THE EFFECT OF CADMIUM STRESS ON GROWTH, SOD ACTIVITY, PROLINE AND ABA CONTENT IN BEAN SEEDLINGS (*PHASEOLUS VULGARIS L.*)

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ABSTRACT

Cadmium (Cd) is one of the widespread environmental pollutants which has toxicity effect and its consumption by animals and humans with their diet can lead to serious diseases. Present study was carried out in order to investigate the effect of Cd stress on the growth, Superoxide dismutase (SOD) activity, Proline and abscisic acid (ABA) content in bean seedlings. In This experiment, 25 bean seeds genotypes were germinated and grown in laboratory conditions consist of solution $CdCl_2$ with concentration of 4mg/lit and distilled water (as a control). Application cadmium caused a significant reduction in germination percentage 9.9%, root and shoot elongation 83.9% and 66.3%, respectively as compared with that of control. While Cd treated plant showed an increase in Sod activity (20.79%), Proline (20.71%) and ABA (41.57%) content in compared to control. Based on results we concluded that, decrease in seed germination and seedling growth by Cd exposure can be in relation with this toxicity and oxidative stress. Also the present results revealed that the increase in the sod activity, proline and ABA content causes to prevent the occurrence of oxidative damage under Cd stress.

Keywords: ABA, bean, Cadmium, growth, oxidative stress, Proline, SOD.

Abbreviations: ABA- Abscisic acid; ROS- reactive oxygen species; SOD- Superoxide dismutase.

Introduction

Heavy metal contamination had influence on the biosphere in many parts of the world (Raskin and Ensley, 2000). The toxicity of different heavy metals and the related tolerance in plants is the subject of numerous studies over the past thirty years which has been considered (Brown JC, Jones WE 1975; Clemens et al., 2002). Generally, environmental stresses

including, heavy metals cause serious problems for many physiological processes and plant growth. Capability of Plant species dealing with these stresses is very different (Yerk, C.D. and Willer, S.C. 1996). No clear concept of the physiological characteristics which are responsible for the inhibition of growth in response to stress, have been introduced (Rascio et al., 1992). Cadmium is one of the most toxic elements presents in environment which this Metal pollutant from industrial activities, mainly phosphate and fertilizers gets into the environment and then transmits to the food chain (Wanger, G.J. 1993). Cadmium is easily absorbed by plant roots and transferred to other parts of plant. Usually excessive accumulation of this element leads to limits plant growth and even death due to reduced enzyme activity (Ouariti et al., 1997), photosynthesis (Lee et al., 1997), and nutrient absorption (Sanita di Toppi, L. and R. Gabbrielli. 1999). One of the biochemical changes that occurs in plants exposed to different conditions of environmental stress is producing reactive oxygen species such as super oxide radical (O_2^-) and hydroxyl radicals (OH). According to the reports of some authors Active oxygen species during oxidative stress leads to damage in biological molecules such as proteins and lipids (Molassiotis et al., 2006). There are evidences that showed tolerance to Damage of environmental stress is correlated with increase of capacity for detoxification of active oxygen species (Foyer et al., 1994). Plants have non-enzymatic and enzymatic defensive systems against toxicity of active oxygen species. One of these defensive systems is activation of antioxidant enzymes including superoxide dismutase enzymes and peroxidase that is located in different parts of the cell. Superoxide dismutase is the only effective enzyme on free radicals that changes them into hydrogen peroxide. Then, the hydrogen peroxide by various enzymes such as peroxidase is converted to water. Increased activity of this enzyme in environmental stresses can raise plant resistance to stress (Fecht et al., 2003). There is several reports show that the cadmium concentration of 5 micro-molar causes lipid peroxidation and reduces antioxidant enzyme activity in bean plants (Sanita di Toppi, L. & Gabbrielli, R. 1999). Proline accumulation has been accepted as an indicator of environmental stress and further plays an important role as a protector. Heavy metal stress enhances proline accumulation (Ali´a P, and Saradhi PP. 1991), the great impact of proline as long as environmental stresses appear, is prevention of degradation enzymes (Shah et al., 2001). Furthermore, other researchers involved in maintaining stability and preventing the breakdown of macro molecules produced during stress in the cell wall and the Clearing of produced hydroxyl during stress in plant, have been reported (Schobert B and Tschesche H, 1978). Such effects have been reported in plants under cadmium stress. It seems that ABA is the internal stimulus for tolerance to environmental stresses which increases under conditions of environmental stress such as drought and salinity (Zeevaart and Creelman., 1998; Zhu et al., 1997).

