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Research Article

HEAVY METAL TOXICITY: CALCIUM IMPROVES TOLERANCE IN CHICKPEA AGAINST CADMIUM WITH ALTERED CARBOHYDRATE METABOLISM

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ABSTRACT
The effect of heavy metal cadmium and its possible alleviation with the use of calcium affecting various metabolites and related enzymes were analyzed. The presence of cadmium in rhizosphere affects the process of photosynthesis due to reduced uptake of water. As a result, the growth of chickpea plants is impaired due to altered sugar metabolism. The present study suggests that calcium ions largely mitigate Cd induced heavy metal toxicity probably by improving absorption and uptake of water and with a restored sugar metabolism. The efficacy of calcium was more when used in higher concentrations.

Keywords: *Legumes, Uptake, Roots, Absorption, Cicer arietinum*

INTRODUCTION
Amongst the heavy metals cadmium is one of the most toxic elements that can detrimentally affect number, diversity and activity of soil organisms. Its ready inflow into our agricultural food chains is a cause of serious concern since Cd^{2+} is taken up by the plant roots of many species for subsequent translocation to shoot as well as seeds (Chardonnens *et al.,* 1998).
Cd induced heavy metal toxicity synthesize specific polypeptides to inhibit plant growth and its toxicity is generally considered 2–20 times higher than that of other heavy metals (Shah and Dubey, 1998). It alters the behavior of many key enzymes involved in various metabolic pathways to accumulate certain metabolites (Vallee and Ulmer, 1972; Alas *et al.,* 1996).
Crop productivity depends upon photosynthesis and the rate of import of its end-products to sink organs. Starch, a temporary storage form of fixed carbon is deposited in the chloroplast as starch granules while sucrose is transported to different organs as the most common photo-assimilate (Verma and Dubey, 2001).
The exposure of plants to heavy metal stress induces ROS formation damaging different macromolecules like proteins, lipids and nucleic acids, thus, affecting both carbohydrate metabolism and mitochondrial respiration (Dixit *et al.,* 2002; Guerrero *et al.,* 2008; Garmash and Golovko, 2009). Abiotic stresses such as drought, cold and salinity leads to major alterations in the carbohydrate metabolism (Gupta *et al.,* 1993; Wanner and Junttila, 1999; Kaur *et al.,* 2000; Gupta and Kaur, 2005) and up regulation of many related genes (Seki *et al.,* 2002).
Sugars regulate growth activities by modulation of gene expression and enzyme activities in both carbohydrate importing and exporting tissues (Coruzzi and Bush, 2001; Kusano *et al.,* 2011).
Calcium an essential macro-nutrient and important component of many signal transduction pathways is taken up by plant roots and transported to shoots via xylem to regulate many physiological and metabolic activities (Tuteja and Mahajan, 2007; Mansoor and Baig, 2014). Its deficiency can result in poor stress tolerance, reduced yield and quality of the crop as well (Dayod *et al.,* 2010; Gilliham *et al.,* 2011). Abiotic stresses like salt, metals, water and heat can induce changes in calcium concentration (Tanaka *et al.,* 1995). It is suggested that calcium plays an important role in providing stress tolerance to plants (Tuteja and Sopory, 2008).
Additives like calcium, glucose, ascorbic acid and reduced glutathione to cadmium containing medium accelerated the growth and chlorophyll content of *Chlorella vulgaris* (Naggar and Sheekh, 1998). Similarly, exogenous application of calcium to cadmium treated *Matricaria chamomilla* L. reduced accumulation of cadmium, reactive oxygen species and the activity of antioxidant enzymes to stimulate

various growth factors (Farzadfar *et al.,* 2013). Cadmium accumulation in plant tissues depend on the level of calcium availability in culture medium (Kurtyka *et al.*, 2008). Ca^{2+} and Cd^{2+} ions compete for binding sites and their concentrations are decisive in the uptake solution for subsequent transport across a membrane (Kim *et al*., 2002; Lu *et al*., 2008, 2010).

Calcium has a protective role against Cd toxicity in tobacco plants (Choi *et al.,* 2001; Choi and Harada, 2005). Calcium plays an important role in antagonizing cadmium induced heavy metal toxicity (Suzuki, 2005; Katoch and Singh, 2014).

