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Amelioration of Cd toxicity by pretreatment of salicylic acid in *Cicer arietinum* L. seedlings

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Abstract

In this study, the ameliorating effect of salicylic acid (SA), serving as a mediator for protecting plants, against cadmium (Cd) toxicity in *Cicer arietinum* was investigated. The seedlings of *Cicer arietinum* treated with increasing Cd concentrations (0, 25, 50, 100 µM) inhibited seedling length, reduced fresh and dry weight, total chlorophyll, carotenoid content and fatty acid methyl ester content. Furthermore, the level of some important parameters like MDA, proline and GSH content related to oxidative stress increased in Cd treated seedlings. Leaves of seedlings pretreated with salicylic acid (0.5 mM), alleviated the toxic effects of Cd by increasing the growth parameters, photosynthetic pigments, GSH and FAME content and decreasing proline and MDA content respectively. The result of the present study reveals the protective role of salicylic acid against Cd toxicity in *C. arietinum*.

Key words

Cadmium, *Cicer arietinum*, Phytotoxicity, Salicylic acid

Introduction

Cadmium (Cd) is a toxic, non essential element with no biological significance. Cd is released from metals, industrial processes, phosphate fertilizers and rock mineralization processes. It readily enters the cells inducing a wide array of visible and metabolic perturbations like chlorosis, browning of leaf tips, inhibition of seed germination, disturbance in photosynthetic metabolism, transpiration rate, water homeostasis and ionic relations, decrease in nutrient uptake, inhibition of cell division and production of reactive oxygen species (Faizan *et al.*, 2011; Lopez-Millan *et al.*, 2009). Increased production of ROS in plants causes oxidative damage to biomolecules like proteins, lipids and nucleic acids resulting in electrolyte leakage and increased lipid peroxidation (Balaknina *et al.*, 2005). Cd induced phytotoxicity has been reported in several crops like rice, gram and barley (Hassan *et al.*, 2005; Tantrey *et al.*, 2010; Vassilev and Lidon, 2011).

Salicylic acid is an endogenous plant growth regulator that has been found to generate a wide range of metabolic and physiological responses in plants thereby affecting their growth and development. It is also considered to be an important signaling molecule in plants which is involved in eliciting specific responses to various biotic and abiotic stresses (Hayat and Ahmad, 2007). Depending on the concentration applied, salicylic acid may produce stimulative or inhibitive effects on plants physiology (Fariduddin *et al.*, 2003; Pancheva *et al.*, 1996). The role of salicylic acid in seed germination, fruit yield, glycolysis, flowering in thermogenic plants, ion uptake and transport, rate of photosynthesis, stomatal conductance and transpiration is well documented (Hayat *et al.*, 2010). Exogenous application of salicylic acid induces the SAR in plants, thereby imparting resistance against various pathogens. Recently, salicylic acid has been effectively found to alleviate the toxic effects of heavy metals, UV radiation, temperature, ozone and salinity stress in plants. The protective role of salicylic acid in attenuating Cd toxicity has been reported in several plants like pea, maize, wheat, barley (Guo *et al.*, 2009; Popova *et al.*, 2009; Krantev *et al.*, 2006, 2008; Metwally *et al.*, 2003).

Cicer arietinum commonly known as chickpea is an important pulse crop, belonging to family Fabaceae, grown and consumed all over the world. Chick pea seeds are considered rich source of dietery proteins due to their good balance of amino acids, high protein availablity and relatively low level of antinutritional factor (Burstin *et al.,* 2011). Although there are several reports available on the protective role of salicylic acid on Cd toxicity in crop plants however, studies on *Cicer arietinum* is meager. In light of above, the present study was undertaken to assess the phytotoxic effect of Cd on *Cicer arietinum* and also report the ameliorating role of salicylic acid in reducing Cd phytotoxicity.

