Acid phosphatase activity in mycorrhizal and non-mycorrhizal Scots pine seedlings in relation to nitrogen and phosphorus nutrition

BARBARA KIELISZEWSKA-ROKICKA

Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland

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Abstract

Mycorrhizal and control seedlings of Scots pine (*Pinus sylvestris* L.) were grown under axenic conditions at various concentrations of nitrogen (0.17 mM, 3.78 mM, 18.92 mM) and phosphorus (0.25 mM, 5.58 mM, 23.5 mM). Enzyme activities of soluble acid phosphatase of excised roots (surface-accessible activity) and of crude enzyme preparations of root tissue (total soluble activity) were analyzed in control and mycorrhizal seedlings. The content of P in pine shoot was also measured. The intermediate supply of nitrogen and phosphorus (N = 3.78 mM, P = 5.58 mM) was optimal for development of ectomycorrhizae. Activity of acid phosphatase was stimulated by higher levels of N in the soil and was higher in mycorrhizal than non-mycorrhizal roots. Pine roots had greater acid phosphatase activity at the reduced than at the higher P concentrations. The presence of mycorrhizae increased the activities of both surface-accessible and total soluble enzyme activities at lower P supply (0.25 mM, 5.58 mM) and decreased it significantly at the highest P concentration (23.5 mM). P content in shoots was higher in mycorrhizal than non-mycorrhizal seedlings.

Key words: *Pinus sylvestris*, *Paxillus involutus*, ectomycorrhizae, ammonium nitrogen, phosphorus, acid phosphatase

INTRODUCTION

Ectomycorrhizal symbiosis can increase nutrient uptake of the host plant (Harley and Smith 1983). Phosphorus can be assimilated only as soluble phosphate. Phosphatases may play a role in the utilization of phosphates which are not directly assimilable by the plant (Bielecki 1973) and in the transport of inorganic phosphate when its availability is limited (see Woolhouse 1969, Boutin et al. 1981), Gianinazzi-Pearson and Gianinazzi 1986). With the aid of autoradiography Kramer and Wilburn (1949) and

Majstřík and Benecke (1969) have shown a greater P uptake by ectomycorrhizal roots than by non-mycorrhizal roots. Finlay and Read (1986) demonstrated that ^{32}P was translocated from mycorrhizal short roots not only to the pine shoot but also to the actively growing hyphal tips and accumulated within the mycelial network. Mycorrhizal associations lead to increased phosphatase activities in host roots (Pearson and Read 1975, Dumas et al. 1986, MacFall et al. 1991b) and concomitantly to higher phosphorus content in plant shoots (Bledsoe and Zasoski 1983, Reid et al. 1983, Pachlewski et al. 1986, MacFall et al. 1991a, Kieliszewska-Rokicka 1992). Acid phosphatase activity in mycorrhizal coniferous trees in relation to phosphate supply was compared by e.g. Alexander and Hardy (1981), Dighton (1983), Dumas et al. (1983, 1986), Berjaud and d'Auzak (1986), Berjaud et al. (1987), MacFall et al. (1991b), who found that acid phosphatase activity in higher plants and mycorrhizal fungi increased when P supply was reduced and decreased with higher P levels.

Studies on the relationship between nitrogen supply and acid phosphatase activity of higher plants are very scarce (O'Connell and Grove 1985, Kieliszewska-Rokicka 1989), although it is well established that mycorrhizal symbiosis is easily disturbed by high levels of inorganic nitrogen (Björkman 1942, Meyer 1985, 1988). Nitrogen fertilization of pine stands caused a decrease of the yield of fruitbodies of mycorrhizal fungi ((Menge and Grand 1978, Ritter and Tölle 1978). However not all the mycorrhizal fungi were reduced by an addition of inorganic nitrogen (Ohenoja 1978). Yields of *Paxillus involutus* and *Lactarius rufus* were increased by nitrogen fertilization.