MATERIAL AND METHODS:

Preparation of samples:

Present experiments were conducted in laboratory of Biotechnology of Karaj Islamic Azad University, faculty of Agriculture and Natural Resources, Iran, spring 2011. 25 bean seeds were prepared from agricultural research institute Khomeyn, Iran, and seeds as the same size and form were selected and then sterilized by 2.5% (v/v) sodium hypochloride for 15min to avoid fungi contamination. All seeds were washed carefully with distilled water.12 seeds of every genotype were germinated in 2 rolled Whatman filter papers and placed in special cultivation vessels including solution CdCl2 with concentration of 4mg/lit and distilled water (as a control). Level of the liquid in vessels was daily examined in order to reduce the inaccuracy of water absorption condition. For providing the optimum temperature of bean development, daily mean temperature during the study was adjusted to 24ºc (75.2ºf).

The process of trait measurement:

The seedling was harvested after 10 days and the germination percentage, root and shoot length and fresh weight were recorded. Root and shoot lengths were obtained with a ruler with the precision of 1mm and the average of 12 seedlings of each genotype noted for every replication. Also the fresh weight of the seedlings was accounted by using a scale with the precision of 0.01gr. For determination of germination percentage, the number of seed emergences in the end of treatment period was counted.

Measurement of antioxidant enzymatic activity, ABA and proline content:

Sampling:

To calculate superoxide dismutase activity, ABA and proline content at first leaves from each seedling were taken and laid in liquid N_2, then stored at -80°C pending biochemical analysis.

Preparation of Enzyme Extraction:

At 10 days of treatment, leaves from each seedling were carefully washed with distilled water and homogenized in 0.16M Tris buffer (pH= 7.5) at 4°C. Then, 0.5 mL of total homogenized solution was used for protein determination by the Lowery et al. (1951) method. Based on the amount of protein per volume of homogenized solution, the following enzymes were assayed in the volume containing a known protein concentration in order to calculate the specific activities of the enzymes.

Determination of Superoxide Dismutase (SOD):

The activity was measured based on Misra and Fridovich (1972), in which the activity was measured on the basis of its ability to inhibit free radical chain oxidation in which o_2^- was a chain propagating radical and the auto oxidation of epinephrine (0.25 mM) was induced. A SOD standard was used for calibration of activity.

Measurement of Proline:

Proline: Proline was determined according to Bates et al. (1973). Leaves and stems (500 mg) were extracted with 3% aqueous 5-sulphosalicylic acid, centrifuged at 5000 rpm. The sample of the supernatant was used for the proline assay and measured at 520 nm. Proline content was expressed as µg g^{-1} fresh weight.

Measurement of Abscisic Acid (ABA):

The initial procedure involved preparing lyophilized plant tissues (0.3 g leaf) which were crushed in liquid nitrogen using a mortar and a pestle and extracted with 3 ml of acetone–water–acetic acid (80:19:1, v/v). The extracts were transferred to two 2-ml tubes and after centrifugation at 13000 rpm for 2 min, the supernatant was collected and the residues were re-extracted with 3ml extraction. The dried sample was reconstituted in 200 ml acetonitrile–water(15:85,v/v)containing 12mM acetic acid (pH3.3). A portion (1–10 ml) of the sample was loaded onto a HP 1100 Series HPLC system equipped with a 100×2.1 mm,5 mm SB-C_{18} LC-MS column using a flow-rate of 0.2ml/min and a Binary solvent system comprising 12mM acetic acid in water (A) and 12mM acetic acid in acetonitrile-Water (90:10,v/v)(B). Typically, the solvent gradient was programmed to change linearly from 15% B to 33% B over the first 10min and then to 100% B over the next 6.7 min before returning to the initial composition at 22 min. For a 10-ml injection of sample prepared with 20 ng internal standard and reconstituted in200 ml initial mobile phase, the limit of detection (LOD) and limit of quantification (LOQ) were calculated from calibration curves and samples using the Quantify module of Mass Lynx version 3.5 software (Zhou et al., 2003).

