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Effect of Chromium on the Axenic Growth and Phosphatase Activity of Ectomycorrhizal Fungi, *Laccaria laccata* and *Suillus bovinus*

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The mycorrhizal fungi are considered as an important tool in afforestation and rehabitation of degraded lands especially in mine spoils, eroded sites and polluted wastelands (Raman and Sambandan, 1998). Ectomycorrhizal fungal inoculated forest trees improve their growth and survival in metal polluted soils than non-mycorrhizal trees (Brown and Wilkins 1985; Jones and Hutchinson 1986). Severe contamination by pollutants such as heavy metals can result in widespread seedling mortality, delay of several decades in revegetation schemes (Richie and Thingvold 1985). Metal tolerant strains of fungi have been developed in some laboratories by repeated sub-culturing in metal contaminated medium (Parry and Wood 1958). The tolerance of ectomycorrhizal fungi growing *in vitro* towards mine metals, Al, Fe, Cu, Zn, Ni, Cd, Cr, Pb and Hg is more important in order to assess their potential for the establishment of ectomycorrhizas in metal contaminated sites (Tam 1995). *Pisolithus arrhizus*, an ectomycorrhizal fungus is highly resistant to toxic elements and grew well on media containing high doses of Cd dust, but considerable changes in glycogen and calcium accumulation were observed (Turnau and Oberwinker 1993; Turnau and Dexheimer 1995). Turnau et al. (1995) reported that increased acid phosphatase activity of *P. arrhizus* in the presence of Cd dust which is a measure of the resistance of the enzyme toxic metals and played an important role in heavy metal detoxification. In the present study, the effects of chromium on the growth and phosphatase activity of two ectomycorrhizal fungi, *Laccaria laccata* and *Suillus bovinus* were investigated.

MATERIALS AND METHODS

Cultures of *Laccaria laccata* (Scop.) Berk. & Br. and *Suillus bovinus* (Fries) O.Kuntze were isolated from sporocarps growing in *Pinus patula* plantations at Nilgiris, Tamil Nadu, India and maintained on Modified Melin Norkrans (MMN) agar medium (Marx 1969; Raman and Mohankumar 1988). Eight mm plugs of fungal colonies were transferred to 250 mL Erlenmeyer flasks containing 100 mL of Palmer Hacskaylo medium (pH 4.5) containing 5 g glucose; 0.5 g NH_4Cl; 0.5 g KH_2PO_4; 5 µg Biotin; 1 mg thiamine HCl and micronutrient solution 2 mL/L. The medium was amended with various concentrations of Cr (0.15, 0.30, 0.45, 0.60, 0.75 and 0.90 m*M*) in the form of CrO_3, flasks were incubated at 25±1°C for 30 d. Five replicates of the treatments were harvested at

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10 d intervals up to 30 d. For each harvest, mycelial dry weight, total protein content, acid and alkaline phosphatase activity were estimated.

The mycelial mat in each flask was transferred to a pre-washed, pre-dried and pre-weighed filter paper and washed thoroughly with distilled water to make it free from any trace of adherent medium and dried in an oven at 80°C for 24 h to a constant weight. Acid and alkaline phosphatase activity of the mycelium was estimated by following the method of Rokicka (1992). Fungal colonies were ground in 0.1 *M* acetate buffer (pH 5.0) containing 0.01 *M* EDTA, 0.01% Triton X 100 and 2.5% PVP. Crude enzyme extracts were obtained after centrifugation at 4°C. The incubation medium of acid phosphatase contained 250 µL of 0.1 *M* acetate buffer (pH 4.8), 50 µL of 0.015 *M* *p*-nitrophenyl phosphate disodium salt and 50 µL of enzyme solution. The reaction was stopped after 30 min by adding 2.5 mL of 0.2 *M* Na_2CO_3. The specific enzyme activity was measured in a Beckman DU 40 spectrophotometer at 410 nm and expressed as µmoles *p*-nitrophenol released/g dry wt/30 min. The reaction mixture for alkaline phosphatase contained 250 µL of 0.1 *M* Tris-HCl (pH 9.7), 50 µL of 0.015 *M* *p*-nitrophenyl phosphate disodium salt and 50 µL of enzyme solution. The reaction was stopped after 30 min by adding 2.5 mL of 0.2 *M* Na_2CO_3. The specific enzyme activity was measured as before and expressed as µmoles *p*-nitrophenol/g dry wt/30 min. The total protein content of the mycelium was estimated by following the method of Bradford (1976) using bovine serum albumin as standard.

