NBU Journal of
Plant Sciences

Vol. 6, No.1March 2012 p.71-76
ISSN No. 0974 6927

Responses of Zn and Cd treatment in soybean and fenugreek

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Abstract
The effect of various doses of zinc and cadmium on morpho-anatomical and physiological parameters of soybean and fenugreek was studied. High concentrations of both Zn as well as Cd resulted in chlorosis, necrosis, retardation of growth and reduction in leaflet size especially in soybean. A characteristic symptom was the formation of a red-brown pigment in the leaves, stem and roots. An increase in the phenolic contents was also observed with increasing Cd doses. The reasons for the formation of pigment have been explored.

Keywords: Cadmium, zinc, toxicity, soybean, fenugreek, anatomy

Zinc and cadmium are major environmental contaminants (Cakmak *et al.*, 2000). They form aerosol which disperse and precipitate out with dust (Friberg *et al.*, 1971) and may be absorbed by leaves and subsequently transported to other organs. Zinc is well known as an essential mineral nutrient for normal growth of plants. The small difference between adequate and toxic zinc is shown in the report of Roberg (1932) that 0.001 mg zinc per 100 ml solution stimulated whereas 0.005 mg retarded the growth of *Chlorella*. Zinc toxicosis has been reported in several plants in green house studies when excessive levels of zinc were added (Chaney, 1973).

Cadmium is not an essential element for plant growth but in spite of this it is accumulated in quite high amounts by different plant species (Aery and Tiagi, 1988). In view of the importance of plants in most food chain (both natural and agricultural) a number of studies have been directed towards cadmium accumulation and its effects on plants. The gross effects of cadmium toxicity have been shown to be chlorosis, necrosis and reduction in growth (Haghiri, 1973; Sandalio *et al.*, 2001; Clemens, 2006; Radha *et al.*, 2010; Wang *et al.*, 2011; Khatamipour *et al.*, 2011; Houshmandfar and Moraghebi, 2011).

Though lot of work has been done on the phytotoxicity of zinc and cadmium little work has been attempted especially on the localisation of these metals in various plant tissues (cf. Aery and Sarkar, 1988) and the concerned anatomical variations and that too in cultivated plants. In order to know the critical and toxic levels of zinc and cadmium for soybean and fenugreek the effect of these two metals added to soil in different doses was studied. The main object was to study the response of soybean and fenugreek in respect of various morpho-anatomical and physiological parameters towards these environ-mental contaminants.

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Materials and Methods

The seeds of *Glycine max* (L.) Merr. var. Gaurav (Soybean) and *Trigonella foenum-graecum* L. (fenugreek) strain UM-34 were used for the study. Garden soil of average fertility was used for the study. Four kilogram of soil was filled in each earthen pot of 30 centimeter height and 25 cm diameter. Nine concentrations of zinc and seven concentrations of cadmium were prepared separately by taking corresponding amounts (calculated on the basis of their molecular weights) of the chemicals per kilogram of air dried soil. The chemicals were mixed thoroughly in the soil. For zinc, zinc sulphate ($ZnSO_4.7H_2O$; E. Merck, GR) and for cadmium, cadmium chloride ($CdCl_2.2H_2O$; Analar, BDH, England) were used. The zinc was applied in the doses of 10, 25, 50, 100, 500, 1000, 2500, 5000 and 7500 µg/g while for cadmium the doses were 5, 10, 50, 100, 250, 500 and 1000 µg of cadmium per gram of air dried soil. Pots without any added metal constituted the control. Ten seeds of each crop were sown equidistantly at 2 centimeter depth in each pot. Before sowing, soybean seeds were inoculated with its *Rhizobium* strain obtained from the microbiological division of IARI, New Delhi.

Watering was done on alternate days for fenugreek and every day for soybean as recommended in agricultural practices. After establishment, seedlings were thinned to five in number for soybean and three for fenugreek in each pot.

Four sets in duplicate were prepared to record observa-tions for each crop in all the four stages of their life span, i.e., seedling, vegetative, flowering and fruiting for both the metals separately for shoot and root length nodule number and weight, leaf area, dry weight, grain yield and morpho-anatomical features. The material was dried in an oven, digested in acid and Zn and Cd contents were determined with the help of an AAS. Total phenol contents were estimated by Folin-Ciocalteau method (Aery, 2010). Results on the effects on morpho-anatomical features are being discussed here.

