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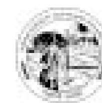

SHORT COMMUNICATION

EFFECT OF LEAD AND COPPER ON ENZYME ACTIVITY IN TWO BRYOPHYTES

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Effect of lead and copper on nitrate reductase activity (NRA), peroxidase (POX) and superoxide dismutase (SOD) in mosses *Rhodobryum roseum* and *Hypnum cupressiforme* grown in different concentrations (control, 5, 10, 50 and 100 mM) for 7 and 15 days was studied. Level of NR activity significantly increased in comparison to control in 5 mM concentartion in 7 days treatment, which decreased if time period and concentration of metals (Pb and Cu) was increased, whereas, oxidative enzymes (i.e. POX and SOD) activity were observed to be significantly higher at all concentrations in 7 and 15 days of treatment period in both the mosses. The result suggests that *Rhodobryum roseum* is less sensitive to Pb and Cu in comparison to *Hypnum cupressiforme*.

***Key words*: Antioxidant, heavy metal, mosses, nitrate reductase activity.**

Bryophytes have a wide spread occurrence and can survive under extreme environmental conditions. At the same time some species are very sensitive to environmental pollution, including metals and have been widely used for biomonitoring (Bruning and Kreeb 1993). The adverse conditions and man made activities prevailing on Kumaon hills has effected bryoflora (Saxena *et al*. 2006). Before inducting a particular moss species for biomapping, it is desirable to study the response of these metals on the physiology of respective mosses to validate the tolerant species (Saxena and Kaur 2005, Saxena and Arfeen 2006). Metals like Pb and Cu are present in nature and are phytotoxic at excessive level (L' Huiller *et al*. 1996). Bryophytes have different counter gradient mechanism by which they accumulate significant concentration of metals in their tissues (Carginale *et al.* 2004) and thus can be deployed for biomonitoring.

Metal stress (Pb and Cu) had a direct effect on NR activity, which is an indicator of nitrogen assimilation and plant growth (Bose and Mishra 1999). The importance of NR activity in the assimilation of nitrate, however, shows high degree of sensitivity to various exogenous and endogenous metabolites (Jaiwal and Singh 1995, Sivasankar and Oaks 1996). Heavy metal stress accelerates the generation of reactive oxygen species (ROS) (Noctor and Foyer 1998). However, detoxification of excess ROS is essential as the high concentration of ROS disrupt the normal physiological and cellular functions (Gille and Sigler 1995). The major ROS scavenging mechanism of plant include POX and SOD activity, which act as defense enzymes and protect plant from oxygen toxicity caused by the pollutants (Polle *et al.* 1990, Allen 1995). Metal tolerance is associated with the capacity of plant to restrict metals to the cell wall and activation of antioxidant defense mechanism. The present study was under taken to asses the response of metals (Pb and Cu) on two mosses *Rhodobryum roseum* and *Hypnum cupressiforme.*

Moss *Rhodobryum roseum* and *Hypnum cupressiforme* were collected in November 2005 from Mukteshwar in Kumaon hills and were brought to

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laboratory in polythene bags. Samples were carefully cleaned and washed to remove soil and adhering dust particles. Green parts of samples were transferred to Petri dishes containing sets of control and different concentrations (5, 10, 50, 100 mM) of $PbNO_3$ and $CuSO_4$. The plates were transferred for 7 and 15 days duration to B. O. D. chamber under white light by two fluorescent white tubes (Philips 20 W TLD. India) with a photon flux density of 52 mE m^{-2} S^{-1} (PAR) and kept at 22$\pm$2°C to provide natural growth environment to plants.

Nitrate reductase activity was determined in freshly harvested plant sample (0.5g) taken in black vials of 20 ml, containing 8.0 ml of 0.1 M sodium phosphate buffer (pH 7.4), 1.0 ml of 0.2 M KNO_3 and 1.0ml of 25% propanol. These vials were sealed and incubated in dark for 30 minutes at 30°C. Nitrite released in incubation mixture due to enzymatic activity was measured by colour development. One ml of 1% sulphanilamide in 1 N HCl and 1.0 ml 0f 0.2 % N-(1-naphthyl) ethylenediamine-dihydrochloride (NED) was added in 1.0 ml of aliquot from the incubation mixture. After 20 minutes, absorbance was read by a spectrophotometer at 540 nm and was calculated as millimole NO_2^- h^{-1} gm^{-1} FW. The colour achieved was due to formation of diazo compound with sulphanilamide and nitrite, which is coupled with NED to give a pink colour (Srivastava 1975).

