Regulation of Growth and Photosynthetic Parameters by Salicylic Acid and Calcium in *Brassica juncea* under Cadmium Stress

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Cadmium, a non-essential and toxic metal, negatively affects plant growth and productivity, and alters the plant's physiological processes necessary for its survival. The present study was designed to explore the individual and combined effects of calcium and salicylic acid (SA) on the morphology and physiology of *Brassica juncea* L. cv. Varuna under cadmium stress. The application of calcium (2 mM) through the soil and/or SA (10^{-5} M) as foliar spray enhanced the growth, photosynthetic parameters, and proline content determined after 45 days of treatment. The application of cadmium (6 mg kg^{-1}) through the soil was toxic and decreased both growth and the photosynthetic parameters. The application of calcium and SA in combination was most effective in alleviating the harmful effects of cadmium on growth and photosynthesis. Calcium and SA clearly induced plant protection mechanisms by enhancing proline and chlorophyll accumulation in the leaves.

Key words: Net Photosynthetic Rate, Proline, Salicylic Acid

Introduction

Abiotic stresses, such as salinity, drought, heat, cold, flooding, and heavy metal contamination, are the major factors that negatively affect crop growth and productivity worldwide. Among the heavy metals, cadmium is a non-essential and toxic metal (Sanita di Toppi and Gabbrielli, 1999), which is rapidly taken up as Cd^{2+} by roots and accumulated in various plant tissues (Amani, 2008) reducing the crop growth and productivity. It easily enters the food chain and therefore poses serious threats to human health (Hall, 2002). The inhibition of root elongation is the most sensitive parameter of Cd^{2+} toxicity (Guo and Marschner, 1995). Cd^{2+} toxicity affects the photosynthesis by inhibiting the biosynthesis of chlorophyll (Lea *et al.*, 2007), chlorophyll content (Hayat *et al.*, 2013) and stomatal opening (Sandalio *et al.*, 2001), and finally slows down the rate of photosynthesis (Hayat *et al.*, 2013). The metal causes an increase in the level of proline (Hayat *et al.*, 2013) that acts as an osmoprotectant, membrane stabilizer, and scavenger of reactive oxygen species (ROS) (Apel and Hirt, 2004).

Calcium taken up as Ca^{2+}, an important signaling messenger and essential nutrient, plays a key role in many plant cellular responses (Miller *et al.*, 2009). It competes with the uptake of Cd^{2+} in plants (Ismai, 2008) and thus alleviates the metal toxicity stress by restoring the plant metabolism and chlorophyll content (Zhang *et al.*, 1998). The first target of heavy metal toxicity in plants is the plasma membrane (Hall, 2002), the damage of which alters the normal Ca^{2+} signal transduction system, which serves as a stress signal mediator (Lynch, 1989). Therefore, application of a moderate amount of exogenous Ca^{2+} can alleviate the plant's environmental stresses by enhancing the plasma membrane stability and restoring the calcium signal transduction system (Cramer, 1985). Several authors have reported the alleviation of cadmium toxicity by exogenous Ca^{2+} in many plants such as beans (Ismai, 2008), faba beans (Siddiqui *et al.*, 2012), and cabbage (Chen *et al.*, 2002).

Salicylic acid (SA) has been recognized as an endogenous plant growth regulator that generates a wide range of metabolic and physiological responses in plants, thereby affecting their growth and development (Hayat *et al.*, 2010). The application of SA has been found to effectively enhance the growth of plants exposed to various stressful environments such as high salinity, heavy metals, temperature extremes, water stress, and irradiance (Hayat *et al.*, 2010, 2014). Exogenous application of SA has been found to increase photosynthetic parameters and plant water relations (Hayat *et al.*, 2014).

The objective of the present study was to assess the ameliorative roles of SA and/or Ca^{2+} against the cadmium toxicity in *Brassica juncea* L. cv. Varuna based on the measurement of growth and photosynthetic parameters.

