Trophic relationships in the rhizosphere : effect of fungal, bacterial and nematode interactions on mineral nutrition of Pinus pinaster seedlings

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INTRODUCTION GENERALE

Les microorganismes agissent comme un puits et une source de N et P disponibles car ils sont responsables des cycles biogéochimiques de N et P. La boucle microbienne est considérée comme un facteur majeur de la minéralisation de l'azote (N) et du phosphore (P) dans les écosystèmes terrestres. Ce rôle présumé de la boucle microbienne est basé sur le fait que les nouvelles populations de bactéries mobilisent des éléments minéraux qui ne sont pas accessibles aux plantes, en particulier N et P, éléments qui sont ensuite rendus disponibles pour la plante par les prédateurs des bactéries (Clarholm, 2005; Kuikman *et al*., 1991). Cependant, la plupart des études ont focalisé sur les effets de la prédation des bactéries par les protozoaires sur la minéralisation de N et la nutrition azotée des plantes herbacées (revu par Bonkowski *et al*., 2004; Krome *et al*., 2009). Cependant, du fait de leur abondance dans la rhizosphère, les nématodes bactérivores pourraient jouer un rôle important dans la boucle microbienne (Ritz and Trudgill, 1999; Bonkowski et al., 2009; Villenave et al., 2004). De plus, ces phénomènes de prédation des bactéries pourraient être importantes et affecter la nutrition minérale des espèces ligneuses mais les données disponibles sur ce sujet sont très peu nombreuses. En effet, nous n'avons trouvé qu'une seule étude portant sur le rôle des protozoaires dans la nutrition minérale de jeunes plants d'Epicéa (*Pice abies*) (Bonskowski *et al*., 2001).

Dans le cas des espèces ligneuses, la boucle microbienne pourrait être plus complexe que chez les herbacées car les racines des arbres sont systématiquement associées à des champignons au travers la formation de la symbiose ectomycorhizienne. Les champignons ectomycorhiziens sont aussi considérés comme un élément clé pour améliorer la nutrition minérale de la plante hôte (Chalot *et al*., 2002; Smith and Read, 2008). Cependant, si l'on observe presque toujours un effet positif de la symbiose (ecto)mycorhizienne sur la nutrition P de la plante, l'effet de l'association sur l'amélioration de la nutrition azotée de la plante a été questionné (Gobert and Plassard, 2008).

De façon intéressante, les racines ectomycorhizées sont aussi associées avec des bactéries qui ont ét appelées "bactéries auxiliaires de la mycorhization" (BAMS, Garbaye, 1994) qui appartiennent à divers genres bactériens (Deveau *et al*., 2007). Le premier effet attribué à ces bactéries a été d'augmenter le taux d'établissment des ectomycorhizes sur le système racinaire (Duponnois and Garbaye, 1991). Cependant, comme souligné par Frey-Klett et al. (2007), ces

bactéries peuvent aussi jouer un rôle important dans le fonctionnement des ectomycorhizes déjà établies en augmentant la croissance des hyphes qui explorent le sol, et/ou en améliorant la mobilisation des éléments minéraux du sol ou encore en exerçant un effet protecteur vis à vis des agents pathogènes des racines. Ceci suggère que dans les conditions naturelles, des bactéries sont toujours associées aux hyphes des champignons ectomycorhiziens. On peut donc s'attendre à ce que les relations entre les populations bactériennes et leurs prédateurs qui vivent dans l'espace mycorhizosphérique peuvent jouer un rôle important pour accélérer la minéralisation d'éléments nutritifs qui pourraient être interceptés par les cellules fongiques puis transférés à la plante.

Dans ce contexte, mes travaux de thèse ont porté sur l'étude du rôle de ces interactions complexes entre champignons ectomycorhiziens, bactéries et leurs prédateurs représentés par les nématodes bactérivores, sur la nutrition N et P de jeunes plantes de Pin. Nous avons utilisé le Pin maritime (*Pinus pinaster*) comme plante hôte. Nous avons choisie cette espèce à cause de son importance économique et de la facilité à obtenir des graines pour réaliser les expérimentations. Les plantes ont été associées à une espèce fongique modèle, le basidiomycète ectomycorhizien *Hebeloma cylindrosporum*. Ce champignon, dont la physiologie est bien connue au laboratoire, forme très facilement des ectomycorhizes avec le Pin maritime. Les plantes, mycorhizées ou non, ont ensuite été inoculées avec une souche bactérienne *Bacillus subtilis* qui a été isolée à partir d'ectomycorhizes prélevées sur le terrain, dans une plantation de Pin maritime, et par l'une ou l'autre de deux espèces de nématodes bactérivores (*Rhabditis sp.* et *Acrobeloides sp.*) isolées du sol de la même plantation de Pin maritime. Etant donné la complexité des interactions à étudier, j'ai mis au point un système expérimental simplifié consistant en une boîte de Pétri remplie d'un milieu solidifié par de l'agarose. Dans une première étape, j'ai quantifié le flux de ^{15}N bactérien et de P à partir de la biomasse bactérienne par l'activité de prédation des nématodes, soit en présence (Chapitre 3) ou en absence (Chapitre 4) de P minéral dans le milieu. Dans ce cas, le P contenu dans la biomasse bactérienne constituait la seule source de P.

Dans une deuxieme étape, j'ai étudié les complémentarités possibles entre les activités de minéralisation du phytate propres à *B. subtilis*, le champignon ectomycorhizien et le nématode bactérivore pour augmenter l'accès du Pin maritime à une source de P particulièrement peu utilisable qui est le phytate, fourni à deux cancentrations (Chapitres 5 et 6). Les résultats obtenus nous ont permis de proposer une nouvelle voie qui pourrait permettre d'augmenter significativement la mobilisation du phytate par les plantes.

GENERAL INTRODUCTION

Soil microorganisms act as a sink and a source of available N and P by mediating key processes in the biogeochemical N and P cycling. The microbial loop is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. This is based on the assumption that newly grown bacteria mobilize nutrients that are not easily accessible to plants, in particular N and P, which are then made available to plants by bacterial grazers (Clarholm, 2005; Kuikman *et al*., 1991). However, most of the studies carried out so far have focused on the role of bacterial grazing by protozoa on N mineralization and N nutrition of herbaceous plants (reviewed in Bonkowski *et al*., 2004; Krome *et al*., 2009). However, due to their abundance in the rhizosphere, bacterial feeding nematodes could play an important role in the microbial loop (Ritz and Trudgill, 1999; Bonkowski et al., 2009; Villenave et al., 2004). In addition, these regulations could also be important to modulate mineral nutrition of woody species, but data obtained so far in this field are very scarce. Indeed, we found only one study addressing the role of protozoa on mineral nutrition of *Picea abies* seedlings (Bonskowski *et al*., 2001).

In the case of woody species, the microbial loop may be more complex than in herbaceous species because roots of woody plants are systematically associated with fungal partners through the formation of ectomycorrhizal symbiosis. Ectomycorrhizal fungi are also considered as a key component for improving mineral nutrition of the host-plant (Chalot *et al*., 2002; Smith and Read, 2008). However, besides the positive effect of the (ecto)mycorrhizal symbiosis almost always observed for plant P nutrition, the effect of this association on plant N nutrition has been questioned (Gobert and Plassard, 2008).

Interestingly, the ectomycorrhizal roots are also associated with bacteria named "mycorrhiza helper bacteria" (MHB, Garbaye, 1994) that belong to a wide range of genera (Deveau *et al*., 2007). The primary effect assigned to MHB was to favor the establishment of ectomycorrhizal symbiosis (Duponnois and Garbaye, 1991). However, as underlined by Frey-Klett et al. (2007), these bacteria can also play important role for the functioning of established ectomycorrhizal roots by improving the growth of the hyphae exploring the soil, and/or by improving the mobilization of nutrient from the soil as well as by protecting the roots against pathogens. This suggests that in natural conditions, bacteria are always

associated with the fungal cells forming ectomycorrhizal associations. Therefore, we could expect that the relationships between bacterial populations and their bacterial grazers in the mycorhizosphere could also be of importance to accelerate mineralization of nutrient elements that could be intercepted by the fungus and translocated to the host plant.

Therefore, during my Ph-D thesis, I addressed the role of these complex interactions between ectomycorrhizal fungi, bacteria and their nematodes grazers on N and P nutrition of Pine seedlings. We used maritime pine (*Pinus pinaster*) as the host plant. This woody species was chosen because it is a species of economic importance, and it is easy to get seeds for experimentation. Plants were associated or not with the model species ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum*. This fungus, whose physiology is well known in our Laboratory, forms easily ectomycorrhizal association with *P. pinaster*. Plants were then inoculated with a bacterial strain *Bacillus subtilis* isolated from ectomycorrhizal roots of *P. pinaster* in the field and one or two species of bacterial feeding nematodes, *Rhabditis sp.* and *Acrobeloides sp.*, also isolated from the same soil samples as ectomycorrhizal bearing *B. subtilis*. Given the complexity of the interactions studied, we set up a simplified experimental system consisting of Petri dishes filled with medium solidified with agarose as the substrate.

In a first step, I quantified the flow of bacterial ^{15}N and P from bacterial biomass to plant via grazing activity of nematodes in a controlled experimental system, either with an external source of mineral P (Chapter 3) or without any external P source except bacterial P (Chapter 4). In a second step, I studied the possible complementarities occurring among the activities of our phytate-hydrolyzing bacterial strain *B. subtilis*, the ectomycorrhizal and the bacterial feeder nematode to improve the utilization of a well-known poorly plant available P source represented by phytate that was supplied at two concentrations (Chapters 5 and 6).

The results obtained enabled us to propose an alternative route to increase significantly the mobilization of phytate for the plant.

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CHAPTER 1. REVIEW OF LITERATURE

1. Importance of soil food web in soil plant nutrient cycling

Soil food web is made up of an incredible diversity of organisms that creates a living, dynamic soil system. According to an estimate, one gram of soil may harbor up to 10 billion microorganisms of possibly thousands of different species (Roselló and Amann 2001).
Beneficial organisms help plants to obtain nutrients and water from soil, to prevent nutrient losses, to protect from pathogens, and to degrade compounds that could inhibit growth. These beneficial organisms include bacteria, fungi, protozoa and nematodes. Maximum nutrient cycling and productivity and minimum nutrient loss make together a healthy soil system. This could only be possible thanks to the complexity of the soil food web. The greater the interactions of decomposers, their predators, and predators of those predators the more tightly nutrients cycle held from stable forms in soils to plants, and back again (Clarholm *et al*., 1985). According to Ferris et al. (2001) highly populated and balanced soil food web could help plants in different ways such as:

- ✓ Create humus by decomposing organic matter
- ✓ Improve soil structure by binding particles together and creating micro aggregates
- ✓ Protect roots from diseases and parasites
- ✓ Retain plant nutrients
- ✓ Slowly release retained nutrients to plant
- ✓ Produce enzymes and hormones that help plants grow and resist stress
- ✓ Decompose pollutants that enter the soil

Plants and soil organism's food web can be divided into different trophic levels according to their size and functions (Figure 1). All food webs are fuelled by the primary producers made of plants, lichens, moss, photosynthetic bacteria, and algae that use the sun's energy to fix carbon dioxide from the atmosphere and constitute the first trophic level.

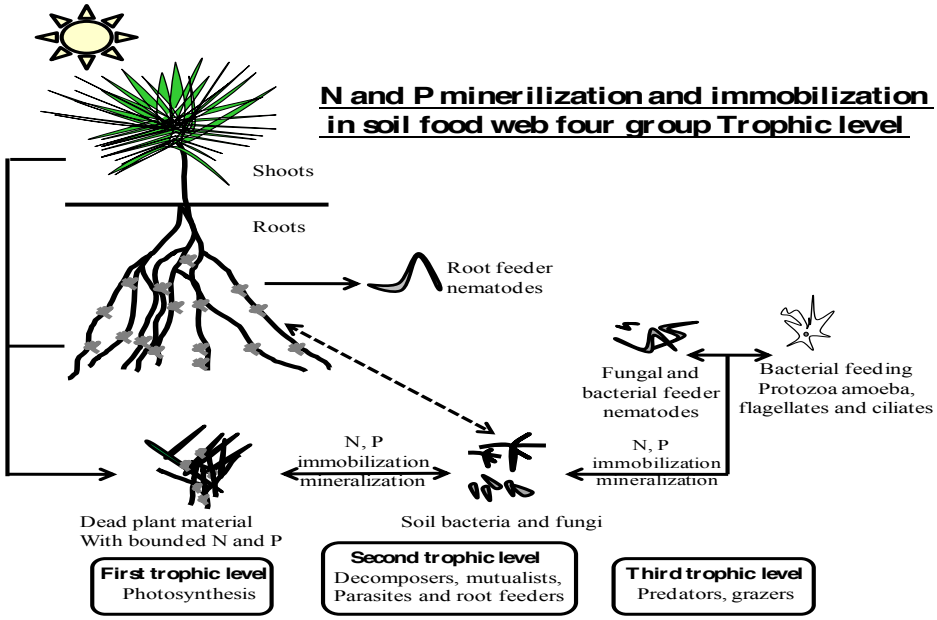

Figure 1. Scheme of N and P mineralization and immobilization in soil food web with three trophic levels.

Second trophic group in soil food web consists of organisms that get energy and carbon by consuming the organic compounds found in plants, other organisms, and waste by-products. Recent evidences also suggest that a major part of the second level trophic food web strongly relies on the C-inputs from plant roots and less so on the carbon and nutrient inputs via leaf litter (Bonkowski *et al*., 2009). The third soil food web trophic level includes consumers of microorganisms in the rhizosphere, because they are at the base of the soil food web channeling the energy to the higher trophic levels via two distinct routes, the fungal and the bacterial energy channel (Moore and Hunt 1988). Finally these third level trophic partners also determine the rates of nutrient cycling and the availability of mineral nutrients to plants (Bonkowski 2004, Bonkowski *et al*., 2009).

2. Components of soil food web

2.1. Bacteria

Bacteria are some of the smallest and most abundant microbes in the soil. In a single gram of soil, there can be billions of bacteria. By taking the genome size of *Escherichia coli* as a unit, Torsvik et al. (1996) calculated the presence of about 6000 different bacterial genomes per gram of soil.

There are an estimated 60,000 different bacteria species, most which have yet to be even named, and each has its own particular roles and capabilities. Most of them live in the top 10cm of soil where organic matter is present. Bacteria are ubiquitous in every habitat on earth including extreme or favourable conditions of life (Pikuta *et al*., 2007). Despite their tiny sizes, bacteria in soil are important in term of abundance and function. Indeed, a single gram of fresh soil contains 10^6-10^9 bacteria (Swift and Anderson. 1993). Bacterial cells are about 0.5– 5.0 μm in length and display typically one of 3 shapes (Figure 2): rod (bacilli), sphere (cocci) and spiral (spirilla). The characteristic shape is maintained by the structure of the bacteria cell walls.

Bacterial diversity is greater than the diversity of any other group of organisms. Bacteria are responsible for diverse metabolic functions that affect soil and plant health. Soil bacteria could be divided in four functional groups. Most are decomposers that consume simple carbon compounds from the litter and root exudates and participate in organic matter decomposition and nutrient cycling (Hättenschwiller *et al*., 2005). The second group is the mutualists that form partnerships with plant (symbiotic or not) and promote the plant growth. Theses bacteria are called Plant Growth-Promoting Rhizobacteria (PGPR) and improve germination rates, root growth, yield, leaf area, chlorophyll content, hydraulic activity, tolerance to drought, shoot and root weights (Lucy *et al*., 2004). The third group is the deleterious for plant growth and plant pathogen bacteria (Suslow and Schroth. 1982, Nehl *et al*., 1997). The fourth group is the chemolitho - autotroph bacteria that obtain energy for example from nitrogen, sulfur, iron mineral compounds. These bacteria play a key role in biogeochemical cycling i.e.

- ✓ block, hold back, nutrients in plant materials and animal waste used as fuel
- ✓ produce new organic compounds that are origins of energy and foods for other organisms.
- ✓ mineralize plant unavailable nutrients such as nitrogen and phosphorus

Deleterious effects include plant disease promotion. Enhancing knowledge of soil bacterial functioning and diversity will aid in the development of sustainable agroecosystems

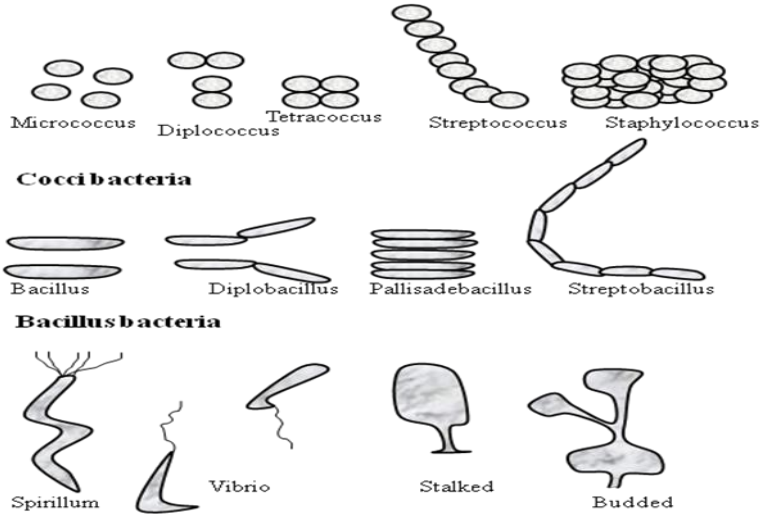

Figure 2. Schematic representations of bacterial shapes and arrangements.

2.2. Fungi and mycorrhizal symbiosis

The fungi, belonging to the kingdom of fungi, are eukaryotic and can be either unicellular (yeasts) or multicellular microorganisms. When multicellular, fungi form most of the time hyphae that are in average a few micrometers (< 10) wide and few tens of micrometers long. The main characteristics of fungi are that they contain chitin in their cell wall instead of cellulose and accumulate glycogene instead of amidon. As they are heterotrophic, they constitute the other way to channel energy in the soil from carbohydrates produced by photosynthesis. Fungal energy channels are considered as slow cycles, because they are favored by acidic soils low in available nutrients, recalcitrant organic materials and high C/N ratio in soil, leading to relatively long generation times (Blagodatskaya and Anderson, 1998; Högberg *et al*., 2007). Fungi have been shown to use organic substrates more efficiently (Holland and Coleman, 1987; Sakamoto and Oba, 1994), i.e. they form more biomass from the same amount of substrate than bacteria (Joergensen and Wichern, 2008). Indeed, estimations of microbial biomass based on biochemical markers derived from cell-wall compounds such as glucosamine (derived from fungal chitin) and muramic acid (derived from bacteria) showed that fungal C constitutes in average 70% of the total microbial C (Joergensen and Wichern, 2008) in most of the situations studied (Table 1).

Table 1. Contribution of fungal C to the total microbial residue C in percent, based on the measurements of glucosamine and muramic acid. Litter includes data on microbial root and straw colonization (adapted from Joergensen and Wichern, 2008).

	Arable	Grassland	Forest	Litter
Mean	75	68	70	76
Standard deviation	6	6	10	14
Number of replicates	267	95	125	301

Soil fungi can be grouped into three general functional groups based on how they get their energy.

1-Decomposers: saprophytic fungi – convert dead organic material into fungal biomass, carbon dioxide (CO_2), and small molecules, such as organic acids. Like bacteria, fungi are important for immobilizing, or retaining, nutrients in the soil.

2- Pathogens or parasites that cause reduced production or death when they colonize roots and other organisms. Root-pathogenic fungi, such as *Verticillium*, *Pythium*, and *Rhizoctonia*, cause major economic losses in agriculture each year.

3- Mutualists – the mycorrhizal fungi – 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 to describe the formation of new root organs resulting from the association between the fungus and the root. As described below, the mycorrhizal association is very important for plant nutrition and for soil food web functioning as it forms with most of the plants.

2.2.1. Classification of mycorrhizal roots

Fungi make variable associations with roots, and based on morphological and anatomical features, mycorrhizal roots can be separated into three main types: ectomycorrhizas, endomycorrhizas and ectendomycorrhizas (Brundrett, 2004; Smith and Read, 2008) (Table 2). Among these types, ectomycorrhizas and arbuscular endomycorrhizas have been the most deeply studied, due to economic importance of their host-plants that are forest trees for ectomycorrhizas (ECM) and 70-90% of land plants, including agriculture plants such as cereals, legumes, potato for arbuscular endomycorrhizas (AM). However, some plant families do not form mycorrhizal association, such as the *Brassicaceae*, *Chenopodiaceae*, *Caryophilaceae* or *Proteaceae* (Vance 2008).

Table 2. Classification scheme of mycorrhiza (adapted from Brundrett, 2004 dans http://mycorrhizas.info/index.html)

Category	Plant type and species	Fungal partner
Ectomycorrhizas	**Exclusively woody species (= 3 to 5% of taxa)**	
	Pinaceae: *Abies*, *Pinus*, *Picea*, *Cedrus*, *Larix* *Fagaceae*: *Fagus*, *Quercus*, *Castanea*, *Salicaceae* (*Salix, Populus, ...*) *Betulaceae* (*Betula, Carpinus, ...)* *Myrtaceae* (Eucalyptus, …)	**Basidiomycota :** *Boletus*, *Pisolithus*, *Laccaria*, *Rhizopogon*, *Amanita*, *Lactarius*, *Russula*, … **Ascomycota :** *Tuber*, *Elaphomyces* **Endogone :** *Endogone sp*
Endomycorrhizas	**Woody and herbaceous species**	
Arbuscular (AM)	**(=80% of taxa)** Pteridophyta, Pinophyta, Magnoliophyta	**Glomeromycota** *Glomus*, *Gigaspora*, *Acaulospora*,
Orchidoid	**Orchids** *Orchidaceae* (*Goodyera*, *Vanilla*, *Orphrys*)	**Basidiomycota :** *Ceratobasidius*, *Marasmius*, *Armillaria, Tulasnella, ...*
Ericoid	**Shrubs, herbs, …** *Ericaceae* , *Empetraceae*, *Epacridaceae*	**Ascomycota :** *Pezizella ericae*
Ectendomycorrhizas	**Shrubs, herbs, …**	
Arbutoid	*Arbutaceae* (*Arbutus*, …) *Pyrolaceae* (*Pyrola*)	**Basidiomycota** *Boletus*, *Laccaria*,
Monotropoid	*Monotropaceae*	*Cortinarius*, …

Ectomycorrhizal fungal species are widely distributed although they make associations with only 3 % of the vascular plant families. Fungi able to form ectomycorrhizas include about 5, 000 to 6,000 species that mostly belong to Basidiomycota with few examples of Ascomycota and Zygomycota (Molina *et al*., 1992, Castellano and Bougher 1994). The roots of 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). In this type of symbiosis, the hyphae develop both outside of the root, by forming a fungal sheath and inside the root, by growing between cortical root cells, in the middle lamella of host cell walls, to form the Hartig net (Fig. 3). Finally, hyphae develop an extraradical network of hyphae that contributes substantially to the fungal C in soil. In forest ecosystems, although ECM hyphae are more difficult to distinguish morphologically from saprotrophic fungi and hyphal length estimates are less reliable, available data suggest hyphal length densities of between 3 to 600 mg^{-1} soil (Jones *et al*., 2009). Using a combination of techniques such as in-growth mesh bags, measurements of fungal markers such as phospholipid fatty acids and ergosterol, ∂13C values and trenching Wallander et al. (2001) were able to distinguish mycorrhizal fungi from soil dwelling saprotrophs. The total amount of ECM mycelium colonising the mesh bags was calculated to be 125–200 kg ha^{-1} and the total amount of ECM mycelium, including ECM sheath was estimated to be 700–900 kg ha^{-1}. Other estimates made in a 13-year old *Pinus pinaster* planted forest using mesh bags and ∂13C indicate that ECM hyphal lengths were 100 (control plots) to 700 (irrigated and P-fertilized plots) times greater than fine root lengths (Bakker *et al*., 2009).

In contrast to ECM fungi, arbuscular mycorrhizal fungi (AMF) belong only to Glomeromycota (Fig. 4). Until recently, most of the 214 currently described species (www.amf-phylogeny.com) were characterized only by spore morphology and the majority of the species have not yet been cultured. Therefore, these last years, a great effort has been devoted to obtain molecular tools in order to detect and to quantify all the diversity of AM fungi in situ and these tools are now available (Krüger *et al*., 2009; Stockinger *et al*., 2010). The AM symbiosis is very old (older than the ECM symbiosis) as first traces of AM plants are dated > 400 million year (Parniske, 2008) and it has been proposed that this symbiosis enabled plants to expand in terrestrial ecosystems (Parniske, 2008, Bonfante and Genre, 2008).

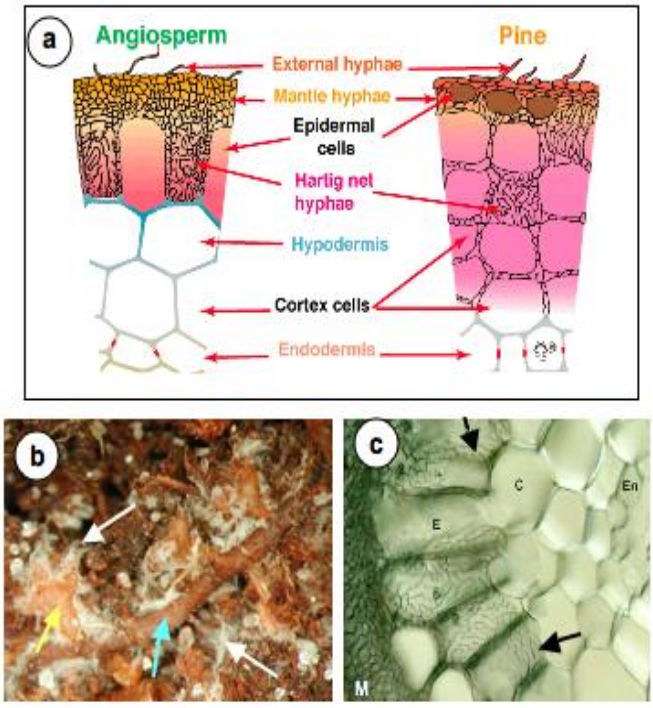

Figure 3. Anatomy and morphology of ectomycorrhizal roots. (a) schematic representation of a cross section of ectomycorrhiza formed in Angiosperm and Gymnosperm roots. In Angiosperm, root colonization is limited to the first layer of cell (epidermis).
(b) Ectomycorrhiza (yellow arrow) with extraradical hyphae (white arrow) on *Pinus pinaster* root (blue arrow) in a soil sample from *P. pinaster* plantation (picture J. Guinberteau); (c) Cross section of a Poplar ectomycorrhiza showing the external fungal sheath (M) and the Hartig net (black arrows). Pictures a, c are from Brundrett, http://mycorrhizas.info/ecmf.html.

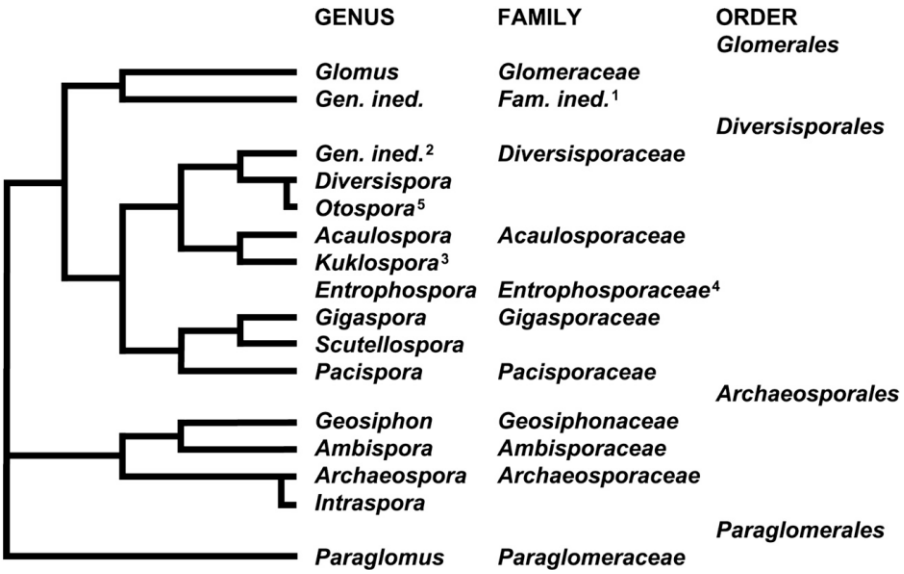

Figure 4. Phylogenetic relationships of taxa in the Glomeromycota published in Krüger et al. (2009).

The morphology and the anatomy of this association are clearly different from the ectomycorrhizae as the hyphae do not form an external sheath around the root. Instead, the hyphae progress into the root cortex to form arbuscules and, in some cases, vesicules. However, as in ECM, the fungus produces extraradical hyphae that explore the soil (Fig. 5) Estimates of hyphal length for AM fungi typically range from 3–30 m g^{-1} soil but 68–101 m g^{-1} soil have been recorded in undisturbed grasslands with permanent plant cover (Leake *et al*., 2004; Jones *et al*., 2009).

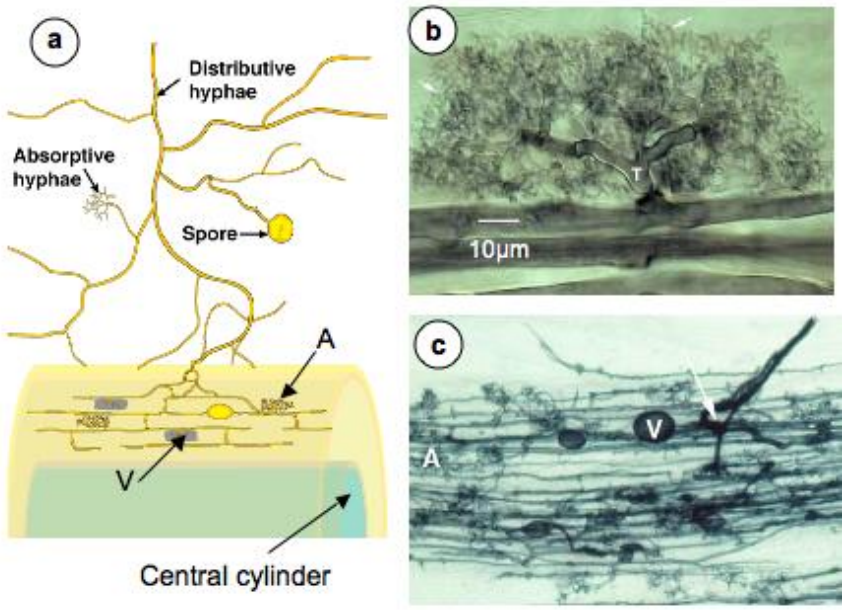

Figure 5. (a) Schematic representation of endomycorrhizal root with hyphae developing out of the root that produce spores and explore the soil to take up nutrients (absortive hyphae). The hyphae also develop inside the root cortex to form arbuscules (A) and, sometimes, vesicules (V). Fungal development is stopped by the endodermis. (b) Picture of a *Glomus mosseae* arbuscule in the host cell. The trunk T of the arbuscule ramifies from intercellular hyphae and gives fine ramifications of the fungal cell (white arrows). (c) Observation of a root associated with *Glomus sp.* with arbuscules (A) and vesicles (V) after coloration with chlorazol black. From Brundrett (http://mycorrhizas.info/vam.html)

2.2.2. Formation of mycorrhizal roots

The process of mycorrhization can be divided into several steps (Martin *et al*., 1997) but the starting point is always the recognition between plants and mycorrhizal fungi to allow the early contacts between the two partners of the symbiosis. As underlined by Sanders (2011) "the problem for both plants and mycorrhizal fungi is how to tell each other to establish a symbiosis and how to do that without setting off some of the plant's complex

defense reactions". One of the last advancement of our knowledge in mycorrhizal signaling comes from the demonstration of the role of small protein produced produced by ECM (Plett *et al*., 2011) and AM (Kloppholz *et al*., 2011) fungi. These mutualistic fungal effector proteins allow symbiosis formation and allow the fungus to manipulate the plant's defense response, respectively. In the case of ECM symbiosis, this achievement was facilitated by the whole genome sequencing of the ECM fungus *Laccaria bicolor* and genome-wide transcriptome studies on *L. bicolor*/poplar ECM (Martin *et al*., 2008). These studies revealed that a protein of 68 amino acids, known as Mycorrhizal induced Small Secreted Protein7 (MiSSP7), highly accumulated in ECM root tips (Martin *et al*., 2008) is produced only when the fungus makes the symbiosis and not when the fungus is in a free-living state (Plett *et al*., 2011). In addition, the fungus released this protein without direct contact with its host-plant (Poplar) or in presence of Arabidopsis thaliana which is not capable of forming any type of mycorrhizal symbiosis, indicating that diffusible plant-produced signals must be released by the root but that they are not specific. Finally, Plett et al. (2011) demonstrate that MiSSP7 enters plant cells, probably by endocytosis, and accumulates in the plant nucleus as described in figure 6. In addition to early signalling, MiSSP7 also controls Hartig net formation by inducing transcripts involved in cell wall remodeling and auxin homeostasis. Taken as a whole, it seems that MiSSP7 is really a fungal effector necessary for ectomycorrhiza formation (Sanders, 2011).

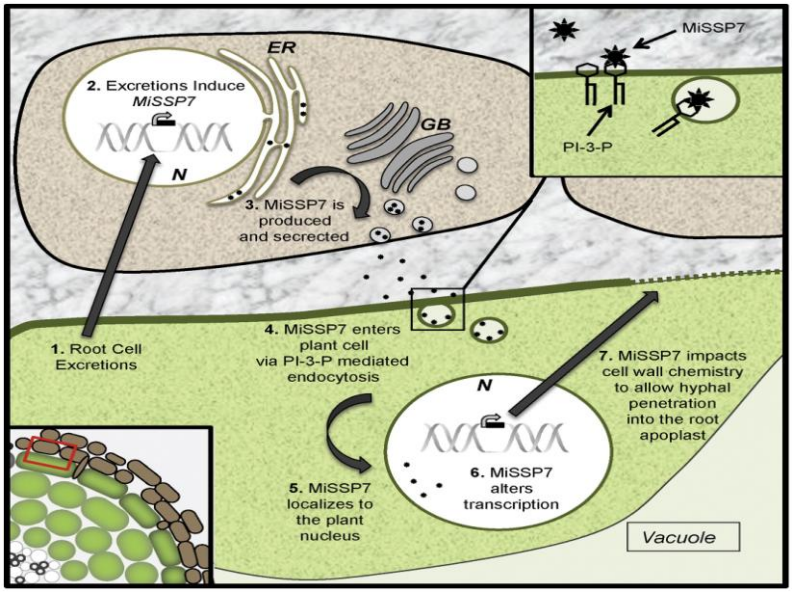

Figure 6. Mode of action of Mycorrhizal induced Small Secreted Protein7 (MiSSP7) produced by the ectomycorrhizal Basidiomycete *Laccaria bicolor* in interaction with poplar roots (from Plett *et al*., 2011).

