

Full Length Research Paper

## Acid phosphatase activity of the pantropical fungus *Scleroderma sinnamariense* in pure culture

Eneke Esoeyang Tambe Bechem

Department of Botany and Plant Physiology, Faculty of Science University of Buea, P. O. Box 63, Buea, Cameroon.

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Ectomycorrhizal fungi have been shown to improve on the phosphorus nutrition of their plant partners and acid phosphatase activities are thought to be important in the uptake process. The effect of nitrogen source and presence/absence of phosphorus in the experimental medium on acid phosphatase activity of *Scleroderma sinnamariense*, an ectomycorrhizal fungus associated with *Gnetum* spp. in Cameroon, was determined in comparison to *Pisolithus tinctorius*. The effect of internal mycelial phosphorus status of the inoculum on acid phosphatase activity was also studied. Results show that the absence of phosphorus in the assay medium stimulated cell bound acid phosphatase activity. Extracellular phosphatase activity was less than cell bound activity by a factor of 100.

**Key words:** Acid phosphatase, cell-bound activity, *Scleroderma sinnamariense*, ectomycorrhizal fungus.

### INTRODUCTION

Some ectomycorrhizal (EM) fungi have the potential to mobilise P from organic polymers (Read and Perez-Moreno, 2003). It is believed that in ecosystems characterised by the retention of P as organic complexes in soil, selection would favour systems with well-developed saprotrophic capabilities. The abilities of EM fungi to access organic P sources depend on the activity of enzymes such as acid phosphatases.

There is evidence that EM fungi produce surface phosphatases which may permit access to poorly soluble organic phosphates in soil. Baghel et al. (2009) demonstrated the production of acid phosphatase by *Cantharellus tropicalis*, a tropical EM fungus. Sharma et al. (2010), went further to illustrate the fact that mycelial growth and the ability of this fungus to produce acid phosphatase was affected by external factors such as pH, temperature, nitrogen, carbon and P sources of the assay medium. These external factors are also believed to influence the efficiency of the EM fungi in supplying its host plant with vital nutrients such as N and P. Read and Perez-Moreno (2003), showed that EM fungal species

could take up P from litter and natural substrates. Several studies have reported the acid phosphatase activity of genera such as *Amanita*, *Suillus*, *Paxillus*, etc (Antibus et al., 1986; Cullings et al., 2008; Kieliszewska-Rokicka, 1992). However, most of these studies were carried out on temperate species. More of such studies are needed on tropical species so as to get a full understanding of the role of these enzymes in EM functioning in tropical forest ecosystems.

In light of the potential role of EM fungi in P nutrition and the vital importance of organic P pools in tropical forest soils, the present study was targeted to evaluate the phosphatase activities in *Scleroderma sinnamariense*, an EM fungus associated with *Gnetum* spp. in Cameroon. Bechem and Alexander (2012a), had shown that *Gnetum* spp. were exclusively ectomycorrhizal in Cameroon. This fungus was able to solubilise insoluble P sources in pure culture (Bechem, 2011). EM *Gnetum* plants also showed better nutrient uptake and growth as compared to non-mycorrhizal plants (Bechem and Alexander, 2012b). Information from these studies would

**Table 1.** Inoculum N/P status and corresponding composition of assay medium on which acid phosphatase activity was evaluated.

Inoculum N/P status	Enzyme assay medium composition
+N <sup>+</sup> + Pi	MMN + N <sup>+</sup> + Pi
+N <sup>+</sup> + Pi	MMN-N <sup>+</sup> -Pi
+N <sup>+</sup> - Pi	MMN + N <sup>+</sup> - Pi
+N <sup>+</sup> - Pi	MMN-N <sup>+</sup> -Pi
+ Peptone +Pi	MMN + Peptone + Pi
+Peptone +Pi	MMN + Peptone - Pi

+N<sup>+</sup>, with ammonium N; -N<sup>+</sup>, without ammonium N; +Peptone, with peptone N; +Pi, with inorganic P; -Pi, without inorganic P.

help in establishing a proper management plan for this plant which is threatened in its natural environment. *Pisolithus tinctorius*, a broad host range EM fungus was included in this study so as to provide comparative data.