Materials and Methods

Plant material and treatment

Healthy seeds of *Brassica juncea* L. cv. Varuna procured from Chola Beej Bandhar, Aligarh, India, were surface-sterilized with 0.01 % $HgCl_2$ solution followed by repeated washings with double distilled water (DDW). The experiment was conducted with 28 earthen pots (diameter, 25 cm; height, 25 cm) filled with sandy loam soil mixed with farmyard manure [organic carbon, 16.78 %; pH, 8.18; EC, 3.70 d Sm^{-1}; the following elements (in mg kg^{-1} soil): total N, 96; P, 7.38; K, 144.5; Fe, 1980; Mn, 182; Cu, 12.7; Zn, 55.4; Cd, 0.34; Ca, 67.8; Mg, 23.0] in a ratio of 9:1. The pots were arranged in such a way that each treatment had four replicates, and within each pot three plants were maintained under simple randomized block design in the net-house of the Botany Department, Aligarh Muslim University, Aligarh, India. Surface-sterilized seeds of *Brassica juncea* L. cv. Varuna were sown in soil, moistened with water, and allowed to germinate. Six days after sowing (DAS), the soil was amended with $CdCl_2$ (32.7 μM; 6 mg Cd kg^{-1} soil) and/or $CaCl_2$ (2 mM) solution. At 30 DAS, plants were sprayed with DDW and/or SA (10^{-5} M). Each seedling was sprayed three times with 1 mL each time. The plants were then sampled at 45 DAS to determine the parameters given below.

Growth biomarkers

The plants were uprooted from each pot, washed under running tap water to remove the adhering soil particles, and then dried by using a blotting sheet. The length of root and shoot was measured by a metric scale, and the fresh weight of the samples was recorded. The leaves of the plants were kept in an oven (80 °C for 48 h) for dehydration and then weighed again to record their dry mass. The leaf area was measured by a portable leaf area meter (AM300; ADC BioScientific, Hoddesdon, Hertfordshire, United Kingdom).

Chlorophyll determination

The chlorophyll concentration in leaves attached to plants was measured by a SPAD chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Osaka, Japan), and is given in relative SPAD units.

Gas exchange parameters

The gas exchange parameters, *i.e.* net photosynthetic rate (P_N), stomatal conductance (g_s), internal CO_2 concentration (C_i), transpiration rate (E), and water use efficiency (*WUE*) were measured using a portable photosynthesis system (LI-6400; LI-COR, Lincoln, NE, USA). The measurements were made in the uppermost fully expanded leaves of a plant under clear sunlight.

Leaf proline content

The proline content in fresh leaves was determined spectrophotometrically using the ninhydrin method given by Bates *et al.* (1973).

Statistical analysis

Treatment means were compared by the analysis of variance using SPSS (SPSS ver. 17 for windows; IBM, Chicago, IL, USA). The standard error between replicates was also calculated.

Results

Growth biomarkers

SA, Cd, and Ca concentrations were chosen on the basis of data provided by Hayat *et al.* (2012, 2013) and Siddiqui *et al.* (2012), respectively.

Growth parameters, *i.e.* length, fresh and dry mass of root and shoot, and leaf area, were significantly

