Original Article

Foliar spray of brassinosteroid enhances yield and quality of Solanum lycopersicum under cadmium stress

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Abstract The presence of cadmium in the soil above a particular level is proposed to check not only plant growth but also productivity and fruit quality. Therefore, in the present study investigations are directed to evaluate the effect of four levels of cadmium (3, 6, 9, 12 mg kg\(^{-1}\)) in interaction with two analogs of brassinosteroids on the growth, fruit yield and quality of tomato. Under greenhouse conditions plants were analyzed for antioxidant system activity and photosynthetic assimilation efficiency. Cd stressed plants exhibited poor growth and biological yield. The metal also had a negative impact on the antioxidant system of the resulting fruits. However, the follow up application of BRs (10\(^{-8}\) M) neutralized the damaging effects of the metal on the plants.

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1. Introduction

Tomato is one of the common garden fruits in India and is cultivated worldwide because of its edible fruits that are rich in antioxidants, such as lycopene. The consumption of tomato is believed to benefit the heart, among other cures. The fruit contains lycopene, one of the most powerful natural antioxidants which are able to fight singlet oxygen and peroxo radicals otherwise both are responsible for damaging DNA in the process that leads to the origin of cancer. Similarly ascorbic acid and \(\beta\)-carotene also act as antioxidant against reactive oxygen species (ROS) and protect the living system from their damaging effect. Therefore it is the need of the hour that researchers should pay more attention toward the factors that favor the production and retardation of these potential agents.

Contamination of soil with heavy metals is the major problem worldwide that poses great environmental threat to biota as the metals accumulate in the soil, exposing plants to their undesirable amounts (Jamali et al., 2007). Although some of the heavy metals are required in smaller quantities, by the organism for maintaining normal physiological processes their excessive accumulation in the cells is always detrimental. Cd is one of the heavy metals that is known to generate toxicity even at a very low concentration. It accumulates in plants during
growth in edible parts, thereby, endangering crop yield and their quality. This causes a potential hazard to human and animal health. Cd is known to cause enzyme inactivation and damages cells by acting as antimetabolite or forms precipitates or chelates with a number of essential metabolites. Cd also alters various physiological and biochemical processes. The cumulative effect of all these altered processes is reflected in terms of reduced biological yield. During the past years several strategies and technologies for treating Cd stressed plants have been developed among them application of plant hormones was found to be most effective and least damaging. Of the plant hormones, a considerable attention has been paid to brassinosteroids (BR). As a rule the BRs regulate a number of physiological processes such as cell elongation and division, ATPase activity and prevention of photosynthetic pigment loss (Sasse, 1997; Musing, 2005; Hasan et al., 2008, 2011; Hayat et al., 2010, 2012) which result in better crop growth, under stressed conditions.

In our previous studies, it was found that root applied BRs enhanced growth, metabolism and yield of tomato under normal growth conditions. Thus the primary objective of the present study was to assess whether the leaf applied BR analog could neutralize the potentially negative influence of Cd contaminated soil on the growth, yield and quality of fruit in two tomato cultivars.

2. Materials and methods

2.1. Hormone preparation

BR analogs (28-homobrassinolide/24-epibrassinolide) were obtained from Godrej Agrovet Ltd., Mumbai. Stock solution (10^{-4} M) was prepared by dissolving the hormone in 0.5 cm³ of ethanol, in a 100 cm³ volumetric flask and the final volume was made up to the mark by using double distilled water (DDW). Surfactant (Tween 20; 0.5%) was added. The concentration (10^{-3} M) of HBL/EBL was prepared by dilution of stock solution in distilled water.

2.2. Plant material

The seeds of Lycopersicon esculentum var. K-25 and Sarvodya were purchased from National Seed Corporation Ltd., New Delhi. Healthy seeds were surface sterilized with 0.01% aqueous solution of mercuric chloride followed by repeated washings with DDW and were sown in earthen pots (25 × 25 cm) containing 0, 3, 6, 9 or 12 mg Cd kg^{-1} soil. These earthen pots were filled with sandy loam soil and farm yard manure, mixed in the ratio of 9:1 to create the nursery. At 20 day stage, the treated seedlings were subsequently transplanted to the maintained pots, under similar conditions as in case of nursery pots. The plants were grown in a net house under natural environmental conditions. The average temperature, humidity and day/night photoperiods were 25 ± 2 °C, 65 ± 5% and 14/10 h, respectively. At 59 day stage, the plants were sprayed with 0 (water) or 10^{-3} M of HBL/EBL. Each seedling was sprinkled thrice on the same day. The nozzle of the sprayer was adjusted in such a way that it pumped out 1 cm³ of DDW or HBL or EBL solutions. The plants were sampled at 90 DAS to assess various photosynthetic and biochemical characteristics. The remaining plants were allowed to grow to maturity (190 DAS) to obtain fruit yield per plant and the contents of lycopene, β-carotene and ascorbic acid, in ripe fruits.

