



Supplementation of Salicylic Acid and Citric Acid for Alleviation of Cadmium Toxicity to *Brassica juncea*

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Received: 11 July 2018 / Accepted: 15 July 2019 / Published online: 24 July 2019
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Abstract

The reported study investigated the interaction between salicylic acid (SA) and citric acid (CA) in cadmium (Cd)-stressed *Brassica juncea* plants. Seedling received Cd (0.6 mM) stress through soil at 5-day stage of growth. SA (0.01 mM) and CA (0.6 mM) treatments were applied at 25 days after sowing. Growth, photosynthesis, oxidative burst, and antioxidant systems were examined at 30-day stage of growth. Growth and photosynthetic parameters reduced significantly in the presence of Cd, and elevated levels of H₂O₂ were indicative of oxidative burst which resulted in decline of cell viability. Foliar spray of SA and CA alone or in combination mitigated the toxic effects generated by Cd and enhanced plant growth parameters. The inhibitory effects of Cd toxicity on width of stomatal pore resulted in reduced internal CO₂ concentration and carbonic anhydrase activity which consequently limited the photosynthetic rate. SA and CA alleviated the inhibitory effect of Cd on photosynthesis by stimulating the stomatal activity and pore size. The Cd-generated oxidative burst was reduced via enhanced antioxidant activity (catalase, peroxidase, and superoxide dismutase) upon follow-up treatment with SA and CA alone or in combination. A combined dose of SA and CA countered Cd-induced damage by reducing levels of reactive oxygen species and strengthening plant antioxidant defense systems, which resulted in membrane stabilization and recovery from stress. Combined dose of SA and CA proved more effective than their individual application towards Cd stress which suggests an effective synergism between the two acids.

Keywords Cadmium · Citric acid · Salicylic acid · Chlorophyll · Catalase

Introduction

Rapidly increasing population, urbanization, industrial discharges, and use of chemical fertilizers in agriculture have contaminated the soil environment. Heavy metals are among the most significant threats to plant growth and development due to their non-biodegradability. It is, therefore, essential to maintain a healthy soil environment for plant growth. Cadmium (Cd), lead, chromium, and mercury are among the most notorious soil and water pollutants and their concentrations are increasing as a result of anthropogenic activities

including mining and smelting operations, and use of metals and metal-containing compounds for domestic as well as agricultural purposes (Nagajyoti et al. 2010). Heavy metals occur in the natural environment, albeit in trace quantities. Increased concentrations above certain levels results in serious adverse effects on plant physiology and productivity (Mahmud et al. 2018). Cd is one of the most toxic heavy metals which enters agricultural fields via excessive use of phosphatic fertilizers, uncontrolled releases from industry, and by land application of Cd-enriched wastewater treatment solids (Murtaza et al. 2015). Cd is readily transferred through the food chain due to its high mobility (Gallego et al. 2012). Humans may ultimately become exposed to Cd as a result of uptake by crop plants (Clemens et al. 2013).

Specific effects of Cd to plants include reduced nutrient uptake, decreased photosynthetic activity, altered nitrogen and sulfur metabolism, and changes in levels of antioxidant enzymes (Benavides et al. 2005; Gill and Tuteja 2011; Zong et al. 2017; Pereira de Araújo et al. 2017). Cd induces an increase in levels of reactive oxygen species (ROS) by

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00344-019-10007-0>) contains supplementary material, which is available to authorized users.

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disrupting the electron transport chain (Wang et al. 2004). Chlorophyll is denatured, which disrupts enzymes of the Calvin cycle, ultimately decreasing the rates of both photosynthesis and gas exchange (Silveira et al. 2015).

Salicylic acid (SA) acts as a plant growth regulator and stress messenger (Ghazijahani et al. 2014). The role of SA in mitigating biotic stress is well established. SA induces a hypersensitive response within the plant upon encountering an invading entity. The response acts as a secondary messenger and increases the resistance in plants to external stimuli (Klessig and Malamy 1994). The protective role of SA against abiotic stress has been reported in various plants (Gondor et al. 2016; Kohli et al. 2018a, b, 2019). Metwally et al. (2003) reported SA-mediated alleviation of Cd toxicity in barley seedlings. SA provides stress tolerance in plants by inducing the expression of genes that encode defense-related compounds like jasmonic acid (JA) and proline (Wani et al. 2017).

Citric acid (CA) is an intermediate product formed during tricarboxylic acid cycle that supplies energy to cells during respiration and in many other biochemical pathways (Hu et al. 2016). Various reports suggest that CA plays a role during phytoremediation of several heavy metals—it detoxifies metal-induced oxidative stresses in the plant by improving antioxidant capabilities (Gao et al. 2010; Yeh et al. 2012; Vagner et al. 2013). Involvement of CA in aluminum detoxification, iron-generated stress, and tolerance towards heavy metal and salinity stress has been reported (Ma and Furukawa 2003; Shlizerman et al. 2007; Gao et al. 2010; Sun and Hong 2011).

Several studies have assessed the role of SA or CA in the presence of specific abiotic stresses, especially heavy metals; however, the combined effects of SA and CA under stressful conditions remain unexplored (Sun and Hong 2011; Ghazijahani et al. 2014; Gondor et al. 2016; Liu et al. 2016; Hassan et al. 2016).

Cd is among the most common toxic metals present in many soil environments and is not readily removed from soil; it is therefore necessary to reduce its phytotoxicity. By virtue of the protective role of SA and CA in response to heavy metal stress, the present experiment was designed with the objective of assessing the interactive role of SA and CA in enhancing plant growth, photosynthesis rate, and antioxidant defense systems. The study would establish the relation between the two acids, i.e., whether they act synergistically or antagonistically to each other.

Materials and Methods

Plants Used for Study

Seeds of *Brassica juncea* var. RGN-48 were obtained from the Indian Agricultural Research Institute (IARI), New Delhi,

India. Healthy uniform-size seeds were tested for percent viability before the start of the experiment. Seeds were surface-sterilized with 5% sodium hypochlorite for 2 min followed by washing at least thrice with distilled water.

Treatment Pattern and Experimental Design

The experiment was conducted using a simple randomized complete block design. Seeds were sown in earthen pots filled with soil and farmyard manure in the ratio of 6:1 and subsequently placed under natural environmental conditions in an open area. Average temperature and humidity were 25 °C and 55%, respectively. Concentrations used in this experiment were selected according to the data provided in Supplementary Material Fig. 1.

Cd (0.6 mM) was applied to soil on the fifth day after sowing (DAS). Twenty-five day-old seedlings were sprayed with either double distilled water (DDW), designated as control; 0.01 mM salicylic acid (SA); or 0.6 mM citric acid (CA) on foliage in the evening. The sprayer nozzle was adjusted so that each plant received a total of 3 mL DDW, SA, or CA. Each treatment was replicated five times. Plants were sampled at 30 DAS in order to determine various growth and biochemical parameters.