Looking at the importance of antagonistic interaction of these two ions it was thought worthwhile to study the interaction of Ca^{+2} and Cd^{2+} affecting accumulation and the utilization of carbohydrates during growth studies of chickpea plants.

The present study was undertaken to examine quantitative changes in starch content, reducing sugars and activity of enzymes related to carbohydrate metabolism in presence of cadmium alone and in combination with calcium.

MATERIALS AND METHODS

Healthy seeds of chickpea (*Cicer arietinum* L. cv. Himachali chana-2) procured from Himachal Agricultural University, Palampur, India were surface sterilized for 2 min by 0.1% $HgCl_2$, washed thoroughly with distilled water and inoculated with appropriate *Rhizobium* strain. The plants were raised in polythene lined earthen pots filled with river sand. Tap water was used for irrigation purposes along with weekly supply of nitrogen containing nutrient medium (Minchin and Pate, 1975) till the establishment of root nodules replacing it thereafter with nitrogen free solution. Both cadmium (as $CdSO_4$: 0.2 and 0.4 mM) and calcium (as $CaCl_2$: 0.5, 1.0 and 2mM) were provided alone and in combinations along with the nutrient solution at 20d interval.

Endogenous level of biomolecules associated with carbohydrate metabolism such as total sugars, reducing sugars and starch were determined including the activities of enzymes such as α-amylase, β-amylase, invertase. The analyses were made using fresh leaves at the reproductive stage (110 DAS, days after sowing).

Carbohydrates

Oven dried leaf tissue was homogenized and extracted in hot ethanol (80%) and centrifuged at 2000 rpm for 10 min. Supernatant was clearly decanted off. Subsequently, 3 ml. of ethanol was added to the residue and re-centrifuged. The extraction was repeated twice to ensure the complete recovery of sugars. Supernatant was subjected to the analysis for total sugars and reducing sugars.

The residue was kept for further analysis of starch. Total sugars were estimated according to the method explained by Yemm and Willis (1954). 4ml chilled anthrone reagent was added to 1ml ethanol (80%) extract. Tubes were shaken gently to mix the solution. These were then covered with glass marbles and immediately placed in boiling water bath for 10min and then cooled in ice bath.

The absorbance of solution was read at 625nm in spectrophotometer against blank containing 80% ethanol. The concentration of total sugars (mg/g DW) was calculated from a standard curve plotted with known concentration of glucose. Reducing sugars were estimated as per method of Sumner (1935).

To 1ml of DNSA reagent, ethanol extract (1ml), prepared as above was added. The reaction mixture was boiled for 12min. 2ml of distilled water was added and absorbance was recorded at 560nm against a blank containing 80% ethanol in place of ethanol extract. The concentration of reducing sugars (mg/g DW) was calculated from a standard curve plotted with known concentration of glucose. Starch content was measured by acid hydrolysis method given by McCreddy *et al.*, (1950).

To the residue, 5ml of distilled water and 6.5ml of 52% perchloric acid was added to extract the starch by placing the samples at 0°C for 20min. The mixture was centrifuged and retained the

extract. The process was repeated 3-4 times using fresh perchloric acid and diluted to final volume 100ml.

To 0.5ml of diluted extract, 4.5ml of distilled water was added followed by addition of 10ml of cold anthrone sulfuric acid reagent in ice bath. The sample mixture was heated at 100°C for 8min. and cooled rapidly to room temperature. The absorbance was measured at 630nm. The final content of starch was calculated from a standard curve plotted with known concentration of glucose.

Enzymes

Activity of α-Amylase was assayed by the method of Shuster and Gifford (1962). Fresh leaf tissue was homogenized in ice cold extraction buffer (0.1M phosphate buffer pH 7.0), centrifuged at 10,000rpm (4°C) and the supernatant treated as enzyme extract. One ml of starch substrate was added to 0.5ml of enzyme extract.

