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# Spatial distribution of phosphatase activity associated with ectomycorrhizal plants is related to soil type

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ABSTRACT

Several ectomycorrhizal fungi, including *Hebeloma cylindrosporum*, actively release large quantities of phosphatase enzymes into their growth medium. We fractionated the phosphatase activity of the ectomycorrhizal association between *H. cylindrosporum* and its host plant, *Pinus pinaster*, with the aim to quantify its spatial and temporal variation in response to contrasting soil phosphorus conditions. Seedlings were grown in mini-rhizoboxes and the phosphomonoesterase activity of rhizosphere soil, released by roots, surface-bound to roots or mycelium was determined spectrophotometrically with the *p*-nitrophenyl phosphate method or microscopically with the ELF-method as a function of culture time. We showed that acid phosphatase activity of the soil and the root increased with mycorrhizal association. We also observed that the phosphatase activity associated with ectomycorrhizal plants was related to soil type. All phosphatase fractions decreased over culture time, except the proportion of hyphae exhibiting phosphatase activity in the extramatrical mycelium, which increased over time. The specific fractions of phosphatase activity associated with the mycorrhizal plants were clearly related to the soil phosphorus type and content. Soils showed an increase in acid phosphomonoesterase activity with mycorrhizal association, supporting a role for this enzyme in the degradation of soil bound phosphorus. The gradually increasing proportion of hyphae in the extramatrical mycelium exhibiting alkaline phosphatase activity, particularly under low phosphorus conditions, indicates an induction of alkaline phosphatase activity by phosphorus limitation.



## 1. Introduction

Many soils are deficient in readily available forms of phosphorus (P) for plant uptake (Hinsinger et al., 2003) and may require the application of P-based fertilisers to remain productive. However, in forest soils, such fertilizer addition is not often applied and the growth of trees will largely rely on the use of naturally occurring P, either in organic or inorganic form. It is thought that part of this P is made available for uptake through acidification of rhizosphere soil and release of organic anions (Hinsinger, 2001; Hinsinger et al., 2003). Another possible mechanism to liberate P from soil is the release of phosphatase enzymes into the environment by many microorganisms, including various soil fungi (Nahas et al., 1982; Bae and Barton, 1989; Haas et al., 1992). This mechanism is of special interest for the *Hebeloma cylindrosporum* Romagnesi (dikaryotic strain D2) – *Pinus pinaster* Soland. *in* Ait. association since previous studies have demonstrated that this symbiosis neither acidifies the rhizosphere soil nor releases significant amounts of organic acids (Casarin et al., 2003, 2004). It has, however, been suggested that a part of the P accumulation by *P. pinaster* in association with *H. cylindrosporum* derives from fungal P uptake (Torres Aquino and Plassard, 2004).

Ectomycorrhizal plants often increase the phosphatase activity in the rhizosphere soil and this is occasionally related to the degradation of labile organic P (see e.g. Liu et al., 2004). However, acid phosphatase (ACP) from roots did not seem to be influenced by P supply in the form of organic or inorganic P (Norisada et al., 2006). For arbuscular mycorrhizal fungi, active release of phosphatases into the soil and its importance in plant P nutrition has been questioned (Joner et al., 2000). Despite that some ectomycorrhizal fungi actively produce and release large quantities of phosphatases into their growth medium when grown in pure culture, especially *H. cylindrosporum* (Leprince and Quiquampoix, 1996; Tibbett et al., 1998, personal observation), studies on quantifying enzyme release when the fungus is associated with its host plant and grown in soil are still lacking. Indeed, localised quantification of phosphatase activity in soil, roots and extramatrical hyphae under varying P conditions is the first step in

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understanding the role of such enzymes in the mobilisation of inorganic P from soil bound P.

In this study, our objective was to quantify the variation of phosphatase activities among the different parts of the ectomycorrhizal association in response to contrasting soil P conditions. We chose the model ectomycorrhizal association between *P. pinaster* and the basidiomycete *H. cylindrosporum*, as this fungal species is an appropriate candidate to study the fate of phosphatase activity from fungal origin. The plants, whether or not mycorrhizal, were cultivated in mini-rhizoboxes (Casarin et al., 2004), which enabled us to measure phosphatase activities in the rhizosphere soil, released or surface-bound to the roots and in the mycelium. Phosphatase activities in the different parts were assessed as a function of culture time ranging from 8 to 16 weeks after plant transfer to mini-rhizoboxes. We hypothesised that *H. cylindrosporum* hyphae, whether associated with the roots in ectomycorrhizal tips or growing into the soil, could display increased patterns of phosphatase activity compared to non-mycorrhizal roots as a function of decreased soil P availability.

## 2. Materials and methods

### 2.1. Experimental set up

Seeds of *P. pinaster* Soland. *in* Ait. (origin Medoc, Landes-Sore-VG source, France) were surface sterilised with $H_2O_2$ (30%) for 30 min and then rinsed thoroughly with sterile dd$H_2O$. The seeds were kept for 48 h in dd$H_2O$ at 4 °C, then air-dried to remove excess water and transferred to water agar plates (15 g $l^{-1}$ agar, 2 g $l^{-1}$ glucose). The water agar plates were sealed with tape, placed on an angle of approximately 45° and stored for one week at 4 °C. After this, the plates were transferred to a growth incubator, and the seeds were left to germinate in the dark at 26 °C.