Growth Biomarkers

Plants were gently removed from each pot along with soil, immersed in DDW and rinsed gently to remove adhering soil particles. Root and shoot lengths were measured using meter scale. Samples were weighed to obtain their fresh and dry mass. Plant tissue was oven-dried at 70 °C for 48 h to measure dry biomass.

Chlorophyll (SPAD Value) and Total Carotenoid Contents

Chlorophyll values were measured using an SPAD chlorophyll meter (SPAD-502; Konica, Minolta Sensing, Inc., Japan), under natural lighting “at mid-day.”

Carotenoid contents were estimated by the method of Maclachlan and Zalik (1963). Fresh leaves (0.1 g) from the interveinal region were ground in 10 mL of 80% acetone using a mortar and pestle. The suspension was decanted and filtered through Whatman no. 1 filter paper into a Buchner funnel. The optical density (OD) of the solution was read at 480 and 510 nm for carotenoid estimation using a UV–Vis spectrophotometer. Total carotenoid contents were calculated using the formula:

Total carotenoids = $7.6(\text{OD } 480) - 1.49(\text{OD } 510)$
 $\times \text{mg g}^{-1}$ fresh weight (FW),

where OD is the optical density of the extract at the given wavelength (480 or 510 nm).

Scanning Electron Microscopy

Stomatal apertures from leaf abaxial surfaces were observed under a scanning electron microscope (JEOL, JSM 6510). Fresh leaves were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h. The leaves were transferred to petri plates to run an ethanol-graded series (50%, 70%, 80%, 90%, and 100%). After dehydrating, the samples were coated with gold–palladium in a sputter coater (JEOL JFC-1600).

Leaf Gas Exchange Traits

Stomatal conductance, intracellular CO_2 concentration, transpiration rate, and net photosynthetic rate were analyzed using a portable infrared gas analyzer (IRGA) photosynthetic system (LI-COR 6400, LI-COR, Lincoln, NE, USA) between 11:00 and 12:00 h under clear skies. Relative humidity, CO_2 concentration, air temperature, and photosynthetic photon flux density (PPFD) were maintained at 85%, $600 \mu\text{mol mol}^{-1}$, 25°C , and $800 \mu\text{mol mol}^{-2} \text{s}^{-1}$, respectively.

Nitrate Reductase Activity

Nitrate reductase (NR) was measured (Jaworski 1971) in fresh leaf samples that were cut into small pieces and dipped into a solution containing phosphate buffer (pH 7.5), KNO_3 , and isopropanol followed by incubation at 30°C for 2 h. Sulfanilamide and *N*-1-naphthylethylenediamine hydrochloride solutions were added afterwards. Absorbance was read at 540 nm on a UV–Vis spectrophotometer. NR activity was expressed on a fresh mass (FM) basis as $\text{n mol (NO}_2\text{) g}^{-1}$ (FM) s^{-1} .

Carbonic Anhydrase Activity

Carbonic anhydrase (CAase) activity was determined by the method of Dwivedi and Randhawa (1974). Fresh leaf samples were cut into small pieces and suspended in a solution of cysteine hydrochloride. Samples were incubated at 4°C for 20 min and then transferred to test tubes containing phosphate buffer (pH 6.8) followed by addition of alkaline

bicarbonate solution and bromothymol blue indicator. The test tubes were incubated at 5°C for 20 min and titrated against 0.05 N HCl after adding 0.2 ml of methyl red indicator. CAase activity was expressed on a FM basis as $\text{mol CO}_2 \text{g}^{-1}$ (FM) s^{-1} .

Proline Content

The proline content of fresh leaves was determined by the method of Bates et al. (1973). Samples were placed in sulfosalicylic acid followed by addition of an equal volume of glacial acetic acid and ninhydrin. The samples were heated at 100°C for 2 h and subsequently transferred to an ice bath. 5 ml of toluene was added to the reaction mixture. The absorbance of the aspirated toluene layer formed was read at 520 nm. Proline content was expressed as mol g^{-1} (FM).

H_2O_2 Content

The content of H_2O_2 in fresh leaves was determined according to the method of Mukherjee and Choudhuri (1983). Leaves of *B. juncea* (0.5 g) were homogenized using a cold mortar and pestle in pre-cooled acetone (5 mL) and the homogenate was centrifuged at $12,000\times g$ for 5 min. 1 mL of supernatant was mixed with 0.1 mL of 5% $\text{Ti(SO}_4\text{)}_2$ and 0.2 mL of 19% ammonia after a precipitate was formed. The reaction mixture was centrifuged at $12,000\times g$ for 5 min. The resulting pellet was dissolved in 3 mL of 2 M H_2SO_4 and absorbance was read at 415 nm. The H_2O_2 concentration was calculated according to a standard curve of H_2O_2 ranging from 0 to $10 \mu\text{M}$. H_2O_2 content was expressed as n mol g^{-1} (FW).

Antioxidant Enzyme Activity

Antioxidant enzyme activities were determined by the method of Khan et al. (2015). Fresh leaves were homogenized in a pre-cooled mortar and pestle with phosphate buffer (pH 7), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, and 2% polyvinylpyrrolidone (PVP). This homogenate was subjected to centrifugation at $12,000\times g$ at 4°C for 20 min and the supernatant was held at -20°C for analysis of catalase (CAT 1.11.1.6), peroxidase (POX 1.11.1.7), and superoxide dismutase (SOD 1.15.1.1). Enzyme activities were expressed on a fresh mass basis as $\text{n mol (H}_2\text{O}_2\text{) decomposed g}^{-1}$ (FM) for CAT, U g^{-1} (FM) for POX, and U g^{-1} (FM) for SOD.

Compound Microscopy

Compound microscopy (Nikon ECLIPSE Ci-E equipped with Nikon digital camera DS-Fi1c) was used for detection of stomatal activity (Siddiqui et al. 2018). At least three leaves were collected from each treatment and immersed in a solution containing NaOH to promote removal of the epidermis. The abaxial surface was exposed under a cover slip to view stomatal aperture under a compound microscope.

Confocal Laser Scanning Microscopy

Cell viability was determined by the method of Siddiqui et al. (2018). Fresh roots were immersed in a solution containing 25 μ M propidium iodide (PI) for 10 min. They were subsequently washed twice with DDW and placed on glass slides for viewing under a confocal laser scanning microscope (Zeiss, LSM 780) at $\times 20$ magnification with maximum excitation at 535–617 nm.

Statistical Analysis

Data were statistically analyzed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Standard error was calculated and analysis of variance (ANOVA) performed to determine the least significance difference (LSD) between treatment means with a level of significance at $p \leq 0.05$.

Results

Growth Biomarkers

Values of all the growth biomarkers (i.e., root and shoot length; fresh and dry biomass) increased markedly after exogenous application of SA (0.01 mM) and CA (0.6 mM) to the Cd-stressed plants as compared to control plants (Fig. 1). Role of SA in promoting growth of *B. juncea* has been established in our previous studies (Hayat et al. 2009, 2012; Fariduddin et al. 2003; Yusuf et al. 2008); hence, the sole treatment of SA was omitted in the present study.