The main objective of this experiment was to assay extracellular and wall bound acid phosphatase activity under the following conditions: (i) different N sources in experimental medium (ammonium, peptone and no N source), (ii) different internal mycelia P status of inoculum (P sufficient and P starved), and (iii) presence/absence of Pi in the experimental medium.

## MATERIALS AND METHODS

The basic approach adapted was the measurement of *p*-nitrophenol (pNP) released from *p*-nitrophenol phosphate by the action of acid phosphatase produced by *S. sinnamariense* and *P. tinctorius*. The *S. sinnamariense* was isolated from *G. africanum* EM root tips from Cameroon, whilst the pure culture of *P. tinctorius* was obtained from Australia. The methodology for this assay was a modification of the one used by Tibbett et al. (1998) and Kropp (1990).

### Starter cultures

Prior to the enzyme assay, each isolate was grown for 30 days on modified Melin-Nokrans (MMN) nutrient agar (Marx, 1969) modified as follows: (i) MMN with ammonium N and inorganic P (+N<sup>+</sup>/+Pi), (ii) MMN with ammonium N and no P (+N<sup>+</sup>/-Pi), and (iii) MMN with peptone N and inorganic P (Peptone/+Pi)

### Preparation of modified universal buffer (MUB)

The buffer stock solution (Skujins et al., 1962) consisted of tris (hydroxymethyl), 3.025 g aminomethane, 2.9 g maleic acid, 3.5 g citric acid, 1.57 g boric acid, 1 M sodium hydroxide (NaOH) solution (122 ml) and distilled water added to give a final volume of 250 ml. The buffer solution used in the assay was prepared by taking 50 ml of the stock solution and titrating with 0.1 M hydrochloric acid (HCl) to a pH of 5.5. Distilled water was then added to give 250 ml.

### Assay media

The assay media was MMN nutrient solution with the following modifications: (i) MMN/+N<sup>+</sup>/+Pi, (ii) MMN/+N<sup>+</sup>/-Pi, (iii) MMN/-N<sup>+</sup>/-Pi, (iv) MMN/Peptone/+Pi, and (v) MMN/Peptone/-Pi.

The substrate in this assay consisted of 0.115 M disodium-*p*-nitrophenyl phosphate in MUB pH 5.5. Mycelia of different N/P

status were used to inoculate assay media in the order shown in Table 1.

Each fungal species with internal mycelial status as indicated on Table 1 was evaluated on the corresponding assay medium. Each inoculum and medium combination was run in three replicates.

## Experimental procedure

In the assay methodology, 25 ml of MMN nutrient solution as modified above was poured into Petri dishes. Each dish was inoculated with a 5 mm diameter fungal mycelium cut from the edge of an actively growing starter culture. Plates were incubated at 30°C in the dark for 30 days. Mycelia were harvested by filtration and filtrate was collected. Recovered mycelial mat was washed several times with MUB pH 5.5. A 5 mm cork borer was flame sterilised, allowed to cool and used to collect a disk of fungal mycelia from the harvested mycelia. The remainder of the mycelia were dried overnight at 80°C, and cooled in a desiccator before weighing.

The disk of mycelia mat and 1 ml filtrate were transferred into test tubes containing 4.5 and 4 ml MUB pH 5.5, respectively. Tubes and content were incubated at 30°C in a hot water bath for 5 min prior to the addition of 1 ml of 0.115 M disodium-*p*-nitrophenyl phosphate. They were then incubated at 30°C for 1 h in the dark. Following incubation, the reaction was stopped by the addition of 4.5 and 4 ml of 1 M NaOH to tubes containing mycelium and filtrate respectively; bringing the final test tube volume to 10 ml. Assayed mycelial disks were filtered from tubes.