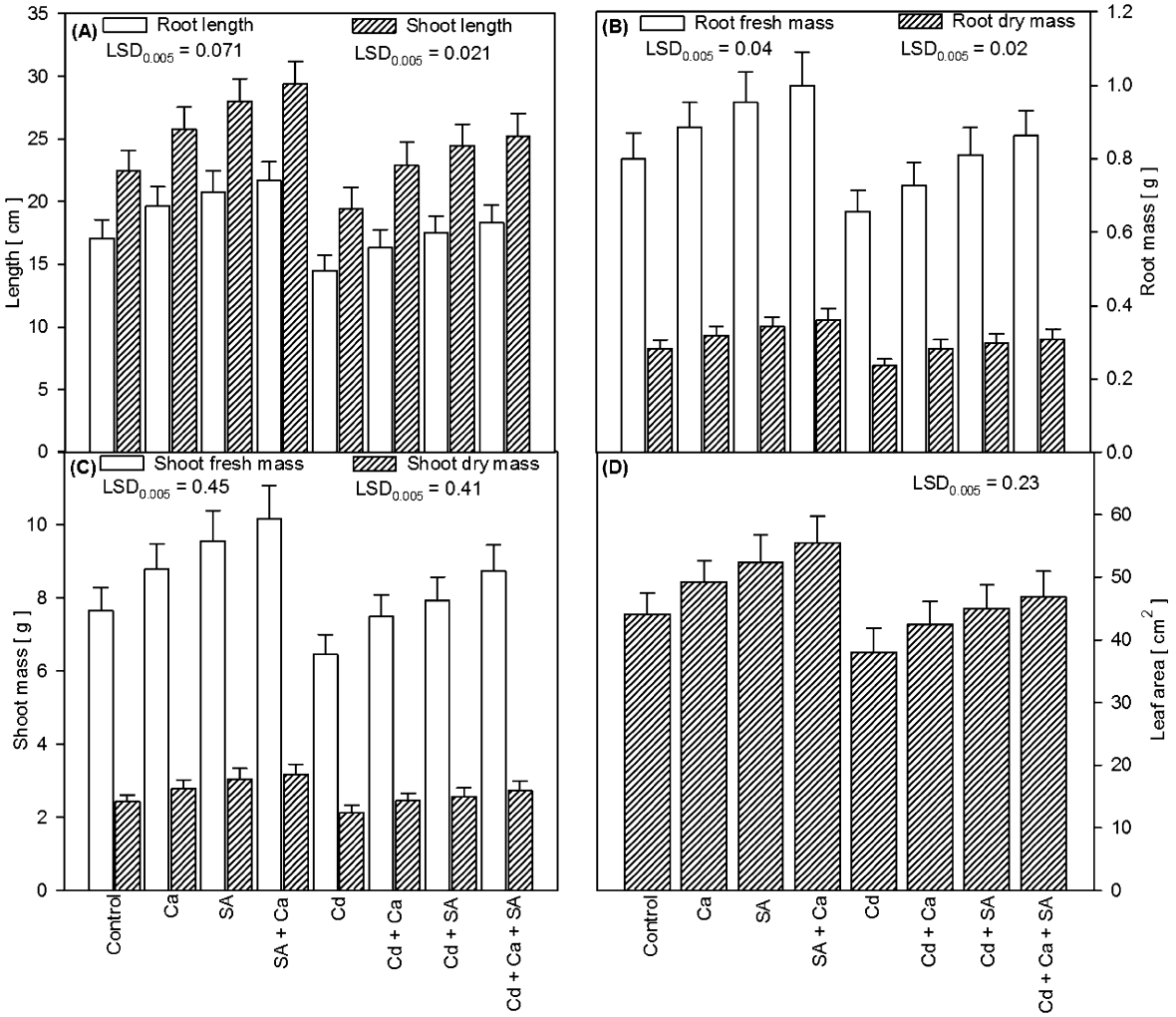


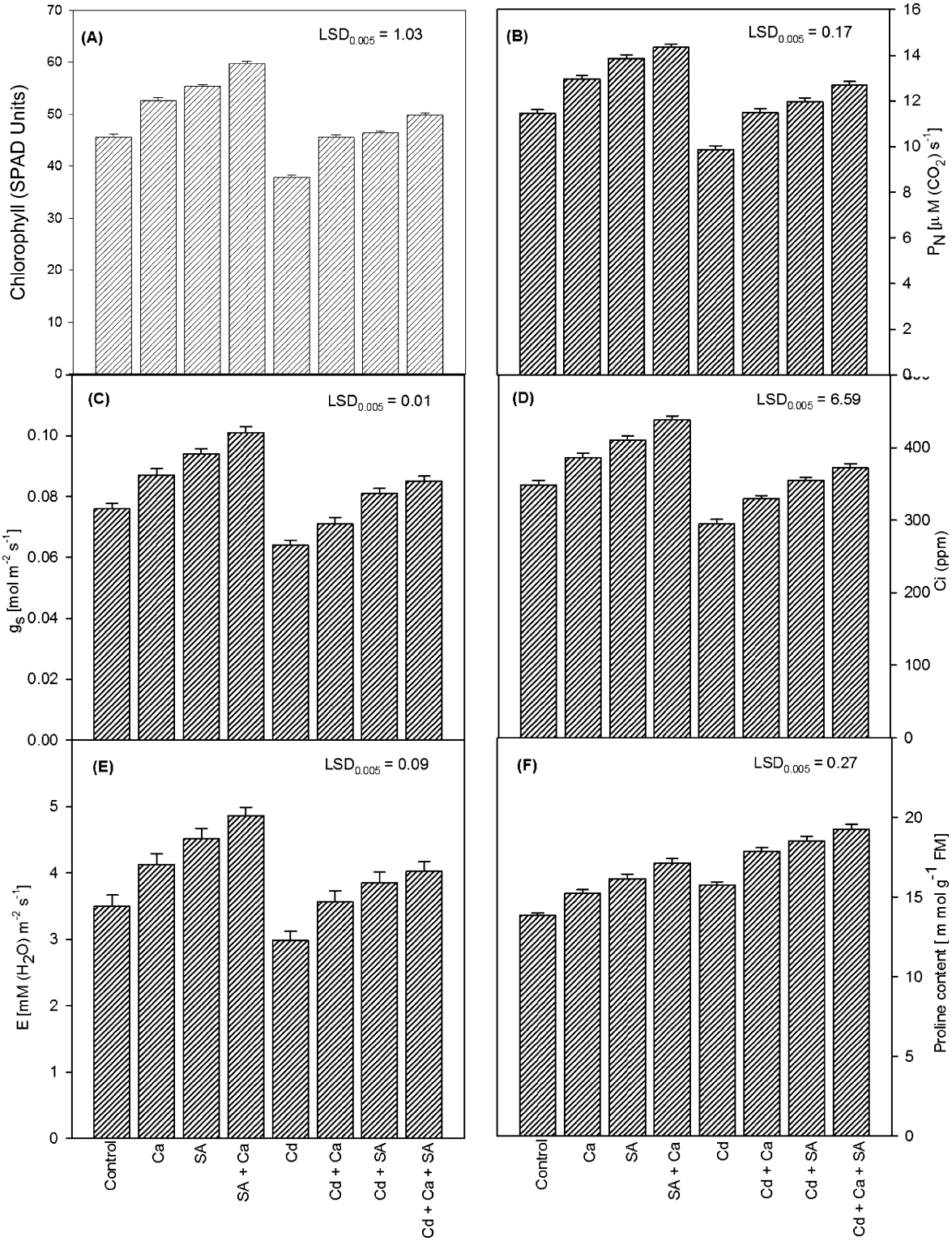
Fig. 1. Effect of Ca^{2+} (2 mM) and/or SA (10^{-5} M) on the Cd (6 mg kg^{-1} soil)-induced changes in (A) length of root and shoot, (B) fresh and dry mass of root, (C) fresh and dry mass of shoot, and (D) leaf area in *Brassica juncea* L. cv. Varuna 45 days after treatment.

($P \leq 0.05$) reduced by 28%, 13.5%, 18%, and 15.5% with the application of Cd^{2+} through soil, at 45-days-stage compared to the control (Fig. 1), while treatment with Ca^{2+} through the soil and spray of SA to the foliage increased the growth of the plants in the absence of Cd^{2+} stress. The toxic effects of Cd^{2+} were significantly overcome in all growth parameters by the application of SA alone or in combination with Ca^{2+} to the metal-stressed plants.

Chlorophyll content

Plants receiving Ca^{2+} through the soil or/and SA as foliar spray contained more chlorophyll at 45-days-

