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Purification Assay of Phosphatases Secreted by Hebeloma Cylindrosporum and Preparation of Polyclonal Antibodies*

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Abstract

Acid phosphatase activities have been measured in a phosphate-depleted medium of *Hebeloma cylindrosporum* mycelia. These activities showed a great variability depending on the substrate (sodium *para* – nitrophenyl phosphate or sodium polyphosphate) and the strain of *H. cylindrosporum* (wild dikaryotic strain or homokaryotic strains issued from this dikaryon). The purification tests were carried out on the phosphatase secreted by the wild dikaryon. After filtration, concentration and desaltation of the culture solution, the phosphatases were separated by a cation exchange chromatography (Carboxy Methyl Trisacryl). Two fractions of phosphatase activities were collected, one of which was eluted using a gradient of NaCl. The electrophoretograms of these fractions showed a high purity and the phosphatase was directly injected to a rabbit. The appearance of antibodies in the serum were detected by the ELISA test. These antibodies are useful to study the regulation of the fungal phosphatase activities in the presence of orthophosphate or the phanerogam-host.

Introduction

The mycorrhizal symbiosis improves the phosphorus nutrition of the host-plant (Harley and Smith, 1983; Mousain, 1989). Higher surface phosphatase activities were generally observed in ectomycorrhizae than in non-mycorrhizal roots (Bartlett and Lewis, 1973; Hamza, 1982; Doumas, 1984; Mousain, 1989). That is one of the hypotheses postulated to explain this improvement. The phosphatases activities of the mycosymbionts would allow the mycorrhizal host plant to mobilize phosphorus from the combined forms present in the soil or inside the cell. These activities have been

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measured essentially in the mycosymbiont mycelium in pure culture (Berjaud et al., 1987; Bousquet, 1987; Mousain, 1989). They would contribute to increase the orthophosphate pool available in the rhizosphere and to enhance membrane carrier process (Bieleski, 1973).

In ectomycorrhizal homobasidiomycetes, acid phosphatase activities show a great variability depending on the substrate and the species or the strain (Mousain et al., 1988). Moreover, a soil phosphorus deficiency increases the mycelium phosphatase activities of these fungi mainly in the cellular environment (Calleja et al., 1980). Orthophosphate is a competitive inhibitor of the aspecific phosphatases (Berjaud, 1986; Bousquet, 1987), and it could also affect the repression syntheses and/or the post-translational secretion of the enzyme (Bousquet, 1987).

In the phosphate deficient medium, the phosphatase activities secreted from twelve homokaryotic strains issued from the same *Hebeloma cylindrosporum* dikaryon have been compared against two phosphorylated substrates. The enzyme purification of the secreted fraction has been done from the dikaryotic parental strain with the objective of preparing antibodies against one of these phosphatases and its use in immunochemical tests. These antibodies are very useful to study the phosphatase regulation by orthophosphate (Bousquet, 1987).

Materials and Methods

Fungal material. Mycelia of *Hebeloma cylindrosporum* Romagnesi (wild dikaryotic strain no 111 and twelve homokaryotic strains) were cultured for 11 days on a medium containing 20 μM Pi, and then for 10 days on Pi-depleted medium (Calleja et al., 1980).

Enzyme purification. After these 21 days of growth, the culture was filtered (Sartorius filter, 0.2 μm) and then lyophilized. Lyophilisates were diluted in a buffer, that was used in all the purification procedures, containing 25 mM acetate and 0.02% NaN_3(pH 5), then desalted by gel filtration chromatography of AcA 202. The desalted fractions were applied to Carboxy Methyl Trisacryl (CM Trisacryl) column chromatography (5 × 22 cm) and eluted with the acetate buffer and a linear gradient of NaCl (0 to 0.4 M).

After chromatography, fractions containing phosphatase activity were concentrated by dialysis against 20% PEG 35 kD (Merck) in acetate buffer.

All procedures were performed at 4°C.

The salinity of the solutions were determined with a chloridorimeter according to Cotlove (1963) and Davidian (1986).

Protein measurement. Total protein of the enzymatic extracts were measured by Coomassie Brillant Blue staining (Bradford, 1976).

Phosphatase activities. Phosphatase activities were determined with sodium *p*-nitrophenyl phosphate (PNPP) or sodium polyphosphate ($Na_{20}P_{18}O_{55}$; Sigma S-6003) buffered with 25 mM sodium acetate (pH 5.0) and 5 mM β-mercaptoethanol. *Para*-nitrophenol and Pi released by PNPP were measured colorimetrically according to Coupé and d'Auzac (1979) and Taussky and Shorr (1953), respectively. Phosphatase activities were expressed in nanokatals per mg of protein (nkat mg^{-1} prot).

