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Alleviation of cadmium toxicity in common bean (*Phaseolus vulgaris* L.) plants by the exogenous application of salicylic acid

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SUMMARY
A two-season pot experiment was conducted on *Phaseolus vulgaris* L. plants to evaluate their response to 1.0 mM salicylic acid (SA) in a growing medium contaminated with 0.25 or 0.50 mM Cd^{2+} ions. Plants were sampled for growth measurements and chemical analyses 45 d after sowing, and to measure the yield of beans at the end of each experiment. Exposing plants to either concentration of Cd^{2+} ions resulted in significant declines in growth, pigment concentrations, relative water content, and nutrient concentrations, and in chlorophyll fluorescence (F_v/F_m) and the performance index (PI) of photosynthesis. However, 1.0 mM SA mitigated Cd^{2+} ion stress and significantly improved each of these parameters. Both Cd^{2+} ion treatments increased proline and Cd^{2+} ion concentrations, electrolyte leakage, and lipid peroxidation (measured as malondialdehyde concentration). However, 1.0 mM SA attenuated the adverse effects of Cd^{2+} ions on these characteristics. Cd^{2+}-induced increases in the activities of several key anti-oxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase were reduced following the exogenous application of 1.0 mM SA. These results confirm that the application of 1.0 mM SA could be used to reduce the adverse effects of Cd^{2+} ion toxicity in bean plants.

Common bean (*Phaseolus vulgaris* L.) is one of the most popular leguminous vegetable crops grown in the Middle East. It acts as a rich source of proteins, carbohydrates, and nutrients in the human diet. Beans are widely cultivated on newly-reclaimed soils in the Middle East, including Egypt. However, newly-reclaimed soils require extensive fertiliser application, especially phosphate (P). When used extensively, the application of P fertilisers results in increased cadmium ion (Cd^{2+}) levels in the soil (McLaughlin *et al*., 2000). In addition, human activities and the mineralisation of natural rocks enriched with toxic metals, including Cd^{2+}, can lead to contamination by Cd^{2+} (Sanitadi *et al*., 1999). Cadmium (Cd^{2+}) ions are easily absorbed by roots and are frequently transported to other plant parts.

Like many other heavy metals, low concentrations of Cd^{2+} ions are highly toxic to cells in living organisms (Clemens *et al*., 1999). Since plants can easily absorb Cd^{2+} ions, these can enter the food chain and cause health disorders in humans. The biochemical activities of living plant cells are also easily affected by Cd^{2+} ions, leading to phytotoxicity. In previous studies, Cd^{2+} ions were shown to suppress growth, induce leaf and root necrosis, and leaf chlorosis (Hernandez and Cooke, 1997; Ahmad *et al*., 2011). They also perturbed chlorophyll metabolism (Baryla *et al*., 2001), inhibited photosynthesis and transpiration (Shi and Cai, 2008; Shi *et al*., 2010), caused imbalances in mineral nutrition (Ahmad *et al*., 2011), disturbed water homeostasis (Poschenrieder *et al*., 1989), induced oxidative stress (Sandalio *et al*., 2009), altered enzyme activities (Hasan *et al*., 2009), and modified gene expression (Herbette *et al*., 2006). Any of these effects can be used as a criterion for Cd^{2+} ion toxicity in plants (Ernst *et al*., 2000). Oxidative stress in plant cells can be caused by Cd^{2+} ion toxicity, because Cd^{2+} ions trigger the synthesis and accumulation of reactive oxygen species (ROS) which cause cellular damage and lipid peroxidation (Shah *et al*., 2001; Ahmad *et al*., 2011) and inhibit the activities of anti-oxidant enzymes involved in the oxidative defence system.

Salicylic acid (SA) is a plant hormone-like substance that has important roles in the regulation of plant growth and development, as well as in seed germination, fruit yield, glycolysis, and flowering (Klessig and Malamy, 1994; Abdou and Mohamed, 2014). In addition, SA affects ion uptake and transport (Harper and Balke, 1981), as well as the rates of photosynthesis, stomatal conductance, and transpiration (Khan *et al*., 2003). Signal molecules such as SA are involved in the expression of specific plant responses to biotic or abiotic stresses involving anti-oxidants. Many recent studies have shown that SA has a protective role against abiotic stresses such as salinity (Borsani *et al*., 2001; Semida and Rady, 2014), drought (Senaratna *et al*., 2000), and Cd^{2+} ion toxicity (Agami and Mohamed, 2013).

Many previous studies have shown that SA can mitigate the harmful effects of heavy metals on plants (Zhou *et al*., 2007; 2009). Drazic and Mihailovic (2005),

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Popova *et al*. (2009), Belkhadi *et al*. (2010), and Agami and Mohamed (2013) showed that the exogenous application of SA can alleviate Cd^{2+} ion toxicity in soybean, pea, flax, and wheat seedlings, respectively. SA-induced protection of plants from oxidative injury caused by heavy metals such as Cd^{2+} has been linked to the enhanced activities of key anti-oxidant enzymes (Wang *et al*., 2006). In addition, Ahmad *et al*. (2011) reported that Cd^{2+} ions increased levels of ROS and therefore activated anti-oxidant systems in mustard (*Brassica juncea* L.) plants and that the exogenous application of 1.0 mM SA alleviated the injurious effects of Cd^{2+} ion toxicity.

The objective of this study was to determine the effects of Cd^{2+} ion stress and the exogenous application of 1.0 mM SA on various morphological and physiological changes in common bean plants. The exogenous application of SA may be a strategy to increase plant tolerance to Cd^{2+} ions by regulating anti-oxidant defence systems and increasing the levels of key metabolites involved in stress tolerance.

