ACTIVITY OF ACID PHOSPHATASE IN THE ECTOMYCORRHIZAL FUNGUS *CANTHARELLUS TROPICALIS* UNDER CONTROLLED CONDITIONS

RK Baghel, R Sharma* & AK Pandey

Mycological Research Laboratory, Department of Biological Sciences, R.D. University, Jabalpur, M.P. India

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BAGHEL RK, SHARMA R & PANDEY AK. 2009. Activity of acid phosphatase in the ectomycorrhizal fungus C*antharellus tropicalis* under controlled conditions. Ectomycorrhizal fungi benefit host plant by absorbing phosphorus with the help of acid and alkaline phosphatases. Acid and alkaline phosphatases are cell wall-bound enzymes. *Cantharellus tropicalis* were grown in axenic cultures to determine the activity of acid phosphatase by a quantitative colorimetric method. The effects of pH and temperature on the activity of surface acid phosphatase were also studied. Results showed that the optimal pH and temperature of the *C. tropicalis* strain were 4 and 40 °C respectively.

Keywords: Alkaline phosphatase, cell wall-bound enzymes, p-NPP, *Dendrocalamus strictus,* pH, temperature

BAGHEL RK, SHARMA R & PANDEY AK. 2009. Aktiviti asid fosfatase dalam kulat ektomikoriza C*antharellus tropicalis* dalam keadaan terkawal. Kulat ektomikoriza memberi faedah kepada tumbuhan perumah kerana ia menyerap fosforus dengan bantuan asid fosfatase serta alkali fosfatase. Asid fosfatase serta alkali fosfatase merupakan enzim yang terikat pada dinding sel. *Cantharellus tropicalis* dibiakkan untuk menentukan aktiviti asid fosfatase menggunakan kaedah kolorimetri. Kesan pH dan suhu terhadap aktiviti asid fosfatase permukaan turut dikaji. Keputusan menunjukkan bahawa pH dan suhu optimum strain *C. tropicalis* adalah masing-masing 4 dan 40 °C.

INTRODUCTION

Ectomycorrhizal associations are essential especially to forest trees growing in soils low in fertility. Ectomycorrhizal fungi can help plants in utilizing phosphorus (P), protect them from root pathogens and provide resistance to drought. There has been considerable interest in possible utilization of ectomycorrhiza as inoculum for nursery inoculation for forest tree regeneration. In the absence of anthropogenic influences, soil nutrients occur in organic compounds usually inaccessible to plants. Nutrient uptake by forest trees depends upon ectomycorrhizal fungi colonizing fine roots. Ectomycorrhizal fungi use organic forms of soil nutrients through production of extracellular enzymes (Aučina *et al.* 2007) as an adaptation for plants to colonize soils (Read & Perez-Moreno 2003).

In forest ecosystems, P is one of the most important growth-limiting nutrients for plants. In soil, there are three main sources of P, namely, in solution as orthophosphate, ionically bound in primary and secondary minerals and bound in organic compounds (Jennings 1995). The major part of soil P (sometimes as much as 90%) is sequestered in the organic compounds phosphomonoesters and phosphodiesters (Nygren 2008). The P uptake by forest trees has been shown to be greatly enhanced in plants colonized by ectomycorrhizal fungi (Conn & Dighton 2000, Courty *et al.* 2005). The phosphatase enzyme capabilities of ectomycorrhizal fungi are continuously distributed between species rather than restricted to a particular taxonomic group (Nygren 2008). Sheathing mycorrhizal fungi have been shown to possess phosphatase enzymes which can hydrolyze inositol hexaphosphate. Phosphatase production by basidiomycete fungi in liquid culture is independent of P in the medium. Saprophytic basidiomycetes tend to incorporate hydrolysed phosphate into their

**Author for correspondence. Present address: 21, Kundan Residency, 4th Mile Tilhari, PO Box Gauraiyaghat via RFRC, Mandla Road, Jabalpur 482 021, Madhya Pradesh, India. E-mail: rsmushrooms@rediffmail.com*

biomass. In contrast mycorrhizal fungi release more hydrolysed phosphate into solution than they absorb (Dighton 1983).

Ectomycorrhizal fungi are able to secrete hydrolytic enzymes involved in the degradation of organic matter (Abuzinadah & Read 1986, Burns & Dick 2002, Lindahl *et al.* 2005). Acid phosphatases solubilize insoluble forms of P not readily available to uninfected plant roots (Tibbett *et al.* 1998a). These enzymes are generally bound to the outer cell walls (Rast *et al.* 2003, Alvarez *et al.* 2004). Phosphatase activities of ectomycorrhizal fungi can vary between species, resulting in different efficiency of P utilization of host plant (Ho & Zak 1979, Dighton 1983). These enzymes are in direct contact with soil environment but are able to adapt to various soil conditions and maintain activity. However, soil components, pH and trace elements can modify the conformation of enzymes and affect their activities (Eivazi & Tabatabai 1977, Geiger *et al.* 1998). Activities of acid phosphatase are found to differ significantly amongst ectomycorrhiza synthesized with different fungi and among different species of the same fungi (Antibus *et al.* 1986, Buée *et al.* 2005, 2007, Courty *et al.* 2006). Ectomycorrhizal phosphatases generally have a pH and temperature optimum approaching that of their native soil.

