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Properties of soluble acid phosphatases in an ectomycorrhizal fungus and in host plants subjected or not to Pi starvation

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SUMMARY

The phosphatases of an ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker & Couch. and of two pines *Pinus pinaster* Ait. and *Pinus halepensis* Mill. were studied in culture media with and without orthophosphate. Isophosphatases of the soluble fraction of each organism were separated by chromatography on DEAE-Sephadex. Lack of phosphate induced two effects : either the increase of one or two particular isoenzymes, or the derepression of new phosphatases in the case of *P. pinaster*. The phosphatase activities of *P. tinctorius* were enhanced by phosphate starvation and these enzymes displayed a lower K_m than those found in the pines. The phosphatases of the fungus were more inhibited by orthophosphate than the phosphatases of *P. halepensis*. The soluble acid phosphatases extracted from organisms previously Pi-starved exhibited a greater ability to hydrolyse the natural phosphorylated compounds present in soils (i.e. pyrophosphate, tripolyphosphate for *P. tinctorius*, pyrophosphate, tripolyphosphate, phytate for the pines).

Additional key words : *Pisolithus tinctorius*, *Pinus halepensis*, *Pinus pinaster*.

RÉSUMÉ

Propriétés de différentes phosphatases chez un champignon ectomycorhizogène et des plantes hôtes carencées ou non en Pi.

Les phosphatases d'un champignon ectomycorhizogène, *P. tinctorius* (Pers.) Coker & Couch., et deux variétés de pins, *P. pinaster* Ait. et *P. halepensis* Mill., ont été étudiées lorsque le milieu de culture est carencé ou non en phosphate. Les différentes isophosphatases de la fraction soluble de chaque organisme ont été séparées par chromatographie sur DEAE-Sephadex. L'absence de phosphate induit deux effets : soit l'augmentation de l'activité d'une ou deux isoenzymes particulières, soit la dérépression de nouvelles phosphatases dans le cas de *P. pinaster*. Les phosphatases de *P. tinctorius* qui augmentent avec la carence en phosphate possèdent un K_m inférieur à ceux trouvés chez les pins, qu'ils soient ou non mycorhizés. Les phosphatases du champignon sont davantage inhibées par le phosphate que celles de *P. halepensis*. Chez le champignon et les pins les phosphatases de la fraction soluble possèdent, en milieu carencé, une plus grande capacité d'hydrolyser les composés phosphorylés normalement présents dans les sols tels le pyrophosphate, le tripolyphosphate et le phytate.

Mots clés additionnels : *Pisolithus tinctorius*, *Pinus halepensis*, *Pinus pinaster*.

I. INTRODUCTION

Many authors have shown that phosphate deficiency causes an increase in phosphatase (Pase) activities in bacteria (TORRIANI, 1960), yeast (VAN RIJN *et al.*, 1972), fungi (KOMANO, 1975 ; CALLÉJA *et al.*, 1980) and higher plants (BOUTIN *et al.*, 1981 ; DOUMAS *et al.*, 1983 ; STROTHER, 1984). Phosphatase activities also increase during development of mycorrhizal infection (MOUSAIN & SALSAC, 1982 ; GIANINAZZI-

PEARSON, 1982 ; DOUMAS, 1984). The ability of the fungal symbiont to produce acid phosphatases (EC 3.1.3.2) has been demonstrated in several studies (HO & ZAK, 1979 ; CALLÉJA & d'AUZAC, 1983). These enzymes appear to play an important role in mycorrhizal utilization of soil phosphorus (BOWEN, 1973). Furthermore, changes in the biochemical properties of certain phosphatases, particularly in the fungal partner, during phosphate deficiency (NINOMIYA *et al.*, 1977) could help to explain the mechanisms involved in the improvement of phosphate nutrition.

II. MATERIAL AND METHODS

A. Organisms and cultivation

Pinus halepensis Mill. and *Pinus pinaster* Ait. were cultivated under the conditions described by DOUMAS (1984). *Pisolithus tinctorius* (Pers.) Coker & Couch., strain F9, was cultivated in a liquid medium (CALLÉJA *et al.*, 1980). The culture medium either contained no phosphate (– P) or 2 mM potassium phosphate (+ P). After 27 days of culture the thalli were collected, washed with 1 % glucose and freeze-dried.

B. Preparation of soluble fraction

Fungus extraction was carried out in 50 mM acetate buffer (pH 5.0); 5 mM mercaptoethanol (MSH); 10 mM ethylenediaminetetracetic acid (EDTA) and 5 mM diethylethiocarbamate (DIECA); 1 % Triton X100. During the grinding, polyvinylpyrrolidone (polyclar AT) was added as 0.1 mg per gram of dry matter of the fungus. The soluble fraction was obtained by the method described by CALLÉJA *et al.* (1980). The soluble phosphatases of pines were obtained using the method described by DOUMAS (1984).