Polyacrylamide gel electrophoresis. It was carried out according to the method of Laemmli (1970) on 10% discontinuous polyacrylamide gel. Phosphatase activities were visualized by incubation at 30°C in 0.1 M acetate buffer (pH 5.0) containing β-naphtylphosphate (1 mg ml^{-1}) and Fast Garnet (Sigma 1 mg ml^{-1}). Protein staining was performed with silver nitrate.

Immunochemical approach. The most active fractions of peak II were concentrated by dialysis, emulsified with Freund's complete adjuvant and then injected intradermally at 10 different sites on the back of a New Zealand rabbit (Vaitukaitis, 1981). The first injection was done with 2.5 ml of enzymatic extract containing 18 μg of protein with a specific activity of 168 U mg^{-1} protein. The blood collected before the first injection (T_0) was used as control.

A booster injection was performed 4 weeks later (T_1) with 3 ml of enzymatic extract containing 64 μg of protein with a specific activity of 240 U mg^{-1} protein. Blood bleeding were repeated periodically (T_2, T_3). After coagulation and centrifugation of the supernatant, the serum was stored at −20°C. Indirect ELISA tests were performed according to Fuhrmann and Wollum (1985) in microplates using 100 μl of protein extract (1 μg protein ml^{-1}).

Results and Discussion

Comparison of acid phosphatase activities excreted by the mycelium of Hebeloma cylindrosporum *stains*

The acid phosphatase activities show a great variability depending on the substrate (sodium *para*-nitrophenyl phosphate or sodium polyphosphate) and the strain of *H. cylindrosporum* (Fig. 1). The strains are distributed in two clusters: (i) cluster with high phosphatase activities, greater than 170 nkat mg^{-1} prot, with half the homokaryotic strains, (ii) cluster with low phosphatase activities, less than 70 nkat mg^{-1} prot or 120 nkat mg^{-1} protein for polyphosphate or PNPP substrate respectively. This last cluster include the other homokaryotic strains and the parental dikaryotic strain (Fig. 1).

Secreted polyphosphatase activity represent about 52 per cent of the secreted PNPPase activity, except for strains no 19 and 22 where this activity is higher than 52 per cent. When analyzing the whole data, a positive relationship is observed

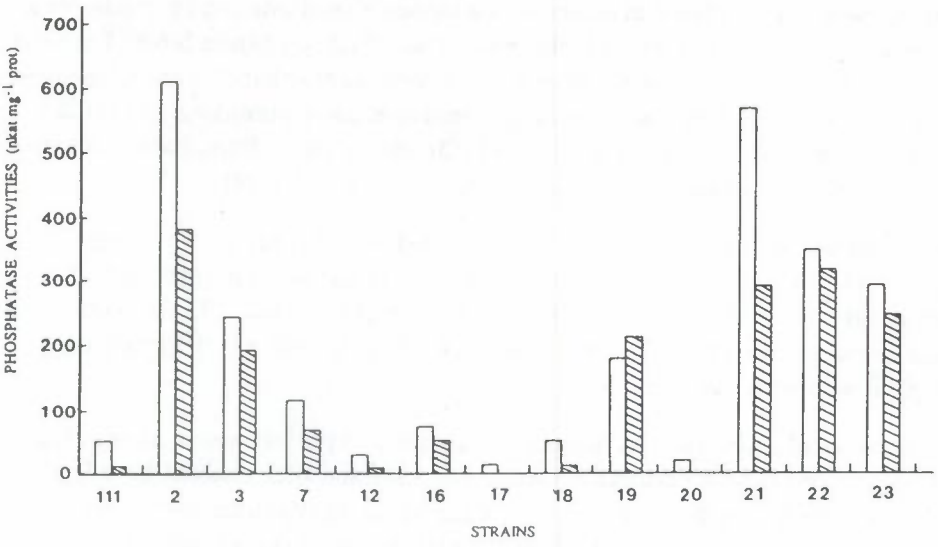

Figure 1. Phosphatases activities secreted by the mycelium of *Hebeloma cylindrosporum*. The average of *para*-nitrophenyl phosphatase activities (□) and polyphosphatase activities (▨) are expressed in nanokatals of Pi per mg of mycelial proteins. The phosphatase activities sharing the same letters into parentheses, are not significantly different ($p < 0.05$). – PNPPase activities: 2(a), 21(a), 22(b), 23(c), 3(d), 19(e) 7(f), 16(fgh), 111(gh), 18(gh), 12(gh), 20(h), 17(h). – Polyphosphatase activities: 2(a'), 22(a'b'), 21(b'c'), 23(b'c'd'), 19(c'd'), 3(c'd'), 7(e'), 16(e'), 18(e'), 111(e'), 12(e'), 20(e'), 17(e'). The correlation between the PNPase and the polyphosphatase activities is significant ($n = 47$, $r = 0.89$, $p < 0.01$). Equation of the regression:

[1] $y = 12.81 + 0.62 \times$ (y: polyphosphatase activities; x: PNPPase activities).

between the secreted PNPPase and polyphosphatase activities (Fig. 1). This result is very interesting from an experimental point of view since the polyphosphatase activity could be infered from the simple and fast measurement of the PNPPase activity.

The secreted phosphatase activities of the dikaryotic parental strain are lower than those of many homokaryotic lineages. The use of controlled dikaryotic strains, obtained from genetically compatible homokaryotic strains would be suceptible to further increase the variability in regards to the original strain.

The capacity of mycorrhizogen fungal strains to use phosphorylated substrates with phosphatases (organic phosphates or inorganic polyphosphates) available in the forest litter, phosphate fertilisants or inside the cell, could increase the phosphate nutrition of the host plant when this property is preserved in the symbiosis. When measuring phosphatase activities in the broth medium of *Hebeloma cylindrosporum*, homokaryotic strains show a high variety. This could be useful in a breeding programme to select efficient strains for the mobilization of phosphorus from organic phosphates and inorganic polyphosphates.

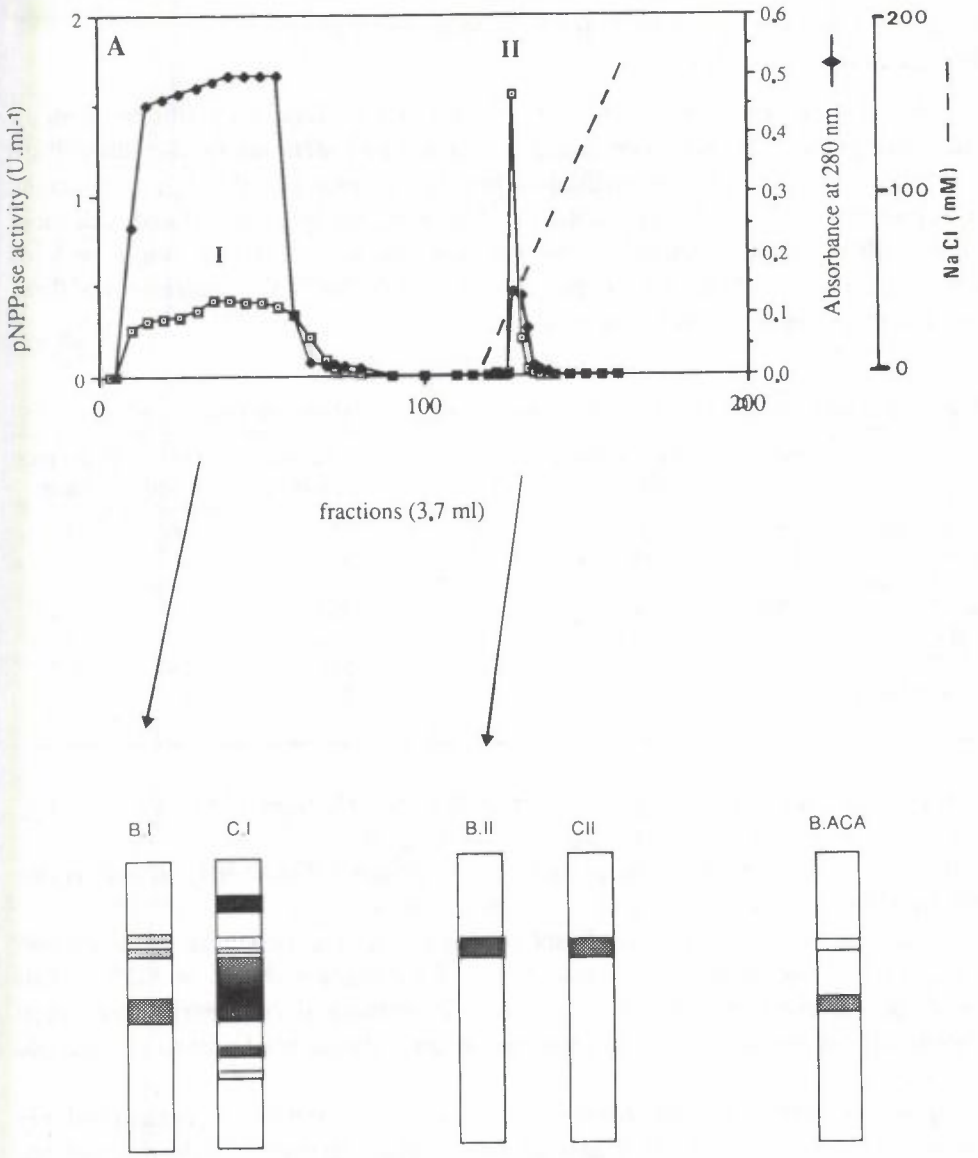

Figure 2. (A) Second chromatography of the secreted phosphatase of *Hebeloma cylindrosporum* on a column of CM Trisacryl. Column, 2.2 × 5 cm; buffer acetate 25 mM 0.02% NaN_3 (pH 5.0); flow rate, 63 ml h^{-1}. (B) Electrophoresis with pic. I (B.I) and II (B.II) and with an extract before chromatography (B. AcA): Phosphatase activity revelation. (C) Electrophoresis with pic. I (C.I) and II (C.II): Protein stained with $AgNO_3$.

Purification of a phosphatase secreted by a dikaryotic strain of Hebeloma cylindrosporum