2.3. Chlorophyll, photosynthesis measurements and leaf water potential (LWP)

The SPAD chlorophyll content in intact leaves was measured by using Minolta chlorophyll meter (SPAD-502; Konica Minolta Sensing Inc, Japan) whereas the transpiration rate (E), internal CO₂ concentration (Ci), stomatal conductance (gs) and net photosynthetic rate (Pn) were measured by Li-COR 6400 portable photosynthesis system (Li-COR, Lincoln, NE, USA). LWP of fresh leaf samples was measured by PSYPRO, water potential system, WESCOR, Inc, Logan, USA.

2.4. Enzyme activities and proline content

The activity of carbonic anhydrase (CA) and nitrate reductase (NR) was determined following the procedure described by Dwivedi and Randhawa (1974) and Jaworski (1971) respectively. Peroxidase (POX) and catalase (CAT) were assayed following the procedure described by Chance and Maehly (1956). The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The proline content in fresh leaf was determined by adopting the method of Bates et al. (1973).

2.5. Lycopene, β-carotene and ascorbic acid

Lycopene and β-carotene content, in the ripe fruits, was determined by the procedure described by Ranganna (1976) and Sadasivam and Manickam (1997), respectively, whereas ascorbic acid content in the fruits was determined following the procedure applied by Raghuramulu et al. (1983).

2.6. Yield characteristics

At harvest (180 DAS), nine plants (three plants from each pot) representing each treatment were randomly sampled and counted for the number of fruits per plant and weighed to assess fruit yield per plant.

3. Results

3.1. Growth

Plants raised in the soil amended with cadmium exhibited a significant reduction in all the growth attributes (shoot and root length, fresh and dry mass and leaf area). The degree of damage caused by the metal in both the cultivars was in proportion to the concentration of the metal. The variety, K-25 had comparatively lesser damage, against different metal concentrations, than Sarvodya. The highest concentration (12 mg kg^{-1}) of metal was found to be most damaging for both the varieties (Fig. 1). However, the spray of either of the brassinosteroid analogs (HBL/EBL) to the foliage of the plants significantly favored growth and also nullified the toxic effect
of the metal in a concentration dependent manner. Out of the two BR analogs EBL was more effective than HBL. The maximum values for fresh and dry mass of shoot, of 71.7%, 61.2%; 82.8% and 78.2% higher were recorded in the un-stressed plants of K-25 and Sarvodya sprayed with the EBL, over their controls, respectively. Other growth attributes also showed similar response.

3.2. SPAD chlorophyll and photosynthetic attributes

Supply of metal to the plants through the soil had an adverse impact on the SPAD chlorophyll content and all the photosynthesis related traits. The degree of damage caused by the metal was in proportion to its concentration (Fig. 2). The spray of brassinosteroid to the foliage significantly overcomes the damaging effects of the metal, more efficiently in K-25 than in Sarvodya. BR analogs completely neutralized the damage caused by the lower three concentrations (3, 6 and 9 mg kg⁻¹) in K-25, whereas in Sarvodya damaging effects of the lower concentration (3 mg kg⁻¹) of Cd was completely neutralized by either of brassinosteroids. The best values for net photosynthetic rate were recorded by the EBL spray alone that were 35.3% and 32.1% higher for K-25 and Sarvodya as compared to their controls, respectively.

3.3. Carbonic anhydrase and nitrate reductase activity

The leaves of the plants raised in the Cd amended soil possessed significantly lower CA and NR activity in both the varieties than their controls. The loss was more visible in the plants fed the highest level of the metal (Fig. 3A, B). However, the foliage of the plants sprayed with either of the brassinosteroid
analogs, nullified the damaging effects of the metal to a limited extent in the plants fed higher Cd levels but completely in plants which received a lower concentration of the metal. Both the brassinosteroid analogs generated almost similar response.