The peroxidase enzyme was extracted from the fresh samples at 4°C with 0.1 M sodium phosphate buffer (pH 7.4). The extract was centrifuged at 5000 rpm for 15 minutes. The supernatant was used as enzyme preparation. Assay medium consisted of 1.0 ml of 100 mM sodium phosphate buffer (pH 6.4), 1 ml of guaiacol (0.29 ml/100 ml), 2.0 ml of distilled water and 1.0 ml of enzyme preparation in a total volume of 5 ml. Reaction was started by adding one drop of 3% H_2O_2 and was recorded spectrophotometrically at 470 nm at 15 second interval till absorbance increases. The enzyme activity was expressed as ΔOD $min^{-1}gm^{-1}$FW and was calculated by taking the difference of absorbance at 1 minute time interval (Putter 1974).

For superoxide dismutase activity 0.5 g of fresh plant sample was homogenised with 1 ml of buffer (pH 7.0), 0.5 ml PVP (0.2%), 0.5 ml EDTA (0.1mM) and 0.5 ml $MgCl_2$ (3 mM). After grinding, mixture was centrifuged at 2500 rpm for 10 minutes at $\pm$ 4°C. The supernatant was stored in freezer as enzyme source. Assay mixture was prepared by taking 0.7 ml buffer (pH 7.8), 0.5 ml Methionine (0.0970 gm in 50 ml DW), 0.7 ml NBT (0.031 gm in 50 ml DW), 0.5 ml EDTA, 0.1 ml enzyme extract and 0.3 ml Riboflavin (50 μM), at last in test tube. The test tubes were kept 30 cm below a light source consisting of two 15 watts tube light for 15 minutes, to develop the colour. A non – irradiated reaction mixture was run in parallel, which did not develop colour and served as control (Beuchamp and Fridovich 1971). Absorbance was taken spectrophotometrically at 560 nm.

Samples were collected in triplicate to conduct the statistical analysis. Value represented as mean $\pm$ standard error (Snedecor and Cochran 1967). ANOVA revealed significant differences in the metal concentration over to control (for $p \leq 0.01$, $p \leq 0.05$) utilizing Dunkun's Multiple Range test (Karmer 1956).

Nitrate reductase enzyme plays a central role in plant primary metabolism and exhibits complex regulation mechanism for its catalytic activity (Viegas and Silveira 2002). NR activity for Pb increases up to 10 mM in *R. roseum* and up to 5mM in *H.cupressiforme*, whereas, same was observed up to 5 mM in case of Cu (Table 1). At higher concentrations, decline in NR activity was observed up to 68 % and 69 % in Pb and Cu, respectively, in *Rhodobryum roseum* and 47 % (Pb) and 60 % (Cu) in *Hypnum cupressiforme* during 7 days of treatment. However, prolonged incubation decreased NR activity even at lower concentrations. It indicated that the activity of enzyme nitrate reductase is inhibited by heavy metals as observed in other species by Gouia *et al*. (2000) and Saxena and Saxena (2002).

The long term treatment of 15 days caused a considerable increase in peroxidase (POX) activity. Results show non significant value between control and 5 mM in *Rhodobryum roseum* and *Hypnum cupressiforme* on giving 7 days treatment of Pb and Cu (Table 1). However, significant increase in POX activity was observed on increasing the concentration of Pb and Cu in studied mosses. The increase of 128 % and nearly 148 % in *Rhodobryum roseum* and 88 % and 89 % in

Table 1. Effect of Pb & Cu on NRA (mmoles NO_2 h^{-1} g^{-1} fw) & POX (ΔOD min^{-1} g^{-1} fw) & SOD (SOD unit g^{-1} fw) parameters in moss *Rhodobryum roseum* and *Hypnum cupressiforme* at different concentrations for 7 and 15 days treatment.