Importance of these small secreted proteins is stressed by the study of Kloppholz *et al*. (2011) carried out in the AM fungus *Glomus intraradices*. As there is no complete AMF genome or published transcriptome data, the authors used a modified version of the yeast secretion sequence trap method to collect proteins secreted by the fungus. They identified a small protein (called secreted protein 7, SP7) containing a signal domain, a nuclear localization domain and tandem hydrophilic repeats. As found for MiSSP7, the protein is located into the plant nucleus. However, this study showed also that SP7 decreased the expression of a transcription factor (called ERF19 in *Medicago truncatula*) that normally activates the expression of defense proteins in plants. This would suggest that SP7 is an AMF effector protein that switches off parts of the plant defense mechanism, constituting a major milestone in understanding the AM symbiosis.

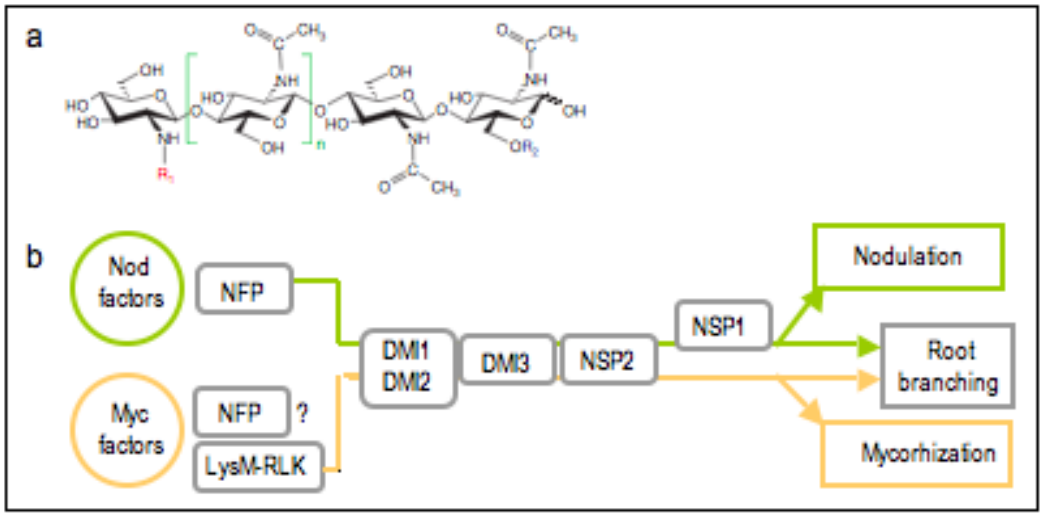

Figure 7. Current knowledge of the genetic control of AM and nodule formation, and root branching (from Maillet *et al*., 2011).
(a) General Myc-LCO structure (Myc factors). For both natural and synthetic Myc-LCOs n=1 or 2, R2=H or SO_3H. For natural Myc-LCOs, R1=C16, C16:1, C16:2, C18:0 or C18:1Δ9Z (oleic acid); for synthetic Myc-LCOs, R1=C16:0 or C18:1Δ9Z (oleic acid). (b) Model for the genetic control of Nod- and Myc-factor activated signal transduction pathways leading to root branching, nodulation and mycorrhization. The symbiotic signalling pathway identified in *M. truncatula* includes genes coding for Nod factor perception (NFP and LYK3), calcium signalling (DMI1, DMI2 and DMI3) and nodulation-specific transcription factors (NSP1 and NSP2).

Regarding AM symbiosis, recent evidence suggests that AM fungi produce other diffusible symbiotic signals. The studies of Maillet et al. (2011) demonstrate that *Glomus*

intraradices secretes symbiotic signals that are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs), which stimulate formation ofAM in plant species of diverse families (Fabaceae, Asteraceae and Umbelliferae). In the legume *Medicago truncatula* these signals stimulate root growth and branching by the symbiotic DMI signalling pathway (Fig. 7). These molecules are active at extremely low concentrations (nM range), making their identification very difficult.

2.2.3. Nutrient exchange in mycorrhizal roots

The fungus is heterotroph for carbon, the plant supplies carbohydrates derived from photosynthesis at the level of common interfaces in mycorrhizal roots, ie in the Hartig net for ECM and arbuscules for AM symbiosis. Mycorrhizal symbiosis constitutes therefore a privileged pathway to drive plant C to the soil through the hyphae network. Data from a range of microcosm-based labeling studies suggest that 7-30% of net C fixation is allocated to ectomycorrhizal mycelium and that 16-71% of this C is lost by respiration (Leake *et al*., 2004; Jones *et al*., 2009). Of the nutrient exchanged between fungal and root cells, those of N and P are the most extensively studied. Regarding N, evidence have been gained in AM symbiosis that a net transfer of N taken up by the extraradical hyphae to carrot roots occurs at the level of intraradical hyphae (Govindarajulu *et al*., 2005). However, in the field, direct and indirect evidence indicate high variability of N transfer among fungal species (Gobert and Plassard, 2008; Chalot and Plassard, 2010). In contrast to N, the positive effect of mycorrhizal symbiosis on P nutrition of the host plant has been repeatedly demonstrated (Smith and Read, 2008). In AM plants, the occurrence of two pathways to take up P from the soil solution has been proposed (Fig. 8) in which direct plant P uptake can be replaced by fungal uptake at different degrees (Smith *et al*., 2003; 2004; 2011, Facelli *et al*., 2010).

Such pattern is strongly supported by the discovery of mycorrhiza-inducible Pi transporters specifically expressed at the level of arbuscules in herbaceous plants (as for example StPT3 in potato, Rausch *et al*., 2001; MtPT4 in Medicago, Harrisson *et al*., 2002) or perennial plants (PtPt10 in poplar, Loth-Pereda *et al*., 2011) among others plant Pi transporters reviewed by Javot et al. (2007a) and Bucher (2007).

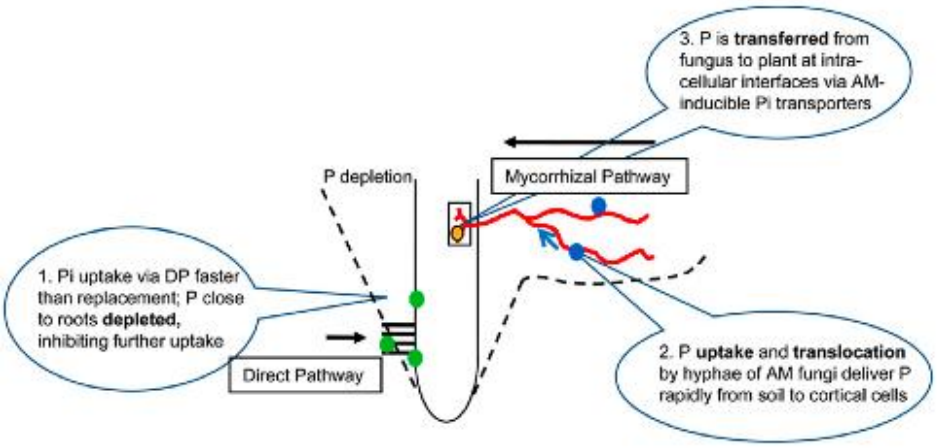

Figure 8. The two pathways of P uptake in an AM root involve different regions of the root, different cell types, and different Pi transporters.
In the direct pathway (DP), Pi is absorbed from the rhizosphere by plant Pi transporters in epidermis and root hairs (green circles) close to the root surface. Uptake is normally faster than replacement by diffusion from the bulk soil, resulting in reduced Pi concentrations (depletion) close to the roots (callout 1). In the mycorrhizal pathway, Pi is taken up into AM fungal hyphae by fungal Pi transporters (blue circles) several centimeters from the root and translocated to intracellular fungal structures (arbuscules and hyphal coils) in root cortical cells (callout 2). Plant Pi transporters, induced in colonized cells (yellow circle), transfer Pi from the interfacial apoplast to plant cortical cells (callout 3) (from Smith et al., 2011).

Finally, figure 9 presents an hypothetical scheme on the mechanisms possibly involved in the P transfer between the fungal and the root cells that occur in the common cell wall space in mycorrhizal roots. So far, the main uncertainties are about the signal regulating polyphosphate hydrolysis, and the fungal Pi transport systems recruited to sustain the efflux of Pi out from the vacuole and the cystosol. However, the deletion of MtPT4 in Medicago plants resulted in a very low rate of AM symbiosis and decaying of the arbuscules (Javot *et al*., 2007b), suggesting that such Pi exchange are required to the formation and development of AM symbiosis. Taken as a whole such intimate regulation of Pi exchanges in mycorrhizal symbiosis could explain why improvement of P nutrition in mycorrhizal plants (Chalot *et al*., 2002; Smith and Read, 2008; Plassard and Dell, 2010) is so often observed.

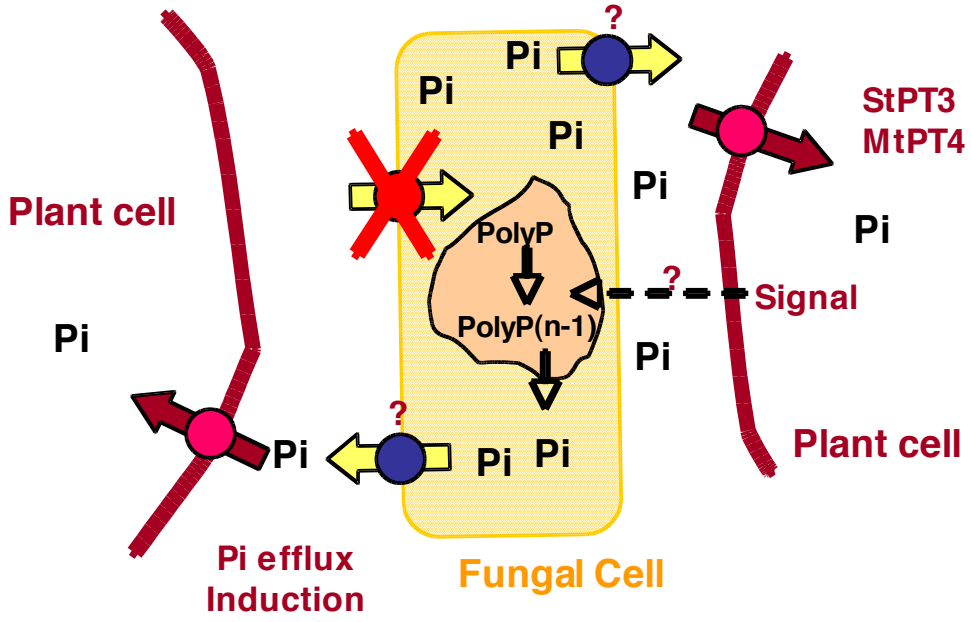

Figure 9. Schematic view of the possible mechanisms leading to fungal P efflux towards the plant cell at root–fungal interface in mycorrhizal organs. Fungal cells transport P taken up from the soil solution as polyphosphates (PolyP) presumably in vacuoles. PolyP are then hydrolyzed in soluble Pi following the production of an unknown signal from plant cell. Pi molecules are then driven into the common cell wall space through unknown mechanisms yet. Pi in the cell wall is taken by mycorrhiza-induced plant Pi transporters (for example StPT3 or MtPT4). Simultaneously, fungal high affinity Pi transporters should be turned off.

2.3. Nematodes

Nematodes, the earth's most abundant metazoa, are ubiquitous in the soil environment. They are sufficiently large to be identifiable by light microscopy and sufficiently small to inhabit water films surrounding soil particles. They are multicellular organisms, unsegmented worm, bilaterally symmetric and have a simple nervous system, a complete digestive system and no respiratory and circulatory systems. Nematodes have a sexual reproduction and a basic life cycle consisting of an egg stage, four juvenile stages where after each stage a molt is occurring, and an adult stage (Byerly *et al*., 1976). There are free-living species with a size ranging from 50 µm to 1-10 mm (Maggenti, 1981). Nematodes are found in different ecosystem including marine and terrestrial habitats where they require a water film for locomotion. About 26600 species have been described (Hugot, 2002) and the number of estimated living species in the Nematoda phylum was evaluated at 500 000 by Hammond (1992). Densities of Nematodes range from $7.6*10^{5}m^{-2}$ in a desert to $2.9*10^{7}m^{-2}$ in a mixed

deciduous forest (Bernard, 1992; Liang and Shi, 2000). Generally, 20–50% of the total number of nematodes present in soil is bacterial-feeders, and the ratio reaches 90–99% at sites of high microbial activity (Griffiths, 1994; Li *et al*., 2001). The nematodes are characterized by an incredible variety of functions that can occur at several trophic levels of the soil food web. Some feed on the plants and algae (first trophic level); others are grazers that feed on bacteria and fungi (second trophic level); and some feed on other nematodes (higher trophic levels) (Yeates *et al*., 1993). The nematodes can be classified into five broad groups based on the morphology of their mouthparts and pharynx that is characteristic of their diet (Figure 10).

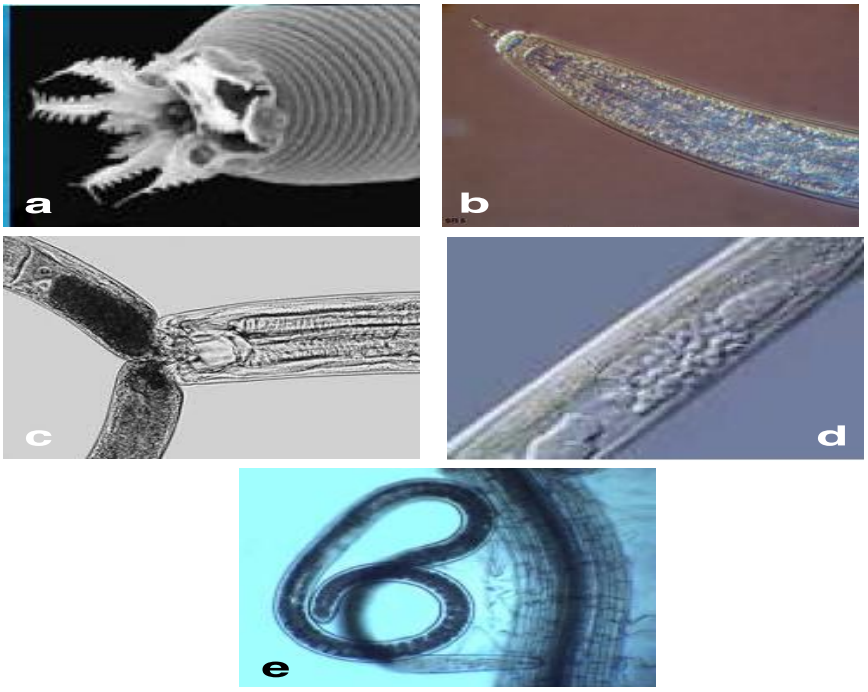

Figure 10. Morphology of nematode's mouthparts and pharynx according to their diet:

- ✓ (a) Bacterial-feeders consume bacteria.
- ✓ (b) Fungal-feeders feed by puncturing the cell wall of fungi and sucking out the internal contents.
- ✓ (c) Predatory nematodes eat smaller organisms whole, or attach themselves to the cuticle of larger nematodes, scraping away until the prey's internal body parts can be extracted.
- ✓ (d) Omnivores eat a variety of organisms or may have a different diet at each life stage.
- ✓ (e) Root-feeders are plant parasites, and thus are not free-living in the soil.

However, the phylogeny of nematodes based on 18SrRNA gene showed 5 mains clusters independent of their feeding habits (Blaxter *et al*., 1998).

Those few species responsible for plant diseases have received a lot of attention, but far less is known about the majority of the nematode community that plays beneficial roles in soil (Villenave *et al*., 2004). The functional roles of nematodes, determined by their metabolic and behavioral activities, may be categorized as their ecosystem services (Ferris, 2010). Among these ecosystem services their functions in soil food webs are of great importance as listed below:

- ✓ Channeling resources derived through herbivory,
- ✓ Channeling resources derived through bacterial and fungal decomposition,
- ✓ Accelerating turnover rates of decomposers,
- ✓ Priming beneficial functions and services of the food web,
- ✓ Regulating opportunists through predation,
- ✓ Regulating arthropod populations with soil life-stages,
- ✓ Serving as prey for higher level predators,
- ✓ Biodegradation of toxins,
- ✓ Influencing plant community composition and succession,
- ✓ Redistributing organisms in the soil matrix,
- ✓ Transporting organisms to new resources,
- ✓ Altering substrates to provide access to other organisms,
- ✓ Sequestering and redistributing minerals, carbon and energy.

2.4. Protozoa

Protozoa belong to the kingdom of Protista and are a paraphyletic group. They are unicellular eukaryotic microorganisms, that can be found in many different ecosystems (oceans, lakes, soils). However they always require a water film for locomotion and feeding. Usually, their sizes vary between 3 µm-250µm but some protozoa can exceed 1 mm in diameter (Westphal and Mühlpfordt, 1976).

There are four morphological types of these unicellular eukaryotes occur commonly in soil:

- ✓ (a) naked amoebas
- ✓ (b) testate amoebas
- ✓ (c) flagellates
- ✓ (d) ciliates.

Soil protozoa are also characterized by their ability to form resistant cysts which permit them to survive to dryness or other adverse conditions.
Most species occur in the upper 10 cm of soil (Janssen and Heijmans, 1998; Ekelund *et al*., 2001). Fresh water protozoa were classified by Pratt and Cairns (1985) on the basis of feeding habits into six groups:

- ✓ (1) photosynthetic,
- ✓ (2) autotrophs,
- ✓ (3) bacterivores/detritivores,
- ✓ (4) saprotrophs, algivores,
- ✓ (5) non-selectiveomnivores,
- ✓ (6) predators.

Identification of protozoa is based on their locomotion and morphological structures and requires sometimes transmission electron microscopy. Approximately 25000 species were described in 1976 (Westphal and Mühlpfordt, 1976) and 50000 currently (Lee *et al*., 2002; Gobat *et al*., 2004). All these trophic groups can be found in soils but a large proportion of protozoa are bacterivores. Bacterivores protozoa exploit the decomposer bacteria as a source of energy, transferring some of the assimilated bacterial production into protozoan tissue and some into energy for metabolic processes and movement. With a function of regulation of bacterial population, protozoa are also an important food source for other soil organisms and help to suppress disease by competing with or feeding on pathogens. In this way the protozoa play a potential role in energy flow of microbial soil food-web.

3. Functionning of soil food web in rhizosphere

3.1. Importance of rhizosphere in microbial populations

Hiltner (1904), defined rhizosphere as the volume of soil under the influence of root, as well as the root itself. Later Marilley *et al*., (1998) described three different fractions depending on distance to the root (Figure 11):

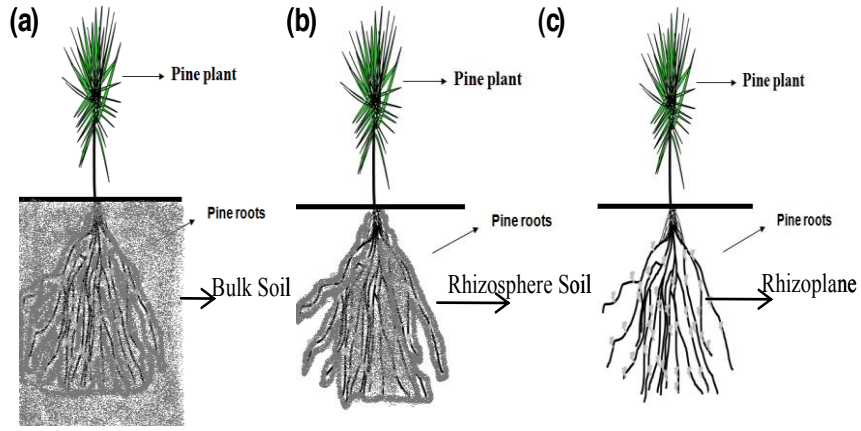

Figure 11. Three schematic rhizosphere fractions (a) Bulk Soil (BS) or Non-Rhizosphere Soil corresponding to soil that is not influenced by the roots, (b) - Rhizosphere Soil (RS) defined by the soil that adheres to the root when the root system is shaken manually, (c)- Rhizoplane-Endorhizosphere (RE) corresponding to the surface and the interior of the root.

Cultivation-based analyses of rhizosphere microorganism's communities have long known to be strongly influenced by the presence of a root. Studies based on the use of growth media steadily showed that microbes populations residing in the rhizosphere are one to two orders of magnitude larger than those residing in bulk soils (Buée *et al*., 2009).

For the soil that is generally poor nutrients and energy sources (Nannipieri *et al*., 2003), the rhizosphere is an active interface where a higher number of microorganisms are found due a strong nutrient flow brought up by rhizodeposition (Whipps and Lynch, 1986, Tyagi, 2007) and is probably the greatest hot spots in soils (Kuzyakov, 2002). Major part of the soil food web organisms relies on the C-inputs from plant roots and less so on the carbon and nutrient inputs via leaf litter (Albers *et al*., 2006; Larsen *et al*., 2007; Pollierer *et al*., 2007; Elfstrand *et al*., 2008). Rhizodeposition is a ubiquitous phenomenon through which release of C compounds occurs from living plant roots into the surrounding soil (Jones *et al*., 2004, 2009). Rhizodeposition results in different chemical, physical and biological characteristics in the rhizosphere compared with those of the bulk soil. The loss of C from root epidermal and

cortical cells leads to a proliferation of microorganisms inside (endophytes), on the surface (rhizoplane) and outside the root (rhizosphere). The biochemical nature of compounds liberated by roots is very diverse: simple and complex sugars, amino acids, organic acids, phenolics, alcohols, polypeptides and proteins, hormones and enzymes (Nguyen, 2003).

The composition of C compound by roots enables a high number of soil microbes to interact more efficiently with roots. This microbe-root interaction can withstand during challenging conditions of rhizosphere life i.e. low-nutrient environments, which are quite common in natural ecosystems (Schloter *et al*., 2000). For example, the interaction with soil fungi lead to the development of mycorrhiza which explore the soil for phosphate, nitrogen and other nutrients and micronutrients much beyond the physical expansion of the root system. The bacteria with their impressive metabolic versatility and originality can better access the often most limiting nitrogen and phosphorus supply (Hartmann *et al*., 2008). Furthermore, the rhizosphere is a quite heavily populated microhabitat which is characterized by competition and even predation among the inhabitants. Therefore, soil organism food web does experience the rhizosphere environment as micro-habitat of great opportunities but also of big challenges.

3.2. Role of soil food web in root architecture and growth

Root architecture is a fundamental aspect of plant productivity. Therefore, breeding attempts have focused on developing larger root systems in crop plants. In addition to abiotic factors, such as patchy supply of different nutrients (Drew, 1975; Zhang and Forde, 2000), water stress (Fukai and Cooper, 1995) and soil compaction (Iijima *et al*., 1991), also biotic components of the root environment strongly influence the architecture of the root system.

For example, rhizosphere bacteria have been shown to affect root growth by the release of signal molecules, such as hormones, toxins or other metabolites (Arshad and Frankenberger, 1998; Phillips *et al*., 2004; Matiru and Dakora, 2005). These complex interactions are further complicated by the fact that protozoan and nematodes grazers are a strong selective force that affects bacterial activity in soils and in the rhizosphere (Blanc *et al*., 2006; Griffiths *et al*., 1999; Ronn *et al*., 2002).

Jentschke et al. (1995) found that the protozoa increased N uptake because hormonal effects resulted in the changes in the root morphology and plant growth; the significant changes in plant roots in the presence of protozoa might be mostly due to the effects of protozoa grazing on the rhizosphere bacteria. Despite the positive effect of protozoa on root architecture mycorrhiza had opposite effects on root architecture (Jentschke *et al*., 1995;

Bonkowski *et al*., 2001). The length of fungal hyphae decreased by 18% in the presence of protozoa, while the presence of mycorrhiza led to reduced numbers of bacteria (−38%) and their respective protozoan grazers (−34%), indicating a significant trade-off in plant carbon allocation between bacterial and fungal rhizosphere colonizers.

Bonkowski et al. (2000) further proved that the non-nutritional effects of protozoa on plant growth might result from directly released hormonal active substances as waste by protozoa or by products from bacterial remaining during predation; this would result in a complex root system with more lateral roots and large surface area. This larger surface area could increase water and nutrient uptake, so enhancing plant growth, which is called the hormonal effect of micro-fauna (Chen *et al*., 2007). In the presence of protozoa, wheat seeding had more and longer L-type lateral roots, which are basic elements to construct a strong fiber root system used for taking up nutrients (Bonkowski and Brandt, 2002). In order to investigate effect of rhizosphere food-web interaction on root growth, Kreuzer et al. (2006) performed a controlled experiment with rice seedlings (*Oryza sativa* L.) growing in Petri dishes on homogeneous nutrient agar. These authors used a simple system consisting of a diverse bacterial community and a common soil protozoa, *Acanthamoeba castellanii*, as bacterial grazer. The results showed that the root systems in presence of protozoa were characterized by high numbers of elongated laterals (those laterals that are a prerequisite for the construction of branched root systems) as compared to high numbers of lateral root primordia and short laterals (which did not grow out of the rhizosphere region of the axile root) in absence of protozoa.

Bacteria-feeding nematodes have the similar grazing habit as observed by protozoa, and they stimulate bacterial activity and modify the composition of the rhizosphere microbial community via grazing selectivity (Djigal *et al*., 2004a, b; Griffiths, 1999). Thus, it is not surprising that bacteria-feeding nematodes affect plant growth through hormonal effects, as described for protozoa. However, although even larger in number in comparison with protozoa, bacteria-feeding nematodes have always been neglected in studies. Mao et al. (2006) reported the promotion effect of bacteria-feeding nematodes on plant (tomato and wheat) root growth, resulting in larger surface area, more tips, but shorter diameter on average. And then further study on the content of auxin in rhizosphere soils by High Performance Liquid Chromatography (HPLC) indicated that, in contrast to the control, the contents of IAA and GA3 increased remarkably with abundant bacteria-feeding nematodes (Mao *et al*., 2007).

Besides direct hormonal effects, NO_3 can also induce hormone like effects on the changes in root form. Since NO_3-N is not only the nitrogen resource, but also the signal substance to induce lateral roots to elongate (Zhang and Forde, 2000). During the study of effects of soil food-web on root growth, attention should be paid to nutritional (NO_3) or non-nutritional effects (hormones).

3.3. Role of soil food web in rhizosphere N cycle

Soil food web organisms have a major role in regulating nitrogen mineralization, as reviewed by Anderson *et al*. (1979). Microbial mineralization and nitrification are generally thought to be the rate limiting steps in the N cycle. Early studies by (Anderson *et al*., 1978; Cole *et al*., 1978; Coleman *et al*., 1977, 1978; Elliott *et al*., 1979; Woods *et al*., 1982) showed that nutrients added to the soil were rapidly immobilized by rhizosphere bacteria (*Pseudomonas* sp.) with only small amounts returned to the soil after carbon supply ceased. Grazing of the bacterial community by higher trophic levels e.g. protozoa, nematodes and invertebrates releases this immobilized N back into the root soil environment and stimulates mineralization rates (Ingham *et al*., 1985; Krome *et al*., 2009; Bonkowski, 2004 Bonkowski *et al*., 2000, ,2009) (Figure 12).

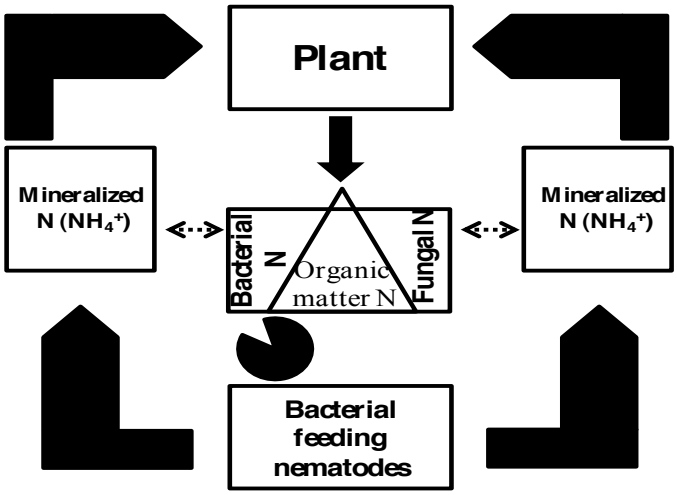

Figure 12. Schematic diagramme of N cycling by bacterial feeding nematodes.

Protozoa, nematodes and their bacterial prey presumably differ little in respect to their C: N: P ratios, but they use only 10–40 % of the prey carbon for biomass production, the excess N and P is assumed to be excreted in inorganic form and may therefore be readily available for other soil organisms including plant roots (Griffiths, 1994; Zwart *et al*., 1994; Graham and Martin, 2007). In a study of protozoan grazing on the micro-organisms, the ryegrass plants

were able to assimilate 44 % of bacterial immobilized N. Thus, plant roots were effective in competing for N released due to protozoan grazing (Ritz and Griffiths, 1987). In presence of protozoa, the amount of shoot N was found to be increased by 18–137 % (Elliott *et al*., 1979), 12–48 % (Clarholm, 1985), 6–38 % (Kuikman *et al*., 1991) and 40–45 % (Jentschke *et al*., 1995) in laboratory experiments. Effects of bacterial feeding nematodes have been found to be similar to those of protozoa with 49% (Ingham *et al*., 1985), 11-14% (Bonkowski *et al*., 2000) but also slight negative effect with -5 % by (Alphei *et al*., 1996) . This is probably due to the comparatively smaller reproductive rate of nematodes and the ability of protozoa to feed on bacteria in soil pores inaccessible to nematodes (Griffiths, 1994). However, indirect contributions of nematodes to N cycling may be even more important than their direct effects because grazing stimulates microbial mineralization processes. De Ruiter et al. (1993) calculated that the contribution of amoebae and nematodes to overall N mineralization in winter wheat was 18 and 5%, but their subsequent deletion from the food-web model resulted in reductions of 28 and 12% of N mineralization for amoebae and nematodes, respectively. Thus grazing of nematodes on soil bacteria is important also in the context of the nitrogen economy of agro-ecosystems, since it has been estimated that nematodes may mineralize 14-124 kg N ha^{-1} y^{-1} under field conditions (Anderson *et al*., 1981). Jentschke et al. (1995) and Bonkowski et al. (2001) studied the effect of interactions among protozoa and ecto-mycorrhizal Norway spruce seedlings (*Picea abies*) that were inoculated with a bacteria or bacteria plus protozoa on plant N uptake. Presumably, synergistic microbial effects maximized uptake of nitrogen up to17% in the combined compared with control treatments without mycorrhiza and protozoa where the additional of hyphal network increased the uptake of protozoa-mobilized nitrogen. However nematodes fungal interaction was observed to be non significant in total shoot N accumulation when Ingham et al. (1985) inoculated blue grama grass (*Bouteloua gracllis*) with bacteria (*Pseudomonas paucimobilis*) and a saprotrophic fungus (*Fusarium oxysporum*).

3.4. Role of ectomycorrhizal symbiosis on N cycling

As mentioned before, the fungi are considered to use organic substrates more efficiently than bacteria and could contribute to N cycling through the release of extracellular enzymes to catalyse the decomposition of soil organic matter. Regarding N mineralisation, the first step is the bereak-down of proteins under the action of proteases. Such ability to release proteases has been demonstrated in ectomycorrhizal fungi from their capacity to use protein sources as

the sole source of N in vitro (Yamanaka, 1999; Lilleskov *et al*., 2002) and extracellular protease activities have been measured in ECM fungi (Smith and Read 2008; Chalot and Brun 1998; Nehls *et al.,* 2001). Although it is not possible to know the exact contribution of ECM fungi in protease activity from soil extracts collected in forests, it is interesting to note that there was a linear relationship between the total free amino concentrations and soil protease activity, across successional soils in Alaska differing in the tree composition (Kielland *et al*., 2007). Remarkably, the lowest levels of protease activity were found in willow and alder forests (predominantly AM-forming species) and the highest levels (12-fold greater than the lowest ones) in spruce forests where ECM fungi were the sole fungal species associated with the roots, suggesting a major contribution of ECM to protease activity *in situ*. Besides this capacity to use protein, ECM fungi displayed also much greater capacities to use peptides or amino acids as N source than their host plant (Finlay *et al*., 1992; Quoreshi *et al*., 1995; Plassard *et al*., 2000; Wipf *et al*., 2002; Guidot *et al*., 2005). Amino acid uptake was demonstrated for several species of ECM fungi forming ECM tips (Chalot and Brun 1998; Wallenda and Read 1999; Boukcim and Plassard 2003). A last step of organic N mineralization would be the release of NH_4^+ into the environment by deaminase action on amino acids. To our knowledge, there is only one study reporting the production of free NH_4^+ in the medium by an ECM fungus, *Hebeloma crustuliniforme* (Quoreshi *et al*., 1995). This net release of NH_4^+ occurs only when the fungus was grown without glucose in the medium, questioning the importance of this activity *in situ* by ECM fungi as they do always rely on carbohydrates supplied by their host-plant. Finally, regarding the use of inorganic N, it is well documented that ECM fungi have a preference for ammonium over nitrate *in vitro* (Rangel-Castro *et al*., 2002; Guidot *et al*., 2005) and in the field (Clemmensen *et al*., 2008). However there is a large variability among ECM fungi to use nitrate, with fungal species growing better with nitrate than ammonium (Scheromm *et al*., 1990; Montanini *et al*., 2002) and unable to use nitrate as the sole source of N (Chalot and Plassard, 2010). This versatility could help ECM fungi to compete with other soil microbes (bacteria and/or non ECM symbiotic fungi) for the various N sources. However, experiments where a new source of ^{15}N is brought to the soil generally demonstrate that soil microbes (other than ECM fungi) are the first sink for the added N (in example, see Clemmensen et al. (2008) regarding the fate of added glycine, ammonium or nitrate), indicating that ECM fungi may not be the faster microorganisms to take new sources of N at short term. However, in some situations such as boreal forests where organic N is the predominant available form, pot experiments and field studies have shown

that up to 80% of all plant N is derived from ECM fungi (reviewed by van der Heijden *et al*., 2008), indicating that mycorrhizal symbiosis could benefit of the N cycling occurring through the microbial loop.

3.5. Role of soil food web in rhizosphere P cycle

Soil food-web organisms are integral to the soil phosphorus (P) cycle and play an important role in regulating the availability of P to plants (Figure 13).

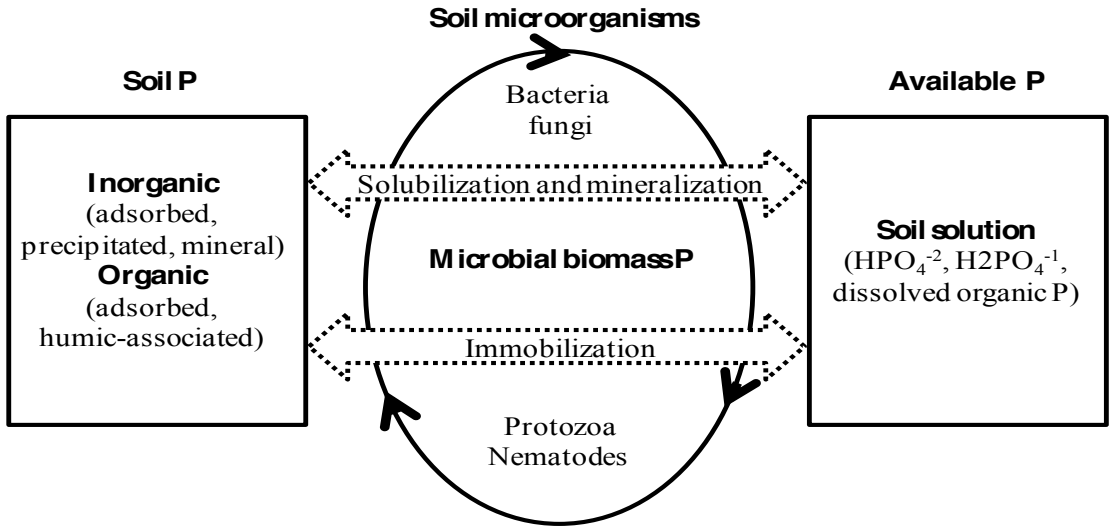

Figure 13. Schematic representation of the importance of microorganisms to P availability in soil. Microorganisms and their interactions in soil play a critical role in mediating the distribution of P between the available pool in soil solution and the total soil P through solubilization and mineralization reactions, and through immobilization of P into microbial biomass and/or formation of sparingly available forms of inorganic and organic soil P. (Redrawn from Richardson and Simpson 2011).