Figure 2 shows the elution profile of a column of CM Trisacryl performed with an enzymatic extract obtained as described in Material and Methods, from media culture of 150 mycelia. Data of each purification step is presented in Table 1. Two fractions with phosphatase activities were obtained. The main one (peak I) is eluted with most of the other proteins excreted in the medium (see UV spectrum) and a peak of phosphatase activity (peak II) was eluted with 123 mM NaCl. Concentrations of these two fractions enable electrophoresis studies.

Table 1. Purification of acid phosphatases secreted from strain no. 111 on CM Trisacryl (pH 5.0).

Steps	Volume (ml)	Total activities (Units)	Proteins (mg)	Specific activities (U/mg protein)	Yield per. cent	Purification degree
crude extract	3549	223	12.5	17.9	100	1
After concentration	121.8	69.2	5.9	11.7	31	—
AcA 202	201	99.8	3.9	25.4	44.7	1.4
CM I	246	75.3	3.52	21.4	33.7	1.2
CM II	14.5	14.3	0.07	191.6	6.4	10.7
Concentration II	2.34	9.3	0.05	202	4	11.3

The electrophoresis of the pic I confirms that phosphatases of this fraction (Fig. 2, BI) are mixed with several other proteins (Fig. 2, CI).

In the second peak, only one phosphatase is detected (Fig. 2, BII) as well as one protein (Fig. 2, CII) corresponding to this phosphatase.

So, we can consider that this phosphatase is mostly representative of the protein fraction of the pic II, despite the low degree of purification shown in Table I. This could be explained by the important loss of activity during purification steps (specifically the concentration step) and the low quantities of total protein in the crude medium.

The optimal pH of the phosphatase activities of peaks I and II were determined (pH 5.4 and 5.8 respectively; Fig. 3). This result is a positive argument that the propositions observed between the two fractions were not the result of a difference of the phosphatase activities due to the reactional medium pH (which was pH 5.6).

Serum obtention against phosphatases secreted by Hebeloma cylindrosporum

The serum titer was determined by ELISA assay. The test performed on several dilutions of serum T_0, T_1, T_3, T_4 gave the results presented in Figure 4. There were no antibodies against phosphatase II in the control serum (T_0) since its titer remained