Fig. 2. Effect of Ca^{2+} (2 mM) and/or SA (10^{-5} M) on the Cd (6 mg kg^{-1} soil)-induced changes in (A) chlorophyll content, (B) net photosynthetic rate, (C) stomatal conductance, (D) internal CO_2 concentration, (E) transpiration rate, and (F) proline content in *Brassica juncea* L. cv. Varuna at 45-days-stage of growth.


(A)
LSD0.005 = 1.03
Chlorophyll (SPAD Units)
(B)
LSD0.005 = 0.17
PN [μM (CO2) s-1]
(C)
LSD0.005 = 0.01
gs [mol m-2 s-1]
(D)
LSD0.005 = 6.59
Ci (ppm)
(E)
LSD0.005 = 0.09
E [mM (H2O) m-2 s-1]
(F)
LSD0.005 = 0.27
Proline content [m mol g-1 FM]
Control
Ca
SA
SA + Ca
Cd
Cd + Ca
Cd + SA
Cd + Ca + SA

stage of growth (15%, 21%, and 43% higher compared to control plants) (Fig. 2A). In the presence of Cd^{2+} in the soil, the chlorophyll content was reduced by about 17%, and this negative effect of Cd^{2+} stress was significantly overcome by the application of SA alone or in combination with Ca^{2+}.