The world's high-quality sources of rock phosphate are finite and this itself justifies the need to develop agricultural systems that are more P efficient. For the development of sustainable agriculture systems, use of microorganisms to increase the availability of P in soil therefore is an attractive proposition (Richardson and Simpson, 2011). This strategy will be very helpful for developing and even for developed countries where access to mineral fertilizers is restricted (Sa´nchez, 2010).

The concept of bacterial and fungal enhancement of P availability to plants is not new. First studies have focused on the capacity of bacteria or fungi to dissolve inorganic soil P made of phosphate ions complexed with cations or oxides to form insoluble or poorly soluble mineral

P. Pioneer work by Gerretsen (1948) showed that pure cultures of soil bacteria could increase the P nutrition of plants under controlled conditions through solubilization of precipitated forms of calcium (Ca) phosphates. The dissolution of soil P by microbial populations is mediated by acidification and/or chelation due to the production of protons, organic anions and siderophores (Hinsinger *et al*., 2011; Marschner *et al*., 2011; Richardson and Simpson, 2011).

Besides mineral P, soil contains P linked to C-containing compounds to form organic P (Po). Among the different forms of organic P extracted from soil, phytate, corresponding to the salt of myo-inositol hexakisphosphate (IP6) is often the dominant form of organic P (Turner, 2007). To be used as a P source, Pi groups linked to C must be released by the action of phosphatases that can be specific for their substrate in the case of phytases. Regarding soil phytate use, although plants are able to mobilise P from phytate from the seed (accounting for ~70% of the total seed P: Raboy, 2007) during germination thanks to endogenous phytases, experimental data obtained so far indicate clearly that the roots have a very poor capacity to use phytate as the sole P source when they are grown in axenic conditions (Richardson *et al*., 2007). In contrast, saprotrophic fungi and bacteria are able to produce phytases. These enzymes have been classified in four classes based on their structural differences and varied catalytic properties by Mullaney and Ullah (2003, 2007). Of these four classes, two are particularly important for phytate cycling in soil. The first one includes histidine acid phytases (HAP) that can be found in fungi (such as *Aspergillus* sp.), plants and bacteria that are active at acidic pH and release 5 of the 6 phosphate groups of phytate (Mullaney and Ullah, 2007). The second one includes ß-Propeller phytase (BPP) found exclusively in bacteria (Lim *et al*., 2007; Jorquera *et al*., 2008b), especially in *Bacillus* sp. (Lim *et al*., 2007; Jorquera *et al*., 2011). These enzymes, mainly studied using *B. subtilis* strains, also called alkaline phytases, are active at neutral pH and release 3 of the 6 phosphate groups of phytate (Mullaney and Ullah, 2003; Lim *et al*., 2007). Pioneer work made by Richardson (2001) showed that the P nutrition of plants supplied with phytate was improved significantly when the agar medium is inoculated with soil microorganisms or a strain of *Pseudomonas fluorescens* with phytase activity. The work of Jorquera et al. (2008a) showed that culturable populations of bacteria isolated from the rhizosphere of plants (ryegrass, white clover, wheat, oat and yellow lupin) grown in Andic soils (with high levels of Po) able to solubilise phytate were always detected, even in higher proportion than bacteria able to solubilise Ca-phosphate (Table 3). Interestingly, some bacterial strains were able to solubilise both mineral and organic P

sources. Such property should allow these bacterial strains to be good competitors among other rhizosphere bacteria.

Table 3. Occurrence of phosphobacteria in the rhizosphere of ryegrass, white clover, wheat, oat, and yellow lupin (from Jorquera *et al.*, 2008a)

	Rhizosphere				
	Perennial ryegrass	White clover	Wheat	Oat	Yellow lupin
PMB	33.8[a] (73.3[b])	38.0 (73.8)	4.1 (7.7)	9.8 (22.1)	0.9 (5.3)
PSB	5.6 (12.2)	3.0 (5.8)	34.0 (63.4)	26.2 (59.3)	8.9 (52.0)
PMPSB	6.7 (14.5)	10.5 (20.4)	15.5 (28.9)	8.2 (18.6)	7.3 (42.7)
Total phospho-bacteria (TP)	46.1a	51.5	53.6	44.2	17.1
THCB ($\times 10^5$ $CFUg^{-1}$)	9.7±2.3[c]	27.7±0.5	13.3±0.3	5.3±2.8	4.3±1.8

PMB Culturable bacteria that mineralize phytate only; *PSB* culturable bacteria that solubilize phosphate only ; *PMPSB* culturable bacteria that mineralize phytate and solubilize phosphate.
TP total phosphobacteria (=PSM+PSB+PSPSB);
THCB total heterotrophic culturable bacteria on LB medium
a Percent in relation to 300 colonies randomly chosen from LB agar
b Percent in relation to TP
c Mean±standard error

Taken collectively, these studies highlight that soil microbes have great capacities to solubilize mineral P and mineralize organic P. Therefore, bacterial and fungal biomass P (mineral and organic P) constitute a significant component of the total soil P and are generally equivalent to, or exceeds that held in plant biomass. Concentrations of microbial phosphorus in soils range between 0.75 for the mineral layer of a sandy Spodosol planted with loblolly pine (Grierson *et al*., 1998) and 184 mg/P kg for the litter layer of a planted pine forest (Ross *et al*., 1999). Microbial phosphorus contributes between 0.5% of the total phosphorus in grassland soils of New Zealand (Chen *et al*., 2000) and 26% of the total phosphorus in the litter layer of an indigenous broadleaved–podocarp forest, also in New Zealand (Ross *et al*., 1999). However, this microbial P is a highly dynamic pool of soil P and is subject to significant change in response to higher tropic-level predation (Turner and Haygarth, 2001;

Bonkowski, 2004). Among these higher trophic-level predators protozoa and nematodes are of great importance. Release of P by protozoan and nematodes grazers from the microbial biomass (bacteria and fungi) and from detrial sources occurs as orthophosphate and could be available to plants (Macklon *et al*., 1997) (Fig. 13)

Despite the fact that a lot of work has been done on fungal and bacterial organic phosphorus transformations no data is available on turnover rate of phosphorus by bacterial feeding nematodes.

3.6. Role of mycorrhizal symbiosis on P cycling

The ability of mycorrhizal fungi on P cycling has been mainly studied in ectomycorrhizal fungi by investigating the capacities of fungal species to produce organic anions (Low Molecular Weight Organic Anions, LMWOAs) on one hand, and phosphatase/phytase activities on the other hand.

Ectomycorrhizal fungal species have been shown to be able to produce a range of LMWOAs (Plassard and Fransson, 2009), among which oxalate appears to be the main compound released, as shown by the pioneer studies carried out by Lapeyrie and colleagues (Lapeyrie, 1988; Lapeyrie *et al*., 1987; 1991). However, ectomycorrhizal fungi studied so far display a huge diversity to produce oxalate occurs among species and even among isolates of the same species. Now, approximately 30 species of ECM fungi belonging to the genera *Cortinarius*, *Lactarius*, *Paxillus*, *Piloderma*, *Pisolithus* and *Suillus* were found to be able to release substantial amounts of LMWOAs (Courty *et al*., 2010). On the other hand, almost no production of organic acids was detected in some ECM species belonging to the genera *Amanita, Cenococcum*, *Hebeloma, Thelephora* and *Tylospora* (Courty *et al*., 2010) or in the species *Laccaria bicolor* (Lapeyrie *et al*., 1991) or *Hebeloma cylindrosporum* (Arvieu *et al*., 2003). The ecological meaning of such diversity has not yet been established. However, the ability to release significant amounts of oxalate in the soil enhanced the P nutrition of the host plant grown in controlled conditions, with simplified (Wallander 2000) or sterilized (Casarin *et al*., 2003, 2004) soil enriched with insoluble mineral P (apatite), underlying the central role played by oxalate in the solubilization of mineral P.

In addition to the fungal capacity, some bacteria associated with hyphae have been shown to display a great capacity for mineral weathering. Indeed, the presence of complex bacterial communities in the ectomycorrhizosphere has been reported many times (Frey-Klett *et al*., 2005). These authors demonstrated, using in vitro assays, that the proportion of isolates

able to mobilize iron or to solubilize phosphorus from inorganic stocks was signi!cantly higher in the mycorrhizosphere than in the bulk soil. Similar results were obtained when studying the *Scleroderma citrinum* mycorrhizosphere (Calvaruso *et al*., 2007; Uroz *et al*., 2007). These authors clearly demonstrated that the bacteria from the mycorrhizosphere were more efficient in weathering biotite than those from the bulk soil and that their proportion was significantly higher. However, it is not known if the absence of LMWOA release by ECM fungi could be compensated by the activity of their associated bacteria.

The second aspect that has been studied is the ability of mycorrhizal fungi to release acid phosphatases in their environment. Depending on the pH of the incubation medium, one can distinguish acid phosphomonoesterase activity (ACP) from alkaline phosphomonoesterase activity (ALP), measured respectively at pH around 5 (ACP) and 7 (ALP). The release of ACP activities into the environment has been shown in many microorganisms, including various soil fungi (Nahas *et al*., 1982; Bae and Barton, 1989; Haas *et al*., 1992). Regarding mycorrhizal fungi, active release of phosphatases into the soil has been questioned for arbuscular fungi (Joner *et al*., 2000). In contrast to AM fungi, ECM fungi have been shown to release ACP in pure culture (e.g. Tibbett *et al*., 1998; Louche *et al*., 2010). As a result, ECM plants often increase the phosphatase activity in the rhizosphere soil or around ECM tips (Buée *et al*., 2005; Courty *et al*., 2006) and this is occasionally related to the degradation of labile organic P (see e.g. Liu *et al*., 2004). However, like what was observed for the production of LMWOAs, varying abilities to release ACP were reported among ectomycorrhizal species and strains (e.g. Matumoto-Pintro (1996) in Quiquampoix and Mousain, 2005; Tibbett *et al*., 1998, Nygren and Rosling, 2009). Regarding phytase activity, results obtained so far indicate a poor ability of ectomycorrhizal fungi to release Pi from phytate. For example, Louche *et al*. (2010) measured rates of phytate hydrolysis ranging from 1 to 8% compared to that measured on artificial substrate (para nitrophenol Phosphate) in the four fractions separated from the culture medium of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. So far the occurrence of bacteria able to mineralize organic P (such as phytate) in the rhizosphere of mycorrhizal hyphae has not been reported.

4. Hypothesis and objectives of thesis

As shown in this literature review, mineral nutrition of plants depends on many functions that can be modified by the organisms leaving either in symbiosis with the roots or in close association with the root, at the level of rhizosphere. In particular, bacteria and fungi may

strongly modify the availability of mineral nutrients through their capacity to solubilize, mineralize or immobilize macronutrients such as N and P that are limiting ecosystem productivity most of the time. In this context, the microbial loop, based on the grazing of bacteria by predators such as protozoa or bacterial-feeding nematodes is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. Although bacterial feeding nematodes are very abundant in the rhizosphere of plants, whether herbaceous or perennial, data regarding their role in the mineralization of N and P are very scarce compared to those obtained on the effect of protozoa on N nutrition of herbaceous plants.

In this context, the main hypothesis of my work was that bacterial feeding nematodes play an important role in the regulation of N and P availability to woody plants. However, the microbial loop in the rhizosphere of woody species should be more complex than in herbaceous species because roots of woody plants are systematically associated with fungal partners through the formation of ectomycorrhizal symbiosis. The fungus can also be in close interaction with bacteria living in its environment, thus increasing the contribution of the bacterial component to mobilization/immobilization of minerals in the mycorrhizosphere. Therefore, during my Ph-D thesis, I addressed the role of these complex interactions between ectomycorrhizal fungi, bacteria and their nematodes grazers on N and P nutrition of Pine seedlings (Fig. 14) with the following objectives:

- ✓ To quantify the flow of bacterial N and P from bacterial biomass to plant via grazing activity of nematodes in a controlled experimental system,
- ✓ To determine the role of nematode grazing activity on plant growth, root architecture of woody seedlings,
- ✓ To identify alternative routes for mobilization of a well-known poorly plant available P source represented by phytate through possible complementarities occurring among activities of phytate-hydrolysing bacterial, ectomycorrhizal and bacterial feeder nematode components.

To fill these objectives we used maritime pine (*Pinus pinaster*) as the host plant. This woody species was chosen because it is a species of economic importance, and it is easy to get seeds for experimentation. Plants were associated or not with the model species ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum*. This fungus, whose physiology is well known in our Laboratory, forms ectomycorrhizal association with *P. pinaster* easily.

Plants were then inoculated with a bacterial strain *Bacillus subtilis* isolated from ectomycorrhizal roots of *P. pinaster* in the field and two species of bacterial feeding nematodes, *Rhabditis sp.* and *Acrobeloides sp.*, also isolated from the same soil samples as ectomycorrhizal bearing *B. subtilis*. Given the complexity of the interactions studied, we set up a simplified experimental system consisting of Petri dishes filled with agarose as the substrate.

The results obtained are presented in four manuscripts, one already published and three in preparation:

1. Grazing by nematodes on rhizosphere bacteria enhances nitrate and phosphorus availability to *Pinus pinaster* seedlings (published in Soil Biology Biochemistry).
2. Phosphorus is required to the operation of microbial loop in the rhizosphere, independently of N availability
3. Phosphorus acquisition from phytate depends on efficient bacterial grazing, irrespective of the mycorrhizal status of *Pinus pinaster*
4. Sodium toxicity and phytate use in the rhizosphere: A helping hand by microbial partners.

The manuscript will comport a chapter describing the material and methods and will end with a chapter of conclusions and perspectives.

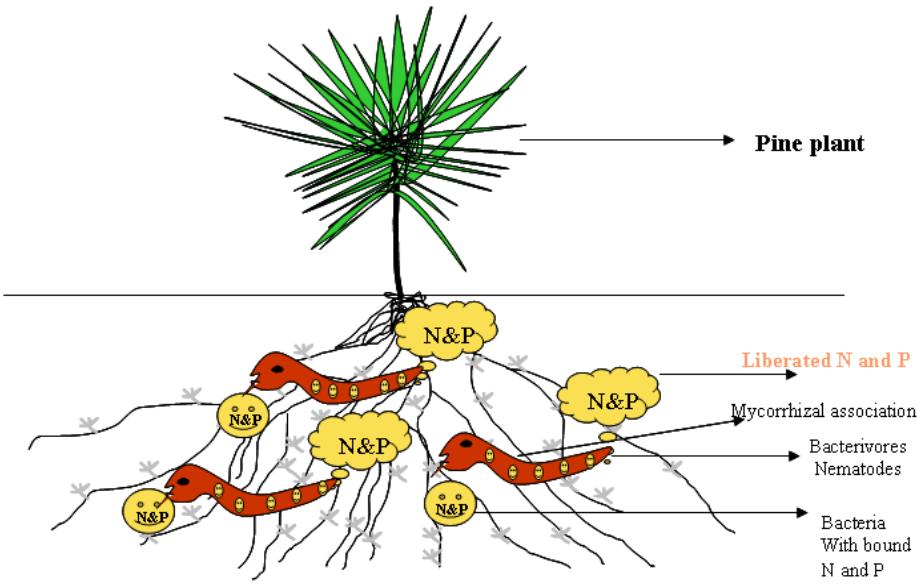

Figure 14. Schematic diagram of hypothesis of work and main mechanisms studied during the thesis.

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CHAPTER 2. MATERIAL AND METHODS

1. Biological material and culture method in routine conditions

1.1. Plant and fungus

1.1.1. Seed germination

Seedlings of Maritime pine (*Pinus pinaster* Soland in Ait. from Medoc, Landes-Sore-VG source, France) were always grown from seeds that were surface sterilized for 30 min. in a 30% (w/w) H_2O_2) solution. After rinsing with sterile distilled water until no bubbles could be seen in the solution, the seeds were kept in water at 4°C for 3 days. They were then taken off from the solution and aligned in Petri plates (Fig. 1) containing agar medium (10g/L) supplemented with glucose 2g/L. The germination took place after two weeks at 24°C, in the dark. The germinated seeds were used when the root size was around 50 mm long and always before the cotyledons were fully expanded, that is when the seed coat was still there.

Figure 1. Picture of a Petri plate with *Pinus pinaster* seeds showing germination after 10 days incubation.

1.1.2. Fungal cultures

A dikaryotic strain (D2) of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* Romagnesi, resulting from the plasmogamy of the two compatible homokaryotic strains (h1 and h7) (Debaud and Gay, 1987) was always used. Stock cultures were produced in Petri plates containing standard nitrate medium (N6) medium (composition given in Table 1) solidified with agar-agar (15g/L). When necessary, liquid cultures were carried out to grow the fungus. Agar plugs (8 mm diameter) of 1-month-old stock cultures were used to inoculate 120 ml-glass flasks containing 40 ml of liquid medium. A nichrome wire was used to

maintain the agar plug at the surface of the medium. Mycelia were grown in the dark, at 24°C, without shaking.

Table 1. Composition of nutrient solutions used for fungal and plant cultures.

	Final concentration in :	
Salt / element	**Nitrate (N6) medium (fungal culture)**	**Nitrate (N1) medium (plant culture)**
KNO_3	6 mM	0.6 mM
$Ca(NO_3)_2$	-	0.2 mM
KCl	4 mM	0.2 mM
NaH_2PO_4	3 mM	-
KH_2PO_4	-	0.2 mM
$MgSO_4,7H_20$	1 mM	1 mM
$CaCl_2$	0.5 mM	-
Fe citrate 1% (W/V)	0.5 ml/L	0.5 ml/L
Micro-nutrients[a]	0.2 ml/L	0.2 ml/L
Thiamine-HCl (100 µg/L)	1 ml/L	1 ml/L
Glucose	5 g/L	-

aMicro-nutrient solution composition, per liter : 2.82 g H3BO3, 98 mg CuSO4 5H2O, 3.08 g MnSO4 H2O, 0.29 g NaMoO4 2H2O, 4.41 g ZnSO4 7H2O (Morizet and Mingeau, 1976). This solution is kept at 4°C.
The pH of media is adjusted to 5.5 with Ca(OH)2 or H2SO4 before sterilization by autoclaving at 120°C, for 20 min.

1.1.3. Mycorrhizal synthesis

Mycorrhizal synthesis between *P. pinaster* seedlings and *H. cylindrosporum* was carried out using young germinated seedlings placed in test tubes as described in Plassard et al. (1994). For plant inoculation, three agar plugs (8 mm diameter) were taken from the margin of an active fungal culture and placed in the test tube, in the vicinity of the root (Fig. 2). Non mycorrhizal plants were prepared by replacing fungal agar plugs with simple agar plugs without any fungus. Each tube received 10 ml of a sterile nutritive (N1) solution (Table 1) that was renewed once a week in sterile conditions. Plants, whether inoculated or not, were placed in a growth chamber under a 16/8h light/dark cycle at 25/18°C, 80% rh, CO_2 concentration of c. 350 mm^3/l and a PAR of c. 400 µmol/m^2/sec (400-700 nm). Ectomycorrhizal short roots (Fig. 2C) can be observed after 1 month of culture in test tubes.

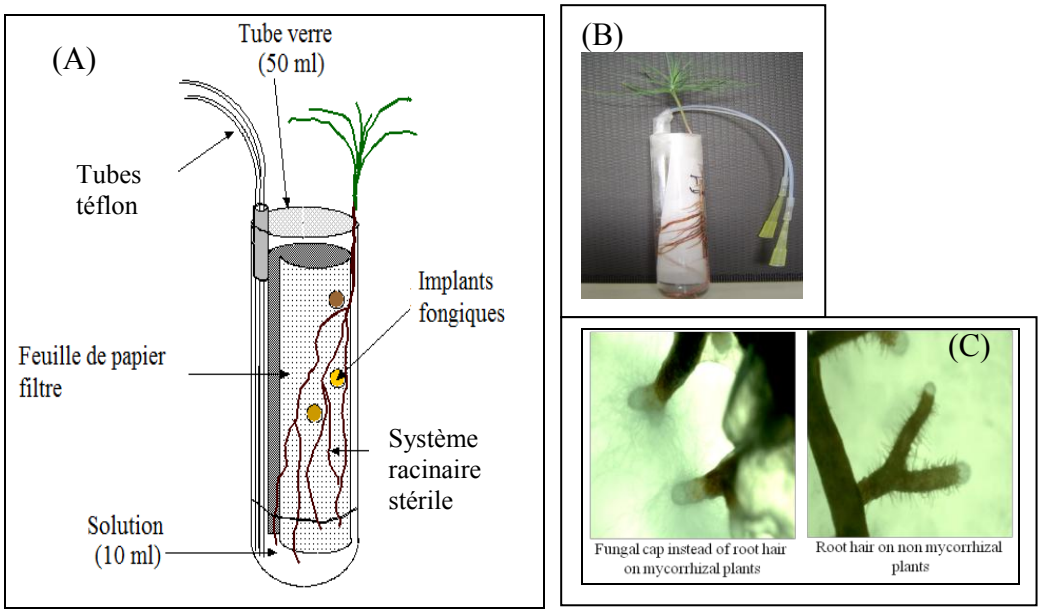

Figure 2. Mycorrhizal synthesis in test tubes.
(A) Scheme of the device, (B) Photo of a test tube with a P. pinaster plant, (C) Photos of H. cylindrosporum ectomycorrhizal short roots (left) characterized by the absence of root hairs and the presence of external hyphae and fungal sheath and non mycorrhizal short roots (right) characterized by the presence of root hairs.

1.2. Bacteria

Two bacterial strains were used in the experiments that were (i) *Escherichia coli* (strain DH10B) to isolate bacteria-feeding nematodes from soil forest soil samples and (ii) a bacterial strain isolated from ectomycorrhizal roots in the field. Both bacterial strains were maintained in Luria Broth (meat peptone 10g/L, NaCl 5 g/L, Yeast extract 5 g/L) (LB) medium. Unless stated, fresh bacterial cultures were prepared by inoculating LB medium with stock solution (made of culture in LB and glycerol, 2/1, v/v, kept at -80°C). The fresh LB medium was then shaken (150 rpm) for 24h at 37°C.

The bacterial strain used for the co-inoculation experiment was chosen among a collection of bacterial strains previously isolated from ectomycorrhizal roots. This collection was kindly supplied by the UMR LSTM (Laboratoire d'Etude des Symbioses Méditerranéennes, Baillarguet, Hérault). Ectomycorrhizal roots were extracted from soil cores collected in a 15-year old *P. pinaster* plot in the Landes Region, near Bordeaux. The plot belongs to an

experimental design comprising three different fertilization regimes that were i) without fertilization, ii) P fertilization, iii) N and P fertilization, whether irrigated or not (Trichet *et al*., 2009; Bakker *et al*., 2009). We selected the plot that was irrigated together with N and P fertilization. Among the 22 individual bacterial colonies available, we chose the strain 111b because it was able to use ammonium and nitrate as source of N. In addition, this strain was shown to be able to dissolve insoluble tricalcium phosphate when grown in pure culture with nitrate as the sole source of N (O. Domergue, LSTM, personal communication). Further study of this strain showed that it was able to use phytate as the sole source of P. Molecular analysis of 16S rDNA indicated that this bacterial strain could be identified as *Bacillus subtilis*. The bacteria were regularly spread on solidified (Agar-agar 15 g/L) LB medium to control the purity of the strain (Fig. 3A). Observation of concentrated solution under microscope showed the characteristic shape of *Bacillus* (Fig. 3B).

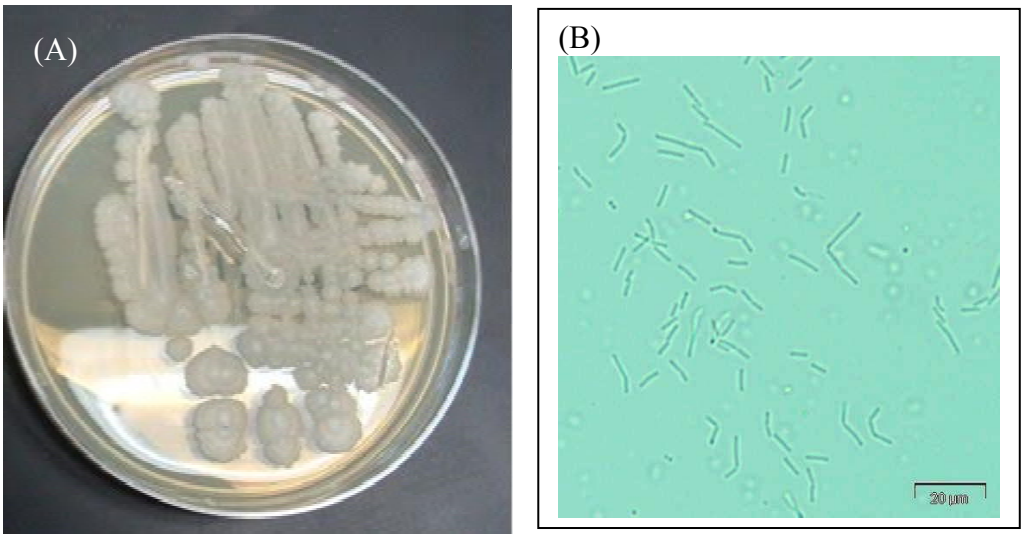

Figure 3. Bacterial cultures of *Bacillus subtilis* (strain 111b).
(A) Culture on solid Luria broth (LB) medium, (B) Concentrated bacteria in liquid LB under 40X microscope lens and zoom.

1.3. Nematodes

1.3.1. Isolation and multiplication of Nematodes

Nematodes were isolated from a soil sample collected in November 2007 in the same plot as the one used to isolate the bacteria associated with ectomycorrhizal roots of *P. pinaster*

trees. They were extracted following the Cobb method (s'Jacob and van Bezooijen, 1986) using the steps described below:

- ✓ Add 200g of soil in a plastic bottle
- ✓ Add 400ml of tap water
- ✓ Shake it for 3 minutes
- ✓ Leave it for 30 sec for settling down the particles
- ✓ Pass the supernatant through a sieve of 1 mm and wash the impurities
- ✓ Add again 400ml of tap water and repeat the same operation twice
- ✓ Remove big particles from the sieve of 1 mm
- ✓ Then successively pass the soil solution through sieves of 500, 250, 200, 100 and 50 µm respectively (repeat the same operation 3 times with sieve of 50 µm)
- ✓ Collect all material passed through sieves and leave it for 5 minutes for particles to settle down
- ✓ Pour the final solution on a fine tissue paper (from Lotus) placed in a Petri plate (90 mm diameter)
- ✓ Leave the device for 48 h. Living nematodes will move from the filter paper into the water.

The Nematodes were then inoculated one by one on Petri plates containing TSA medium containing 1% agar (w/v), 3 g l^{-1} Tryptic Soy Broth (Fluka ref 22092) supplemented with cholesterol (final concentration of 5 mg/L) added in cooled, autoclaved medium and already inoculated with 0.4 ml of fresh *E. coli* cultures.

Four days after inoculation, bacterial-feeding nematodes had grown on the agar plates (Fig. 4) whereas nematodes with other feeding behavior died. Female nematodes were recognized as they had plenty of eggs. They were inoculated on new plates to start monospecific breeding. Twenty plates were prepared with one female having plenty of eggs.

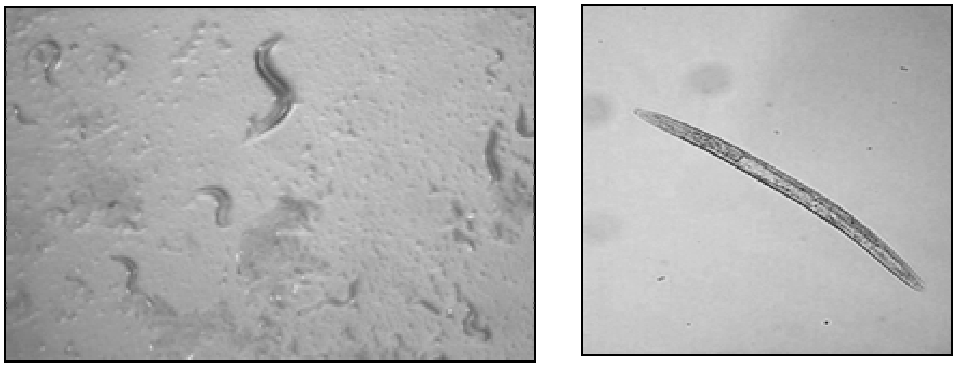

Figure 4. Photograph of nematodes after isolation in Petri plate and under binocular microscope at two magnifications. (Scale 1mm)

1.3.2. Sterilization and testing of Nematodes with *Bacillus subtilis* (111b)

In order to have monoxenic breeding of nematodes, sterilization of nematodes was carried out using the procedure given below:

- ✓ Select 4 Petri plates with a lot of eggs or a lot of females with eggs
- ✓ Add 5ml of sterilized water to collect all the nematodes and their eggs from plate
- ✓ Transfer this material into a centrifugation tube of 15 ml
- ✓ Centrifuge it for 8 minutes at 4000 rpm (2058 rcf) at 4°C
- ✓ Remove the supernatant from the tube
- ✓ Add successively and rapidly the following solutions:

  6ml distilled water
  3ml NaOCl (26%)
  0.5ml 1M NaOH
- ✓ Incubate the tube for 22 minutes to be sure that all nematodes are killed
- ✓ Centrifuge it again 4000 rpm (2058 g/min) at 4°C
- ✓ Remove the supernatant
- ✓ Wash immediately the eggs by adding 10ml of sterilized water
- ✓ Centrifuge it again 4000 rpm (2058 g/min) at 4°C and repeat twice the washing procedure
- ✓ Put the water solution with in a Petri plate and leave it for 24 hours.

The ability of each sterilized nematode population to feed on the *Bacillus subtilis* strain 111b was checked in sterile conditions. Two families of nematodes were selected for further experiments, belonging respectively to the *Rhabditidae* and *Cephalobidae* family as determined after DNA extraction and sequencing (see section 4.2 below). Nematodes were maintained by transferring every month individuals on new TSA plates containing *B. subtilis*. Nematodes multiplied in the dark, at 24°C. Solutions containing nematodes to be used in the inoculation experiments were prepared by removing them from the breeding TSA plates by washing the surface with a sterile NaCl solution (1%). They were washed from most *B. subtilis* by centrifugation (1000 rpm = 515 g/min, 5 min) and re-suspended in sterile deionized water.

2. Specific culture conditions

2.1. ^{15}N labeling of bacteria

Labelling of bacterial organic N was carried out by growing the bacterial cells in a synthetic medium (Table 2). The ^{15}N source was added as $(^{15}NH_4)_2SO_4$ labelled at 77%. The amount of ^{15}N used in the co-inoculation treatment was calculated to bring 2 mg of ^{15}N per plant representing the total N contents of plants at the time of inoculation. Bacteria were grown at 26°C with constant shaking (150 rpm) until the complete exhaustion of ammonium from the medium that occurred typically after 4 days of culture (Fig. 5). Bacteria were centrifuged at 5000 g, 10 min and the bacterial pellet was washed twice in 10 mM $CaCl_2$ solution to eliminate any remaining ^{15}N-NH_4^+. Bacteria were then re-suspended in sterile deionized water that was further used for filling Petri dishes in co-inoculation experiment.

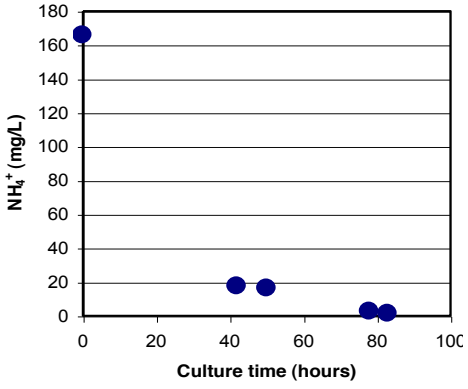

Figure 5. Depletion of ammonium from the culture medium during the culture of B. subtilis (strain 111b). Ammonium was supplied as $(^{15}NH_4)_2SO_4$ labelled at 77%.

Table 2. Synthetic medium for ^{15}N labelling of *Bacillus subtilis*.

Salt / element	Final concentration	
KCl	0.8 g/L	11 mM
MgSO4, $7H_2O$	0.5 g/L	2 mM
$CaCl_2$	0.5 g/L	5 mM
$(NH4)_2SO_4$	1.08 g/L	8 mM
KH_2PO_4	0.175 g/L	1 mM
Glucose	5 g/L	
Micro-nutrient solution[a]	4 ml/L	
Vitamin solution[b]	1 ml/L	

[a]Micro-nutrient solution composition, per liter : 125 mg H_3BO_3, 67.5 mg $CuSO_4$ $5H_2O$, 25 mg KI, 500 mg $MnSO_4$ H_2O, 50 mg $NaMoO_4$ $2H_2O$, 650 mg $Fe_2(SO_4)_3$, 100 mg $ZnSO_4$ $7H_2O$, 30 mg $CoCl_2$ $6H_2O$, 25 mg $NiSO_4$ $6H_2O$. This solution is autoclaved 40 min at 110°C.
[b]Vitamin solution composition, per liter: 5 g panthotenate, 20 g inositol, 2 g nicotinic acid, 250 mg pyridoxal hydrochloride, 250 mg thiamine hydrochloride, 10 mg of biotine. This solution is sterilized by filtration at 0.2 µm.

2.2. Phytate medium

The ability of *B. subtilis* and *H. cylindrosporum* to use phytate in pure culture was measured in a synthetic medium containing nitrate as the sole N source and buffered at pH 7 with MOPS (3-[N-morpholino]propane-sulfonic acid, Sigma ref M-1254) (see the basal composition in Table 3). Indeed, preliminary experiments showed that the bacterial growth in presence of phytate was highly sensitive to the pH of medium, with maximal growth occurring at pH around 7. Depending on the experiment, phytate (inositol hexakisphosphate, sodium salt, Sigma, ref P0109) was added at two concentrations that were 1 mM (Chapter 5) or 6 mM (Chapter 6). This phytate source, chosen because of its low contents of free orthophosphate (Pi) amounting to 5.37 mmol Pi mol^{-1} of phytate, contained 12 moles of Na^+ per mole of phytate. Such a high content of sodium resulted in a pH value of aqueous solution of phytate (10 mM) around 10. Therefore, it was very important to check the pH of culture medium and to buffer it. On the other hand, preliminary experiments showed that this phytate salt was highly unstable during autoclaving. Consequently, the medium without phytate was first autoclaved (115°C, 40 min) and cooled to 55°C before adding filtered (0.2 µm pore size)

sterilized solution of phytate at the suitable concentration. Culture media of the same basal composition (Table 3) supplemented with free Pi (1 mM) supplied as KH_2PO_4 were used to compare the efficiency of the bacteria and the ectomycorrhizal fungus to use phytate compared to mineral P (Chapter 5).