Results and Discussion

Visual Toxicity Symptoms:

Effect of zinc on soybean:

Chlorosis, thickening of leaf and necrosis started appearing in the lower pair of leaflets at zinc doses of 2500, 5000 and 7500 µg/g even in the seedling stage. Chlorosis first started appearing on the margins and on the adaxial surface of leaf which extended towards the midrib. Base and tips of leaves became necrotic. The above symptoms increased with concentration of zinc and age of the plant. In the fruiting stage at a zinc level of 1000 µg/g, lower 3-4 leaflet pairs became completely chlorotic. Zinc doses of 5000 to 7500 µg/g also decreased stem elongation which resulted in stunted growth.

Another symptom of zinc toxicity in soybean was a characteristic deep red-brown pigment formation in the roots especially in the plants treated with high zinc (5000 µg/g and 7500 µg/g) doses.

Effect of cadmium on soybean:

At higher doses of cadmium, i.e., at 250 to 1000 µg/g, there was a retardation of growth, reduction in leaflet size and shortening of stem internodes. Additional stems with abnormal small-sized trifoliate leaves developed in plants at 250, 500 and 1000 µg/g cadmium doses. Further, 10 to 1000 µg/g cadmium levels showed an increasing degree of red-brown pigmentation in the roots of the plants (Figure 1A).

Formation of red-brown pigment was also observed in leaves which formed in the veins at 250 to 1000 µg/g cadmium dose in seedling stage. With the increasing age, red-brown pigment deposition spread along the major veins and petioles of primary leaves and was more apparent on the adaxial surface where it appeared earlier. Necrotic spots could also be observed on the leaves. The extent of pigment formation was always higher at higher cadmium treatments than at lower concentrations.

Table 1 Cadmium toxicity symptoms

Cd dose	Symptoms
0 & 5 µg/g	No visual toxicity symptoms.
10 µg/g	Red colouration at petiole base and base of primary leaf and lower trifoliate leaves.
50 µg/g	Red colouration at petiole base of all leaves; red colouration extended slightly from base to the margin or tip of the primary as well as trifoliate leaves.
100 µg/g	Symptoms present in the 50 µg/g Cd dose; primary as well as lower 3-4 trifoliate leaflets showed signs of redness.
250 & 500 µg/g	Leaves smaller than control, curved, chlorotic with slight to prominent reddish tinge in veins.
1000 µg/g	Leaves comparatively more small, thick, somewhat leathery, completely yellowish with red veins; leaf curvature more prominent

Table: 2 Total phenol contents in soybean under the influence of different cadmium concentrations

Cadmium Concentrations (µg/g)	Total phenols (mg/g)
Control	2.45
5	2.30 (-6.12)
10	2.70 (+10.20)
50	2.95 (+20.40)
100	3.25 (+32.65)
250	3.45 (+40.81)
500	3.85 (+57.14)
1000	3.85 (+57.14)

Leaflets also showed intraveinal chlorosis which increased with age and applied cadmium concentrations. At acute toxicity levels (1000 µg/g) the first and second leaves appeared pale-yellow and largely devoid of chlorophyll (Figure 1A). The chlorosis was accompanied by a severe laminar deformation followed by abscission in some cases.

Effect of zinc and cadmium on fenugreek:

Higher concentration of zinc and cadmium affected both root as well as top growth. Plants showed stunted growth, with less expansion of internodes, increased diameter of stem and thick leathery leaves. Cadmium was found inhibitorier to the growth than zinc. Roots showed red-brown pigmentation. Here plants could not survive even after 100 µg/g in fruiting stage which showed early lethality as compared to zinc. Leaves were not retained on the plant and were shed due to early leaf fall.

Anatomical Toxicity Symptoms:

Anatomical studies were carried out by cutting free hand sections to study the deposition pattern of red-brown pigment in the leaf, stem as well as root and to observe any histological changes under the influence of toxic doses of cadmium.

In a T.S. of the root of soybean in control (Figure 1B) no traces of any pigmentation were visible. The pith region was intact and no disorganization has occurred.

