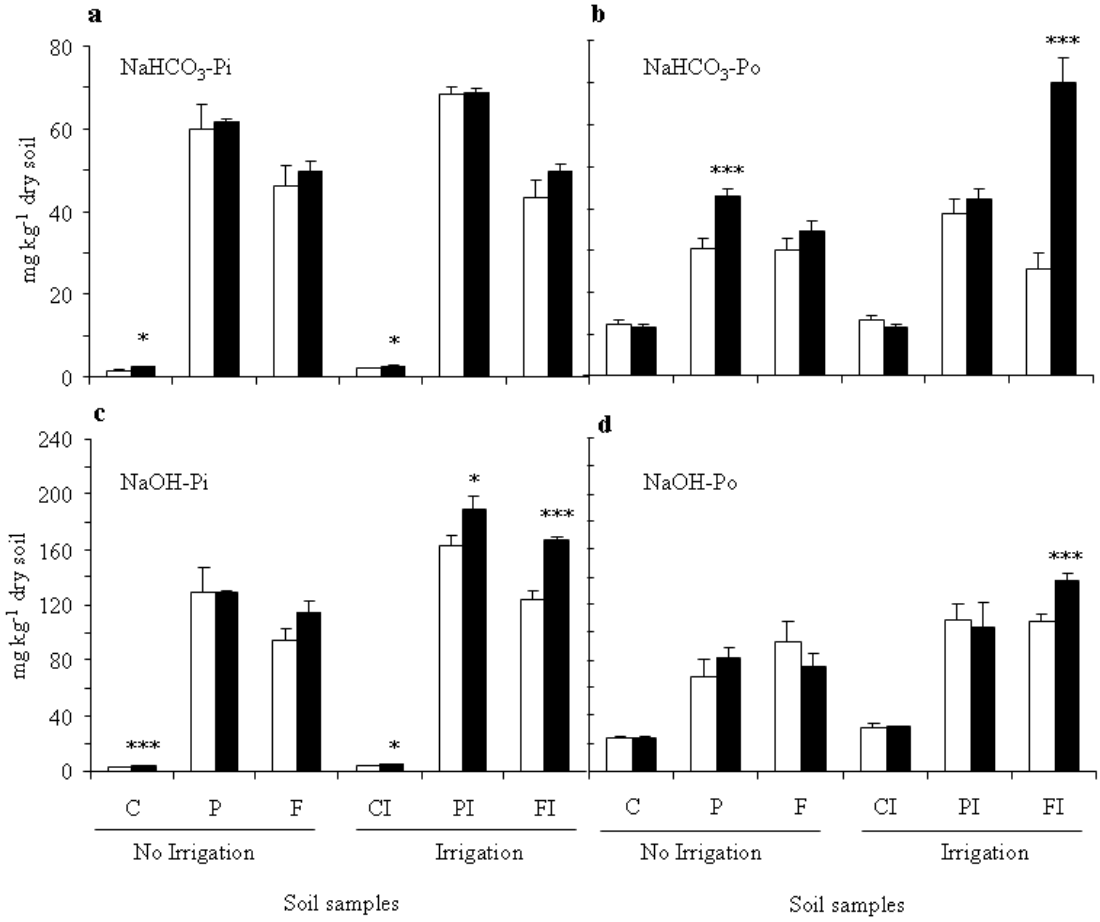

Figure 1: Concentrations of $NaHCO_3$ Pi and Po (a and b respectively) and NaOH extractable Pi and Po (c and d respectively) from the air dried soils after eight months of *P. pinaster* growth experiment in rhizobox. White bars of histogram denote means of P concentration with seedlings while black bars denote P concentrations without plants. 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, $P > 0.05$.

Po in control (C and CI) treatments represented the dominant form (> 80 %) of extractable P regardless of the extraction type, whereas in fertilized soil samples (Fig. 1b and d) Pi was the dominant fraction (20-50 %). As a whole, Po was slightly higher in irrigated soil samples than in non-irrigated ones. In general plants were able to deplete both bicarbonate and hydroxide extractable Po, when compared to the concentrations of Po with and without young seedling of *P. pinaster*, but the depletion was significant (t-test, $P < 0.05$) only in irrigated and complete fertilizer (FI) treatment. Data also revealed significant depletion of $NaHCO_3$ extractable Po in P treatment (Fig. 1b). Figure 1 showed that in fertilized soils NaOH extractable Pi was the dominant pool followed by NaOH extractable Po, $NaHCO_3$ extractable Pi and $NaHCO_3$ extractable Po in decreasing order.

3.2. Ectomycorrhizae and phosphatase activity

The comparison average phosphatase activity of ectomycorrhizal root tips for different soil treatments was 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 275 % to 353 % (Fig. 2). The decrease of phosphatase activity was also strong (183 %) when plants were grown in CI soil samples. Finally, the decrease of phosphatase activities was the lowest (62 %) in ECM tips developed in soils sampled from FI plot (Fig. 2). Molecular identification of ECM tips indicated that *Rhizopogon luteolus* was the dominant fungal species, as it represented 80 % of the total analysed morphotypes independent of the treatment. The remaining 20% of ECM tips were identified as *Sphaerosporella brunnea.*

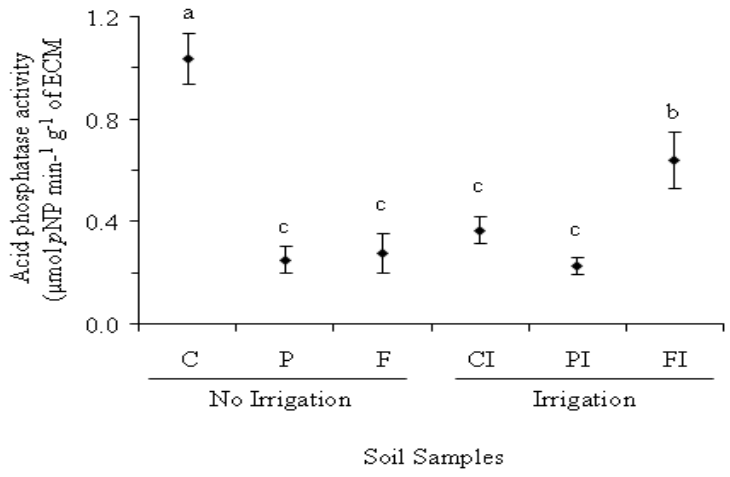

Figure 2: Acid phosphatase activity secreted by ECM root tips (n = 8-25) of *P. pinaster* from different soils. Different letters represent least significant differences of Fisher ($P < 0.05$) and bars are standard errors.

