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Detoxification mechanism of heavy metal stress in *Boerhavia diffusa* L.

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Abstract:

Effect of heavy metals such as cadmium, chromium, mercury and lead on morphology, anatomy and secondary metabolite distribution of *Boerhavia diffusa* is investigated by cultivating rooted propagules in Hoagland nutrient solution, artificially contaminated with known quantities of these metals. Cellular distortion and rupture, ill developed-xylem and- phloem are the morphological changes observed in the root and stem due to all metals and these changes vary from metal to metal. Increased cell wall thickening, trichome development on stem and accumulation of stained masses in some cells and vessels are observed in the root and stem. Stained masses indicate the accumulation of the heavy metals. Increase in the synthesis of phenolics is another impact of heavy metal treatment Concerted activity of bioaccumulation potential, enhanced phenolics synthesis, trichome development on the epidermis of the stem etc. are presumed to be the strategy of *B. diffusa* towards detoxification of heavy metal stress.

Key Words: *Boerhavia diffusa*, bioaccumulation potential, heavy metal stress, detoxification, trichomes, phenolics

Introduction:

Heavy metals are important environmental pollutants and many of them are toxic even at very low concentrations. Pollution of the biosphere with toxic metals has been accelerated dramatically since the beginning of the industrial revolution (Foy *et al*., 1978; Nriogo, 1979; Liang *et al.,* 2011; Shivahare, Sharma, 2012; Abdussalam *et al*., 2013 and Swapna *et al*., 2014). The primary sources of environmental pollution are the burning of fossil fuels, the mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, and sewage (Kabata- Pendias and Pendias, 1999*).* There is vast literature of analytical data relating to metal uptake and bioaccumation illustrating the scale of differences between species and genotypes and between metals in the field and laboratory studies ranging from trace nutrient elements to toxic heavy metals (Lepp, 1981; Prasad, 1997; Cseh, 2002). Heavy metals like cadmium, chromium, mercury, lead etc. are having no beneficial properties for the plant growth but are highly reactive and consequently toxic to plants.

General symptoms of cadmium toxicity in plants are growth inhibition, low biomass production, impaired water relations, respiration, photosynthesis and nitrogen metabolism (Sergin and Ivanov, 2001; Perfus-Barbeoch *et al.*, 2002; Linger *et al.*, 2002; Tran and Popova, 2013). Chromium toxicity in plants is observed at multiple levels such as reduced yield, inhibited growth of leaves and roots, (Clijsters and Van Assche, 1985; Bishnoi *et al*., 1993; Shanker *et al*., 2005). Major impact of mercury has been reported on plant functions such as uptake and distribution of minerals (Beauford *et al*., 1977), bioaccumulation (Velasco-Alinsug *et al*., 2005). Effect of lead on translocation (Tomsig and Suszkiw, 1991), rapid root growth inhibition (Gzyl *et al*., 1997; Malkowski *et al*., 2002) and bioaccumulation (Arazi *et al*., 1999; Kim *et al*., 2002; Axtell *et al*., 2003) have been investigated in many plants.

Boerhavia diffusa is a medicinal plant widely used as an important component of many Ayurvedic preparations. About 45 Ayurvedic preparation are manufactured using *B. diffusa* as an important ingredient (Sivarajan and Balachandran, 1994). This wild plant grows profusely in polluted areas such as road side, railway track, banks of drainage, near public comfort stations and wet lands which are used for waste disposal and hence highly polluted. This taxon is well adapted to diverse soil conditions also. Notwithstanding, physiological impacts, bioaccumulation pattern and mechanism of detoxification of these toxic heavy metals have not yet been investigated in cultivated plants in general and medicinal plants in particular. Hence the objective this investigation is the elucidation of detoxification mechanism in relation to adaptation and metabolism of *B. diffusa* cultivated in nutrient medium artificially contaminated with known quantities of Cadmium, Chromium, Mercury and Lead.