Statistical analysis:

This experiment was carried out in the form of factorial in randomized complete block design (RCBD) by three replications per treatment. All data were analyzed using SAS Institute Inc., Version 9.1 software. And first analyzed by ANOVA to determine significant ($P\forall \leq 0.01$) cadmium treatment and genotype effects Mean comparison was conducted using the Duncan's Multiple Range Test (DMRT) at 1% level of probability.

Result and discussion

Effect of cadmium chloride on root and shoot length of bean seedling:

The effects of $CdCl_2$ on growth parameters were assessed and the obtained results were given in Table1. The results of analysis of variance indicated that the main effect and interactions of studied traits were significant ($P<0.01$). According to figure 1 it showed that Cd treatment causes reduction of 83.9%, 66.3% in root and shoot length of bean seedling, respectively by comparison with control. Among the genotypes, Emerson and Wa4502-1, Jules and Ks-41126 respectively have the most account of root and shoot length while Shokofa and Pak, Shokofa and G-11867 have the least one in Cd treatment (figs. 3 and 4). The result of present study revealed that Cd was an inhibitor factor on root and shoot length growth and the effect of Cd on root length growth was more obvious than shoot length growth. These gained resulted in relation with the influence of Cd on limitation and reduction of growth of plant species is in conformity with the findings of other researchers. For example, application of Cd reduced the shoot length in bean and alfalfa (Bhardwaj et al., 2009; Aydinalp and Marinova, 2009) and also Mihalescu et al (2010) were of the opinion that the reduction in root length and height of the plant was due to Cd accumulation in Zea mays. Furthermore they induced that such a decrease was directly proportional with the increase of metal concentration. The reduction in root length affected by Cd treatment in bean (Bhardwaj et al., 2009) and alfalfa (Aydinalp and Marinova, 2009) was recorded that these results are similar to our outcomes.

The reason for the reduced seedling growth in metal treatments could be as a result of the reduction in meristematic cells present in this region and some enzymes contained in the cotyledons and endosperm. Cells become active and begin to digest and store food which is converted into soluble form and transported to the primary root and shoot tips for enzyme amylase which converts starch into sugar and proteases act on proteins. So, when activities of hydrolytic enzymes are affected, the food does not reach to the primary root and shoot, thereby affecting the seedling length (Kabir et al., 2008). Several authors reported that, the inhibition of root elongation caused by heavy metals may be due to metal interference with cell division, including inducement of chromosomal aberrations and abnormal mitosis (Radha et al., 2010; Liu et al., 2003), which can be effected on seedling growth and explain the inhabitation of seedling growth in this investigation.

Seed germination percentage and fresh weight:

The results of analysis of variance (table.1) indicated that the main effect and interactions of studied traits were significant ($P<0.01$). According to figure 3 and 4, it showed that Cd treatment causes reduction of 9.9% in seed germination percentage by comparison with the control (fig. 2) and Zodras, Pak and Taylor had the most amounts while Jules had the least one under Cd stress (fig. 5). Findings of this investigation were in conformity with the results of other researchers (Hoshmandfar and Moraghebi, 2011). For instance, Rahman et al. (2010) reported that treatment with nickel and cobalt caused a reduction in seed germination in

Chickpea plant. According to Shafiq et al. (2008) decrease in seed germination of plant can be characterized to the accelerated breakdown of stored food materials in seed by the application of Cd. Furthermore, Reduction in seed germination can be attributed to changes of selection permeability properties of cell membrane. Growth is the best indices plant reaction to environmental stress. Mean fresh weight in genotypes under Cd treatment reduced 42.3% by comparison with control (fig 7). Among the genotypes, G-14088 and G-11867 had maximum and minimum amount for fresh weight respectively (fig. 6). Bhardwaj et al. (2009) in their investigation on the bean plant observed a reduction in total biomass and fresh weight of seedling and also similar reports have been found by other researchers in *T. sativum* (Ouzounidou et al., 1997) and *Lens esculanta* (Mesmar and Jaber, 1991). Reduction in fresh weight may be referred to toxicity of $CdCl_2$, thereby this toxic material can breakdown normal physiological mechanisms and finally have negative influences on biomass.

