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CHLOROPHYLL AS A BIOLOGICAL MARKER OF STRESS FOLLOWING APPLICATION OF HEAVY METALS (Pb, Ni AND Cd) IN MOSS *THUIDIUM CYMBIFOLIUM*

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Plant chlorophyll concentration is an indicator of environmental conditions such as air borne heavy metals. The overall goal of this assessment was, therefore, to evaluate the effect of three heavy metals (Pb, Ni and Cd) on chlorophyll content of the moss *Thuidium cymbifolium.* A drastic reduction in chlorophyll content was noticed after 3 and 6 days of Pb exposure, whereas after 15 days the pigment content was more or less similar to untreated plants, indicating the induction of tolerance mechanism. Interestingly Ni treatment of 3 days increased pigment content gradually from 0.6562 mg g^{-1} fr. wt. in control to 0.7212 mg g^{-1} fr. wt. and 0.8238 mg g^{-1} fr. wt. in 0.01 and 0.1 M concentration, respectively. The phytotoxic effect of Ni was most apparent after 15 days of treatment reducing significantly by 63 % at 0.2 M concentration. *In vitro* exposure of moss for 72 hrs to Cd showed no significant reduction in pigment content. Most astounding and remarkable was to note the increase in chlorophyll content of moss leaves even when the exposure period was increased to 6 days. The longer exposure period of 15 days reduced the chlorophyll content to 33 % at 0.2 M treatment. The results of the present study showed that Pb had the most significant effect on chlorophyll content followed by Ni and Cd. The result further confirms the use of chlorophyll content as biological marker of heavy metal stress.

Keywords: Cadmium; Chlorophyll; Lead; Nickel; *Thuidium cymbifolium.*

Introduction

Plants are exposed to various unfavorable conditions that may alter plant metabolism. It seems likely that plants exposed to natural stresses are predisposed to be injured by anthropogenic stresses. Of special significance is the rise in heavy metals that originate frequently from industrial and agricultural activities [1]. Metals like Pb, Ni and Cd spread around road sides from vehicles and contaminate vegetation [2]. As a result these heavy metals accumulate in plants and affects plant metabolism. Each metal has a different mode of action; however, in general these metals are known to inhibit plant growth and reduce photosynthesis by inhibiting chlorophyll production [3,4].

The loss of chlorophyll from plants (chlorosis) have long been used to assess injury induced by varied air pollutants, and is believed to signal a decline in photosynthetic capacity and plant productivity. Mosses have been used frequently as biomonitors; nevertheless, little experimental attention has been paid to the effects of heavy-metal uptake on the growth and physiological processes of bryophytes. Such studies are quite useful to validate tolerant species that can be further used for bio-mapping studies in field. The present study therefore aims to outline our current knowledge on the phyto-toxic effect of heavy metals (Pb, Ni and Cd) on chlorophyll content of moss *Thuidium cymbifolium* to validate its tolerance potential.

The presently used test plant *T. cymbifolium* is pleurocarpous, ectohydric moss with extensive branching. No literature is so far available on the use of this moss for eco-physiological studies under *in vitro* conditions. Further the use of three different metals will allow us to get a comparative picture of their individual effect on chlorophyll content of the moss. The present study is aimed, to indicate if, chlorophyll content of the moss could be used as biological marker of heavy metal stress.

Materials and Methods

Sample collection: Samples of pleurocarpous moss *Thuidium cymbifolium* were collected from Artola-Jageswar road (1870 m) in December 2003 in poly-bags from a uniform area of 50 cm^2. After collection, adhering substrate and litter were removed by means of a forceps and finally washed with running tap water. Dilute solutions of heavy metals $CdCl_2$, $Pb(CHCOO_3)_2$ and $NiSO_4$ were used to determine plant sensitivities to metal toxicity. Moss samples were treated with different concentrations (0.01, 0.1 and 0.2 M) for 3, 6 and 15 days respectively. After treatment period plant tissues were used for physiological

analysis. All the treatments had three replications.