Materials and Methods

Growth conditions of seedlings and experimental design: Chickpea (*Cicer arietinum* L. cv. ispanyol) seeds were soaked in tap water for 5hr, surface sterilized with 0.5 % (m/v) sodium hypochlorite for 30 min, rinsed several times with distilled water and then germinated on moist cotton placed in sterilized dishes. Seeds germinated for three days were sown in plastic pots filled with sand and topsoil. They were grown by being soaked in tap water for 3 times a week. The plants were grown in controlled growth chambers having photoperiod of 16 hr day/8hr night, 27± 2º C temperature and relative 60-70 % humidity, respectively. Seedlings that showed abnormal growth were eliminated. Before treatment, roots of the seedlings were washed with deionised water and placed between the canals of the sponge lids of jars that contained basic Hoagland nutrient solution (30%). Fifteen-day-old seedlings were used for experimental purpose. Nutrient solution was aerated twice a day. 0.5 mM SA concentration prepared in deionized water and $CdCl_2$ (0, 25, 50 and 100 µM) concentrations prepared in nutrient solution were used as test solutions. The seedlings were divided into eight groups, each consisting of 10 seedlings. The solutions were applied to the roots of seedlings using hydroponic method. Four set of seedlings were placed in jars containing deionised water and remaining four sets of seedlings were pretreated with 0.5 mM of salicylic acid (SA) solution for 24 hrs. After pre-treatment with SA, the seedlings were left for one day, washed and transferred into the jars containing 0, 25, 50 and 100 µM cadmium solutions. After one day treatment with $CdCl_2$, the seedlings were harvested for studying various growth and biochemical parameters.

Growth parameters of seedlings and biochemical analysis: In order to determine the growth parameters, seedling length (%) and fresh and dry weight (%) of seedlings were calculated. To establish the dry weight of seedlings, the seedlings, which were duly packed and marked, were kept in drying oven and their final weight was measured (Kacar, 1972). Chlorophyll and carotenoid content in leaves of control, Cd treated and pretreated SA seedlings were estimated by following the method of Arnon (1949). Using the absorbance values, total chlorophyll and carotenoid contents were estimated by the formula given by Witham *et al.* (1971). Proline concentration in the samples was estimated by the method of Bates *et al.* (1973) and expressed in µmol g^{-1} f.wt. The level of lipid peroxidation in the plant tissue was quantified by estimating MDA content following the method of Heath and Packer (1968). The MDA content in samples was expressed in nmol g^{-1} f.wt. The glutathione (GSH) content in plant extract was estimated following the method of Ellman (1959) and Griffith (1980). The absorbance of reaction mixture was read at 412 nm and the GSH content was expressed as µg g^{-1} f.wt. Tissue extraction and measurement of amounts were carried out in a modified manner at 412 nm after (Ellman, 1959; Griffith, 1980). For the analyses of fatty acid, leaf tissue used and analyzed in gas chromatography (Christie, 1990; Hara and Radin, 1978). The amount of fatty acids methyl esters (FAME) in the samples was expressed in µmol g^{-1} f. wt. Results were determined in terms of weight % of total. Three replicates for each treatment were maintained. All physiological analyses were replicated three times for each treatment. For each analyses 0.5 g of leaf tissue used.

Statistical analyses: Results were analyzed using one-way ANOVA (SPSS 15.0 Evaluation Version Production Mode Facility). The difference between treatments were considered significant at $p<0.01$-0.05. Duncan test was performed to compare means.

Results and Discussion

The seedlings of *C. arietinum* treated with different concentrations of Cd reduced seedling length, fresh and dry weight significantly in a concentration dependent manner. As compared to Cd treatment, pretreatment of seedlings with SA showed 3.06 and 1.77 % increase in seedling length, 1.76 and 4.8 increase in fresh weight, 4.81 and 6.52 % increased in dry weight of seedlings treated with 50 and 100 µM Cd, respectively (Table 1). The results revealed not significant ($p \geq 0.05$) regulatory effect of SA on Cd toxicity in chickpea seedlings. While control and 25 µM Cd +SA treated seedlings showed decrease in growth parameters. This may be as a result of complex behaviors of SA, which is considered as a plant hormone (Fariduddin *et al.,* 2003; Hayat and Ahmad, 2007). Growth inhibition in plants in response to Cd toxicity has been reported earlier in tomato, chickpea and pea seedlings (Çanakcý and Dursun, 2012; Faizan *et al.,* 2011; Popova *et al.*, 2009). However, pretreatment of SA significantly alleviated Cd toxicity on growth parameters. Salicylic acid is known to play a key role in regulating growth and

Table 1 : Growth parameters of *Cicer arietinum* L. seedlings under Cd toxicity, without (-) and with (+) SA (0.5 mM) pretreatment

Cd (µM)	SA pretreatment (0.5 mM)					
	Seedling length (%)		Seedling fresh weight (%)		Seedling dry weight (%)	
	-SA	+SA	-SA	+SA	-SA	+SA
Control	100±1.36^{a}	97.40±1.51^{ab}	100±4.63^{a}	94.91±3.06^{a}	100±5.71^{a}	98.29±4.55^{a}
25	97.21±1.49^{ab}	95.20±1.47$^{b¤}$	90.41±4.96^{ab}	89.52±3.10$^{b¤}$	97.71±6.24^{a}	95.72±3.66^{a}
50	91.59±1.42$^{bc¤}$	94.40±1.45$^{b¤}$	84.43±3.11$^{bc¤}$	85.92±2.19$^{b¤}$	88.88±6.92^{ab}	93.16±3.38^{a}
100	90.80±0.86$^{c¤}$	92.41±1.04$^{bc¤}$	80.83±2.57$^{c¤}$	84.73±2.52$^{bc¤}$	78.63±3.11$^{b¤}$	83.76±4.02$^{b¤}$