Paxillus involutus (Batsch) Fr. an ectomycorrhizal fungus with a wide host range, frequently found in cold and temperate zones of Eurasia and North America can grow on media rich in available nitrogen (Laiho 1970, Ohenoja 1978). In mature pine stands with nitrogen deficiency this fungus was replaced by fungi characteristic for raw humus (Laiho 1970). Higher nitrogen levels also stimulated the growth of *P. involutus* mycelia in agar cultures (Kieliszewska-Rokicka 1992). The growth stimulation of mycorrhizal fungi by nitrogen fertilization depended not only on the level but also on the source of nitrogen. In field experiments fertilization with calcium ammonium nitrate stimulated the frequency of sporophore development of *P. involutus* more than urea (Laiho 1970). Nitrogen source influenced significantly the solubilization of phosphate by different mycorrhizal fungi *in vitro* (Lapeyrie et al. 1991). Most of the fungi tested by these authors were able to solubilize phosphate only in the presence of ammonium, however one strain of *P. involutus* was almost as efficient on nitrate as on ammonium nitrogen media.

The purpose of this study was to compare the acid phosphatase activity of mycorrhizal and non-mycorrhizal pine roots and to find a relation between varied N and P supply, mycorrhizal development and P content in shoots.

MATERIALS AND METHODS

PLANT AND FUNGAL MATERIAL

Seedlings of *Pinus sylvestris* (L.) were obtained from seeds collected in a seed orchard of Institute of Dendrology at Kórnik. The seeds were surface-sterilized with 3% H_2O_2 for 30 minutes and germinated at room temperature.

The mycelium of mycorrhizal fungus *Paxillus involutus* (Batch) Fr. was isolated in 1988 by M. Rudawska (Institute of Dendrology, Kórnik), from a sporocarp collected in a mixed forest at Kórnik. Methods of isolation and cultivation were as described by Rudawska (1980, 1986a, b).

ECTOMYCORRHIZAL SYNTHESSES

The ectomycorrhizal syntheses were performed under axenic conditions in Roux flasks containing a peat-perlite mixture (1:4 v/v) saturated with nutrient solutions according to Björkman (1942) which differed in their nitrogen (ammonium form) and phosphorus content. Five nitrogen and phosphorus combinations were used: N_0P_1 , N_1P_1 , N_5P_1 , N_1P_0 , N_1P_4 , where $N_0 = 0.17$ mM, $N_1 = 3.78$ mM, $N_5 = 18.92$ mM, $P_0 = 0.25$ mM, $P_1 = 5.58$ mM, $P_4 = 23.5$ mM). The nutrient solutions were supplemented with glucose (2.5 g L^{-1}), thiamine (50 μ g L^{-1}) and biotine (5 μ g L^{-1}).

The medium was initially inoculated with 3 inocula (5 mm diameter) cut from the edge of a 14-day-old fungal agar plate culture. After the fungus had become established three aseptically germinated 10-day-old seedlings of Scots pine were transferred to the flasks which were placed in a greenhouse with supplemental light (sodium lamps, 250 W/ m^2 , distance 1.0 m) at a 16 h photoperiod. Control flasks did not contain mycelium. The experiment lasted for 6 month.

At the end of the period roots were examined under the binocular microscope at a magnification 4-10x and number of mycorrhizal short roots were counted. Total length of root was determined with the intersection method according to Böhm (1979). Fresh and dry weight of shoot and root were measured. Data in the Table 1 are means of at last 10 replicates and were determined with the Student's t-test ($p = 0.01$).

PREPARATION OF SOLUBLE ENZYME FRACTION

Pine roots were ground in liquid nitrogen and washed with 80% ice cold acetone followed by anhydrous ice cold acetone to obtain acetone powder. Soluble proteins were extracted with 100 mM acetate buffer pH 5.0 containing 0.01% Triton X 100, 10 mM EDTA and 2.5% polyclar AT. After centrifugation the supernatant was used as a soluble enzyme fraction.