Shoot length declined by 20% upon exposure to Cd as compared to control plants. However, treatment with SA or CA alleviated Cd-generated toxicity; growth of plants treated with SA and CA improved by 14.9% and 11.9%, respectively, over the control. The combined dose of SA and CA proved the most beneficial in mitigating toxicity symptoms by enhancing shoot length by 30.9% as compared to the control plants and 63.8% when compared to the sole Cd-treated plants (Fig. 1A). A 20% decline in root length

occurred in Cd-treated plants. CA enhanced root length by 20.9% in non-stressed plants and improved growth by 38.8% in Cd-stressed plants as compared to sole Cd-treated ones. Similarly, SA improved root length by 43.8% in Cd-stressed plants as compared to the control. The combined SA and CA treatment protected plants from Cd stress and improved root length by 61.2% when compared to those treated with Cd (Fig. 1B). Shoot fresh mass (SFM) declined by 22.2% in Cd-treated plants and an increase of 23.7% was observed in CA-treated plants. SA and CA improved growth in Cd-stressed plants by 15.9% and 9%, respectively, over the control. The combined SA and CA treatment proved beneficial and completely neutralized toxicity effects (Fig. 1C). Cd reduced root fresh mass (RFM) by 30.6%; however, follow-up treatment with SA or CA improved RFM by 3.4% and 1.1%, respectively, over the control. The combined SA and CA treatment completely neutralized Cd-generated toxic effects by increasing RFM by 23.8% as compared to the control (Fig. 1D). A decrease of 25.3% in shoot dry mass (SDM) occurred in Cd-treated plants, whereas a 24% increase was observed in CA-treated plants compared to the control. When SA or CA were applied as a follow-up treatment to Cd-stressed plants, a recovery of 8.3% and 1.9%, respectively, over the control, was observed. The combined SA and CA treatment yielded the best response and increased SDM by 40.9% compared to the control (Fig. 1E). A decrease of 27.7% of root dry mass was observed in Cd-treated plants. The SA and CA treatments completely neutralized the toxic effects of Cd and increased root dry mass (RDM) of Cd-treated plants by 13.8% and 3.9% compared to the control (Fig. 1F). Decrease in leaf area was significant in the presence of Cd as compared to control plants (Fig. 2A). Foliar application of SA, CA, and SA + CA to Cd-stressed plants resulted in increased leaf area by 36.4, 34 and 49.4%, respectively, over Cd-stressed plants. Highest leaf area values were noted in the plants treated with combined SA and CA.

Chlorophyll and Carotenoid Contents

Cd-treated plants experienced a marked reduction in chlorophyll content; however, foliar application of SA and/or CA to the plants significantly increased chlorophyll content (SPAD value) by 41.7% and 35.2%, respectively (Fig. 2B). Therefore, the Cd-induced reduction was completely overcome by the follow-up treatment with SA and/or CA alone and in combination.

Cd-generated stress decreased total carotenoid contents by 20% (Fig. 2C). The follow-up treatment of SA and/or CA mitigated the toxic effects of Cd. Treatment with the combined SA and CA resulted in highest chlorophyll values in plants treated with Cd only and also in water-treated plants.

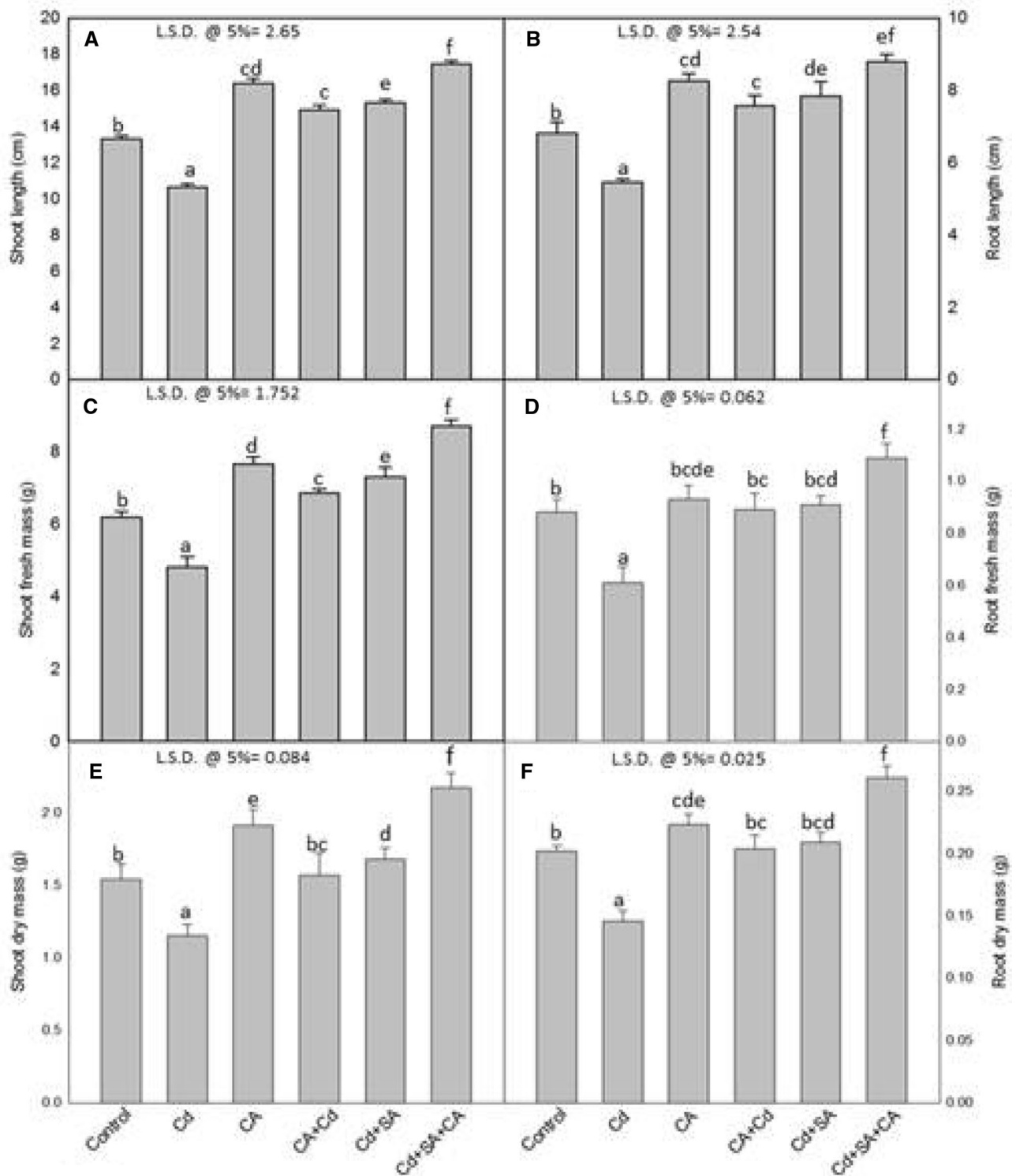


Fig. 1 Effect of 0.01 mM salicylic acid (SA), 0.6 mM citric acid (CA), SA (0.01 mM)+CA (0.6 mM) in the presence of 0.6 mM cadmium (Cd) stress on the length of shoot (a) and root (b) fresh mass of shoot (c) and root (d), dry mass of shoot (e) and root (f) of mustard at