MATERIALS AND METHODS

Plant material and growing conditions

Common bean (*Phaseolus vulgaris* L. 'Bronco') seeds were obtained from the Agricultural Research Center, Department of Vegetable Crops, Giza, Egypt. Healthy seeds were surface-sterilised in 5% (v/v) NaClO for 5 – 10 min. On 3 February 2012 and on 1 February 2013, three sterilised seeds were sown in each of 36, 40 cm-diameter plastic pots filled with acid-washed and rinsed sand. The pots were arranged in an open greenhouse with average day and night temperatures of 18º ± 3ºC and 10º ± 2ºC respectively. The relative humidity ranged from 60.4 – 65.2%, and day-length varied from 11 - 12 h.

Hoagland's nutrient solution (0.5× full-strength; Hoagland and Arnon, 1950) was supplied to all the pots for 7 d before the different cadmium ion (Cd^{2+}) treatments (0, 0.25, or 0.50 mM $CdCl_2$ in Hoagland's nutrient solution) were initiated and applied every 2 – 3 d, throughout the experiments. Salicylic acid (SA; 1.0 mM) was sprayed to run-off using a 2 l manual sprayer. The SA was mixed with 0.1% (v/v) Tween-20 as a surfactant and spreading agent, and was applied three times at 10, 24, and 38 d after sowing (DAS). The concentrations of $CdCl_2$ and SA, and the times of spraying, were based on results from preliminary studies (data not shown). Concentrations of $CdCl_2$ > 0.50 mM proved lethal to bean plants. Therefore, concentrations below the lethal limit (i.e., 0.25 and 0.50 mM) were used in these experiments.

The experiments were laid out in a completely randomised design with six replicates (i.e., each replicate = 1 pot). Plants were collected to measure all morphological, biochemical, and physiological factors at 45 DAS. At the end of the experiment (60 DAS), bean yields were measured.

Growth, bean yield, and water-use efficiency (WUE) measurements

At 45 DAS, the shoots and roots of six bean plants from each treatment were separated and washed in distilled water and their fresh weights (FWs) were measured. For dry weight (DW) determinations, each plant was dried to constant weight at 70ºC for 48 h, then weighed. At the end of experiment (60 DAS), the green bean pods on each plant (n = 6) were collected, counted, and weighed.

Water-use efficiency (WUE) values (in g of pods l^{-1} of water applied) were calculated for the different treatments at the end of each experiment, according to the following equation (Jensen, 1983):

$$WUE = \frac{Pod\ yield\ (\text{g pot}^{-1})}{Water\ applied\ (\text{l pot}^{-1})}$$

Salicylic acid (SA), cadmium ion (Cd^{2+}), and photosynthetic pigment concentrations

The SA concentration (in mg kg^{-1} FW) in the third fully-expanded leaf from the top of each plant in each replicate (pot) was determined following the methods of Siegrist *et al*. (2000) and Metwally *et al*. (2003) using an HPLC system equipped with a fluorescence detector (LC-2010 AHT; Shimadzu, Tokyo, Japan).

Powdered dried plant tissue samples (i.e., shoots, roots, and pods) were used to measure the concentrations of Cd^{2+} ions (in mg kg^{-1} DW) using an atomic absorption spectrophotometer (Model 3300; Perkin-Elmer, Akron, Ohio, USA; Chapman and Pratt, 1961).

Total chlorophyll and carotenoid concentrations (in mg g^{-1} FW) were measured and calculated according to Arnon (1949). Leaf discs (0.2 g from each replicate of each treatment) were homogenised in 50 ml of 80% (v/v) acetone and centrifuged at 10,000 × *g* for 10 min at room temperature. The absorbance of each acetone extract was measured at 663, 645, and 470 nm using a UV-160A UV-visible recording spectrometer (Shimadzu, Kyoto, Japan).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured on a sunny day using a portable fluorometer (Handy PEA; Hansatech Instruments Ltd., Kings Lynn, UK). One leaf of the same age was chosen on each of the six plants in each treatment. The fluorescence measurements made included the maximum quantum yield of PS II (F_v/F_m) calculated according to Maxwell and Johnson (2000) as follows:

$$F_v/F_m = (F_m - F_o) / F_m$$

The performance index (PI) of photosynthesis, based on equal absorption (PI_{ABS}), was calculated according to Clark *et al*. (2000).

Proline concentrations, relative water contents, and electrolyte leakage measurements

Proline concentrations in the main root and in the third fully-expanded leaf from the top of each plant (n = 6) from each treatment were measured by colourimetry according to Bates *et al*. (1973). Proline was extracted from 0.5 g DW of each leaf or root sample by grinding in 10 ml of 3% (w/v) sulphosalicylic acid. The mixture was then centrifuged at 10,000 × *g* for 10 min at room temperature. Two ml of the supernatant was added to a test tube and 2 ml of a freshly prepared acid-ninhydrin solution (Bates *et al*., 1973) was added. The tubes were

incubated in a water bath at 90ºC for 30 min and the reactions were terminated in an ice-bath. Each reaction mixture was extracted with 5 ml of toluene, vortex-mixed for 15 s, and allowed to stand for at least for 20 min in the dark at room temperature to allow separation of the toluene and aqueous phases. Each upper toluene phase was then carefully collected into a clean test tube and the absorbance of the toluene fraction was read at 520 nm. The proline concentration in each sample was determined using a standard curve based on analytical-grade proline, and was expressed in mg kg^{-1} DW.