Materials and Methods:

Boerhavia diffusa plants were cultivated in Hoagland nutrient solution artificially contaminated with known quantities of Cadmium chloride (30 µM), Potassium dichromate (400 µM), Mercuric chloride (10 µM) and Lead acetate (600 µM). Culture techniques, treatment and sampling pattern were done according to Abdussalam *et al.* (2013). For anatomical studies, uniformly cut pieces of root and stem of control and treated plants samples after four days of growth were fixed in FAA, dehydrated through alcohol TBA series and embedded in paraffin wax (Johansen, 1940; Berlyn and Miksche, 1976). Using Rotary Microtome (Leica Model RM 2125RT) individual blocks were cut at 10µm and sections were used for anatomical staining. Sections were stained with aqueous Toluidine Blue according to the procedure of Khasim (2002) deparaffinised in xylene and mounted in DPX. Photomicrographs were taken by using Nikon Microscope (Model, ECLIPSE E 400) fitted with Nikon Digital Camera and Image Analyser. Total phenolics were estimated using Folin-Denis Reagent (Folin and Denis, 1915). Cadmium, chromium, mercury and lead content of the root, stem and leaf tissues were analyzed by Atomic Absorption Spectrophotometry in the samples prepared according to the method of Allan (1969).

Results:

Anatomical Studies

Anatomy of root showed cellular damage due to Cd treatment in *B. diffusa*. In the roots, piliferous layer and cortex cells were completely torned compared to control plants. Roots tissue treated with chromium showed general thinning and complete damage of piliferous layer and cortex. Treatment with mercury resulted in a general reduction of cells size in the roots compared to the control and other treatments. Piliferous layer and cortex were fully damaged and cell number was reduced in the mercury treated tissues. Lead treatment showed comparatively slight adverse effect in the root structure. Partial damage of the piliferous layer was also observed. (Fig. 1 &2).

Cadmium treatment in the stem tissue was completely break resulted in ill-developed phloem tissue. Stem of *B. diffusa* treated with chromium showed complete damage of epidermis, hypodermis, secondary phloem and outer part of sclerenchymatous conjunctive tissue. Epidermal and cortical cells showed partial damage. Some of the epidermal cells were modified into trichome like appendages which were multicellular and filled with stained patches (Fig.1). General reduction of epidermal and hypodermal cell size was observed in the mercury treated stem. Some epidermal cells were modified to multicellular trichomes (Fig.2). In stem of the lead treated tissue, epidermal and hypodermal cells showed partial cell wall breakage.

Due to the cadmium treatment, the stem secondary phloem and cambium cells were completely damaged. Partial damage was observed in sclerenchyma and conjunctive tissue (Fig1). More cell wall thickening was observed in the outer regions of paranchymatous pith. The meduallary vascular bundles were intact. Some pith cells and vessels showed stained masses accumulated in the lumen indicating the accumulation of cadmium (Fig.1). Cell wall

thickening of parenchyma and xylem vessels was another impact whereas the number of xylem vessels was reduced due to the chromium treatment compared to the control (Fig.1). More cell wall thickening was seen in schlerenchymatous conjunctive cells and paranchymatous pith. Secondary xylem vessels showed reduction in size and number. Accumulation of stained masses were seen in some cells of pith and vessels in the chromium treated tissues. Mercury treatment showed highly reduced Sclerenchymatous layer but cells were slightly thick walled. Paranchymatous pith region was damaged showing some breakage or lysis of cell wall resulting in the formation of many cavities. Accumulation of stained masses was observed in the sclerenchymatous conjunctive tissue and secondary phloem. Secondary phloem and outer layer of sclernchymatous conjunctive tissue were damaged due to the lead treatment. Cell wall thickening and reduction of cell size were seen in the conjunctive tissues. Pith cells were slightly distorted. The number of secondary xylem vessels was reduced. Medullary bundles were almost intact. Some vessels and pith cells contained stained masses due to the lead treatment (Fig. 1 & 2).

Phenolics

Phenolic content of root tissue was increased insignificantly in the plants treated with cadmium and the increase was significant in the roots treated with chromium and mercury (Table 1), whereas lead showed a doubling compared to the control. The stem tissue also followed more or similar trend in the distribution of phenolics as that of root tissue. Phenolic content of leaf tissue of *B. diffusa* plant treated with cadmium remained intact whereas significant increase was observed in the leaf tissue of plants treated with chromium, mercury and lead ($P<0.01$). Among the treatments, the increase in the phenolics of leaf tissue was in the order Cd< Cr<Hg<Pb.

Bioaccumulation of Metals

Accumulation of cadmium in the root tissue was continuously increased proportional to the duration of treatment. Comparatively very low amount of cadmium was present in the stem tissue which started accumulating 4^{th} day onwards and gradually increased. First day onwards, chromium started accumulating in the root tissue and considerable quantities were accumulated and the increase was linear during growth. Accumulation of mercury in the root tissue was started 4^{th} day onwards and the rate of increase was linear and proportional to the concentration. After first day of treatment lead was present only on the root tissue and stem showed the accumulation on 4^{th} day (Table 2).