Determination of the chlorophyll: 500 mg of the fresh moss samples were homogenized in the ice cold 80% acetone with a mortar and pestle. The extracts were centrifuged at 5000 rpm, chlorophyll content was quantified by reading the absorbance at 652 nm. Total chlorophyll contents were expressed as mg g^{-1} fr. wt. [5]. Figure in the text indicate mean values ± S.E.

Results and Discussion

Fig. 1 illustrates both short term and long term treatment of the three metals lead, nickel and cadmium on the total chlorophyll content of the moss.

After 3 days of treatment total chlorophyll content at the lowest Pb concentration (0.01 M) was reduced by 23% and by 97 % at 0.2 M Pb concentration. The deleterious effect of lead was more pronounced after 6 days of treatment, at the highest concentration (0.2 M) the chlorophyll content was almost undetectable. Interestingly after 15 days of treatment there was no significant difference in chlorophyll content in relation to the untreated plants.

A quite different trend was observed for both Ni and Cd. Total chlorophyll content after 3 days Ni treatment increased gradually from 0.6562 mg g^{-1} fr. wt. in control to 0.7212 mg g^{-1} fr. wt. and 0.8238 fr. wt. in 0.01 and 0.1 M concentration respectively, decreasing slightly to 0.7725 mg g^{-1} fr. wt. at highest concentration. After 6 days of treatment, the chlorophyll content of moss showed a declining trend more or less similar trend was noticed after treatment period of 15 days. A drastic reduction of 63 % was noticed at 0.2 M concentration.

Following Cd treatment of 3 days no significant change in total chlorophyll in comparison to untreated plants was observed. Quite interestingly it is worth noting an increase in total chlorophyll content of the moss after 6 days Cd treatment. Even an exposure period of 15 days did not cause noticeable change in chlorophyll content of Cd treated plants, except for 0.2 M concentration where the total chlorophyll content was reduced from 0.3084 mg g^{-1} fr. wt. in control to 0.2058 mg g^{-1} fr. wt. at highest concentration.

After 3 days of Pb treatment, total chlorophyll content of the moss decreased and the deleterious effect of lead was more pronounced after 6 days of treatment. Lead in excess concentration, similarly as other heavy metals, decreases chlorophyll concentration in leaves either by inhibition of chlorophyll biosynthesis or by induction of its degradation [3,6]. By way of mechanism, it has been observed that lead (Pb) inhibits delta-amino levulinic acid dehydratase activity [7], the enzyme responsible for the synthesis of delta-amino levulinic acid, an important intermediate in chlorophyll biosynthesis.

The substitution of the central magnesium (Mg) in chlorophyll by heavy metals (Hg, Cd, Cu, Ni, Zn and Pb) *in vivo* is also an important type of damage in metal stressed plants. Furthermore, it is suggested that high concentration of lead decreases tissue hydration by 50 % in comparison to untreated plants [7] and as a result this metal induced water stress might be responsible for reduced chlorophyll biosynthesis. This decrease in chlorophyll content by Pb is consistent with studies conducted earlier on lichen *Ramalina duraiei*[8] and on moss *Tortula ruralis*[9]. Interestingly after 15 days of treatment there was no significant difference in chlorophyll content in relation to the untreated plants. This result points to the induction of tolerance mechanism in moss due to continuous exposure of moss to Pb.

Interestingly, after 3 days of Ni treatment, a gradual increase in chlorophyll content was noticed. An increase in chlorophyll concentration has been observed experimentally in *Hordeum vulgare* at 1 ppm concentration[10]. Nevertheless the experimental concentrations used in present study are too high, suggestive of enhanced tolerance mechanism in mosses compared to higher plants. However, significant decrease in chlorophyll content of the moss *Sphagnum cuspidatum* was reported by Saxena and Saxena[11], where the highest Ni concentration used during experimentation was 0.1 M. It has been suggested that substitution for essential metals in a number of metal-activated enzymes by nickel may explain some of the growth responses reported in the presence of nickel [12].