Table 3. Composition of nutrient solutions used to assess the capacity of the bacterial and the ectomycorrhizal fungus to use phytate as sole source of P.

	Final concentration in:	
Salt / element	**Phytate medium**	**Pi medium**
P source		
- $(Na)_{12}$Phytate[a] (Sigma, P0109)	6 mM	-
- KH_2PO_4	-	1 mM
KNO_3	1 mM	1 mM
MgSO4, $7H_2O$	2 mM	2 mM
$CaSO_4$	4 mM	4 mM
MOPS[b] (pH 7)	50 mM	50 mM
Glucose	55 mM	55 mM
Thiamine-HCl (100 mg/L)	50 µg/L	50 µg/L
Fe-citrate 1% (W/V)	0.5 ml/L	0.5 ml/L
Micro-nutrient[c]	0.2 ml/L	0.2 ml/L

a: Add sterilized phytate by filtration (0.2 µm) after medium autoclaving, in cooled medium at 50-60°C.
b: Adjust the pH of concentrated MOPS (50mM) at pH 7 with 1N KOH
c: Morizet and Mingeau (1976) Micro-nutrient solution (see Table 1)
Sterilize the medium by autoclaving at 120°C, 20 min

2.3. Co-inoculation media

As described in Table 4, different co-inoculation media were used, differing mainly by the P source supplied in the solid medium. Generally speaking, medium composition had to cope with several constraints. First, the N source should be different from ammonium (supposed to be released by grazing of bacteria) and should not induce acidification. Therefore, nitrate was always used as it could be used by the bacteria, the ectomycorrhizal fungus and the plant. Second, the P concentration had to be controlled.

Preliminary experiments showed that agar-agar (Agar HP 696, from kalys, www.kalys.com) contained a high concentration of free Pi (40.71 µmol P g^{-1} dry wt) in contrast to agarose (Eurobio, molecular grade) that contained only 1.4 µmol P g^{-1} dry wt. Therefore, agarose was systematically used for medium solidification. Depending on the aim of co-inoculation experiment, different sources of P were supplied, varying from insoluble mineral P (tri calcium phosphate) in experiment 1, no added P in experiment 2, and phytate sodium salt in experiments 3 and 4.

Table 4. Composition of nutrient solutions used for co-inoculation experiments.

	Final concentration in Co-inoculation experiment n°:			
Salt / element	**1**	**2**	**3**	**4**
P source				
- $(Na)_{12}$Phytate[a] (Sigma, P0109)	-	-	1 mM	6 mM
- KH_2PO_4	-	-	-	-
- Tri Calcium phosphate	4 g/L	-	-	-
KNO_3	1 mM	2 mM	2 mM	2 mM
MgSO4, $7H_2O$	2 mM	2 mM	2 mM	2 mM
CaSO4	4 mM	4 mM	4 mM	4 mM
MOPS[b] (pH 7.0)	-	50 mM	50 mM	50 mM
Glucose	-	-	-	-
Thiamine-HCl (100 mg/L)	50 µg/L	50 µg/L	50 µg/L	50 µg/L
Fe-citrate 1% (W/V)	0.5 ml/L	0.5 ml/L	0.5 ml/L	0.5 ml/L
Micro-nutrient[c]	0.2 ml/L	0.2 ml/L	0.2 ml/L	0.2 ml/L
Cholesterol (5g/L)[d]	1 ml/L	1 ml/L	1 ml/L	1 ml/L
Agarose (Eurobio, molecular grade)	7 g/L	10 g/L	10 g/L	10 g/L
pH of the medium	7.5	7	7	7

a: Add sterilized phytate by filtration (0.2 µm) after medium autoclaving, in cooled medium at 50-60°C.
b: Adjust the pH of concentrated MOPS (50mM) at pH 7 with 1N KOH
c: Morizet and Mingeau (1976) Micro-nutrient solution (see Table 1)
d: Cholesterol dissolved in pure ethanol, added after medium autoclaving, in cooled medium at 50-60°C.
Sterilize the medium by autoclaving at 120°C, 20 min

2.4. Co-inoculation experiment design

Unless stated, experiment of co-inoculation consisted of 6 treatments with 6-7 replicates per treatment that were:

a: Non-mycorrhizal (NM) *P. pinaster* seedlings,

b: NM *P. pinaster* seedlings + *B. subtilis*, strain 111b,

c: NM *P. pinaster* seedlings + *B. subtilis* + Nematodes (one or two species),

d: Mycorrhizal (M) *P. pinaster* seedlings associated with the ectomycorrhizal fungus, *H. cylindrosporum*, strain D2,

e: M *P. pinaster* seedlings+ *B. subtilis*, strain 111b,

f: M *P. pinaster* seedlings+ *B. subtilis* + Nematodes (one or two species).

Co-inoculation experiment was carried out in square Petri dishes (12 x 12 cm) (Fig. 5) filled with 70 ml of solid medium of varying composition (Table 4). Petri dishes were manufactured by making a hole to enable us to place the shoots outside. The medium was then poured at an angle of 10° to give more support to roots in the opposite direction of the hole. When applicable, bacteria and nematodes were supplied as liquid inoculum (0.5 ml/plant) that were spread over the surface of agarose medium. The root system of 1 to 2-month old pine seedling grown in test-tubes was then placed on the top of the solid medium. A support was given to the plant stem with a cotton plug sterilized twice (120°C, 30 min). Finally the plates were sealed with a sticky tape to protect the plant from contamination. All the plates were placed horizontally in the growth chamber (Fig. 5) with the same conditions as those previously described. They were covered with aluminum foil to minimize root system lighting. The weight of each plate (medium + plant) was recorded at the time of transfer in the growth chamber and then weekly during the experiment. If necessary, plant transpiration was compensated by adding sterile water corresponding to the weight loss calculated from initial weights.

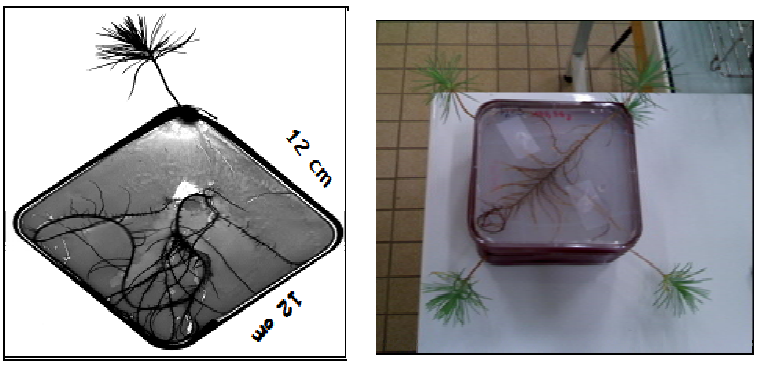

Figure 6. *Pinus pinaster* plants in Petri plate during co-inoculation experiments.

3. Quantitative assessment of microbial populations

3.1. Bacteria

Total populations of bacteria (alive or dead) were counted using haemocytometry. We used a "cellule de Malassez" (Fig. 7) consisting of a grid engraved on the glass slide surrounded by 2 draining channels enabling to put a known volume of solution to be counted. The grid had 100 rectangles of 0.01 mm^3 each that were made of 20 small squares of 50 µm.

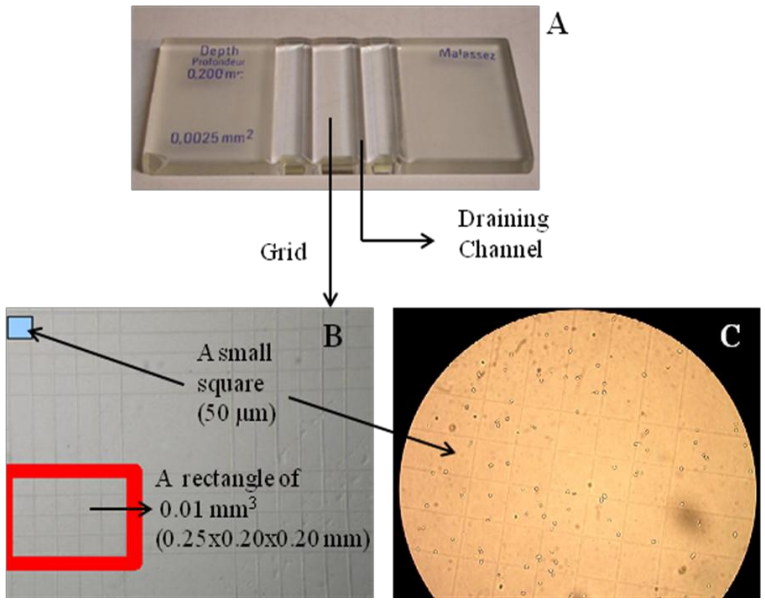

Figure 7. Description of Haemocyter ("cellule de Malassez")
A: general view of the counting slide consisting of one grid and 2 draining channels, B: enlargement of the grid with rectangle (red border) composed of 5x4 small squares (in blue), C: picture of squares containing *B. subtilis* cells taken at lens 40X.

The method for counting is as follows:

- ✓ Place 15µl cell suspension on an engraved grid and let the preparation stand for 1-2 minutes to allow the cells to settle at the bottom.
- ✓ Put the cover glass over the grid carefully so that no air bubble enters between the slides and cover glass. The centre portion of the slide has etched grids with precisely spaced lines. The cover-slip is positioned 100 µm above the slide.
- ✓ Slide the cover glass backwards and forwards until the colored rings are visible as the two surfaces of cover glass and slide come into close contact.

- ✓ Count the bacterial cells in ten rectangles in the middle of the slide.
- ✓ Use the 10X objective lens first, since the counter chamber is far thicker than a standard microscope slide. Then switch to the 40X objective.
- ✓ Record the data as "average number of the cells per mm^2" by dividing the total number of cells by the number of rectangles counted.

The number of cells per ml of bacterial solution (Counts) can be calculated using the formula given on the link (http://www.changbioscience.com/cell/hemo.html):
Counts = average number of cells counted per square mm*dilution factor*10,000 (conversion of 0.1 mm^3 to 1 mL).
Haematocytometry was used to quantify bacterial density at the beginning (in solutions used for inoculum) and at the end the co-inoculation experiment. In this case, solid medium from each square Petri plate was first cut in pieces and transferred to pots with 10 ml of deionized sterile water in sterile conditions. Each pot was shaken by hands for 5 min and aliquot of solution (1 ml) was taken after 2 h. This solution was used to numerate bacterial cells after adequate dilution (at least 1/10000).

3.2. Nematodes

The remaining solution (9 ml) and solid medium (around 70 ml) collected from the Petri plates were then transferred on a filter paper for 48 h to collect the population of living nematodes that moved through the filter into the water (50 ml) beneath. Nematodes were further concentrated by gravitation in a fraction of 5 ml. Nematodes were either counted immediately by using a stereo-microscope to determine their final density or fixed to determine their biomass. Fixation was carried out by mixing the solution with nematodes and fixation solution (4% formaldehyde) heated at 70°C. Slides were then made and observed under microscope. The dimensions (width and length) of nematodes were recorded using the software cell B (http://www.microscopy.olympus.eu/microscopes/Software_cell_B.htm). The body weight (µg) of each individual was calculated using a formula proposed by Andrassy (Andrassy, 1956 in Zuckerman *et al*., 1967) based on an average specific gravity of 1.084 for nematodes.

[Body weight of nematodes (µg) = a^2 x b/1600000 where a is the greatest body width and b the body length in microns]

3.3. Ectomycorrhizal degree

Ectomycorrhizal degree of plants was quantified from the amount of chitin contained in *P. pinaster* roots using the colorimetric method described by Vignon et al. (1986). Chitin assay can be done on the roots, substrates as well as mycelia in pure culture (as for example in Plassard *et al*., 2000). Chitin is specific of fungal cell walls (it replaces cellulose) and the assay is performed in two steps: (i) the polymer is completely hydrolyzed into glucosamine residues with a strong hot acid, (ii) the glucosamine residues are then measured colorimetrically. The colored assay involves two different stages: the glucosamine residues are first deaminated by HNO_2 to produce 2-5 anhydromannoses, molecules that possess a free aldehyde group on carbon 1. These compounds then react with MBTH, a specific reaction of aldehyde groups, and give a blue colour in the presence of $FeCl_3$ (Tsuji *et al*., 1969a,b). After acid hydrolysis it is possible to assay aldehyde groups that do not result from the deamination of amino sugars. The absence of HNO_2 during incubation enables one to quantify the amount of aliphatic aldehydes present in the solution. Therefore, the quantity of amino sugars is be calculated by the difference between the absorbance values obtained with and without the action of HNO_2.

3.3.1. Acid hydrolysis

The acid hydrolysis was performed by mixing dry root samples with 6N HCl (10 mg of dry weight/ mL HCl 6N) in screw cap tubes (glass tubes or eppendorf tubes). The tubes left open were incubated first for 3 h at room temperature. After that, the tubes were closed by screwing firmly their cap and were placed for then 16 h at 80 °C. The hydrolysis was stopped by a passage in a bath of cold water.

3.3.2. Colorimetric assay

The colored reaction has to be carried out at pH comprised between 4 and 5. Therefore it is necessary to adjust the pH of the solution by adding 1.25 M sodium acetate (0.5 ml 6N HCl / 2.5 ml CH_3COONa). This mixture is used for colored reaction.

The total absorbance (aldehydes + hexosamines) is measured by mixing 0.5 ml of the solution containing residues of glucosamine with 0.5 ml of $KHSO_4$ 5% (w/v) and 0.5 ml of $NaNO_2$ 5%

(w/v). This mixture is strongly shaken to allow the formation of HNO_2. The absorbance due to aliphatic aldehydes is measured in the same experimental conditions by replacing only $NaNO_2$ by 0.5 ml of water.

The mixture is allowed to stand for 15 min (deamination time).

Then 0.5 ml of ammonium sulfamate ($H_2NSO_3NH_4$) 12.5% (w/v) is added to the tube to destroy the excess of nitrous acid. A few seconds after addition of sulfamate, the solution should foam and rise in the tube. This strong reaction is fundamental to the success of the color reaction. After vigorous stirring for 5 minutes, 0.5 ml of MBTH (3-methyl-2-hydrazone benzothiozolone hydrochloride) 0.5% freshly prepared (0.5 g/100 ml) is added. The tubes are shaken a few seconds and left one hour at room temperature without agitation before the addition of hydrated ferric chloride ($FeCl_3$, $6H_2O$) * 0.5% freshly prepared (0.83 g/100 ml). The tubes are again shaken and left at least 30 min at room temperature. The absorbance of the two series of samples was read at 653 nm against a sample without glucosamine as blank, and remains stable for more than 20 h. The concentration of glucosamine in the solution is calculated using standard solutions with concentrations ranging from 1 to 30 µg glucosamine per ml established from a concentrated solution (10 mg/ml) stored at -20°C.

4. Molecular methods

4.1. Bacteria

Identification of the strain 111b was carried out by molecular analysis of 16S rDNA gene. PCR reaction was carried out by putting directly bacteria picked from colony in the PCR mix. Amplification was run with Taq polymerase (GoTaq® Flexi DNA Polymerase, Promega) using the primer pair: GC-338F forward (5'-CCTACGGGAGGCAGCAG-3') with a GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') and 518R reverse (5′-ATTACCGCGGCTGCTGG-3′) from Eurogentec SA (Liège, Belgium). The thermo cycling pattern used was 94°C 2 min (one cycle); 94°C 30 s, 65°C 30 s and 72°C 30 s (25 cycles, with a decrease of 0.5°C/cycle); 94°C 30 s, 55°C 30 s, 72°C 30 s (10 cycles) and 72°C for 10 min (one cycle). After checking the presence of one single band of ca 180 bp on gel electrophorese, PCR products were sequenced and identified to species level by launching a query through blast of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.2. Nematodes

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

5. Plant and medium analysis

5.1. Root parameters and plant harvest

The effects of various modes of inoculation on root development were measured in each experiment by scanning the root system either during the course of experiment without disturbing the plant (Irshad *et al*., 2011, Chapter 3) or at end of the experiment, after extraction and cleaning of the root system from solid medium. Root systems were scanned using WinRHIZO (http://www.regentinstruments.com/products/rhizo/Rhizo.html) (Fig. 8) and root length, root branching (forks and tips), root diameter and surface area were determined with the help of this software. After completion of root scanning, plants were separated into roots and shoots (needles plus stem). Amounts of biomass were measured on fresh or freeze-dried material. Dried plant parts were then milled before carrying chemical assays (N, P, chitin assay).

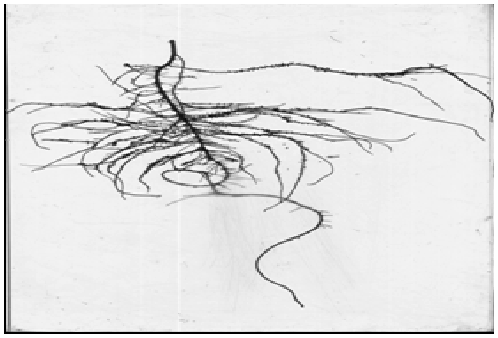

Figure 8. Example of *P. pinaster* root system scanned with WinRhizo at the end of co-inoculation experiment.

5.2. Solution extraction from solid medium

At the end of the co-inoculation experiment, plugs (8mm diameter) of solid medium were collected in triplicates throughout the Petri plate as illustrated in Figure 9. Each agarose plug was placed on a filter (made by cutting a micropipette tip of 1ml) in an eppendorf tube (1.5 ml) to extract the solution by centrifugation (5 min, 13000 rpm). The solutions were then kept at -20°C until further use.

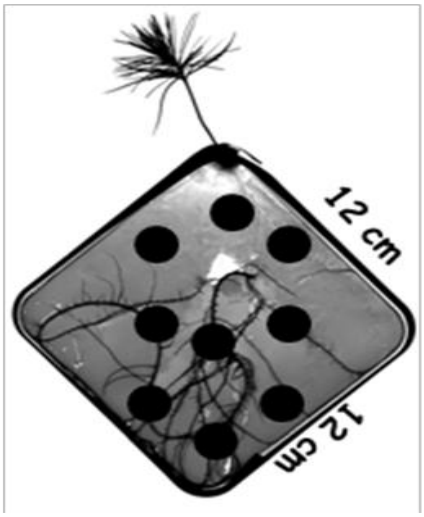

Figure 9. Scheme indicating the strategy to collect agar plugs (8mm diameter) for subsequent analysis of the medium.

6. Chemical measurements

6.1.1. Nitrogen

6.1.1.1. ^{15}N and total N

When ^{15}N labeling was used, total N contents and ^{15}N abundance were measured on plant samples using a mass spectrometer (Tracer Mass; Europa Scientific, Crewe, UK). In this case, the sample must contain at least 100 µg of total N (corresponding to 10-15 mg dry weight) for accurate measurement of ^{15}N atom excess. When plants were not labeled with ^{15}N, their total N contents were measured by elemental analyzer (CHN Fisons / Carlo Erba Na 2000, Milan, Italy). In contrast to ^{15}N, preliminary assays showed that sample weight should be comprised between 1 to 3 mg of dry matter to get a complete combustion of the samples and to avoid overestimation of the actual N concentration of samples.

6.1.1.2. Free ammonium

Concentrations of free NH_4^+ in culture solution were measured using the phenolic colorimetric method of Berthelot (Martin *et al.*, 1983). Briefly, the assay is carried out on 0.2 ml solution (7<pH>1) with [NH_4^+] ranging from 1 to 10 mg N/L corresponding to the linearity of the standard curve. When the sample is aqueous, it is mixed first with 1 ml of reactive 1 (R1: phenol 1% (v/v), Na Nitroprussiate 50.6 mg/L) and then with 1 ml of reactive 2 (R2: NaOH 0.125 M, Na_2HPO_4 0.25 M and NaOCl 0.12%) and mixed. When the sample is acidic, it has to be neutralized first by adding 1 ml of R2 into the sample (to produce NH_3 from NH_4^+, which is the reactive species with phenol) followed by the immediate addition of R1 to avoid the lost of NH_3 in the atmosphere. In both cases, the mixture is incubated at 37°C for 30 min, preferably under fume hoods to avoid toxic phenolic vapors. Absorbance of the blue solution is read at 625 nm.

6.1.1.3. Nitrate

Nitrate concentrations were measured in the solution extracted from agarose plugs using NO_3^-microelectrodes. They were made as previously described in Plassard et al. (2002) using glass capillaries containing a filling fibre (Clark Electromedical, GC150F). After pulling and silanization, electrodes were back-filled with 0.4 µL of an ion-selective sensor. NO_3^- sensor

was adapted from Zhen, Smith and Miller (1992) and contained 0·5% methyltridodecylammonium nitrate (MTDDA NO_3^-), 0·084% methyltriphenylphosphonium bromide (MTPPB) and 99·4% n-phenyloctyl ether (NPOE). This modified NO_3^- sensor enabled us to increase the linearity range down to 10 µM NO_3^-. The pipettes were then backfilled with 0.1 M KCl + 0.1 M KNO_3. Tips were broken to a diameter of 5–10 µ m to reduce microelectrode resistance to 1–3 G Ω in solutions containing 20 µM KNO_3. Microelectrodes were calibrated first in KNO_3 solutions containing 0·2 mM $CaSO_4$. They gave linear responses down to 10 – 5 µM with slopes of linear regressions varying between 56 and 59 mV per logarithmic unit of concentration. For measurements, the tip of the microelectrode was inserted into the solution contained in the eppendorf tube together with a salt bridge to ensure the closure of electrical circuit. The bridge was made of a silicone tube (3 mm diameter) filled with KCl 2M solution solidified by Agar 1.5 % (w/v). Readings were made using the same experimental device described in Plassard et al. (1999).

6.1.2. Phosphorus

Total P contents were determined after mineralization of tissues with H_2SO_4 36N as described in Torres Aquino and Plassard (2004). Briefly, the sample (10 mg dry wegiht) and acid (0.1 ml) are placed in a pyrex test tube (160 mm height, 15 mm diameter). The tube is then placed into a metal block heater (Liebisch, http://www.liebisch.com/) placed in a fume hoods. The sample is heated progressively to 310°C. After 10 min, the tube is withdrawn from the heater to add 0.2 ml of ultrapure H_2O_2 (110 vol, not stabilized with phosphate) to oxidize carbon. The tube is replaced at 310°C for 10 min. The addition of H_2O_2 is repeated until the solution becomes transparent. The mineralized solution is made only of concentrated H_2SO_4 that is diluted 36 times before assaying P concentration according to Ohno and Zibilske (1991) with malachite green.

6.1.3. Sodium

Sodium was determined by atomic absorption spectrophotometry in the same H_2SO_4 mineralized extracts as for P determination. The standard solutions of NaCl (0 – 2 mg/L) were prepared in the same H_2SO_4 solution.

7. Statistical analysis

Unless otherwise stated, the results are given as mean ± standard deviation. The differences between means were analyzed by factorial ANOVA followed by Tukey's HSD post-hoc test using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Kolmogorov Smirnov test and, where necessary, the data was either square root or log10 transformed prior to analysis to meet the assumptions of ANOVA. The comparison of means between NM and M samples at the beginning and the end of experiment was carried out using the Student's t test.

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CHAPTER 3

GRAZING BY NEMATODES ON RHIZOSPHERE BACTERIA ENHANCES NITRATE AND PHOSPHORUS AVAILABILITY TO *PINUS PINASTER* SEEDLINGS

The microbial loop is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. This is based on the assumption that newly grown bacteria mobilize nutrients that are not easily accessible to plants, in particular N and P, which are then made available to plants by bacterial grazers (Clarholm, 2005; Kuikman et al., 1991). Such mechanism could be of great importance in ecosystems where nutrient availability is strongly limiting biomass production as in the case of the forest ecosystem "Landais" (Trichet et al., 1999). Despite its nutrient-poor sandy soil, this ecosystem of 1 million ha is planted with *Pinus pinaster* and is economically important as it produces ca 20% of French softwood (Bert and Danjon, 2006).

So far, most research has been performed with annual plants and protozoa, highlighting the role of protozoa on N mineralization, root architecture and plant growth (Bonkowski, 2004; Krome et al., 2009). Only two studies have reported the effect of protozoa on growth and mineral nutrition of woody species (*Picea abies*: Bonkowski et al., 2001; Jentschke et al., 1995) and no results are available for the effect of nematodes on woody plants, despite the ecological importance of nematodes in soil (Ritz and Trudgill, 1999).

Our hypothesis was that nematode grazing on the bacteria developing in the rhizosphere of *P. pinaster* could enhance N and P availability to plants.

The objectives of this chapter were the following:

- ✓ To quantify the flow of N from bacterial biomass to plant shoots via grazing activity of nematodes in a controlled experimental system.
- ✓ To determine the role of grazing activity on root architecture of seedlings.
- ✓ To quantify the role of grazing on growth and P nutrition of seedlings.

This study was carried out using a bacterial strain (*Bacillus subtilis*) and a grazer nematode (*Rhabditis* sp.) species that were isolated from soil samples collected in "The Landes" forest.

We set up a simplified sterile experimental system to grow the plants, whether or not inoculated with bacteria or bacteria + nematodes. Bacteria were labeled with ^{15}N in order to follow the fate of bacterial N, and inoculated in roots of two months old pine seedlings. As it is thought that nematode grazing will result in the production of ammonium, the solid medium contained nitrate to avoid confusing effects between N supplied in the medium or from bacterial grazing. Finally, phosphorus was supplied as insoluble tricalcium phosphate (TCP) unavailable to plants to highlight any effect of bacteria or bacterial grazing on plant P nutrition. After 35 days of co-inoculation the plant shoots were examined for their N and P status and results were compared by ANOVA one factor among different treatments.

The results obtained in this work have been published in the journal "*Soil Biology and Biochemistry*" (Irshad et al., 2011).

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CHAPTER 3

GRAZING BY NEMATODES ON RHIZOSPHERE BACTERIA ENHANCES NITRATE AND PHOSPHORUS AVAILABILITY TO *PINUS PINASTER* SEEDLINGS

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1. Abstract

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

Key words: Trophic relationship, mineral nutrition, woody plant, inorganic P, soil bacteria, bacterial-feeding nematodes, *Rhabditis* sp., *Bacillus subtilis*.

2. Introduction

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

Most research has been performed with annual plants and protozoa, highlighting the role of protozoa on N mineralization, root architecture and plant growth (Bonkowski, 2004; Krome et al., 2009). Few studies have been carried out with woody plants. So far as we are aware, only two studies have reported the effect of protozoa on growth and mineral nutrition of Picea abies (Bonkowski et al., 2001; Jentschke et al., 1995) and no results are available for the effect of nematodes on woody plants, despite the ecological importance of nematodes in the soil (Ritz and Trudgill, 1999). Bacterial-feeding nematodes are a major component in the soil. For example, of the whole nematode population extracted from a mixed deciduous forest, 20–50% of nematodes were bacterial-feeders (Bernard, 1992; Liang and Shi, 2000). Furthermore, a high proportion of bacterial-feeding nematodes (90-99%) are found at sites of high microbial activity, such as the rhizosphere (Griffiths, 1994; Li et al., 2001a, b). Studies carried out with various bacterial-feeding nematode species showed that these organisms increased N mineralization and plant growth of herbaceous species (Djigal et al., 2004a, b; Ingham et al., 1985).

In forest ecosystems, N and P nutrition is always a growth limiting factor (Aerts and Chapin 2000; Comerford et al., 2002). However, according to Joergensen and Wichern (2008), the mean value of the bacterial contribution to the total microbial C in forest soils (30±4.3%, mean±standard deviation, n=125) is close to that calculated in agricultural situations (25±2%,

mean±standard deviation, n=267). This bacterial biomass may constitute an important pool of nutrients in forests if mobilized by predation activity (Osler and Sommerkorn, 2007). This mobilization of mineral nutrients sequestered in the microbial biomass may be of particular importance in poor nutrient forest ecosystems such as the Landes forest in south-west France. This forest ecosystem, consisting of stands of maritime pine (Pinus pinaster Soland in Ait.), is used for intensive wood production and requires integrated management strategies to maintain optimal soil fertility (Trichet et al., 1999; 2008).

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

3. Materials and methods

3.1. Plant production

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

3.2. Bacteria

The bacterial strain used in this study was isolated from pine roots collected from a 15-year old stand of *P. pinaster* in the Landes Region, near Bordeaux. This strain was chosen because it was able to use either nitrate or ammonium as the sole source of N in pure culture. This isolate was identified as *Bacillus subtilis* from DNA sequencing. To label their N pool with

^{15}N, the bacteria were grown in a synthetic medium containing 5.2 mM $(^{15}NH_4)_2SO_4$ labeled at 77%, macronutrients (11 mM KCl, 2 mM $MgSO_4.7H_2O$, 5 mM $CaCl_2$, 1 mM KH_2PO_4), 5 g l^{-1} glucose and 50 mM MOPS (3-[N-morpholino]propane-sulfonic acid, Sigma ref M-1254) to maintain the pH of the medium close to 7 after the complete assimilation of NH_4^+ by the bacteria. After autoclaving (115°C, 40 min), micronutrient (4 ml l^{-1}) and vitamin (1 ml l^{-1}) solutions were added to the medium as described by Tatry et al. (2009, Supplementary material). The volume of medium was calculated to provide 2 mg of ^{15}N of bacterial origin per plant, which was roughly the amount of total N contained in the plants at the beginning of the inoculation experiment. The bacteria were grown on a shaker (150 rpm) at 26°C for 83 h, until the NH_4^+ had completely disappeared from the medium measured using the Berthelot method (Martin et al., 1983). The medium was centrifuged (5000 g, 10 min) and the bacterial pellet was washed twice in 10 mM $CaCl_2$ solution to eliminate any remaining ^{15}N-NH_4^+. The bacteria were then re-suspended in 8 ml of 10 mM $CaCl_2$ solution before use.

3.3. Isolation, multiplication and identification of nematodes

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

Genus identification of the Rhabditidae selected was realized by molecular analysis of 18S rDNA gene. DNA was extracted from single nematodes placed in a volume of lysis buffer (Tris-HCl 200 mM, NaCl 200 mM, ß-mercaptoethanol 1% (vol/vol), proteinase K 0.8 $g.l^{-1}$) and ultrapure water (25 µl each). After centrifugation (1min, 5000g), the mixture was incubated at 58°C for 1 h in shaken conditions (900 rpm). After cooling at -20°C for 1 h, the mixture is incubated again at 58°C overnight. Three microlitres of the DNA extract were used for PCR amplification with Taq polymerase (GoTaq® Flexi DNA Polymerase, Promega) using the primer pair: F3730 forward (5'-ACGGGGGGAGGGCAAGTCTGGTGCCA-3')

and F3729 reverse (5'-TTTACGGTAGAACTAGG-3') from Eurogentec SA (Liège, Belgium). The thermo cycling pattern used was 95°C for 2 min (one cycle); 95°C for 30 s, 61°C for 30 s and 68°C for 30 s (25 cycles) and 68°C for 10 min (one cycle). After checking the presence of one single band of ca 500 bp on gel electrophorese, PCR products were sequenced and identified to genus level by launching a query through blast of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4. Experimental design

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

Six to seven replicates were set up for each treatment (S, B and BN) to give a total of 19 plants. The experiment was carried out in square Petri dishes (12 x 12 cm) in sterile conditions with a hole to allow the plant shoots to develop outside the dish. Each Petri dish was filled with 70 ml of solid nutrient medium containing 1.5 % agarose (Eurogentec Molecular Biology Grade), 1 mM $MgSO_4.7H_2O$, 50 µg l^{-1} thiamine hydrochloride, 0.5 ml l^{-1} 1% Fe citrate, 0.2 ml l^{-1} micronutrient solution (Morizet and Mingeau, 1976), 1 mM KNO_3 as the sole source of N and 4 g l^{-1} insoluble $Ca_3(PO_4)_2$ (TCP) from Fluka (CAS 7758-87-4) as the sole source of P. The quantity of TCP (4g l^{-1}) was calculated to give 54 mg of insoluble inorganic phosphorus per plant for the inoculation medium. After autoclaving (115°C, 40 min), the medium was poured at an angle of 10° to facilitate root placement in the direction opposite the hole. After the medium solidified, 0.5 ml of ^{15}N-labeled bacterial suspension (equal to 2 mg of ^{15}N per plant) was added to the plates for treatments (B) and (BN) and 0.5 ml of nematode suspension (equal to 40 nematodes per plant) was added to the plates for treatment (BN). Each solution was spread on the surface of the medium before laying out the root system of the pine seedlings. Each Petri dish contained only one seedling. The plates were sealed with adhesive tape to protect the plant from contamination and placed in the growth chamber in the conditions described above. All the plates were placed horizontally and covered with aluminum foil to minimize the exposure of the root system to the lighting in the growth chamber. The plants were watered when necessary by adding a volume of sterile deionised water to maintain the moisture level. The plants were allowed to grow for 35 days.

3.5. Sampling and analytical procedures

On days 15 and 25 and the end of the experiment, the undisturbed root systems were scanned using WinRHIZO (http://www.regentinstruments.com/products/rhizo/Rhizo.html) without opening the dishes to measure the root length. The other root architecture parameters - surface area, diameter, number of tips and forks of the roots - were measured only at the end of the experiment. The aerial parts (needles plus stem) were separated from the roots and freeze-dried to determine the dry biomass. As it was difficult to clean the medium off the root systems, they were not used for subsequent measurements. The shoots were ground and the total nitrogen and ^{15}N abundance were measured using a mass spectrometer (Tracer Mass; Europa Scientific, Crewe, UK).

The total P in the aerial parts was determined after mineralization with H_2SO_4 36N as described by Torres Aquino and Plassard (2004). The free orthophosphate concentration (in aerial parts) was assayed in the mineralized solution using malachite green as described by Ohno and Zibilske (1991). At the end of the experiment, the whole agarose was placed on a filter paper for 48 h to collect population of living nematodes that moved through the filter into the water beneath.

The nematodes collected were counted using a binocular microscope to determine their final density.

3.6. Statistical analysis

Unless otherwise stated, the results are given as mean ± standard deviation (n= 6 or 7). The differences between means were analyzed by factorial ANOVA followed by Tukey's HSD post-hoc test using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Kolmogorov Smirnov test and, where necessary, the data was either square root or log10 transformed prior to analysis to meet the assumptions of ANOVA.

4. Results

4.1. Nematode abundance

The abundance of nematodes increased significantly during the experiment, with a final average density of 198 ± 15 *Rhabditis sp.* plant^{-1}. On average, the population increased by a

factor of 5 during the experiment. Visual observations indicated that juveniles were dominant 35 days after inoculation, indicating good growth conditions for these organisms.