The absorbance of liberated pNP in the filtrates, was measured at 410 nm using a CE 373 Linear Readout Grating Spectrophotometer manufactured by CECIL instruments. This was measured against a range of standards made from 0 to 10 mg/l by diluting pNP in equal parts of modified universal buffer and 1 M NaOH. A number of controls, which were run alongside the experiment, consisted of: (i) Same volume of buffer and substrate only, (ii) buffer and filtrate only, and (iii) buffer and mycelium only. Each treatment was replicated three times.

For wall bound phosphatases, the dry weight from each assayed mycelial disk was required to calculate cleaved substrate on a mass basis. For extracellular phosphatases, total fungal dry weight in each Petri dish was required to calculate cleaved substrate on a mass basis. Consequently, the assayed mycelia disks together with the unassayed portion of mycelium were dried overnight at 80°C and cooled in a desiccator prior to weighing. The enzymatic activity was expressed as μmol pNP released per hour per gram fungal dry weight at 30°C.

## Statistical analysis

T-test was run to evaluate the effect of inoculum N and P status as well as assay medium N, P status on both cell bound and extracellular acid phosphatase activity.

**Table 2.** Mean total dry weight (mg) of *Scleroderma* and *Pisolithus* isolates grown over 30 days at 30°C on MMN with different N sources and with or without inorganic P. Each value is a mean of three replicates.

Internal mycelia N/P status of inoculum	Enzyme assay medium composition	<i>Scleroderma</i>	<i>Pisolithus</i>
+N <sup>+</sup> + Pi	MMN + N <sup>+</sup> + Pi	20.8 (5.1)	22.73 (0.9)
+N <sup>+</sup> + Pi	MMN-N <sup>+</sup> -Pi	3.4 (0.8)	4.10 (1.1)
+N <sup>+</sup> - Pi	MMN + N <sup>+</sup> - Pi	19.8 (4.7)	5.57 (1.3)
+N <sup>+</sup> - Pi	MMN-N <sup>+</sup> -Pi	2.93 (0.7)	3.0 (0.7)
+ Peptone +Pi	MMN + Peptone + Pi	25.27 (6.3)	11.43 (2.7)
+Peptone +Pi	MMN + Peptone - Pi	10.07 (2.5)	8.03 (1.8)

Values in parentheses represent the standard error of the mean. +N<sup>+</sup>, with ammonium N; -N<sup>+</sup>, without ammonium N; +Peptone, with peptone N; +Pi, with inorganic P; -Pi, without inorganic P.

## RESULTS

### Growth

*Scleroderma* and *Pisolithus* produced measureable biomass following growth for 30 days on all assay media at 30°C. The biomass yields (mg dry weight) are shown in Table 2. *Scleroderma* produced a maximum yield of 25.27 ( $\pm$  6.3) mg on peptone-N whilst *Pisolithus* gave a maximum yield of 22.73 ( $\pm$  0.9) mg on ammonium-N. Both P sufficient and P starved *Scleroderma* and *Pisolithus* produced little biomass on medium devoid of N and P source. Growth of P starved *Scleroderma* on medium devoid of a P source was better than growth of P starved *Pisolithus* on the same medium (Table 2).

### Phosphatase activity on ammonium based media

Cell bound phosphatase activity of *Scleroderma* and *Pisolithus* were comparable (Figure 1a and b). The greatest activity was shown by P starved mycelium (N/no P) assayed in medium lacking inorganic P (N/no P). For both species, this activity was greater ( $P < 0.05$ ) than any of the other treatments. It appears that the absence of N from the assay medium suppressed activity that is activity of P starved inoculum in P starved assay medium was greater than the activity of P starved inoculum in both N and P starved assay medium. The presence of P in the medium also suppressed activity that is activity of P starved inoculum in P starved assay medium was greater than the activity of N and P sufficient inoculum in N and P sufficient assay medium (Figure 1).