3.3. Pinus pinaster *growth and mineral nutrition*

Plant analysis indicated significant ($P < 0.05$) increase in shoot biomass of plants only in FI (+ 145 %) treatment compared to C treatment. Whereas, root biomass was significantly increased (120, 65 and 182 %) in F, PI and FI soil samples respectively, compared to control (C) plants (Table 2). Finally, for both shoot as well as root biomass, significant increase was occurring only in FI soil samples. Considering total biomass of plants (Table 3), complete fertilizer treatments (F and FI) only gave significant increases compared to control conditions (C). Concentrations of N differed by a factor lesser than 2 between all treatments for both roots and shoots, and were the highest in both irrigated and non-irrigated controls (C and CI) (Table 2). In contrast to N concentrations, P concentrations varied over a factor of 6 between all treatments. The analysis of variance ($P < 0.05$) showed that the concentrations of P measured in shoot and roots were the highest in PI treatment and the lowest in control treatment (C and CI). However, soils from P, F and FI treatments also significantly increased plant P concentrations compared to control (C and CI) treatments (Table 2).

Table 2: Accumulation of biomass and concentrations of N and P in roots and shoot parts of *P. pinaster* plants grown for 8 months in rhizoboxes containing soil samples from different provenances (see Table 1 for details). Analysis of variance was performed and means are compared using LSD Fisher. Different letter in the same column give significant difference ($P < 0.05$) of soil samples (n = 8) with standard error in brackets.

Soil Samples	**Dry biomass (g plant^{-1})**		**Concentrations of N**		**Concentrations of P**	
			mg g^{-1} dry weight			
	Shoot	**Roots**	**Shoot**	**Roots**	**Shoot**	**Roots**
C	0.41b	0.29d	6.80a	4.68a	0.32c	0.40c
	(0.05)	(0.03)	(0.59)	(0.18)	(0.01)	(0.01)
P	0.57b	0.40cd	3.71b	4.05b	1.47b	2.06b
	(0.05)	(0.05)	(0.15)	(0.10)	(0.14)	(0.20)
F	0.51b	0.64b	3.94b	3.58b	1.15b	1.96b
	(0.05)	(0.05)	(0.13)	(0.13)	(0.18)	(0.13)
CI	0.47b	0.37cd	6.12a	4.70a	0.28c	0.43c
	(0.07)	(0.04)	(0.39)	(0.18)	(0.02)	(0.02)
PI	0.50b	0.48bc	3.60b	3.67b	1.86a	2.71a
	(0.03)	(0.02)	(0.15)	(0.10)	(0.18)	(0.07)
FI	0.91a	0.82a	3.49b	3.65b	1.39b	1.80b
	(0.11)	(0.08)	(0.10)	(0.15)	(0.13)	(0.10)

Net accumulation of N and P occurring in plants are given in figure 3 for shoot and roots and in Table 3 for the whole plant. Figure 3a shows that the net accumulation of N in shoots was systematically lower compared to roots in fertilized treatment, whereas the accumulation was higher in shoot of control plants both with and without irrigation. Data indicated significantly higher net accumulation of N in shoot of C, CI and FI treatment ($P < 0.05$), whereas N accumulation in roots was only significantly higher in FI plants followed by F treatment (Fig. 3a). Finally, total net accumulation of N was increased significantly only in plants of FI soil samples (Table 3).