4.2. Shoot biomass and root parameters

A significant increase in shoot growth was observed in *P. pinaster* seedlings inoculated with bacteria and nematodes compared to plants grown in sterile conditions, whereas the presence of bacteria only induced a slight, non-significant increase in the shoot biomass compared to the S plants (Fig. 1a). The evolution of the total root length as a function of time indicated the root growth during the whole experimental period (Fig. 1b). Compared to the initial value, the final root length increased by factor of 2.3 in the S plants and by a factor of 3.0 in B or BN plants (Fig. 1b). However, the treatment did not significantly modify the root length recorded at each date (Fig. 1b) or the root elongation rate calculated over the whole experiment of 3.5 ± 0.9 cm day^{-1} $plant^{-1}$in the S treatment, 4.4 ± 0.9 cm day^{-1} $plant^{-1}$ in the B and 4.5 ± 0.4 cm day^{-1} $plant^{-1}$ in the BN treatments. The other root parameters (surface area, diameter, tips and forks) of *P. pinaster* seedlings were not significantly modified either in the B or the BN treatments (Table 1).

Table 1. Effect of bacteria (B. subtilis) and bacterial-feeding nematodes (Rhabditis sp.) on root parameters (surface area, diameter, number of tips and forks) of P. pinaster seedlings after 35 days. Values are the means of 6-7 replicates per treatment and differences between treatments for a given parameter were analyzed with ANOVA. The values of P and F and degree of freedom (df) are given and means accompanied by different letters are significantly different at $p \leq 0.05$.

	Root parameters			
Treatments	Surface area (cm^2 $plant^{-1}$)	Diameter (mm $plant^{-1}$)	Tips (number $plant^{1}$)	Forks
Sterile	54a	0.76a	286a	442a
Bacteria	65a	0.81a	387a	509a
Bacteria+Nematodes	53a	0.74a	400a	564a
P-values	0.13	0.41	0.19	0.51
F-values	2.29	0.93	1.83	0.90
df	2	2	2	2

4.3. ^{15}N accumulation in shoot biomass

The ^{15}N accumulation in shoots originating from bacterial 15N differed significantly between treatments (Fig. 2). The greatest accumulation of 15N occurred in BN plants with a value close to 14 µg plant-1. However, B plants accumulated very low amounts of 15N with a value of 0.21 µg plant-1, a value not significantly different from the S plants.

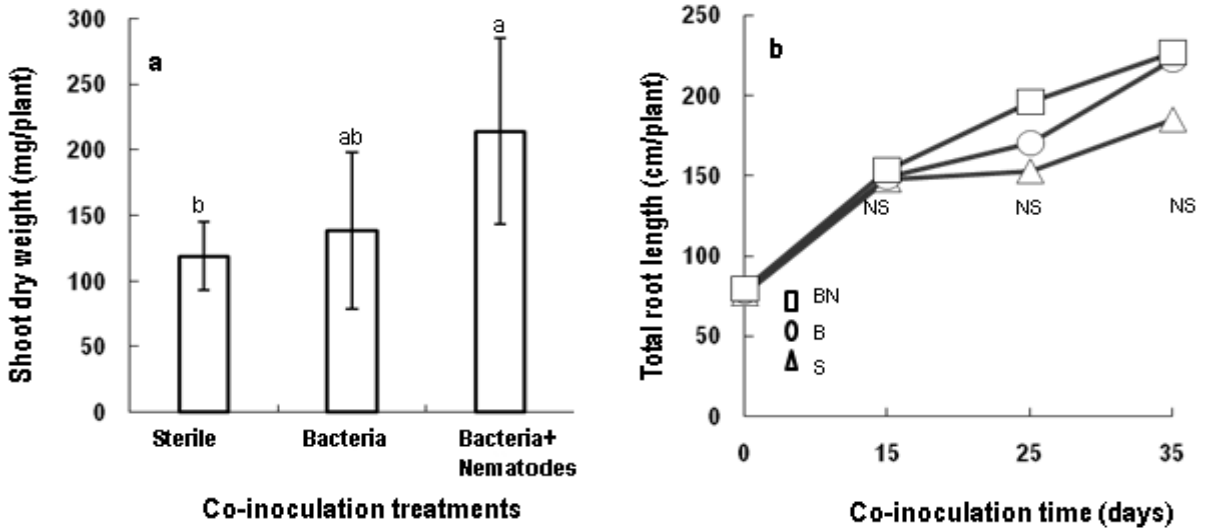

Figure 1. Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp.) (a) on dry shoot biomass of P. pinaster seedlings (b) on the variation of total root length of P. pinaster seedlings after 35 days of the inoculation experiment. Bars with different letters in figure 1a show significant differences (Tukey's minimum significant difference test, P≤ 0.05). In figure 1b no significant differences were found (NS). Each point or bar is the mean of 6-7 replicates per treatment (S: sterile, B: bacteria, BN: bacteria + nematodes).

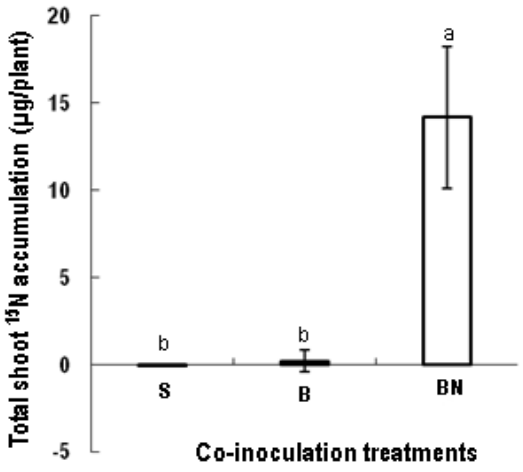

Figure 2. Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis sp.)* on ^{15}N accumulation in shoots of *P. pinaster* seedlings after 35 days of co-inoculation treatments (S: sterile, B: bacteria, BN: bacteria + nematodes). Bacteria were labeled with ^{15}N and supplied to the plants at a rate of 2 mg ^{15}N per plant in B and BN treatments. Each bar is the mean of 6-7 replicates per treatment and error bars are the standard deviations. Bars with different letters are significantly different (Tukey's minimum significant difference test, P≤ 0.05).

4.4. Nutrient accumulation in shoot biomass

The BN treatment significantly increased the N concentration in the shoots by a factor of 1.25 (relative to the S treatment) and 1.33 (relative to the B treatment) as well as increasing the accumulation of total N in the shoots by factors of 1.65 and 1.74 compared to the Sand B treatments, respectively (Table 2). Although no significant effect of B or BN treatments on the P concentration in the shoots was measured, the presence of both nematodes and bacteria significantly increased the accumulation of total P in the shoots by factors of 1.92 and 1.66 compared to S and B treatments, respectively (Table 2).

Table 2. Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis sp.*) on the accumulation of N and P in shoots of *P. pinaster* seedlings after 35 days. Values are the means of 6-7 replicates per treatment and differences between treatments for a given parameter were analyzed with ANOVA. The values of *P* and *F* and degree of freedom (df) are given and means accompanied by different letters are significantly different at $p \leq 0.05$.

	Element concentration (mg g^{-1} dry weight)		Element accumulation (mg $plant^{-1}$)	
Treatments	N	P	N	P
Sterile	7.69b	3.20a	1.08b	0.39b
Bacteria	7.21b	3.17a	1.02b	0.45b
Bacteria+Nematodes	9.64a	3.45a	1.78a	0.75a
P-values	0.003	0.322	0.021	0.009
F-values	8.53	1.22	4.94	6.27
df	2	2	2	2

5. Discussion

5.1. Nematode population

The nematode population used in this study reached an average of 198 individuals per plant which corresponds to around 3 nematodes ml^{-1} of agar medium. This level is very low compared with the values given in the soil microcosm where there were higher numbers of Pelodera (Rhabditidae family 49 g^{-1} of non rhizospheric soil and 912 g^{-1} of rhizospheric soil) after 105 days (Ingham et al. 1985). However, it was shown that different nematode species

belonging to the Cephalobidae family had significant differences in growth rate in soil columns (Djigal et al., 2004a). In that study, populations of *Cepholobus pseudoparvus* and *Acrobeloides nanus* reached an average of 4 and 15-18 individuals g^{-1} soil respectively after 7 weeks of incubation. In addition to nematode densities that were close to those observed for *C. pseudoparvus*, we observed a high proportion of juveniles as reported in the same study (Djigal et al. 2004a). Taken together, these results suggest that our experimental conditions were suitable for nematode development.

5.2. Effects of bacteria and bacteria plus nematodes on *P. pinaster* development

Our results indicated that the presence of *B. subtilis* isolated from the rhizosphere of *P. pinaster* alone in combination with the opportunistic bacterial-feeding nematodes *Rhabditis sp.* also isolated from same soil from a *P. pinaster* stand had a significant effect on the plant shoot biomass. This result agrees with the findings of Ingham et al. (1985) for the herbaceous species *Bouteloua gracilis* grown with bacterivorous nematodes and bacteria. However, this positive effect on growth has not always been observed. Other studies with nematodes (e.g Djigal et al. 2004b) or protozoa (e.g Bonkowski et al. 2001) reported no significant effect of inoculation on shoot biomass. Only a slight, non-significant positive effect of bacteria, either alone or with nematodes, was observed on the root elongation rate and root branching of *P. pinaster* seedlings. This differs from previous studies carried out on herbaceous species such as tomatoes (Mao et al., 2006), rice (Kreuzer et al., 2006), watercress (*Lepidum sativum*) (Bonkowski and Brandt, 2002) and *Arabidospis thaliana* (Krome et al., 2009) where highly significant increases in root branching or root biomass were observed within the few days after inoculating plants with bacteria and protozoa. The results obtained with *Picea abies* also showed that, after 44 weeks of growth, the values for root biomass, root length and root branching were significantly increased by the presence of bacteria and amoebae compared to control plants (Bonkowski et al., 2001). The duration of our experiment, which was shorter than that for another woody plant, *P. abies*, may explain why the effect of inoculation treatments (B or BN) on the root architecture of maritime pine was non-significant in this case.

5.3. Effects of inoculation on the fate of bacterial ^{15}N and N nutrition of *P. pinaster* seedlings

The presence of nematodes was required to measure significant accumulation of ^{15}N in *P. pinaster* shoots compared to the S plants. However, the amount of ^{15}N was only 0.7 % of the total amount in the bacteria added to the medium, indicating that the grazing of bacteria by nematode populations may result in a low net bacterial N release available for plants. Ferris et al. (1998) reported that nematodes belonging to Rhabditidae were able to mineralize soil N at the rate of 0.0058 µg N nematode^{-1} d^{-1}. Assuming that the average number of nematodes during the experiment was 100 individuals plant^{-1}, the final amount of ^{15}N which would result from the grazing activity of ^{15}N labeled bacteria is 20 µg plant^{-1}. This value is of the same order of magnitude as the ^{15}N accumulation measured in this experiment.

In addition to ^{15}N accumulation, nematode activity induced a significant increase in unlabeled N accumulation in *P. pinaster* shoots. These results are similar to previous studies using other plant species (Alphei et al., 1996; Bonkowski et al., 2001; Bonkowski, 2004; Djigal et al, 2004a; Djigal et al, 2004b; Jentschke et al., 1995; Kuikman et al., 1991), indicating that pine plants benefit from the presence of nematodes in the rhizosphere. Plants grown alone or with bacteria only had a lower total accumulation of N in the shoots compared to the combined treatment (BN). This means that bacteria alone, even if they were able to develop on nitrate, were not able to provide an N source usable by the plant. Compared to the S or B plants, the additional amount of N accumulated in plants with nematodes and bacteria was 0.7 mg plant^{-1}. This is close to the amount of nitrate supplied in the medium (around 1 mg N plant^{-1}). These calculations indicate that the presence of nematodes enabled plants to take up most of N from the medium that was then preferentially allocated to the shoots.

This improvement of N uptake was not due to better exploration of the medium by roots, as the root surface area was similar in all treatments. However, even without modification of root architecture, the nematodes may facilitate the uptake of nutrients not directly available to the roots by their ability to transfer bacteria throughout the medium. The bacterial cells may develop in unexploited, substrate-rich microsites (Ferris, 2010). After ingestion of these nutrient-rich bacteria, nematodes might move close to the roots (Anderson et al., 1982; Gould et al., 1981; Knox et al., 2004; Wasilewska et al., 1975). The excretion and defecation products of nematodes may result in increased availability of unlabeled N for plant growth. Another hypothesis is that the presence of nematodes is able to enhance the root capacity to

take up nitrate from the medium. However, the mechanism behind this hypothesis remains to be determined.

5.4. Effects of inoculation treatments on P nutrition of *P. pinaster* seedlings

In addition to the increase in N accumulation, P accumulation in the shoots was also significantly enhanced by the presence of nematodes. This suggests that the availability of P limited the growth in S and B plants. This might be due to the absence of soluble orthophosphate (Pi) available for uptake as all the phosphorus was supplied as insoluble mineral phosphorus (Tri Calcium Phosphate). The presence of bacteria did not make the P available. This may indicate that, even if the bacteria were able to use P from the medium, their P content was not available to plants as shown by early studies (Anderson et al., 1979; Cole et al., 1978; Coleman et al., 1977 and 1978; Elliott et al., 1979; Woods et al., 1982). The presence of nematodes greatly increased P availability to the plant as shown by Djigal et al., (2004b) with *Zeldia punctata* and maize. This might be due to a higher rate of respiration in this treatment than in the other two treatments, owing to the presence of nematodes, bacteria and roots. Indeed, as underlined by Chen et al. (2007), most of the carbon resources grazed from bacteria by micro-fauna is used for respiration. This will result in greater CO_2 concentrations in treatments with nematodes than without nematodes. This will in turn acidify the medium and solubilize insoluble TCP to increase Pi availability to plants. Another way to increase P availability is the release of bacterial P from grazing into the medium, either as inorganic or organic P. Organic P might be mineralized either from the enzymes released by nematodes (Chen et al., 2007; Hu et al., 1999) or by plant roots (Li et al., 1997).

6. Conclusion

A sterile experimental system was used to quantify the effect of different combinations of organisms on plant nutrition that may occur in natural conditions in the rhizosphere of a woody plant, *P. pinaster*. The duration of the experiment (35 days) was sufficient to measure significant effects of the various inoculation treatments on the nutrient flow. The results showed that grazing by nematodes of bacteria was able to induce different effects on plant growth and development through an increase of shoot biomass and no significant effect on root architecture. However, the results support the hypothesis that the bacterial-feeding nematodes are a key factor for determining mineral nutrition to this forest species. Their

presence significantly enhanced N and P availability to *P. pinaster* seedlings, probably by improving plant use of nitrate and insoluble P supplied into the medium. Further experiments should be carried out to uncover the mechanisms behind these positive effects by considering various concentrations of N and P similar to those occurring in natural ecosystems. This study shows that the manipulation of soil faunal populations can exert a significant effect on the development of plants, by improving the ecology and enhancing plant growth.

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CHAPTER 4

PHOSPHORUS IS REQUIRED TO THE OPERATION OF MICROBIAL LOOP IN THE RHIZOSPHERE, INDEPENDENTLY OF N AVAILABILITY

In the previous chapter the results showed that grazing by nematodes of bacteria was able to induce different effects on plant growth and development through an increase of shoot biomass and no significant effect on root architecture. However, the results support the hypothesis that the bacterial-feeding nematodes are a key factor for determining mineral nutrition to this forest species. Their presence significantly enhanced N and P availability to *P. pinaster* seedlings, probably by improving plant use of nitrate and insoluble P supplied into the medium. This experiment was carried out to uncover the mechanisms behind these positive effects by considering the key role of phosphorus availability without giving any external P source to the medium. This was based on the assumption of Turner and Haygarth, (2001) that significant amounts of N and P can be released as ammonium and orthophosphate from the microbial biomass in response to higher tropic-level predation. Further Richardson and Simpson (2011) reviewed that P held within soil microorganisms constitutes a significant component of the total soil P and is generally equivalent to, or exceeds that held in plant biomass. Therefore, in this chapter, we supplied P only as bacterial P assimilated during the growth period used to label bacterial N with ^{15}N. Given the potential importance of ectomycorrhizal fungi on P nutrition of the host plant (Plassard and Dell, 2010), we added ectomycorrhizal plants in the experiment design.

Our hypothesis was that nematode grazing on bacteria, supplied as the sole source of P, could be able to make bacterial P available to the plant. In addition, ectomycorrhizal association may also enhance P uptake by *Pinus pinaster* seedlings.

The main objectives of this chapter were:

- ✓ To assess if the grazing by nematodes of ^{15}N-labeled bacteria representing the only source of P modified the cycling of N and of P to plants,
- ✓ To quantify the role of ectomycorrhizal association on N and P accumulation in plants.

We used the same type of experimental system to grow the plants, whether or not associated with the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*, and inoculated with bacteria or bacteria + nematodes. Bacteria were labeled with ^{15}N in order to follow the fate of bacterial N. The solid medium contained agarose to control P availability that was mainly supplied as bacterial P. Plants were grown for 45 days and analyzed for their biomass production, ^{15}N, total N and total P accumulation in roots and shoots, together with root architecture parameters.

The results obtained in this study are presented as a manuscript entitled "Phosphorus is required to the operation of microbial loop in the rhizosphere, independently of N availability" prepared for submission to Soil Biology Biochemistry.

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CHAPTER 4. PHOSPHORUS IS REQUIRED TO THE OPERATION OF MICROBIAL LOOP IN THE RHIZOSPHERE, INDEPENDENTLY OF N AVAILABILITY

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1. Abstract

Soil bacteria act as a sink and a source of available N and P to plants by mediating key processes in the biogeochemical N and P cycling. The grazing of bacteria by predators such as bacterial-feeding nematodes may accelerate mineralization and availability of N and P from bacterial biomass through the operation of microbial loop. However, little is known on the conditions required for the operation of the microbial loop, especially P availability. This study was undertaken to assess if nematode grazing upon the sole bacterial P could sustain the microbial loop and enhance N and P availability to the woody species *Pinus pinaster* whether or not associated with an ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*. Young seedlings were grown for 45 days in a simplified sterile experimental system with bacteria (*Bacillus subtilis*) and bacterivorous nematodes (*Rhabditis* sp.) isolated from soil samples collected from a 15-year old stand of maritime pine. Nitrogen was provided as nitrate (1.96 mg plant^{-1}) and ^{15}N-labeled bacterial N (1.86 mg plant^{-1}) whereas P was only as bacterial P (330 µg plant^{-1}). The results showed that despite all plants were able to grow and to use nitrate from the medium (up to 100% in non mycorrhizal plants with bacteria, with or without nematodes), they were not able to use bacterial P. The ^{15}N flow from the bacteria to the plant shoots was not affected by the mycorrhizal fungus and was only significant when nematodes were present. However, despite a high abundance of nematodes, the ^{15}N flow represented less than 0.5% of initial bacterial ^{15}N, with an average value of 7±2 µg plant^{-1} of ^{15}N. These values of N mineralization are much lower than those calculated from published rates of mineralization by bacterial-feeding nematodes. These results suggest therefore that P availability is a key factor for the operation of the microbial loop to improve N and P availability from bacterial biomass.

Key words: Trophic relationship, mineral nutrition, woody plant, ectomycorrhizal association, bacterial P, bacterial-feeding nematodes, *Rhabditis* sp., *Bacillus subtilis*.

2. Introduction

Soil microorganisms act as a sink and a source of available N and P by mediating key processes in the biogeochemical N and P cycling. In addition, bacterial and fungal demand for N and P seems to prevail over geochemical sorption when competing for available P in soils with high sorption capacities (Ehlers et al., 2010). Phosphorus held within soil microorganisms constitutes a significant component of the total soil P and is generally equivalent to, or exceeds that held in plant biomass (Richardson and Simpson, 2011).
Despite the fact that P concentration in the soil solution is very low (1-10 μM Hinsinger, 2001), it is evident that fungi and bacteria effectively compete with plants for available orthophosphate from soil solution and also represent a significant pool of immobilized P that is temporarily unavailable to plants (Richardson and Simpson, 2011). However, significant amounts of N and P can be released as ammonium and orthophosphate from the microbial biomass in response to higher tropic-level predation (Turner and Haygarth, 2001; Bonkowski, 2004). These higher trophic-level microbial grazers includes protozoa and nematodes which can play a major role in releasing nutrients sequestered in the bacterial biomass in the rhizosphere (Bonkowski et al., 2009; Villenave et al., 2004). These predators themselves are likely to benefit from increased plant growth since bigger plants may allocate more carbon underground and support a greater root biomass resulting in increased prey (bacteria) density (Bonkowski and Brandt, 2002; Bonkowski, 2004; Phillips et al., 2003).

Significant amounts of N and P can also be released from soil microorganisms (bacteria and fungi) without net change in the size of the microbial biomass pool due to recycling and turnover, but the outcome may depend on nutrient availability in the soil, especially that of phosphorus. Sundareshwar et al. (2003) reported that, whereas the plant community was limited by nitrogen, the bacterial community from the same soil was limited by phosphorus. On the other hand, Techau et al. (2004) established that phosphate-limited plants increased their root exudation which stimulated mycorrhizal colonization as well as population of free-living rhizosphere organisms. This may in turn have resulted in a higher mineralization rate and nutrient uptake despite the fact that differential responses to nutrients found among trophic groups and their subsequent interactions limit our predictive capability. Moreover,

nutrient limitation should affect the balance of energy and elements in living biological systems and the development of the ecosystem through competition for nutrient resources.
Productivity of forest ecosystems is most of the time limited by P availability (Plassard and Dell, 2010) and this is particularly true for the Landes Forest in south-west of France (Trichet et al., 1999; 2008). This forest ecosystem, used for intensive wood production, consists of stands of maritime pines (*Pinus pinaster* Soland in Ait.) planted on spodosols very poor in available mineral P (Ali et al., 2009). However, microbial P was shown to represent an important proportion of total P amounting up to 53% in some situations (Achat et al., 2010). Acceleration of P cycling through the grazing of bacteria by bacterial-feeding nematodes could therefore enhance mineral P availability to the trees. In addition to the activity of bacterial-grazing organisms, the mycorrhizal association could enhance plant P accumulation by increasing the uptake of any liberated mineral P through the fungal hyphae and its subsequent transfer to the host plant (Aquino and Plassard, 2004; Plassard and Dell, 2009).

In a recent study, we demonstrated that the grazing of bacteria by bacterial-feeding nematodes was able to increase N and P accumulated in shoots of young non mycorrhizal seedlings of *Pinus pinaster* (Irshad et al., 2011). However, in this study, P could come from the solubilization of calcium phosphate supplied to the medium or/and from the release of bacterial P after grazing activity. Here, we addressed this question by supplying bacterial P as the only source of P in the same simplified experimental system (Irshad et al., 2011) using *P. pinaster* seedlings, whether or not inoculated with bacteria (*Bacillus subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp.). We studied also the potential importance of the mycorrhizal association on the fate of P, by using *P. pinaster* seedlings, whether or not associated with the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*. Together with P, the fate of bacterial N was followed by inoculating the plants with ^{15}N-labeled bacteria. The effects of grazing by nematodes on root architecture, plant growth and mineral nutrition (N and P) were quantified, both in root and shoots of plants.

3. Material and methods

3.1. Fungal and plant material

Seedlings of maritime pine (*Pinus pinaster* Soland in Ait. from Medoc, Landes-Sore-VG source, France) were obtained from germination of seeds carried out in sterile conditions (Irshad et al., 2011). For mycorrhization, a dikaryotic strain (D2) of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* Romagnesi, resulting from the plasmogamy of the two compatible homokaryotic strains (h1 and h7) (Debaud and Gay, 1987) was used. Mycelia were grown in the dark, at 24°C in standard nitrate (N6) medium, on agar-solidified-medium (Louche et al., 2010). Mycorrhizal synthesis was performed in test tubes (Casarin et al., 2004) and plants, whether inoculated or not, were placed in a growth chamber under the same conditions as described in Ali et al. (2009) for three before the co-inoculation experiment. Each tube received 10 ml of a sterile nutritive solution (Irshad et al., 2011) weekly.

3.2. Bacterial strain and nematode

Previously isolated bacteria (*Bacillus subtilis*) were maintained in Luria Broth for stock solutions. For experimentation the bacteria were grown in a complete synthetic medium as described by Irshad et al. (2011) containing ^{15}N supplied as ammonium sulfate. Bacteria were grown in shaken conditions (150 rpm) at 28°C for 44 h. At maximum optical density the cultures were centrifuged (5000 g, 10 min) and the pellet was washed twice in sterile deionized water to eliminate any remaining $^{15}(NH_4)_2SO_4.2H_2O$. Bacteria were then re-suspended in 30 ml of sterile deionized water before use. Cell density of this solution was determined using dilution (1/1000) deposited on a hemocytometer (0.2mm depth). Calculations of bacterial density were performed using the link http://www.changbioscience.com/cell/hemo.html. Unless stated, initial bacterial density of the solution used to inoculate plates was $0.6x10^6$ cells ml^{-1}.

Previously isolated nematodes (*Rhabditis* sp.) were maintained by transferring every month individuals on new TSA plates containing *B. subtilis* cultures added to solid medium (TSA) containing 1% agar, 3 g l^{-1} Tryptic Soy Broth (Fluka ref 22092) supplemented with cholesterol (5 µg l^{-1} of media). The genus of the selected nematode was identified from molecular analysis of 18S rDNA gene as *Rhabditis* sp. (Irshad et al., 2011). Nematodes

multiplied in the dark, at 24°C. They were then removed from the breeding TSA plates by washing the surface with sterile deionized water. They were washed from most *B. subtilis* by centrifugation (1000 rpm, 5 min) and re-suspended in sterile deionized water to a density of 340±92 nematodes ml^{-1} for inoculation in plates.

3.3. Experimental design for co-inoculation

Plants of two status, either non mycorrhizal (NM) or mycorrhizal (M) with *H. cylindrosporum*, were then set up with three inoculation treatments: (1) no inoculation (control conditions (c)), (2) inoculation with *B. subtilis* (+bac) and (3) inoculation with *B. subtilis* and *Rhabtidis* sp. (+bac+nem). The experiment was carried out in square Petri dishes (12 x 12 cm) in sterile conditions with a hole to allow the plant shoots to develop outside the dish. Each Petri dish was filled with 70 ml of solid nutrient medium containing 1.0 % agarose (Eurobio Molecular Biology Grade), 2 mM $MgSO_4.7H_2O$, 4 mM $CaSO_4$, 50 µg l^{-1} thiamine hydrochloride, 0.5 ml l^{-1} 1% Fe citrate, 50mM MOPS, 0.2 ml l^{-1} Morizet & Mingeau solution of micronutrients (1976), 2 mM KNO_3 as the sole source of N, and without any external source of P except contained in agarose (Pi estimated equal to 43.4 µg P g^{-1} dry weight of agarose, giving an amount of 30.4 µg Pi per plant) plus cholesterol (5 µg l^{-1}). The medium without cholesterol was autoclaved (115°C, 40 min) and cooled to 55°C before adding solution of cholesterol. The medium was then poured at an angle of 10° to facilitate root placement in the opposite direction of the hole. After solidification, plates from both NM and M treatments (+bac and +bac+nem) received 1 ml of bacterial suspension (equal to 0.6 ± 0.1 $\times 10^6$ cells $plant^{-1}$) and plates from treatment (+bac+nem) received 0.5 ml of nematode suspension (equal to 170 ± 46 nematodes $plant^{-1}$). Both of these inoculums contributed a negligible amount of free Pi (1.53µg/$plant^{-1}$). Each plate from treatments without bacteria and nematodes (control) received 1.5ml sterile deionized water to give the same level of moisture to all plants. Each solution was spread on medium surface before laying the root system of pine seedlings. Each Petri dish contained only one seedling. Finally the plates were sealed with a sticky tape to protect the plant from contamination. Plants were placed in the growth chamber (see above for the conditions). All the plates were placed horizontally and covered with aluminum foil to minimize root system exposure to lightning in the growth chamber. To

maintain the moisture level the plants were watered every week by adding a volume of sterile deionized water corresponding to their weight loss. Plants were allowed to grow for 45 days.

3.4. Medium nitrate measurement

At the end of the co-inoculation experiment, immediately after opening the plates, agarose plugs (8mm in diameter) were collected in zones away (bulk) or close from the roots, either around growing apices (young roots) or brown roots (mature roots) to measure nitrate concentration remaining in the medium. Each agarose plug was placed on a filter (made by cutting a micropipette tip of 1ml) in an eppendorf tube and centrifuged (5 min, 13000 rpm) at 4°C to extract their solution. Given the small volumes of solution extracted, nitrate-selective microelectrodes were used to measure nitrate concentration. Microelectrodes were made and calibrated as previously described in Plassard et al. (2002). The concentrations of nitrate were calculated using the values of voltage output and the calibration equation.

3.5. Plant analysis

Root parameters (length, surface area, number of tips and forks) were measured on cleaned root systems scanned with the software WinRHIZO (http://www.regentinstruments.com/products/rhizo/Rhizo.html). Shoots (needles plus stem) and roots were freeze-dried for dry biomass determination and were then milled before carrying out N and P determination. Total nitrogen and ^{15}N abundance were measured using a mass spectrometer (Tracer Mass; Europa Scientific, Crewe, UK).

Total P contents were determined after mineralization of tissues with H_2SO_4 36N as described in Torres Aquino and Plassard (2004). Free orthophosphate concentration was assayed in mineralized solution according to Ohno and Zibilske (1991) with malachite green. All these analysis were carried out first on 3-month old NM and M plants (data given in table 1) used to set up the inoculation experiment and at the end of the experiment.

Table 1. Root architecture (length, surface area, number of tips and forks) and amounts of total biomass, total N and total P measured in three-month old P. pinaster plants of different status, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*, at the time of transfer for inoculation experiment. Values are the means (n=6) with standard deviation between brackets.

		Root architecture						
Plant status	Plant parts	Length (cm plant^{-1})	Surface area (cm^2 plant^{-1})	Tips (number plant^{-1})	Forks	Dry weight (g plant^{-1})	Total N (mg plant^{-1})	Total P
NM	Roots	239 (63)	61 (21)	946 (165)	1364 (400)	0.12 (0.03)	0.75 (0.24)	0.30 (0.09)
	Shoots					0.27 (0.07)	2.19 (0.51)	0.55 (0.17)
M	Roots	233 (90)	62 (20)	1022 (321)	1401 (643)	0.12 (0.02)	0.92 (0.21)	0.27 (0.07)
	Shoots					0.24 (0.08)	1.83 (0.58)	0.56 (0.18)

3.6. Bacterial and nematode populations

Remaining solid medium was first cut in pieces and transferred to pots with 10ml of deionized sterile water in sterile conditions. Each pot was shaken by hands for 5 min and aliquot of solution (1 ml) was taken after 2 h. This solution was used to numerate bacterial cells with a hemocytometer after adequate dilution (at least 1/10000). The living nematodes population was obtained for counting by transferring remaining solid medium on a filter paper as described by Irshad et al., 2011. Nematodes were counted immediately by using a stereo-microscope to determine the final density and then fixed to determine their biomass. Fixation was carried out by mixing the solution with nematodes and fixation solution (4% formaldehyde) heated at 70°C. Slides were then made and observed under microscope. The dimensions (width and length) of nematodes were recorded using the software cell B (http://www.microscopy.olympus.eu/microscopes/Software_cell_B.htm). The body weight (µg) of each individual was calculated using a formula derived by Andrassy (1956) based on an average specific gravity of 1.084 for nematodes.

[Body weight of nematodes (µg) = a^2 x b/1600000 where a is the greatest body width and b the body lenght in microns]

3.7. Statistical analysis

Unless otherwise stated, the results are given as mean ± standard deviation (n= 6). The differences between means were analyzed by one way ANOVA followed by Fisher's HSD post-hoc test using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Kolmogorov Smirnov test and, where necessary, the data was either square root or log10 transformed prior to analysis to meet the assumptions of ANOVA. The comparison of means from NM and M plants between the beginning and the end of experiment was carried out using the Student's t test.

4. Results

4.1. Assimilation of $^{15}NH_4^+$ and phosphorus in bacterial biomass

Bacillus subtilis was grown in pure culture and the evolution of [NH_4^+] and Pi remaining in the culture medium was measured. After 44h of culture, most of ammonium and P had disappeared from the medium. Assuming that all ammonium and Pi depleted from the medium was assimilated by the bacteria, and N and P contained in bacteria at the time of inoculation was close to zero, we calculated that 1ml of bacterial suspension ($0.6\pm0.1\times10^6$ cells) assimilated 1.84 mg $^{15}NH_4^+$and 330µg P from the medium.

4.2. Plant parameters

4.2.1. Root architecture

Four parameters were recorded to study the effect of the inoculation treatments on root architecture (table 2). Except for NM plants with no inoculation treatment (treatment c), all plants were able to increase their root length and their root surface area during the experiment. Despite that these parameters were not significantly affected by plant status and inoculation treatments (two-way ANOVA analysis, data not shown), one-way ANOVA showed that NM plants grown with bacteria and nematodes presented increased root length (+60%) and surface area (+61%) compared to those recorded in control plants. The ramification of the root system, estimated by the number of tips per plant, did not significantly increase in NM plants

grown either in control (c) or with bacteria (+bac) during the 45 d experiment (Table 2) although neither the plant status nor the inoculation treatments modified the average numbers of tips (not shown). Despite the fact that all plants increased their numbers of forks during the experiment, the only significant difference was between (+bac+nem) NM plants having 50% more forks than M plants grown in control conditions (Table 2).

Table 2. Root growth parameters (length, surface area, number of tips and forks) measured in *P. pinaster* plants of different status, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*. Two months old plants were supplied with nitrate in solid medium for 45 d and received different inoculation treatments that were either no addition (c or m), or addition of a bacteria only, *Bacillus subtilis*(+bac) or together with the bacteria-feeding nematode, *Rhabditis* sp (+bac+nem or +bac+nem) on both sides of NM and M plants.

		Root growth parameters (plant^{-1})			
Plant status	Treatments	Lengths (cm)	Surface area (cm^2)	Number of Tips	Number of Forks
NM	c	312b	75b	1374a	2455*ab
	+bac	393*ab	100*ab	1407a	2856*ab
	+bac+nem	500*a	121*a	1851*a	3549*a
M	m	375*ab	95*ab	1412*a	2360*b
	+bac	400*ab	93*ab	1628*a	2927*ab
	+bac+nem	389*ab	98*ab	1641*a	3088*ab
P-Values		0.26	0.21	0.36	0.34

Data are means (n=6) and were compared using one-way ANOVA followed by the Fisher's minimum significant difference test. For each parameter, means significantly different at $P < 0.05$ have different letters. Asterisk shows that all the parameters measured differed significantly from the means measured in plants sampled before the experiment given in Tab. 1 (comparison of means, Student t test).

4.2.2. Plant biomass and N accumulation

The effect of inoculation on dry biomass accumulation of roots and shoots of *P. pinaster* seedlings is shown in figure 1a. Despite the fact that all plants were able to accumulate a net amount of dry biomass during the experiment, two-way ANOVA showed that there was no significant effect of mycorrhizal status and inoculation on root or shoot biomass (not shown). However, one-way ANOVA showed that in NM plants, root biomass in control plants was

significantly lower than that measured in the plants of the two other treatments (+bac and +bac+nem) whereas shoot biomass was the same in all inoculation treatments (Fig. 1a). Biomass measured in roots and shoots of M plants was the same whatever the inoculation treatments. The greatest differences between NM and M plants were recorded in shoot biomass of plants inoculated with bacteria, where the presence of the fungus decreased by 33% the shoot weight relative to NM plants. Finally, net accumulation of dry biomass at the whole plant level was not significantly different between the treatments (Fig. 2a).

