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Heavy metal Cd affecting nodulation and leghaemoglobin proteins in soybean and chickpea

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Abstract

Cadmium, one of the heavy metal soil pollutants, affects nitrogen fixing efficacy of legumes adversely. Nodulation in soybean reduces with Cd induced stress. The heavy metal effect in terms of root nodule number gets substantiated in cumulative manner from vegetative to flowering to pod filling stage of the crop. Increased amino acid content of soybean root nodules was highest at flowering stage and dose dependent also. The increase in level of this metabolite also coincided with nodular senescence and age related changes. Leghaemoglobin content of root nodules lowers significantly both in soybean and chickpea with higher doses of Cd. Brown coloration of root nodules was an indicator of the Lb degradation noticed at flowering and pod filling growth stages in the presence of heavy metal. Such changes lower crop productivity due to ineffective nodulation and the lesser availability of nitrogen in Cd polluted soils.

Keywords: Leghaemoglobin, N_2-fixation, amino acids, abiotic stress, nodular metabolism.

Introduction

Heavy metals, obnoxious pollutants of soil and water, are highly toxic elements to the living organisms. They have caused widespread contamination of the soils in Northern India in the recent times [1, 2, 3]. Cadmium (Cd) is one of the most toxic heavy metal trace elements which causes Itai-Itai (Ouch-ouch) disease in humans [4]. It is primarily released into the environment through human activities [5]. On an average, 25,000- 30,000 tons of Cd is released into the environment every year [6]. The dispersal of metal-rich waste around mining and smelting plants, secondary metal refining, waste incineration manufacturing, and automobiles are amongst the human activities introducing Cd to the agricultural soils [7]. The use of phosphate fertilizers and sewage sludge amendments also contaminate the soils [8].

Biological nitrogen fixation is an efficient source of nitrogen for sustainable agricultural production, contributing nearly 139-175 million tons of nitrogen in the terrestrial environment on annual basis [9]. The available literature points that symbiotic nitrogen fixation is badly affected in the presence of various trace elements, wherein reduction in the number of rhizobia, plant vigor, nodulation, altered expression of various enzymes of nitrogen fixation, leghaemoglobin content and nitrogenase activity have been reported [10, 11, 12]. A comparative study of heavy metals toxicity has indicated Cd being most toxic to symbiotic relationship in legumes [13, 14]. Eivazi [15] reported a decrease in dry matter accumulation and symbiotic nitrogen fixation in metal contaminated soils.

Leghaemoglobin (Lb), a haemo-protein in the N_2-fixing root nodules of leguminous plants facilitates diffusion of oxygen to endo-symbiotic bacteria *Rhizobium* and *Bradyrhizobium* [16]. Barring a few reports, only a scarce data is available on the quantitative and qualitative effects of heavy metals on leghaemoglobin and enzymes of nitrogen fixation in the root nodules of leguminous plants. The study was aimed at assessing effect of heavy metal Cd induced toxicity on nodulation, Lb content and related metabolites in the two legume crops soybean and chickpea.

Materials and Methods

The seeds of Soybean (*Glycine max* (L.) Merr. cv. PK-416) and Chickpea (*Cicer arietinum* L. cv. PBG1) procured from Punjab Agricultural University, Ludhiana, Punjab (India) were sown after inoculating with standard rhizobial culture broths each for soybean (*Bradyrhizobium* sp.) and chickpea (*Rhizobium* Ca-181) obtained from Department of Microbiology, Punjab Agricultural University, Ludhiana (India) and Bio-fertilizer Technology Centre, Department of Microbiology, CCS Haryana Agricultural University, Hisar, respectively. These cultures were maintained in yeast extract mannitol agar (YEMA) medium [17] in sterilized test tubes (25×150 mm) at 28±2 ºC in the incubator. Before use, they were re-cultured in broth of the same medium without agar-agar in conical flasks kept on rotary shaker for 2-3 days.

Plants were raised in sand cultures in unglazed earthenware pots (25 cm inner diameter) lined with pricked polythene bags. Each pot was filled with approximately 5 kg of thoroughly washed river sand. The seeds were surface sterilized with mercuric chloride (0.1%) followed by thorough washing under tap for 30 minutes. Thereafter, the seeds were coated with thick slurry of rhizobial broth culture in activated charcoal and acacia gum and kept overnight before sowing. Each pot was sown with 8-10 seeds at equal distance and depth. They were irrigated with tap water and supplied with nitrogen containing Minchin and Pate nutrient solution [18] at weekly intervals (@ 500ml per pot till the establishment of root nodules. Thereafter, the nutrient solution supplied was nitrogen free. Nickel (10µg $litre^{-1}$) was added to the medium as a micronutrient to prevent urea accumulation in the leaves [19]. Three healthy and vigorously growing seedlings were chosen after thinning. Cd was supplied along with the nutrient solution at weekly intervals in three different doses of 4, 20 and 40µM as cadmium sulphate ($CdSO_4.4H_2O$; MW- 208.47). This was in the range of environmentally relevant concentration of 0.2, 1 and 2 mg kg^{-1} of sand.