The relative water content (RWC) of the third fully-expanded leaf from the top of each plant (n = 6) from each treatment was determined using five leaf discs (2 cm^2 each), excluding the midrib. The leaf discs of each plant were weighed quickly (FW) and immediately floated on double-distilled water in a Petri dish for 24 h in the dark to saturate them. Any adhering water was blotted and the turgid weight (TW) was recorded. The DW of the discs was then recorded after dehydrating them at 70ºC for 48 h. The RWC was then calculated using the following formula (Hayat *et al*., 2007):

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

The total concentration of inorganic ions that leaked out from each treated bean leaf sample was measured according to Dionisio-Sese and Tobita (1998). Eighteen leaf discs (three leaf discs per plant × six plants per treatment) were put in a boiling tube containing 10 ml of deionised water and the electrical conductivity was measured (EC_0). The tube was heated to 60ºC in a water bath for 25 min and the EC was measured again (EC_1). The tube contents were then boiled for 10 min and the EC was again recorded (EC_2). Electrolyte leakage was then calculated using the formula:

$$Electrolyte\ leakage\ (\%) = \frac{EC_1 \times EC_0}{EC_2 \times EC_0} \times 100$$

Lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2) measurements

Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA), according to Madhava Rao and Sresty (2000). A 0.5 g FW sample of the third fully-expanded leaf from the top of each plant (n = 6) from each treatment was homogenised in 2.5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at 15,000 × *g* for 10 min at 4ºC. Four ml of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added to every 1.0 ml of the supernatant. The mixture was then centrifuged for 15 min at 10,000 × *g* and the absorbance of the supernatant was read at 532 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate MDA concentrations.

H_2O_2 concentrations were determined at the National Research Center, Egypt, according to Velikova *et al.* (2000). A 0.5 g FW sample of the third fully-expanded leaf from the top of each plant (n = 6) from each treatment was homogenised in 5 ml of 0.1% (w/v) TCA. The extract was centrifuged at 18,000 × *g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer, pH 7.0 and 1.0 ml of 1 M KI solution. The absorbance of the supernatant was read at 390 nm.

Anti-oxidant enzyme assays

The third fully-expanded leaf from the top of each plant (n = 6) from each treatment was excised and weighed immediately. Each 1.0 g sample was ground with a pestle in an ice-cold mortar containing 10 ml of 50 mM phosphate buffer, pH 7.0. The homogenate was then centrifuged at 20,000 × *g* for 30 min at 4ºC. The supernatant was filtered through two layers of cheese-cloth and used to measure various anti-oxidant enzyme activities. The total protein concentration in each crude leaf extract was measured according to Bradford (1976).

Superoxide dismutase (SOD) activity was measured according to Dhindsa and Matowe (1981). Each 2.8 ml reaction mixture contained 0.2 ml of 200 mM methionine, 0.1 ml of the crude enzyme extract, 0.5 ml of 1 M Na_2CO_3, 0.5 ml of 2.25 mM nitroblue tetrazolium (NBT), 0.5 ml of 3 mM EDTA, 0.5 ml of 75 mM riboflavin, and 0.5 ml of double-distilled water, and was placed under a 15 W florescent lamp for 10 min at 25º – 28ºC. Blank A contained the same reaction mixture, but was kept in the dark. Blank B contained the same reaction mixture, but without the crude enzyme extract, and was placed in the light alongside the test samples. The reactions were terminated by turning-off the light. The absorbance of each sample, and Blank B, were read at 560 nm against Blank A, and the percentage difference in the reduction in the blue colour between Blank B and each sample was calculated. A 50% colour reduction was considered to be 1 enzyme Unit (EU) of SOD activity, and was expressed in EU mg^{-1} total soluble protein (TSP).

Catalase (CAT) activity was measured following the method of Aebi (1984). CAT activity was determined by measuring the loss of H_2O_2 by following the decline in absorbance at 240 nm. Each reaction was carried out in a final volume of 2 ml containing 1.7 ml of reaction buffer, 0.1 ml of 3 mM EDTA, 0.1 ml of crude enzyme extract, and 0.1 ml of 3 mM H_2O_2. The reaction was incubated at 20ºC for 10 min. CAT activity was calculated based on extinction coefficient (ϵ) for H_2O_2 of 0.036 mM^{-1} cm^{-1} at 240 nm, and expressed in EU mg^{-1} TSP. One EU of CAT activity was the amount needed to degrade 1 µmol of H_2O_2 min^{-1} at 25ºC.

The ascorbate peroxidase (APX) activity in leaves was determined according to Nakano and Asada (1981). Each 1.0 ml reaction mixture contained 0.6 ml of reaction buffer, 0.1 ml 0.5 mM of ascorbate, 0.1 ml of 0.1 mM H_2O_2, 0.1 ml of 0.1 mM EDTA, and 0.1 ml of crude leaf extract. APX activity was calculated based on the decrease in absorbance at 290 nm using an extinction coefficient for ascorbate of 2.8 mM^{-1} cm^{-1}, and was expressed in EU mg^{-1} TSP. One EU of APX activity was considered to be the amount needed to decompose 1 µmol of ascorbate min^{-1} at 25ºC.

Glutathione reductase (GR) activity was determined following the method of Foster and Hess (1980). Each 1.0 ml reaction mixture contained 0.1 ml of crude enzyme extract, 0.75 µl of 100 mM potassium phosphate buffer, pH 7.0, containing 1.0 mM EDTA, 150 µM NADPH, and 500 µM oxidised glutathione. GR activity was determined by measuring the decline in absorbance at 340 nm. An extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1} was used to determine GR activity which was expressed in µmol NADPH oxidised mg^{-1} TSP min^{-1}.

Determination of macro- and micro-nutrient concentrations

Shoot and root N concentrations (in mg g^{-1} DW) were measured according to Hafez and Mikkelsen (1981). An Orange-G dye (Sigma Chemical Co., St. Louis, MO, USA) solution was prepared by dissolving 1.0 g of 96% (w/w) assay-dye in 1.0 l of distilled water with 21.0 g citric acid and 2.5 ml of 10% (v/v) thymol in ethyl alcohol as an inhibitor of microbial growth. Each powdered plant sample (0.2 g) was placed in a centrifuge tube, 20 ml of the dye reagent was added, and the tube was shaken for 15 min. After filtration through a Whatman No. 1 filter paper, the solution was diluted 100-fold with distilled water and the absorbance was measured at 482 nm. N concentrations were calculated using the formulae:

$$N\ (\%) = 0.39 + 0.954 \times Dye\ absorbed\ (\text{g } 100\ \text{g}^{-1})$$

and

$$Dye\ absorbed\ (\text{g } 100\ \text{g}^{-1}) = (a - b / a)\ (cfv / w) \times 100$$

where a was the absorbance of the dye reagent at 482 nm without any plant material (the blank), b was the absorbance of the dye reagent solution at 482 nm with plant material, c was the concentration of the dye reagent (1.0 g l^{-1} distilled water), f was the purity factor of the dye reagent (96%), v was the volume of the dye reagent used for each sample (20 ml), and w was the weight of the dry powdered sample (0.2 g).