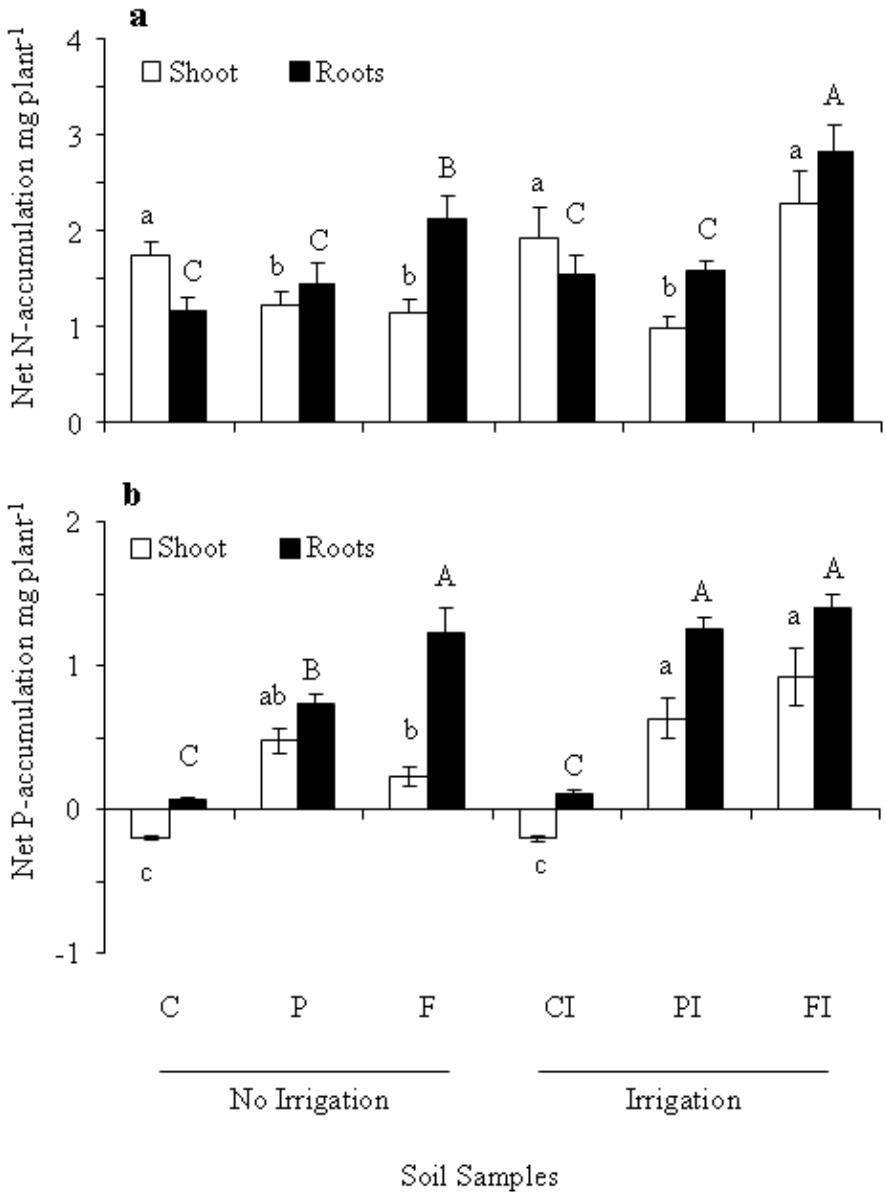

Figure 3: Net accumulation of N (a) and P (b) in shoot and roots of young seedlings of *P. pinaster* after eight months of growth chamber experiment in rhizoboxes. Analysis of variance was performed and means of eight plants grown in different soil samples are compared using LSD fisher model ($P < 0.05$). Different letters explain the significant differences for shoot (small letters) and roots (capital letters). Bars represent standard error of eight replications.

Fig. 3b indicated significantly higher net accumulation of P in shoot dry biomass of plants grown in PI and FI soil samples compared to those grown in non irrigated F and P as well as control treatments. Remarkably, there was a net decrease of P accumulation in shoots of plants grown in C and CI soils while in root parts a very low average net accumulation of P was calculated. Similarly, accumulation of P in roots of F, PI and FI soil plants was significantly higher than control soil plants whereas non irrigated P soil samples caused significantly low accumulation of P in the roots of plants. As shown in Table 3, total net accumulation of P per plant was significantly higher in fertilized soil samples as compared to control, where it was negative and close to zero. Nevertheless, irrigation resulted to increase significantly (56 and 60 %) the net P accumulation in PI and FI plant compared with P and F plants respectively (Table 3).

Table 3: Total biomass, net N and P accumulation per plant and [N:P] ratios of shoot and roots separately of *P. pinaster* grown in rhizoboxes during 8 months in soil samples (see Table 1 for details). Values are means (n = 8) and asterisk sign denote the least significant difference of Fisher over control treatment. Means with different letters denote least significant difference ($P < 0.05$).

Soil sample	Total biomass (g plant^{-1})	Net N-accumulation (mg plant^{-1})	Net P-accumulation (mg plant^{-1})	[N:P] shoot	[N:P] roots
C	0.70 c	2.90 b	-0.13 d	21.1 a	11.80 a
P	0.96 bc	2.66 b	1.20 c	2.74 b	2.14 b
F	1.15 b	3.25 b	1.45 bc	3.71 b	1.84 b
CI	0.84 bc	3.46 b	-0.09	22.4 a	11.10 a
PI	0.98 bc	2.55 b	1.88 ab	2.08 b	1.36 b
FI	1.73 a	5.10 a	2.32 a	2.67 b	2.05 b

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. 4a). This relationship explained the high net accumulation of N with increasing plant growth and vice versa (Fig. 4a). Compared to N, the value of regression coefficient between net P accumulation and total biomass per plant for all soil samples was decreased but remained significant (51 %). However, when fertilized and control treatment were used separately, the

coefficient of linear regression was increased to 56 % for fertilized soil samples and 93 % for only unfertilized control (C and CI) treatments (Fig. 4b).

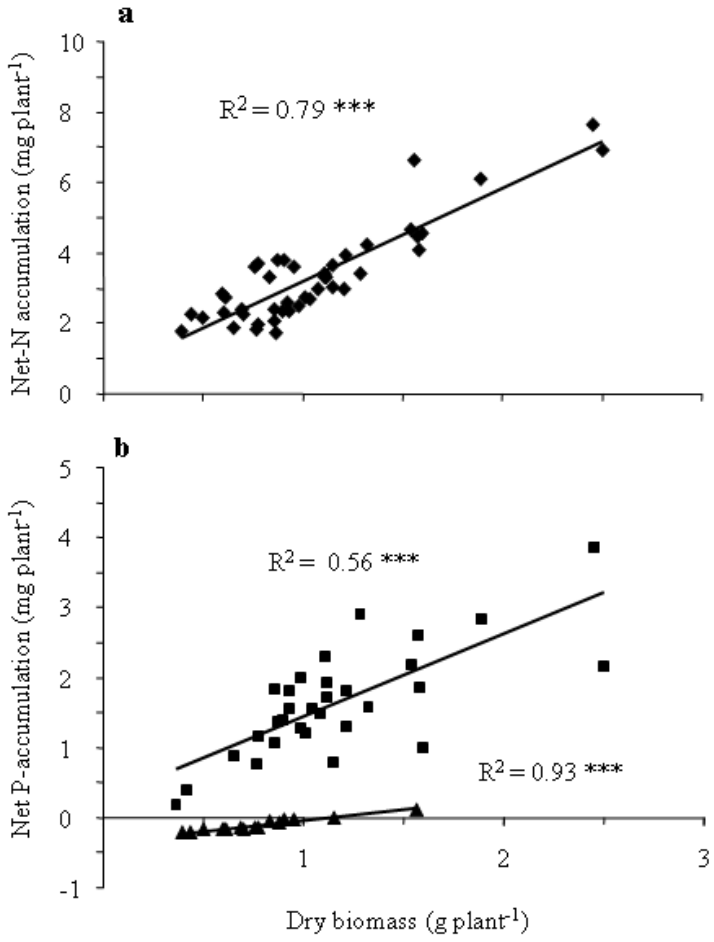

Figure 4: Relation of total net accumulation of N (a) and P (b) per plant with total dry biomass of *P. pinaster* plant. Regression line extended over triangles denotes control (C and CI) soil plants and regression line over squares denote fertilised soil plants (b). Values in brackets represent the coefficient of linear regression (R^2) and asterisks denote significance of relationships (*** = P< 0.001).