Accessible enzyme activity

Roots of mycorrhizal seedlings were divided into two groups:

1) root tips with evident mycorrhizae, 2) root tips without mycorrhizae. They were washed and weighed. The incubation medium contained 1.9 ml of 0.1 M acetate buffer pH 4.8, 100 μ L of 40 mM p-nitrophenyl phosphate and 20 mg fresh weight of root tips.

Total enzyme activity

The soluble enzyme fraction was used for determination of total acid phosphatase activity. The incubation medium contained 250 μ L of 0.1 M acetate buffer pH 4.8, 50 μ L of 15 mM p-nitrophenyl phosphate and 50 μ L of enzyme preparation. Incubation took place at 35°C for 30 minutes for both accessible and total enzyme activity. The reaction was stopped by adding 2.5 mL of 0.2 M Na_2CO_3 . Absorbance of free p-nitrophenol was determined at 400 nm. Accessible enzyme activity was expressed as nanokatal (20 mg root tips)⁻¹ and total enzyme activity as nanokatal (mg protein)⁻¹. Protein content was determined according to the method of Bradford (1976).

PHOSPHORUS ANALYSIS

Phosphorus content in pine needles was measured with the method of Kuttner and Lichtenstein (1932) as modified by Humphries (1956). The values given in the Table 2 are means of 4 replicates and were determined with the Student's t-test ($p = 0.01$).

RESULTS

GROWTH OF SEEDLINGS

Growth of shoot was low at the lowest N supply ($N_0 = 0.17$ mM) and it was stimulated by increased levels of N ($N_1 = 3.78$ mM, $N_5 = 18.92$ mM). Seedlings grown at the lowest N concentration had the highest growth parameters of roots and their growth was reduced by higher N levels. After 6 months of cultivation under acenic conditions there were no significant differences in growth between mycorrhizal and non-mycorrhizal seedlings (Table 1).

ECTOMYCORRHIZAL DEVELOPMENT

The most abundant ectomycorrhizae was found on roots of inoculated seedlings receiving the intermediate concentrations of nitrogen and phosphorus

Table 1

The development of *Pinus sylvestris* (L.) seedlings after 6 months of cultivation under axenic conditions with different combinations of nitrogen and phosphorus supply

N/P con- centration	Inocu- lation	Fresh weigh [mg]		Dry weight [mg]		Total length of roots [cm]	Average num- ber of mycorr- hizal root tips
		shoot	root	shoot	root		
N ₀ P ₁	—	108.0 a	129.3 c	36.0 a	35.9 b	89.0 e	0
N ₀ P ₁	+	98.7 a	129.3 c	31.9 a	30.7 b	67.5 de	16
N ₁ P ₁	—	214.7 bc	93.4 bc	51.8 b	16.1 a	44.3 c	0
N ₁ P ₁	+	228.7 bc	94.6 bc	57.3 b	18.0 a	40.9 bc	55
N ₅ P ₁	—	255.2 bcd	77.0 ab	64.8 c	14.6 a	20.4 ab	0
N ₅ P ₁	+	278.0 d	80.9 ab	63.9 c	12.2 a	13.7 a	18
N ₁ P ₀	—	185.0 b	105.8 c	56.6 bc	17.5 a	52.4 c	0
N ₁ P ₀	+	180.0 b	65.7 c	56.8 bc	12.7 a	21.9 a	30
N ₁ P ₄	—	213.0 bc	114.5 cd	55.6 bc	15.8 a	46.9 cd	0
N ₁ P ₄	+	180.6 b	137.3 d	47.1 b	16.8 a	51.9 cd	38

The N and P concentrations in nutrient solutions were used according to Björkman (1942). Data are means of at least 10 replicates. Values in each column followed by the same letter do not differ significantly ($p=0.01$).