30 days after sowing (DAS). All the data are the mean of five replicates ($n=5$) and vertical bars show standard error (\pm SE). Values of bars with the same letter are not significantly different, $P < 0.05$ Duncan's multiple range test

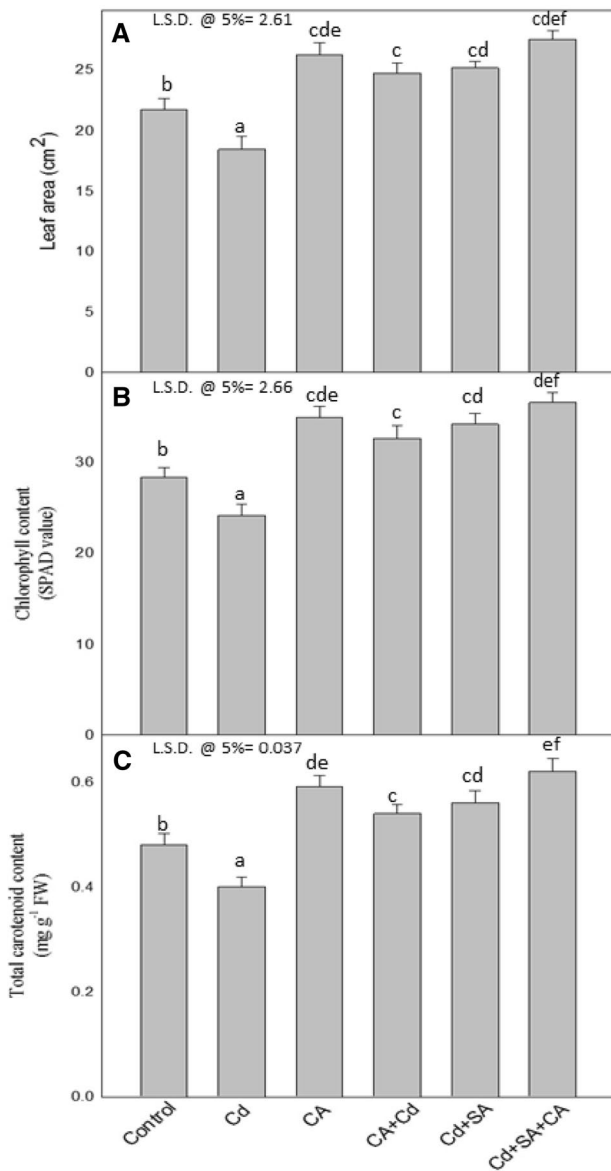


Fig. 2 Effect of 0.01 mM salicylic acid (SA), 0.6 mM citric acid (CA), SA (0.01 mM)+CA (0.6 mM) in the presence of 0.6 mM cadmium (Cd) stress on the **a** leaf area and **b** chlorophyll content (SPAD value), **c** total carotenoid content at 30 days after sowing (DAS). All the data are the mean of five replicates ($n=5$) and vertical bars shows standard error (\pm SE). Values of bars with the same letter are not significantly different, $P < 0.05$ Duncan's multiple range test

Leaf Gaseous Exchange, CAase, and NR Activity

Application of CA enhanced the photosynthetic rate (P_N) as compared to the control. The presence of Cd in the soil reduced P_N and stomatal conductance (Gs) in the plants as compared to control (Fig. 3A, B), with losses of 18 and 19.5%, respectively. Treatment of Cd-stressed plants with SA and CA significantly increased values of P_N by 52.1%

and 42.44%, respectively, over sole Cd-treated plants. The values for Gs increased by 42.42% and 36.36% upon SA and CA treatment, respectively, with reference to the plants that received only Cd treatment. Combined dose of SA and CA enhanced P_N (37.9%) and Gs (57.5%) in Cd-stressed plants as compared to plant treated with only Cd. The presence of Cd in the soil resulted in decreases of both internal CO_2 concentration (C_i) and transpiration rate (E); however, subsequent application of SA, CA, and SA + CA improved C_i by 17, 14, and 30%, and E by 16, 12, and 31% over the control, respectively (Fig. 3C, D). The treatments, therefore, resulted in complete recovery of the plants from Cd stress.

CAase and NR activity declined in plants that received Cd only as compared to the control (Fig. 3E, F) but improved by subsequent application of SA and CA. Highest values for CAase and NR activities were recorded in plants supplemented with SA and CA (27 and 31%, respectively, greater than the control).

Antioxidants and H_2O_2 Content

Activities of CAT, POX, and SOD increased under Cd stress and increased further upon exposing plants to SA and/or CA (Fig. 4A–C). Values for CAT, POX, and SOD in plants treated with SA and CA were 51, 63, and 50% greater than those of the control plants. Proline content increased in leaves of plants that received Cd, SA, and CA (Fig. 4D). Values were 42.4, 37.5, and 63% greater than the control by application of SA, CA, and SA + CA, respectively. The SA + CA treatment resulted in highest values for proline content. Increased H_2O_2 content was determined in Cd-treated plants (Fig. 4E). However, treatment of plants with CA and/or SA reduced H_2O_2 generated by Cd. The maximum decrease in H_2O_2 content (19%) was recorded in plants in the combined SA and CA treatment, compared with stressed plants treated with Cd only.

Microscopic Studies

Compound and scanning electron microscopic studies showed clear evidence of Cd toxicity to stomatal openings by affecting stomatal pore size (Figs. 5 and 6). However, recovery was observed in the SA + CA treatment as evidenced from increases in stomatal pore size. Confocal microscopic study of roots revealed that Cd stress resulted in membrane damage as expressed by increase numbers of stained nuclei (DNA). However, plants treated with SA and CA exhibited recovery from Cd-induced toxicity as revealed by the reduction in number of stained nuclei as compared to sole Cd-treated plants (Fig. 7a–c).