Discussion:

Plants absorb, distribute and accumulate essential and non essential metals internally in different ways. Localization of many metals is seen mostly in roots and stems and or metals are stored in non-toxic form for future distribution and use. One of the mechanisms of tolerance in plants apparently involves binding of potentially toxic metals on cell walls of roots, stem and leaves, away from sensitive sites within the cell or storing them in a vacuolar compartments. The metal form appears to have a decisive role in metal absorption and transfer to various parts of the plant body and /or other organisms. It is of great interest to note that plant species which have no exclusion mechanism in the roots, absorb and translocate large quantities of metals and accumulate them in their growing parts, especially in their leaves, without showing any toxicity symptoms, via a sort of internal resistance which varies from plant to plant or metal to metal (Prasad, 1997; Orcutt and Nilsen, 2000; Fodor, 2002; Pilon –Smits, 2005).

Anatomy of root and stem of *Boerhavia diffusa* shows cellular damage due to the treatment with cadmium, chromium, mercury and lead. Since the plants are grown in nutrient culture medium containing these metals, the roots are in direct contact with the toxic metal ions and the root growth is adversely affected resulting in cellular damage and general thinning and brittle texture of roots. Among the four metals, accumulation in the form stained

masses are seen in the roots of plants treated with cadmium, chromium and lead. Maximum bioaccumulation also was shown by quantitative estimation (Table 2). Damage of secondary phloem and reduced size and number of vessels, are found to be the reason for comparatively low translocation of these metals to leaves and hence adversely affect the influx of photosynthates to the stem and root, culminating in general growth retardation. Development of trichomes in the stem epidermis of plants treated with chromium and mercury (Figs. 1 &2) is found to be related to the escape of the accumulated metals in the stem as suggested by Dominguez- Solis *et al*. (2004) who reported the involvement of trichomes in the efflux of accumulated cadmium in *Arabidopsis thaliana*.

An important impact of heavy metal toxicity in *B. diffusa*, is accumulation of phenolic content in the plant body (Table 1). Since phenolics are the precursor of lignin (Buchanan *et al*., 2000), increased phenolic content due to heavy metal stress is directly related to lignin synthesis resulting in woody texture which is found to be an impact rather, a defensive mechanism of heavy metal stress in plants. Induction of phenolic compound biosynthesis has been reported in maize in response to aluminium (Winkel-Shirley, 2002), in pepper due to copper (Diaz *et al*., 2001) and in green gram exposed to cadmium (Dietz and Schnoor, 2001). The anatomical changes such as woody and brittle nature of stem and root and cell wall thickening observed in *B. diffusa* (Fig.) due to lignification induced by heavy metal stress are in conformity with the views of Michalak (2006) who reported *de novo* synthesis of soluble phenolics takes place under heavy metal stress and these compound act as intermediates in lignin biosynthesis.

Phenolic compound have been attributed to function also as antioxidants in plants under stressful condition (Michalak, 2006). According to this author, flavenoid group of phenolics can directly scavenge molecular species of reactive oxygen species (ROS) due to their ability to bind with phenolic molecules. Significant increase of phenolic compounds (Table 1) which may act as antioxidants is found to be another defensive mechanism in *B. diffusa.* This observation is in conformity with the views of Posmyk *et al.* (2009) who suggested that in red cabbage, accumulation of phenolics, anthocyanine and other isoflavinoides is an effective strategy against reactive oxygen species under copper stress. Antioxidant property of phenolic compound is due to their high tendency to chelate metals, because phenolics possess hydroxyl and carboxyl groups which effectively bind with metal ions (Morgen *et al*., 1997). Polyphenols possess ideal structural chemistry for scavenging activity and have been shown to be more effective than ascorbate under *in vitro* condition (Rice –Evans *et al.*, 1997). According to Lavid *et al*. (2001) direct chelation of chromium, lead and mercury by binding to phenolics occur in *Nymphaea alba*. In *B. diffusa* coincident of abundant occurrence of phenolics and maximum accumulation of all heavy metals in general and chromium and lead in particular in the roots reveal the chelation of these metals by phenolics resulting in the sequestration and reduced translocation to the aerial parts. More or less similar occurrence of abundant phenolics and bioaccumulation of all metals except cadmium is found also in the leaves of *B. diffusa*.