At zero time 0.2ml of aliquot was removed from this and added 3ml of KI. The absorbance was recorded at 620nm. Then, the reaction mixture left was incubated at 25°C, and then after every 30min. removed the aliquot and repeated the color developing process (violet blue). Blank was run simultaneously without having substrate. In control the enzyme extract was substituted with 0.5ml of distilled water.

The enzyme activity was expressed as µg/min/mg protein in terms of decrease in OD at 620nm. The activity of β-amylase was assayed described by Shuster and Gifford (1962). Homogenization of fresh plant material was done in ice cold 0.067M phosphate buffer pH 6.0, centrifuged at 10,000rpm (4°C) and the supernatant treated as enzyme extract.

Reaction mixture containing 0.2ml enzyme extract and 1.0ml of freshly prepared starch solution was incubated at 30°C for 1h, and the reaction was terminated by adding 1ml of DNSA. After that, tubes were kept in boiling water for 10min. and cooled at room temperature. Two ml of distilled water was added to each tube and absorbance was recorded at 560nm.

Control for every reaction mixture was run simultaneously to check the level of endogenous sugar where reaction was terminated by adding 1ml DNSA reagent before incubation. Standard curve was prepared using known concentrations of glucose.

The activity of enzyme invertase was assayed according to Hawker and Hatch (1965) and Nygaard (1977). Plant material was homogenized in chilled sodium acetate buffer (0.2M pH 4.8) containing polyvinyl pyrrolidone) centrifuged at 10,000g (4°C) and the supernatant used as enzyme extract.

Reaction mixture was prepared by adding 0.6ml of 0.2M acetate buffer pH 4.8, 0.3ml of 0.4M sucrose solution in 0.1ml of enzyme extract.

In control tubes, sucrose was added only when enzyme preparation has been inactivated by boiling for 5min.

After incubation at 30°C for 30min 1ml of 3-5ml DNSA was added to reaction mixture, thereafter, tubes were placed in boiling water bath for 10min. and cooled to room temperature. The entire samples were diluted to 5ml to read absorbance at 560nm.

RESULTS AND DISCUSSION

Carbohydrates

Total content of sugars in leaves was decreased with cadmium treatment and percentage decline was 50.27% and 56.39% of the level of control in 0.2 and 0.4mM treatments, respectively. Treatment combination of $Ca_{0.5}$ was not effective to check loss of sugars.

A significant recovery in sugar content was observed when treated with higher concentrations of calcium. The losses in sugar content of leaves were reduced to 38.6% ($Ca_{1.0}$) and 20.9% ($Ca_{2.0}$) in $Cd_{0.2}$; and 33.4% ($Ca_{1.0}$) and 22.6% ($Ca_{2.0}$) in $Cd_{0.4}$ over the control levels.

The efficacy of combination treatment was more with higher calcium levels in restoring the total sugars though none of the treatment could restore sugar content to the level of untreated ones (Figure 1).

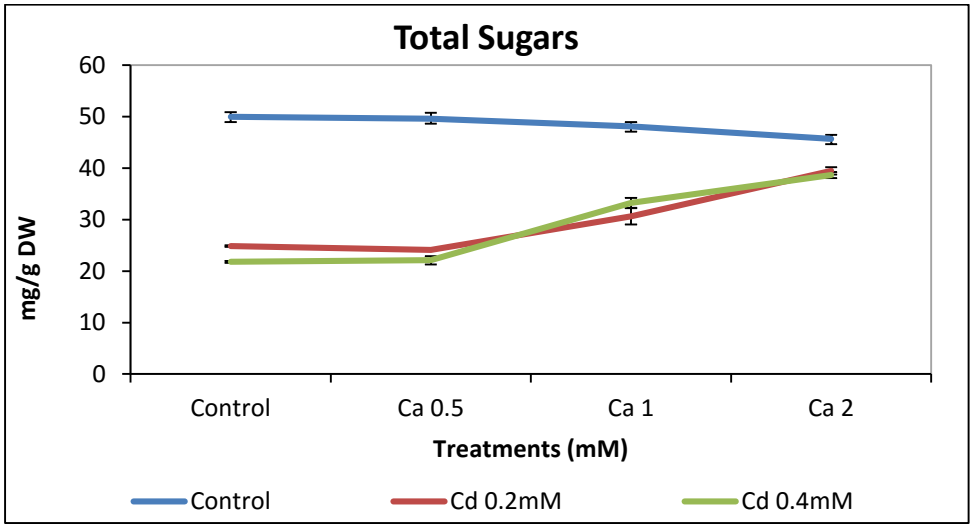

Figure 1: Effect of Cadmium and Calcium Treatments on Total Content of Carbohydrates, in Chickpea Leaves