¤ Compared to the control group; at p<0.01- 0.05 probability levels. Values are mean of ten replicates ±SE

productivity in plants. The results of present study are in agreement with the earlier reports on the protective role of SA on Cd induced toxicity. Hayat *et al.* (2005) reported increase in the leaf number, fresh and dry weight per plant of wheat seedlings raised from pre-soaked grains in low concentration of SA.

Similarly, the growth promoting effect SA against Cd toxicity has been observed in maize, pea, barley, soyabean, wheat and *Brassica juncea* plants (Krantev *et al.*, 2008; Popova *et al.*,2009; Khan *et al*., 2003; Shakirova *et al*., 2003; Fariduddin *et al.,* 2003). Liu *et al.,* (2003) propounded that the inhibiting effect of Cd on plant growth may result due to its cytotoxic effect on cell division. In the present study, visible symptoms like chlorosis associated with pigment destruction and necrotic stains resulting from cell destruction in leaves was also observed due to Cd toxicity.

Similar to growth parameters, Cd treatment inhibited chlorophyll and carotenoid content in seedlings of *C. arietinum* in a dose dependent manner. While pretreatment of 0.5µM of SA ameliorated Cd toxicity in 50 and 100 µM Cd treated seedlings. Due to pretreatment, 6.26 % ($p<0.05$) to 6.33 % ($p\geq0.05$) increase in total chlorophyll content and 7.10 % to 6.11 % ($p\geq0.05$) increase in carotenoid content was found in 50 and 100 µM Cd treated seedlings as compared to Cd treatment (Table 2a). Reduction in photosynthetic pigments in response to Cd toxicity and its amelioration by exogenous application of SA is well documented in crop plants (Popova *et al*., 2009). Recently, Çanakci and Dursun (2012) in their study observed enhanced pigment content in tomato seedlings pretreated with SA as compared to Cd treated tomato seedlings. SA is supposed to increase the functional state of the photosynthetic apparatus in plants either by mobilization of internal tissue nitrate or chlorophyll biosynthesis.

Proline accumulation in plant tissues results due to increase in proline biosynthesis or decrease in proline degradation (Yoshiba *et al.,* 1997). Proline is known to accumulate in plants under various stress conditions (Vassilev and Lidon, 2011; Çanakci and Dursun, 2011). Although its functional mechanism is not quite known, it is suggested to be an adaptive response against abiotic stresses (Maggio *et al.*, 2002). The enhanced proline and MDA contents in Cd treated seedlings as compared to control seedlings in the present study indicated that these seedlings were under oxidative stress. On the other hand, exogenous application of SA was found to alleviate oxidative stress in Cd treated *Cicer* seedlings. It was found that maximum proline and MDA content decreased by 20.64 % and 24.83% in 50 µM Cd+SA treated seedlings as compared to 50 µM Cd-SA treatment (at $p<0.05$) (Table 2a and 2b). Similar results have been reported earlier in maize, barley, pea and rice plants (Krantev *et al.*, 2006/2008; Metwally *et al.*, 2003; Popova *et al.*, 2009; Guo *et al.*, 2009). Free radicals increasing with oxidative stress, increase lipid peroxidation in plant cells (Quariti *et al.*, 1997; Dietz *et al.*, 1999). This condition indicates that membrane integrity and membrane structure have been impaired.

ROS generated in cells are highly reactive in nature and destroy the normal cellular function and metabolism. Plants have various defense mechanisms by which they can scavenge these ROS. GSH is a non-enzymatic antioxidant that quenches increasing free radicals (ROS, H_2O_2 etc) in cells as a result of oxidative stress in plants (Mohan and Hosetti, 2006; Hasan *et al.*, 2009). In the present study, GSH content increased with increase in Cd treatment in *Cicer* seedlings and maximum GSH content was found in 100 µM Cd treatment. In a study conducted on sunflower plant, it was reported that reduced glutathione amount in leaves increased proportionally with increasing Cd concentration (Hatata *et al.*, 2008). SA pre-application increased GSH amount in roots and shoots against Cd toxic effect and alleviated oxidative damage due to low level of H_2O_2 and MDA in rice (Guo *et al.*, 2009). SA pre-application (50 µM Cd+SA) however, increased GSH content by 35.97% ($P<0.05$) (Table 2b). Antioxidant enzymes activities are stimulated by Cd treatment and was associated with changes in reduced glutathione contents (GSH) suggesting that Cd can be added to the list of stresses such as drought, heat shock, ozone and SO_2 (Hatata *et al.*, 2008). Also SA pre-treatment elevated non-enzymatic antioxidants and