C. Purification of soluble phosphatases

The soluble phosphatases of *P. tinctorius* and of the two pines were obtained by filtration on an Ultrogel AcA202 column (IBF) and then separated by ion-exchange chromatography on DEAE-Sepharose CL6B column (IBF). The proteins were eluted with a linear gradient (0 – 0.4 M) of NaCl. The buffer used for these two steps was a 25 mM acetate buffer (pH 5.0); 5 mM MSH in the case of the fungus and a 25 mM triethanolamine-HCl buffer (pH 7.5); 5 mM MSH in the case of the pines.

D. Phosphatase assay

Acid phosphatase activity was measured in the standard way with p-nitrophenylphosphate (pNPP) by the method described by COUPÉ & d'AUZAC (1979). The inorganic phosphate released was determined by the TAUSSKY & SHORR (1953) method. Activities are expressed as micromoles of Pi released per minute ($\mu\text{mol ml}^{-1} \text{min}^{-1}$).

III. RESULTS

A. DEAE-Sepharose chromatography of fungus and pine soluble acid phosphatases

According to the spectra obtained from chromatography on DEAE-Sepharose of the phosphatases of *P. halepensis* and *P. tinctorius*, phosphate deficiency induced a similar effect in both organisms, i.e. a preferential increase in the activity of one of the phosphatases: Pase T1[–] in the *Pisolithus* and Pase H1[–] in the pine (DOUMAS *et al.*, 1983) (fig. 1 and fig. 2). In

Figure 1

DEAE-Sepharose chromatography of soluble acid phosphatases from *Pisolithus tinctorius*.

T1⁺, T2⁺, T3⁺: phosphatases from *Pisolithus* cultivated on a Pi-supplied medium.

T1[–], T2[–], T3[–]: phosphatases of *Pisolithus* cultivated on a Pi-deprived medium.

Figures above the peaks indicate the NaCl molarities corresponding to their elution.

Chromatographie sur DEAE-Sepharose des phosphatases acides solubles de *Pisolithus tinctorius*.

T1⁺, T2⁺, T3⁺: phosphatases de *Pisolithus* cultivé sur un milieu non carencé en Pi.

T1[–], T2[–], T3[–]: phosphatases de *Pisolithus* cultivé sur un milieu carencé en phosphate.

Les chiffres au-dessus des pics indiquent les molarités de NaCl correspondant à leur élution.

P. pinaster phosphate deficiency mainly increased the activity of Pase P1[–] but two other isoenzymes were derepressed by the deficiency (Pase P0 and Pase P2[–]) (fig. 3).

B. Comparison of fungus and pine soluble acid phosphatases

The Michaelis-Menten constants (Km) are shown in Tables 1a, 1b and 1c. The Km values varied between 0.08 and 0.6 mM. Lack of phosphate did not seem to modify the affinity of the enzymes of *P. tinctorius* for pNPP. In the case of the pines, the phosphatases present during phosphate starvation showed little more affinity for pNPP than their analogues occurring in the non-starved pines. Examination of the phosphatases most influenced by phosphate deficiency (Pase T1[–] of *Pisolithus*, H1[–] of *P. halepensis*, P1[–] of *P. pinaster*) showed that Pase T1[–] of the fungus had a lower Km value than Pase H1[–] and P1[–]. Inhibition constants (Ki) values showed that the phosphatases of *Pisolithus* were more sensitive to inorganic phosphate concentrations than phosphatases of *P. halepensis*.

Figure 2

DEAE-Sepharose chromatography of soluble acid phosphatases from *Pinus halepensis*.

$H1^-$, $H2^-$, $H3^-$: phosphatases from *P. tinctorius* cultivated on a Pi-deprived medium.

$H1^+$, $H2^+$, $H3^+$: phosphatases from *P. tinctorius* cultivated on a Pi-supplied medium.

Chromatographie sur DEAE-Sepharose des phosphatases acides solubles de *Pinus halepensis*.

$H1^-$, $H2^-$, $H3^-$: phosphatases issues de *P. tinctorius* cultivé en milieu carencé en Pi.

$H1^+$, $H2^+$, $H3^+$: phosphatases issues de *P. tinctorius* cultivé en milieu non carencé en Pi.

Table 1

K_m for pNPP (mM) and K_i for KH_2PO_4 (mM) of acid phosphatases of *Pisolithus tinctorius* (table 1a), *Pinus halepensis* (table 1b) and *Pinus pinaster* (table 1c) cultivated on a phosphorus-supplied (+P) or a phosphorus-deprived medium (–P).