Similarly, reducing sugars declined when treated with cadmium. A decrease of 35.8% and 41.3% was observed in Cd 0.2mM and 0.4mM treatments over the control. The content of reducing sugars increased when plants were treated with cadmium in combination with calcium. The loss in content of reducing sugars was reduced to 24.4%, 23.4% and 23.5% in $Cd_{0.2}$ + $Ca_{0.5,\ 1.0,\ 2.0}$ and; 35.7%, 25.7% and 24.5% in $Cd_{0.4}$ + $Ca_{0.5,\ 1.0,\ 2.0}$ of the level of controls, respectively. The recovery in reducing sugars content of leaves was partial even with higher calcium treatments (Figure 2).

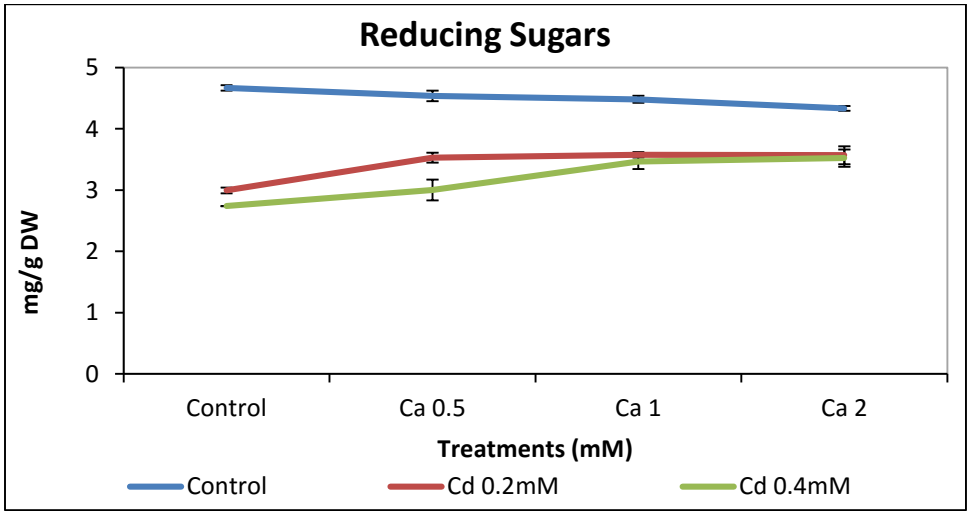

Figure 2: Effect of Cadmium and Calcium Treatments on Content of Reducing Sugars in Chickpea Leaves

Starch content of leaves decreased in the plants with cadmium treatments. The decrease was about 46.5% and 47.1% of control levels in 0.2mM and 0.4mM Cd treatments, respectively. A linear increase in content of starch was observed with combination treatment of calcium depending upon its concentration. The reduction in starch content was reduced to 45.8%, 42.4 and 33.0% in $Cd_{0.2}$ + $Ca_{0.5,\ 1.0,\ 2.0}$ treatments and; 43.5%, 43.3% and 36.0% in $Cd_{0.4}$ + $Ca_{0.5,\ 1.0,\ 2.0}$ treatments, respectively. Lowest level of reducing sugars was observed in $Cd_{0.4}$ treatment followed by $Cd_{0.2}$ while highest levels were observed in untreated plants (Figure 3).