3.4. Antioxidative enzyme activity and proline content

The activity of antioxidative enzymes and the proline content were higher in the leaves of the plants raised in the metal amended soil than the control. This increased further with the increase in the level of the metal. Either of the brassinosteroid analogs had an additive effect (Fig. 3C–F). Variety K-25 showed higher activity in response to all the treatments than Sarvodya, reflecting its resistant nature. Maximum values were recorded from the leaves of the plants that received the highest level of metal (12 mg kg$^{-1}$) with EBL spray. It showed 118.4%, 85.5%; 52.1%, 36.5%, 126.0% and 88.8% higher POX, CAT and SOD values in K-25 and Sarvodya over their respective controls. Proline content also exhibited a similar response.

3.5. Leaf water potential

Leaf water potential of both the varieties under metal stress decreased, compared with control plants (Fig. 4A). This loss was in proportion to the concentration of the metal. Moreover, the spray of either of the brassinosteroid analogs was found effective in neutralizing the ill effect of metal more effectively in K-25 than Sarvodya. In case of K-25 the spray of either of the analogs completely neutralized the damage caused by the...
lower two concentrations (3 and 9 mg kg\(^{-1}\)) of the metal but in Sarvodya, the toxic effect generated only by the lowest concentration (3 mg kg\(^{-1}\)) of the metal was completely neutralized by the hormone. Moreover, EBL proved better than HBL, and generated maximum values in both the varieties.

3.6. Lycopene \(\beta\)-carotene and ascorbic acid content in fruits

At harvest, fruits developed on the plants grown in metal amended soil possessed significantly lower values for lycopene and \(\beta\)-carotene contents. This loss, increased further with an increase in the level of metal (Fig. 4B, C). Variety K-25 showed comparatively better resistance against metal stress than Sarvodya. Moreover, the follow-up treatment of the stressed plants with either of the brassinosteroid analogs, improved the values to completely neutralize the toxic effect generated by the metal in both the varieties, more effectively in K-25 than Sarvodya. However, the ascorbic acid content in the fruits had a significant reduction in response to the hormone whereas the values increased with the increased level of metal in the soil. Sarvodya possessed higher ascorbic acid than K-25 (Fig. 4D). Moreover, the fruits borne on the cadmium fed plants possessed lower lycopene and \(\beta\)-carotene contents.

3.7. Number of fruits and fruit yield

Plants grown in the metal amended soil possessed a comparatively lower number of fruits and fruit yield per plant, at harvest. This rate of fruit loss was in proportion to the concentration of the metal (Fig. 4E, F). However, treatment
with either of the brassinosteroid analogs to the foliage, favored the number of fruits and fruit yield per plant. The toxic effect generated by the lower concentrations (3 and 6 mg kg\(^{-1}\)) was completely neutralized by the hormone but that of the higher metal level (9 and 12 mg kg\(^{-1}\)) was partly overcome. K-25 was comparatively resistant and more responsive toward hormone treatment.

4. Discussion

Plant adjustments against supra-optimal levels of soil Cd naturally lead to compromise in certain fundamental plant processes associated with the uptake and assimilations. These include plant water relations, photosynthesis and mineral nutrition which result in hampered energy extraction and economy as evident from the leakage of free radicals.

Cd decreases membrane potential hampering absorption of water and minerals (Harnandez et al., 1996) which is obvious from the decrease in fresh and dry mass of root and shoot and their lengths (Fig. 1). However, BRs favor plant basic processes, evident as growth promotion which appears as a result of the additive effect of re-established water status plus induced activity of ATPase pump (Khripach et al., 2003) in vacuoles (metal detoxification) or acid growth promotion. Turgor driven cellular enlargement presents the integrated picture as healthy growth of plant in terms of fresh mass, expanded (photosynthetic) leaf area and length of shoot and root (Fig. 1) in Arabidopsis thaliana (Li et al., 1996) and Pisum sativum (Nomura et al., 1997) biosynthetic mutant. BRs applied exogenously to varied groups of plants improved the growth of the root and/or that of the shoot (Hayat et al., 2001a; Bajguz and Tretyn, 2003; Fariduddin et al., 2004, 2005; Ali et al., 2008; Hasan

![Figure 4 Effect of brassinosteroids](image-url)
Chlorophyll molecules (Harpaz-Saad et al., 2007). Cd is well known to inhibit the activity of these two enzymes (Okabe et al., 1999), and their transcription and/or translation that may generate a significant impact on the expression of specific genes (Khripach et al., 2008). One of the prominent effects of Cd in the vicinity of biological membranes is evident as alteration in membrane potential and membrane phase change which renders the capacity to retain water status (Barcelo and Poschenrieder, 1990) creating water deficit (Barcelo et al., 1986; Lefèvrea et al., 2010). However, BRs have a favorable impact on the leaf water potential of the plants, even if given as a follow up treatment with Cd (Fig. 4).