All the parameters adopted for the present investigation provide a detoxification mechanism shown by *B. Diffusa* towards Cd, Cr, Hg and Pb. The optimal concentrations selected for culture studies were standardised by observing about 50% growth retardation (Abdussalam *et al*, 2013) and hence it seems that despite the plants are under stress, their survival is controlled by some detoxification process. Observations such as morphological/anatomical modifications and cell wall thickening due to lignifications which is directly linked with increased phenolics synthesis are found to be important mechanisms of detoxification rather adaptations of *B. diffussa* towards Cd, Cr, Hg and Pb. Similarly bioaccumulation also is another mechanism of detoxification since localisation of these metals are restricted only to some cells/tissues permitting plant growth and survival inspite of some toxicity symptoms.

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Table: 1 Effect of Heavy Metals on Phenolics in *Boerhavia diffusa* (mg g^{-1} dry weight)

Treatments	Tissues	Interval (Days)					
		0	4	8	12	16	20
Control	Root	0.45±0.03	0.68±0.02	0.79±0.01	0.93±0.03	1.21±0.03	1.38±0.02
	Stem	0.68±0.01	0.98±0.05	1.54±0.10	1.68±0.01	1.73±0.04	1.89±0.10
	Leaf	0.79±0.04	0.92±0.02	1.24±0.10	1.58±0.09	1.67±0.06	1.81±0.12
Cadmium	Root	0.45±0.02	0.73±0.05	0.95±0.05	1.24±0.10	1.59±0.04	1.67±0.09
	Stem	0.68±0.03	1.12±0.09	1.47±0.06	1.68±0.09	1.98±0.03	2.10±0.08
	Leaf	0.79±0.02	1.14±0.10	1.27±0.08	1.38±0.08	1.59±0.08	1.78±0.10
Chromium	Root	0.45±0.04	0.84±0.03	1.26±0.02	1.58±0.10	1.94±0.09	2.10±0.08
	Stem	0.68±0.04	0.93±0.02	1.45±0.10	1.73±0.02	1.98±0.08	2.51±0.13
	Leaf	0.79±0.02	1.27±0.04	1.59±0.08	1.81±0.08	2.33±0.10	2.85±0.10
Mercury	Root	0.45±0.01	0.95±0.02	1.59±0.10	1.74±0.09	2.36±0.10	2.59±0.12
	Stem	0.68±0.02	0.95±0.02	1.62±0.09	1.84±0.12	2.40±0.11	2.83±0.23
	Leaf	0.79±0.02	1.03±0.04	1.56±0.10	1.89±0.14	2.62±0.11	3.54±0.14
Lead	Root	0.45±0.01	0.54±0.05	1.52±0.10	2.04±0.18	2.85±0.12	3.94±0.10
	Stem	0.68±0.04	0.69±0.03	1.26±0.20	1.89±0.09	2.64±0.12	3.87±0.12
	Leaf	0.79±0.07	1.12±0.01	1.86±0.02	2.58±0.09	3.62±0.12	3.86±0.13

Table: 2 Bioaccumulation pattern of Heavy Metals in *Boerhavia diffusa* cultivated in Hoagland solution containing known quantities of Cd, Cr, Hg and Pb.

Interval of sample collection (Days)	Tissues	Bioaccumulation of Heavy metals(µg/g) Dry weight			
		Cadmium (5.5µg) *	Chromium (58.8 µg) *	Mercury (2.715µg) *	Lead (227.6 µg) *
1	Root	0.454 (8.2)	3.48 (5.9)	0.116 (4.27)	0.230 (0.09)
	Stem	NDR	NDR	NDR	NDR
	Leaf	NDR	NDR	NDR	NDR
4	Root	0.970 (17.6)	3.850 (6.5)	0.310 (11.4)	0.830 (0.35)
	Stem	0.220 (4.0)	0.192 (0.32)	NDR	0.100 (0.04)
	Leaf	NDR	NDR	NDR	NDR
8	Root	1.670 (33.4)	5.000 (8.5)	0.410 (15.1)	6.080 (2.61)
	Stem	0.280 (5.0)	2.790 (4.7)	0.110 (4.1)	0.230 (0.09)
	Leaf	NDR	NDR	NDR	NDR