3.4. Diagnostic analysis

N:P ratio (Table 3) indicated higher absolute values in control treatments (~ 20 and ~ 12, respectively) for shoots and roots, 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 very 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 significantly affect N:P ratio both in fertilized as well as in 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 P treatment was taken as reference (100 %) since

soils at studied site were limited for phosphorus nutrition. Figure 5 presents vector nomograms with data from all treatments. However, the arrows and full lines indicate only the treatments which showed significant dispersion of N (Fig. 5a) and P (Fig. 5b) contents, concentrations or biomass in shoot relative to the reference treatment (P). As shown in Figure 5a, plants from C and CI treatments showed luxury consumption of N, with relatively high accumulation of N in shoot. Plants from PI and F treatment showed no vector because of non significant N accumulation compared to reference P treatment. Plants from FI treatment showed a sufficiency response, as relative biomass and N contents significantly increased without increasing relative N concentration in shoots. The vector nomogram for P nutrient (Fig. 5b) showed significant depletion of relative P contents and P concentration with non-significant increase in shoot biomass of control plants. These conditions indicate the re-translocation of P in control plants. The plants in PI treatment indicate low level of luxury consumptions resulting into accumulation of P without significant change of biomass. In FI treatment, P contents and plant biomass 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.

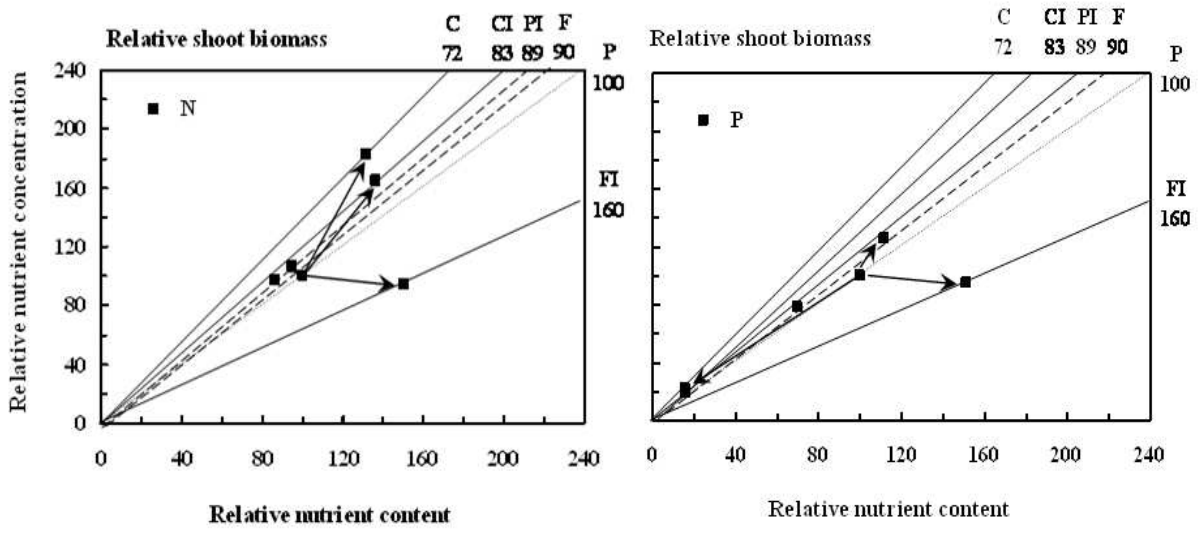

Figure 5: Vector nomograms of changes in dry biomass, N (a) and P (b) contents as well as concentrations in the shoot of eight months old *P. pinaster* seedlings grown in rhizoboxes. The mean (n = 8) values of shoot dry biomass, N and P contents and concentration of P treatment was taken 100 %. The mean values of other treatments relative to P treatment are plotted in vector nomogram for diagnosis. The arrow head lines and complete lines represent significant effects whereas the dashed lines represent non significant effect compared to the reference P treatment.

4. Discussion

4.1. Soil P Fractions

Application of mineral P fertilizers not only augmented significantly the pools of Pi but also Po in both bicarbonate and hydroxide extracts. The major pools of the soil P (NaOH extractable Pi and Po) suggested that the applied P could be either associated with oxides of Al and Fe (Fontes and Weed, 1996; Barroso and Nahas, 2005), or immobilized into organic P forms (Holford, 1997). Despite of it, sufficient amount of Pi was readily available (bicarbonate Pi) for plant uptake in fertilized soils. In contrast, in control treatments availability of P was highly limited due to low total and plant available P contents in these soils. These data are in accordance with the findings of Bonneau (1995) and Trichet et al. (2009) that P is limiting in these soils. The bicarbonate extractable Pi in fertilized treatments with and with out irrigation did not change but hydroxide extractable Pi was significantly higher in irrigated fertilized (PI and FI) treatments compared to non irrigated fertilized treatments (P and F). These higher concentrations could be due to change of soil reaction (dissolution) that governs the release and diffusion of P (Watanabe et al., 1960) throgh dissolution of Pi associated with Al and Fe oxides, which depends on the availability of water in soil (Holford and Patrick, 1979). Moreover, the volume of the soil that is occupied by water affects the cross-sectional area through which the P can diffuse (Barber, 1980). Similarly, slightly higher concentrations of Po pools (bicarbonate and hydroxide) could be due to increased microbial biomass (Ruppel and Makswitat, 1999) that might increase with optimum water supply in soils. The lower concentrations of Pi (bicarbonate and hydroxide) in F treatment compared to P treatment, despite of 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), could be due to the intensive development of forest floor annual vegetation (*Phytolacca* spp) only observed in F plots (personal observation). It could induce accelerated recycling rate and redistribution of Pi accumulated in aerial parts of annual species during the growth season, returning to the soil in winter. In addition, the root compartment of these vegetations may also play a role in Pi recycling, distribution and redistribution, 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 these forest soils. Similarly, Ali et al. (2009) suggested that the increased spatial distribution of Pi between line and interline position in *P. pinaster* stand, with NPK application could be due to understory vegetation in these soils. Consequently, Pi concentrations were higher in P treatment compared to F treatment. The depletion of Pi in rhizoboxes with plant indicated

that plants were able to take up P from soil. However, the significant depletion of hydroxide extractable Pi in irrigated fertilized treatments might be due to the modification of overall soil health resulting into more dissolution of Al and Fe associated P in soils (Holford and Patrick, 1979) compared with non-irrigated fertilized soils. The depletion of Pi by plants in control treatments was significant (t-test, $P < 0.05$) but it was comparatively low when compared to fertilized treatments. This small fraction of Pi in these P deficient soils might be taken up predominantly by soil microorganisms (Plante, 2007) and less promisingly by plants.