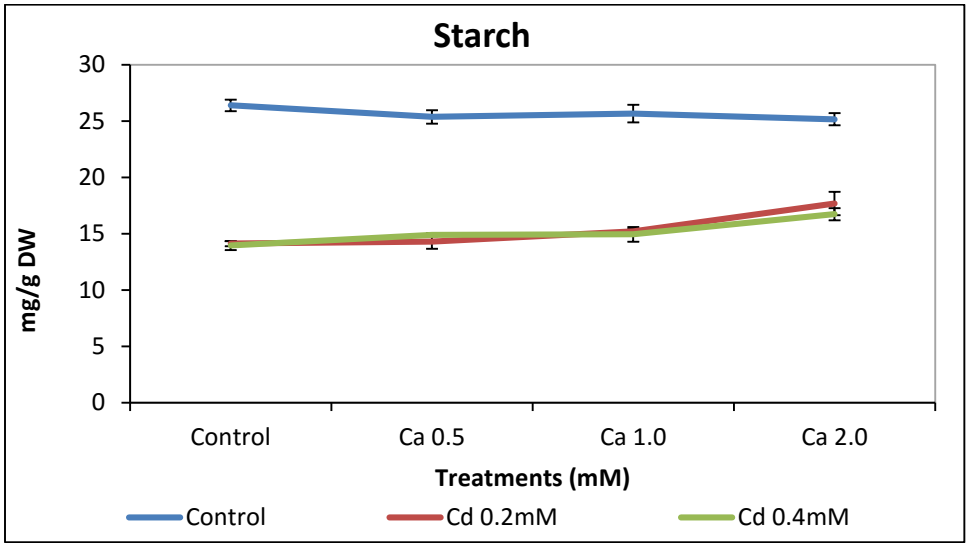

Figure 3: Effect of Cadmium and Calcium Treatments on Starch Content in Chickpea Leaves

Enzyme Activity

Cadmium caused significant decline of about 55.88% and 61.55% was measured in the activity of invertase in comparison to control in 0.2mM and 0.4mM Cd treatments, respectively. The enzyme activity improved with the application of calcium and the increase was proportionate to its concentration levels showing activity of 51.9%, 46.93, 39.82% in $Cd_{0.2}$ + $Ca_{0.5,\ 1.0,\ 2.0}$ and; 56.13%, 50.28%, 48.24% in $Cd_{0.4}$+ $Ca_{0.5,\ 1.0,\ 2.0}$, respectively in comparison to control. The activity of enzyme could only be restored partially even with high calcium concentrations (Figure 4).

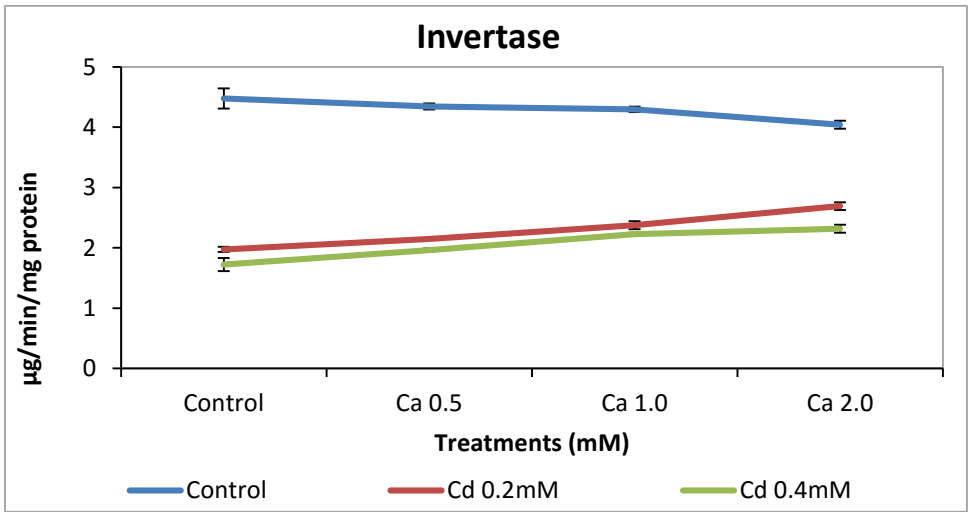

Figure 4: Effect of Cadmium and Calcium Treatments on the Activity of Enzyme Invertase in Chickpea Leaves

The activity of enzyme α-amylase decreased with cadmium showing a decline of about 36.97% and 46% in 0.2mM and 0.4mM treatments, respectively of the level of control.