Table1: **Analysis of Variance for experimental traits under normal and cadmium stress conditions**

		MS						
Sov	**DF**	**Root**	**Shoot**	**Seed percentage**	**Weight**	**Sod**	**Proline**	**ABA**
Rep	2	$11.83^{n.s}$	$1.855^{n.s}$	$59.71^{n.s}$	$0.39^{n.s}$	47.07^{**}	61.6^{**}	38.55^{**}
A(Cd level)	1	2376.2^{**}	2013.4^{**}	2334.1^{**}	2625.7^{**}	1305.7^{**}	610.3^{**}	556.1^{**}
B(genotype)	24	8.51^{**}	6.98^{**}	748.48^{**}	60.93^{**}	178.9^{**}	127.9^{**}	27.49^{**}
A*B	24	10.31^{**}	7.23^{**}	$174.27^{n.s}$	23.02^{**}	19.37^{**}	14.75^{**}	10.45^{**}
Error	98	0.755	1.008	139.09	2.49	5.20	1.74	2.59
C.V%		15.79	13.58	14.12	10.13	7.28	6.15	14.41

ns and **: Non significant and significant at 1% levels of probability, respectively

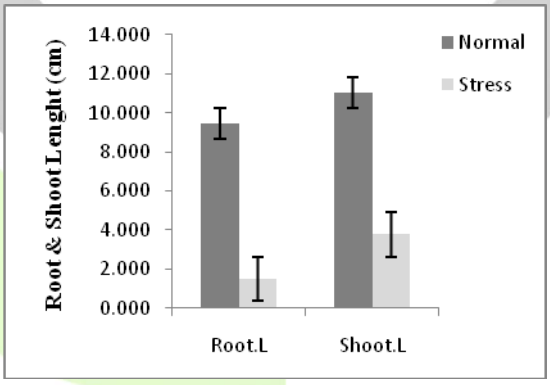

Fig. 1: Comparison of Root and Shoot length in normal and stress conditions. Each bar represents mean (±SE) of three different samples.

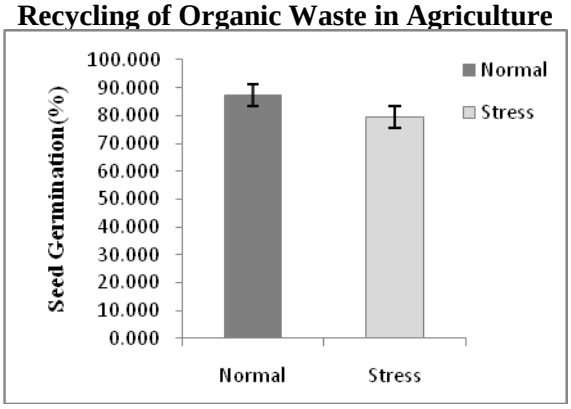

Fig. 2: Comparison of Seed germination in normal and stress conditions. Each bar represents mean (±SE) of three different samples.

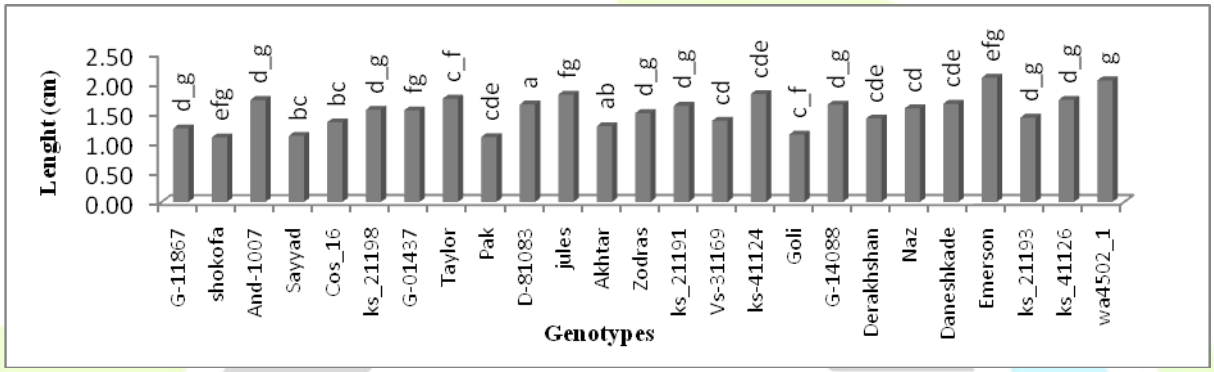

Fig. 3: Mean Comparison of Root length in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT.