The depletion of $NaHCO_3$ extractable Po in fertilized treatments with young seedlings could be the result of net microbial mineralization of Po compounds due to low C/P ratio (C/P < 200), (Plante, 2007) and high microbial biomass. Interestingly, the depletion of bicarbonate and hydroxide extractable Po in FI treatment was highly significant (t-test, $P > 0.001$). This effect could be attributed to the high fungal biomass and phosphatase activity in these soils. Previously, Ali et al. (2009) reported high fungal biomass in the rhizosphere of *P. pinaster* with complete fertilizer application (NPKCaMg) in these soils. Similarly, increase in fungal biomass was recorded with phosphorus (Bakker et al., 2009) and nitrogen application (Parrent and Vilgalys, 2007) in the field of *P. pinaster* and *Pinus taeda* respectively. Conversely, in control treatments the high C/P ratio (C/P > 300) could have induced net immobilization, as indicated by higher Po concentration in rhizoboxes with plants (Plante, 2007). However, these trends are less obvious for NaOH extractable Po, suggesting two different pools of Po 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 independent of 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 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 *Sphaerosporella brunnea* considered as a common contaminant in nurseries of mycorrhizal plants (García-Montero et al., 2008), however their presence was proportionally low. Therefore, it can be suggested that the phosphatase activity assayed in ECM morphotypes belonged to *R. luteolus*. Acid phosphatase activity of ECM assayed using *p*NPP as a substrate was different in different soil samples. The availability of P resulted to decrease phosphatase activity of ECM morphotypes grown in soils (Kroehler et al.,

1988; Antibus et al., 1992; Chen et al. 2002; Ali et al., 2009) or with the ectomycorrhizal fungus grown in pure culture (Bousquet et al. 1986). Irrigation is supposed to increase P availability and high P availability resulted in decreased phosphatase activity in CI and PI treatments compared with C and P treatments. However, additional application of nitrogen fertilizers coupled with irrigation (FI treatment) resulted in increased phosphatase activity measured in ECM tips. Our results are in agreement with those found in ECM tips of *Pinus sylvestris* (Kieliszewskarokicka, (1992), Baar et al.1997) and 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 with nitrogen fertilizer application coupled with irrigation. These results suggest that the levels of phosphatase activities are not regulated only by P availability.

4.3. Plant growth and nutrient uptake

Soil samples with complete fertilizers (F) gave significant biomass increase (+ 64 %) when compared to control soil (C), and irrigation application to these complete fertilized soils (FI) further increased biomass production (+ 50 %) compared to F treatment. Both irrigation and fertilization increased maximum total biomass up to 147 % 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, Pi availability in soil (Fig. 1) was highly limited and was clearly observable in plant P analyses in tissues. Although, the phosphatase activity in C treatment was highest, it was not enough to increase net P accumulation. In this study, the concentrations of P in tissues were lower than critical limit (0.6-0.7 mg g^{-1}) at which photosynthetic limitation might have occurred in *P. pinaster* seedlings (Lousteau et al. 1999a, b; Delzon et al. 2005). These limitations could have resulted into significantly low 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 total accumulation of P in plants was significantly higher than in C treatments, but the increase in total biomass was not significant compared to control treatments. Indeed, application of P as well as irrigation (PI) induced even higher accumulation of P in seedling, with significant increase of only root biomass. This suggested that plants showed excess of P accumulation without additional biomass production in both P and PI treatments. In treatment of complete fertilization and irrigation (FI), plants showed maximum accumulation of N, P as well as biomass and were significantly higher than F

treatment. It suggested that FI treatment increased nutrients availability, which resulted into high shoot growth and light absorption (Trichet et al. 2008).

Interestingly, in FI and F treatments, the apparent significant differences of biomass and nutrient accumulation were due to irrigation, while irrigation during our experiment was similar and uniform for all treatments. It suggested that the observed effects were not only due to the water availability itself during the growth period, but also due to some other factors occurring in the field, which were different only in FI treatment. These changes could be associated with soil properties and soil microbial populations and biomass including fungi and bacteria. In this study, the observed significant increase in ECM phosphatase activity (Fig. 3) and significant mobilization of hydroxide and bicarbonate extractable Po in FI treatment (Fig. 1) compared to other fertilized treatments could be used as evidence for high microbial activity in FI treatment. Apart from microbial influences, high nutrients availability and irrigation could increase *P. pinaster* growth by decreasing stomatal limitations and increasing photosynthetic capacity (Lousteau et al. 1999b; Delzon et al. 2005).

N:P ratio and vector analysis was used as another 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 treatment. The high values of N:P ratio both in shoots and roots are in agreement with the results of Lockaby and Conner (1999), who reported the deficiency of P at N:P ratio > 10 in wetland 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 and plant growth was limited by P in control treatments. Moreover, it diagnosed high accumulation of N in control but this high N concentration was not sufficient to overcome the P deficiency and produce high biomass. 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.

In conclusion, the limitation P availability in control soils was confirmed by both analytical as well as diagnostic approach. Similarly, the sufficiency and steady growth with significantly high biomass production in FI treatment was also confirmed. Results also showed that in treatment of irrigation along with complete fertilization both bicarbonate and hydroxide extractable Po were depleted significantly, which could be due to significantly high phosphatase activity of ECM compared to other fertilized treatments. In 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 role of fertilizers, irrigation or soil microbial communities on site fertility and plant productivity.

