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Original Research Article

Possible involvement of non-protein nitrogenous metabolites in conferring in vitro lead tolerance to *Dalbergia sissoo* Roxb.

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Abstract: *Dalbergia sissoo* Dehn. is planted on the roadsides all over India. A heavy load of lead pollution on these sites due to various reasons is well documented. An investigation was thus, conducted to study the *in vitro* differential action of lead on morphological and biochemical parameters of *D. sissoo* shoots regenerated from young nodal explants. *D. sissoo* has been observed to tolerate very high concentration of Pb ($150mgl^{-1}$) *in vitro*, through its stress alleviation mechanism, which possibly involves detoxification of heavy metal generated ROS species by nitrogenous metabolites, and by accumulation of antioxidant - carotenoids and phenolics. The accumulation of stress alleviating proteins could not be found in the present investigation.

Keywords: *Dalbergia sissoo*; Lead (Pb) pollution; Bioindicator; Growth; PVP (polyvinylpyrrolidone)

Introduction

Environmental pollution is one of the major problems world is facing today. Unmanaged human activities such as livestock grazing, logging and international fire can result in environmental degradation. Burning of fossil fuel, flooding, mining and eutrophication, further deteriorate the environment. Degradation of environment adversely affects germination, growth and biodiversity of plants. Heavy metals are of special interest with respect to toxicological importance to human health, plants and animals (1, 2). Lead is one of the best known heavy trace elements, with a long history of toxicity. Its exposure is becoming a great concern because of its toxic nature, wide occurrence and long life in biological system. The major source of lead in soil are usually derived from weathered bedrock, parent material from lead mine, smelting operations, use of lead arsenate, use of tetramethyl lead as anti-knocking additive to petrol (3). Lead is a common heavy metal and can be found in batteries, ceramics, chemicals and fertilizers. It is also used in a number of products including gasoline, hair dyes, leaded glass, newsprint, paints, pesticides, pottery and rubber toys. In general, Chhattisgarh soils (with higher clay content) are reported to contain 12.8 -$545\mu gg^{-1}$ Pb (4). Inhibition to germination and retardation of plant growth has been reported due to lead toxicity (5, 6, 7).

Plants near roadways have relative increase of Pb deposition due to vehicles using leaded petrol (8, 9). Lead toxicity has become important due to its constant increase in the environment. The effects of heavy metals on the environment and human health have been widely studied (10).

Heavy metals are defined as elements with metallic properties (ductility, conductivity, stability as cation etc.) and atomic weight >20 or in other words with a density higher than $5g\ cm^{-3}$ (11).

In vitro response of lead in *D. sissoo* callus or any other plant have not been reported previously, although, fragmentary reports on effects of different atmospheric lead concentrations on several plants (plant parts) including *D. sissoo* are available (12,13). Since, systematic experiments with lead (Pb) in field or pots may lead to contamination of soil, *in vitro* studies have been preferred. Thus, this work was aimed to study the effect of different concentrations of lead on the growth indices and possible mechanism of lead tolerance in *D. sissoo in vitro.*

Material and Methods

Nodal parts of young shoots of 4-5 year old plant of *D. sissoo* were used as explants. The size of the explant was about 1.5-2.0cm. Nodal explants were collected and kept in running tap water for 30 minutes for

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removal of surface contaminants followed by sterilization with 2–3 drop of tween-20 for 15 minutes and subsequent sterilization with 1% aqueous solution of Bavistin (fungicide) for 15-20 minutes. The explants were rinsed with double distilled water 3-4 times to remove all traces of sterilants. The explants were then disinfected with 0.1% (w/v) aqueous solution of mercuric chloride for 8-10 minutes and further rinsed with sterile double dist water 3-4 times. Nodal parts contai axillary bud in the month of April-May were cultured on semi-solid media containing MS salts and vitamins (14) with 3% (w/v) sucrose, 0.1% PVP and different concentrations of auxin and kinetin with PbAc (lead acetate). The pH of medium was adjusted to 5.7 with 1N NaOH or 1N HCl. Flasks were plugged with non-absorbent cotton plug and sterilized at 121° C and 1.06kgcm^{-2} pressure for 15 minutes. The cultures were maintained in incubation chamber by providing proper light and temperature using PAR lamps and timer. The constituents of MS medium used in these studies were purchased from (HIMEDIA) in a pre-mixed powder form.