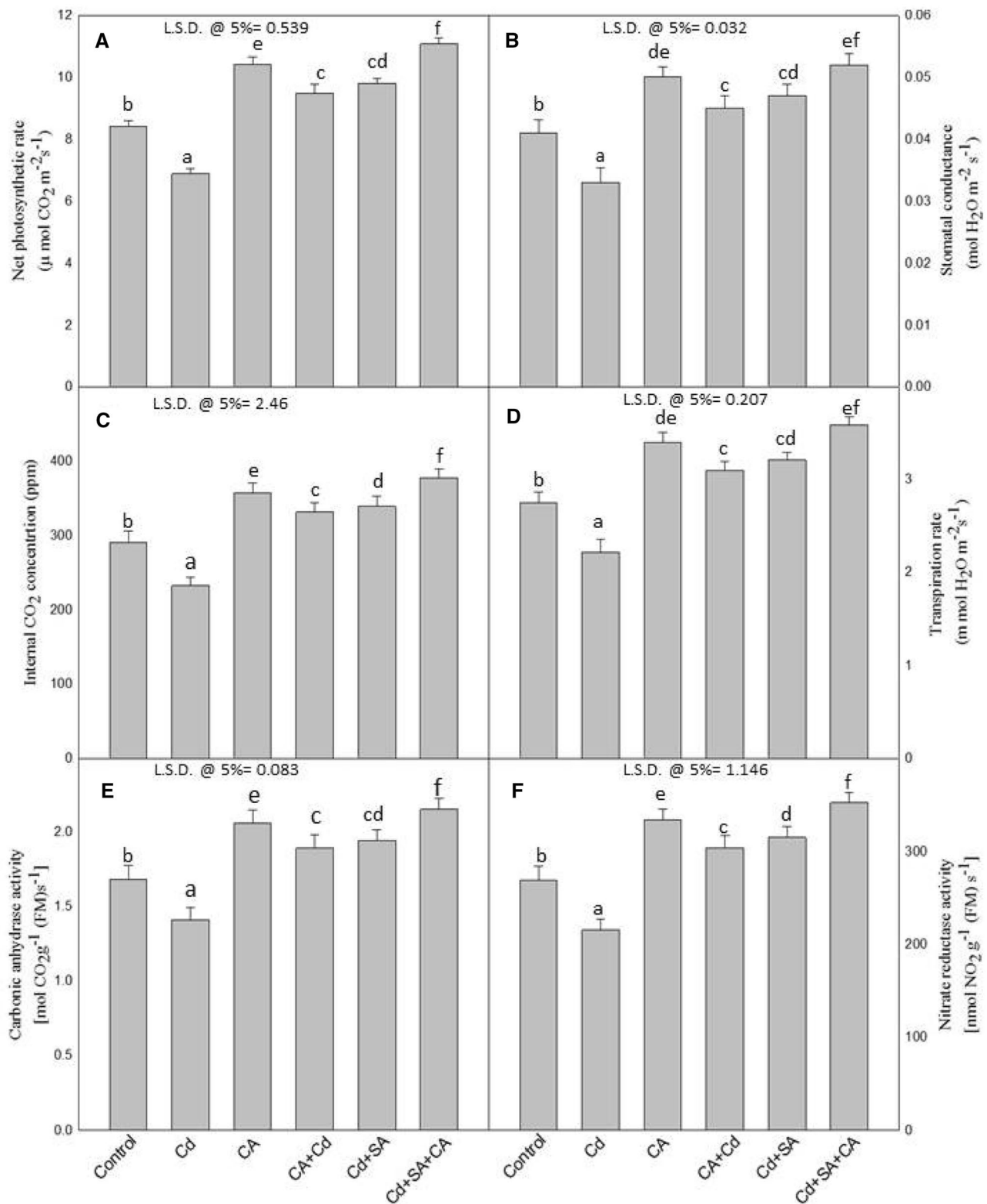


Fig. 3 Effect of 0.01 mM salicylic acid (SA), 0.6 mM citric acid (CA), SA (0.01 mM)+CA (0.6 mM) in the presence of 0.6 mM cadmium (Cd) stress on the **a** net photosynthetic rate (P_N), **b** stomatal conductance (G_s), **c** internal CO_2 concentration (C_i), **d** transpiration rate (E), **e** carbonic anhydrase (CAase) activity, and **f** nitrate reduc-

tase (NR) activity of mustard at 30 days after sowing (DAS). All the data are the mean of five replicates ($n=5$) and vertical bars shows standard error (\pm SE). Values of bars with the same letter are not significantly different, $P < 0.05$ Duncan's multiple range test