The dried powdered plant shoots and roots were also used to measure concentrations of other nutrients (P, K, Fe, Mn, and Zn) using a Perkin-Elmer Model 3300 atomic absorption spectrophotometer, according to Chapman and Pratt (1961).

Statistical analysis

Simple analysis of variance (ANOVA) was used to analyse the data. Significant differences between means were compared using Fisher's least-significant difference (LSD) test at a probability level of 95% ($P \leq 0.05$).

RESULTS

Effect of cadmium (Cd^{2+}) ions and salicylic acid (SA) on growth, bean yield, and leaf pigment concentrations

Exposure of common bean plants to either Cd^{2+} ion concentration resulted in a marked reduction in the FWs of both shoots and roots (Table I). Shoot FWs were reduced by 15.1% at 0.25 mM Cd^{2+}, and by 35.8% at 0.50 mM Cd^{2+} compared to the water-treated controls. Root FWs were reduced by 26.8% and 46.5% at 0.25 mM and 0.50 mM Cd^{2+}, respectively, compared to the water controls. The DWs of shoots and roots also showed significant declines under Cd^{2+} ion stress (11.1% and 32.1% in shoots, and 20.5% and 38.5% in roots at 0.25 mM and 0.50 mM Cd^{2+}, respectively). However, significant increases in shoot FWs and DWs were noticed following exogenously applied SA under both Cd^{2+} regimes. A 12.7% increase in shoot FW and a 15.8% increase in root FW were observed following the application of 1.0 mM SA plus 0.50 mM Cd^{2+} compared to 0.50 mM Cd^{2+} alone. Shoot and root DWs also showed 7.3% and 16.7% increases, respectively, at the same concentrations of Cd^{2+} plus SA compared to Cd^{2+} alone.

The same trend was observed for bean pod yields (Table I). The numbers of pods pot^{-1} and pod yields pot^{-1} decreased by 14.0% and 15.3%, respectively, under 0.25 mM Cd^{2+} ion stress, and declined by 35.1% and 42.1%, respectively, under 0.50 mM Cd^{2+} ion stress compared to the water-only controls. However, 1.0 mM SA increased these yields by 32.4% and 61.5% compared to 0.50 mM Cd^{2+} alone.

The inhibition of bean plant growth following the application of 0.25 or 0.50 mM Cd^{2+} was associated with a significant reduction in total chlorophyll concentration (Table II), particularly at 0.50 mM Cd^{2+}. However, exogenous application of 1.0 mM SA alleviated this harmful effect of Cd^{2+} ions. Carotenoid concentrations were also reduced by 0.25 mM Cd^{2+} ion stress compared to the water-only controls. Appling 1.0 mM SA also ameliorated the negative effect of Cd^{2+} ions on carotenoid concentrations.

Effect of Cd^{2+} ions and SA on free proline concentrations, water-use efficiency (WUE), relative water content (RWC), electrolyte leakage (EL), lipid peroxidation (MDA), and hydrogen peroxide (H_2O_2) concentrations

Exposure of common bean plants to 0.25 or 0.50 mM Cd^{2+} increased proline concentrations (Table II). The maximum increases in proline concentrations were 89.6% and 97.2% in roots and leaves, respectively, in 0.50 mM Cd^{2+} compared to the water-only controls. However, the exogenous application of 1.0 mM SA increased root and leaf proline concentrations by 120.8% and 138.8%, respectively, in common bean plants exposed to 0.50 mM Cd^{2+}.

Exposing common bean plants to 0.25 or 0.50 mM Cd^{2+} significantly reduced their WUE (Table III). Exogenous SA treatment of bean plants, in the absence of Cd^{2+} ion stress, significantly increased their WUE compared to the water-only controls. The maximum reduction in WUE was 40.7% under 0.50 mM Cd^{2+}. However, the application of 1.0 mM SA increased the WUE by 50% compared to bean plants exposed to 0.50 mM Cd^{2+} alone.

RWC values decreased in common bean plants exposed to Cd^{2+} ion stress, particularly 0.50 mM Cd^{2+} (Table III). However, the application of 1.0 mM SA

TABLE I

*Effect of 1.0 mM salicylic acid (SA) on selected growth traits [shoot and root fresh weights and dry weights (g $plant^{-1}$)], and yield [number of pods pot^{-1} and pod yield (g) pot^{-1}] in bean (*Phaseolus vulgaris *L. 'Bronco') plants grown under cadmium ion stress*

Treatment (mM)	Shoot FW	Shoot DW	Root FW	Root DW	Number of pods pot^{-1}	Pod yield (g pot^{-1})
Control (0 Cd)	15.9 ± 1.2b†	8.1 ± 0.6a	7.1 ± 0.5b	3.9 ± 0.2a	11.4 ± 0.9b	40.4 ± 2.9b
SA (1.0) alone	17.1 ± 1.4a	8.4 ± 0.8a	8.0 ± 0.5a	4.1 ± 0.2a	12.2 ± 1.1a	45.9 ± 3.3a
0.25 Cd^{2+}	13.5 ± 1.2d	7.2 ± 0.6c	5.2 ± 0.3d	3.1 ± 0.2c	9.8 ± 0.9c	34.2 ± 2.4d
0.25 Cd^{2+} + SA	14.5 ± 1.1c	7.8 ± 0.5b	5.9 ± 0.4c	3.6 ± 0.3b	11.1 ± 0.9b	41.6 ± 3.1b
0.50 Cd^{2+}	10.2 ± 0.8f	5.5 ± 0.4d	3.8 ± 0.3f	2.4 ± 0.2e	7.4 ± 0.5d	23.4 ± 2.0e
0.50 Cd^{2+} + SA	11.5 ± 1.0e	5.9 ± 0.4d	4.4 ± 0.3e	2.8 ± 0.2d	9.8 ± 0.8c	37.8 ± 2.7c

†Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case letter are significantly different by Fisher's least-significant difference test (LSD) at $P \leq 0.05$.