In comparison to control (Figure 1D) a T.S. of the root of cadmium treated (1000 $\mu g g^{-1}$) plant showed (Figure 1C) a completely disorganised pith and disorganization in patches in the cortex. A heavy deposition of red-brown pigment in patches in the epidermis, cortical, endodermal, pericycle and pith cells adjoining metaxylem elements was observed. The epidermal and cortical cells showed the maximum pigment deposition. Though the pigment deposition led to the filling of some of the parenchyma cells completely (Figure 1C), the deposition was primarily in the intercellular spaces and cell walls of the parenchyma cells. Often the cell wall and the deposits protruded some distance into the cell cavity. No deposition was observed in the xylem vessel lumens.

Similarly, in comparison to control (Figure 1F) a T.S. of the stem of the cadmium treated (1000 µg/g) plant also showed a completely disorganised pith and heavy deposition of red-brown pigment in the epidermal, Table: 3 Showing relation among the soil applied cadmium concentrations and Cd contents in different plant parts at maturity in soybean and fenugreek

Cadmium conc. (µg/g)	Cadmium concentrations (µg/g) in plant parts					
	Soybean			Fenugreek		
	Shoot	Leaf	Root	Shoot	Leaf	Root
Control	0.48	0.58	0.75	0.28	0.38	0.68
5	3.15	3.58	6.30	10.37	11.35	12.17
10	6.30	11.89	54-59	12.69	12.70	12.83
50	18.48	30.09	99.73	21.19	31.70	40.97
100	28.51	35.82	191.52	57.69	64.78	80.67
250	44.42	47.28	355.62	-	-	-
500	45.85	60.18	696.43	-	-	-
1000	117.50	141.86	829.70	-	-	-

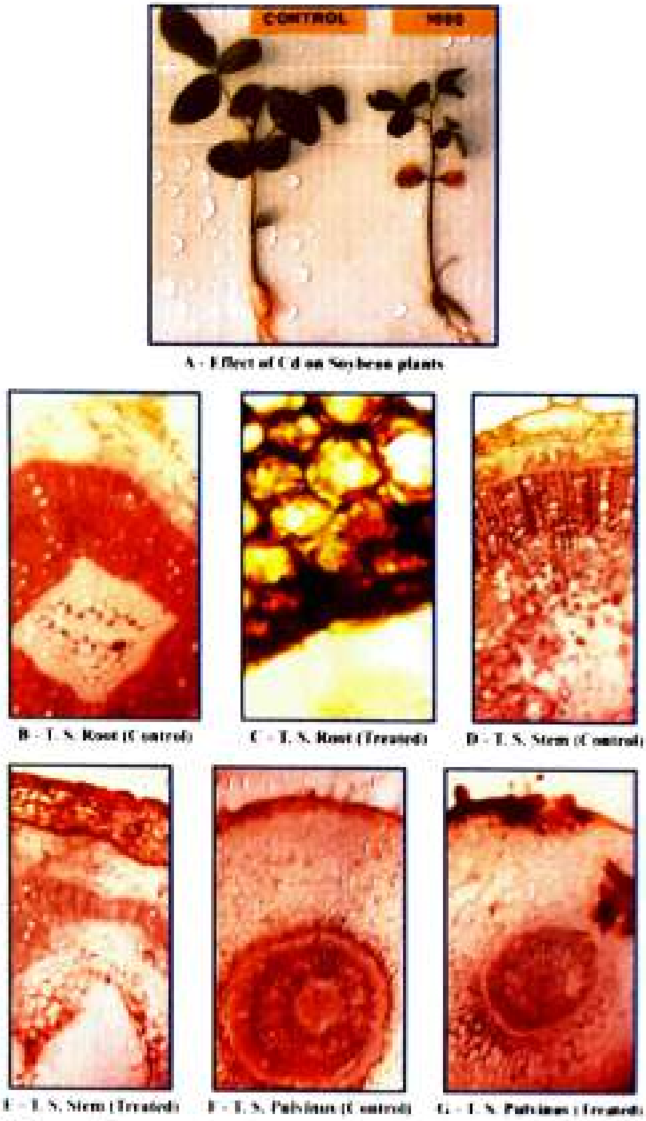
cortical, endodermal and margins of disorganised pith regions. The red-brown pigment completely masked the green colour of chlorenchymatous cortical cells. No such deposition was observed in the pericycle, phloem and xylem regions (Figure 1E). Moreover, the, pattern of secondary vascular tissue formation was found to be somewhat irregular. At some places the amount of secondary xylem was much more than at other places (Figure 1E).