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General conclusions and perspectives

1. Conclusion

The results obtained after different studies, permitted to reply the objectives of thesis. These were to characterise different pools of P and to evaluate the contribution of Po in total P of the soils of Landes. Secondly it was to find the relations between phosphatase activity of ectomycorrhizae and phosphorus status of the soils Relationship between phosphatase activity and the Pi availability in the soils was also studies. Thirdly, the role of mineral nutrition and phosphatase activity on the growth of *P. pinaster* was studied in field as well as in growth chamber. The important results of the different studies are summarised in this section.

I.1. P fractions in soil planted with P. pinaster

The forest of *P. pinaster* is established in Landes of Gascogne, on almost homogenous substrate, i.e. sandy spodosol soils. These soils showed a large variation of P fractions (bicarbonate, hydroxide and H_2SO_4). The mean total P (ignition method) contents were 61 mg kg^{-1} in spring 2006 and 41 mg kg^{-1} in autumn 2006. However, it presented a large variation between different soil plots used in this study. The values ranged between 29-149 mg kg^{-1} in spring and 14-78 mg kg^{-1} in autumn. This suggested a large variation of P contents within the forested plots as well as between the seasons. Organic P was major fraction of total P (upto 80 %), with high inta-site variability in the soils of Landes. On an average, hydroxide extractable Po was the dominant fraction of total P (26 % in spring and 51 % in autumn). Similarly, about 6 % and 16 % of total P, in spring and in autumn respectively, was present as bicarbonate extractable Po (Chapter 2).

Inorganic P concentrations were highly limited in these soils and concentrations of bicarbonate extractable Pi ranged between 2-10 mg kg^{-1} dry soils (Chapter 2). Fertilizers application significantly increased Pi fractions in these soils (Chapter 2-5). Seasonal variation was also observed. The concentration of bicarbonate and hydroxide extractable Pi was higher in spring compared to autumn. Whereas, bicarbonate and hydroxide extractable Po was significantly higher in autumn compared to spring. These seasonal variations suggested the mobilization of Po in spring and immobilization in autumn.

The soils from an experimental site, managed with annual application of fertilizers, with or without irrigated, called “Parcelle L” were used in short term rhizobox experiments (6-8 months) in growth chamber. The analyses of soils showed that both Pi and Po concentrations in soils were significantly higher in P as well as NPK treatments compared to

unfertilized control treatments. The comparisons of Pi concentrations in soils from line and interline tree position showed very low spatial distribution of applied P between line and interline, particularly in P treatments. However, a significant spatial distribution of P applied in interline position was observed in NPK treatments. It means that the difference of Pi concentrations between line and interline was low in NPK treatment compared to P treatment, instead of receiving similar dose of P in interline tree position. It suggested the role of enhanced growth of annual forest floor vegetation as well as high fungal biomass observed in these soils compared to P and control treatments.

1.2. Factors controlling the variance of P fraction in soils

A number of soil as well as plant variables were studied to evaluate their role to the variation of P fraction in soils. Both soil and plant properties significantly contributed to control over 50 % of the variance in partition of variation (chapter 2). The concentrations of bicarbonate and hydroxide extractable Pi were positively linked to each other, which were positively controlled by H^+ and negatively by Al_{ox} and age since last fertilization. Organic fractions of P were positively linked to total N but total N itself was highly correlated with organic matter (heat loss weight) and total C contents in soils. The parameters used to describe exchange of Pi between soils solid and solution interface showed that Pr1min was significantly correlated with organic matter, total N, cation exchange capacity and total C. It suggested that organic matter could reduce fixation of P or inhibit adsorption of P on inorganic surfaces. Mechanism responsible for inhibiting adsorption could be large humic molecules and organic acids produced during organic matter decomposition, adhered at the surface of metal hydrous oxides, and chelation of low molecular weight organic acids with Al and Fe, preventing the fixation of P at adsorption sites (Iyamuremye and Dick 1996; Brady and Weil 2002; Santruckova et al. 2004). Al_{ox} was not only significantly correlated with Pr1min but also with n parameter, indicating that Al_{ox} concentration controls both the reaction at first minutes of isotopic exchange at solid-liquid interface as well as the reaction after first minute of isotopic exchange.

1.3. Ectomycorrhizae of P. pinaster *and their phosphatase activity*

The inspection of ECM fungal morphotypes in Landes ecosystem showed relatively low total ECM morphotypes (19 morphotypes) as compared to other pine oak forest (Palmer et al. 1994; Tuininga 2000). This low ECM richness could be due to mono-specific *P. pinaster* forest and forest management practices. However, ECM morphotypes in mono-

specific forest plots showed high variation in terms of ECM evenness, richness as well as their phosphates activity (chapter 3). The evenness (abundance) of ECM morphotypes in the studied plots was significantly controlled by water contents, P fertilization or P availability. The results suggested that the evenness of majority of ECM was high with low water contents and low Pi-Olsen. On the other hand, plants growing in rhizobox with intact soils (Parcelle L) showed the colonization of only *Rhizopogon luteolus* (chapter 4, 5). It was suggested that *R. luteolus* was the only mycorrhizal fungus with highly resistant spores in these soils. The latter is known to possess high resistance and viability in the soil to form ectomycorrhizal association (Massicotte et al. 1994, Colgan and Claridge 2002, Bruns et al. 2009).

Phosphatase activity of ECM collected from field experiments was significantly controlled (canonical correspondence analyses), by water contents, Pi-Olsen, P fertilization and plant age. Similarly, both ECM abundance and phosphatase activity were positively controlled by fine root parameters and fine roots were negatively linked with P availability and P fertilization in soils (chapter 3). High phosphatase activity was observed in soils with low Pi availability as compared soil with high Pi availability or fertilizers treatments (chapter 4, 5). However, the phosphatase activity was increased in soil with irrigated NPK treatment, though the level of Pi concentrations or P application was similar. It suggested that although P availability decreased phosphatase activity (Antibus et al. 1992, Chen et al. 2002), the addition of N could induce high phosphatase activity (Wang et al. 2008) in soils.