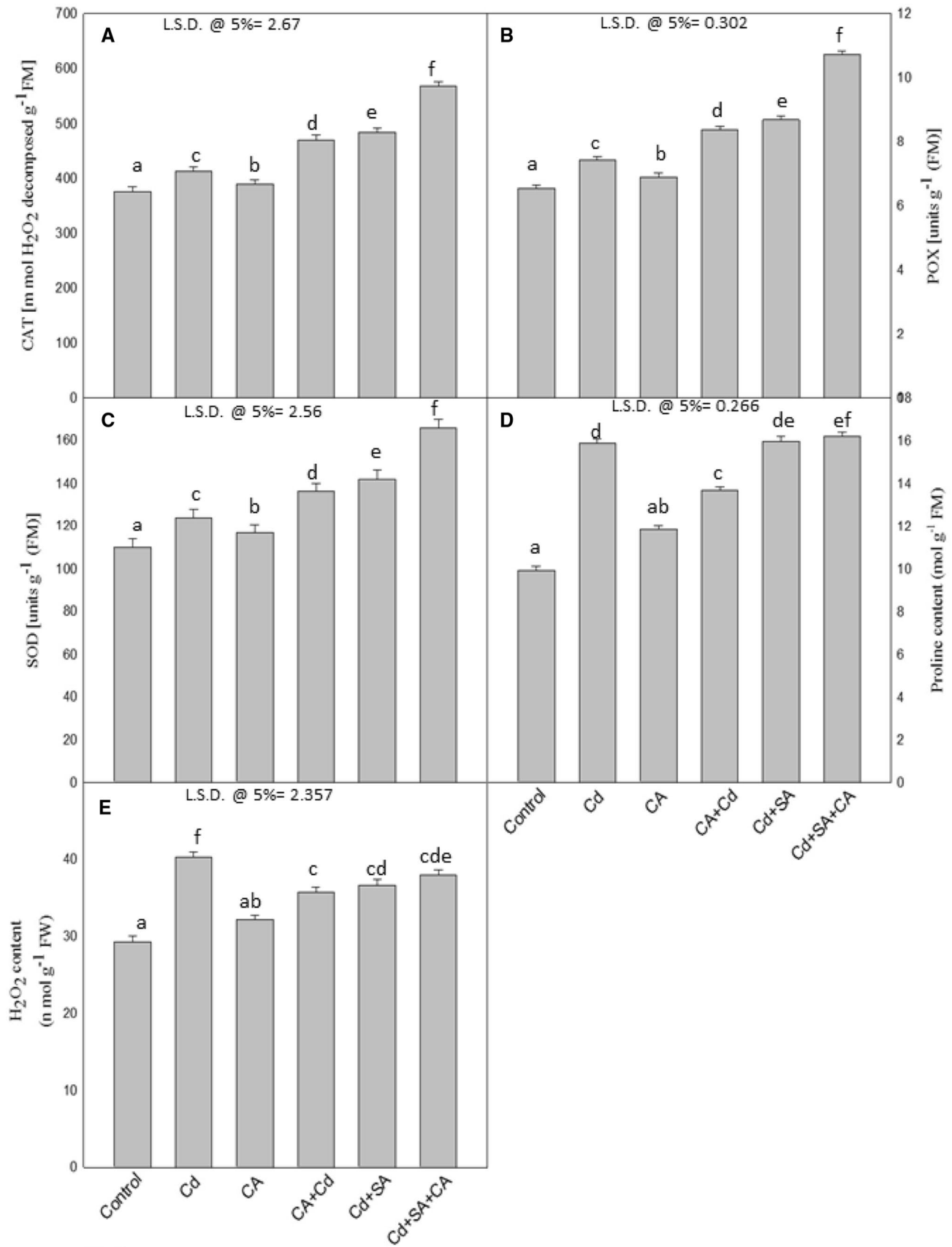


Fig. 4 Effect of 0.01 mM salicylic acid (SA), 0.6 mM citric acid (CA), SA (0.01 mM)+CA (0.6 mM) in the presence of 0.6 mM cadmium (Cd) stress on **a** catalase activity, **b** peroxidase activity, **c** superoxide dismutase, **d** proline, and **e** hydrogen peroxide content of mustard at 30 days after sowing (DAS). All the data are the mean of five replicates ($n=5$) and vertical bars shows standard error (\pm SE). Values of bars with the same letter are not significantly different, $P<0.05$ Duncan's multiple range test

Discussion

Cd is recognized as one of the most toxic heavy metals in soil that destabilizes membrane integrity and nutrient status; moreover, it inhibits chlorophyll biosynthesis (Benavides et al. 2005) which ultimately results in reduced plant growth (Wahid et al. 2007). Cd toxicity has been reported among various plant groups (Gallego et al. 2012; Ahmad et al. 2015; Al Mahmud et al. 2017; Kaur et al. 2017; Lu et al. 2018; Kaya et al. 2019).

Growth parameters in *B. juncea* such as root and shoot length, plant fresh and dry weights, and leaf area decreased upon exposure to Cd (Figs. 1A–F and 2A), which is in agreement with the findings of Irfan et al. (2014). Ahmad et al. (2015) reported that Cd adversely affects plant growth by interfering with mineral uptake, plant water relations, and photosynthesis. However, SA and/or CA application promoted the recovery of the Cd-stressed plants and the SA + CA spray proved to be most effective in mitigating Cd-related toxicity symptoms. Lu et al. (2013) reported higher biomass production by CA treatment in *Sedum alfredii* plants growing under Cd stress. Positive results of SA and CA under salinity, heat, and alkalinity stress have also been reported (Ahmad et al. 2018; Sun and Hong 2011; Hu et al. 2016). Alleviation of Cd toxicity by application of SA in rice, mustard, and flax has also been documented (Guo et al. 2007; Belkhadi et al. 2010; Ahmad et al. 2011).

The photosynthetic apparatus is a primary target of Cd toxicity to plants (Balakhnina et al. 2005). Chlorophylls and carotenoids are important photosynthetic pigments involved in the light-harvesting process, and carotenoids also protect chlorophyll from photo oxidative damage by reducing levels of ROS (Behera et al. 2002). It is essential to maintain the proper balance of these pigments for optimal light energy capture required to initiate photosynthesis (Wahid and Ghazanfar 2006). Estimating leaf chlorophyll content is useful in predicting rate of photosynthesis (Dalio et al. 2011). Cd application (0.6 mM) decreased SPAD chlorophyll and carotenoid levels in *B. juncea*; however, foliage-applied CA and/or SA not only neutralized the toxic effects of Cd but also increased chlorophyll and carotenoid contents even beyond those of the control (Fig. 2B, C) which contributed in improving photosynthesis rate and related parameters. Similar trends of recovery upon exposure to heavy metal stress were reported in *B. juncea* (Kaur et al. 2018; Parashar

et al. 2014; Yusuf et al. 2012), where SA and CA alleviated the deleterious effects of heavy metals on photosynthetic pigments. The loss of chlorophyll content may be due to Cd-mediated inhibition of amino levulinate synthesis that acts as one of the precursors of chlorophyll biosynthesis and is responsible for photoreduction of protochlorophyllide to chlorophyllide (Parmar et al. 2013).

Cd-induced decrease in chlorophyll and carotenoid contents (Fig. 2B, C) might have resulted in decreased P_N ; moreover, the decrease in Gs and pore size further slowed P_N (Fig. 3A–C). However, application of CA and/or SA mitigated the harmful effects of Cd stress on mustard plants. The combined dose of CA and SA proved optimal in mitigating the toxic effects of Cd as compared to plants treated with either SA or CA. Similar effects of SA and CA towards heavy metal stress have been reported in *B. juncea* (Kaur et al. 2018; Parashar et al. 2014; Yusuf et al. 2012).

Metals impart toxic effects to photosynthetic rate as they inhibit chlorophyll synthesis and induces its degradation (Zengin and Munzuroglu 2005; Parmar et al. 2013; Liu et al. 2014). Figure 3A–D reveals lower values for gas exchange parameters P_N , Gs, E, and C_i due to Cd; however, this damage was mitigated by follow-up treatment with CA and/or SA, which is supported by the results of Ghazijahani et al. (2014). The decline of photosynthesis upon Cd exposure may be the expression of (i) closing of stomata; (ii) increase in stomatal resistance; and/or (iii) decrease in stomatal density which lowers the rate of gas exchange (Ying et al. 2010). A similar decrease in the size of stomatal pores in the presence of Cd was observed in the present study. The follow-up treatment with CA + SA, however, mitigated the effects of Cd by increasing pore size (Fig. 5a–f), which is in agreement with the findings of Kaur et al. (2018) and Sandalio et al. (2001). SA increases leaf potassium ion concentration in the presence of abiotic stress (Delavari et al. 2010). Changes in stomatal aperture upon CA application may be a result of changes in K^+ levels in and around the guard cells as K^+ is known to play a major role in the opening and closing of stomata (Smith and Stewart 1990).

Plants exhibit tolerance to heavy metals via activation of compounds involved in metal chelation such as phytochelatin (PCs), metallothioneins (MT), organic acids (citrate and malate), and amino acids (Anjum et al. 2015; Manara 2012; Rauser and Curvetto 1980). Salicylic acid enhances tolerance towards oxidative stress by increasing the phytochelatin content in *Zea mays* via regulation of glutathione reductase and phytochelatin synthase activity (Szalai et al. 2013). Citrate is itself a ligand and exhibits strong affinity for Cd as compared to other organic acids (Anjum et al. 2015). CA suppresses Cd transport to above-ground plant parts by forming complexes and facilitating Cd uptake from solution to root, followed by storage of Cd–citric complexes in vacuoles. PC synthesis terminates as soon as those metal ions