While studying P utilization by ectomycorrhizal fungi, Ho and Tilak (1988) adapted a simple method to assess the acid phosphatase activity of large isolates. The purpose of the present study was to determine the effectiveness of this technique and to estimate the acid phosphatase activity of *Cantharellus tropicalis,* an edible mushroom in Central India, and determine optimum conditions of pH and temperature for its ectomycorrhizal activity with *Dendrocalamus strictus.*

MATERIALS AND METHODS

Culture conditions

The culture of *C. tropicalis* was obtained from fruit bodies collected from *Dendrocalamus* forest of Baiyar-Balaghat, Madhya Pradesh during forays in mushroom seasons in the rainy season of 2007. The culture was deposited at the Regional Fungal Germplasm Collection Centre (FGCC), Department of Biological Sciences, Rani Durgavati University, Jabalpur.

Estimation of acid phosphatase

Pure culture of *C. tropicalis* was grown in Petri dishes containing agar medium (0.05 g $CaCl_2$, 0.025 g NaCl, 0.5 g KH_2PO_4, 5.25 g $(NH_4)_2HPO_4$, 0.154 g $MgSO_4.7H_2O$, 100 µg Thiamine HCl and 1.5 g agar (Hi-Media, India) per 1000 ml of distilled water) adjusted to 5.5 with 5 N HCl or 1 mM and/or 5 M NaOH. The experiment was conducted in triplicates and each inoculation was carried out using one 9-mm mycelial plug of actively growing culture of mushroom. Sterilized discs of Whatman filter paper No. 1 (2-cm diameter) were transferred aseptically into 0.1 M p-nitrophenyl phosphate (p-NPP) solutions prepared in modified universal buffer (pH 5.5). The buffer was prepared by titrating 120 ml of stock buffer (7.26 g tris-hydroxymethyl amino methane buffer, 6.96 g maleic acid, 8.4 g citric acid, 3.7 g boric acid, 4 ml 0.5 M NaOH, made up to 120 ml with distilled water) (Skujins *et al.* 1962) and allowing it to saturate. A disc of Whatman paper (9-mm diameter) was placed aseptically over the fungal colony in each plate and incubated for two hours at 26 °C. After incubation the colour of the disc turned yellow due to hydrolysis of p-NPP caused by acid phosphatase activity of fungus. The intensity of colour was compared against a series of standards developed by dissolving different concentrations of p-nitrophenol (10, 50, 100, 300, 500, 1000 mM) in modified universal buffer (Ho & Tilak 1988).

The above method for qualitative assessment of acid phosphatase activity was successfully tried with some modifications using *C. tropicalis* isolate. The liquid medium with 0.1 M p-NPP (50 ml/1000 ml) was poured into Petri dishes and inoculated with cultures of *C. tropicalis.* After incubation for 5, 10 and 15 days, the liquid media turned yellow due to the secretion of acid phosphatase. The intensity in colour was observed against a white paper to differentiate the activity of young mycelia near the edge of colony from the dead or slow growing mycelia at the centre.

Acid phosphatase activity at different pH values and temperatures

Measurement of enzyme activity was done according to Tibbett *et al.* (1998a) and Antibus *et al.* (1986) with slight modifications according to laboratory requirements. The mycelia of *C.*

tropicalis, separated from culture medium by filtration, were washed in modified universal buffer. Mycelia were placed in 30 ml screw cap test tubes followed by 4 ml of modified universal buffer of different pH (1–7) and 2 ml of p-NPP solution (made in the same buffer). The test tubes were then incubated at 37 °C for two hours. Higher pH values, up to 12, were also tested for possible alkaline phosphatase production.

Optimum temperature was determined by incubating acid phosphatase with buffered p-NPP at different temperatures, namely, 5, 10, 15, 20, 30, 35, 40 °C for 60 min. After incubation 4 ml 0.5 M NaOH was added to the screw cap test tubes and the contents were mixed well for a few seconds before filtering the supernatant through Whatman No. 1 filter paper. The yellow colour complex of p-nitrophenyl was measured using a spectrophotometer (Scigenics 118) at 410 nm. The amount of p-nitrophenyl released was calculated by referring to a calibration graph and comparison with a standard curve.