Figure 1b shows that all plants but M ones not inoculated (control) were able to accumulate N during the growth period in the roots. Effects of plant status and of inoculation treatments were significant (two-way ANOVA, not shown): the presence of the mycorrhizal fungus lowered N accumulation and the presence of bacteria, with or without nematodes increased N accumulation. One-way ANOVA showed that the presence of bacteria (+bac) and bacteria + nematodes (+bac +nem) increased N accumulation by 41% and 129% respectively compared to the control treatment of NM and M plants (figure 1b). N accumulation in shoots did not vary with plant status or inoculation treatments (not shown) and plants from only two treatments (+bac+nem NM plants and c M plants) were able to accumulate more N in the growing period. The calculation of net total N accumulation showed a slight but non significant effect of treatments, with a maximum value of 2.20 mg N measured in NM plants from (+bac +nem) treatment (Fig. 2a).

4.2.3. P accumulation

Figure 1c shows that plants did not significantly accumulate more P during the growth period neither in roots nor shoots. However, some variability of root P accumulation was found at the end of experiment, with a significantly higher value measured in roots of NM plants (+bac) and M plants (+bac+nem) than in NM plants grown in control conditions. P accumulation in shoots was not affected by any of the treatments. Accordingly, the net increment in P uptake that has occurred during experiment was found close to zero, with no effect of the treatments (Fig. 2a).

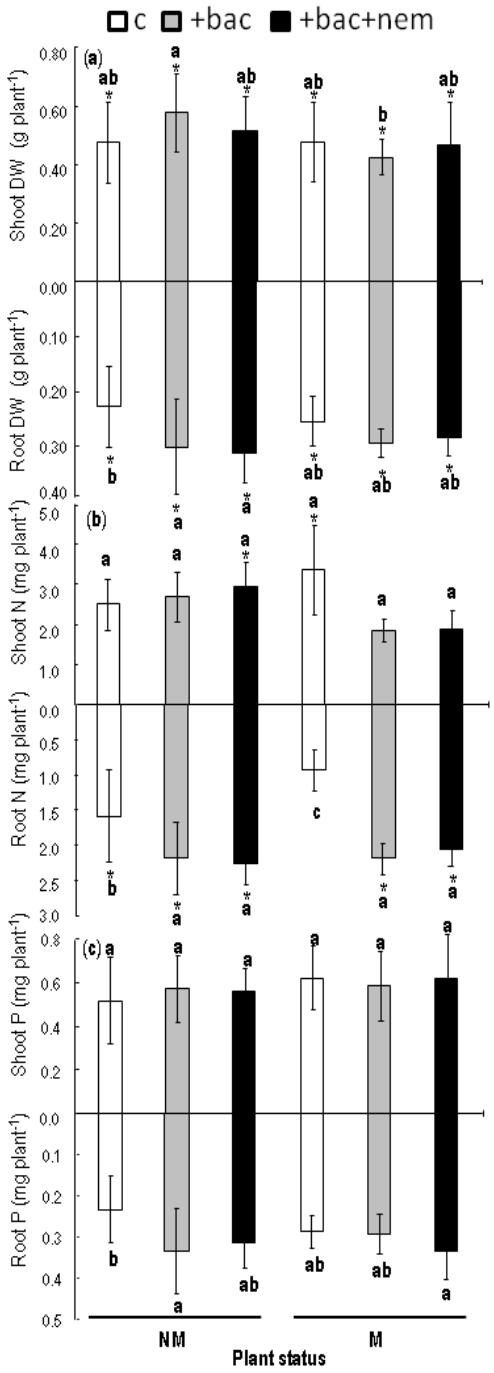

Figure 1. Total accumulation of dry biomass (a), N (b) and P (c) in roots and shoots of *P. pinaster* plants of different status, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum* and cultivated with different inoculation treatments that were either no addition of organisms (c), or addition of a bacteria *B. subtilis* (+bac) or together with the bacteria-feeding nematode *Rhabditis* sp. (+bac+nem). Three month old plants were grown for 45 d in solid medium containing 2 mM nitrate (1.96 mg plant^{-1}) plus bacterial N (1.86 mg ^{15}N plant^{-1}) and bacterial P (330µg plant^{-1}) as the sole source of P.

Bars correspond to means (n=6). Data measured in roots or shoots were compared using one-way ANOVA followed by the Fisher's minimum significant difference test and bars accompanied with different letters are significantly different ($P < 0.05$). Bars accompanied with an asterisk indicate that the mean is significantly different from the initial value measured in three-month old plants (see Tab. 1) using a Student's t-test of mean comparison ($P < 0.05$).

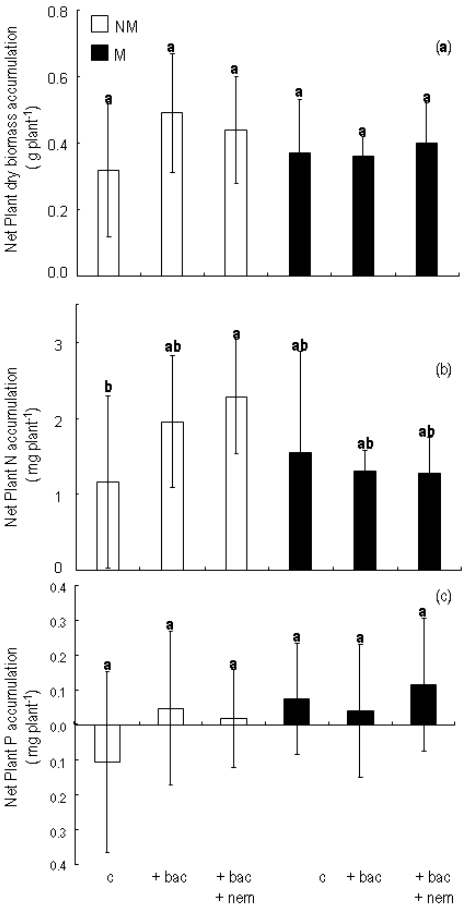

Figure 2. Total plant net accumulation of dry biomass (a), N (b) and P (c) of *P. pinaster* plants of different status, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum* and cultivated with different inoculation treatments that were either no addition of organisms (c), or addition of a bacteria *B. subtilis* (+bac) or together with the bacteria-feeding nematode *Rhabditis* sp. (+bac+nem). Three month old plants were grown for 45 d in solid medium containing 2 mM nitrate (1.96 mg plant-1) plus bacterial N (1.86 mg 15N plant-1) and bacterial P (330µg plant-1) as the sole source of P. Bars correspond to means (n=6). Data were compared using one-way ANOVA followed by the Fisher's minimum significant difference test and bars accompanied with different letters are significantly different ($P < 0.05$).

4.2.4. ^{15}N accumulation in root and shoot biomass

Figure 3 reports the average values of ^{15}N accumulation in shoots and roots from ^{15}N contained in bacterial biomass. In shoots, inoculation treatments had a significant effect (P<0.000) whereas plant status had no effect. The greatest shoot accumulation occurred in NM and M plants grown with bacteria and nematodes (+bac+nem) with a value close to 7.33 and 7.95 µg plant^{-1}. In contrast, plant shoots grown with bacteria, (+bac) accumulated low amounts of ^{15}N of 1.55 and 2.50 µg plant^{-1} in NM and M plants, respectively. In roots, also inoculation treatments had a significant effect (P<0.04) whereas plant status had no effect. Accumulation of ^{15}N in roots was increased by a factor 10 compared to that in shoots, with the lowest amount of ^{15}N (41 µg plant^{-1}) occurring in roots of NM plants grown with bacteria

only. In contrast with ^{15}N accumulation in shoots, ^{15}N accumulation in roots was not affected by the three other treatments. In addition, there was a significant relationship between ^{15}N accumulation and total root length of plants (not shown).

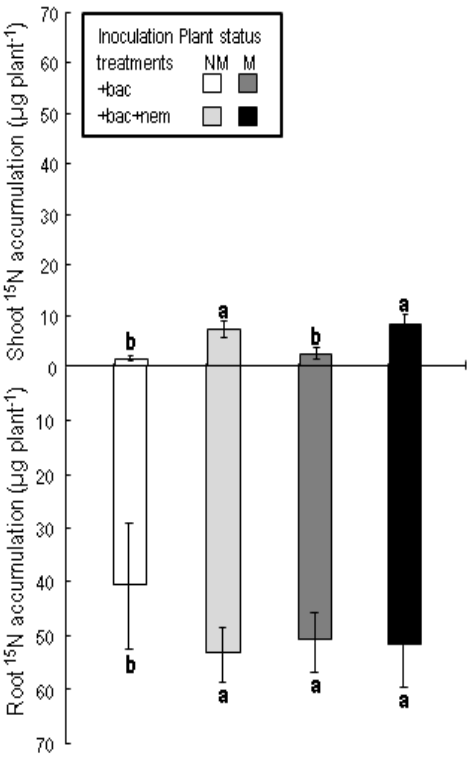

Figure 3. Effect of bacteria (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp.) on ^{15}N accumulation in roots and shoots of *P. pinaster* seedlings after 45 days of co-inoculation treatments, either with bacteria alone (+bac) or with bacteria and their grazer nematodes (+bac+nem). Plants were of two statuses, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum.* The solid medium contained 2 mM nitrate (1.96 mg plant^{-1}) plus bacterial N (1.86 mg ^{15}N plant^{-1}) and bacterial P (330µg plant^{-1}) as the sole source of P. Each bar is the mean of 6 replicates per treatment and error bars are the standard deviations. Bars with different letters are significantly different (Fisher's minimum significant difference test, P≤ 0.05).

4.3. Nitrate concentration in medium

The nitrate concentration measured in medium at the end of experiment in different zones around roots was found non-significant within treatments (table 3) despite great variations of nitrate concentrations. A slight decrease in nitrate concentrations was observed in different zones following the pattern i.e bulk zone > mature root zone > young roots zone. However this decrease in nitrate concentration was non-significant except the M plant treatment (+bac) with values 1.6, 0.4 and 0.24 mM nitrate in bulk, mature root and young root zone.

Table 3. Total nitrate disappearance from the medium among different zones of plant roots of different status, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum* and cultivated with different inoculation treatments that were either no addition of organisms (c), or addition of a bacteria *B. subtilis* (+bac) or together with the bacteria-feeding nematode *Rhabditis* sp. (+bac+nem). Three-month old plants were grown for 45 d with 2 mM nitrate (1.96mg N) plus bacterial biomass N ($^{15}NH_4^+$ 1.83 mg) and P (330 µg P) as the sole source of P in solid medium.

		Nitrate concentration (mM)			
Plant status	Treatments	Bulk zone	Mature root zone	Young root zone	P-values between zones
NM	c	2.10aA	0.52aA	0.3aA	0.18
	+bac	0.32aA	0.44aA	0.26aA	0.24
	+bac+nem	0.42aA	0.28aA	0.26aA	0.69
M	m	0.82aA	0.36aA	0.22aA	0.35
	+ bac	1.60aA	0.40aB	0.24aB	0.03
	+bac+nem	1.20aA	0.46aAB	0.22aB	0.10
P-values between treatments		0.48	0.95	0.35	

Data measured in medium were compared using one-way ANOVA followed by the Fisher's minimum significant difference test performed either between treatments for a given zone (small letters) or between zones for a given treatment (capital letters).

4.4. Abundance of bacteria and nematodes

Compared to the initial amounts of bacteria added to each inoculated plant that were 0.6±0.1 x10^6 cells, bacterial populations increased in the rhizosphere of plants after 45 d of co-inoculation by factors ranging from 7 to 11 (table 4). However, no significant difference was found in bacterial population among different accumulation treatments and plant status. The number of nematodes estimated after 45 d of treatment was not significantly different between NM and M plants, with mean values of 2280 and 1355 nematodes plant^{-1} in NM and M plants, respectively (table 4). The biomass of nematodes calculated randomly by taking 500 individuals depends on their numbers with a value of 35 and 21 µg plant^{-1} in NM and M plant roots, respectively.

Table 4. Abundance of bacteria (*B. subtilis*) and bacterial-feeding nematode (*Rhabditis* sp) populations 45 days after inoculation in the rhizosphere of *P. pinaster* plants, either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum.* The solid medium contained 2 mM nitrate (1.96 mg plant^{-1}) plus bacterial N (1.86 mg ^{15}N plant^{-1}) and bacterial P (330µg plant^{-1}) that was the sole source of P. The initial populations were 0.6x 10^6±0.1 bacteria and 170±46 nematodes per plate.

Plant status	Treatments	Bacterial abundance	Nematodes abundance	Nematodes biomass
		Numbers plant^{-1}		(µg plant^{-1})
NM	+bac	6.5×10^6a		
	+bac+nem	4.0×10^6a	2280a	35
M	+bac	5.4×10^6a		
	+bac+nem	4.1×10^6a	1355a	21
P-Values		0.26	0.09	

Data are means (n=6) and were compared using one-way ANOVA followed by the Fisher's minimum significant difference test. For each parameter, means significantly different at $P < 0.05$ have different letters.

* Biomass of nematodes was calculated from the size of 500 individuals randomly sampled from the whole population.

5. Discussion

5.1. Root architecture and plant growth

The effect of grazing on root architecture was significant only in NM plants, with values of length and surface area of roots increased in +bac+nem compared to non inoculated plants. However, this positive effect was not observed on root biomass that remained the same between inoculation treatments, indicating that the presence of nematodes induced a thinner, more extended root system of NM plants than in sterile conditions. This slower and thinner root growth might be due to productive environments as stated by Poorter and De Jong (1999). Compared to our previous data (Irshad et al., 2011) where no effect of inoculation treatments with the same organisms was observed on root architecture of NM *P. pinaster* seedlings, the duration of culture time increased from 35 d to 60 d may explain the positive effect of nematode grazing on root architecture, as already observed in *Picea abies* grown with bacteria and amoebae compared with control plants (Bonkowski et al., 2001). In contrast,

the presence of the fungus prevented the positive effect of nematode grazing on root architecture, confirming the results obtained by Jentschke et al. (1995) in *Picea abies*.

Shoot biomass of NM plants was not significantly modified by inoculation treatments, especially by the presence of nematodes and bacteria, contrary to what we observed in our previous study (Irshad et al., 2011). The only difference is the absence of mineral P in the present experiment suggesting that P availability may play a prominent role to determine the effect of nematode grazing on shoot growth. The presence of the fungus, alone or with inoculation treatments, did not improve shoot biomass compared to NM plants, giving even the lowest shoot biomass in +bac treatment. This may be due to a high C drain to the fungus when bacterial populations are present (Bonkowski, 2004), resulting in turn, to a growth decrease of the host plant.

5.2. Total N and ^{15}N accumulation

All NM plants were able to accumulate higher amounts of total N than those contained in plants before experiment. However, N remained accumulated mostly in the roots, except when nematodes were present. In this case, around 30% of N taken up during the experiment was allocated to the shoots. In contrast to our previous data (Irshad et al., 2011), we did not observe the positive effect of nematode grazing on shoot N accumulation of NM plants. Huhta et al. (1998) concluded that the effect of soil fauna on plant growth is not always positive and the outcome of an interaction may depend on biotic and abiotic conditions such as food-web structure and resource availability (Bronstein, 1994). This suggests that enhanced shoot N accumulation by bacterial grazing may depend on other environmental factors, such as P availability.

All M plants were also able to take up supplementary N during the course of experiment. However, the pattern of N allocation between roots and shoots was strongly dependent upon inoculation treatments as non inoculated M plants allocated new N entirely to the shoots whereas M plants from +bac and +bac+nem allocated almost no N to their shoots.

Calculation of net N accumulation showed that it was at maximum 2 mg N $plant^{-1}$ in NM plants with bacteria and nematodes, amounting roughly to half of the total N supplied into the medium either as nitrate (1.96 mg $plant^{-1}$) and bacterial N (1.86 mg $plant^{-1}$). Measurements of ^{15}N in roots and shoots showed that N accumulated by all plants was almost unlabeled,

indicating that it originated from nitrate supplied in the medium. This assumption is corroborated by the decrease of nitrate concentrations measured in the solution extracted from the solid medium at the end of the experiment. In addition, the highest nitrate concentrations were measured in bulk medium sampled in NM control plants presenting a small root system and accumulating low amounts of N compared to NM plants with bacteria and nematodes. These data suggest that the diffusion of nitrate could have been limited in the solid medium and that root nitrate uptake depended on root exploration of the medium. These observations suggest also that nitrate was taken up directly by the roots of plants, as previously demonstrated in P. pinaster (Plassard et al., 2002). Although the net N accumulation in M plants was not significantly different from that occurring in NM plants, it tended to be lower when plants are inoculated with bacteria alone or with nematodes. This decrease may be due to N immobilization in fungal hyphae as shown previously with this fungal species grown with nitrate (Plassard et al., 2000).

5.3. P accumulation

In this experiment, the exclusive source of P was contained in the bacteria. Our results showed that the plants were not able to accumulate supplementary P during the culture duration, whatever the plant status and the inoculation treatment. These results indicate that the bacterial P remained locked in the bacteria. This suggests also that the positive effect of nematode grazing upon P accumulation in shoots observed previously (Irshad et al., 2011) originated from the mineral P supplied in the medium. However, despite the restricted P availability, P. pinaster seedlings were able to accumulate net biomass during the experiment. These results are in agreement with the high plasticity of maritime pine reported by Ali et al. (2009). This tree species was shown to be able to produce biomass even when grown with very low P availability by diluting the initial P contents.

5.4. Populations of bacteria, nematode grazers and ^{15}N and P mineralization

Direct count of bacterial cells showed that bacterial populations significantly increased during the experiment, indicating that they were able to multiply. However, this increase (of 6 in average) was very low compared to that obtained in presence of a P source (of 45 in average) (see Chapter 5) indicating that the low P availability had hampered bacterial multiplication,

despite a high N availability. This result is in agreement with previous studies showing that bacterial populations in ecosystems are very sensitive to phosphorus limitation (Sundareshwar et al., 2003). In our experiment, the only source of P was that contained in the medium (measured at 30 µg plant^{-1}), suggesting that the bacteria may have used this P source to multiply.

On the other hand, nematodes populations were also significantly increased during the experiment, independently of the plant status. Such an increase of bacterial-feeding nematodes was observed in pea plants grown in P deficient compared to P enriched conditions (Techau et al., 2004). However, the biomass of nematodes was very low (amounting to 28 µg plant^{-1} for 1800 nematodes plant^{-1} on average) compared to that obtained with high P availability (amounting to 300 µg plant^{-1} for 4000 nematodes plant^{-1} on average, see chapter 5), indicating that the nematodes were also limited in their growth. This could be due to low food availability represented by bacterial populations as described by Aescht and Foissner (1992) and Ettema and Bongers (1993) that low microbial biomasses lead to less nematodes abundance.

Compared to our previous results where NM *P. pinaster* shoots accumulated 15 µg of ^{15}N from bacterial biomass mineralization in presence of 198 nematodes (Irshad et al., 2011), the increase of nematodes populations to 2280 individuals did not even maintain the same level of ^{15}N mineralization. However, using the rate of 0.058 µg N mineralized per nematode (Ferris et al., 1988), and assuming an average number of nematodes of 1100 (NM plants) and 677 (M plants), the overall mineralization from N-labelled bacterial biomass should have been of 287 and 176 µg ^{15}N per plant in NM and M treatments, respectively. These values not measured in the plants, whether mycorrhizal or not. This could be explained by two main hypotheses. The first one is that nematodes are able to mineralize bacterial ^{15}N at the rate mentioned by Ferris et al. (1998) but mineralized N remained locked in microbial populations. The second one is that the actual rate of mineralization is much lower than this value due to a low turn-over rate of bacterial populations limited by P availability in the medium. This hypothesis are in agreement with the findings of Bouwman et al. (1994) as he found the higher bacterial production in the +P treatments with that of large numbers of bacterial feeding nematodes, especially Rhabditidae.

Further this low P availability prevented the plants to benefit from bacterial P, either without or with bacterial feeding nematodes. The presence of the mycorrhizal fungus did not change this situation. These results suggest that the grazing activity of nematodes that have occurred upon the bacterial populations was not sufficient to produce a net release of P from bacterial biomass available to plants. This indicates also the importance of a P source for the action of microbial loop, whether or not directly available to plants.

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CHAPTER 5

PHOSPHORUS ACQUISITION FROM PHYTATE DEPENDS ON EFFICIENT BACTERIAL GRAZING, IRRESPECTIVE OF THE MYCORRHIZAL STATUS OF *PINUS PINASTER*

We observed (chapter 4) that P plays a key role in microbial food web nutrient flow. The results suggest that in the absence of external P source nematodes grazers are not able to exert a positive influence on plant growth. Similarly, grazing activity did not show any P liberated from bacterial biomass phosphorus. Further no significant role of plant-ectomycorrhizae interface was found when linked with bacteria and their grazer nematodes in absence of P source.

Up to now, plants grown in axenic conditions have been shown to exhibit a poor capacity to use phytate as the sole source of P (Hayes et al., 2000; Richardson et al., 2000; 2001a, b) suggesting a low capacity to release phytase into the external medium. This low capacity seems to be shared by the ectomycorrhizal species as data obtained so far (Mousain et al., 1988, Louche et al., 2010) indicated that phytase from these symbiotic fungi represent only a small fraction of their total phosphatase activity. In contrast, some bacteria such as *Bacillus subtilis* are known to secrete phytase enzyme (Mullaney and Ullah, 2003; 2007). We hypothesized that the interactions between phytate mineralizing bacteria and mycorrhizal fungi together with bacterial grazer nematodes could be able to improve plant P use from phytate. We tested this hypothesis by supplying phytate as the sole source of P in the solid medium used to grow *P. pinaster* seedlings, whether or not ectomycorrhizal, alone or with B. subtilis and their nematode grazers represented by two families (Rhabditidae and Cephalobidae) differing by their multiplication rates.

The objectives of this chapter were to evaluate:

- ✓ The role of bacterial capacity to mobilize and to make phytate P available to plants.
- ✓ The measurements of differential effects of grazing by two nematodes families on same bacteria and provision of Pi to plants by bacterial turnover.

- ✓ Mycorrhization degree of plant roots and their contribution in facilitating plant for Pi absorption.
- ✓ Population abundance of bacteria and nematodes in presence and absence of carbon source.

We used the same experimental set-up as previously. Phytate was supplied at 1 mM and plants were grown for two months.

The results obtained in this study are presented as a manuscript entitled "*Phosphorus acquisition from phytate depends on efficient bacterial grazing, irrespective of the mycorrhizal status of Pinus pinaster*" prepared for submission to Plant and Soil.

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CHAPTER 5. PHOSPHORUS ACQUISITION FROM PHYTATE DEPENDS ON EFFICIENT BACTERIAL GRAZING, IRRESPECTIVE OF THE MYCORRHIZAL STATUS OF *PINUS PINASTER*

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1. Abstract

Phosphorus from phytate, although constituting the main proportion of organic soil P, is unavailable to plants. Despite the well-known effects of rhizosphere trophic relationships on N mineralization, no work has been done yet on P mineralization. We hypothesized that the interactions between phytate-mineralizing bacteria, mycorrhizal fungi and bacterial grazer nematodes are able to improve plant P use from phytate. We tested this hypothesis by growing *Pinus pinaster* seedlings in agar containing phytate as P source. The plants, whether or not ectomycorrhizal with the basidiomycete *Hebeloma cylindrosporum*, were grown alone or with a phytase-producing bacteria *Bacillus subtilis* and two bacterial-feeder nematodes, *Rhabditis* sp. and *Acrobeloides* sp. The bacteria and the nematodes were isolated from ectomycorrhizal roots and soil from *P. pinaster* plantations. Only the grazing of bacteria by nematodes enhanced plant P accumulation. Although plants increased the density of phytase-producing bacteria, these bacteria alone did not improve plant P nutrition. The seedlings, whether ectomycorrhizal or not, displayed a low capacity to use P from phytate. In this experiment, the bacteria locked up the phosphorus, which was delivered to plant only by bacterial grazers like nematodes. Our results open an alternative route for better utilization of poorly available organic P by plants.

Key-words: phytase, ectomycorrhiza, root architecture, *Bacillus subtilis*, *Hebeloma cylindrosporum*, Rhabditidae, Cephalobidae.

2. Introduction

Phosphorus (P) is of vital importance for plant production due to the plant metabolic requirements for growth and replication. However, P is taken up by plants and microorganisms only as orthophosphate (Pi), represented by the anion $H_2PO_4^-$ and HPO_4^{2-}. Due to its strong capacity to make complexes with cations and to its low mobility, concentrations of free Pi in the soil solution are generally low and estimated between 1 to 10 µM (Hinsinger 2001). Therefore, P limits the productivity of plants in many terrestrial ecosystems and is often the first or second element limiting aboveground net primary productivity of forests (Plassard and Dell 2010).

Remarkably, besides the low level of available free Pi, soils contain a high amount of P that is linked to C-containing compounds to form organic P (Po). Typically, Po contributes from 30 – 65%, sometimes >90%, of the total soil P (Harrison 1987) and is present under a wide range of compounds, including mono- and di-phosphate esters. Among these Po compounds, *myo*-inositol hexakisphosphate (phytate) holds a particular place as it has been found to accumulate in soil (Turner et al. 2002; Turner 2007) indicating that it may be difficult to degrade either by roots and soil organisms. This form of phosphorus is found in many ecosystems, including forest ecosystems (Turner et al. 2002; Turner, 2007). Finding strategies to increase the use of P contained in phytate by plants is of particular importance because this Po form, making up 65-75% of P in the seeds of cereals or legumes (Raboy 2007), is also one of the primary P species of manure produced by monogastric animals (Peperzak et al. 1959; Barnett 1994; Turner and Leytem 2004). Such a strategy would increase the recycling of P to enhance or maintain plant production while decreasing the use of mineral P of which high grade phosphate rocks are definitely expected to be exhausted within decades from now on; (Cordell et al. 2009).

To be used by plants and microorganisms, phosphate groups of phytate must be released by phytases. Based on their structural differences and varied catalytic properties, four classes of phytases have been proposed. These include (1) members of histidine acid phytases (HAP) , found in fungi, plants and bacteria (2) ß-Propeller phytase (BPP) found in bacteria, especially in *Bacillus* sp., (3) cysteine phytase (CPhy) found in bacteria from anaerobic environments and (4) purple acid phytase (PAP) found in plants (Mullaney and Ullah 2003; 2007). The ability to mobilise phytate in soil solution will rely on the capacity of organisms to produce the enzymes into the external medium or at least in the cell wall space. Up to now, plants

grown in axenic conditions have been shown to exhibit a poor capacity to use phytate as the sole source of P (Hayes et al. 2000; Richardson et al. 2000; 2001a; Richardson et al. 2001b) suggesting a low capacity to release phytase into the external medium. This low capacity seems shared by the ectomycorrhizal species as data obtained so far (Mousain et al. 1988; Louche et al. 2010) indicated that phytase from these symbiotic fungi represent only a small fraction of their total phosphatase activity.

The first strategy used to enhance plant access to P from phytate was gene technology through plant transformation with phytase genes of fungal (*Aspergillus niger* - Richardson et al. 2001a) or bacterial (*Bacillus subtilis* B-propeller - Yip et al. 2003) origin. Transgenic plants were able to produce phytase activity in their external medium and to take up significant amounts of P from phytate. However, this strategy is not always sucessful when plants are grown in soil (George et al. 2005; Richardson et al. 2007). In addition, it is only applicable to plants easily transformable and could thus be limited to these species.

Another strategy that has not been studied yet would rely on the exploitation of the interactions between plants, mycorrhizal fungi, bacteria and their grazers in the rhizosphere – the food web relationships- combined with the bacterial ability to degrade phytate. Plant rhizosphere was shown to contain bacteria able to mineralize phytate *in vitro* (Jorquera et al. 2008a, b) and plant inoculation with bacteria able to degrade phytate in pure culture was shown to increase plant access to P from myo-inositol hexakisphosphate (Richardson and Hadobas 1997; Hayes et al. 2000; Richardson et al. 2001b). However, this positive effect of plant inoculation by phytate-using bacteria could be strongly increased by the presence of bacterial grazers, through the microbial loop in which plant exudates stimulate bacterial growth that will in turn liberate nutrients through their grazing by bacterial grazers. In addition to the bacteria, mycorrhizal fungi could also exert a positive role by enhancing the soluble mineral P uptake by the host plant from the supplementary P released into the medium (Plassard and Dell 2010; Smith et al. 2011).

Of the bacterial grazers, protozoa and nematodes have been shown to play a major role releasing nutrients sequestered in the bacterial biomass in the rhizosphere, especially regarding N cycling (Bonkowski, Villenave and Griffiths 2009; Villenave et al. 2004). However the contribution of soil fauna to the release of P from the microbial biomass has not yet been determined (Marschner et al. 2011). Elucidation of the possible role of the food web on P cycling from recalcitrant organic P sources such as phytate through the activity

bacterivorous nematodes appears highly relevant as these organisms are a major component of the food web in the soil, especially in forest situations. For example, of the whole nematode population extracted from a mixed deciduous forest, 20–50% of nematodes were bacterial-feeders (Bernard 1992). Furthermore, recent studies showed that the proportion of bacterial- over fungal-feeder nematodes increased with root densities of two perennial species, tending to values ranging from 0.7 (*Pinus nigra*) to 0.9 (*Kunzea ericoides*) (Dickie et al. 2011), underlying the importance of the bacterial compartment in these conditions. Despite this ecological significance, the impact of predation on P cycle at the rhizosphere level has almost not been studied (Ingham et al. 2005).

In this work, we hypothesized that the interactions between phytate mineralizing bacteria and mycorrhizal fungi together with bacterial grazer nematodes are able to improve plant P use from phytate. We tested this hypothesis by growing *Pinus pinaster* seedlings in agar containing phytate as P source. Plants, whether or not associated with the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*, were grown alone or with a phytase-producing bacteria *Bacillus subtilis* and two bacterial grazer nematodes belonging to *Rhabditis* sp. and *Acrobeloides* sp. The bacteria and the nematode were isolated from ectomycorrhizal roots and soil from *P. pinaster* plantations.

3. Material and Methods

3.1. Fungal and plant material

A dikaryotic strain (D2) of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* Romagnesi, resulting from the plasmogamy of the two compatible homokaryotic strains (h1 and h7) (Debaud and Gay 1987) was used. Mycelia were grown in the dark, at 24°C in standard nitrate (N6) medium, either on agar-solidified-medium or in liquid culture without shaking (Louche et al. 2010). Seedlings of maritime pine (*Pinus pinaster* Soland in Ait. from Medoc, Landes-Sore-VG source, France) were obtained from germination of seeds carried out in sterile conditions (Irshad et al. 2011). Mycorrhizal synthesis was performed in test tubes (Casarin et al. 2004) and plants, whether inoculated or not, were placed in a growth chamber under the same conditions as described in Ali et al. (2009) for two months before the co-inoculation experiment.

3.2. Bacterial strain and nematodes

The bacterial strain was isolated from pine roots collected from a 15-year old *P. pinaster* stand in the Landes Region, near Bordeaux, and was identified as *Bacillus subtilis* from DNA sequencing (Irshad et al. 2011). The bacteria were grown in Luria Broth for stock solutions and in a synthetic medium for inoculation of plants. The synthetic medium contained macronutrients (1 mM KNO_3, 11 mM KCl, 2 mM $MgSO_4.7H_2O$, 5 mM $CaCl_2$, 0.2 mM KH_2PO_4, 4 mM $CaSO_4$), 55 mM glucose and 50 mM MOPS (3-[N-morpholino]propane-sulfonic acid, Sigma ref M-1254) to maintain a pH close to 7. After autoclaving (115°C, 40 min), this medium was complemented with micronutrient (4 ml l^{-1}) and vitamin (1 ml l^{-1}) solutions given in Tatry et al. (2009, Supplementary material). Bacteria were grown in shaken conditions (150 rpm) at 28°C for 40 h. At maximum optical density (600 nm), the cultures were centrifuged (5000 g, 10 min) and the pellet was washed twice in sterile deionized water to eliminate any remaining P. The bacteria were then re-suspended in 30 ml of sterile deionized water before use. Cell density of this solution was determined using a dilution 1/1000 deposited on a hemocytometer (0.2mm depth). Calculations of bacterial density were performed using the link http://www.changbioscience.com/cell/hemo.html. Unless differently stated, the initial bacterial density of the solution used to inoculate plates was $1.3x10^{-7}$ cells ml^{-1}.

Nematodes were isolated from the soil of the same 15-year old stand of *P. pinaster* using the Cobb sieving method (s'Jacob & van Bezooijen 1986). Bacterial-feeding nematodes were selected by growing them on *B. subtilis* cultures added to solid medium (TSA) containing 1% agar, 3 g l^{-1} Tryptic Soy Broth (Fluka ref 22092) supplemented with cholesterol (5 µg l^{-1} of media). Monoxenic populations of nematodes were obtained by sterilizing the eggs of a single gravid female with Na0Cl. Selected nematodes were identified from molecular analysis of 18S rDNA gene as *Rhabditis* sp. (Irshad et al. 2011) and *Acrobeloides* sp. The nematodes were maintained by transferring individuals every month on new TSA plates containing *B. subtilis*. The nematodes multiplied in the dark, at 24°C. They were then removed from the breeding TSA plates by washing the surface with a sterile NaCl solution (1%). They were washed from most *B. subtilis* by centrifugation (1000 rpm, 5 min) and re-suspended in sterile deionized water to a density of 214±12 (*Rhabditis* sp.) and 196±10 (*Acrobeloides* sp.) nematodes ml^{-1} for inoculation in plates.

3.3. Experimental design for co-inoculation

Plants of two status, either non-mycorrhizal (NM) or mycorrhizal (M) with *H. cylindrosporum*, were then set up with three inoculation treatments: (1) no inoculation (none), (2) inoculation with *B. subtilis* (+bac), and (3) inoculation with *B. subtilis* and *Rhabtidis* sp. (+bac+nem1) or *Acrobeloides* sp. (+bac+nem2). The experiment was carried out in square Petri dishes (12 x 12 cm) in sterile conditions with a hole to allow the plant shoots to develop outside the dish. Each Petri dish was filled with 70 ml of solid nutrient medium containing 1.0 % agarose (Eurobio Molecular Biology Grade), 2 mM $MgSO_4.7H_2O$, 4 mM $CaSO_4$, 50 µg l^{-1} thiamine hydrochloride, 0.5 ml l^{-1} 1% Fe citrate, 50mM MOPS, 0.2 ml l^{-1} Morizet & Mingeau solution of micronutrients (1976), 2 mM KNO_3 as the sole source of N, 1mM phytate (inositol hexakisphosphate, sodium salt, Sigma, ref P0109) and cholesterol (5 µg l^{-1}). The medium without phytate and cholesterol was autoclaved (115°C, 40 min) and cooled to 55°C before adding filtered (0.2 µm pore size) sterilized solution of phytate and cholesterol. Before the experiment, agarose and phytate were checked for their free Pi contents that were 43.4 µg P g^{-1} dry wt of agarose and 182.1 µg P $mmol^{-1}$ of phytate. Taken together, the solid medium contained 43 µg free Pi $plate^{-1}$. The medium was then poured at an angle of 10° to facilitate root placement in the opposite direction of the hole. After solidification, plates from both NM and M treatments, (+bac) as well as (+bac+nem) treatment, received 1 ml of bacterial suspension (equal to 1.3 ± 0.1 $\times 10^7$ cells $plant^{-1}$). On top of that, plates from treatment (+bac+nem) received 0.5 ml of nematode suspension (equal to 107±6 and 98±5 nematodes $plant^{-1}$ for *Rhabditis* sp. and *Acrobeloides* sp., respectively). Both of these inoculums contributed a negligible amount of free Pi (1.53µg/$plant^{-1}$). Each plate from treatments without bacteria and nematodes (none) received 1.5ml sterile deionized water to give the same level of moisture to all plants. Each solution was spread on the medium surface before laying the root system of pine seedlings. Each Petri dish contained only one seedling. Finally, the plates were sealed with a sticky tape to protect the plant from contamination. The plants were placed in the growth chamber (see above for the conditions). All the plates were placed horizontally and covered with aluminum foil to minimize root system exposure to light in the growth chamber. To maintain the moisture level, the plants were watered every week by adding a volume of sterile deionized water corresponding to their weight loss. The plants were allowed to grow for two months.