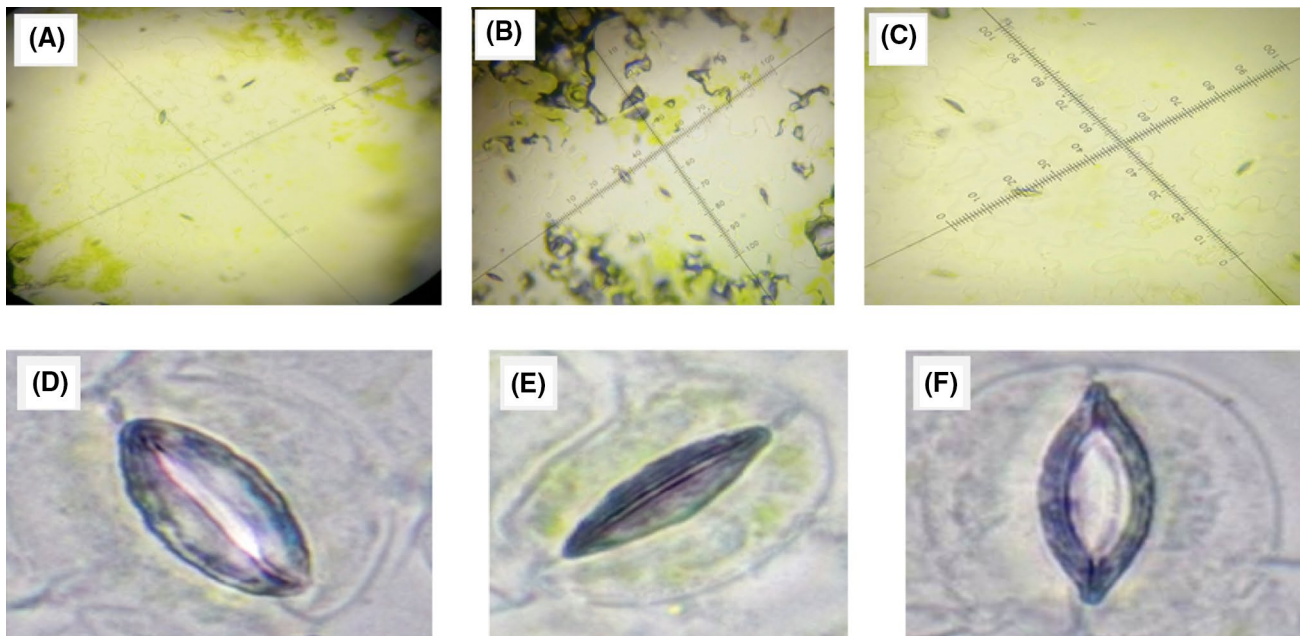
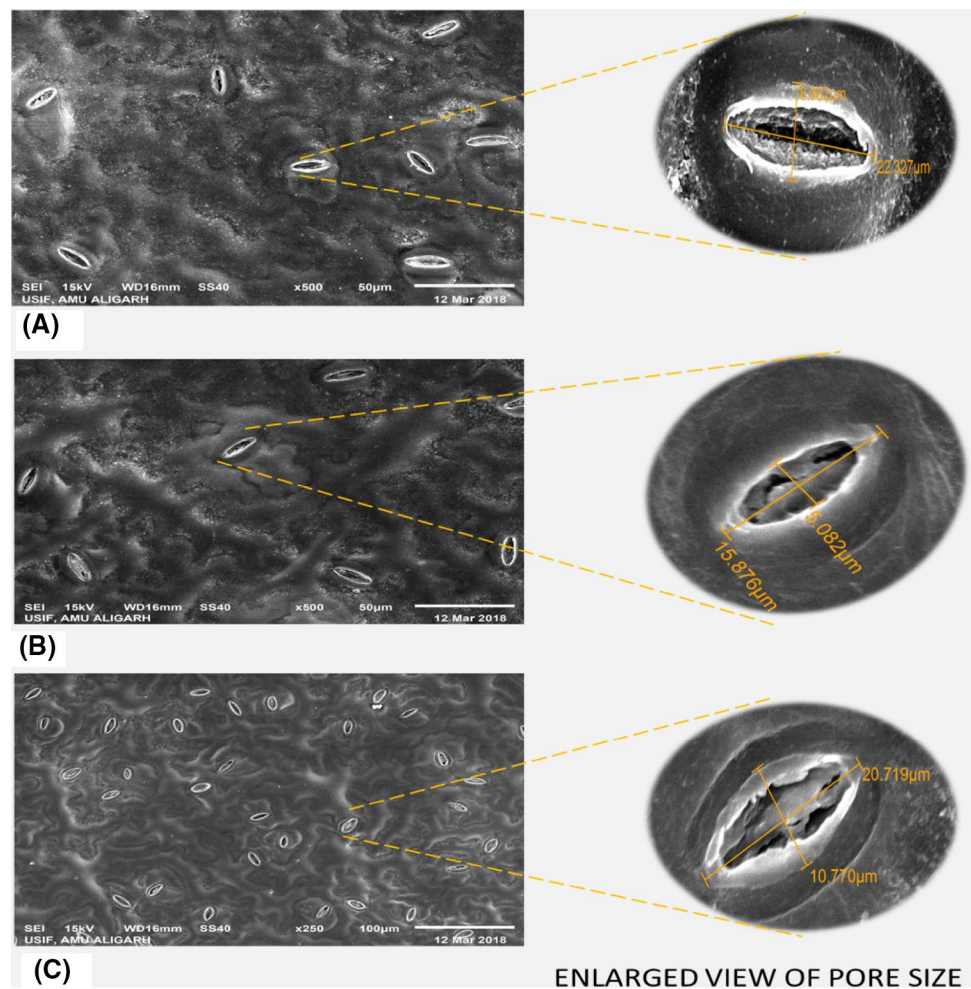


Fig. 5 Compound microscopic images of stomata of *Brassica juncea* treated with **a** double distilled water (control), **b** cadmium (Cd; 0.6 mM), **c** Cd+salicylic acid (SA; 0.01 mM)+citric acid (CA; 0.6 mM) at $\times 10$ magnification. **d–f** are magnified ($\times 40$) images of **a–c**, respectively

Fig. 6 Scanning electron microscope showing change in stomatal aperture of *Brassica juncea* treated with **a** double distilled water (control), **b** cadmium (Cd; 0.6 mM), and **c** Cd+salicylic acid (SA; 0.01 mM)+citric acid (CA; 0.6 mM)