Extracellular phosphatase activity (Figure 2a and b) was less than cell bound activity by a factor of 100. Again the greatest activity was shown by P starved mycelia assayed in medium lacking inorganic P. The absence of N from the assay medium appeared not to affect extracellular activity to the same extent as cell bound activity.

### Phosphatase activity on peptone based media

In the case of *Scleroderma*, cell bound activity was about 500 times greater than extracellular activity. Cell bound

activity was almost completely suppressed by the presence of Pi in the assay medium (Figure 3a), but there was no effect of Pi on the low levels of extracellular phosphatase detected (Figure 3c).

Cell bound activity of *Pisolithus* (Figure 3b) was less than that of *Scleroderma* and completely suppressed by Pi. In contrast, extracellular activity of *Pisolithus* (Figure 3d) was five times that of *Scleroderma* and was suppressed by Pi.

## DISCUSSION

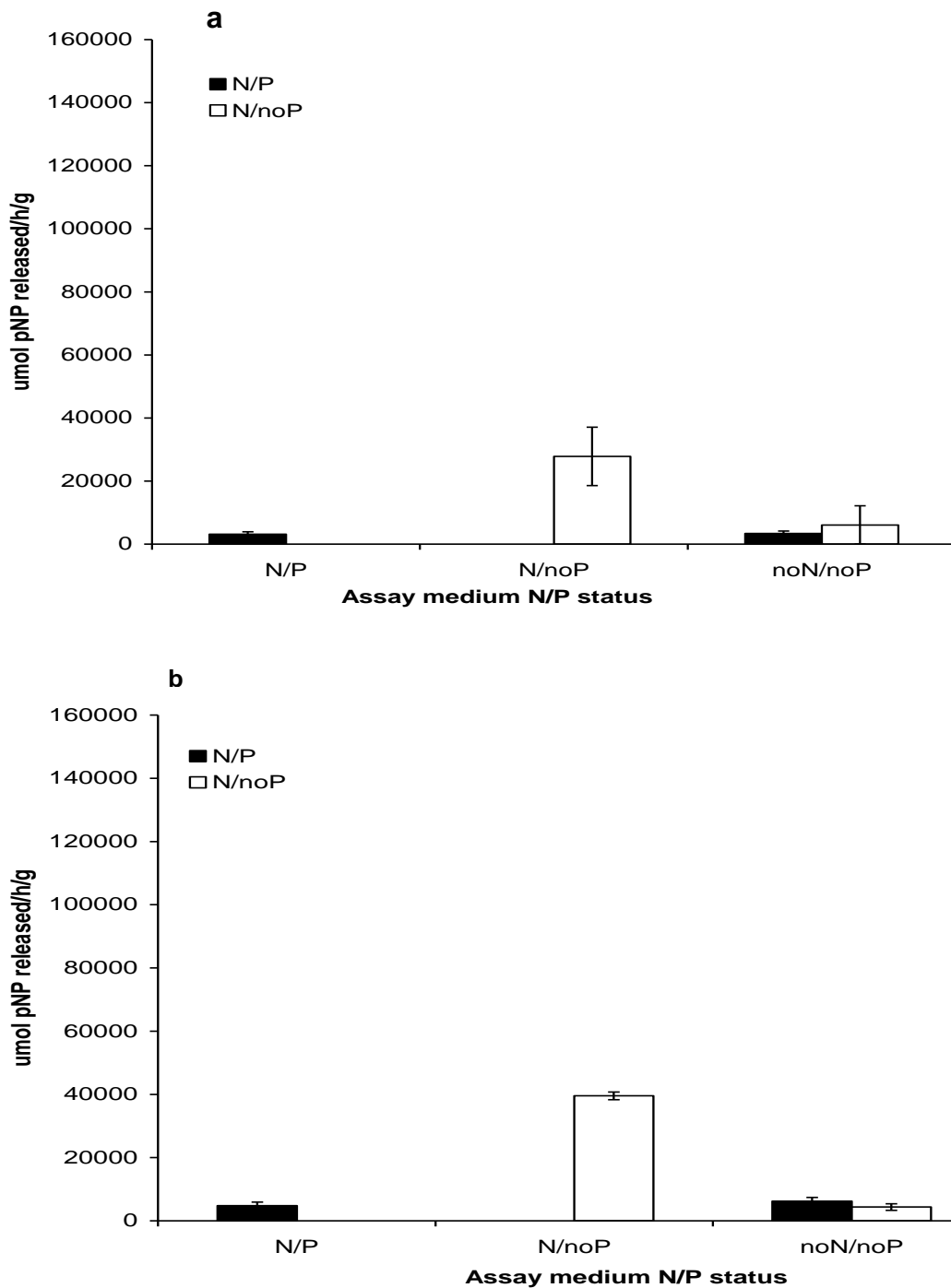
### Fungal growth

In this study, both fungi produced measurable biomass on all media and inoculum combinations. However, growth was poor in assay medium with no N and P source, an observation, which reinforced the point that N and P were necessary for proper growth and development of living organisms including EM fungi. P starved *Scleroderma* showed better growth than the P starved *Pisolithus* on assay solution lacking inorganic P with presence of either ammonium-N or peptone-N. This observation suggested that the *Scleroderma* isolate had a larger internal store of P than *Pisolithus*. Overall, *Scleroderma* grew best in medium containing peptone-N whilst *Pisolithus* showed maximum growth on ammonium-N. This ability of a fungus to grow better on organic N sources had also been observed in other similar studies like Abuzinadah and Read (1988) and Sharma et al. (2010).

### Acid phosphatase

The *Pisolithus* isolate showed a wider range of variation in extracellular enzyme activity than the *Scleroderma* isolate. However, for cell bound enzyme activity, a wider variation was observed with the *Scleroderma* isolate.

The results in this study indicate that levels of *p*-nitrophenol phosphatase (*p*NPPase) activity of some species of EM fungi grown in pure culture were partially influenced by the N and P source as well as status of the inocula and assay medium. Sharma et al. (2010) had a