After 6 days of treatment, the chlorophyll content of moss decreased up to 32 % in comparison to untreated plants at higher concentrations (0.1 M and 0.2 M) and was analogous to control at 0.01 M treatment. It is probable that Ni might have been immobilized by the negative charges of the cell walls [13,14] and only to a limited degree or at a slow rate transported into the cell interior. Since, the plasma membrane controls the entry of metals in to the cytoplasm; maintenance of membrane integrity under metal stress is the crucial factor in determining the tolerance of plants to metals [15]. It has been shown earlier [14] that nickel damages cell membrane at concentrations greater than 0.2 M, signifying Ni tolerance in moss.

Phytotoxicity of Ni was apparent after treatment period of 15 days. A similar decrease in chlorophyll concentration on Ni treatment in the leaves of plants has been reported by several workers [16,17]. Nickel too like other heavy metals reduces the chlorophyll biosynthesis or induces its degradation [18,19]. Nickel also causes changes in chloroplast ultrastructure [20]. Reduction and swelling of thylakoids in cabbage was reported by Molas [17] on its exposure to excess of nickel sulfate.

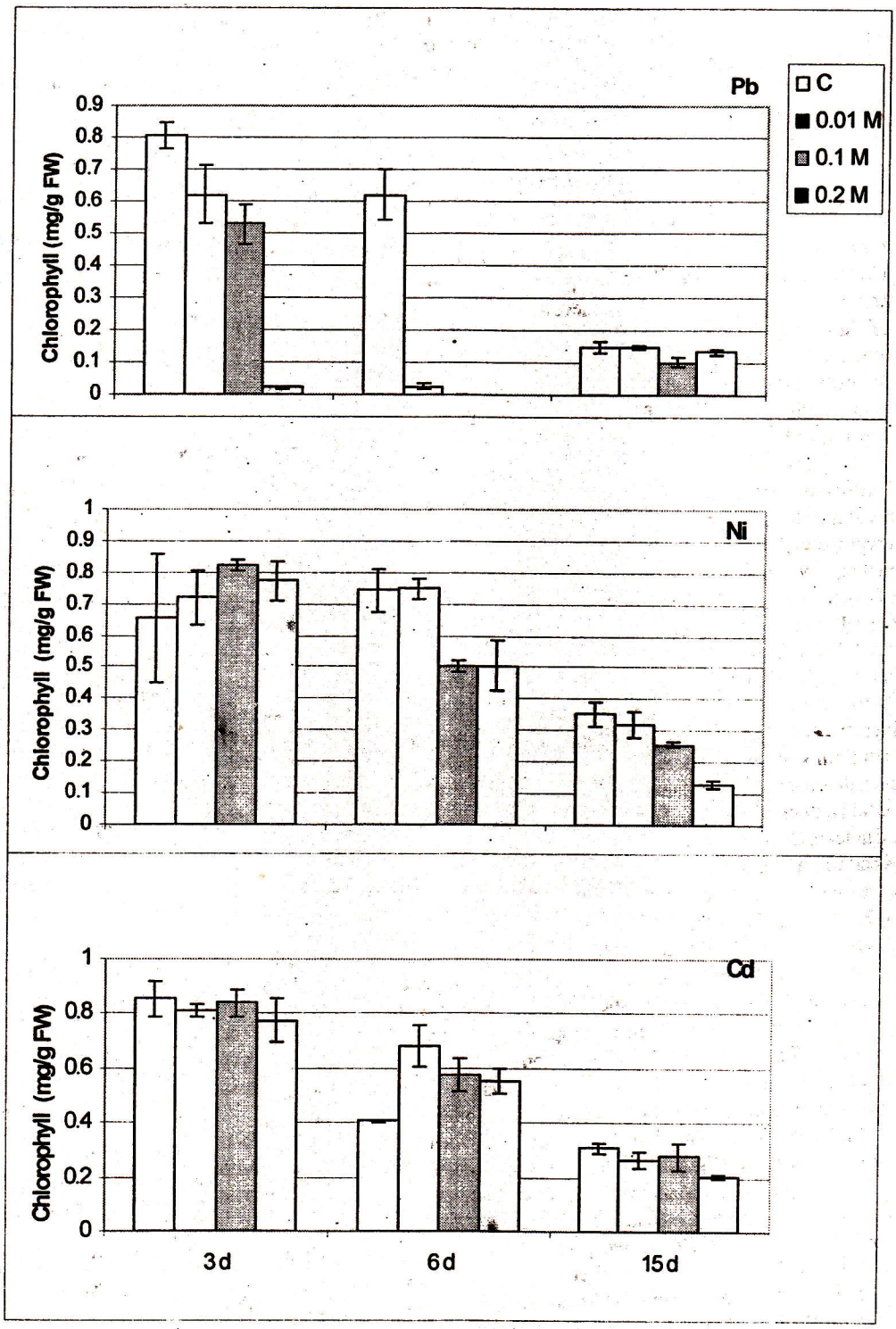

Fig. 1. Effect of different concentrations of Pb,Ni and Cd on chlorophyll content (mg g^{-1} fr.wt.). Each value is the mean of 3 replicates ± S. E.

Following Cd treatment of 3 days no significant change in total chlorophyll in comparison to untreated plants was observed. Cadmium like lead has no biological function and is extremely toxic, even at low concentrations and is easily assimilated by plants. It induces various visible symptoms of phytotoxicity e.g., chlorosis and inhibits growth [21]. However in present study the results were some-what complementary to *in vitro* experiments conducted earlier on chlorophyll content of the leaves. It is probable that in moss *Thuidium cymbifolium* an efficient Cd detoxification mechanism played by vacuolar compartmentalization, must have prevented free circulation of Cd ions in the cytosol and forced them into a limited area may be in vacuole [22], thus determining low stress intensity. This might explain why no significant decrease in total chlorophyll content following 3 days Cd treatments was noticed