Gas exchange parameters

Gas exchange parameters, *i.e.* net photosynthetic rate (P_N), stomatal conductance (g_s), internal CO_2 concentration (C_i), and transpiration rate (E), were significantly reduced by Cd^{2+} stress at 45-days-stage, the decrease being 14%, 15.8%, 15.2%, and 14.8%, respectively, compared to control plants (Figs. 2B – E). The toxic effects caused by Cd^{2+} were effectively overcome by either Ca^{2+} or SA alone or their combination at 45-days-stage. Plants receiving the combination of SA and Ca^{2+} exhibited the highest values of the gas exchange parameters at 45-days-stage, and the parameters were increased by 25.1%, 32.9%, 25.9%, and 38.9%, while SA alone increased the parameters by 20.9%, 23.7%, 17.8%, and 28.9% at 45-days-stage with respect to water-sprayed control plants.

Leaf proline content

An increase in the proline content was observed in plants that had received Cd^{2+}, Ca^{2+}, and SA by 13.9%, 9.9%, and 16.7%, respectively, in comparison to control plants. The maximum value of the proline content (increase of 39%) was found in the plants receiving the interactive treatment of Cd^{2+}, Ca^{2+}, and SA (Fig. 2F).

Discussion

Cadmium is an important phytotoxic element that causes growth inhibition and even plant death. In our present study, cadmium fed through the soil caused significant damage to plants as expressed in terms of reduced growth (length of shoot and root, fresh and dry mass, and leaf area) (Fig. 1). The cause of this damage may be the loss of cellular turgor, inhibition of cellular activities, and reduction of cell enlargement (Sanita di Toppi and Gabbrielli, 1999). Cadmium also becomes associated with the cell wall and middle lamella, which increases the cross-linking of pectins and results in the inhibition of cell growth (Poschenrieder *et al.*, 1989).

Follow-up treatment with SA (in the presence and absence of Ca^{2+}) improved the growth of plants, the combination of Ca^{2+} and SA being most effective (Fig. 1). This may be explained on the basis of the roles of Ca^{2+} and SA in signal transduction. SA acts at the level of transcription and/or translation thereby increasing the activity of various enzymes necessary for the growth of plants (Hayat *et al.*, 2010). SA also enhances the accumulation of abscisic acid and indole-3-acetic acid that improve the protective and growth-promoting effect of SA (Sakhabutdinova *et al.*, 2003). The ameliorative effect of Ca^{2+} is related to the enhancement of potassium ion concentration and the decrease of Cd ion concentration in the roots (Kurtyka *et al.*, 2008). Likewise, Rivetta *et al.* (1997) reported that the negative effects of Cd^{2+} on radish growth could be reversed by Ca^{2+} application.

Photosynthetic parameters and the chlorophyll content are affected by metal stress of plants and used as indicators of the severity of the stress (Küpper and Kroneck, 2005). In the present study, Cd^{2+} application through the soil caused a decrease in various photosynthetic parameters (P_N, g_s, C_i, and E) and the chlorophyll content (Figs. 2A – E). It has been suggested that thylakoid membrane leakage under Cd^{2+} stress (Najeeb *et al.*, 2011) might be responsible for the decrease in photosynthesis. Another possible reason may be that the closure of stomata in response to the decreasing partial pressure of CO_2 in the stroma under Cd^{2+} stress (Barcelo and Poschenrieder, 1990) becomes the direct cause of the loss of stomatal conductance (g_s), internal CO_2 concentration (C_i), and transpiration rate (E). Therefore, a cumulative effect of all these altered processes leads to a decrease in the photosynthetic rate.

Furthermore, Cd^{2+} enhances the activity of chlorophyllase that initiates the degradation of chlorophyll (Reddy and Vora, 1986), and it also causes a decline in the synthesis of δ-amino-levulinic acid and the protochlorophyllide reductase complex (Stobart *et al.*, 1985) leading to a decrease in the chlorophyll concentration (Fig. 2A) and the photosynthetic rate (P_N) (Fig. 2B), as observed in other studies as well (Hayat *et al.*, 2013). Application of SA to either unstressed or Cd^{2+}-stressed plants increased all the photosynthetic parameters (Figs. 2B – E). The exogenous application of SA has been shown to raise thephotosynthetic pigment concentration and the activities of Rubisco and PEP carboxylase (Singh and Usha, 2003). The improvement of all these characteristics ultimately leads to an increase in the net photosynthetic rate. Moreover, Ca^{2+} acts as signaling molecule and is involved in the opening and closing of stomata (McAinsh and Pittman,

2009). It thus improves the stomatal conductance as observed in this study.