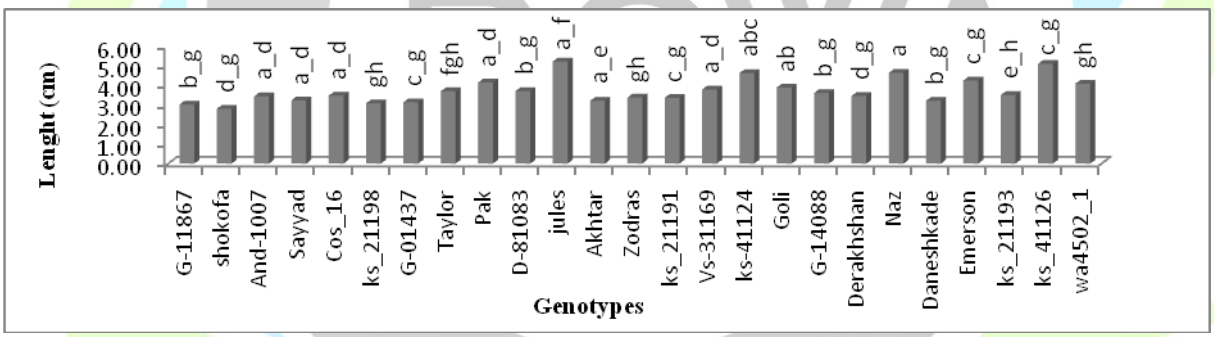

Fig. 4: Mean Comparison of Shoot length in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT.

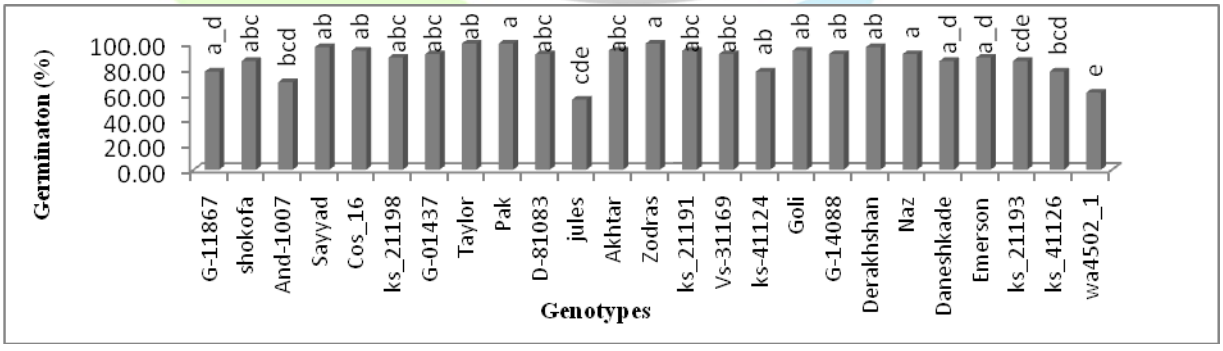

Fig. 5: Mean Comparison of seed Germination in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT.

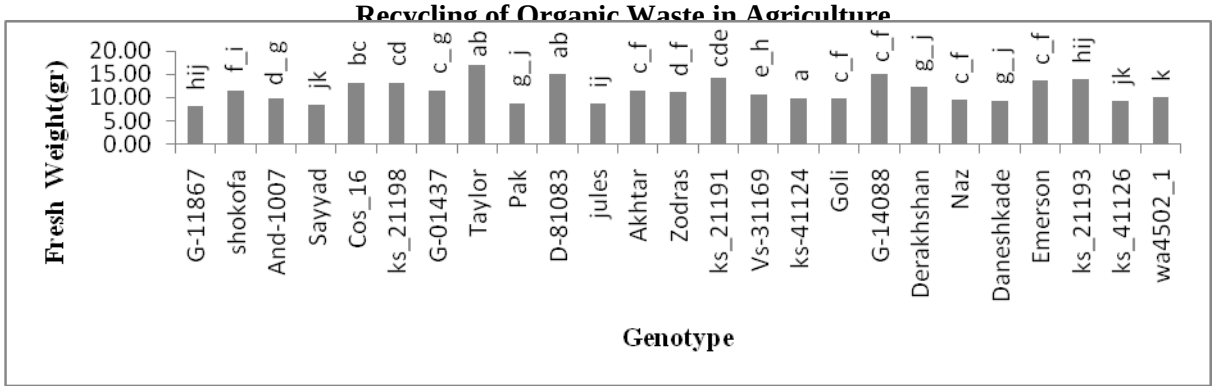

Fig.6: Mean Comparison of Fresh Weight in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT.