The data were subjected to analysis of variance and the significant differences among the means were compared with Duncan's new multiple range test (DMRT) using SPSS pc+ software (Snedecor and Cochran, 1967) at P = 0.05 level.

RESULTS AND DISCUSSION

The results of the mycelial dry weight are presented in Table 1. The mycelial growth decreased in all the concentrations except in 0.15 m*M* where the growth increased in both the test fungi. *L. laccata* yielded more mycelia in all concentrations of Cr when compared with *S. bovinus*.

The effect of Cr on acid phosphatase activity of *L. laccata* and *S. bovinus* is presented in Table 2. Changes in the dry matter content is a prime indicator to the tolerance of fungi. Generally, both the test fungi showed increased acid phosphatase activity in all the treatments when compared with control. Increasing concentration of Cr induced the enhanced acid phosphatase activity. At 20th d of incubation, both the test fungi showed increased acid phosphatase activity than on 10th and 30th d. In both the test fungi, maximum acid phosphatase activity was observed in 0.90 m*M* concentration.

The effect of Cr on alkaline phosphatase activity of *L. laccata* and *S. bovinus* is presented in Table 3. In *L. laccata*, the alkaline phosphatase activity increased in 0.60 m*M* concentration than other treatments and control. On 10th d, higher

Table 1. Mycelial dry weight of ectomycorrhizal fungi at different concentrations of chromium.

Conc. of Cr (m*M*)	Mycelial dry weight (mg)					
	L.laccata			*S.bovinus*		
	10^{th} d	20^{th} d	30^{th} d	10^{th} d	20^{th} d	30^{th} d
Cont	93^{c} (±1)	101.3^{b} (±3.5)	120.3^{d} (±3.5)	3.3^{ab} (±0.6)	30.3^{d} (±1.5)	65.3^{e} (±0.6)
0.15	97^{d} (±2)	111.7^{c} (±3.5)	122.3^{d} (±3.1)	7^{e} (±1)	48.3^{f} (±1.5)	70.3^{f} (±2.5)
0.30	74.3^{b} (±1.5)	96^{b} (±1)	107^{bc} (±4)	6^{de} (±1)	39.3^{e} (±2.5)	48.3^{d} (±1.5)
0.45	67.3^{a} (±0.6)	89.3^{a} (±2.5)	100.3^{a} (±2.5)	5.3^{cd} (±0.6)	21.7^{c} (±1.5)	31.3^{c} (±3.5)
0.60	75.0^{b} (±1)	95.7^{b} (±3.1)	102^{ab} (±3)	4^{bc} (±1)	20.3^{bc} (±2.5)	26.7^{b} (±2.5)
0.75	90.3^{c} (±2.5)	99^{b} (±4)	110^{c} (±3)	3^{ab} (±1)	18.3^{b} (±1.5)	23.3^{b} (±1.5)
0.90	91^{c} (±2)	98^{b} (±4)	109.6^{c} (±5.0)	2^{a} (±0)	7.3^{a} (±0.6)	10.7^{a} (±1.5)

Means sharing a common letter within the row are not significantly different at P=0.05 level.

alkaline phosphatase activity was recorded in 0.60 m*M*. Alkaline phosphatase activity in *S. bovinus* decreased in all the treatments when compared with control except in 0.60 m*M* on 30th d.

Total protein content of the test fungi in different concentrations of Cr is presented in Table 4. In *L. laccata*, the protein content increased in all the concentrations. Maximum protein content was noticed in 0.45 m*M* on 10th d. In *S. bovinus*, increased level of protein was observed in all the concentrations except in 0.30 m*M*. Maximum protein content of *S. bovinus* was observed in 0.75 m*M* concentration on 10th d (Table 3). The metal tolerance of higher plants aided by mycorrhizal fungi is poorly understood. However, some reports have come up on ericaceous mycorrhizas (Bradley et al. 1982) and ectomycorrhizas (Brown and Wilkins 1985; Denny and Wilkins 1987) which prove that the metal tolerance of higher plants by their mycobionts is through accumulation of metals in the walls of extramatrical hyphae and extrahyphal slime by which passage of metals to the shoot is restricted. Decreased level of growth of *P. tinctorius* has been shown in chromium amended medium (Tam 1995). The higher mycelial dry weight yields of *L. laccata* and *S. bovinus* at 0.15 m*M* concentration of Cr than the control indicated the ability of these fungi to detoxify this metal. However, Cr is not known to be a micronutrient for fungal growth, so it affected the growth in all other concentrations. Highest acid phosphatase activity is displayed by *Hebeloma crustuliniforme* and *L. laccata* among the ectomycorrhizal fungi (Ho and Zak 1979; Ho and Tilak 1988). The stimulation of acid phosphatase activity was recorded in *Alyssum bertolonii* in 0.01 m*M* of Ni (Gabbrielli et al. 1989). *L. laccata* has been shown with high acid

Table 2. Acid phosphatase activity of ectomycorrhizal fungi at different concentrations of chromium.