In order to cope with environmental stresses, plants have developed certain adaptive mechanisms. One such mechanism is the accumulation of compatible organic solutes such as proline (Kavi Kishor *et al.*, 2005). Therefore the increase in the proline content in plants subjected to Cd stress was expected (Fig. 2F). Several reports have suggested the antioxidant character of proline, scavenging ROS particles and acting as singlet oxygen quencher (Matysik *et al.*, 2002). Treatment of plants with SA and/or Ca^{2+} in the presence or absence of stress enhanced the proline content (Fig. 2F). The increase of the proline content by Ca^{2+} application is in agreement with the reports of Nayyar and Walia (2003) and Arshi *et al.* (2010) who found that Ca^{2+} reduces stress effects by elevating the contents of proline and glycine betaine in wheat and *Cichorium intybus* L. seedlings. Enhancement of proline levels by Ca^{2+} and SA indicates the ameliorative role of this compound against Cd^{2+} stress.

Conclusion

Cd^{2+} stress (6 mg kg^{-1} soil) has been found to cause a significant reduction in growth and photosynthetic parameters in *Brassica juncea* L. cv. Varuna. At the tested concentrations, SA reduced the toxic effects of Cd^{2+} more efficiently than Ca^{2+}, while the combination of the two completely overcame Cd^{2+} toxicity.

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Amani A. L. (2008), Cadmium induced changes in pigment content, ion uptake, proline content and phosphoenolpyruvate carboxylase activity in *Triticum aestivum* seedlings. Aust. J. Basic Appl. Sci. **2**, 57 – 62.

Apel K. and Hirt H. (2004), Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. **55**, 373 – 399.

Arshi A., Ahmad A., Aref I. M., and Iqbal M. (2010), Effect of calcium against salinity-induced inhibition in growth, ion accumulation and proline contents in *Cichorium intybus* L. J. Environ. Biol. **31**, 939 – 944.

Barcelo J. and Poschenrieder C. H. (1990), Plant water relations as affected by heavy metal stress. J. Plant Nutr. **13**, 1 – 37.

Bates L. S., Waldeen S. P., and Teare I. D. (1973), Rapid determination of free proline for water stress studies. Plant Soil **39**, 205 – 207.

Chen X. T., Wang G., and Liang Z. C. (2002), Effect of amendments on growth and element uptake of Pakchoi in a cadmium, zinc and lead contaminated soil. Pedosphere **12**, 243 – 250.

Cramer G. R. (1985), Displacement of Ca^{2+} by Na^{+} from the plasma lemma root cells. Plant Physiol. **79**, 207 – 211.

Guo Y. and Marschner H. (1995), Uptake, distribution and binding of cadmium and nickel in different plant species. J. Plant Nutr. **18**, 2691 – 2706.

Hall J. L. (2002), Cellular mechanism for heavy metal detoxification and tolerance. J. Exp. Bot. **53**, 1 – 11.

Hayat Q., Hayat S., Irfan M., and Ahmad A. (2010), Effect of exogenous salicylic acid under changing environment: A review. Environ. Exp. Bot. **68**, 14 – 25.

Hayat S., Maheshwari P., Wani A. S., Irfan M., Alyemeni M. N., and Ahmad A. (2012), Comparative effect of 28 homobrassinolide and salicylic acid in the amelioration of NaCl stress in *Brassica juncea* L. Plant Physiol. Biochem. **53**, 61 – 68.

Hayat S., Hasan S. A., Alyemeni M. N., and Ahmad A. (2013), Synergy of photosynthesis and antioxidant system potentiate the growth of tomato genotypes under cadmium stress. Life Sci. J. **10**, 232 – 240.

Hayat S., Hayat Q., Alyemeni M. N., and Ahmad A. (2014), Cumulative effect of proline and salicylic acid on the cadmium induced changes in *Cicer arietinum* L. Fres. Environ. Bull. **23**, 330 – 340.

Ismai M. A. (2008), Involvement of Ca^{2+} in alleviation of Cd^{2+} toxicity in common bean (*Phaseolus vulgaris* L.) plants. Res. J. Agric. Biol. Sci. **4**, 203 – 209.