TABLE II

*Effect of 1.0 mM salicylic acid (SA) on photosynthetic pigment concentrations [total chlorophyll and carotenoid (mg g^{-1} FW)] in leaves and roots and leaf proline concentrations (mg kg^{-1} DW) of bean (*Phaseolus vulgaris *L. 'Bronco') plants grown under cadmium ion stress*

Treatment (mM)	Total chlorophyll conc.	Carotenoids conc.	Root proline conc.	Leaf proline conc.
Control (0 Cd)	1.24 ± 0.09a[†]	0.32 ± 0.02c	44.3 ± 1.4e	38.7 ± 1.2e
SA (1.0) alone	1.21 ± 0.08a	0.39 ± 0.02b	63.9 ± 2.1d	56.8 ± 1.8d
0.25 Cd^{2+}	1.19 ± 0.09a	0.24 ± 0.01d	44.8 ± 1.5e	39.2 ± 1.4e
0.25 Cd^{2+} + SA	1.25 ± 0.08a	0.33 ± 0.02c	75.7 ± 2.6c	68.8 ± 2.1c
0.50 Cd^{2+}	0.89 ± 0.06b	0.40 ± 0.03b	84.0 ± 2.9b	76.3 ± 2.5b
0.50 Cd^{2+} + SA	1.15 ± 0.07a	0.44 ± 0.03a	97.8 ± 3.3a	92.4 ± 2.8a

[†]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case letter are significantly different by Fisher's least-significant difference test (LSD) at $P \leq 0.05$.

attenuated the adverse effects of Cd^{2+} stress on RWC.

Electrolyte leakage (EL) increased significantly under both Cd^{2+} treatments, while the application of 1.0 mM SA application prevented this Cd^{2+}-induced rise in EL (Table III).

MDA concentrations increased by 25.7% under 0.25 mM Cd^{2+} stress, and by 74.3% under 0.50 mM Cd^{2+} stress compared to the water-only controls. This was reduced by 15.9% and 16.4%, respectively, following the application of 1.0 mM SA (Table III).

H_2O_2 concentrations showed 50.3% and 104.2% increases under 0.25 mM and 0.50 mM Cd^{2+}, respectively, compared to the water-only controls. However, exogenous application of 1.0 mM SA caused reductions of 21.5% and 30.3% compared to the water-only controls, respectively (Table III).

Effect of Cd^{2+} ions and SA on endogenous Cd^{2+} and SA concentrations, and on chlorophyll fluorescence

Although SA was found at low concentrations in the leaves of control plants (Table IV), exposure of common bean plants to 0.25 or 0.50 mM Cd^{2+} significantly increased the endogenous level of SA, particularly in 0.50 mM Cd^{2+}-treated plants. However, exogenously-applied 1.0 mM SA significantly reduced endogenous SA concentrations in Cd^{2+}-treated plants.

Higher concentrations of Cd^{2+} ions were found in bean roots than in shoots and pods (Table IV). Cd^{2+} was not detected in roots, shoots, and pod tissues in the Cd^{2+}-free growth medium. In contrast, significant increases in Cd^{2+} ion accumulation were observed in plants treated with 0.25 mM or 0.50 mM Cd^{2+}. Cd^{2+} treated-plants also treated with exogenous SA showed a significant reduction in Cd^{2+} ion concentrations in both shoots and roots, and further reductions in their pods.

Significant increases of the maximum quantum yield of photosynthesis (F_v/F_m) and in the photosynthetic performance index (PI) were found in SA- and in SA plus Cd^{2+}-treated plants compared to Cd^{2+}-only treated plants (Table IV).

Effect of Cd^{2+} ions and SA on anti-oxidant enzyme activities

Table V shows that SOD activity increased with an increase in Cd^{2+} concentration in the growth medium. SOD activity increased by 18.7% and by 30.9% under 0.25 mM and 0.50 mM Cd^{2+} stress, respectively, compared to the water-only controls. However, the exogenous application of 1.0 mM SA caused significant reductions (15.1% and 5.0%, respectively) in these rises in SOD activity in Cd^{2+}-treated plants.

CAT activity was reduced under Cd^{2+} ion stress. However, the exogenous application of 1.0 mM SA reduced the decline in CAT activity by 14.1% in 0.50 mM Cd^{2+}-treated bean plants.

APX activity increased under Cd^{2+} ion stress compared to the water-treated controls. The maximum APX activity was observed under 0.50 mM Cd^{2+} stress. The application of 1.0 mM SA reduced the increase in activity of APX in Cd^{2+}-treated plants by 15.4% under 0.25 mM Cd^{2+} ion stress.

Glutathione reductase activity also increased under Cd^{2+} stress, by 17.0% in 0.50 mM Cd^{2+}-treated plants; however, exogenous 1.0 mM SA reduced the increase in GR activity in all Cd^{2+}-treated bean plants.

Effect of Cd^{2+} ions and SA on macro- and micro-nutrient concentrations

Common bean plants exposed to either Cd^{2+} ion concentration showed a marked reduction in shoot and root concentrations of N, P, K, Fe, Mn, and Zn (Table VI). N concentrations declined with an increase in Cd^{2+} stress (i.e., 14.0% under 0.25 mM Cd^{2+} and 32.6% under 0.50 mM Cd^{2+} in shoots; 6.5% under 0.25 mM Cd^{2+} and 21.5% under 0.50 mM Cd^{2+} in roots) compared to the water-only controls. However, the exogenous application of 1.0 mM SA restored N concentrations in both shoots and roots.