(N₁ = 3.78 mM, P₁ = 5.58 mM). The inoculated seedlings supplied with nutrient solutions containing 3.78 mM nitrogen (N₁) and the lowest or the highest phosphorus concentrations (P₀ = 0.25 mM and P₄ = 23.5 mM respectively) developed less mycorrhizal short roots (about 30%) than those grown at the intermediate (N₁P₁) levels of the nutrients (Table 1). At the lowest and the highest levels of applied nitrogen (N₀ = 0.17 mM and N₅ = 18.92 mM respectively) and simultaneously the intermediate phosphorus concentration (P₁ = 5.58 mM) root colonization by *Paxillus involutus* was of 70% lower than at the N and P levels optimal for mycorrhizae formation (N₁ = 3.78 mM, P₁ = 5.58 mM).

ACCESSIBLE ACID PHOSPHATASE

Activity of the surface-accessible acid phosphatase of non-inoculated pine roots was stimulated by the highest P concentration (P₄ = 23.5 mM) but not by N level. Ectomycorrhizae modified strongly the surface-accessible phosphatase activity (Fig. 1). At lower phosphorus concentrations (P₀ = 0.25 mM, P₁ = 5.58 mM) roots of mycorrhizal seedlings exhibited higher accessible phosphatase activity than roots of non-inoculated seedlings. Greater ability to hydrolyse p-nitrophenyl-phosphate as compared to control seedlings was shown not only by roots with evident mycorrhizae but also by non-mycorrhizal short roots from the same inoculated seedlings. With high phosphorus supply (P₄) non-mycor-

rhizal roots of inoculated seedlings had lower accessible phosphatase activity than the control ones, and the roots with mycorrhizal associations showed much decreased activity of this enzyme.

Fig. 1. Effect of different nitrogen and phosphorus concentrations ($N_0 = 0.17$ mM, $N_1 = 3.78$ mM, $N_5 = 18.92$ mM, $P_0 = 0.25$ mM, $P_1 = 5.58$ mM, $P_4 = 23.50$ mM) in the culture medium on the surface-accessible p-nitrophenyl phosphatase activity of control roots (non-inoculated) and roots inoculated with the mycorrhizal fungus *Paxillus involutus*. Error bars = standard deviation

TOTAL ACID PHOSPHATASE

Total specific acid phosphatase activity of control roots was stimulated by higher levels of nitrogen and influenced by a supply of phosphorus (Fig. 2). Relatively low activity of this enzyme was detected in roots of control seedlings grown in the presence of low nitrogen levels ($N_0 = 0.17$ mM and $N_1 = 3.78$ mM) and the intermediate supply of phosphorus ($P_1 = 5.58$ mM). The highest nitrogen concentration ($N_5 = 18.92$ mM) as well as the lowest and at the highest P concentrations ($P_0 = 0.25$ mM and $P_4 = 23.5$ mM, respectively) stimulated the total phosphatase activity. The presence of mycorrhizae increased significantly the activity of the total soluble acid phosphatase at lower phosphorus supply as compared to the non-inoculated seedlings. At the highest P concentration ($P_4 = 23.5$ mM) the activity of total soluble acid phosphatase in roots of mycorrhizal and non-mycorrhizal seedlings was almost equal.

Fig. 2. Effect of different nitrogen and phosphorus concentrations (see: Fig. 1) on the total soluble p-nitrophenyl phosphatase activity of control roots (non-inoculated) and roots inoculated with mycorrhizal fungus *Paxillus involutus*. Error bars = standard deviation

PHOSPHORUS CONTENT IN PINE NEEDLES

Phosphorus content (mg shoot⁻¹) was increased by added nitrogen rates from 0.17 mM up to 18.92 mM, as could be expected with the growth response of fertilized seedlings. Phosphorus concentrations in shoots were higher in both inoculated and non-inoculated seedlings grown in the medium with the highest level of phosphorus. Fungal colonization increased significantly the concentration of P (Table 2).