A significant increase in activity was observed with the application of calcium to lower activity loss to 27.3%, 13.9%, 12.9% in $Cd_{0.2}$ + $Ca_{0.5,\ 1.0,\ 2.0}$ and; 32.5%, 23.6%, 16.1% in $Cd_{0.4}$ + $Ca_{0.5,\ 1.0,\ 2.0}$, respectively of the level of control. The restoration of enzyme activity was maximum calcium used in higher concentrations (Figure 5).

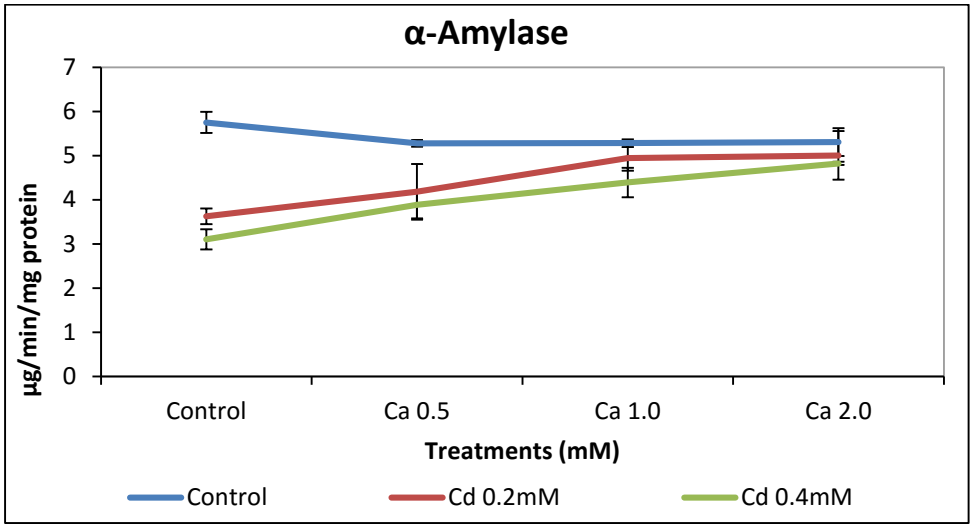

Figure 5: Effect of Cadmium and Calcium Treatments on the Activity of Enzyme α-Amylase in Chickpea Leaves

Similarly, a decrease in the activity of β-amylase was noted in Cd treated plants showing decline of about 48.4% ($Cd_{0.2}$) and 59.2% ($Cd_{0.4}$) in comparison to control. Combination of $Cd_{0.5}$ and $Ca_{2.0}$ was able to restore enzyme activity. The decline in enzyme activity with calcium application was only 8.5% in $Cd_{0.2}$+$Ca_{2.0}$ and 21.22% in $Cd_{0.4}$+$Ca_{2.0}$ treatments in comparison to control (Figure 6).

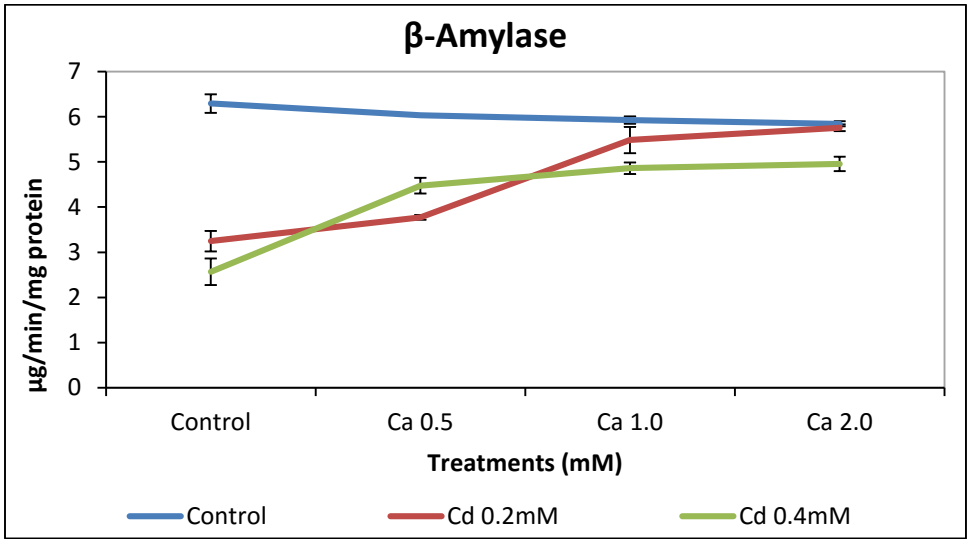

Figure 6: Effect of Cadmium and Calcium Treatments on the Activity of Enzyme β-Amylase in Chickpea Leaves