In cadmium treated (1000 µg/g) plants a T. S. of the pulvinus showed a red-brown pigment deposition mainly in the epidermal region and that too in patches (Figure 1G). The epidermal hairs, epidermis, as well as 1-2 cortical layers adjoining epidermis got heavy but patched deposition of the pigment. Pigment deposition was also observed in patches in the cortical zone at the place of departure of leaf trace and endodermal cells. No such deposition was visible in vascular zone as well as pith which remained intact (Figure 1G).

In the T.S. of petiole, (Figure 2A) in the control, the cells are neither disorganised nor showed any pigmentation. In cadmium treated (1000 µg/g) plants a heavy deposition of red-brown pigment in the epidermis, upper cortical layers, as well as pith region was observed (Figure 2B). The xylem and phloem bundles were free from pigmentation. Moreover, a slight to marked elongation of cells both in the cortical and pith region was also observed (Figure 2B & C).

A T.S. of the primary leaf of cadmium treated (1000 µg/g) plant showed heavy deposition of red-brown pigment in the epidermal and adjoining mesophyll cells both on the adaxial, as well as the abaxial side (Figure 2 E). This deposition was highly prominent in the midrib region. Even the hairs present both on abaxial and adaxial sides in this region got impregnated with the pigment (Figure 2 E). This gave the red-brown colour to the veins of primary leaf and trifoliate leaflet as visual symptoms. A slight disorganization of tissues, both below and above the vascular bundle occurred. In contrast to control (Figure 2D) the cells above and below the vascular bundle in the T.S. the leaf of cadmium treated plants gave a storied appearance (Figure 2F). Xylem and phloem parenchyma also showed slight deposition of red -brown pigment. No pigment was present in the lumen of xylem vessels.

Several studies have shown the effect of heavy metals on plants resulting in reduced growth and phytomass accumulation (Marques *et al.*, 2000; Sandalio *et al.*, 2001). However, few studies are available in the literature on visual symptoms of zinc and Cd toxicity. John *et al.* (1972) observed chlorosis of leaves and stunted growth in radish plants due to addition to 50 mg of Cd to 500 g of silt loam soil. However, no such symptoms could be observed for lettuce. Rauser (1973) reported colouring of leaf veins in white bean (*Phaseolus vulgaris*) plants grown under conditions of zinc toxicity. Root *et al.* (1975) has reported the presence of a Cd induced inter-veinal chlorosis and correlated it with a decrease in chlorophyll content in corn. Haghiri (1973) has shown in soybean a progressive browning of the veins which ended in chlorosis and correlated it with increased Cd treatment in soybean. Boggess and Koeppe (1977) and Boggess *et al.* (1978) studied a number of soybean varieties grown on soil amended with $CdCl_2$ or sewage sludge. Cd toxicity symptoms appeared from slight effects such as a red to red-brown colouration at the junction of the leaf blade and petiole to severe leaf curling and extensive reddening of the leaf veins, chlorosis and finally a brittle condition which was followed by abscission (Boggess and Koeppe, 1977). Brisson *et al.* (1977) reported toxicity symptoms not only due to heavy metals but also to infection by certain pathogens. Visual symptoms developed by plants in response to heavy metals have also been reported by Fontes and Cox (1998). Srighar *et al.* (2005) and Vollenweider *et al.* (2006) have studied the effects of Cd on the leaf anatomy of *Brassica juncea* and *Salix viminalis*, respectively. Gomes *et al.* (2011) studied the effect of heavy metals on biomass production, photosynthetic capacity, and anatomical changes in roots and leaves of *Brachiaria decumbens*. Reduction in size and number of conducting elements of the xylem in response to heavy metals has been reported by Sandalio *et al.* (2001). Chandra *et al.* (2010) has studied the effects of Cd and Cr on the structure of root and stem as well as accumulation of Cd and Cr in the plant body of *Vigna unguiculata* and *Vigna radiata*. Cvetanovska *et al.* (2010) has studied the anatomical and physiological disorders in tobacco (*Nicotiana tabacum* L.) after intoxication with copper, cadmium and lead. Hussain (2010) has reported the presence of safranin stained masses in the xylem vessels of stem of *Bacopa monnieri* (L.) Pennell under the effect of $HgCl_2$ contaminated water.