Kavi Kishor P. B., Sangam S., Amrutha R. N., Laxmi P. S., Naidu K. R., Rao K. R. S. S., Rao S., Reddy K. J., Theriappan P., and Sreenivasulu N. (2005), Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr. Sci. **88**, 424 – 438.

Küpper H. and Kroneck P. M. H. (2005), Heavy metal uptake by plants and cyanobacteria. In: Metal Ions in Biological Systems (Sigel A. and Sigel H., eds.). Marcel Dekker, New York, USA, pp. 97 – 142.

Kurtyka R., Małkowski E., Kita A., and Karcz W. (2008), Effect of calcium and cadmium on growth and accumulation of cadmium, calcium, potassium and sodium in maize seedlings. Pol. J. Environ. Stud. **17**, 51 – 56.

Lea P. J., Sodek L., Parry M. A. J., Shewry P. R., and Halford N. G. (2007), Asparagine in plants. Ann. Appl. Biol. **150**, 1 – 26.

Lynch J. A. (1989), Salinity stresses increases cytoplasmic activity in maize root protoplasts. Plant Physiol. **90**, 127 – 180.

Matysik J., Alia, Bhalu B., and Mohanty P. (2002), Molecular mechanism of quenching of reactive oxygen species by proline under stress in plants. Curr. Sci. **82**, 525 – 532.

McAinsh M. R. and Pittman J. K. (2009), Shaping the calcium signature. New Phytol. **181**, 275 – 294.

Miller G., Suzuki N., Cifti-Yilmaz S., and Mittler R. (2009), Reactive oxygen species homeostasis and signaling during drought and salinity stresses. Plant Cell Environ. **33**, 453 – 467.

Najeeb U., Jilani G., Ali S., Sarwar M., Lu L., and Zhou W. J. (2011), Insight into cadmium induced physiological and ultra-structural disorders in *Juncus effuses* L. and its remediation through exogenous citric acid. J. Hazard. Mater. **186**, 565 – 574.

Nayyar H. and Walia D. P. (2003), Water stress induced proline accumulation in contrasting wheat genotypes as affected by calcium and abscisic acid. Biol Plant. **46**, 275 – 279.

Poschenrieder C., Gunse B., and Barcelo J. (1989), Influence of cadmium on water relations, stomatal resistance, and abscisic acid content in expenditure bean leaves. Plant Physiol. **90**, 1365 – 1371.

Reddy M. P. and Vora A. B. (1986), Changes in pigment composition, Hill reaction activity and saccharide metabolism in bajra (*Pennisetum typhoides* S & H) leaves under NaCl salinity. Photosynthetica **20**, 50 – 55.

Rivetta A., Negrini N., and Cocucci M. (1997), Involvement of Ca^{2+} calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination. Plant Cell Environ. **20**, 600 – 608.

Sakhabutdinova A. R., Fatkhutdinova D. R., Bezrukova M. V., and Shakirova F. M. (2003), Salicylic acid prevents the damaging action of stress factors on wheat plants. Bulg. J. Plant Physiol. (special issue), 314 – 319.

Sandalio L. M., Dalurzo H. C., Gomez M. C., Romero Puertas M. C., and del Rio L. A. (2001), Cadmium induced changes in the growth and oxidative metabolism of pea plants. J. Exp. Bot. **52**, 2115 – 2126.

Sanita di Toppi L. and Gabbrielli R. (1999), Response to cadmium in higher plants. Environ. Exp. Bot. **41**, 105 – 130.

Siddiqui M. H., Al-Whaibi M. H., Sakran A. M., Basalah M. O., and Ali H. M. (2012), Effect of calcium and potassium on antioxidant system of *Vicia faba* L. under cadmium stress. Int. J. Mol. Sci. **13**, 6604 – 6619.

Singh B. and Usha K. (2003), Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. Plant Growth Regul. **39**, 137 – 141.

Stobart A. K., Griffits W., Bukhari L., and Sherwood A. (1985), The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. Physiol. Plant. **63**, 293 – 298.

Zhang S. G., Gao J. Y., Song J. Z., and Weng Y. J. (1998), Salt stress on the seedling growth of wheat and its alleviation through exogenous $Ca(NO_3)_2$. Triticale Crops **18**, 60 – 64.