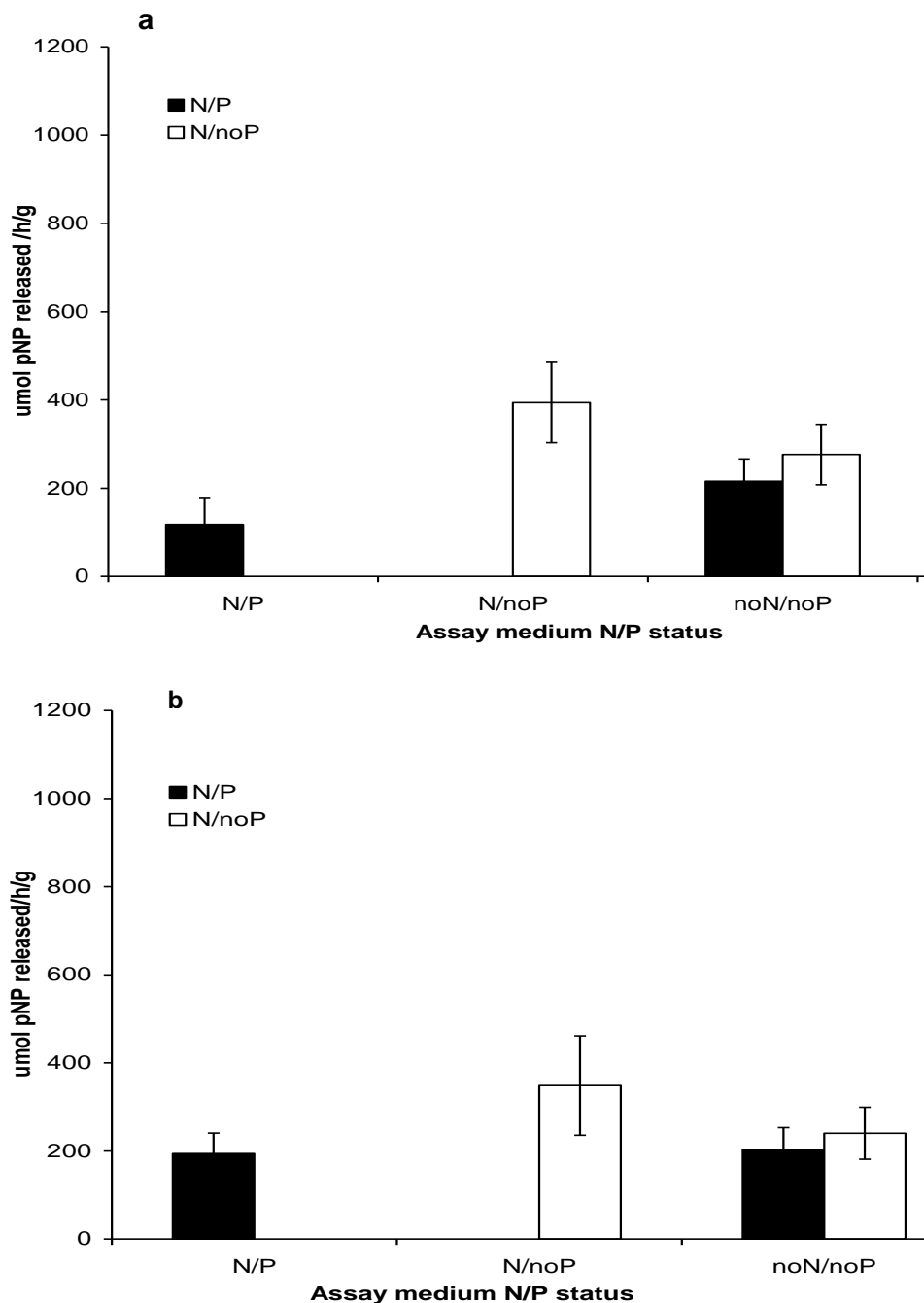
**Figure 1.** Variations in cell bound acid phosphatase activity of *Scleroderma* (a) and *Pisolithus* (b) following 30 days of growth on different N and P treatments. Each value represents a mean of three replicates. Vertical bars represent the standard error of mean. N and P in legend represent inoculum N, P status.

similar observation in which acid phosphatase production by *Cantharellus tropicalis* varied with N, P and carbon sources of the medium.

Cell bound phosphatase activity of *Scleroderma* was greatly influenced by the presence of inorganic P and peptone-N in the inoculum. This was demonstrated in the

absence of inorganic P and presence of peptone-N in assay solution. On the other hand, the activity of this enzyme in *Pisolithus* was only slightly affected by presence of inorganic P and absence of an N source in the assay medium.