3.4. Use of phytate by the fungus, the bacteria and the nematodes without plant

To check their capacity to use phytate as a source of P, the fungus and the bacteria were grown in pure culture. *H. cylindrosporum* was grown in a flask containing 40 ml of liquid medium of the same composition as the co-inoculation medium supplemented with 55 mM glucose. Phosphorus was added either as KH_2PO_4 (1mM) or phytate (1mM). The fungus was grown for 24 days in the dark, as described above. The cultures of *B. subtilis* were also carried out in liquid medium of the same composition as the co-inoculation medium supplemented with 55 mM glucose and with P either as KH_2PO_4 (1 mM) or sodium phytate (6 mM). The bacteria were grown at 28°C, with shaking (150 rpm) for 120h.

We also checked the capacity of the bacteria and their grazers (*Rhabditis* sp.) to develop in the co inoculation medium, supplied in the same conditions as for plant culture, with no additional C source added to the solid medium. Square Petri dishes containing 70 ml of solid medium with 1 mM phytate were inoculated with bacteria ($1.3 \pm 0.1 \times 10^6$ bacteria plate^{-1}) only or with bacteria and nematodes (107 ± 6 individuals plate^{-1}), as described previously for plants. The plates were sealed with sticky tape and placed in the growth chamber and protected from light. They were used to measure the bacterial and nematode populations after every two weeks until 60 days of incubation (n=3 plates/time/treatment).

3.5. Plant analysis

Root parameters (length, surface area, number of tips and forks) were measured on cleaned root systems scanned with the software WinRHIZO (http://www.regentinstruments.com/products/rhizo/Rhizo.html). Shoots (needles plus stem) and roots were freeze-dried for dry biomass determination and were then milled before carrying out P determination and chitin assay. Total P contents were determined after mineralization of tissues with H_2SO_4 36N as described in Aquino & Plassard (2004). Free orthophosphate concentration was assayed in mineralized solution according to Ohno and Zibilske (1991) with malachite green. All these analyses were carried out first on 2-month old NM and M plants (data given in Table S1) used to set up the inoculation experiment and at the end of the experiment. Chitin assay was performed at the end of the experiment on 10 mg of roots heated in 1 ml of 6N HCl according to the method described in Vignon et al. (1986).

3.6. Bacterial and nematode populations

The solid medium of each Petri dish was first cut into pieces and transferred to pots with 10 ml of deionized sterile water in sterile conditions. Each pot was shaken by hand for 5 min and aliquot of solution (1 ml) was taken after 2 h. This solution was used to numerate bacterial cells with a hemocytometer after adequate dilution (at least 1/10000). The remaining solution and solid medium were then transferred on a filter paper for 48 h to collect the population of living nematodes that moved through the filter into the water (50 ml) beneath. The nematodes were further concentrated by gravitation in a fraction of 5 ml. They were counted immediately by using a stereo-microscope to determine the final density and then fixed to determine their biomass. Fixation was carried out by mixing fixation solution (4% formaldehyde) heated at 70 °C with the solution containing the nematodes (10/1, v/v). Slides were then made and observed under microscope. The dimensions (width and length) of nematodes were recorded using the software cell B ((http://www.microscopy.olympus.eu/microscopes/Software_cell_B.htm). The body weight (µg) of each individual was calculated using a formula derived by Andrassy (Andrassy 1956 in Zuckerman, Brzeski and Deubert 1967):

Body weight of nematodes (µg) = a^2 x b / 1600000

where a is the greatest body width and b the length in micrometers.

3.7. Statistical analysis

Unless otherwise stated, the results are given as mean ± standard deviation (n= 6 at T0 and 7 at the end of experiment). The differences between means were analyzed by factorial ANOVA followed by Tukey's HSD post-hoc test using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Kolmogorov Smirnov test and, where necessary, the data was either square root or log10 transformed prior to analysis to meet the assumptions of ANOVA. The comparison of means between NM and M samples at the beginning and the end of experiment was carried out using the Student's t test.

4. Results

4.1. Use of phytate by microbial partners in pure culture

The growth and total P accumulation measured in *H. cylindrosporum* thalli displayed huge differences when grown for 24 days in the same liquid medium containing either soluble orthophosphate (Pi) or phytate as the sole source of P (Table S2). Biomass production, total P

contents and accumulation in Pi-grown mycelia were 5, 1.5 and 8 times higher than in phytate-grown mycelia, respectively (Table S2). In addition, the amounts of P accumulated in phytate-grown mycelia (on average 33 µg total P per thallus) were of the same order of magnitude as the contamination brought by phytate salt (7 µg per flask) and agar plug (23 µg per flask). Taken together, these results indicated that the fungus displayed a poor ability to use phytate as a P source. The bacteria *B. subtilis* were able to grow whatever the P source supplied into the medium (Fig. S1). Although phytate-grown bacteria grew 33% less than Pi-grown bacteria, they were able to increase external [Pi] in the medium in addition to their own growth (Fig. S1b). These results confirmed the phytate-mineralizing capacity of this bacterial strain.

As shown in Table 1, bacterial populations increased by a factor of around 6 after only 15 days of incubation. They remained stable over the last 45 days, with or without the bacterial-feeding nematodes *Rhabditis* sp., although the presence of nematodes significantly increased the number of bacterial populations after 60 days of incubation. Similarly to that of bacteria, the population of active nematodes dramatically increased by a factor of 10 during the first 15 days of incubation. After this peak of growth, nematode abundance did not change significantly with incubation time (Table 1).

Table 1. Evolution of populations of phytate mineralizing bacteria (*Bacillus subtilis*) and their nematode grazer (*Rhabditis* sp.) in solid medium grown in sterile conditions containing phytate as the sole source of P and C. The initial populations were 1.3x 10^6±0.1 bacteria and 107±7 nematodes per plate.

	Days	15	30	45	60	*P*-value
Bacterial cells (x10^6 plate^{-1})	none	ND	ND	ND	ND	
	+bac	6.3±0.6a^A	6.0±0.3b^A	6.4±0.1a^A	6.4±0.3b^A	0.59
	+bac+nem	6.7±0.4a^A	6.9±0.3a^A	6.9±0.9a^A	7.5±0.1a^A	0.39
	P-value	0.36	0.01	0.31	0.00	
Nematodes (x 10^3 plate^{-1})	+bac+nem	1.03±0.21^A	0.82±0.18^A	0.87±0.44^A	0.92±0.33^A	0.85

Data are means ± standard deviation (n=3). Different small letters denote significant effect of inoculation treatments and capital letters denote significant effect of time given by one way ANOVA followed by the Tukey's minimum significant difference test.

4.2. Effect of food web complexity on root development, plant growth and mineral nutrition

Whatever the treatment, the plants displayed a significant root development as all values of root parameters (length, surface area, number of tips and forks) measured in plants sampled at the time of transfer to the phytate-containing medium were significantly lower than those measured at the end of experiment (Table 2). The values of root parameters recorded at the end of experiment were first analyzed using two-way ANOVA showing that there was no interaction between plant status and inoculation treatments on all parameters measured except the numbers of root tips (Table 2). Generally speaking, plant status induced a significant effect on root growth, as all parameters measured in NM plants were higher than those measured in M plants (Table 2). Conversely, inoculation treatments induced a significant effect on root parameters due to a positive effect of nematodes on root extension assessed by length, surface area and ramification degree given by tip and fork numbers. More precisely, in non-mycorrhizal plants, nematode activity induced a significant increase of root length (+30% and +34%, for *Rhabditis* sp. and *Acrobeloides* sp., respectively) and root surface area (+54% for *Rhabditis* sp. only) compared to the average value of sterile and + bacteria plants (Table 2). However, nematode activity induced a significant increase of root ramification compared only to the + bacteria treatment as the numbers of tips and forks were increased respectively by 31% - 27% (*Rhabditis* sp.) and by 46% - 36% (*Acrobeloides* sp.). In contrast, inoculation treatments did not modify the root parameters measured in M plants, except the number of tips that was significantly enhanced by +59% (*Rhabditis* sp.) and +84% (*Acrobeloides* sp.) by nematodes compared to sterile M plants.

In agreement with root development, root dry matter was increased in all plants during the culture on phytate-containing medium compared to the initial root dry weight of plants (Fig. 1a). In contrast, shoot dry biomass was not significantly increased in all treatments, as sterile NM or M plants, and M plants with bacteria did not produce shoot growth. Only the presence of *Rhabditis* sp. induced a high production of shoot biomass in NM and in M plants (Fig. 1a). Statistical analysis showed that the plant status did not modify root or shoot dry weights, contrary to the inoculation treatments which induced strong changes in root and shoot growth (Table 3, Fig. 1a). Indeed, the plants, whether or not mycorrhizal, accumulated the same amounts of root and shoot dry biomass when grown in sterile conditions or with bacteria only. Compared to the averaged amounts of biomass calculated in the plants grown in sterile

conditions or with bacteria alone, only the presence of *Rhabditis* sp. induced a strong increase of both roots (+44 %) and shoots (+50 %) in NM plants and only a significant biomass increase (+38%) of shoots in M plants. (Fig. 1a).

Total P accumulation was significantly increased in all plants grown in phytate- containing medium compared to the P amounts accumulated in plants at the time of transfer except in the M plants inoculated with bacteria (Fig. 1b). As was the case for biomass accumulation, the mycorrhizal status did not modify significantly the P accumulation in roots and shoots, contrary to the inoculation treatments (Table 3, Fig. 1b). This was due to the dramatic effect of both the nematode species as the amounts of P accumulated in roots or shoots of the plants grown without nematodes (none and +bac) were not significantly different (Fig 1b). Compared to the averaged P amounts calculated in plants grown in sterile conditions and with bacteria only, the nematodes strongly increased P accumulation in both NM and M plants. On average, the increase was +174% (roots) and +176% (shoots) in NM plants and +205% (roots) and +147% (shoots) in M plants (Fig. 1b).

Plotting the individual values of total plant P accumulation against the root surface area (Fig. 2a) showed that plant P accumulation in NM plants, whether grown in control conditions (treatment none) or inoculated with bacteria only (treatment +bac), increased linearly with the root surface (r^2= 0.27, p=0.05). The presence of the fungus did not change this relationship in absence of nematodes. In contrast, the values measured in NM and M plants grown in the presence of either nematode species did not fit in with the relationship established in the absence of nematodes. An increase of root surface went with a proportional increase of accumulated P in NM plants whereas M plants exhibited a more variable and stronger increase of P accumulation relative to their root surface (Fig. 2a). As shown in figure 2b, the mean values of the ratio of P accumulation per unit of root surface calculated in NM and M plants without nematodes were not significantly different, indicating that the fungus alone was not able to enhance P uptake from the medium. However, the two species of nematodes differently modified the ratio of P accumulation per unit of root surface. In NM plants, only plants with *Acrobeloides* sp. displayed a significant increase of this ratio, plants with *Rhabditis* sp. were intermediate compared to plants without nematodes. In contrast, with M plants, despite a high variability of the data, the two species of nematodes increased significantly the average values of accumulated P per unit of root surface area compared to plants without nematodes, with the highest average calculated in plants grown in the presence

of *Acrobeloides* sp. As shown in figure 2c, M plants exhibited significant higher amounts of glucosamine in their roots, due to the fungal capacity to colonize the root system. M plants from the two nematode treatments presented the highest values of glucosamine contents together with the largest variability of individual data compared to plants from the other treatments. Taken together, the higher variability of plant P accumulation observed in M plants than in NM plants in presence of nematodes (Fig. 2a), suggests that the roots may not be solely responsible for P uptake and that the mycelium could play a significant role.

Table 2. Root growth parameters (length, surface area, number of tips and forks) measured in *P. pinaster* plants of different status, either non-mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*. Two-month old plants were supplied with phytate in solid medium for 60 d and received different inoculation treatments that were either no addition (none), or addition of a phytate-mineralizing bacteria only, *Bacillus subtilis* (+bac) or of the bacteria with the bacteria-feeding nematodes, *Rhabditis* sp.(+bac+nem1)or *Acrobeloides* sp. (+bac+nem2).

Plant status	Inoculation treatments	Length (cm plant^{-1})	Surface area (cm^2 plant^{-1})	Tips (number plant^{-1})	Forks
NM	none	588abc	135b	2559bc	5554ab
	+bac	527bc	136b	2363cd	4930b
	+bac+nem1	722a	211a	3099ab	7074a
	+bac+nem2	745a	180ab	3452a	6723a
M	none	448b	128b	1869d	3895b
	+bac	481bc	127b	2546bc	4718b
	+bac+nem1	577abc	152b	3009ab	5318b
	+bac+nem2	635ab	147b	3448a	5446ab
One-way ANOVA *P*-value		0.000	0.000	0.000	0.000
Two-way ANOVA) Variation source		*P*-values			
Plant status		0.000	0.000	0.181	0.000
Inoc. treatments		0.000	0.000	0.000	0.000
Interaction		0.568	0.160	0.028	0.210

Data are means (n=7) and were compared using one-way ANOVA followed by the Tukey's minimum significant difference test. For each parameter, means significantly different at $P < 0.05$ have different letters. A two-way ANOVA was also performed to assess the individual and combined effects of plant status and inoculation treatments using the Tukey's minimum significant difference test. All the parameters differed significantly from the means measured in plants sampled before the experiment and given in Table S1 (comparison of means, Student's t test).

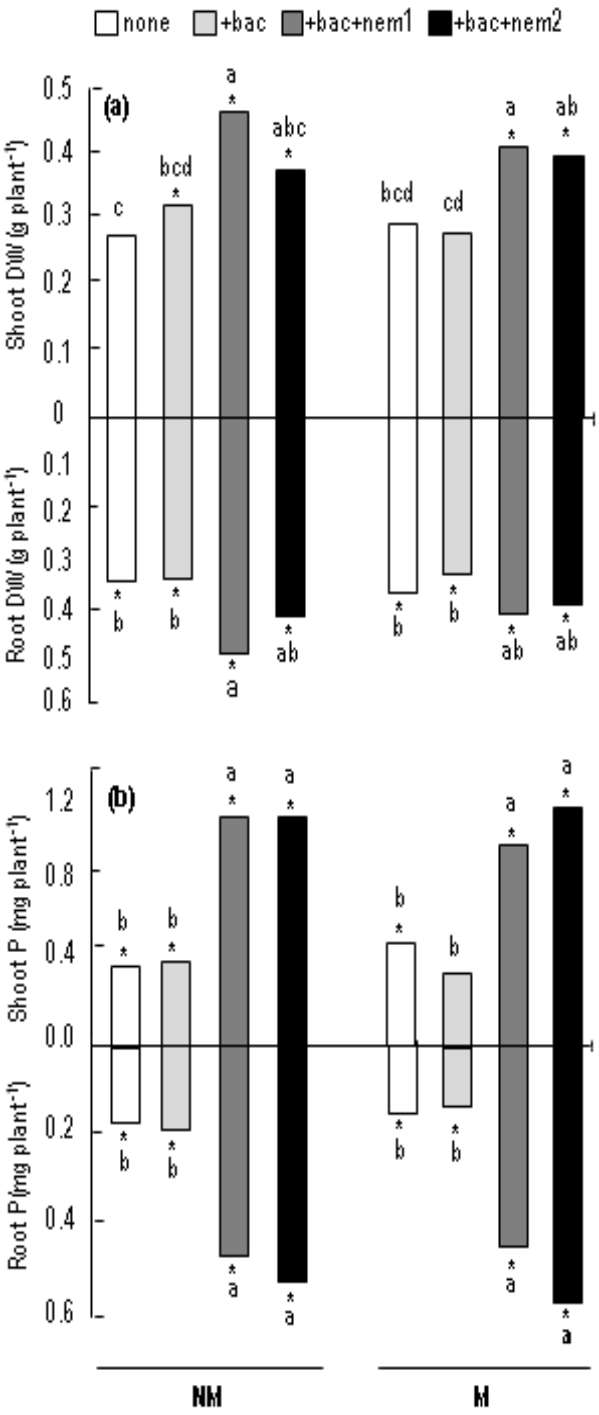

Figure 1. Total accumulation of dry biomass (a), and (b) P in roots and shoots of *P. pinaster* plants of different status, either non-mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum* and cultivated with different inoculation treatments that were either no addition of organisms (none), or addition of a phytate-mineralizing bacteria *B. subtilis* (+bac) or of the bacteria together with the bacteria-feeding nematodes *Rhabditis* sp. (+bac+nem1) or *Acrobeloides* sp. (+bac+nem2).

Two-month old plants were grown for 60 d with phytate as the sole source of P in solid medium. Bars correspond to means (n=7). Data measured in roots or shoots were compared using one-way ANOVA followed by the Tukey's minimum significant difference test and bars accompanied with different letters are significantly different ($P < 0.05$). Bars accompanied with an asterisk indicate that the mean is significantly different from the initial value measured in two-month old plants ($P < 0.05$) using a Student's t-test of mean comparison. The results of a two-way ANOVA performed to assess the individual and combined effects of plant status and inoculation treatments using the Tukey's minimum significant difference test are also given in roots and shoots.

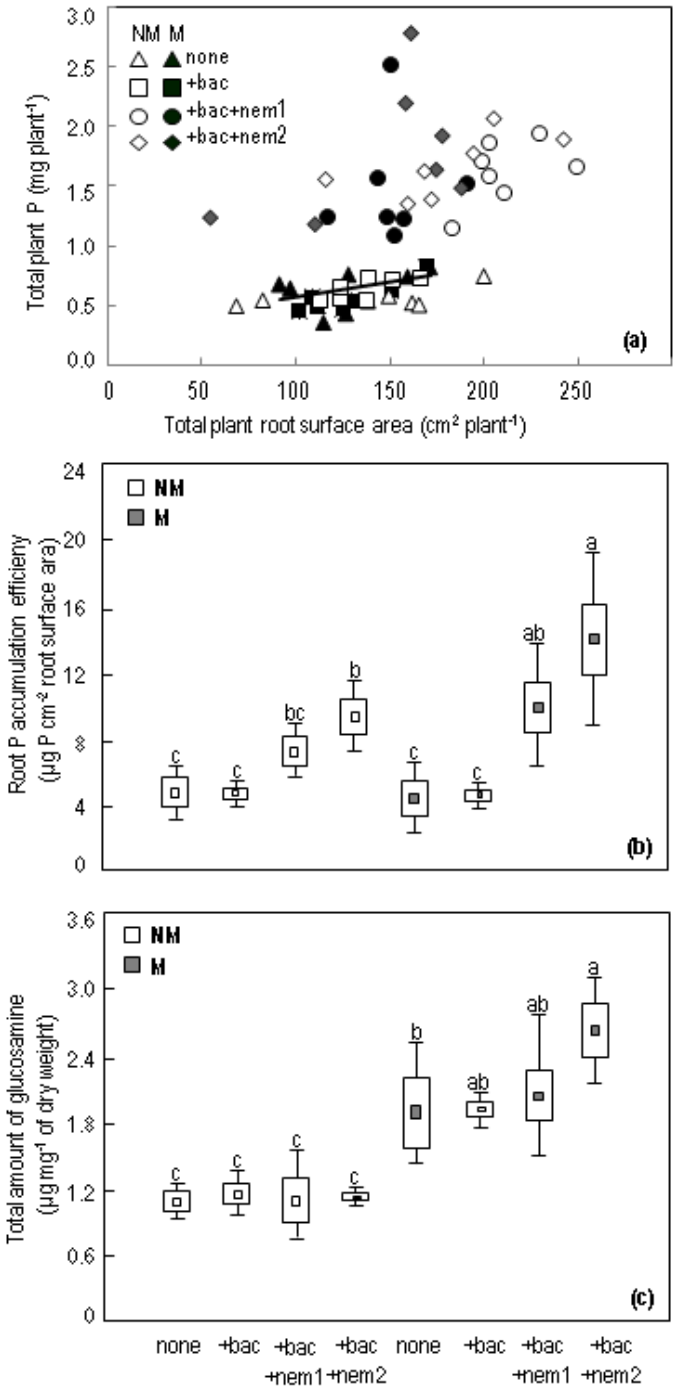

Figure 2. Relationship between (a) total P accumulation and root surface area (b) P absorption efficiency per root surface (cm^2) and (c) glucosamine (µg/ mg^{-1} of dry weight) measured in *P. pinaster* plants of different status, either non-mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*, supplied with phytate in solid medium for 60 d. Two-month old NM or M plants were cultivated with different inoculation treatments that were either no addition (none), or addition of phytate-mineralizing bacteria only (+bac) or together with bacterial feeding nematodes (+bac+nem1 and +bac+nem2).

Table 3. ANOVA table of *P*-values on the effect of plant status (non-mycorrhizal or mycorrhizal with the ectomycorrhizal fungus *H. cylindrosporum*) or inoculation treatments (no addition, addition of a phytate-mineralizing bacteria *Bacillus subtilis* or addition of *B. subtilis* plus the bacteria-feeding nematodes, *Rhabditis* sp. (+bac+nem1) or *Acrobeloides* sp. (+bac+nem2) on plant biomass and mineral nutrient accumulation of *Pinus pinaster* grown for 60 d on solid medium with phytate as the sole source of P (data given in Fig. 1).

	Plant biomass		Total P accumulation	
Variation source	Roots	Shoots	Roots	Shoots
Plant status	0.79	0.53	0.86	0.79
Inoc. treatments	0.000	0.000	0.000	0.001
Interaction	0.30	0.37	0.86	0.74

4.3. Evolution of bacterial and nematode populations in presence of *P. pinaster* seedlings

Compared to the initial amount of bacteria, 1.3±0.1 x10^6 cells, added to each inoculated plant bacterial populations dramatically increased in the rhizosphere of plants after 60 d of co-inoculation by factors ranging from 36 to 54 (Table 4). The NM plants presented the highest level of bacterial populations and the presence of nematodes tended to decrease it. The presence of the fungus together with nematodes significantly decreased bacterial populations by 32 % compared to NM plants with +bac treatments. Compared to their initial population, the nematodes *Rhabditis* sp. increased by a factor of 45 and 27 in NM and M plants respectively. The populations of *Acrobeloides* sp. were twice as high as those of *Rhabditis* sp., and increased by a factor of 91 and 75 in NM and M plants respectively, during the growth period. Although the nematodes *Rhabditis* sp. were more abundant in NM (+66%) than in M plants, their biomass was not significantly different (Table 4). This is due to the higher number of juveniles observed with NM than with M plants.

Table 4. Abundance of phytate-mineralizing bacterial (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis* sp (+bac+nem1) or *Acrobeloides* sp. (+bac+nem2) populations 60 days after inoculation in the rhizosphere of *P. pinaster* plants, non-mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*, supplied with phytate in solid medium. The initial inoculated populations plant $^{-1}$ for bacteria and nematodes were 1.3x 10^6±0.1, 107±6 (*Rhabditis* sp.) and 98±7 (*Acrobeloides* sp.) respectively.

Plant status	Treatments	Bacterial cells ($x10^6$ plant^{-1})	Nematodes ($x\ 10^3$ plant^{-1})	Nematode biomass (µg plant^{-1})
NM	+bac	70±22a		
	+bac+nem1	60±19ab	4.82±0.70b	351±62*
	+bac+nem2	62±18ab	8.91±0.97a	559±74
M	+bac	54±14ab		
	+bac+nem1	48±9b	2.90±0.26c	258±40
	+bac+nem2	47±7b	7.37±0.79a	479±63
	P-values	0.00	0.00	

Data are means ± standard deviation (n=7) and were compared using one-way ANOVA followed by the Tukey's minimum significant difference test. For each parameter, means significantly different at $P < 0.05$ have different letters.
* Biomass of nematodes was calculated from the size of 500 individuals randomly sampled from the whole population.

5. Discussion

5.1. Effect of plant and ectomycorrhizal symbiosis on bacterial and nematode populations

The presence of the plant in the culture system increased bacterial populations in absence of nematodes (compare Tables 1 and 4). This suggests that the growth of bacteria with phytate as the sole source of C and P was limited. The solid medium contained initial low amounts of Pi (assayed at 50 µg per plate) that may have supported the rapid growth of the bacteria observed after 15 d (Table 1). It may also have contained some available C sources that contributed to the initial growth of the bacteria. However, once these available sources were used by the bacteria, the growth stopped, indicating that phytate was not a good source of C for *B. subtilis*. Indeed, the capacity to use phytate as the unique C and P source is limited to 0.5 % of culturable bacteria isolated from soil (Richardson and Hadobas 1997). This unability to use phytate as a C source could be due to the action mode of BPP-phytase (PhyC) secreted by *B.*

subtilis. The enzyme produces both Ins(2,4,6)P3 and Ins(1,3,5)P3 as the major products (Greiner et al. 2002) unusable by bacteria as a C source (Tzvetkov and Liebl 2008). Conversely, when the plant was cultivated with *B. subtilis*, a large number of bacterial cells were observed, especially in absence of the mycorrhizal fungus and nematodes, indicating that the plant was able to supply the C source. The fungus and the nematodes decreased significantly the size of bacterial populations in the medium. Such a negative effect could be due to the competition for available C between the fungus and the bacteria, and/or the grazing pressure exerted by these bacteria-feeding nematodes (Ingham et al. 1985; Fu et al. 2005).

As it did for bacteria, the presence of the plant also strongly increased the size of the nematode populations of *Rabditis* sp., especially when they are non-mycorrhizal, with a high number of juveniles. This reflects the abundant bacterial resource (Aescht and Foissner 1992; Ettema and Bongers 1993). Conversely, the negative effect of the mycorrhizal symbiosis on nematode abundance (-66%) could also derive from the competition for plant C source between the fungus and the bacterial cells, decreasing in turn the resource for nematodes. On the other hand, the populations of *Acrobeloides* sp. were more abundant than those of *Rabditis* sp in NM and M plants. Cephalobidae (such as *Acrobeloides* sp) are "general opportunist" whereas Rhabditidae are "enrichment opportunist" (Bongers 1990). As discussed by Blanc et al. (2006), under enriched conditions such as fertilizer or manure input in soil or breeding on nutrient agar, Rhabditis multiply more than Cephalobidae. However on the long term and in limited resources conditions as in natural soil, Cephalobidae may present higher populations than enrichment opportunists.

5.2. P accumulation and plant growth

Although the total amounts of P accumulated in NM plants grown in sterile conditions or with bacteria alone were higher than the initial ones, they were not significantly different. These results indicated a low capacity of plants to use P from phytate, as claimed by numerous authors (Adams and Pate 1992; Findenegg and Nelemans 1993; Hayes et al. 2000; Richardson et al. 2000, 2001a, b). The presence of phytate mineralizing bacteria did not increase significantly the net amount of P in NM plants. This result demonstrates that the bacteria alone developing in the rhizosphere were not able to provide more P to the plants despite the observed increase of free Pi in liquid culture (Fig. S1). This observation does not agree with previous studies showing that the P nutrition of plants supplied with phytate was improved significantly when the agar medium was inoculated with soil microorganisms or a strain of

Pseudomonas fluorescens with phytase activity (Richardson et al. 2001b). Our experimental conditions favored the growth of bacteria and this may have led to a rapid immobilization of Pi in bacterial biomass remaining unavailable for plants. The presence of *H. cylindrosporum*, whether or not with bacteria, did not modify P availability to plants. This is in agreement with the poor ability of the fungus to use phytate as the sole source of P (Table S1) and its low ability to release phytase in its culture medium (Louche et al. 2010).

The grazing activity of both nematode species upon the phytate-mineralizing bacteria dramatically increased total P accumulation in roots and shoots. The observed P accumulation and growth in shoots of NM plants was comparable to the values obtained when plants were grown in solid medium containing a non limiting P availability supplied as KH_2PO_4 (Irshad, unpublished results). This indicates that the grazing activity upon populations of phytate-mineralizing bacteria could be as efficient as the production of transgenic plants expressing and releasing into the external medium the *Aspergillus* phytase (Richardson et al. 2001a).

However, NM plants with *Acrobeloides* sp. did not significantly accumulate more biomass in roots and shoots than plants grown in sterile conditions or with bacteria alone despite the supplementary amount of P accumulated in plants. These plants presented also the highest level of nematode population that may have drained more carbon exudates than when the plants were grown with the other nematode species. Finally, this high carbon allocation to the food web (Bonkowski et al. 2001) could have resulted in less carbon storage in plants and less growth. The same mechanism could apply in M plants where the presence of *Acrobeloides* sp. did not increase plant biomass compared to control plants despite a higher plant P accumulation. Here, abundance of *Acrobeloides* was not significantly decreased by mycorrhizal association and the level of fungal colonization, estimated from glucosamine concentrations in roots, was the highest. Therefore, this high density of nematodes together with ectomycorrhizal hyphae may also have lead to competition for carbon at the expense of plant growth.

5.3. Effect of food web on root growth and P acquisition

Our results showed that M plants presented values of root length and surface area lower than those measured in NM plants. This agrees with the effect of mycorrhizal symbiosis (Jentschke et al. 1995) and of this fungal species (Aquino and Plassard 2004). However, the presence of nematodes induced always a positive effect on root growth parameters, whatever the status of the plant, mycorrhizal or non-mycorrhizal. These results are not in agreement with the studies

of Jentschke et al. (1995) and Bonkowski et al. (2001) who reported the persistence of the negative effect of ectomycorrhizal symbiosis on root development of Norway spruce seedlings (*Picea abies*) after plant inoculation with either bacteria or bacteria plus protozoa. Our results showed that nematodes exerted a positive influence on root growth and ramification in the presence ectomycorrhizal fungus, suggesting that they could have a different role from protozoa regarding this aspect.

The presence of nematodes also strongly modified the relationship between the root surface area and total P accumulation by simultaneously increasing the surface area and P accumulation, especially in NM plants. The simplest hypothesis to explain this change is that the P availability from phytate to the roots was strongly increased by the grazing activity of nematodes. This effect could arise from several causes. Firstly, this could come from the liberation of free Pi or other sources of organic P accumulated in bacteria and released into the medium by grazing. The free Pi will be directly available whereas the organic P could be easily used by the phosphatases released by roots as shown in wheat and Arabidopsis (Richardson et al. 2000; 2001a). Secondly, enhanced Pi availability by grazing could result from the higher turn-over of bacterial populations in the presence of nematodes than in their absence. Indeed, although the final number of bacteria was lower in plants with nematodes than without nematodes, more abundant bacterial populations have been necessary to support the nematode growth. Therefore, together with their growth, the bacterial populations could have used more phytate resulting in turn in a higher release of free Pi into the medium than with no nematodes present. Also, due to their moving and transporting capacities of bacteria (Chen et al. 2007), the nematodes may have greatly increased the capacity of the bacteria to access phytate throughout the whole solid medium. Finally, plant P could have arisen from nematode life cycling and dead organisms as it was shown that NM *Betula pendula* seedlings were able to accumulate P from nematode necromass (Perez-Moreno and Read 2001). All these effects could have occurred during the experiment, resulting in the observed enhanced P availability to the plants.

The presence of the ectomycorrhizal fungus modified even more strongly the relationship between the root surface area and plant P accumulation by enhancing the root efficiency to take up phosphorus from the medium. All the effects leading to an enhanced P availability by nematode grazing proposed in NM plants may apply in M plants. However, the fungus could add a supplementary effect, which is a better exploration of the medium than plant roots. Such

a positive effect of this fungal species was reported by Aquino and Plassard (2004) in *P. pinaster* plants grown at the surface of a thin layer of soil. It was attributed to a greater medium exploration by the hyphae compared to roots together with a "fungal pathway" of Pi uptake in M plants. This means that although an agar medium was used in our experiment, medium exploration by hyphae in M plants or by roots in NM plants is an important feature to take up Pi, indicating that Pi was probably not freely mobile in the medium.

In conclusion, our data strongly support the possible role played by the microbial loop to enhance P mineralization from organic P and P availability to plants. This positive role is based on the assumption that newly grown bacteria are able to mobilize nutrients that are not easily accessible to plants (such as phytate), which are then made available to plants by bacterial grazers (Clarholm, 2005; Kuikman, Jansen and Van Veen 1991). Although the mechanisms leading to the release of available Pi from the grazing activity of nematodes upon phytate-mineralizing bacteria remain to be elucidated, our data demonstrated for the first time the importance of the microbial loop to enhance P availability from phytate, which is recognized as the most poorly available organic P compounds to plants (Richardson et al. 2000). Our results open a new route to manage P nutrition of plants that needs now further studies at the field level.

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Supplimentary data

Table S1. Root architecture (length, surface area, number of tips and forks) and amounts of total biomass, total N and total P measured in two-month old *P. pinaster* plants of different status, either non-mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum,* at the time of transfer in solid medium containing phytate as the sole source of P. Values are the means (n=6) with standard deviation between brackets.

		Root architecture					
Plant status	Plant parts	Length (cm $plant^{-1}$)	Surface area (cm^2 $plant^{-1}$)	Tips (number $plant^{-1}$)	Forks	Dry weight	Total P
NM	Roots	250 (30)	81 (11)	670 (89)	1627 (329)	152 (28)	0.07 (0.02)
	Shoots					258 (20)	0.23 (0.03)
M	Roots	252 (34)	82 (13)	906 (195)	2102 (314)	172 (15)	0.08 (0.01)
	Shoots					282 (30)	0.31 (0.07)

Table S2. Growth and P accumulation in the ectomycorrhizal fungus *Hebeloma cylindrosporum* grown for 24 days in liquid medium containing either orthophosphate or organic P supplied as KH_2PO_4 1 mM (Pi) or sodium-phytate 1 mM (Phytate). Given values are means (n=7) and different small letters denote significant effect of treatments (one way ANOVA followed by Tukey's test).
The medium contained nitrate (6 mM) as the sole N source and glucose (55 mM) as carbon source.