Lead was supplied as Pb $(CH_3COO)_2$ in the medium at the concentration of 25, 50, 75, 100 and 150 mgl^{-1}. Eight week old callus, raised as above, and the regenerated shoots, were analyzed for selected morpho-biochemical attributes such as, morphological nature of callus, its dry weight, *in vitro* shoot length and frequency of shoot-multiplication; proline (15), protein (16), phenolics (17), nitrogen (18), Ca^{2+}(19), Pb, chlorophylls (20) and carotenoids (21) in 8-week old callus.

All the biochemical parameters were estimated by using Shimadzu spectrophotometer (UV-1800). Tolerance index of 8-week old regenerated shoots was calculated by modifying protocol of (22).

$$\text{TI (Tolerance index \%)} = \frac{\text{The average shoot length of experimental group}}{\text{The average shoot length of control group}} \times 100$$

Lead analysis:

After 2 months of treatment, callus was dried at 70°C for 48 h, then pulverized and digested in HNO_3: $HClO_4$:: 4:1. Five ml of acid mixture was added in 200 mg dried callus, kept for 24 h and then heated on water bath at 90°C for 1h. After completion of digestion 1ml aliquot was made to 10ml with Distilled Water (D.W.) and analyzed for lead concentration by using Atomic absorption spectrophotometer (LABINDIA AA7000).

Results and Discussion

Callus was raised on an optimized medium for shoot regeneration from nodal explants of *D. sissoo* young shoots, (MS+ 0.5mgl^{-1} BAP+ 0.5 mgl^{-1} IAA+ 0.1 % PVP). After eight weeks of induction, callus exhibited maximum shoot regeneration in control set, whereas, lead treatment (0-150mgl^{-1}) resulted in reduced shoot growth (frequency and length) as compared to control (Table.1).

Table.1. Callus response and Shoot regeneration after 8-weeks of inoculation on MS medium supplemented with different growth regulators and PVP with and without lead acetate in *D. sissoo*

Media + PVP (%)	PGRs (mgl^{-1}) BAP+IAA	Concentration of PbAc (mgl^{-1})	Callus morphology	Degree of callus response	% frequency of shoot regeneration	Mean no. of shoots/explant ±SD#	Mean length of shoot (cm) ±SD#
MS+0.I	0.5+0.5	0	Compact, whitish green	++	80	1.26±0.524	3.35±0.789
MS+0.1	0.5+0.5	25	Compact, whitish green	++	60	0.85±0.506	2.79±0.508
MS+0.1	0.5+0.5	50	Compact, whitish green	++	53.80	0.76±0.571	2.78±1.03
MS+0.1	0.5+0.5	75	Compact, whitish green	++	50.00	0.72±0.443	2.75±0.723
MS+0.1	0.5+0.5	100	Compact, whitish green	+	50	0.73±0.450	2.51±0.803
MS+0.1	0.5+0.5	150	Compact, whitish green	+	46.15	0.70±0.437	2.27±1.169

++ = good response
+ = poor response

However, the callus did not exhibit any morphological changes in the presence of lead, though the degree of callusing reduced beyond 100mgl^{-1} Pb supplementation. Callus attained maximum dry weight (43.11% above control) at 150mg/l supplementation of Pb to the medium (Fig.2). However, looking at the Pb uptake status, a decline in accumulation of Pb in callus started at 150mgl^{-1} external concentration, though it yet remained quite high (Fig.1). Interestingly, Pb did not accumulate (was not taken up) till 75mgl^{-1}concentration of it was added to the medium. This concentration is much above the permissible limit of WHO (23), i.e. only 3.0mgl^{-1}.

The callus responded to higher concentrations of Pb by a sudden increase in uptake (up to 100mgl^{-1}) accompanied with a steep decline in cellular Ca^{2+} content. This may be explained on the basis of competition between non- redox ions at Ca^{2+} permeable channels on the membrane. Such altered transport function for lead (Pb) has been demonstrated in roots by (24) and (25).

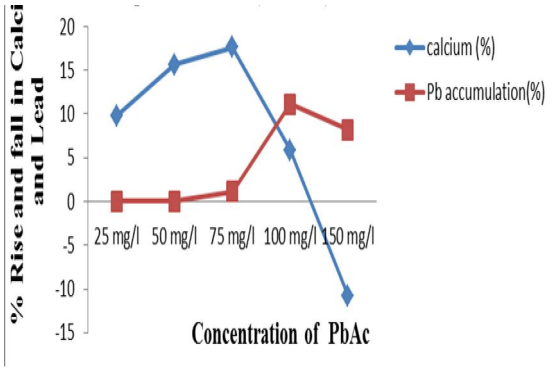

Fig.1: % loss or gain in calcium and lead accumulation in *D. sissoo* calli on supplemented with MS+0.5mg/l BAP+0.5mg/l IAA+0.1% PVP + Lead (25-150mg/l) as compared to control (Lead Free)