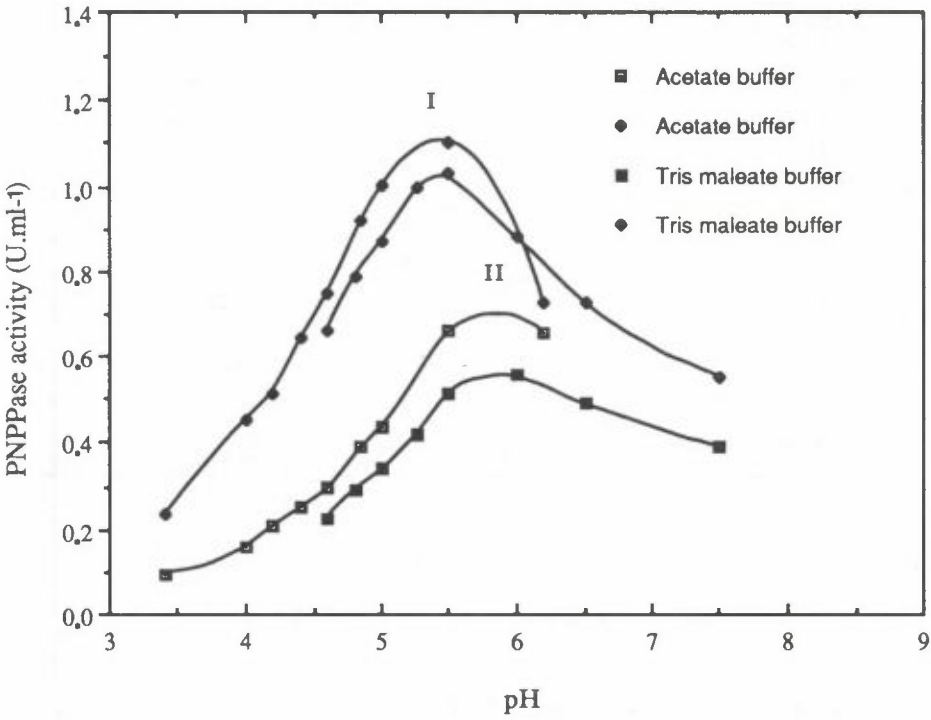

Figure 3. Effects of pH on the activity of phosphatases I and II.

negligible and constant whatever the dilution. The serum collected just before the booster injection (T_1) gave a high ELISA test response despite the rather small quantity of phosphatase injected a month earlier. The high immunogenicity power of the phosphatase II was confirmed by the titer of the last three samples collected 2 (T_1), 5 (T_2) and 7 (T_3) weeks after the booster injection.

The antibody production reached a steady state about three weeks after the booster injection and the titer of the serum diluted 2000 times were great enough to enable the immunochemical studies of these phosphatases.

The immunochemical specificity of this serum for phosphatase II (or for the total secreted phosphatase) has to be verified by immunoblotting in comparison with the total secreted protein and the total protein of the soluble fraction. A high specificity of this serum for the secreted phosphatase of the strains belonging to *Hebeloma cylindrosporum* should authorize the use of immunochemical techniques such as ELISA assay for the characterization of isolates of this species. Moreover, polyclonal antibodies and immunochemical techniques allow the detection of the enzyme even in its inactive form. Then, precise studies can be made on the regulation of the secretion

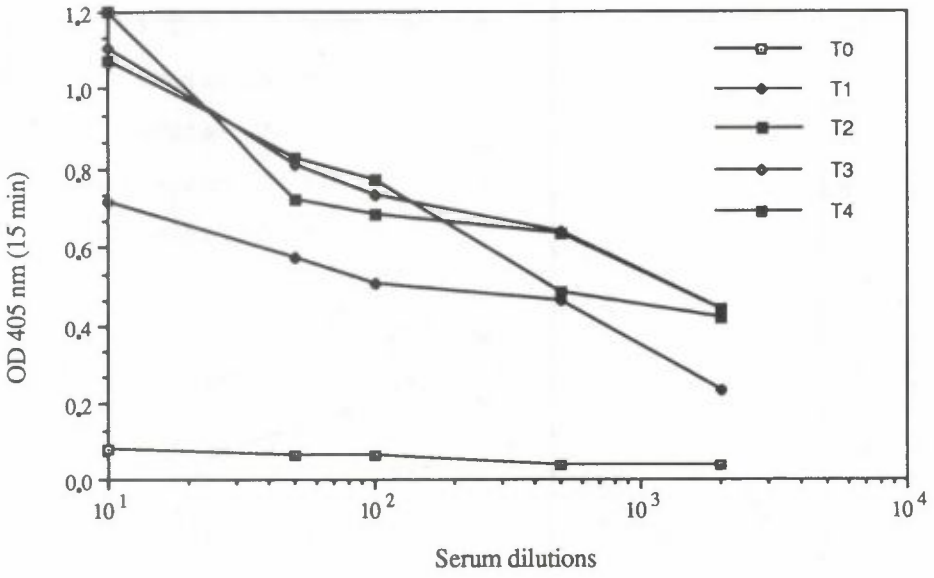

Figure 4. Serum titration using the ELISA test.

of phosphatases by main effectors (external Pi, symbiosis) and their localization in the mycorrhizae generated by *Hebeloma cylindrosporum* in association with the natural plant hosts.