Concentrations of P and K also decreased by 50.0% and 37.8% in shoots, and by 52.4% and 39.8% in roots, respectively, under 0.50 mM Cd^{2+} compared to the water-

TABLE III

*Effect of 1.0 mM salicylic acid (SA) on water-use efficiency [WUE (g pods l^{-1} applied water)], relative water content (RWC; %), electrolyte leakage (EL; %), malondialdehyde (MDA) concentration (mmol g^{-1} FW), and hydrogen peroxide (H_2O_2) concentration (µmol g^{-1} DW) in bean (*Phaseolus vulgaris *L. 'Bronco') plants grown under ion cadmium stress*

Treatment (mM)	WUE	RWC (%)	EL (%)	MDA	H_2O_2
Control (0 Cd)	0.81 ± 0.04b[†]	76.8 ± 2.3a	8.4 ± 0.2d	3.5 ± 0.1d	1.89 ± 0.08d
SA (1.0) alone	0.92 ± 0.04a	70.4 ± 2.4b	9.8 ± 0.3c	3.7 ± 0.1d	1.32 ± 0.05e
0.25 Cd^{2+}	0.69 ± 0.02c	67.2 ± 2.2b	11.3 ± 0.3b	4.4 ± 0.2c	2.84 ± 0.10b
0.25 Cd^{2+} + SA	0.82 ± 0.03b	74.9 ± 2.4a	10.1 ± 0.2c	3.7 ± 0.1d	2.23 ± 0.11c
0.50 Cd^{2+}	0.48 ± 0.02d	48.7 ± 1.3d	14.3 ± 0.4a	6.1 ± 0.2a	3.86 ± 0.15a
0.50 Cd^{2+} + SA	0.72 ± 0.03c	56.6 ± 1.6c	11.8 ± 0.3b	5.1 ± 0.2b	2.69 ± 0.12b

[†]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case letter are significantly different by Fisher's least-significant difference test (LSD) at $P \leq 0.05$.

TABLE IV

*Effect of 1.0 mM salicylic acid (SA) on shoot, root and, pod cadmium ion (Cd^{2+}) concentrations (mg kg^{-1} DW), leaf SA (mg kg^{-1} FW), chlorophyll fluorescence (F_v/F_m) and the photosynthetic performance index (PI) of bean (*Phaseolus vulgaris *L. 'Bronco') plants grown under cadmium ion stress*

Treatment (mM)	Leaf Cd^{2+}	Root Cd^{2+}	Pod Cd^{2+}	SA	F_v/F_m	PI
Control (0 Cd)	ND*	ND	ND	24.8 ± 0.9e	0.52 ± 0.03b	0.34 ± 0.02d
SA (1.0) alone	ND	ND	ND	27.9 ± 1.2e	0.74 ± 0.05a	2.36 ± 0.11a
0.25 Cd^{2+}	6.7 ± 0.2c†	14.5 ± 0.4b	6.2 ± 0.2b	45.8 ± 1.9c	0.30 ± 0.01c	ND
0.25 Cd^{2+} + SA	4.5 ± 0.1d	8.0 ± 0.2c	3.9 ± 0.1c	38.2 ± 1.6d	0.71 ± 0.04a	1.92 ± 0.10b
0.50 Cd^{2+}	10.9 ± 0.4a	19.3 ± 0.4a	9.0 ± 0.2a	87.3 ± 2.7a	0.10 ± 0.01d	ND
0.50 Cd^{2+} + SA	8.4 ± 0.3b	15.3 ± 0.4b	4.6 ± 0.1c	52.4 ± 2.1b	0.56 ± 0.04b	1.21 ± 0.08c

†Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case letter are significantly different by Fisher's least-significant difference test (LSD) at $P \leq 0.05$.
*ND, not detectable.

only controls. The application of 1.0 mM SA helped restore concentrations of these elements in plants.

Significant reductions were also found in Fe, Mn, and Zn concentrations in the shoots and roots of bean plants at either Cd^{2+} ion concentration. These reductions were more pronounced under 0.50 mM Cd^{2+} stress. However, significant restorations in these nutrients concentrations were observed following the application of 1.0 mM SA.

DISCUSSION

The addition of Cd^{2+} ions to the growth medium, particularly 0.50 mM Cd^{2+}, resulted in marked reductions in the FWs and DWs of both shoots and roots of common bean plants. Inhibited growth was associated with Cd^{2+}-induced reductions in the concentrations of chlorophyll (Table II), relative water contents (Table III), and nutrient levels (Table VI). The reduction in growth caused by Cd^{2+} stress could have been due to inhibition of cell division and the rate of elongation of cells, which occur mainly by irreversible inhibition of the proton pump responsible for these processes (Liu *et al.*, 2004). In addition, the reduction in chlorophyll concentration by Cd^{2+} treatment is considered one reason for growth reduction in bean plants. The reduction in chlorophyll content may be attributed to increased activity of the chlorophyll-degrading enzyme, chlorophyllase under Cd^{2+} stress conditions (Reddy and Vora, 1986).

Cd^{2+} ion treatment reduced plant DWs by 54% in *P. vulgaris* (Rady, 2011). As in our study, the ameliorative effect of exogenous SA on bean plants grown under Cd^{2+} ion stress has been reported in different crop plants under abiotic stress conditions. This has been attributed to the positive roles of SA in nutrient uptake (Ahmad *et al.*, 2011), water relations (Agami and Mohamed, 2013), stomatal regulation (Arfan *et al.*, 2007), photosynthetic capacity and growth (Popova *et al.*, 2009), and anti-oxidant systems (Ahmad *et al.*, 2011; Agami and Mohamed, 2013). In the present study, the increased tolerance to Cd^{2+} stress appeared as improved growth, higher concentrations of photosynthetic pigments, and subsequent yields (Table I). This could have been due to Cd^{2+} being retained in the roots and only a small amount of Cd^{2+} being transported to the shoots (Caltado *et al.*, 1983).