Table 2(a) : Chlorophyll (a+b), carotenoid and proline content in leaves of *Cicer arietinum* L. seedling under Cd toxicity, without (-) and with (+) SA pretreatment

Cd (µM)	SA pretreatment (0.5 mM)					
	Chlorophyll (a+b) (mg g^{-1} f.wt.)		Carotenoid (mg g^{-1} f.wt.)		Proline (µmol g^{-1} f.wt.)	
	-SA	+SA	-SA	+SA	-SA	+SA
Control	2.877±0.11^{a}	2.693±0.14^{ab}	0.362±0.01^{a}	0.394±0.02^{a}	2.57±0.14^{a}	2.67±0.12^{a}
25	2.520±0.13$^{bc¤}$	2.443±0.18c$^{d¤}$	0.341±0.03^{abc}	0.335±0.05^{abc}	2.83±0.17^{ab}	2.53±0.13^{a}
50	2.236±0.06$^{def¤}$	2.376±0.06c$^{¤*}$	0.350±0.01^{ab}	0.375±0.01^{a}	4.02±0.22$^{d¤}$	3.19±0.41$^{bc¤*}$
100	2.053±0.07$^{f¤}$	2.183±0.16$^{ef¤}$	0.278±0.05$^{c¤}$	0.295±0.02$^{bc¤}$	3.99±0.12$^{d¤}$	3.40±0.50$^{cd¤}$

¤ Compared to the control group, *Compared to the associated group; at p<0.01- 0.05 probability levels. Values are mean of three replicates ±SE

Table 2(b) : MDA, GSH and FAME content in leaves of *Cicer arietinum* L. seedling under Cd toxicity, without (-) and with (+) SA pretreatment

Cd (µM)	SA pretreatment (0.5 mM)					
	MDA (nmol g^{-1} f.wt.)		GSH (µg g^{-1} f.wt.)		FAME (µmol g^{-1} f.wt.)	
	-SA	+SA	-SA	+SA	- SA	+SA
Control	51.97±8.77^{a}	53.85±8.62^{ab}	20.16±2.84^{a}	26.05±2.76$^{a¤}$	2.077±0.04^{a}	2.011±0.03^{ab}
25	68.17±7.47^{abcd}	61.91±5.07^{abc}	26.55±4.83^{ab}	33.10±4.92$^{b¤}$	2.022±0.02^{ab}	2.033±0.02^{a}
50	77.70±7.43$^{d¤}$	58.40±7.63^{ab*}	28.27±4.45$^{b¤}$	38.44±5.25$^{c¤*}$	2.013±0.03^{ab}	2.036±0.02^{a}
100	81.65±8.12$^{d¤}$	74.61±8.07$^{cd¤}$	31.49±5.48$^{b¤}$	34.16±4.00$^{b¤}$	1.980±0.03$^{b¤}$	1.928±0.03$^{bc¤}$

¤ Compared to the control group, *Compared to the associated group; at p<0.01- 0.05 probability levels. Values are mean of three replicates ±SE

concentrations of GSH in roots and shoots. Due to its membrane transmittance regulating and membrane integrity protecting characteristics in cells (El-Tayeb, 2005), SA may have reduced the toxic damage of Cd in chickpea seedlings. This increase in GSH levels is suggestive of SA playing a particular role in glutathione biosynthesis (Freeman *et al.*, 2005). Therefore, differences in glutathione amounts can be placed on an enzymatic basis (Yoshida *et al.*, 2009). Being able to logically discuss the increase of GSH amounts despite the toxic damage of Cd requires studies on all antioxidant enzymes. On contrary, Dixit *et al.,* (2001) reported that cadmium treatment decrease GSH content in roots and leaves of pea plant. In another study conducted on rice it was found that GSH content increased under low Cd concentrations and decreased at higher concentrations, as compared to roots of seedling pretreated with SA (Choudhury and Kumar, 2004).

In this study, the effect of free radicals on fatty acid composition in leaves of chickpea was investigated. The amount of fatty acid methyl esters (FAME) decreased significantly in Cd treated seedlings as compared to control seedlings. While pretreatment of SA enhanced the amount of FAME in 25 and 50 µM Cd treated seedlings of *Cicer arietinum* (Table 2b). Ivanova *et al.,* (2008) stated that oxidative stress caused decreases in FAME amounts in maize leaves.