Total Lb content of root nodules was estimated by the method of Hartree [20] based on conversion of haematin to pyridine haemochromogen. The total free amino acid content of nodules was measured following Lee and Takahashi [21]. One way analysis of variance (ANOVA) was applied to all the results using the software BioStat Professional Package Release 5.2.5.0 (AnalystSoft, Robust Business Solutions, Vancouver, Canada) to statistically analyze the significance of treatments.

Results

Root nodulation got severely affected and their number reduced drastically in Cd treated soybean plants (Fig. 1). The number decreased 3, 7 and 17% (vegetative phase); 23, 30 and 50% (flowering phase) and 30, 35 and 74% (pod filling stage) at 4, 20 and 40 µM of Cd treatment, respectively. Under these three treatment levels, percentage reduction in the number of root nodules from vegetative to flowering to pod filling stage was 9 and 15% in 4 µM; 15 and 18% in 20 µM and 31 and 63% in 40 µM of Cd. The pinkish red colour of control root nodules turned brown at flowering and pod filling stage at 20 and 40 µM of Cd, probably inducing earlier nodular senescence.

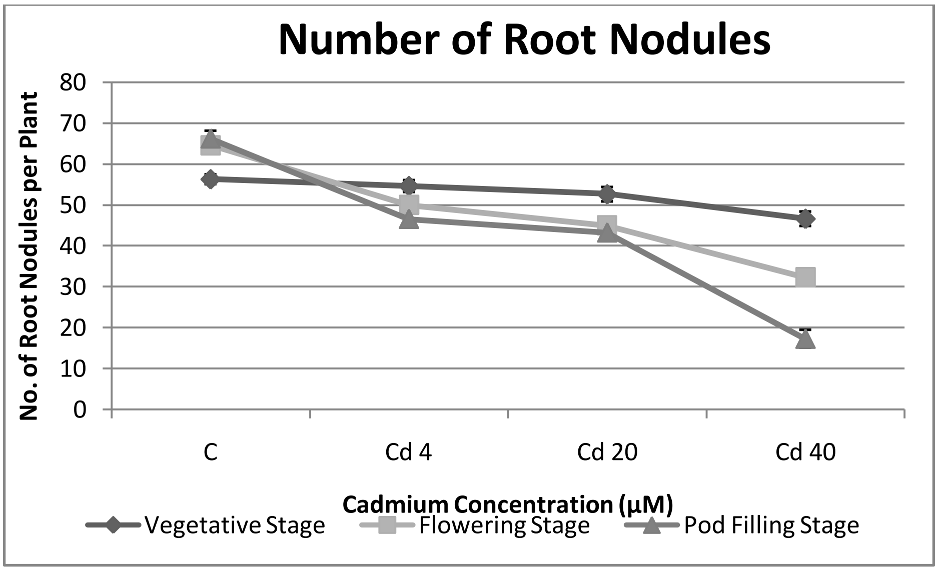

Fig. 1: Effect of Cd on root nodule number in Soybean

Total Lb content of soybean root nodules decreased significantly in a dose dependent manner. At vegetative phase, the decrease was 60, 80 and 88% in comparison to control plants at 4, 20 and 40µM Cd stress levels, respectively. The trend continued at flowering stage to record total losses to 13, 46 and 79%, respectively. Similarly, total decrease at pod filling stage noticed was 35, 50 and 84% at three Cd treatment levels, respectively (Table 1).

Table 1: Effect of Cd on leghaemoglobin content (µg hemin gm^{-1} nodule fresh mass) of soybean nodules.

Treatment → Stages↓	Control	Cd 4 µM	Cd 20 µM	Cd 40 µM
Vegetative Stage	142.69^{a} ± 2.62	56.96^{b} ± 1.12	28.68^{c} ± 0.77	**16.38^{d} ± 0.86**
Flowering Stage	194.42^{a} ± 5.80	167.76^{b} ± 3.70	104.56^{c} ± 1.70	**40.09^{d} ± 2.91**
Pod filling Stage	**76.52^{a} ± 2.25**	**50.65^{b} ± 0.57**	**37.72^{c} ± 0.57**	**12.07^{d} ± 1.76**

Values are mean ± standard errors, n=3. Different superscript letters on mean values along the rows indicate significant differences within $P<0.05$ according to Tukey's HSD range test.