Conc. of Cr (m*M*)	Acid phosphatase activity (µmoles p-nitrophenyl/g dry wt./30 min.)					
	L.laccata			*S.bovinus*		
	10^{th} d	20^{th} d	30^{th} d	10^{th} d	20^{th} d	30^{th} d
Cont	578.0^{a} (±14.0)	682.3^{a} (±5.1)	478.0^{a} (±3.0)	43.6^{a} (±1.52)	56.0^{a} (±3.0)	60.3^{c} (±3.51)
0.15	642.3^{d} (±4.5)	720.3^{b} (±2.5)	497.3^{b} (±4.5)	119.7^{b} (±2.5)	80.6^{b} (±4.5)	27.3^{a} (±1.5)
0.30	611.3^{c} (±2.5)	741.3^{c} (±5.0)	502.0^{b} (±3.0)	161.3^{c} (±2.5)	101.3^{c} (±3.5)	25.0^{a} (±2.0)
0.45	598.3^{b} (±4.5)	767.3^{d} (±2.5)	517.7^{c} (±2.5)	216.6^{d} (±3.2)	142.6^{d} (±1.6)	71.3^{d} (±2.5)
0.60	670.7^{e} (±7.0)	689.0^{a} (±6.0)	498.0^{b} (±3.0)	217.7^{d} (±4.0)	148.3^{e} (±2.5)	54.0^{b} (±2.0)
0.75	678.0^{e} (±7.0)	789.7^{e} (±2.5)	520.6^{c} (±9.5)	277.7^{e} (±6.0)	213.3^{f} (±1.5)	125.0^{e} (±6.0)
0.90	701.6^{f} (±4.5)	811.6^{f} (±3.5)	589.6^{d} (±0.5)	678.6^{f} (±5.7)	369.3^{g} (±3.5)	150.3^{f} (±1.5)

Means sharing a common letter within the row are not significantly different at P=0.05 level.

Table 3. Alkaline phosphatase activity of ectomycorrhizal fungi at different concentrations of chromium.

Conc. of Cr (m*M*)	Alkaline phosphatase activity (µmoles p-nitrophenyl/g dry wt./30 min.)					
	L.laccata			*S.bovinus*		
	10^{th} d	20^{th} d	30^{th} d	10^{th} d	20^{th} d	30^{th} d
Cont	13.3^{e} (±1.5)	5.3^{a} (±0.6)	12^{b} (±2)	14.3^{e} (±0.6)	8.6^{d} (±1.5)	4.3^{b} (±0.6)
0.15	6.7^{cd} (±1.5)	6.0^{a} (±1.0)	8.3^{a} (±0.6)	8.0^{c} (±1)	5.3^{b} (±0.6)	3.3^{ab} (±1.5)
0.30	7.6^{d} (±0.6)	9.7^{b} (±1.5)	14.0^{b} (±2)	10.3^{d} (±1.5)	7.3^{cd} (±0.6)	3.0^{ab} (±1)
0.45	3.7^{b} (±0.6)	14.7^{c} (±1.5)	21.3^{c} (±1.5)	6.6^{c} (±0.6)	8.0^{d} (±1.0)	3.3^{ab} (±0.6)
0.60	25.0^{f} (±2)	11.3^{b} (±1.5)	8.7^{a} (±1.5)	11.7^{d} (±0.6)	5.7^{bc} (±0.6)	7.7^{c} (±1.5)
0.75	1.3^{a} (±0.6)	6.3^{a} (±0.6)	12.7^{b} (±1.5)	3.3^{a} (±0.6)	5.3^{b} (±0.6)	4.7^{b} (±0.6)
0.90	5.0^{bc} (±1)	5.3^{a} (±0.6)	6.6^{a} (±1.5)	5.0^{b} (±1)	3.3^{a} (±1.5)	2.0^{a} (±1)

Means are sharing a common letter within the row is not significantly different at P=0.05 level.

Table 4. Total protein content of ectomycorrhizal fungi at different concentrations of chromium.