The analysis of fatty acid composition in Cd treated seedlings of *Cicer s*howed significant changes (Table 3). As compared to control, Cd treatment decreased the percentage composition of unsaturated fatty acids (linolenic acid, linoleic acid and palmitoleic acid) with an exception to oleic acid that increased upto 50 µM Cd treatment and increased the amount of saturated fatty acids (palmitic and stearic acid). Pretreatment of SA however showed contrary results by decreasing the amount of palmitic and stearic acid and increasing the amount of palmitoleic , oleic, linolenic and linoleic acids (upto 50µM Cd) in Cd treated seedlings respectively.

In a study conducted on tomato seedlings, Quariti *et al.* (1997) reported that cadmium treatment affected membrane lipids by increasing palmitic acid (16:0) and decreasing linoleic acid (18:2) and linolenic acid (18:3). Further their study inferred that heavy metals altered the fatty acid desaturation processes. Moradkhani *et al.,* (2012) showed that SA application reduced the Cd effect on lipid unsaturation by increasing the percentage of linolineic acid and decreasing the percaentage of palmitic acid by same proportion in sunflower.

Popova *et al.* (2009) in their study showed that high concentrations of Cd application increased the linoleic acid (18:2) and decreased the linolenic acid (18:3) amount

Table 3 : Fatty acid content in leaves of *Cicer arietinum* L. seedling under Cd toxicity without (-) and with (+) SA (0.5mM) pretreatment (% weight of total)

Cd (µM)	SA pretreatment (0.5 mM)											
	Palmitic acid (16:0)		Palmitoleic acid (16:1)		Stearic acid (18:0)		Oleic acid (18:1)		Linoleic acid (18:2)		Linolenic acid (18:3)	
	-SA	+SA	-SA	+SA	-SA	+SA	-SA	+SA	-SA	+SA	-SA	+SA
0	11.56 ±0.52^{a}	11.71 ±0.22^{a}	2.943 ±0.67^{a}	2.416 ±0.80^{ab}	1.676 ±0.27^{a}	1.526 ±0.33^{a}	1.523 ±0.35^{a}	1.963 ±0.43^{a}	9.703 ±0.38^{a}	9.696 ±0.28^{a}	72.46 ±0.46^{a}	72.16 ±0.77^{ab}
25	12.32 ±0.24^{ab}	11.94 ±0.33^{ab}	2.683 ±0.58^{ab}	2.590 ±0.62^{ab}	1.910 ±0.49^{ab}	1.740 ±0.35^{ab}	1.773 ±0.37^{a}	2.189 ±0.50^{a}	9.196 ±0.34^{ab}	9.370 ±0.21^{a}	70.75 ±0.56$^{bc¤}$	70.96 ±1.10^{ab}
50	12.67 ±0.49$^{b¤}$	11.70 ±0.46^{a*}	2.046 ±0.37^{ab}	2.670 ±0.63^{ab}	2.226 ±0.51^{ab}	2.043 ±0.42^{ab}	1.753 ±0.26^{a}	2.106 ±0.55^{a}	8.936 ±0.29$^{b¤}$	9.070 ±0.43^{ab}	71.33 ±0.42$^{b¤}$	71.48 ±0.65^{ab}
100	13.75 ±0.46$^{c¤}$	12.62 ±0.53$^{b¤*}$	1.960 ±0.29b$^{¤}$	2.193 ±0.33^{ab}	2.138 ±0.14b$^{¤}$	1.780 ±0.56^{ab}	1.213 ±0.35^{a}	1.607 ±0.24^{a}	9.266 ±0.44^{ab}	8.933 ±0.44^{ab}	70.57 ±0.54$^{bc¤}$	71.98 ±0.45^{ab}

¤ Compared to the control group, * Compared to the associated group; at p<0.01 - 0.05 probability levels. Values are mean of three replicates ± SE

in pea plant. In another study on *Zea mays*, it was reported that as Cd concentration increased, saturated fatty acid (16:0, 18:0) amount in membranes increased and linolenic acid (18:3) amount decreased. In the same study, it was also reported that SA pre-application alleviated these changes in fatty acid composition and that fatty acid composition was not much effected by low Cd concentrations (Ivanova *et al.*, 2008).

The results of the current study suggest that pre-application of SA during the growth period may help the seedlings to avoid cumulative damage upon Cd exposure. SA may activate Cd tolerance mechanisms different from Cd distribution and antioxidant defence or would enhance repair processes. However, further studies with chickpea seedlings are needed to deepen insights into this topic

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