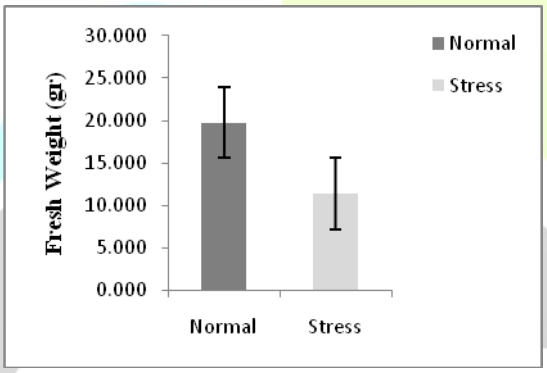

Fig. 7: Comparison of Fresh Weight in normal and stress conditions. Each bar represents mean (±SE) of three different samples

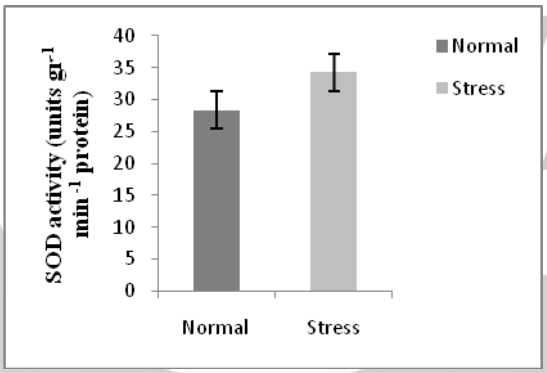

Fig. 8: Comparison of SOD activity in normal and stress conditions. Each bar represents mean (±SE) of three different samples

Effect of Cd on Superoxide dismutase activity (SOD):

Results showed significant difference ($P \leq 0.01$) for SOD activity in Cd treatment of genotypes (table.1) Also, significant difference ($P \leq 0.01$) was observed for activity of SOD in cadmium × genotype interactions (table1). According to fig. 1 it showed that Cd treatment causes increase of 20.79% in sod content as compared to control (fig. 8). In Cd treatment condition, the highest and lowest SOD obtained from G-01437 and Cos-16 genotypes, respectively (fig. 9). Cd toxicity causes the change of oxidant level in plants, including the generation of toxic reactive oxygen species (ROS) such as H_2O_2, OH and O_2 thereby induces oxidative stress (Choudhary and Panda, 2004). Some of the initial responses of plants to environmental stress are free radical and ROS generation, which stimulated by metals

(Halliwell and Gutteridge, 1993), and this can strongly disrupt normal metabolism through oxidative damage to cellular components. In order to repair the damage initiated by ROS, plants have a defensive antioxidant system including super oxide dismutase enzyme. SOD is considered a key enzyme in the process of ROS scavenging. Therefore, increase SOD activity in plant cells indicated that it plays an effective role in controlling the cellular level of these ROS and/or repairing oxidative damage (Miller et al.; 2008). In this experiment increase in activity of super oxide dismutase against free radicals by cadmium stress was observed. Similar increase in SOD activity has also been reported by other researches (Scebba et al., 2006; Mobin and Khan, 2007).