Conc. of Cr (m*M*)	Total protein content (mg/ g dry weight)					
	L.laccata			*S.bovinus*		
	10^{th} d	20^{th} d	30^{th} d	10^{th} d	20^{th} d	30^{th} d
Cont	1.47^{a} (±0.2)	1.2^{a} (±0.3)	0.77^{a} (±0.2)	2.5^{b} (±0.4)	2.7^{a} (±0.2)	2.8^{ab} (±0.3)
0.15	2.93^{c} (±0.2)	1.97^{b} (±0.2)	1.77^{c} (±0.1)	5.5^{e} (±0.1)	3.9^{b} (±0.2)	3.5^{c} (±0.2)
0.30	2.37^{b} (±0.2)	1.47^{a} (±0.2)	1.1^{b} (±0.1)	2.2^{a} (±0.1)	2.8^{a} (±0.3)	2.5^{a} (±0.3)
0.45	4.77^{d} (±0.3)	1.5^{a} (±0.1)	2.77^{e} (±0.1)	6.2^{f} (±0.2)	4.9^{c} (±0.2)	3.6^{e} (±0.1)
0.60	2.73^{bc} (±0.4)	1.4^{a} (±0.2)	1.23^{b} (±0.2)	4.8^{d} (±0.1)	3.9^{b} (±0.2)	3.1^{b} (±0.2)
0.75	5.97^{e} (±0.2)	2.37^{c} (±0.4)	2.77^{e} (±0.3)	7.2^{g} (±0.2)	6.8^{e} (±0.3)	7.2^{e} (±0.3)
0.90	1.77^{a} (±0.2)	2.23^{bc} (±0.2)	2.43^{d} (±0.2)	2.9^{c} (±0.2)	5.7^{d} (±0.3)	5.7^{d} (±0.2)

Means are sharing a common letter within the row is not significantly different at P=0.05 level.

phosphatase activity and tolerance to high concentration of Cu and Ni (Periasamy and Raman 1995). In the present study, *L. laccata* showed progressively increased acid and alkaline phosphatase activities than *S. bovinus* grown in different concentrations of Cr than the control.

Phytochelatins (class III metallothioneins) are short metal-induced sulfhydryl-rich peptides possessing the general structure: (g-GluCys)n-Gly with n=2-11. They are synthesized from glutathione in plants and fungi exposed to Ag, Bi, Cd, Cu, Hg, Ni, Sn, Sb, Te, W, Zn and anions such as SeO_4^{-2}, SeO_3^{-2} and AsO_4^{-3}. The non-inducers of phytochelatins are Na, Mg, Al, Ca, V, Cr, Mn, Fe, Co and Cs (Rauser 1995). In *Pisolithus tinctorius* mycelium treated with Al and Cr, the amount of glutathione and enzymes (Glutathione-S-transferase, Glutathione peroxidase and Glutathione reductase) involved in glutathione metabolism did not differ significantly and Al and Cr did not induce the production of metal binding proteins (Srinivasan 2000). Gluathione is the precursor of phytochelatin synthesis. Even though, Cr is non-inducer of phytochelatins, *L. laccata* and *S. bovinus* showed increased protein content. The increased protein content is due to the increased production of phosphatase enzymes to over come stress. Stress alleviation through metal accumulation in polyphosphate granules in hyphae of *P. tinctorius* has been found for Cu and Zn, but has not evident for Al, Ni, Cd,

Cr or Hg (Tam 1995). Turnau et al (1995) reported that the phosphatase activity of *P. arrhizus* played an important role in heavy metal detoxification. In the present study, intra and extracellular acid and alkaline phosphatase activities of *L. laccata* and *S. bovinus* grown in Cr amended medium enhanced on 20th and 10th

d, respectively, when compared with control. High acid phosphatase activity and tolerance to high concentrations of Cu and Ni were reported in *Laccaria laccata* (Periasamy and Raman, 1995) and *Amanita muscaria* (Raman et al. 1998). Turnau and Dexheimer (1995) observed increased acid phosphatase activity in *P. arrhizus* in the presence of Cd dust especially in young cells, extracellular activity increased, which is a measure of the resistance of the enzyme to toxic metals. This continued the function in resting and immobilized cells and liberated as HPO_4^{2-} that precipitated stoichiometrically with metal ions M^{2+} to form $MHPO_4$, tightly bound to the cell surface. Hence, these fungi may be useful in reforestation of chromium polluted soils and heavy metal polluted soils. The compatibility of fungal strain and host plant is more important to the success of the relationship in the presence of high heavy metal concentrations than fungal adaptation to the metal (Denny and Wilkins 1987). Among these two fungi, *L. laccata* having maximum acid phosphatase activity and tolerance to high concentration of chromium is a potential fungus for reclamation of tannery effluent polluted soils. However, further work on the chromium accumulation in plants associated with *L. laccata* is needed before field application.

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