K_m pour le pNPP (mM) et K_i pour KH_2PO_4 (mM) des phosphatases acides de *Pisolithus tinctorius* (table 1a), de *Pinus halepensis* (table 1b) et de *Pinus pinaster* (table 1c) cultivés sur un milieu avec phosphate (+P) ou sans phosphate (–P).

* Pases mainly enhanced by phosphate starvation.

* Pases principalement exaltées par la carence en phosphate.

Table 1a

	+P		–P	
	Ti ⁺ *	T2 ⁺	Ti [–]	T2 [–]
K_m	0,08	0,62	0,18	0,56
σ	0,02	0,17	0,12	0,43
K_i	3,5	3,6	2,5	0,45
σ	0,68	0,6	0,5	0,04

Table 1b

	+ P		– P		
	H2 ⁺	H3 ⁺	H1 [–] *	H2 [–]	H3 [–]
Km	0,5	0,25	1,5	1	0,5
Ki	—	10,4	11	9,5	9,5

Figure 3

DEAE-Sepharose chromatography of soluble acid phosphatases from *Pinus pinaster*.

$P1^-$, $P2^-$, $P3^-$: phosphatases from *P. pinaster* cultivated on a Pi-deprived medium.

$P1^+$, $P3^+$: phosphatases from *P. pinaster* cultivated on a Pi-supplied medium.

Chromatographie sur DEAE-Sepharose des phosphatases acides solubles de *Pinus pinaster*.

$P1^-$, $P2^-$, $P3^-$: phosphatases issues de *P. pinaster* cultivé en milieu carencé en Pi.

$P1^+$, $P3^+$: phosphatases issues de *P. pinaster* cultivé en milieu non carencé en Pi.

Table 1c

	+P	–P	
	P3 ⁺	P1 [–] *	P3 [–]
K_m	0,3	0,4	0,3

C. Specificity of the phosphatases

All the phosphatases studied in this paper are non specific as is generally the case for acid phosphatases. However these enzymes do not hydrolyse the different substrates at the same rate. The data for the pines and the fungus all indicated that the phosphate deficiency modified the hydrolysis efficiency of some substrates and especially those found naturally in the soil (trime-taphosphate, pyrophosphate, tripolyphosphate and phytate). This phenomenon was more marked for the phosphatases which were mainly increased by the phosphate deficiency ($H1^-$ for *P. halepensis*, $P1^-$ for *P. pinaster* and $T1^-$ for *P. tinctorius*) (fig. 4). We note that the hydrolysis of pNPP was less than that of the other substrates in the case of the fungal symbiont.

Figures 4

Specificity of soluble acid phosphatases from *Pisolithus tinctorius* (4a), *Pinus halepensis* (4b) and *Pinus pinaster* (4c).

Enzyme activities, expressed as a percentage of the activity measured with *p*-nitrophenyl phosphate (pNPP).

Activité spécifique des phosphatases acides solubles de *Pisolithus tinctorius* (4a), de *Pinus halepensis* (4b) et de *Pinus pinaster* (4c). Les activités enzymatiques sont exprimées en pourcentage de l'activité mesurée avec le *p*-nitrophényl phosphate (pNPP).

1 : pNPP ; 2 : trimeta P ; 3 : tripoly P ; 4 : *o*-P-serine ; 5 : bis pNPP ; 6 : phytate ; 7 : pyro P ; 8 : ATP ; 9 : ADP ; 10 : AMP ; 11 : 1-naphtyl P ; 12 : 2-naphtyl P ; 13 : glucose 1P ; 14 : glucose 6P ; 15 : β -glycero P.

IV. DISCUSSION AND CONCLUSIONS

These data show that during phosphate deficiency the fungus seems to be able to hydrolyse the phosphorylated substrates naturally occurring in the soil with a better efficiency than the pines. We can notice however that the phosphatases of *P. tinctorius* cannot hydrolyse the phytate, which is a widespread phosphorus substrate in the soils. Conversely the phosphatases of *P. pinaster* and even more those of *P. halepensis*

noticeably hydrolyse this compound. It should be interesting to research for the significance of this fact in natural conditions.

The phosphatases may play a role in releasing phosphate which is not directly assimilable by the plant (BIELESKI, 1973). One can suppose that the fungus possesses a better system of adaptation to limiting conditions than the pines since the soluble phosphatases of *P. tinctorius* have slightly more affinity for a model substrate such as pNPP and more activity for natural substrates than those of the pines. This could indicate that the phosphatases have some importance in the transport of P_i when its availability is limited (BOUTIN *et al.*, 1981 ; WOOLHOUSE, 1969). These results suggest that phosphatase activities could be a good criterion for the selection of fungi for ectomycorrhizal inoculation in plant nurseries.

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