Extracellular phosphatase activity of *Scleroderma* was

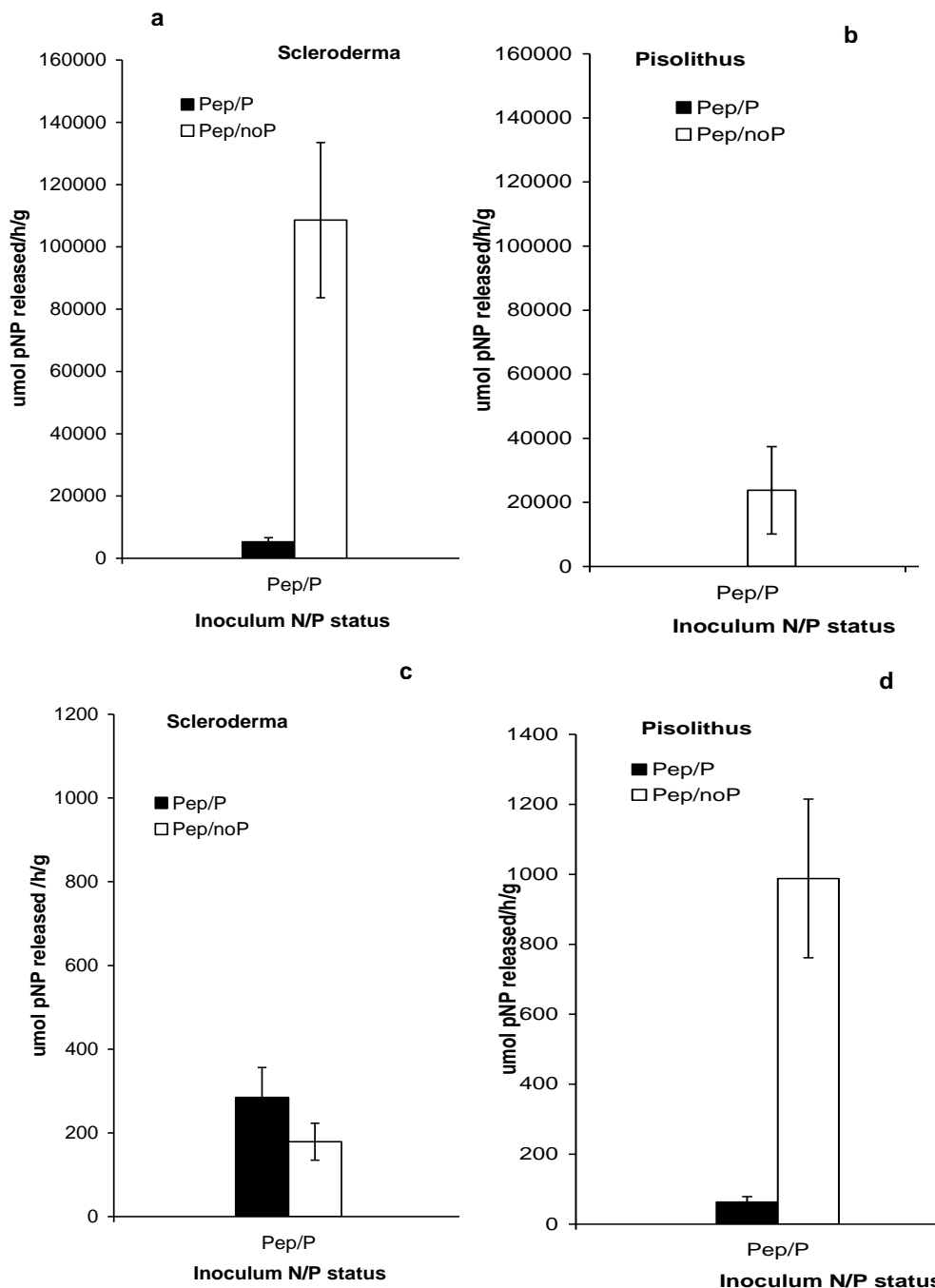


**Figure 2.** Extracellular phosphatase activity of *Scleroderma* (i) and *Pisolithus* (ii). Vertical bars represent standard error of mean. Each point is a mean of three replicates. N and P in legend denote the inoculum N and P status.

very much affected by the presence of peptone as inoculum and assay solution N source. Although this observation was similar to that in *Pisolithus*, in addition, the presence of inorganic P in inoculum and absence of P in assay medium also affected the activity of this enzyme.