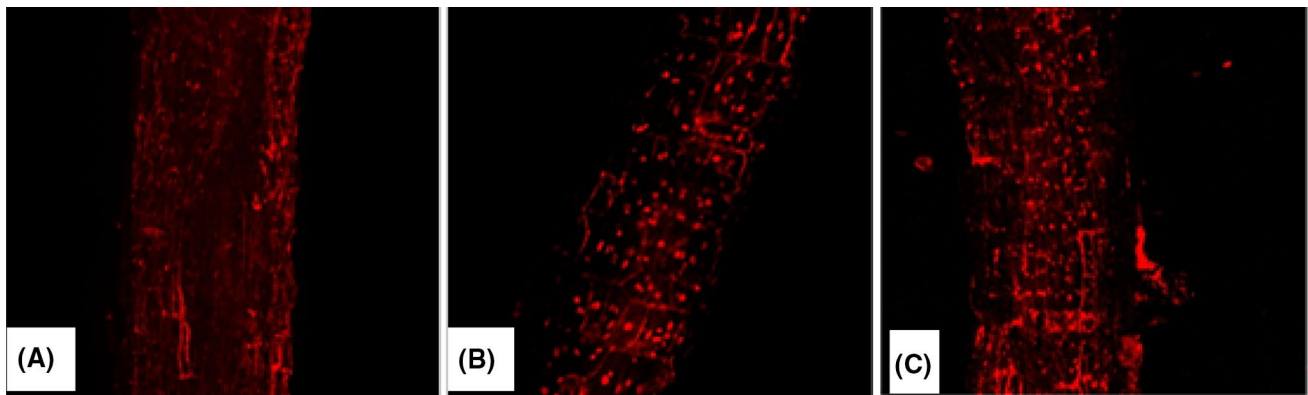


Fig. 7 Confocal microscope roots of mustard plant treated with **a** double distilled water (control), **b** cadmium (Cd; 0.6 mM), **c** Cd+ salicylic acid (SA; 0.01 mM)+ citric acid (CA; 0.6 mM)

activating a PC synthase are bound to a chelator. Citric acid reduces PC content by forming Cd–citrate complexes, leading to decline in Cd ion concentrations (Loeffler et al. 1989).

Metal-sensitive groups (SH– or histidyl groups) interact readily with heavy metals ultimately resulting in inactivation of enzyme catalytic activity (Kneer and Zenk 1992; De Miranda et al. 1990; Vallee and Ulmer 1972). Cd restricts the activity of carbonic anhydrase which is involved in the conversion of atmospheric CO_2 to HCO_3^- during photosynthesis; concurrently, the value for NR (i.e., the enzyme responsible for assimilation of exogenous nitrate) activity declines (Fig. 3E, F). The presence of Cd in soil with consequent loss of CAase and NR activity among different plants has been reported numerous times (Hayat et al. 2007; Khan et al. 2008; Anuradha and Rao 2009; Erdal and Turk 2016). The reduced activity of CAase might be an outcome of stomatal closure and reduced Ci (Figs. 3C and 6). The decline in CAase activity along with lower Gs, E, and Ci values due to Cd stress reduces the availability of CO_2 for rubisco (a major enzyme of the Calvin cycle) and marks the restriction of P_N (Fig. 3A–E). Leaf NR activity is considered a useful indicator of nitrogen status of the plant (Srivastava 1980). As nitrogen is a critical component of phytochelatin structure (Zenk 1996), the enhanced activity of NR in the presence of the combined dose of SA and CA could eventually strengthen the heavy metal detoxification system and resultant growth of the plant.

Biotic and abiotic stress results in overproduction of ROS. The species OH^\cdot , O_2^- , and H_2O_2 cause oxidative stress when present in excess quantities (Gossett et al. 1994; Meneguzzo et al. 1999). Hence, in order to promote normal cell function the balance between ROS generation and degradation must be maintained. Such balance is achieved through the actions of antioxidant machinery including enzymatic (superoxide dismutase, catalase, and peroxidase) and non-enzymatic (proline and carotenoids) antioxidants (Schutzendubel and Polle 2002; Ahmad

et al. 2010). Cd induces the generation of free radicals and oxidative stress in plants (Khanna et al. 2019; Kaur et al. 2019; Alyemeni et al. 2018; Ahmad et al. 2016). It possesses the ability to boost H_2O_2 generation in plants (Fig. 4E) by suppressing or disrupting the antioxidant defense system (Benavides et al. 2005; Srivastava et al. 2004). SA and CA reduced H_2O_2 overproduction in Cd-stressed plants (Fig. 4E) by virtue of the enhanced activities of CAT and POX which convert toxic H_2O_2 to water (Fig. 4A–C). Our finding is supported by Zhang et al. (2011) who reported that exogenous application of SA reduces H_2O_2 accumulation in *Phaseolus* and *Vicia* sp. Reduced H_2O_2 accumulation in Cd-stressed plants by SA or CA application has also been reported by Belkadhi et al. (2014), Ehsan et al. (2014) and Mahmud et al. (2018).

Plants have evolved several defense systems to withstand stressful conditions, where antioxidant enzymes (e.g., SOD, POX, and CAT) act as a first line of defense. These enzymes catalyze the detoxification of ROS and minimize the adverse effects caused by abiotic stress (Hasanuzzaman et al. 2012). Therefore, in the present study (Fig. 4A–C) and in others (Meng et al. 2009; Ehsan et al. 2014; Irfan et al. 2014) the presence of Cd stimulated an increase in the activities of antioxidant enzymes. Moreover, the levels of these antioxidant enzymes increased further in plants supplied with CA and/or SA (Fig. 4E). Similarly, the use of CA/SA to minimize the toxic effects of Cd is reported in different plant species (Zhang et al. 2011; Afshan et al. 2015; Semida et al. 2015; Hassan et al. 2016; Lu et al. 2018).

Proline, a non-enzymatic antioxidant, supports organisms under adverse conditions in mitigating the harmful effects of ROS (Chen and Dickman 2005). Proline accumulation increases in the presence of stress (Hayat et al. 2007) which further increases upon SA and CA application, corroborating the findings of Kaur et al. (2017), Mahmud et al. (2018), and Parashar et al. (2014). Higher proline levels maintain the water balance in plants to withstand stress conditions

(Zengin and Munzuroglu 2005). Increased SOD, CAT, and POX activities along with elevated proline content accelerate the detoxification of harmful H_2O_2 to non-harmful H_2O thus reducing oxidative stress (Najeeb et al. 2011; Kang et al. 2013). SA also decreases the impact of oxidative stress caused by Cd by inhibiting accumulation of Cd in plant cells (Noriega et al. 2012).

Confocal microscopic study further confirmed that enhanced ROS levels caused membrane damage in Cd-stressed plants (Fig. 7a–c). However, SA and/or CA proved beneficial in mitigating Cd-induced damage by accelerating the antioxidant machinery and reducing the ROS levels.

Conclusion

Cd stress in *B. juncea* resulted in growth restriction via reduced photosynthesis and increased oxidative damage. However, the exogenous application of SA and/or CA to foliage of stressed plants minimized the harmful effects of Cd by enhancing the antioxidant machinery which attenuates the Cd-induced oxidative burst. Increased stomatal conductance and aperture improved the CO_2 availability inside the cells and enhanced its capacity to bind with rubisco by elevating the carbonic anhydrase activity which ultimately resulted in enhanced photosynthetic rate. Moreover, the combined dose of SA and CA to standing mustard plants proved optimal in protecting plants from Cd stress.

Acknowledgements We thank the Department of Botany and Aligarh Muslim University for providing the platform to perform this experiment. We thank the authors for their contribution to this experiment. Thanks to Dr. John Pichtel, Ball State University, USA, for assistance with editing the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that this research work conducted without any financial relationship and authors have no conflict of interest among them.

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