Rhodobryum roseum							
Treatment		**7 Days**			**15 Days**		
Concentration	**Metals**	**NRA**	**POX**	**SOD**	**NRA**	**POX**	**SOD**
CONTROL		323.263±1.034*	0.804±0.002[ab]	7.751±0.058[cdef]	483.160±1.741	0.991±0.007	8.932±0.005
5 mM	Pb	387.635±4.070	0.810±0.017[a]	8.564±0.001[c]	324.460±2.129	1.146±0.011	9.346±0.021
	Cu	413.457±1.543*	0.818±0.010[b]	8.646±0.004[d]	318.324±4.652	1.012±0.006	12.176±0.061
10 mM	Pb	432.236±1.736	1.835±0.060	9.077±1.666[e]	246.264±1.785	2.011±0.007	11.284±0.060
	Cu	256.720±2.307	1.994±0.002	9.585±0.008[f]	211.555±5.618	2.784±0.002	13.302±0.001
50 mM	Pb	143.580±1.604	1.160±0.005	12.113±0.007	146.587±3.607	1.537±0.004	18.274±0.059
	Cu	173.558±1.757	1.254±0.013	12.038±0.022	154.381±2.277	1.691±0.008	14.458±0.004
100 mM	Pb	101.204±0.579	0.550±0.014	13.264±0.005	71.575±0.708	0.422±0.001	20.450±0.014
	Cu	98.572±4.761	0.594±0.060	16.534±0.004	79.241±2.229	0.554±0.005	18.012±0.005
Hypnum cupressiforme							
Treatment		**7 Days**			**15 Days**		
Concentration	**Metals**	**NRA**	**POX**	**SOD**	**NRA**	**POX**	**SOD**
CONTROL		271.557±2.494	0.502±0.030*	6.390±0.052	381.427±0.371	0.683±0.007[a]*	7.426±0.015
5 mM	Pb	354.555±0.579	0.656±0.003	9.041±0.023	220.157±1.158	12743±0.008	10.742±0.024
	Cu	283.415±1.741	0.648±0.002	7.381±0.024	278.502±0.579	1.035±0.020	9.886±0.059
10 mM	Pb	250.495±0.355	0.945±0.004	11.054±0.031	169.510±0.578	1.350±0.028	13.179±0.005
	Cu	257.589±1.008	0.951±0.005	13.202±0.058	247.923±1.187	1.864±0.011	11.380±0.023
50 mM	Pb	198.393±0.600	0.555±0.003*	12.548±0.057	98.684±1.697	0.657±0.004[a]	15.180±0.024
	Cu	247.330±1.293	0.667±0.004	14.260±0.034	132.404±0.947	0.747±0.012	17.399±0.115
100 mM	Pb	142.382±1.140	0.358±0.008	15.370±0.020	49.596±1.187	0.433±0.191	18.537±0.075
	Cu	108.429±0.654	0.534±0.017*	22.869±0.075	98.741±1.179	0.610±0.023*	25.749±0.025

Values are represented as mean ± SE, Significance test of ANOVA & DMR test has been done at 1% & 5% significance level, * = value is not significantly different at $p \leq 0.05$ as compared to control site in vertical column, Values superscripted same alphabet in vertical column are not significantly different at 1% and 5% significance level as compared to control site

Hypnum cupressiforme was found in POX at 10 mM in 7 days treated samples of Pb and Cu, respectively. POX activity increases up to 10 mM in 15 days treatment but declined at 50 mM and 100 mM concentrations. The decline in POX activity reflects cell damage due to toxicity at higher concentration.

In *Rhodobryum roseum,* non significant increase in peroxidase activity was observed up to 10 mM over control after 7 days treatment of Pb. However, significant difference was exhibited after 15 days treatment with different concentration of Pb and Cu in both the mosses. In 15 days treatment SOD activity increased up to 56 % (Pb) and 50 % (Cu) in *Rhodobryum roseum,* whereas, it was 59 % (Pb) and 71 % (Cu) more in *Hypnum cupressiforme*. SOD activity also increased at higher concentration during prolonged incubation period (Table 1 and 2). The same

trend for SOD was reported earlier in the lichen *T. nepalense* by Cuny *et al.* (2004).

Present investigation was an attempt to investigate the possible effect of Cu and Pb on NR, peroxidase and SOD activity in mosses *Rhodobryum roseum* and *Hypnum cupressiforme*. Greater toxicity to Cu was observed in comparison to Pb, even at low concentrations. Copper is highly toxic to plant even at a micro molar range of exposure (Cabral 2003). Studies in future may highlight a better understanding of the mechanisms adopted by these moss species in response to concentration of metals and can be recommended for biomapping.

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