This study shows that a source of N in the assay solution is vital for the activation of cell bound acid

phosphatase activity. Overall, activity was slightly higher when a P source was absent in the assay medium. This observation confirmed findings by others that the production of acid phosphatase was enhanced in the absence of Pi in EM fungi (Kropp, 1990; Antibus et al., 1992; Tibbett et al., 1998). The highest expression of both cell bound and extracellular acid phosphatase activity was observed following growth with peptone as sole



**Figure 3.** Cell bound (a, b) and extracellular (c, d) acid phosphatase activity of *Scleroderma* and *Pisolithus* in medium containing peptone as sole N source. Vertical bars represent standard error of mean. Each point is a mean of three replicates. The Pep and P in the legend denotes the assay medium N and P status.

N source but in the absence of Pi. This indicated that inorganic P was an important regulator of both cell bound and extracellular phosphatase activity. Such activity was also dependent on the N source.

Previous studies with *Scleroderma* demonstrated that this fungus preferred peptone as sole N source whilst the *Pisolithus* isolate preferred ammonium-N. A similar growth

response was observed in this experiment and this showed that growth was not proportional to acid phosphatase production. Such an observation leads one to think that P may not have been the only limiting factor of mycelia growth in this experiment.

The ability to restrict secretion when a product is plentiful and to increase production where product is limited,

suggests that EM fungi have an economic regulation of phosphatase production brought into harmony by environmental Pi concentration and therefore aimed at external (soil) substrates (Tibbett et al., 1998). With P being one of the least available plant nutrients found in soil, phosphatases are believed to be very important in their uptake. Phosphatases produced by plants and microbes are presumed to convert organic P into available inorganic P, which is then absorbed by plants. Therefore the observations from this experiment would shed some light into the possible mechanisms by which these fungi in mycorrhizal associations facilitate their host plants with P uptake in a P-limiting environment.

In this study, cell bound phosphatase was the most active enzyme, an observation similar to that of McElhinney and Mitchell (1993). This is probably because cell bound phosphatases are thought to be most important in cleavage and procurement of Pi (Calleja et al., 1980). Nonetheless, in the study carried out by Tibbett et al. (1998), it was observed that extracellular phosphatases accounted for the largest fraction of pNPPase. They put forward an argument that intimate contact between substrate and enzyme was not the only factor required for enzyme activity. They emphasised the fact that proper alignment of both substrate and enzyme was also vital for substrate to be transformed to product.

Acid phosphatases are thought to be vital in the uptake of phosphate and eventual transfer to the plant host. The fact that both fungi could express acid phosphatase activity in pure culture does not necessarily mean that they could access unavailable P sources when in symbiosis. There is a possibility that the phosphatases produced in symbiosis might be slightly different from those produced in pure culture. But some researchers like Gianinazzi-Pearson and Gianinazzi (1989) showed that acid phosphatases produced by EM hyphae are the same as those produced by the fungus in pure culture.

Since P acquisition is one of the most important functions of the EM symbiosis, acid phosphatase activity is of considerable interest in selecting strains for inoculation experiments.

In view of the association of *Gnetum* plants with organic soils of low P status, the demonstration that *S. sinnamariense* (the EM endophyte of *Gnetum* spp. in Cameroon) can produce high acid phosphatase activity in organic environments with low Pi may be of considerable importance in the establishment and growth of *Gnetum* plants in tropical soil communities. In such an environment, absorption of P would be enhanced by the mycorrhizal system. The phosphatase released by the mycorrhizal fungal mantle and attached mycelium ramifying through the soil would probably catalyse the hydrolysis of complex P compounds found in the organic layer, into more readily absorbed forms. Organic acids produced by the fungus may lead to the solubilisation of insoluble P sources, which would be taken up by the fungus and eventually translocated to the plant host. This

improved P nutritional status would make the plant fitter so as to compete for other limiting factors in its environment.

The results of this study indicate that the N and P status of the mycorrhizosphere is an important determinant of acid phosphatase activity. For there to be any meaningful and concrete conclusion, more of such studies are needed on tropical mycorrhizal fungi species.

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