D. sissoo is reported to be a lead bio indicator plant (26), yet its tolerance index declines with increase in Pb concentration beyond 50 mg/l and stabilizes between 100-150 mgl^{-1} (Fig. 2). However, with increasing concentration of Pb, increase in Chl a, b, carotenoid, phenolics and nitrogen content; and decline in protein and proline content (Fig. 3, 4) indicate that in *D. sissoo* the probable mechanism of lead tolerance could be through accumulation of carotenoids and phenolics as ROS scavengers (27, 28) besides, nitrogenous compounds other than proteins. Proline contributes under lower concentration of Pb only, in ROS quenching (29), but does not participate in Pb stress alleviation beyond 50mgl^{-1}, rather declines sharply with increasing lead concentration. Continuous increase in N- content might be indicative of the accumulation of metabolites eg. -Glutathione, reducing the Pb- generated oxidative stress from the cells. Such accumulation of glutathione with Ni stress has been demonstrated in shoots of *Thalspi* hyper accumulator species by (30).

Thus, the present investigation brings out that *D. sissoo* can tolerate very high concentration of Pb through its stress alleviation mechanism which involves detoxification of heavy metal generated ROS species by nitrogenous metabolites, possibly glutathione, as demonstrated by other workers in Ni hyper accumulating system, and by accumulation of antioxidant carotenoids and phenolics. The accumulation of stress alleviating proteins could not be found in the present investigation. However, protein and proline appear to combat Pb stress upto 50 mgl^{-1}. Increase in dry weight by more than 40 % above control along with chlorophylls, indicates accumulation of biomass under active synthetic metabolic state and because of hyper accumulation of Pb (only 11.09 % accumulation). Besides, 150mgl^{-1} appears to be slightly toxic as it challenges the Ca^{2+} ion concentration in the callus severely, though still within the limits of tolerance.

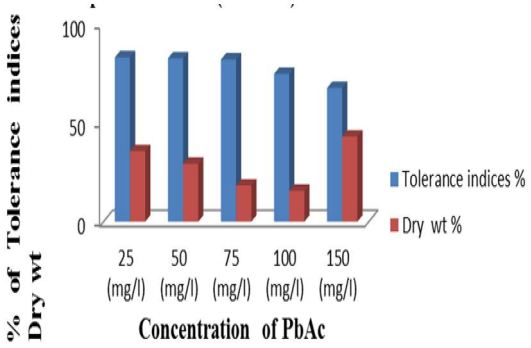

Fig.2: % loss or gain in Tolerance index and dry weight in *D. sissoo* calli on media supplemented with MS+0.5mg/l BAP+0.5mg/l IAA+0.1% PVP + Lead (25-150mg/l) as compared to control (Lead Free)

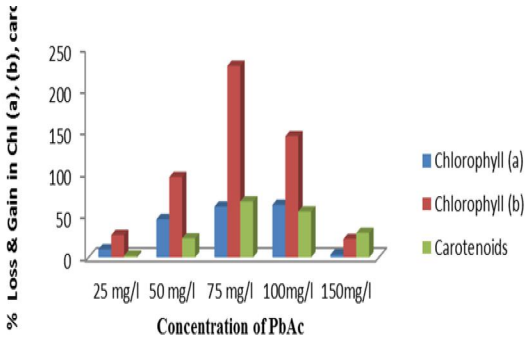

Fig.3: % loss or gain in Chlorophyll a, b and carotenoid content in *D. sissoo* calli on media supplemented with MS+0.5mg/l BAP+0.5mg/l IAA+0.1% PVP + Lead (25-150mg/l) as compared to control (Lead Free)

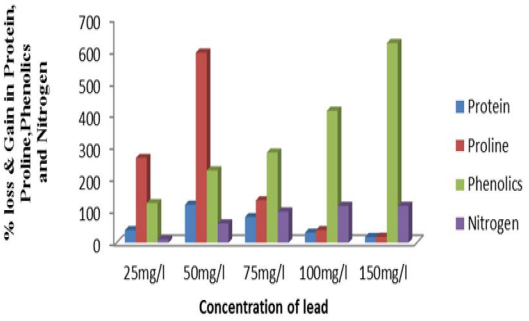

Fig.4: % loss or gain in protein, proline, phenolcs, nitrogen in *D. sissoo* calli on media supplemented with MS+0.5mg/l BAP+0.5mg/l IAA+0.1% PVP + Lead (25-150mg/l) as compared to control (Lead Free)

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