The activity of CA is largely determined by photon flux density, concentration of CO2, availability of Zn (Tiwari et al., 2005) and genetic expression (Kim et al., 1994). Cd is a well-known antagonist of Zn uptake (Wang et al., 2009). The stress generated by Cd decreases the partial pressure of CO2 in the stroma by inducing stomatal closure (Barcelo and Poschenrieder, 1990) that causes a decrease in the activity of CA.

The process of nitrate reduction depends on three main factors (a) the substrate (NO3− level in the cytoplasm (b) the plasma membrane/cytosolic level of functional nitrate reductase (NR) and/or (c) the activity level of functional NR. Each of these factors is, directly or indirectly dependent on the metabolic sensors and/or signal transducers (Campbell, 1999). The major rate limiting step in the whole process of nitrate reduction is the conversion of nitrate to nitrite (Salisbury and Ross, 1992) which is catalyzed by nitrate reductase (NR). The observed decrease in NR activity (Fig. 3A) is an impact of the metal on the activity of plasma membrane proton pump (Obata et al., 1996) and the membrane fluidity (Mehang, 1993), restricting the uptake of nitrate, the inducer and the substrate of NR (Hernandez et al., 1996; Campbell, 1999). However, treatment with BRs, alone or as a follow-up treatment to the Cd-stressed seedlings elevated the activity of CA and NR, by speeding up the assimilation of CO2 (Yu et al., 2004) and increasing the concentration of NO3 by acting at the membrane level (Mai et al., 1989). This could be an additional impact of BRs on the expression of specific genes (Khripach et al., 1999) detoxifying the metal externally or partitioning it internally in vacuoles. Moreover, BRs are also involved in the transcription and/or translation that may generate a significant impact on the activity of these two enzymes (Okabe et al., 1984; Khripach et al., 2003).

Chlorophyllase is an enzyme that regulates the turnover of chlorophyll molecules (Harpaz-Saad et al., 2007). Cd is well known to enhance the activity of chlorophyllase, therefore, promotes degradation of the chlorophyll molecules rather than synthesis (Reddy and Vora, 1986). Cd also decreases the biosynthesis of δ-aminolavulinic acid and protochlorophyllide reductase complex (Stobart et al., 1985), involved in chlorophyll biosynthesis. The cumulative suppressive effect of these two reactions, therefore, resulted in the loss of SPAD chlorophyll content (Fig. 2A). A follow up treatment of Cd-stressed plants with BRs neutralized the damaging effects. BRs fed to the non-stressed plants significantly increased the pigment content that supports the findings of others (Braun and Wild, 1984; Bhatia and Kaur, 1997; Hayat et al., 2000, 2001a; Fariduddin et al., 2003; Yu et al., 2004; Ali et al., 2006, 2007). The BL induced transcription and/or translation of the enzymes involved in chlorophyll biosynthesis associated with a decrease in the level of catabolizing enzymes seems to be the potent reason to such an observation (Bajguz, 2000). Cd induced stomatal closure decreases the partial pressure of CO2 in the sub-stomatal chamber (Barcelo and Poschenrieder, 1990) which declined the values of g, Ci, and E (Fig. 2C, D and F). A cumulative effect of all these altered processes leads to a decrease in the photosynthetic rate (Fig. 2B), that is further supported by the decrease in carbonic anhydrase activity in the present study (Fig. 3B). Low CO2 decrease in photosynthetic pigment (SPAD chlorophyll value) and the activity of CA are the additional factors to cause lower Pn rate. This is further strengthened by an observed positive correlation between CA and Pn (Fig. 5A and B). Also, Cd has a damaging effect on the activity of Rubisco (Siedlecka et al., 1997) which will naturally have a negative impact on CO2 reduction.

The activity of antioxidant system increased in response to Cd (Fig. 3D–F), but the cellular ROS production seemingly generated a higher degree of oxidative stress, leading to growth loss, as compared to control plants (Fig. 1) as indicated by the accumulation of proline content, the stress marker in the present study (Fig. 3C). It has been hypothesized that the ratio of total antioxidant activity and total cellular reactive radicals (TAA/TCR) decreases under heavy metal (Cd) stress. However, this decline in growth parameters was the result of the interaction of Cd and plant genotype. The ratio (TAA/TCR) in case of sensitive varieties appears to be lesser as compared to resistant varieties.