P source	DW (mg/thallus)	Total P concentration (mg/g DW)	Total P accumulation (mg/thallus)
Pi	50a	5.3a	0.269a
Phytate	10b	3.4b	0.033b
P-values	0.00	0.01	0.00

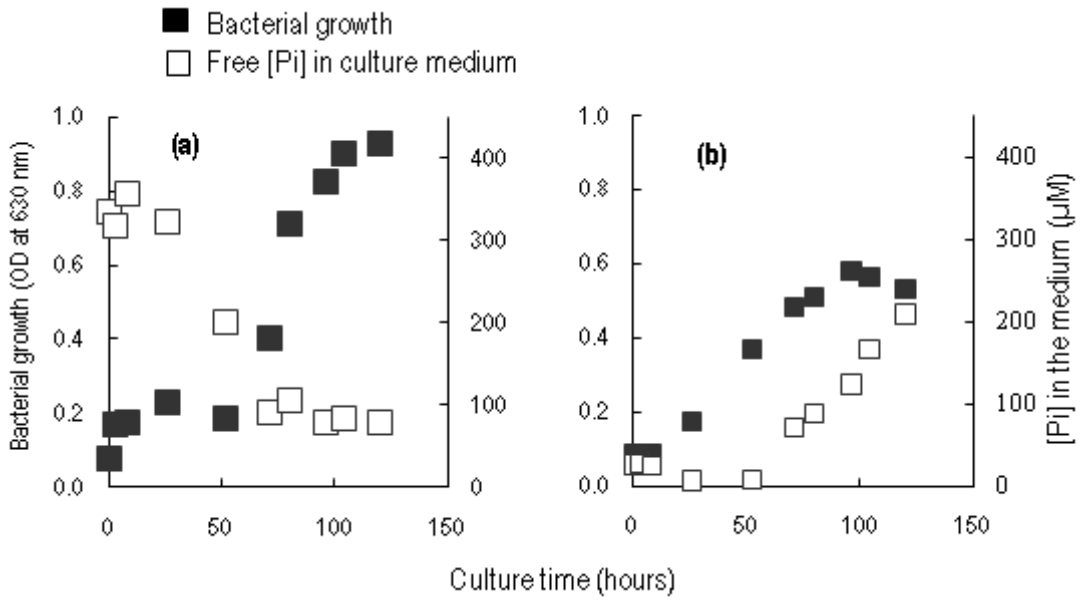

Figure S1. Bacterial growth (estimated from OD measurements at 630 nm) and Pi concentration in liquid medium inoculated with the phytate-mineralizing bacteria, *Bacillus subtilis* and containing (a) KH_2PO_4 (1mM) or (b) sodium-phytate (6mM) as the sole source of P. The culture medium contained nitrate (1 mM) as the sole source of N and glucose (55 mM) as carbon source.

CHAPTER 6

SODIUM TOXICITY AND PHYTATE USE IN THE RHIZOSPHERE: A HELPING HAND BY MICROBIAL PARTNERS

The results of chapter 5 showed that use of phytate as an external P source increased significantly due to the contribution of nematodes grazing in plant P provision. However, experiments that aimed at screening the bacterial capacity to develop on phytate use ultrapure dodecasodium phytate salt supplied at a concentration of 4g l^{-1} (see for example Jorquera *et al*., 2008, 2011; Chapter 5). Dissolution of the phytate salt will produce a concentration of 72 mM of free sodium in the medium indicating that bacterial species such as *Bacillus sp.* (Jorquera *et al*., 2011; Chapter 5) are able to cope with such high Na concentration. In contrast, such high concentrations of sodium may hamper plant growth by interfering with calcium, potassium and magnesium uptake (Sale-Rastin, 1976; Greenway and Munns, 1980; Franklin *et al*., 2002). However, no data regarding salt tolerance in *Pinus pinaster* are available.

In this study, we examined the hypothesis that phytate mineralizing bacteria and their grazer nematodes, combined with the benefit of mycorrhizal symbiosis to take up inorganic P (Pi), could play a role to reduce sodium toxicity and increase P accumulation in plants when sodium phytate is supplied at high concentration in the medium.

The objectives of this chapter were to evaluate:

- ✓ The activity of food web microorganisms in higher phytate concentration.
- ✓ The role of fungi, bacteria and their nematodes grazers in plant survival in the presence of higher concentration of sodium liberated from phytate during efficient bacterial grazing.

Young seedlings of *Pinus pinaster* , whether or not associated with *H. cylindrosporum*, were grown alone or with B. subtilis and nematodes (*Rhabditis* sp.) in the same petri plate system with 6mM phytate for 60 days.

The results obtained in this work are presented in a manuscript entitled "*Sodium toxicity and phytate use in the rhizosphere: A helping hand by microbial partners*" for submission in "*Soil Biology and Biochemistry*" as a short communication.

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CHAPTER 6. SODIUM TOXICITY AND PHYTATE USE IN THE RHIZOSPHERE: A HELPING HAND BY MICROBIAL PARTNERS

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1. Abstract

One particular aspect of sodium toxicity to plants is encountered with the supply of sodium phytate to rhizosphere bacteria for measuring their capacity to release phytase. Previous studies also reported the effect of mycorrhizal association on decreased sodium uptake by plants grown in saline soils. In this regard we aimed that phytate mineralizing bacteria and their grazer nematodes, combined with the benefit of mycorrhizal symbiosis to take up inorganic P (Pi), could play a role to reduce sodium toxicity and increase P accumulation in plants when sodium phytate is supplied at high concentration in the medium. The results obtained showed low Na levels in shoots of non mycorrhizal *Pinus pinaster* seedlings than seedlings associated with ectomycorrhizal fungus *Hebeloma cylindrosporum* whether associated or not with phytate-mineralizing bacteria (*Bacillus subtilis*). A significant remarkable role of nematodes grazing activity in reducing Na toxicity and increasing P availability to plant shoots was observed. No significant, increase of total shoot P accumulation was measured in NM and M plants without nematodes. Contrarily, the bacterial grazer nematodes (*Rhabditidis* sp.) remarkably increased plant P independent of the plant status. This reduction of plant Na uptake and increase in P uptake by bacterial grazing activity could be an important resistance mechanism for plants growing in phytate containing salinized soils.

Key words: *Hebeloma cylindrosporum*, *Rhabditidis* sp., *Bacillus subtilis*, *Dodecasodium phytate*, Na toxicity, P uptake, Microbial abundance.

2. Introduction

Salinity can have a dramatic effect on plants due to its osmotic action and direct ionic toxicity (Greenway and Munns, 1980). Sodium chloride inhibits both stomatal conductance (Renault *et al*., 1999) and root hydraulic conductance (Carvajal *et al*., 2000). Studies reporting the

effect of arbuscular mycorrhizal association (AM) are rather contradictory with decreased sodium uptake and reduce plant yield losses by plants grown in saline soils (Al-Karki, 2000; Azcon and El Atrash, 1997) and no differences in salt tolerance in citrus seedlings (Hartmond *et al*., 1987; Graham and Syversten, 1989). Ectomycorrhizal (ECM) fungi grown *in vitro* displayed specific differences to cope with NaCl supply as shown by Bois et al. (2006). Hydrophylic species such as *Hebeloma crustuliniforme* or *Laccaria bicolor* tended to accumulate more sodium than hydrophobic species as *Suillus tomentosus* at all NaCl concentrations studied (up to 300 mM). However, several studies reported that when associated with the plant, ectomycorrhizal fungi decreased sodium concentration in shoots of plants subjected to external Na concentrations of 13 (Bucking and Heyser, 2000), 25 (Mushin et al., 2002: Nguyen et al., 2006) or 60 mM (Calvo Polanco *et al*., 2008). Therefore, these results suggest that ECM fungi could help the plant to cope with sodium toxicity.

Broth media used to grow bacteria contain high concentrations of sodium supplied as NaCl (i.e. 86 mM in Luria Broth) indicating that they can cope with high level of sodium. One particular aspect of sodium toxicity is encountered with the supply of sodium phytate to screen bacteria for their capacity to release phytase. Typically, experiments use ultrapure dodecasodium phytate salt as the sole source of P that is supplied at 4g l^{-1} (see for example Jorquera *et al*., 2008, 2011; Irshad *et al*., 2011b). Dissolution of the phytate salt will produce a concentration of 72 mM of free sodium in the medium indicating that bacterial species such as *Bacillus sp.* (Jorquera *et al*., 2011; Irshad *et al*., 2011b) are able to cope with such high Na concentration. Therefore, in addition to the use of phytate, bacteria could also play a role to decrease Na toxicity to plants.

As shown previously (Irshad *et al*., 2011b), nematode grazing activity on phytase-producing bacteria was required to get a positive effect on *Pinus pinaster* growth supplied with 1 mM phytate (equivalent to 12 mM Na) as the sole source of P in solid medium. The presence of the ectomycorrhizal fungus *Hebeloma cylindrosporum* increased the net amount of P accumulated in pine seedlings, and this was attributed to a better exploration of the medium. In this study, we hypothesized that phytate mineralizing bacteria and their grazer nematodes, combined with the benefit of mycorrhizal symbiosis to take up inorganic P (Pi), could play a role to reduce sodium toxicity and increase P accumulation in plants when sodium phytate is supplied at high concentration in the medium.

Biological material was the same as described by Irshad et al. (2011a) consisting of combinations made of *Pinus pinaster* seedlings, an ectomycorrhizal fungus (*Hebeloma cylindrosporum*), population of bacteria (*Bacillus subtilis*) displaying phytase activity and one of their predators (nematode *Rhabditis sp*). Co-inoculation experiment was carried out as described previously (Irshad *et al*., 2011b) with phytate as the sole source of P. The only modification was the concentration of phytate that was 4 g l^{-1} (corresponding to 6 mM phytate and 72 mM sodium). Two-month old *P. pinaster* seedlings were used to set up the experiment with the following treatments: uninoculated plants (S), plants inoculated with bacteria (B) and plants inoculated with bacterial and nematodes (BN) on both sides of non mycorrhizal (NM) and mycorrhizal (M) plants. Two months later plants were harvested. Abundance of bacteria and nematodes and nematode biomass were determined as described previously (Irshad *et al*., 2011b). Plants were separated from the medium and shoots were weighed and ground after freeze-drying. Shoot material (50 mg) was mineralized with H_2SO_4 36N as described by Aquino and Plassard (2004). Concentrations of total P and total sodium were assayed in the acidic extract using malachite green (Ohno and Zibilske, 1991) or atomic absorption spectrophotometry, respectively.

Unless otherwise stated, the results are given as mean ± standard deviation (n= 6). The differences between means were analyzed by factorial ANOVA followed by Tukey's HSD post-hoc test using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Kolmogorov Smirnov test and, where necessary, the data was either square root or log10 transformed prior to analysis to meet the assumptions of ANOVA. The comparison of means between NM and M samples at the beginning and the end of experiment was carried out using the Student's t test.

As shown in Table 1, bacterial populations increased during the culture by factors of 10 and 8 in NM and M plants, respectively. Nematodes did not modify the final abundance of bacteria, contrary to the ECM fungus that decreased it significantly. Nematodes population increased more than bacteria, by factors of 33 in NM plants and 24 in M plants. However, the differences in nematode numbers and their biomass between NM and M plants were not significant.

In absence of nematodes, shoot biomass did not significantly increased compared to that measured in plants used to set up the experiment, whatever the mycorrhizal status (Table 2).

In contrast, the presence of nematodes grazers induced a significant increase of +66% and +113% in NM and M shoots biomass (Table 2).

Table 1. Abundance of bacterial (*B. subtilis*) and bacterial-feeding nematodes (*Rhabditis sp.*) populations 60 days after inoculation in the rhizosphere of *P. pinaster* plants, non-mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*, supplied with with 72 mM Na supplied as dodeca Na phytate salt as the sole source of P. The initial inoculated populations were 4.4x 10^6±0.1, 206±14 (*Rhabditis* sp.) plant $^{-1}$ for bacteria and nematodes, respectively.

Plant status	Treatments	Bacterial cells (x10^6 plant^{-1})	Nematodes (x 10^3 plant^{-1})	Nematode biomass (µg plant^{-1})
NM	SB	44±4a		
	SBN	46±6a	6.66±0.97a	129*
M	SB	34±5b		
	SBN	32±7b	4.88±0.79a	79
P-values		0.00	0.09	

Data are means ± standard deviation (n=6) and were compared using one-way ANOVA followed by the Tukey's minimum significant difference test. For each parameter, means significantly different at $P < 0.05$ have different letters.
* Biomass of nematodes was calculated from the size of 500 individuals randomly sampled from the whole population.

Compared to the initial values, total amounts of Na accumulated in shoots increased significantly during the experiment whatever the inoculation treatments. However, as shown by two-way ANOVA, the effect of plant status was significant, and M plants accumulated more Na than NM plants on average (Table 2). The mycorrhizal status induced a significant increase of Na concentrations as shown by two-way ANOVA. The following decreased order of Na concentrations in shoots of M plants was SB>S>SBN. In contrast, there was not effect of inoculation treatments on Na concentrations measured in shoots of M plants. Regarding total P accumulation, no significant increase of shoot P was measured in NM plants without nematodes. On the contrary, shoot P accumulation in M plants was significantly increased during the experiment, with the strongest effect measured in SBN treatment (Table 2). However, total P concentrations increased during the experiment and both mycorrhizal status and inoculation treatments strongly affected the P concentrations. The presence of nematodes increased the P concentrations in NM and M plants. However, M plants with nematodes

presented the highest level of P concentration. In addition, only the nematodes induced a wide clear zone that appeared around the roots of NM plants (Figure 1) and M plants (not shown).

Photo 1. Root growth with phytate solubilization circle in solid medium inoculated with the phytate-mineralizing bacteria, *Bacillus subtilis* and containing sodium-phytate (with 72 mM Na supplied as dodeca Na phytate salt as the sole source of P). The clear solubilization zone around roots was obtained in treatment bacteria+nematodes (BN) and the opaque plate was with bacteria in treatment (B).

Despite the high level of Na in the medium bacterial populations were able to develop and the values recorded are of the same order of magnitude than those measured in NM or M plants grown with 6 mM Na (Irshad *et al*., 2011b). Similarly, the presence of *H. cylindrosporum* decreased the abundance of bacteria, presumably due to a competition for plant C source. The high concentration of Na did not hamper the development of nematode population compared to the results obtained previously, confirming a great tolerance of *B. subtilis* and *Rhabditis sp.* to sodium.

The presence of the fungus did not decreased Na concentrations measured in shoots, compared to NM plants. These results are not in agreement with the data reported so far, indicating that ectomycorrhizal association decreased Na concentration in shoots, even at high Na concentration (ie Calvo Polanco *et al*., 2008). However, as shown by Bois et al. (2006) these effects could be fungal dependent. On the other hand, the mycorrhizal association significantly increased P concentrations even in absence of nematodes, suggesting that the fungus may be able to hydrolyse phytate due to its high concentration.

As observed previously, the presence of nematodes had a dramatic effect on phytate hydolysis, as illustrated by the increase of P accumulation in shoots of NM and M plants and the appearance of a clear zone around the roots. This clear zone may be due to enhanced bacterial activity able to release alkaline phytase active on calcium phytate (Lim *et al*, 2007) and increased root P uptake. Conversely, the re-precipitation zone could be due to an excess of free inorganic P with calcium in the medium resulting from enhanced phytate hydrolysis and not taken up by the roots. Finally grazing activities increased P accumulation. This

supplementary P uptake may have enabled the plant to cope with high Na concentrations as suggested by Mushin et al. (2002). Finally, besides the fact that nematodes predation can increase plant N uptake by increasing N mineralization (Ingham *et al*., 1985; Djigal *et al*., 2004), our results demonstrated that nematode grazing on phytate mineralizing bacteria also enhanced tolerance to sodium toxicity of high sodium phytate salt concentration by increasing plant P uptake.

Table 2. Dry shoot biomass, Na and P accumulations in shoots of *Pinus pinaster* seedlings grown for 60 days in solid medium with 72 mM Na supplied as dodeca Na phytate salt as the sole source of P. Seedlings were either non mycorrhizal (NM) or mycorrhizal (M) with the ectomycorrhizal fungus *H. cylindrosporum*, and with different inoculation treatments that were either no addition of organisms (S), or addition of *Bacillus subtilis* (B) or together with the bacterial feeder nematode *Rhabditis sp.* (BN).

Plant status	treatments Inoculation	Shoot dry weight (mg plant^{-1})	Na (mg plant^{-1})	Na (mg g^{-1} dry weight)	P (mg plant^{-1})	P (mg g^{-1} dry weight)
NM	S	307b	2.11a*	5.94b*	0.46d	4.02c*
	SB	325b	1.75a*	5.58b	0.55cd	5.23c*
	SBN	526a*	2.67a*	4.38b*	1.23bc*	7.90b*
M	S	245b	2.46a*	13.18ab*	0.90bcd*	8.73b*
	SB	234b	3.79a*	17.58a*	1.25b*	8.94b*
	SBN	511a*	3.37a*	7.39b*	2.36a*	11.84a*
One-way ANOVA *P-values*		0.000	0.099	0.001	0.000	0.000
Two-way (ANOVA) (*P-values*) Variation source						
Plant status		0.105	0.025	0.000	0.000	0.000
Inoc. treatments		0.000	0.389	0.062	0.000	0.000
Interaction		0.650	0.266	0.175	0.108	0.612

Data are means (n=6) and were compared using one-way ANOVA followed by the Tukey's minimum significant difference test. For each parameter, means significantly different at $P < 0.05$ have different letters. A two-way ANOVA was also performed to assess the individual and combined effects of plant status and inoculation treatments using the Tukey's minimum significant difference test. Values accompanied with an asterisk indicate that the mean is significantly different from the initial value measured in two-month old plants ($P < 0.05$) using a Student's t-test of mean comparison.

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GENERAL CONCLUSIONS AND PERSPECTIVES

1. Conclusions

In this thesis, we argue that the nutrient mineralization and turnover through bacterial grazing is an important missing piece in our understanding of nutrient cycling in terrestrial ecosystems. We carried out several experiments to increase our knowledge about several poorly understood phenomena regarding the microbial nutrient flow occurring in the rhizosphere through three main objectives.

Firstly, we aimed at evaluating and quantifying the flow of bacterial N and P from bacterial biomass to plant via grazing activity of nematodes in a controlled experimental system. Secondly, we aimed at determining the role of nematode grazing activity on plant growth and root architecture of woody seedlings.

Thirdly, we aimed at quantifying the evolution of bacteria and nematodes in different nutrient environments with or without *Pinus pinaster* roots. In particular, we wanted to assess if the estimation of nematode biomass could be a powerful indicator of rich/poor nutrient environment.

1.1. Direct contribution of bacteria and nematode grazers in N & P mineralization and mineral nutrient acquisition by plants

First of all, we set up a sterile experimental system to use different combinations of organisms that were the host plant, *Pinus pinaster*, whether or no associated with an ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum*, grown in sterile conditions or with bacteria (*Bacillus subtilis*) isolated from ectomycorrhizal roots sampled in a maritime pine plantation and bacterial-feeding nematodes (*Rhabditis* sp.) also isolated from the same pine plantation.

To quantify the flow of bacterial N to the plant through nematode grazing, we used ^{15}N labeled bacteria supplied either with an insoluble source of mineral P (TCP) or with the bacterial P as the sole source of P. The results showed that nematode grazing on bacteria was

required to measure a net accumulation of ^{15}N in plant shoots of non mycorrhizal *P. pinaster* seedlings (Figure 1). This accumulation amounted to 0.7% of N contained in bacterial biomass (Irshad et al., 2011). Interestingly, this percentage of ^{15}N accumulation from ^{15}N bacterial biomass was of the same order of magnitude than the values measured in plant shoots sampled at the surface of intact soil cores that had received bacteria labeled with ^{15}N (up to 0.5 % after 11 d of incubation, Crotty et al., 2011). In addition, this study that kept intact as most as possible all the populations of living organisms in the soil, showed that nematodes, together with Collembola (Entomobryomorpha) and Acari (Oribatida) were the three groups that were significantly enriched in both ^{13}C and ^{15}N from bacterial origin, indicating that these organisms consumed the most of bacteria in more realistic environmental conditions than in our experiment (Crotty et al., 2011).

However, our studies showed also that P availability is an important environmental factor that determined the fate of bacterial ^{15}N. Indeed, results presented in Chapter 4 suggest that the grazing activity of nematodes that have occurred upon the bacterial populations was not sufficient to produce a net release of P from bacterial biomass available to plants. Despite the fact that the populations of bacteria and of nematodes increased during the co-inoculation experiment, the net accumulation of ^{15}N in shoots of pine plants, whether ectomycorrhizal or not, did not increase compared to the first experiment. This strongly suggested the importance of a P source for the action of microbial loop, whether or not directly available to plants.

Besides increased bacterial N mineralization, the data presented in Chapter 5 strongly support the important role played by the bacterial grazer nematodes to enhance P availability from a recalcitrant source of organic P represented by phytate (Figure 1). Indeed, the plant alone or associated with the ectomycorrhizal fungus *H. cylindrosporum* was poorly able to use this source of organic P. Based upon the demonstrated capacity of *B. subtilis* to use phytate *in vitro*, we studied the effect of this bacteria inoculated either alone or with two species of bacterial-feeding nematode on P accumulation in plants, whether or not mycorrhizal. The results showed that only the presence of one or the other nematode species was able to increase dramatically up to 250% plant P accumulation. This positive role is based on the assumption that newly grown bacteria are able to mobilize nutrients that are not easily accessible to plants (such as phytate), which are then made available to plants by bacterial grazers.

Although the mechanisms leading to the release of available Pi from the grazing activity of nematodes upon phytate-mineralizing bacteria remain to be elucidated, our data demonstrated for the first time the importance of the microbial loop to enhance P availability from phytate, which is recognized as the most poorly available organic P compounds to plants.

1.2. Role of fungi, bacteria and their nematodes grazers on plant growth and root architecture

Our results showed that grazing by nematodes of bacteria was able to induce different effects on plant growth and development through an increase of shoot biomass and no significant effect on root architecture (Irshad et al. 2011). However, plant growth stimulation by grazing activity was strongly dependent on nutrient status of medium. For example in the absence of external source of P no difference in plant biomass was found either in the presence of nematodes grazing activity (see Chapter 4). In contrast, grazing by nematodes on bacteria supplied with phytate at 1 mM dramatically increased root growth and modified root architecture (Chapter 5), resulting in an improved exploration of the medium by the root system, especially in non mycorrhizal plants.

In general ectomycorrhizal infection tended to decrease the plant biomass and root growth. More precisely the ectomycorrhizal association reduced root growth and surface area especially in absence of bacterivorous nematodes. On the other hand bacteria alone or in association with ectomycorrhizal fungus did not improve the plant biomass and root architecture with an exception of increase in number of root tips (Chapter 5). Such a negative effect on growth was observed by Jentschke et al. (1995) with a different fungus strain and and Aquino and Plassard (2004) with the same fungus strain. Their results are in agreement to ours with a strong negative effect on the growth of *P. pinaster* roots associated with the same fungal strain. Former studies dealing with the effect of mycorrhizal symbiosis on root growth reported negative, positive or no effects. Although the factors responsible for the fungal effects on root growth and development remain largely unknown, a competition for the carbon compounds between the roots and the fungal cells gained by the fungus could explain the decrease in root growth, as proposed by Bonkowski (2004).

1.3. Evolution of bacteria and nematodes in different nutrient environment during plant growth

Using a direct counting of *B. subtilis* cells, we observed that in the absence of the plant and P as an external source in the culture system no increase in bacterial populations was found even in absence of nematodes (Chapters 4 and 5). In contrast, when the plant was grown with phytate as P source, a large number of bacterial cells were observed, especially in absence of the mycorrhizal fungus and nematodes (Chapter 5). The fungus and the nematodes decreased significantly the size of bacterial populations in the medium (Chapter 6). The same trend was found on nematodes abundance without a carbon source provided by the plant. In addition, when the medium contained no other P source than bacteria, we observed that the populations of nematodes increased dramatically compared to the medium containing mineral P (Chapters 3 and 4, Table 1). However, compared with the two other experiments carried out with phytate either at 1 or 6 mM, the biomass of nematodes estimated when no external source of P was added to the medium, was very low (Table 1). Finally, our results showed no significant difference in nematodes numbers with different P concentrations whereas their biomass was found highly variable (Table 1). Taken together, these results indicate that the biomass of nematodes could be a good indicator of environment P availability for bacteria there are feeding upon.

Table 1. A sum up of results of nematodes numbers and biomass within different nutrient environments only in non mycorrhizal plants calculated on an average time of 45 days.

Thesis Chapter	Nutrient environment with (constant N but variable in P)	Nematode populations (counts plant^{-1})	Nematode biomass (µg plant^{-1})
Four	No P	2280±553	35
Five	As phytate (1 mM)	3621±525	263
Six	As phytate (6 mM)	4997±730	97

As it did for bacteria, the presence of the plant also strongly increased the size of the nematode populations especially when they are non-mycorrhizal, with a high number of juveniles. The mycorrhizal symbiosis induced differential effects on nematodes abundance depending on nematodes species i.e. A negative impact on *Rhabditis* sp. (-66%) while no effect on *Acrobeloides* sp (Chapter 5).

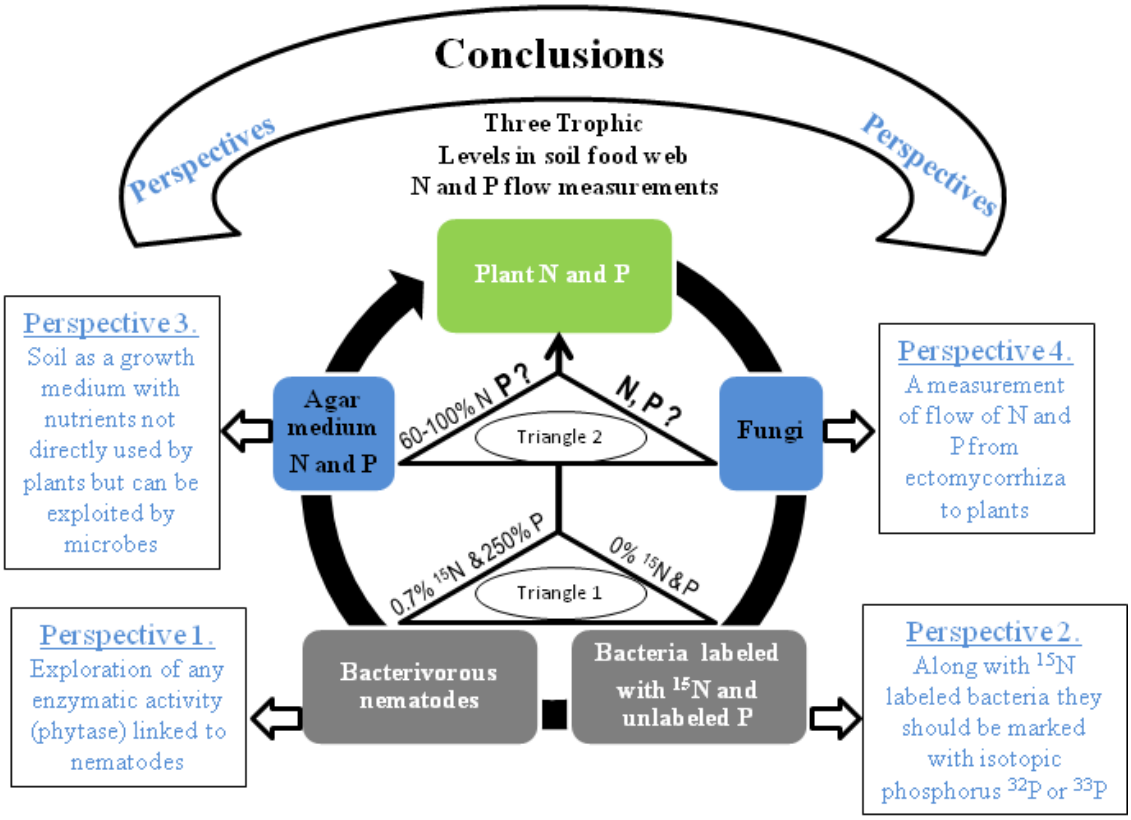

Figure 1. Schematic diagram of N and P flow measurements to plant through three trophic level soil food web microbial partners.
Triangle 1. Represents the bacterial and nematode compartments and N and P flow from it to plant parts. Triangle 2. Represents fungal and medium N and P compartments and nutrient flow from it to plant parts. The main black circle with the arrow represents plant exudates supporting the growth of microorganisms.

2. Perspectives

The conclusions derived from this work interrogate four important aspects that should be focused in the future research plan (Figure 1).

2.1. Nematode enzymatic activity

Firstly, the identification of any phytase enzyme activity linked with bacterivorous nematodes should be carried out. As far as we are aware, no phytase gene has been described in *Caenorhabditis elegans* whose whole genome is available in public data banks. However, some variability may exist between nematode species to produce enzymatic activities. This was shown for the reduction of carboxymethylcellulose (CMC) that was catalyzed by homogenates of phytoparasitic and some myceliophagous nematodes but not by those from animals (*Trichinella spiralis*) and aquarium parasites (*Panagrellus* sp.) or from biocontrol agents (*Neodiplogaster* sp.) (Dropkin, 1963). Therefore, we cannot exclude that the bacterivorous nematode species we have used were not able to produce phytase activity and this should be measured.

2.2. Bacterial phosphorus labeling ($^{32/33}$P)

Secondly, the flow of bacterial P during bacterial grazing should also be quantified. This could be done by labeling bacterial biomass with isotopic phosphorus $^{32/33}$P as along with ^{15}N labeling. Due to its high sensitivity, $^{32/33}$P labeling could also help us to get more knowledge about the feeding behavior of the bacterivorous nematodes. For that purpose, experiments where nematodes are collected after various time of incubation will be necessary to build the curve of P use from bacterial $^{32/33}$P and/or N use from bacterial ^{15}N. Such experiments should therefore help us to quantify the rate of bacterial turnover, as affected by the presence of the different partners that are the plant, the fungus and the nematode grazers as a function of the P source supplied to the medium.

2.3. Soil utilization as growth medium

Our experiments showed that the presence of the bacterial grazers was determinant to improve plant P availability when phytate, a poorly available source of P for the plant and the fungus,

was supplied as the sole source of P in the agar medium. These first experiments were necessary to quantify the beneficial effect of bacteria and predation on plant P availability. In a second step, it will be necessary to add soil in our experimental system as it is well-known that the availability of phytate to enzyme hydrolysis by fungal phytases (belonging to the HAP) may be restricted by the presence of iron and aluminum oxides (Giaveno et al., 2008; 2010). However, the effect of bacterial phytases belonging to beta-propeller phytases such as the enzyme released by *Bacillus subtilis* on phytate mineralization in soil samples has not been studied yet. Our hypothesis is that these enzymes, active on calcium phytate may be more efficient in soil conditions than fungal enzymes (HAP) much more active on free phytate than on complexed phytate (Giaveno et al., 2010). This will in turn, enable the bacteria to access soil phytate for releasing free Pi available to plants and/or for their own growth. In this latter case, nematode grazing upon the bacteria should help the cycling of bacterial P to the plants. These hypotheses could be tested using rhizoboxes developed by Casarin et al. (2004) combined to the same experimental design we used in the thesis (plants whether or not mycorrhizal, whether or not inoculated with bacteria and nematodes). The release of bacterial phytase in soil, together with the active bacteria, could be monitored using bacteria tagged with GFP (Green Fluorescent Protein). Depending on the soil treatment (sterile vs native, supplemented or not with phytate), the contribution of bacterial phytase to plant P availability could therefore be quantified in conditions closer to the natural ones than in agar medium.

2.4. Contribution of ectomycorrhizal symbiosis to N and P flow to the plant

We have shown that the fungus may play different roles on N and P nutrition of the host-plant. To increase our knowledge about the actual role of the fungus upon N and P transfer from the bacterial N and P source, with or without bacterivorous nematodes, it will be necessary to separate physically the access of N and P source from the root system. One can imagine providing labeled bacteria with ^{15}N or $^{32/33}P$ in a multi compartment system where mycelium and bacteria will be isolated from the root system. The distance of the bacterial source from the fungus could be varied (as that used by Thonar et al., 2011) to estimate the strength of the fungus to get new source of N and P. Ultimately, the effect of the fungus, whether positive or not, upon N and P accumulation from bacterial origin could be quantified.

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Trophic relationships in the rhizosphere: effect of fungal, bacterial and nematode interactions on mineral nutrition of *Pinus pinaster* seedlings

Abstract: Soil microorganisms act as a sink and a source of available N and P by mediating key processes in the biogeochemical N and P cycling. The microbial loop, based upon the grazing of bacteria by predators such as bacterial-feeding nematodes, is thought to play a major role in the mineralization of nutrients such as nitrogen (N) and phosphorus (P) in terrestrial ecosystems. However, little is known about the impact of grazing by nematodes on mineral nutrition of ectomycorrhizal woody plants. Different studies were undertaken to quantify the role of nematode grazing on bacteria on the root growth and architecture, mineral nutrition (N and P) of a woody species, *Pinus pinaster*, whether or not associated with the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*. Plants were grown in a sterile simplified experimental system, whether inoculated or not with *Bacillus subtilis* and bacterial-feeding nematodes (belonging to Rhabditidae or Cephalobidae families) that were isolated from ectomycorrhizae and from soil of a *P. pinaster* plantation. The effect of nematode grazing on plant growth and the fate of bacterial ^{15}N towards plant shoots was strongly dependent upon medium P availability. In addition, nematode grazing was required to enable the plant to access P from phytate, a well-known poorly available P source to plants but that was used by bacterial populations of *B. subtilis* due to its ability to release phytase in the medium. These results open an alternative route to increase the use of phytate for plant P nutrition.
Key words: bacterial-grazers nematodes, trophic relationships, mineral nutrition, bacterial ^{15}N, woody plant, ectomycorrhizal symbiosis, mineral P, organic P, phytate-mineralizing bacteria, *Bacillus subtilis*, *Pinus pinaster*, *Hebeloma cylindrosporum*, *Rhabditis* sp., *Acrobeloides* sp.

Relations trophiques dans la rhizosphère : effet des interactions entre champignon ectomycorhizien, bactéries et nématodes bactérivores sur le prélèvement minéral du Pin maritime (*Pinus pinaster*)

Résumé: Les microorganismes agissent comme un puits et une source de N et P disponibles car ils sont responsables des cycles biogéochimiques de N et P. La boucle microbienne, basée sur la prédation des bactéries par les microprédateurs tels que les nématodes bactérivores, est considérée comme un facteur majeur de la minéralisation de N et de P dans les écosystèmes terrestres. Cependant, peu de données sont disponibles sur l'impact de la prédation par les nématodes sur la nutrition minérale des plantes ligneuses ectomycorhizées. Différentes expérimentations ont été conduites pour quantifier le rôle de la prédation des bactéries par les nématodes sur l'architecture et la croissance racinaire, la nutrition minérale (N et P) d'une espèce ligneuse, *Pinus pinaster*, associée ou non avec le basidiomycète ectomycorhizien *Hebeloma cylindrosporum*. Les plantes ont été cultivées dans un système expérimental simplifié et stérile, et inoculées ou non avec *Bacillus subtilis* et des nématodes bactérivores (de la famille des Rhabditidae ou des Cephalobidae) isolés à partir d'ectomycorhizes et de sol provenant d'une plantation de Pin maritime. L'effet de la prédation sur la croissance des plantes et le devenir du ^{15}N bactérien vers les parties aériennes dépend très fortement de la disponibilité en P du milieu. De plus, la prédation des bactéries est indispensable pour permettre à la plante d'utiliser le P du phytate, une source de P organique très peu disponible pour la plante mais très facilement utilisable par *B. subtilis* car cette bactérie est capable de libérer de la phytase dans le milieu. Ces résultats ouvrent de nouvelles perspectives pour améliorer l'utilisation du phytate pour la nutrition phosphatée des plantes.
Mots clés : nématodes bactérivores, relations trophiques, nutrition minérale, ^{15}N bactérien, plante ligneuse, symbiose ectomycorhizienne, P minéral, P organique, bactéries minéralisatrice du phytate, *Bacillus subtilis*, *Pinus pinaster*, *Hebeloma cylindrosporum*, *Rhabditis* sp., *Acrobeloides* sp.