12	Root	2.179 (39.5)	20.79 (35.3)	0.750 (27.6)	8.870 (3.8)
	Stem	0.937 (17.6)	6.070 (10.3)	0.340 (12.5)	0.380 (0.16)
	Leaf	NDR	0.110 (0.18)	NDR	0.087 (0.03)
16	Root	2.230 (40.5)	23.26 (39.5)	0.970 (35.7)	87.85 (37.7)
	Stem	1.190 (21.6)	6.140 (10.4)	0.410 (15.1)	11.08 (4.7)
	Leaf	NDR	0.142 (0.24)	0.110 (4.1)	0.230 (0.09)
20	Root	2.550 (46.3)	26.02 (44.2)	1.110 (40.0)	107.1 (46.0)
	Stem	1.300 (23.6)	3.410 (5.8)	0.714 (26.2)	26.90 (11.5)
	Leaf	0.104 (18.9)	0.200 (3.4)	0.220 (8.10)	0.377 (0.16)

***Note:** 5.5 µg cadmium (250 ml of 30 µm $CdCl_2$), 58.8 µg chromium (250 ml of 400 µm $K_2Cr_2O_7$), 2.715 µg mercury (250 ml of 10 µm $HgCl_2$) and 227.6 µg lead (250 ml 0f 600 µm CH_3-$COO)_2$ Pb $3H_2O$).Values in the parenthesis are percentage of accumulation of each metal

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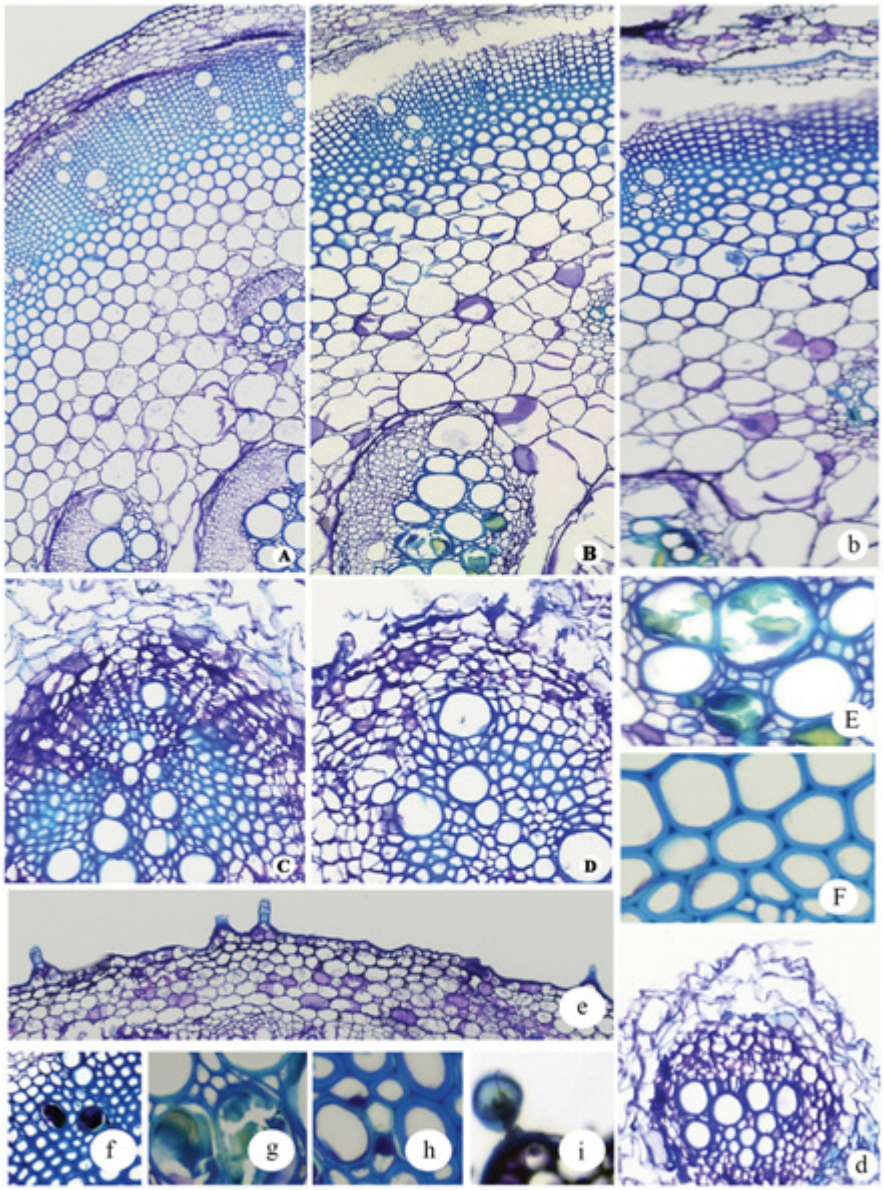