Besides chlorophyll, Cd decreases the level of accessory pigments/carotenoids (Ekmecki et al., 2008) probably by interfering with the synthesis of concerned enzymes. Present study observed (Fig. 4B and C) the decline of lycopene and β-carotene in the mature fruits under Cd stress. However, BRs significantly increased the level of lycopene and β-carotene in the stressed and non-stressed fruits (Fig. 4B and C). Unlike lycopene and β-carotene the BRs significantly decreased the ascorbic acid content in the fruits (Fig. 4D). Similar loss in ascorbic acid contents under BRs has also been reported, earlier in tomato (Vardhini and Rao, 2002; Ali et al., 2006). In natural course, the fruit ripening is associated with the speeding up of the degradation of photosynthetic membranes (thylakoid membrane) and chlorophyll pigments but carotenoids, including lycopene and β-carotene accumulate (Grierson et al., 1987). The biosynthesis of carotenoids is also associated with the ethylene mediated gene expression during ripening process (Fray and Grierson, 1993; Gray et al., 1994; Ronen et al., 1999; Lois et al., 2000; Giovannoni, 2001). Therefore, the plausible reason for the observed increase in lycopene and β-carotene pigments seems to be the ethylene mediated alterations as BRs stimulate ethylene biosynthesis by inducing increased levels of S-adenosylmethionine and l-aminocyclopropane-1-carboxylic acid (Scholagnhauser et al., 1984; Khripach et al., 1999).

A decrease in the number of fruits and fruit yield per plant in the plants, treated with Cd, is possibly because of the slower
pace of CO₂ fixation and partitioning of photosynthates to fruits/sinks. This gets support from the observed significant correlation between root dry mass and fruit yield as well as shoot dry mass and fruit yield (Fig. 5 C–F). However, BRs alone or as a follow-up treatment to the Cd-stressed plants significantly improved yield attributes. In natural course, the fruit bearing capacity of the plants is mainly determined by the density of the flowers retained to set fruits, BRs slow down the process of senescence before and/or after pollination (Zhao et al., 1987; Sugiyama and Kuraishi, 1989; Iwahori et al., 1990), therefore, in our observations the BR treated plants bore a larger number of fruits (Fig. 4E and F). Similarly, a synthetic BL analog (TS-303) improved the fruit setting in tomato (Kamuro and Takatsuto, 1999). Moreover, the slower rate of senescence also allowed the attachment of the leaves to the mother body for a longer duration with an elevated level of photosynthesis (Hayat et al., 2000, 2001a, b; Yu et al., 2004) and speeded translocation rate of photosynthates to the sink (Fujii et al., 1991; Petzold et al., 1992; Fujii and Saka, 2001). Ali et al. (2006) also reached a similar conclusion in tomato.

It has been observed from the present study that EBL excels over HBL in its effects. The mechanism behind this response is yet not well elucidated. Nevertheless, it has been assumed that this effect is due to differences in the structure of these two analogs. Most of the BRs carry an S-oriented alkyl (a methyl or ethyl) group at C-24 of side chain. 24-epiBL although is

Figure 5 Correlation coefficient values between (A,B) carbonic anhydrase activity and net photosynthetic rate, (C,D) fruit yield and shoot dry mass, (E,F) fruit yield and root dry mass respectively in K-25 and Sarvodya.
among the exceptions along with 24-epiCS carries an R-oriented alkyl on the side chain of steroid nucleus. It could be concluded that the steric hindrance due to cross-linking of EBL molecule at its receptor binding site leads to a more distorted three dimensional conformational state as compared to HBL. This thermodynamically acquired new stable state of BR1-1 seems to be more actively involved in triggering a wide array of signaling cascades over HBL. However, how this binding facilitates the kinase activity inhibition of BIN-2 transcription factor is still elusive. Furthermore, how BKI-1 dissociates with BAK-1 to avoid the binding with BR1-1 at membrane or within endosomes induced after a series of reactions to elicit internalization of receptors is also elusive.

5. Conclusion

Anthropogenic activities raised the soil cadmium level above the contaminated level of 3–10 mg kg⁻¹. Cd footprints are more obvious in the urban outskirts, where crop growth is severely compromised. This severe setback hindered yield and quality of produce as a consequence of significant alterations at the level of photosynthesis and antioxidant responses. EBL and HBL, from a class of plant hormones known as BRs, are potent regulators of Cd toxicity and growth enhancers supporting photosynthesis, and balance supra-optimal leakage of reactive radicles with antioxidant biochemical machinery to reset the interface under cadmium contamination. Foliar spray clearly facilitated on-spot regulation of BR mediated stress as the first line of defense. BRs definitely emerge as a potent applied alleviator in arable lands now and in future.

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