A - Effect of Cd on Soybean plants

B - T. S. Root (Control) C - T. S. Root (Treated) D - T. S. Stem (Control)

E - T. S. Stem (Treated) F - T. S. Pulvinus (Control) G - T. S. Pulvinus (Treated)

Figure 1 See text for details

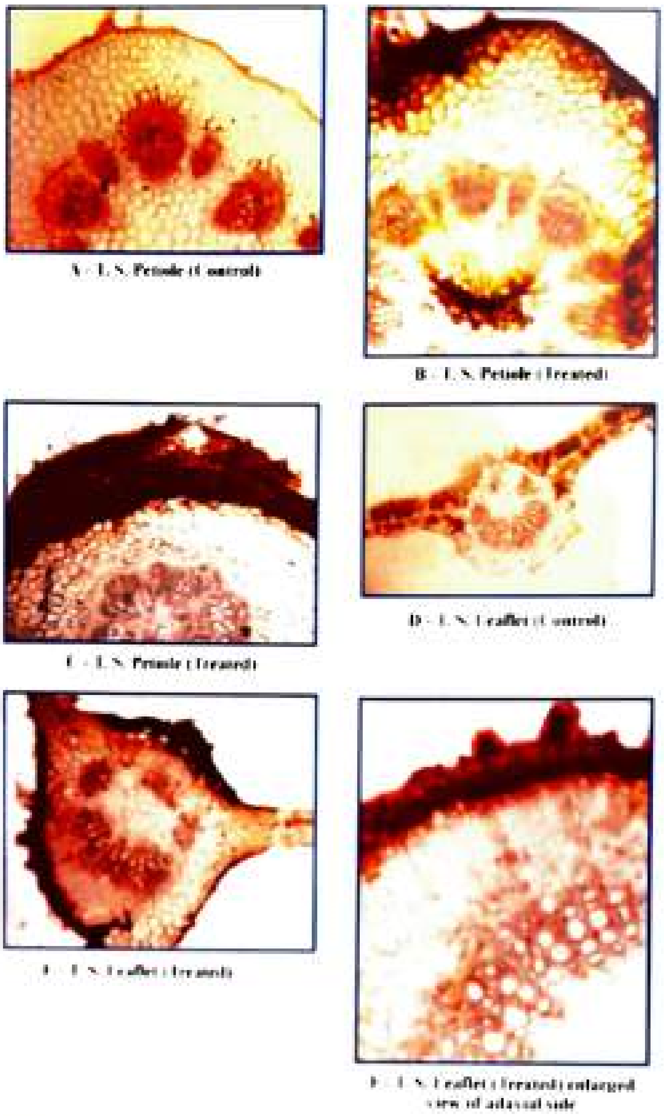
In the present studies no characteristic toxicity symptoms could be observed in fenugreek. High zinc doses caused stunted growth, less expansion of internodes, increased diameter of stem and thick leathery leaves. Higher doses of Cd caused the development of red-brown pigmentation in roots and early senescence.

In soybean, though higher doses of zinc (2500-7500 µg/g) led to necrosis and stunted growth and chlorosis which started appearing at the adaxial surface of leaves, no red-brown pigment formation in the leaves as reported by Rauser (1973) for *Phaseolus vulgaris* and soybean could be observed in the present study.

An interesting observation not appreciated till date was the characteristics deep red- brown pigment formation in the roots of soybean plants treated with higher doses of zinc and Cd (Figure 1A).

Cadmium toxicity symptoms in soybean were much more marked than zinc. Though the symptoms were in a continuous series it could be possible to know the Cd toxic levels only by observing the symptoms (Figure 1& 2). The symptoms are being presented in Table-1.