Fig. 1 Histochemical localisation of Cadmium & Chromium in the Stem and Root of *Boerhavia diffusa*

Cadmium

A. Control Stem; B. Treated Stem; C.Control Root; D. Treated root.
E. Cadmoum localisation; F. Cell wall thickening.

Chromium

A. Control Stem; b. Treated Stem; C.Control Root; d. Treated root. e & i. Epedermis with trichomes; f & g. Chromium localisation; h Cell wall thickening

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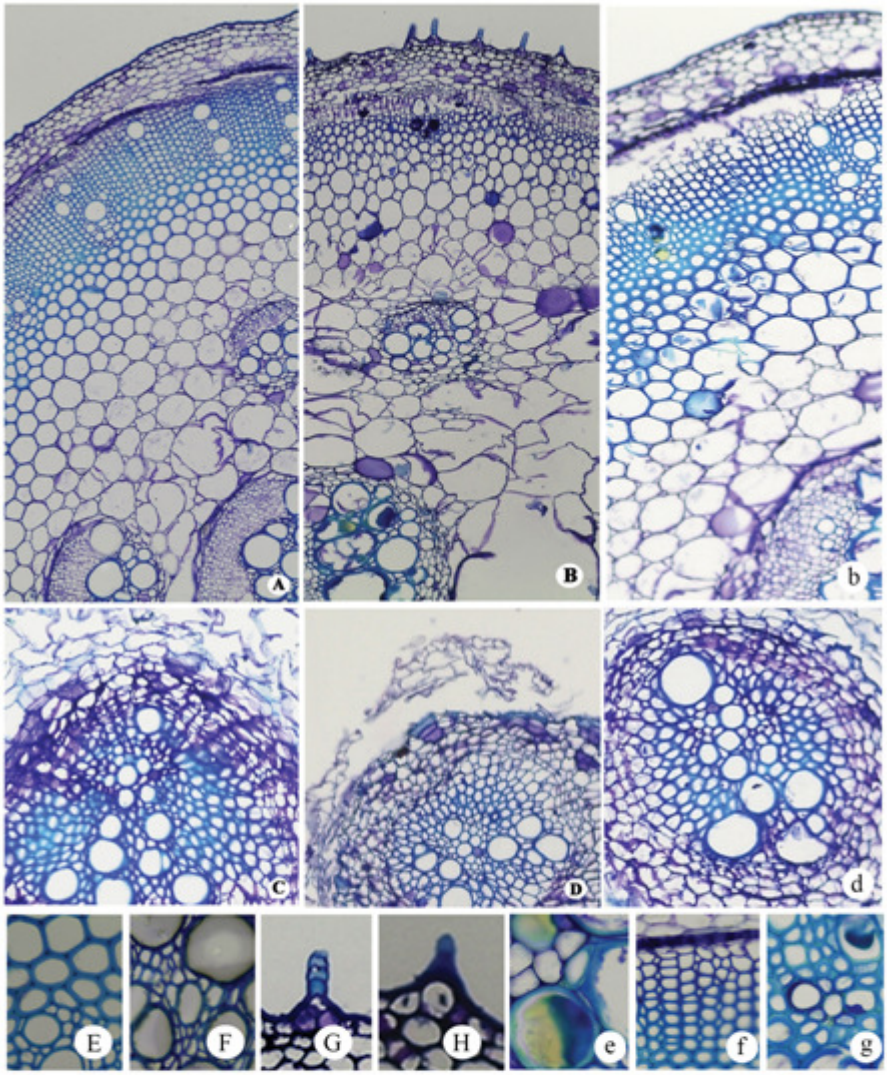

Fig. 2 Histochemical localisation of Mercury & Lead in the Stem and Root of *Boerhavia diffusa*

Mercury

A. Control Stem; B. Treated Stem; C. Control Root; D. Treated Root

E & F. Cell wall thickening ; G & H. Epidermis with trichome

Lead

A. Control Stem; b. Treated Stem; C. Control root; d. Treated root.

e & f. Localisation of lead; g. Cell wall thickening

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