1.4. Mineral nutrition and growth of P. pinaster

As per soil characteristic of Landes, P availability was observed highly limited. The significant increase of plant growth ($P < 0.05$) was observed in P fertilized plots compared to control plots in field. While in rhizobox experiments, although the accumulation of P in seedlings of fertilized treatments (P or NPK) was high, the accumulation of biomass was not as high as P in plants, grown in soils from interlines tree positions. The results suggested high plasticity of *P. pinaster* to the accumulation of P in tissues. Otherwise, these could be explained by the luxury consumption and toxic effects of P accumulation as suggested by Lambers et al. (2008). This could have been due the low capacity of plants to down-regulate their P-uptake capacity, as suggested by Shane et al. (2004).

Interestingly, growth of seedlings was significantly high in NPK treatment and even higher in irrigated NPK treatment compared to control. Instead of irrigation, the significant increase of biomass in irrigated NPK treatment compared to only NPK treatment was suggested due to microbial or biogeochemical properties. Regular irrigation practices at this

plot might have contributed to induce microbial or biogeochemical changes before rhizobox experiment. However, water supply was uniform during rhizobox experiment (Chapter 5). Hence, the observed biomass difference between seedlings of NPK and irrigated NPK treatments were not due to the water supply during the rhizobox experiment but to some pre-rhizobox changes that have occurred in irrigated NPK soils.

In summary, vectors analysis diagnosed the luxury consumption of N and re-translocation or acute deficiency of P in plants grown in control soils. The plants in P treatment soils showed luxury consumption of P, without significant increase of biomass. However, the steady growth response was diagnosed in irrigated NPK treatment, where significantly high accumulation of N, P as well as biomass was observed. The significant depletion of Po pools observed only in these soils could further explain our interpretation, that microorganisms played an important role in plant growth and P mobilization.

2. Perspectives

The conclusion derived from this work, interrogate three important aspects that should be focused in the future research plan. Firstly, is the phosphatase activity measured in ECM morphotypes in contrasting soils are due to one or several proteins; secondly, what is the origin of acid phosphatases occurring in the ectomycorrhizae; thirdly, what are the Po compounds susceptible to different ECM or bacterial phosphatase? In order to reply these questions following research strategies should be adopted.

2.1. Regulation of phosphatase activity

The results of current study (chapter 3) showed that multiple factors control phosphatase activity of ECM. Antibus (1992) determined the decrease of phosphatase activity with increasing Pi concentrations. Wang et al (2008) reported the increase of phosphatase activity with application of N fertilizers in soils. These results could be due to the activity of different phosphatases, coded by different genes that could be regulated by different environmental factors. Therefore, it is important to determine how many phosphatase enzymes are responsible for the measured *p*NP phosphatase activity. This question could be addressed using proteomic approaches aiming at separating phosphatase enzymes from ectomycorrhizal roots. After separation by gel electrophoresis or Fast Protein Liquid Chromatography (FPLC) chromatography, the specific revelation of *p*NP phosphatase activity should enable us to estimate the actual variability of active proteins showing a phosphatase activity as a function of soil treatments.

2.2. Synthesis of fungal or bacterial cDNA libraries to search the genes responsible for acid phosphatase activity in ectomycorrhizae

The alternative strategy which can be used to search, if several phosphatases are released by the ECM fungal species or their associated bacteria. It is to synthesise cDNA libraries using RNA extracted from ectomycorrhizal roots. cDNA libraries are commonly used to track the genes of interest. It is now possible to produce cDNA libraries from low quantities of RNA using commercially available kits (SMART™ technology). The schematic representation of cDNA synthesis is given in following figure.

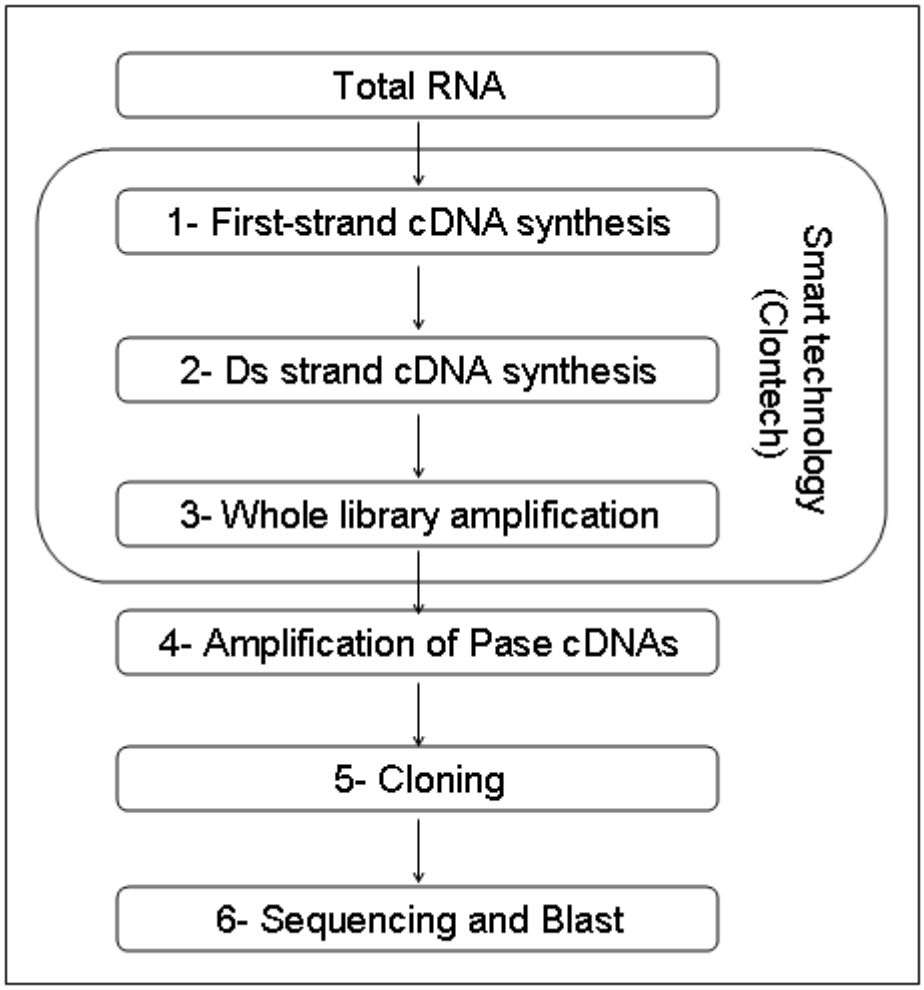

Figure 1: Schematic representation of synthesis of the cDNA library from total RNA extracted from ECM samples collected in the field. After synthesis of and amplification of the whole population of cDNA using the Smart technology (http://www.clontech.com), cDNA(s) encoding acid phosphatases genes will be identified after their amplification with specific (degenerated) primers. After cloning, the sequences will be compared with available data in public databases.