The red-brown pigment has been found to be readily solubilized by extraction with 0.1 % methanolic HCl (Cunningham *et al.*, 1975). It could be a polyphenolic



A - T. S. Petiole (Control)

B - T. S. Petiole (Treated)

C - T. S. Petiole (Treated)

D - T. S. Leaflet (Control)

E - T. S. Leaflet (Treated)

F - T. S. Leaflet (Treated) enlarged view of adaxial side

Figure 2 See text for details

substance and probably a mixture of cyanidin glycosides (Harborne, 1967).

In the present studies, a steady increase in the amount total phenols was observed (Table-2) with increasing Cd doses. This further corroborates that the compound is a phenolic one.

It appears that the formation of pigment in certain cells is a response to higher metal contents. High cadmium levels have been observed in the leaves of Cd-treated plants (Table-3). Whether Cd is a part of this phenolic compound is not known although Jaakola and Ylaranta (1976) reported that cadmium is concentrated in the veins of radish and Cunningham *et al.* (1975) found that in soybean the movement of Cd was restricted to the major veinal areas which coincided with the deposition of anthocyanin pigment. Further, in X-ray films, localized plugging of vascular tissue along major veins in the leaf and at the stem nodes has been observed and tissues beyond blocked veins accumulated less cadmium than regions below the blockade points (Cunningham *et al.*, 1975).

Aery and Sarkar (1988) localized the accumulated zinc in soybean and fenugreek with the help of dithiozone and found it to accumulate along the cell wall suggesting an apoplastic movement. An apoplastic movement of cadmium also has been observed by Cunningham *et al.* (1975). Occurrence of metals such as Cd has been verified in the apoplast (cell walls and intercellular spaces) of the cortical parenchyma of roots (Wójcik *et al.*, 2005).

Cadmium toxicity caused cell degeneration in root tissues. The cortex has wide intercellular spaces suggesting that greater metal deposition occur in the roots (Table-3) and lower transfer to the shoot part (Gomes *et al.*, 2011). Leaf curling may be a strategy to reduce the transpiration area on the surface (Turner and Jones, 1980).

Gomes *et al.* (2011) observed thickening of walls of xylem elements and cortical parenchyma of roots in *Brachiaria decumbens*. This may help to maintain the hydraulic safety of the root which is essential for its activity and constitutes a barrier to water loss by reflux (Gomes *et al.*, 2011). Further, the binding of heavy metals in the cell wall reduces the amount of cytosolic heavy metals and thus has a protective action (Vázquez *et al.*, 1992; Wójcik *et al.*, 2005).

Chlorosis observed in leaves may be related to lower chlorophyll contents. The effect of metal ions on pigment production has been investigated in several species (Chugh and Sawhney, 1999; Horváth et al., 1996).

Aery (1994) have shown leaf iron as well as chlorophyll contents to be negatively correlated with leaf cadmium and leaf Zn. The negative correlation between leaf Fe x leaf Zn, leaf Fe x leaf Cd and positive correlation between leaf Fe x chlorophyll contents suggest that chlorosis arises due to reduction in the adsorption and the translocation of Fe resulting in Fe deficiency at the site of chlorophyll synthesis in leaves (Ambler *et al.*, 1970).

Caststelfranco and Beale (1983) have suggested that during chlorophyll synthesis, the conversion of Mg-Proto-Me to Protochlorophyllide (Pchlide) requires iron, as Mg-Proto (-Me) accumulates in Fe deficient plants (Spiller *et al.*, 1982). Abnormally high Mg-Proto/Pchlide has been found in etiolated tissues treated with compounds such as α,α1, dipyridyl that decrease the activity of Fe by forming bidentate complex (Vicek and Gassman, 1979).

Moreover, Labbe and Hubbard (1960) have described an enzyme from rat liver which is considered to be responsible for the insertion of iron into the protoporphyrin chelate to give haem. This mechanism might be of universal significance and if so could be involved in chlorophyll synthesis if ferrous protoporphyrin is a precursor of the magnesium compound. Metal toxicity might result from competition between the metal and ferrous ions at the enzyme site.

Cunningham *et al.* (1975) have indicated the possibility of clogging of phloem element which results in restricted transport of nutrients from cotyledons which ultimately resulted in growth reduction, chlorosis and inhibition of lateral root formation. This would result in unusual accumulation of sugars at the point of blockage which would induce formation of anthocyanin as indicated by Harborne (1967). The deposition of red-brown pigments in the stem and leaves might be due to the above reason.

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