Table 2

Influence of nitrogen and phosphorus nutrition on the phosphorus content in needles of control and mycorrhizal Scots pine seedlings

N/P concentrations	Inoculation	%P	mg P/shoot
N ₀ P ₁	—	0.33 bc	0.12 ab
N ₀ P ₁	+	0.38 cd	0.12 ab
N ₁ P ₁	—	0.37 cd	0.19 cd
N ₁ P ₁	+	0.44 d	0.25 e
N ₅ P ₁	—	0.35 bcd	0.23 de
N ₅ P ₁	+	0.38 cd	0.24 de
N ₁ P ₀	—	0.18 a	0.10 a
N ₁ P ₀	+	0.26 ab	0.15 abc
N ₁ P ₄	—	0.36 cd	0.20 c
N ₁ P ₄	+	0.56 e	0.26 e

Data are mean of 4 replicates.

Values in each column followed by the same letter do not differ significantly ($p = 0.01$).

DISCUSSION

Both nitrogen and phosphorus supply influenced the development of ectomycorrhizae of roots of Scots pine seedlings grown in aseptic cultures. The intermediate supply of nitrogen and phosphorus ($N_1 = 3.78$ mM, $P_1 = 5.58$ mM) was optimal for ectomycorrhizal development. The average numbers of mycorrhizal short roots per seedling were decreased at the lowest and the highest N and P levels. The effect of nitrogen supply on mycorrhizae was more pronounced than that of phosphorus level (Table 1).

Inhibition of mycorrhizal associations in connection with high fertility has also been observed by other authors (Richard and Wilson 1963, Marx et al. 1977, Menge et al. 1977, Beckjord et al. 1985, MacFall et al. 1991a). The ectomycorrhizal fungus *Paxillus involutus* had shown better growth with ample supply of available nitrogen than in N-poor forest stands (Laiho 1970). It also grew more than other mycorrhizal fungi on media rich in nitrogen (Ohenoja 1978). In agar culture the growth of *P. involutus* mycelia was stimulated by higher nitrogen concentrations in the range between 0.17 mM and 18.92 mM (Kieliszewska-Rokicka 1992). However, a decrease of Scots pine mycorrhizae development was observed in the presence of higher nitrogen levels under axenic conditions (Rudawska 1986b).

Enzyme activities of both surface-accessible and total soluble acid phosphatase in mycorrhizal and non-mycorrhizal pine roots were influenced by phosphorus and nitrogen level (Fig. 1, 2). The surface-accessible enzyme activity of non-inoculated pine roots was increased at the highest (P_4) level of phosphorus (Fig. 1). At the lowest concentration of phosphorus (P_0) the activities of both surface-accessible and total soluble acid phosphatase were higher than at the intermediate (P_1) concentration and the same nitrogen (N_1) supply (Fig. 1, 2). This result was in general agreement with work of other authors, who have found an increase of acid phosphatase activity in higher plants and mycorrhizal fungi when P supply was reduced and a decrease with higher P levels (i.e. Dighton 1983, Doumas et al. 1983, Mousain and Salsac 1984, O'Connell and Grove 1985, Bousquet et al. 1986, Berjaud et al. 1987). MacFall et al. (1991b) observed that surface-accessible acid phosphatase activity of non-mycorrhizal red pine (*Pinus resinosa*) roots increased with increasing additions of superphosphate to the soil and the lowest activity was measured in P-unamended soil.

The presence of mycorrhizae modified the activity of acid phosphatase. At the lowest concentration of phosphorus (P_0) the accessible acid phosphatase activity in inoculated roots increased significantly. In the highest phosphorus concentration (P_4) the accessible activity was much lower than in non-inoculated roots and the total activity was the same as in control roots. Similar results were presented by MacFall et al. (1991b) with mycorrhizal roots of red pine with *Hebeloma arenosa*, which had greater enzyme activity than non-mycorrhizal

roots of seedlings grown in soil with no added P and soil amended with 34 mg/kg P. However these authors did not obtain any significant differences in the enzyme activity in the mycorrhizal and non-mycorrhizal roots of seedlings grown in soil supplemented with 136 mg/kg P. A n t i b u s et al. (1981) showed greater activity of acid phosphatase in roots of *Salix rotundifolia* colonized by various mycorrhizal fungi than in non-mycorrhizal ones, whereas roots colonized by *Hebeloma pussillum* had enzyme activity compared with non-mycorrhizal roots.