In chickpea root nodules, total Lb content decreased as in case of soybean. The total loss was 12, 16 and 41% respectively at vegetative stage; 21, 28 and 43% at flowering stage and 15, 39 and 54% at the pod filling stage at 4, 20 and 40µM Cd treatment levels, respectively (Table 2).

Table 2: Effect of Cd on total leghaemoglobin content (µg hemin gm^{-1} nodule fresh mass) of chickpea nodules.

Treatment → Stages↓	Control	Cd 4 µM	Cd 20 µM	Cd 40 µM
Vegetative Stage	104.79[a] ± 1.36	92.14[b] ± 1.41	88.16[b] ± 0.97	**61.92[c] ± 1.04**
Flowering Stage	130.73[a] ± 1.73	102.90[b] ± 0.88	94.15[b] ± 1.08	**74.94[c] ± 2.22**
Pod filling Stage	**107.41[a] ± 1.03**	**90.93[b] ± 1.06**	**65.61[c] ± 0.87**	**49.13[d] ± 2.13**

Values are mean ± standard errors, n=3. Different superscript letters on mean values along the rows indicate significant differences within P<0.05 according to Tukey's HSD range test.

Total amino acid pool of the root nodules in soybean exhibited a considerable and significant Cd dose dependent increase. The increase was 45, 72 and 118% in comparison to control with Cd treatments (in 4, 20 and 40 µM), respectively at vegetative stage. The pooledup levels were 62, 91 and 127% at flowering and then reclined to 10, 52 and 76% at pod filling stage with the same Cd treatments. This metabolite also depicted age related increase in the content of amino acid profile (Table 3).

Table 3: Effect of Cd on total content of amino acids (mg g^{-1} fresh weight) in soybean root nodules.

Growth Stage	Control	4 µM Cd	20 µM Cd	40 µM Cd
Vegetative Stage	10.06[a] ± 0.217	14.58[b] ± 0.375	17.3[c] ± 0.389	**21.96[d] ± 0.242**
Flowering Stage	10.72[a] ± 0.329	17.34[b] ± 0.426	20.44[c] ± 1.017	**24.36[d] ± 0.183**
Pod filling Stage	**18.66[a] ± 0.375**	**20.58[b] ± 0.466**	**28.28[c] ± 0.356**	**32.9[d] ± 0.361**

Values are mean ± standard errors, n=3. Different superscript letters on mean values along the rows indicate significant differences within P<0.05 according to Tukey's HSD range test.

Discussion

Root nodules are the important sites for conversion of atmospheric N_2 into ammonia in a legume symbiotic relationship. The heavy metals have been previously reported to adversely affect root [22, 23], root hair formation and nodule initiation [24]. A drastic reduction in the number of root nodules as result of heavy metal exposure resulting decreased nodule initiation and their number might be affecting C/N metabolism in the Cd induced phytotoxicity. The de-coloration of pinkish red nodules in control plants to brownish indicates the oxidative changes in the root nodules accompanied degradation of proteins. A stimulation of nodular senescence related parameters like ethylene, ammonia and protease have also been reported with metal toxicity [25]. A considerable reduction in Lb content of nodules is the result of degradation of haeme-protein and an initiation of early nodular senescence as also reported earlier [26, 27]. Many reports have correlated Cd induced toxicity to nodule senescence [28, 29, 30]. Heavy metal toxicity expresses through reactive oxygen species (ROS), jasmonates (JA) and ethylene [31, 32] and these molecules are involved in the metal toxicity induced programmed cell death through caspase like proteases [33]. The heavy metals induce oxidative modification of proteins [34] and increase hydrolytic activity of protease [35] resulting piling up of amino acid content of nodules. The deformation and degeneration of the existing nodules in presence of heavy metal Cd accompany Lb degradations which appear to have a direct relation with earlier nodular senescence and set in of programmed cell death.

Conclusions

Cadmium adversely affects the N_2-fixing efficacy of legume crops. The root nodule decline accompanied the browning of pinkish colored red nodules with Cd exposure. Lb degradation initiates an earlier nodular senescence resulting pooled up levels of amino acids. Such changes lower crop productivity due to ineffective nodulation and the lesser availability of nitrogen in Cd polluted soils.

Conflict of Interest

None

Acknowledgement

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