The application of SA increased the RWC, resulting in increased WUE (Table III). Choudhury and Panda (2004) reported that Cd^{2+} concentrations in SA-primed rice roots were lower than in non-SA-primed roots. They also showed that an SA-induced differential accumulation of Cd^{2+} was one of the potential physiological effects of SA on plants.

The F_v/F_m ratio and PI were used as non-invasive measures to determine the functionality of the photosynthetic machinery. F_v/F_m and PI were significantly reduced by Cd^{2+} ion stress; however the exogenous application of SA significantly improved these parameters in the leaves of Cd^{2+}-treated plants (Table IV). The lowest F_v/F_m ratio (0.21) was observed in the leaves of bean plants treated with 0.50 mM Cd^{2+} alone, while the highest F_v/F_m ratio (0.84) was observed in the leaves of plants treated with SA alone. F_v/F_m values in SA plus Cd^{2+}-treated plants was significantly higher than in Cd^{2+}-only treated plants, indicating that SA reduced the level of Cd^{2+}-induced photo-inhibition by protecting photo-system II. The F_v/F_m ratio has been correlated with the efficiency of photosynthesis. A reduction in this ratio provided an indicator of the extent of photo-inhibitory damage caused by the incident photon flux density when plants were subjected to a wide range of environmental stresses (Bjorkman and Demming, 1987).

The production of H_2O_2 increased markedly in bean plants after Cd^{2+} treatment. Increased H_2O_2 concentrations due to Cd^{2+} have also been reported in other species (Kuo and Kao, 2004; Ahmad *et al.*, 2011). In this study, the application of SA reduced the

TABLE V

*Effect of 1.0 mM salicylic acid (SA) on leaf superoxide dismutase (SOD; EU mg^{-1} protein), catalase (CAT; EU‡ mg^{-1} protein), ascorbate peroxidase (APX; EU mg^{-1} protein), and glutathione reductase (GR; EU mg^{-1} protein) activities in bean (*Phaseolus vulgaris *L. 'Bronco') plants grown under cadmium ion stress*

Treatment (mM)	SOD	CAT	APX	GR
Control (0 Cd)	123 ± 6c†	173 ± 11a	5.0 ± 0.2b	5.3 ± 0.3c
SA (1.0) alone	129 ± 8c	160 ± 10b	4.9 ± 0.2b	5.2 ± 0.2c
0.25 Cd^{2+}	146 ± 7b	149 ± 8c	5.2 ± 0.1b	5.7 ± 0.3b
0.25 Cd^{2+} + SA	124 ± 7c	139 ± 8d	4.4 ± 0.1c	4.7 ± 0.1d
0.50 Cd^{2+}	161 ± 9a	128 ± 7e	5.8 ± 0.3a	6.2 ± 0.4a
0.50 Cd^{2+} + SA	153 ± 8ab	110 ± 5f	5.3 ± 0.2b	5.8 ± 0.3b

†Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case letter are significantly different by Fisher's least-significant difference test (LSD) at $P \leq 0.05$.
‡EU, enzyme units (see Materials and Methods section).

Table VI
*Effect of 1.0 mM salicylic acid (SA) on selected macro- and micro-nutrient concentrations (mg kg^{-1} DW) in the shoots and roots of bean (*Phaseolus vulgaris *L. 'Bronco') plants grown under cadmium ion stress*

Tissue/Treatment (mM)	N	P	K	Fe	Mn	Zn
Shoots						
Control (0 Cd)	2.36 ± 0.17a†	0.48 ± 0.02a	2.67 ± 0.12a	313 ± 24a	204 ± 14a	85 ± 4a
SA (1.0) alone	2.23 ± 0.12a	0.49 ± 0.03a	2.61 ± 0.10a	293 ± 21a	215 ± 17a	66 ± 4b
0.25 Cd^{2+}	2.03 ± 0.10b	0.36 ± 0.02c	2.36 ± 0.08a	212 ± 15c	164 ± 12b	44 ± 3d
0.25 Cd^{2+} + SA	2.31 ± 0.11a	0.43 ± 0.01b	2.59 ± 0.11a	243 ± 20b	214 ± 15a	65 ± 4b
0.50 Cd^{2+}	1.59 ± 0.08c	0.24 ± 0.01d	1.66 ± 0.06c	141 ± 13e	104 ± 7c	23 ± 2e
0.50 Cd^{2+} + SA	2.04 ± 0.11b	0.37 ± 0.02c	2.03 ± 0.09b	182 ± 11d	163 ± 10b	54 ± 3c
Roots						
Control (0 Cd)	2.14 ± 0.09a	0.63 ± 0.04a	2.94 ± 0.14a	364 ± 28a	283 ± 19a	115 ± 5a
SA (1.0) alone	2.08 ± 0.10a	0.61 ± 0.03ab	2.86 ± 0.13a	373 ± 26a	263 ± 16a	116 ± 7a
0.25 Cd^{2+}	2.00 ± 0.08a	0.54 ± 0.03c	2.59 ± 0.09bc	282 ± 19c	232 ± 12b	86 ± 3b
0.25 Cd^{2+} + SA	2.16 ± 0.11a	0.59 ± 0.02b	2.81 ± 0.10ab	313 ± 26b	264 ± 18a	94 ± 4b
0.50 Cd^{2+}	1.68 ± 0.08b	0.30 ± 0.01e	1.77 ± 0.07d	182 ± 16e	153 ± 11c	53 ± 2d
0.50 Cd^{2+} + SA	1.96 ± 0.09a	0.48 ± 0.02d	2.37 ± 0.10c	252 ± 22d	223 ± 14b	74 ± 3c

†Values are means ± SE (n = 6). Mean values in each column followed by different lower-case letters for each tissue are significantly different by Fisher's least-significant difference test (LSD) at $P \leq 0.05$.

concentration of H_2O_2 and reduced Cd^{2+}-induced oxidative injuries in bean plants. This may have been due to the effect of SA as an anti-oxidant, counteracting the generation of H_2O_2 under Cd^{2+} stress (Ahmad *et al*., 2011).