RESULTS AND DISCUSSION

The disc assay conducted in this study showed differences in the activities of accessible acid phosphatase between test plates of *C. tropicalis* of different ages (0, 5, 10, 15 days). The highest activity, indicated by the dark yellow colour of cultures, was observed in 15-day-old plates while the 5- and 10-day-old plates had intermediate to white and also dark yellow cultures (results not shown). There was a difference in colour between young cultures and those near the peripheral regions of growth.

Cantharellus tropicalis show maximum growth in acidic culture medium (Sharma 2008) and this was also observed in this study (Table 1). The p-nitrophenol phosphatase (p-NPPase) activity of *C. tropicalis* isolate showed a stable activity at a pH range of 3–4 with optimum activity at pH 4 (4.16 mg g^{-1} mdw) even though the mycelial biomass production was less compared with pH 3 (170 vs 180 mg respectively). The activity of p-NPPase for *C. tropicalis* isolate dropped significantly above pH 4.0 (Table 1). Experiments conducted at higher pH up to 12 to detect any alkaline phosphatase activity gave negative results. Surface-bound phosphoesterases activities of *Paxillus involutus, Austropaxillus boletinoides, Descolea antartica, Cenococcum geophilum* and *Pisolithus tinctorius* have been reported at pH 3–7 (Alvarez *et al.* 2005). Investigations by Antibus *et al.* (1986) indicated that *Hebeloma*, *Paxillus*, *Entoloma* and *Cenococcum geophilum* were typified by sharp decrease in phosphatase activity above pH 5.

Table 1 Effects of pH and temperature on the acid phosphatase activity of *Cantharellus tropicalis*

pH study			Temperature study		
Enzyme incubation pH	Mycelial dry weight (mg)	Enzyme activity (mg g^{-1} mdw)	Incubation temperature (± 2 °C)	Mycelial dry weight (mg)	Enzyme activity (mg g^{-1} mdw)
1	180 ± 0.0 a	0.30 ± 0.01 a	5	540 ± 0.1 a	3.73 ± 0.05 a
2	160 ± 0.0 b	0.97 ± 0.03 b	10	510 ± 0.1 b	3.57 ± 0.05 b
3	180 ± 0.0 a	3.90 ± 0.28 cd	15	430 ± 0.0 c	4.50 ± 0.14 c
4	170 ± 0.1 c	4.16 ± 0.54 d	20	520 ± 0.1 d	5.67 ± 0.33 d
5	150 ± 0.0 d	3.71 ± 0.15 cf	25	280 ± 0.0 e	6.66 ± 0.08 e
6	110 ± 0.0 e	3.81 ± 0.26 cf	30	390 ± 0.0 f	11.13 ± 0.81 f
7	110 ± 0.0 e	3.55 ± 0.18 f	35	370 ± 0.0 g	8.80 ± 0.33 g
8–12	–	–	40	390 ± 0.0 f	18.48 ± 0.55 h

Initial pH was 5.5; culture was 15 days old; results are average of mycelial dry weights (mdw) with standard deviations; values within a column followed by the same letters do not differ significantly at $p < 0.05$.

Surface acid phosphatase activities were found to differ among different assay temperatures. Activity of p-NPPase was highest (18.48 mg g^{-1} mdw) at 40 °C and significantly reduced at 35 °C and lower (Table 1). When temperature increased, adaptive phenotypic changes occur in the Arrhenious activation energy (E_c) of several enzymes (Gaham & Patterson 1982). Little information exists concerning mechanisms of temperature acclimation in mycorrhizal fungi. However, acid phosphatase activities in soils have been reported at assay temperature as low as 20 °C (Bremner & Zantua 1975). Increase in enzyme production at low temperatures may be caused by cell plasma membrane confrontation and consequent leakage of intracellular p-NPPase, as observed for arctic fungal strains (Tibbett *et al.* 1998b, c). Nevertheless, there is a need to work on ecological significance of extracellular p-NPPase production at low temperatures.

Phosphatase activity is a good criterion to be used for the selection of ectomycorrhizal mushroom for forest inoculations of nursery seedlings (Trappe 1977). This demonstration of acid phosphatase of *C. tropicalis* is the first report of an acid phosphatase activity in tropical ectomycorrhizal fungi. The method described for preliminary evaluation of acid phosphatese activity is useful in screening large number of ectomycorrhizal fungal isolates for phosphatase activity. Our results show that pH and temperature affect the activity of surface pNPPase. However, it will be interesting to know the relationship between acid phosphatase activity in pure cultures and mycorrhizal formation capacity of ectomycorrhizal fungi, the *in vitro* capacity of different strains of species or different species of genus and the *in vivo* behaviour of acid phosphatase in decomposing organic phosphate in the forest. For future study, enzyme activity of crushed mycelia should be tested as many species express high enzyme activity in the interior parts of mycelia due to the breakdown of phospholipids in cell walls of old hyphae (Nygren 2008). This study will help in understanding the mechanism by which ectomycorrhizal fungi may increase nutrient uptake through production of surface-bound acid phosphatase.

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