Discussion

The presence of cadmium adversely affects growth by decreasing water transport to leaves, impairing transpiration rate, ultra-structural changes in cell organelles and altering the behavior of key enzymes of various metabolic pathways (Shah and Dubey, 1998). Stressful conditions adversely affect metabolism of sugars. Accumulation of sugars contributes for the regulation of internal osmolarity and protection to biomolecules and membranes (Hayashi *et al.*, 1997; Sinniah *et al.*, 1998). In addition to the sugars controlling osmoregulation, they also enable the plant to maximize their carbohydrate reserves supporting basal metabolism during unfavorable environment (Hurry *et al.*, 1995; Dubey and Singh, 1999). In the

present study a significant decline starch, total sugars and the content of reducing sugars was noticed with cadmium application. Similar, observations were made in several studies in the presence of cadmium (Costa and Spitz, 1997; Devi *et al.,* 2007; John *et al.,* 2008). The changes in carbohydrates and sugar content of plants were associated with osmotic regulation of guard cells that control stomata movement and regulate plant water flux (Talbott and Zeiger, 1993). Further, the accumulation and deprivation of carbohydrates were directly correlated with modification of photosynthetic processes (Moya *et al.,* 1993). The negative effect of heavy metals on carbon metabolism was suggested a possible interference in the reactive centre of ribulose biphosphate carboxylase (Stiborova *et al.,* 1987). Reduced leaf surface humidity accompanying lower transpiration rates lead to a significant rise of leaf surface temperature (3.1 ^{o}C) as adaptation avoiding unfavorable conditions (Chosden *et al*., 2014).

In the present study activities of enzymes like invertase, α-amylase and β-amylase declined in cadmium stressed chickpea plants. Reduced activity of such enzymes and the reducing sugars content were noticed under conditions of heavy metal stress including Cadmium (Verma and Dubey, 2001; Prado *et al.,* 2010). The enzyme invertase plays an important role in phloem loading and unloading by maintaining steep sucrose gradient (Lohaus *et al.,* 1995). The principal carbon source in growing rice seedling was sucrose and its level in the tissues had a strong influence on related enzymes activity (Ricard *et al.,* 1998).

The degree of inhibition in Cd induced heavy metal stress depends on the calcium level present in the uptake medium (Greger and Bertell, 1992). Ca^{2+} increases the resistance of plant tissues which is an important ubiquitous secondary messenger in many of signal transduction pathways in plants. Ca^{+2} offers more protection against Cd^{2+} induced toxicity to the content of proteins, carbohydrates and photosynthetic oxygen evolution and also lowering the uptake of Cd^{2+} significantly in *Chlorella vulgaris* (Naggar and Sheekh, 1998).

Calcium improves the growth of plants by ameliorating heavy metals induced toxicity (Marschner, 1995, Hagemeyer, 1999; Qiu *et al.,* 2005; Farzadfar *et al.,* 2013; Katoch and Singh, 2014). More uptake and accumulation of Cd was reported in the absence of Ca nutrition in environment (Wan *et al.,* 2011). High levels of $CaCl_2$ around the Ca^{+2} channels reduce Cd^{+2} uptakes in competition with its ion influx (Yan *et al.,* 1992; Stohs *et al.,* 2000).

Thus, present study suggests that the presence of cadmium in rhizosphere affects the process of photosynthesis due to reduced intake of water. As a result, the growth of chickpea plants is impaired due to altered sugar metabolism. Further, calcium ions largely mitigate Cd induced heavy metal toxicity probably by improving absorption and uptake of water and restoring sugar metabolism. The efficacy of Calcium was more when used in higher concentrations.

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