The accumulation of MDA, a product of lipid peroxidation, is commonly used as an indicator of oxidative stress. Monteiro *et al*. (2009) and Ahmad *et al*. (2011) observed increases in MDA concentrations due to Cd^{2+} stress in lettuce and in mustard. Zhang *et al*. (2007) also demonstrated increased MDA concentrations in the leaves of *Bruguiera gymnorrhiza* exposed to a variety of heavy metals, and suggested lipid peroxidation as a biomarker for metal stress. Our data showed that the Cd^{2+}-induced increase in MDA concentration in bean plants could be reduced by SA application (Table III).

Oxidative stress induced by Cd^{2+} ions led to increased activities of several anti-oxidant enzymes in plants (Ahmad *et al*., 2011). Increases in the activities of SOD, APX, and GR followed the application of Cd^{2+} in *Bacopa monnieri* (Mishra *et al*., 2006), *Triticum aestivum* (Khan *et al*., 2007), *P. vulgaris* (Rady, 2011), and *Triticum aestivum* (Agami and Mohamed, 2013), whereas CAT activities declined in *B. monnieri* (Mishra *et al*., 2006), *Phragmites australis* (Iannelli *et al*., 2002), and *P. vulgaris* (Rady, 2011).

In this study, Cd^{2+} ions increased the activities of all anti-oxidant enzymes except CAT, while the exogenous application of SA reduced the activities of all four anti-oxidant enzymes tested in Cd^{2+}-treated plants. SA is an iron-chelating molecule that can directly scavenge hydroxyl radicals (Dinis *et al*., 1994). Cd^{2+} ions induced higher levels of SA in bean plants which may have functioned as anti-oxidants to reduce levels of ROS. Guo *et al*. (2007) reported that exogenously-applied SA increased the Cd^{2+} tolerance of rice by increasing the activities of enzymes involved in the anti-oxidant defence system. Furthermore, Senaratna *et al*. (2000) reported that SA could induce anti-oxidant activity under various stresses. Mba *et al*. (2007) observed that the activity of SOD increased in cabbage by increasing the external concentration of Cd^{2+}, but addition of SA to the culture medium reduced the activity of SOD. Glutathione reductase is known to catalyse several vital steps in the ascorbate-glutathione cycle. It maintains a high GSH:GSSG ratio, which is required for the regeneration of ascorbate and for the activation of a number of enzymes involved in CO_2-fixation (Noctor and Foyer, 1998). GR activity increased following Cd^{2+} treatment in our study, but was reduced by the application of SA.

In this study, the application of Cd^{2+} caused a marked increase in endogenous SA concentrations in the leaves of bean plants. A similar Cd^{2+}-induced increase in SA has been reported in pea and mustard (Popova *et al*., 2009; Ahmad *et al*., 2011). Exogenous application of SA to non-Cd^{2+} treated bean plants increased their endogenous SA content; however, applying SA to Cd^{2+}-treated plants resulted in a decreased accumulation of endogenous SA.

The beneficial effects of exogenous SA appeared as positive changes in many biochemical parameters. Proline concentrations increased markedly in Cd^{2+}-treated bean plants, and the application of SA further increased the levels of proline in these plants. Proline accumulation is an indicator of stress tolerance (Ashraf and Foolad, 2007). Under stress conditions, proline acted as an osmoprotectant (Hartzendorf and Rolletschek, 2001), a membrane stabiliser (Bandurska, 2001), and a scavenger of ROS (Matysik *et al*., 2002). The increased activities of anti-oxidant enzymes and increased proline concentrations resulted in an increase in the ability of bean plants to tolerate Cd^{2+} stress in this study. Increased accumulations of proline in response to Cd^{2+} toxicity have been reported in *T. aestivum*, *Vigna radiate*, *Helianthus annuus*, and *P. vulgaris* (Dhir *et al*., 2004; Zengin and Munzuroglu, 2006; Rady, 2011; Agami and Mohamed, 2013).

The application of Cd^{2+} led to a deficiency in macro- and micro-nutrients in bean plants, which may have caused other changes in plant metabolism. Cd^{2+} ions significantly disturb ionic homeostasis, but the application of SA alleviated this. The Cd^{2+}-induced decrease in K concentration may be attributed to decreased K^+ ion uptake, caused by the antagonistic effect of Cd^{2+} ions (Murphy *et al*., 1999). N, P, Fe, Mn, and Zn concentrations in the leaves of bean plants declined under Cd^{2+} stress, but exogenous application of SA mitigated the negative effects of Cd^{2+} on these mineral nutrients. Similar results have been reported previously (Larbi *et al*., 2002; Ramos *et al*., 2002; Ahmad *et al*., 2011).

CONCLUSIONS

Exogenous 1.0 mM salicylic acid (SA) increased the specific activities of several key anti-oxidant enzymes

(superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase) and the concentrations of several non-enzymatic anti-oxidants (carotenoids, proline, and SA itself) in common bean plants grown under 0.25 mM or 0.50 mM Cd^{2+} ion stress, as well as under Cd^{2+} stress-free conditions. However, the effect of exogenous 1.0 mM SA on plant anti-oxidant systems was greater under Cd^{2+} ion stress, suggesting that this increased anti-oxidant activity may be responsible, at least in part, for the greater tolerance of SA-treated bean plants to Cd^{2+} stress, by protecting the photosynthetic machinery, leading to improved plant growth.

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