Studies in moss *Rhytidiadelphus squarrosus*[23] had demonstrated that this moss showed physiological resistance to experimentally supplied Cd; suggesting that cryptogamic plants that experience elevated metal levels in their environment are less sensitive to intracellular level of experimentally supplied Cd. It is also probable that this Cd tolerance in *Thuidium cymbifolium* might be due to altered intracellular Cd uptake kinetics or due to increased extracellular binding affinity for Cd. The exact mechanism of toxic metal exclusion by *Thuidium* needs further investigation. It is also worth noting, that in present study cadmium was supplied as cadmium chloride and it has been demonstrated [23] that accompanying anion may, upto certain extent, effect both intra-cellular and extra-cellular Cd uptake. Cd uptake is slightly higher when Cd is supplied as cadmium acetate rather than chloride, nitrate or sulfate, since higher pH of the acetate solution reduces competition by H^+ ions for binding sites. These possibilities might explain for the Cd tolerance observed in moss *Thuidium.*

Quite interestingly, it is worth noting, an increase in total chlorophyll content of the moss after 6 days Cd treatment. A slight increase in chlorophyll content was reported earlier at 0.1 µM Cd concentration[24]. The results of the present study support few earlier findings [25,26], that there is some potentially positive impact of Cd on plant growth. The main explanation, for this enhancement in chlorophyll content, might be due to increased availability of Fe for chlorophyll-heme biosynthesis. Even an exposure period of 15 days did not cause noticeable change in chlorophyll content of Cd treated plants. These results are in general agreement with earlier findings on moss *Tortula ruralis*[9], who reported 40 % reduction (0.1 M) in chlorophyll content after two weeks treatment. This reduction might be due to inhibition of chlorophyll synthesis [6, 27].

It is evident from present results that amongst the three metals undertaken (Pb, Cd, Ni); Pb had the most deleterious effect on total chlorophyll content of the leaves, suggesting it has been taken up in to the moss cells; clearly indicating that Pb was more phyto-toxic than either Cd and Ni. The main reason for present observation is probably the induction of metal binding protein of both Cd and Ni. There is further need to investigate other eco-physiological techniques as well in lab that will allow selection of appropriate variables to indicate heavy metal stress.

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