We know already that acid phosphatase genes identified so far in fungi (Bernard et al. 2002) and in the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* (Louche

unpublished) possess highly conserved sequences. Degenerated primers will be used from these highly conserved sequences in step 4 (Figure 1) to specifically amplify phosphatase genes present in the cDNA library. After cloning of amplification products, sequencing of clones putatively containing phosphatase genes will be performed. The cloning of complete phosphatase genes and their comparison with published sequences should enable us to assess the gene variability as a function of highly contrasted fertilization regime (such as those applied in Parcelle L). A re-analysis of the new sequences encoding phosphatase genes should enable us to design new primer sequences that will be used to associate one ectomycorrhizal fungal species with one phosphatase sequence. These data could then be used to assess our working hypothesis that several phosphatases could be expressed by the fungi. Further, the new genes could be expressed in heterologous system to produce the corresponding protein. It will be possible to establish their substrate specificity or and their ability to release Pi from Po extracted from soil samples.

Similarly, it could be possible to construct a cDNA library from mRNA extracted from prokaryotes as described in Figure 1. This will require the elimination of polyadenalyted mRNA of eukaryotes from total RNA extract. Following the same strategy of primer design targeted to conserved sequences among bacterial acid phosphatases, might give indications about the possible contribution of bacterial populations associated with the ectomycorrhizal roots to hydrolyse Po fractions.

2.3. Identification or characterization of Po compounds

In this study, the net depletion of Po has occurred in the rhizoboxes with *P. pinaster* seedlings, specifically in the irrigated NPK treatments (chapter 5). Although, the phosphatase activity was highest in non fertilized soils, the hydrolysis of Po was not significant between rhizoboxes with and without seedlings. It suggested that the quality of Po compounds was different in different treatments. It means that control soil may contain Po which was recalcitrant or difficult to hydrolyse. While irrigated NPK soils contained other forms of Po which were easily hydrolysable. We hypothesize that in irrigated NPK treatments, the recalcitrant Po compounds were transformed into hydrolysable Po forms through microbial transformations, the intensity of these microbial transformations may depend on water and nutrient availability in the field. Afterwards, the new pool of Po could be librated by trophic interactions occurring among the soil microbial populations and microbe feeders such as nematodes or protozoa. In this way, the transformed Po could be easily hydrolysed by ECM

acid phosphatase. Figure 2 explains the hypothetical schematic representation of this new cycle of Po in soil.

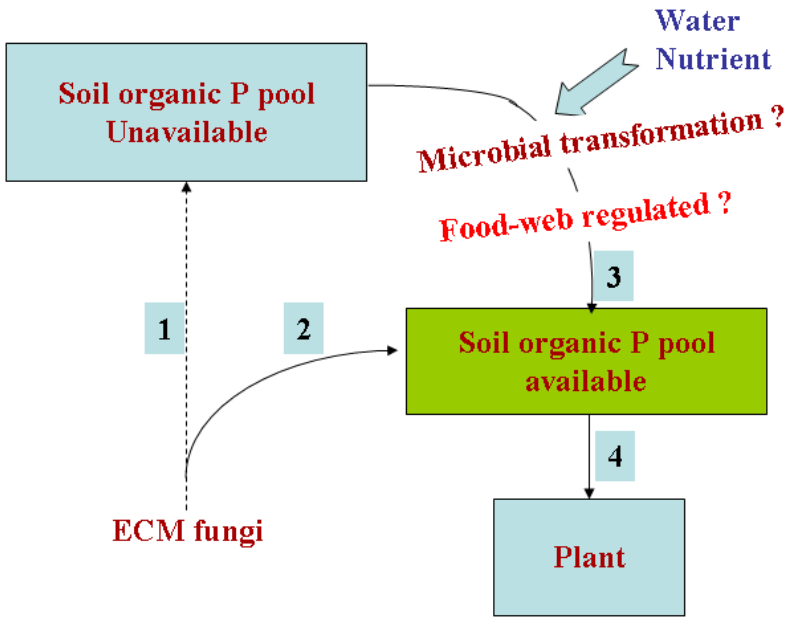

Figure 2: Schematic representation with new insight into the role of ectomycorrhizal fung in which the hydrolysis of soil organic P pool by acid phosphatase released by ECM fungi may not be operating. Therefore, the soil organic P pool can be qualified as "unavailable" to ECM fungi (1). The soil organic P pool could become available to ECM fungi (2) after microbial transformation and trophic relationships occurring in the mycorhizosphere (3). Finally, the mobilisation of this new soil organic P pool by ECM fungi may benefit to the plant (4).

The hypothesis can be checked first by determining the composition of Po compounds in different soil extracts including control and irrigated NPK treatments. The forms of Po compounds could be determined by using nuclear magnetic resonance (NMR) or high pressure liquid chromatography (HPLC). Po will be determined before and after the incubation of soil extracts with purified acid phosphatase from an ectomycorrhizal species able to release huge amounts of acid phosphatase in its culture medium (*Hebeloma cylindrosporum*, (Louche unpublished data) or from phosphatase produced by the expression of genes identified in cDNA libraries. expressed.

Unfortunately, it will not be possible to carry out a new experiment with soil samples from Parcelle L with different fertilizer and irrigation treatments as it was damaged by the storm events in spring 2009. The other possibility that could be utilized is to take soil samples from a 93 year old (Baudes) forest stand. This site was characterised by high organic matter as well Po pools. Soil samples from this stand could be used in rhizobox to grow young seedlings. Fertilization amendments (P and NPK) could be applied to check whether these conditions are able to activate or reactivate the microbial activity and trophic interactions. Thereafter, the evolution of Po forms in these soils will be studied to determine the role of soil

microorganisms and to assess whether it is possible to change the availability of recalcitrant Po pool in Baudes soils.

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