The existence of a specific probe of these phosphatases could allow for their study in the natural environment which could then lead into knowing the exact role of the fungal phosphatases in the hydrolysis of phosphorylated compounds in the soil.

Finally, and if the specificity of the antiserum prepared against secreted phosphatase is confirmed, rapid, easy and sensitive characterization of mycorrhizal mycelium *in situ* open a new approach for ecological studies of ectomycorrhizal fungi in their natural environment.

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REFERENCES

Bartlett, E.M. and Lewis, D.H. 1973. Surface phosphatase activity of mycorrhizal roots of beech. *Soil Biol. Biochem.*, **5**: 249–257.

Berjaud, C. 1986. Effet de la carence en phosphate sur les phosphatases acides d'un champignon ectomycorhizogène *Pisolithus tinctorius. Thèse Doct. (nouveau régime) (spécialité: Biologie et Physiologie Végétales)*, U.S.T.L., Montpellier.

Berjaud, C., Doumas, P., Coupé, M., and Auzac, J. (d'). 1987. Properties of soluble acid phosphatases in an ectomycorrhizal fungus and in host plants subjected or not to Pi starvation. *Agronomie*, **7** (2): 95–99.

Bieleski, R.L. 1973. Phosphate pools, phosphate transport, and phosphate availability. *Annu. Rev. Plant Physiol.*, **21**, 225–252.

Bousquet, N. 1987. Etude immunoenzymatique de phosphatase acides de champignons ectomycorhiziens. *Thèse Doct. (nouveau régime) (spécialité: Biologie et Physiologie Végétales)*, U.S.T.L., Montpellier.

Bousquet, N., Cleyet-Marel, J.C., Berjaud, C., and Mousain, D. 1987. Use of immunoenzymatic techniques for characterization of ectomycorrhizal fungi. *Bulletin OEPP*, **17** (2): 306–307.

Calleja, M., Mousain, D., Lecouvreur, B., and Auzac, J. (d'). 1980. Influence de la carence phosphatée sur les activités phosphatases acides de trois champignons mycorhiziens: *Hebeloma edurum* Metrod., *Suillus granulatus* (L. ex Fr.) P Küntze et *Pisolithus tinctorius* (Pers.) Coker et Couch. *Physiol. Vég.*, **18**, 489–504.

Bradford, M.N. 1976. A rapid and sensitive method for quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Cotlove, E. 1963. Determination of the true chloride content of biological fluids and tissues. II. Analysis by simple, non isotopic methods. *Anal. Chem.*, **35**, 101–105.

Davidian, J.C. 1986. L'excrétion de H^+ par la racine: contrôles ioniques et métaboliques de la pompe à H^+ de la racine d'orge. *Thèse Doct. Etat (Sciences)*, U.S.T.L., Montpellier.

Doumas, P. 1984. Influence de la carence en phosphate et de la mycorhization sur les phosphatases racinaires de deux espèces du genre *Pinus* (*P. halepensis et P. pinaster*). *Thèse Doct. 3ème Cycle Agronomie (option: Nutrition minérale)*, U.S.T.L., Montpellier.

Fuhrmann, J. and Wollum, A.G. 1985. Simplified enzyme-linked immunosorbent assay for routine identification of *Rhizobium japonicum* antigens. *Appl. Environ. Microbiol.*, **49**, 1010–1013.

Hamza, P. 1982. Mycorhization et activités phosphatases acides chez le Pin maritime. *D.E.A. Physiologie de la nutrition des végétaux*, U.S.T.L. et I.N.R.A., Montpellier.

Harley, J.L., and Smith, S.E. 1983. *Mycorrhizal symbiosis.* Academic Press, London, New York.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Mousain, D., Bousquet, N., and Polard, C. 1988. Comparaison des activités phosphatases

d'homobasidiomycètes ectomycorhiziens en culture in vitro. *Eur. J. For. Pathol.*, **18**, 299–309.

Mousain, D., 1989. Etude de la nutrition phosphatée de symbiotes ectomycorhiziens. *Thèse Doct. Etat (Sciences)*, U.S.T.L., Montpellier.

Taussky, H.H., and Shorr, E. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **202**, 675–685.

Vaitukaitis, J.L. 1981. Production of antisera with small doses of immunogen: multiple intradermal injections. *Methods Enzymol.* **73**, 46–52.