After germination, seedlings of which the cotyledons were still covered by the seed coat and with a root length of at least 4 cm were selected and transferred to sterile test-tube systems (previously described by Plassard et al., 1994). Mycorrhizal plants were obtained by placing three agar plugs containing mycelium of *H. cylindrosporum* Romagnesi (dikaryotic strain D2) close to the root system. The mycelium originated from stock cultures which were maintained according to Lambilliotte et al. (2004). Non-mycorrhizal plants were prepared with agar plugs without mycelium. After ca. two months, when the plants of the mycorrhizal treatment were colonised, plants of similar development were selected and transferred to mini-rhizoboxes (previously described by Casarin et al., 2003, 2004; Torres Aquino and Plassard, 2004). The average shoot weight for non-mycorrhizal plants was 0.332 g and for mycorrhizal plants it was 0.372 g ($n = 6$; *t*-test did not show significant differences). The average root weight for non-mycorrhizal plants was 0.354 g and for mycorrhizal plants it was 0.472 g ($n = 6$; *t*-test did not show significant differences). One plant was placed per mini-rhizobox. In total 16 mini-rhizoboxes with mycorrhizal plants and 8 with non-mycorrhizal plants were set up for each soil treatment for the full experimental period.

Soils had been air-dried, crushed gently and sieved at 210 μm. Soils were mixed with deionised water (1/1.2, w/w) in plastic bags and autoclaved two times for 40 min at 115 °C with five days interval, thus eliminating soil respiration (Casarin et al., 2003). Each compartment of a mini-rhizobox was filled with a 0.5 mm layer of soil that was spread on one side of a glass plate and air-dried. Three soils with varying P levels were used as treatments; one soil classified as a chromic luvisol according to the FAO system contained average levels of available inorganic P ("Med P"). The two other soils were obtained from a Mediterranean chromic cambisol originally poor in available P ("Low P"). This Low P soil was supplemented with 350 mg P $kg^{-1}$ soil as $KH_2PO_4$ (Casarin et al., 2004; Torres Aquino and Plassard, 2004) to obtain the third soil with high level of available P ("High P"). Available P concentrations were measured as bicarbonate extractable $P_i$ and were 3, 22 and 50 mg P $kg^{-1}$ of dwt soil in Low P, Med P and High P, respectively.

The mini-rhizoboxes were placed in near vertical position in a container, filled with nutrient solution without P (containing 0.3 mM $Ca(NO_3)_2$, 0.4 mM $KNO_3$, 1.0 mM $MgSO_4$, 0.2 mM KCl, 8.3 mg $l^{-1}$ Séquestrène® 138FE 100SG to supply Iron as Fe-EDDHA and 100 μg $l^{-1}$ Thiamine HCl) and placed in a phytotron (16/8 h light/dark cycle, 25/20 °C, 80/100 relative humidity and PAR of approximately 350 μmol $m^{-2}$ $s^{-1}$). Plants were exposed to light, but roots and the soil part of the mini-rhizobox were kept in the dark. Nutrient solution of the container was replaced weekly supplying 50 ml of nutrient solution per plant in order to maintain a level of nutrients that was sufficient to sustain plant growth in these small soil volumes. Destructive harvests of mini-rhizoboxes were performed at four time periods; at 8, 12, 14 and 16 weeks after setting up of the mini-rhizoboxes, four mycorrhizal and two non-mycorrhizal replicates were harvested.

### 2.2. Plant and fungal development

Upon harvesting, the mini-rhizoboxes were opened and a picture was taken to register the fungal and root development. Soil surface covered by the hyphae was determined using image analysis as described in Torres Aquino and Plassard (2004). Shoots and roots were harvested and their weights were determined. Part of the root system was stored at 4 °C until it was used for determination of phosphatase activity. For mycorrhizal systems all roots collected were covered with a sheet of mycelium and the sample included multiple mycorrhizal root tips. The extramatrical mycelium was carefully recovered from the surface of the thin soil layer with fine forceps and stored in ethanol at 4 °C before being used for determination of phosphatase activity. A sample of soil was taken from under the roots (non-mycorrhizal plants) or from under the roots and extramatrical mycelium (mycorrhizal plants) and used for determination of phosphatase activity. Soil from under the extramatrical mycelium and the rest of the soil were collected, weighed and frozen until P determination. Following P determination, water content of each sampled soil was determined by drying it at 105 °C for 16 h.

### 2.3. Phosphorus analysis

Readily available P content in soil was determined according to the method developed by Olsen et al. (1954). Briefly, de-frosted soil (0.5 g) was mixed with 10 ml of 0.5 M $NaHCO_3$, pH 8.5. The soil suspension was shaken end-over-end for 30 min before centrifugation (20 min at 20,000 *g*). After dilution, free Pi was assayed in the supernatant using the malachite green method according to Ohno and Zibilske (1991).

### 2.4. Analysis of phosphatase activity

Acid phosphomonoesterase activity was determined for three different fractions in the mini-rhizoboxes by *p*-nitrophenyl phosphate (*p*NPP) using a modified procedure based on that of Tabatabai and Bremner (1969). Strictly spoken, the capacity of *p*NPP hydrolysis, a phosphomonoester compound that normally is stable in soil, to *p*-nitrophenol in an acid environment was determined. This method is commonly used as an indicator for ACP activity (see e.g. Tibbett et al., 1998; Buée et al., 2005; Courty et al., 2006). The three fractions measured were the ACP activity 1) present in soil samples,