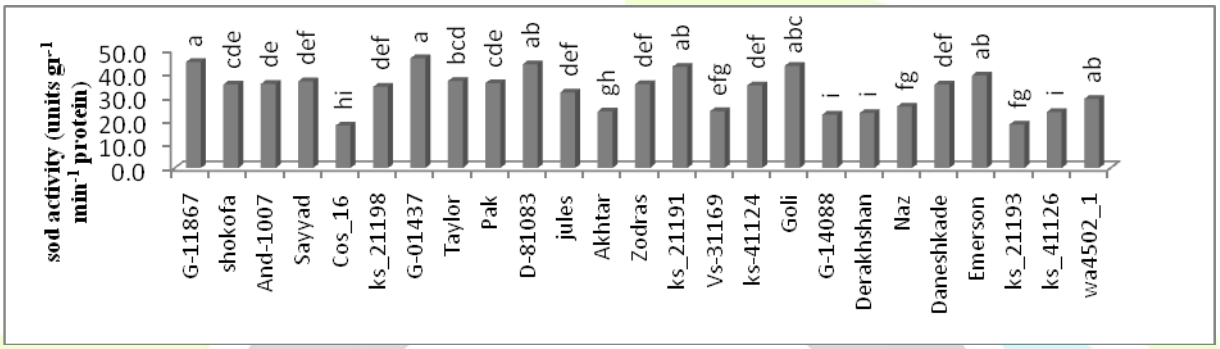

Fig.9: Mean Comparison of SOD activity in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT

Effect of Cd on free proline content:

Results showed significant difference ($P \leq 0.01$) for free proline content in Cd treatment of genotypes (table.1) Also, significant difference ($P \leq 0.01$) was observed for activity of proline in cadmium × genotype interactions (table1). According to fig. 1 it showed that Cd treatment causes increase of 20.71% in free proline content in comparison with control. In Cd treatment condition, the highest and lowest proline content obtained from G-01437, Pak and Cos-16, Ks-21193 genotypes, respectively. Proline is accumulated in presence of heavy metal and is considered to be an index of special stress resistance. The Proline accumulation in Cd treated seedlings can be regarded as one of the most sensitive responses to water deficiency and osmotic stress (Ashraf and Harris, 2004). Proline accumulation is mostly influenced by the water stress component of Cd toxicity (Schat et al., 1997) on the other hand (Kastori et al., 1992) reported that Proline accumulation is not dependent of any water-stress component. Beside, proline can behave as an antioxidant and decrease the damage of free radicals by prevention of lipid peroxidation so maintenance the membranes. Our findings revealed that proline content has increased under Cd treatment stress condition, which was in agreement with the report of other researchers about Proline accumulation in many plant species (Shah and Dubey, 1997; Mishra and Dubey, 2006).

Effect of Cd on abscisic acid content (ABA):

Results showed significant difference ($P \leq 0.01$) for ABA content in Cd treatment of genotypes (table.1) Also, significant difference ($P \leq 0.01$) was observed for activity of ABA in cadmium × genotype interactions (table1). According to fig. 1 it showed that Cd treatment causes increase of 41.57% in ABA content in comparison with control. Among the genotypes under Cd treatment, the highest and lowest ABA content obtained from Vs-31169, Taylor and Jules, Cos-16, Ks-21193 genotypes, respectively. Environmental stress is associated with the level increase of ABA in plants. The processes of dynamic balance between biosynthesis and degradation, which lead to the determination of the amount of ABA and these processes affect by various environmental factors. In this connection, ABA content was increased under Cd stress condition in different plant species (Hsu et al., 2006; Fediuc et al., 2005).In this study we observed that the level of ABA in plants exposed to Cd treatment was increased .also, This result was in accord with results of Omer et al. (2008) who reported that increase in ABA levels caused by heavy metal can be utilized in statement of ceasing water uptake from roots to shoots.

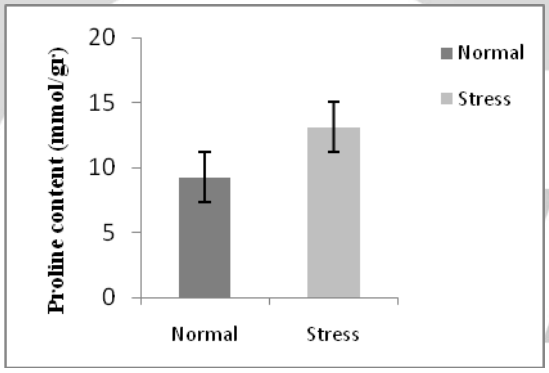

Fig. 10: Comparison of proline content in normal and stress conditions. Each bar represents mean (±SE) of three different samples