The possible reasons for the conflicting results of the cited authors could be: 1) different phosphorus concentrations used in the experiments, 2) different requirements of higher plants and mycorrhizal fungi for phosphorus and nitrogen nutrition, 3) differences in phosphatase activity among mycorrhizal fungi, and 4) type of nitrogen nutrition used in the experiments. It was found that acid phosphatase activity of mycorrhizal fungi grown *in vitro* differ among species (H o and Z a k 1979, M o u s a i n et al. 1988) and even within species (P l a s s a r d et al. 1987, K i e l i s z e w s k a - R o k i c k a 1992). According to T r a p p e (1977) acid phosphatase activity might be a good criterion useful for selecting fungi for ectomycorrhizal inoculation in nurseries, however it appears to be very important to recognise that the requirement of fungi may differ with respect to the nutrients level in the root environment.

Relations between nitrogen supply, phosphatase activity and phosphorus content in plant tissues were studied more often in non-mycorrhizal than mycorrhizal plants. A stimulation of the acid phosphatase enzyme activity by a higher N level have been shown in experiments with non-mycorrhizal karri (*Eucalyptus diversicolor* F. Muell.) seedlings by O'Connell and Grove (1985).

Data presented in this paper (Fig. 1, 2, Table 2) show that the higher levels of nitrogen stimulated the activity of soluble acid phosphatase which was released by pine roots to the substrate. This could increase the pool of available phosphorus and thus enhance the P content in pine needles.

As it was pointed by L a i h o (1986) the mycorrhizal infection does not cause changes in the overall pattern of nutrient uptake, although many details are greatly affected and mycelium hairs covering short roots make trees as efficient as microbes in the competition for nutrients.

Finlay and Read (1986) showed that in mycorrhizal plants P was absorbed by roots and transported both to the plant and to the mycelial network and it could be accumulated in the substrate areas with high P and N concentrations. P export from root to shoot is considered to be an active process controlled by P supply and the content of P in the shoot and dependent on certain ion carriers, probably proteins. S c h j o r r i n g and J e n s e n (1987) showed that the amount of P utilized from reserves was strongly reduced or almost eliminated in the presence of inhibitors of protein synthesis.

It is thus possible that N supply and N status of plants are important factors

influencing release as well as transport of phosphorus. Mycorrhizal associations could increase those effects especially in the pine roots with most abundant mycorrhizal development growing at optimal nitrogen supply.

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Aktywność kwaśnej fosforazy w mikoryzowych i niemikoryzowych siewkach sosny zwyczajnej w zależności od nawożenia azotem i fosforem

Streszczenie

Siewki mikoryzowe sosny zwyczajnej (*Pinus sylvestris* L.) wyhodowano w sterylnych warunkach w obecności grzybni grzyba mikoryzowego *Paxillus involutus*. Kontrolne siewki rosły w sterylnym podłożu bez grzybni. Źródłem pokarmów dla obu symbiontów była pożywka, która w poszczególnych wariantach doświadczenia zawierała różne ilości azotu i fosforu.

Analizowano aktywność kwaśnej fosforazy wydzielanej na zewnątrz przez odcięte korzenie kontrolnych i mikoryzowanych siewek oraz aktywność rozpuszczalnej kwaśnej fosfatazy w surowym preparacie enzymatycznym korzeni siewek. Oznaczano także zawartość fosforu w pędach. Rozwój mikoryzy był wyraźnie zależny od stężenia azotu i fosforu w pożywce. Średnie stężenia azotu i fosforu (N, P_1) były najbardziej korzystne dla symbiozy mikoryzowej. Wyniki wskazują na związek między mikoryzą, aktywnością kwaśnej fosfatazy i zawartością fosforu w pędach siewek sosny.