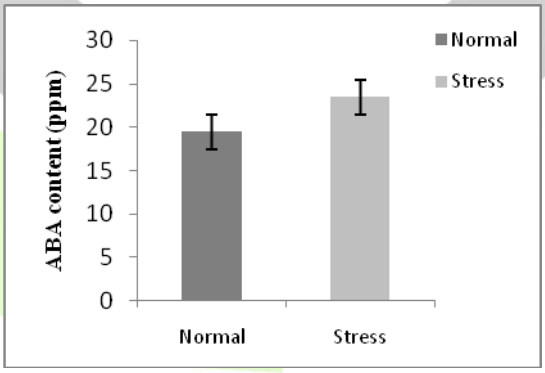

Fig. 11: Comparison of ABA content in normal and stress conditions. Each bar represents mean (±SE) of three different samples

CONCLUSION:

Overall, the results of our investigation showed that the toxicity of cadmium in bean seedlings impressed. This information can be considered a contributing step in exploring of the tolerance range of bean genotypes at 4mg/lit concentration of treated cadmium And the fact that the response of bean seedlings strongly depends on genetic variation among the genotypes. The data evaluating the growth responses in both normal and cadmium stress allow the suggestion that Cos-16, D-81083, Akhtar, Taylor, G-14088 and Naz genotypes are more tolerant than other genotypes. Results of findings can be a useful indicator of metal tolerance to plantation of these genotypes in metal contaminated zones. Finally, in the metal contaminated areas, additional research is necessary to provide further focus concerning the special relationship between metal stress and the antioxidant response.

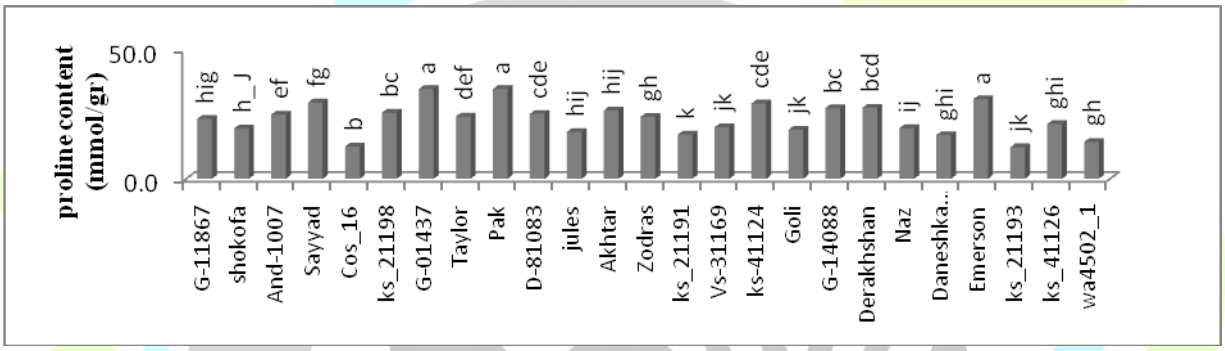

Fig.12: Mean Comparison of proline content in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT

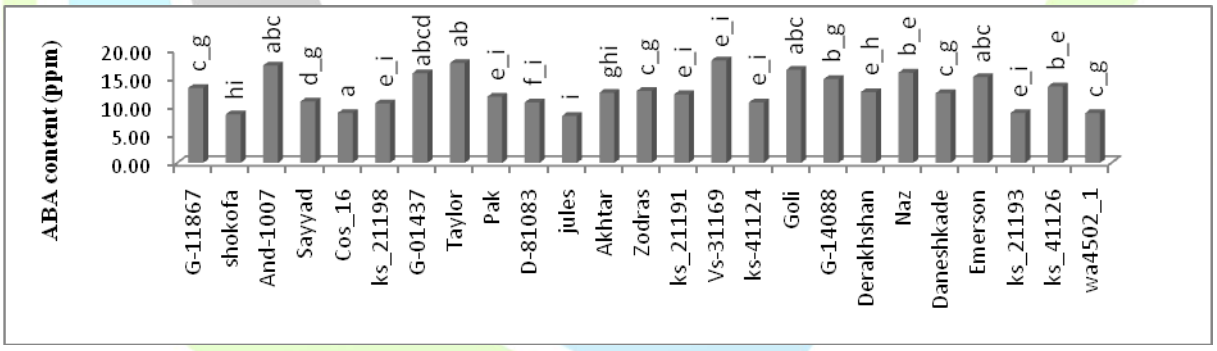

Fig.13: Mean Comparison of ABA content in different genotypes under stress condition for a given means within each column of each genotype followed by the same letter are not significantly different at p =1% according to DMRT

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