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ORIGINAL ARTICLE

Role of calcium in AMF-mediated alleviation of the adverse impacts of cadmium stress in *Bassia indica* [Wight] A.J. Scott



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Abstract The aim of this study was to evaluate cadmium stress induced changes in the growth, lipid peroxidation and antioxidant activity of *Bassia indica* associated with arbuscular mycorrhizal fungi (AMF) and their amelioration by calcium application. Cadmium stress can cause alterations in the physiological and biochemical processes in plants. A calcium application combined with an AMF treatment resulted in the reduction of lipid peroxidation and the production of hydrogen peroxide, thereby mediating the mitigation of cadmium induced oxidative stress. The activity of antioxidant enzymes increased with cadmium application, whereas AMF inoculation combined with a calcium application further enhanced their activity. An increase in the content of non-enzymatic antioxidants such as ascorbate, reduced glutathione (GSH), oxidized glutathione (GSSG) and S-nitrosoglutathione (GSNO) in AMF-inoculated and calcium-treated plants further suggests their role in strengthening the antioxidant defense system that results in maintained growth. The application of calcium combined with the AMF treatment caused a significant reduction in lipid peroxidation and in the production of hydrogen peroxide, thereby mediating the mitigation of the cadmium induced oxidative stress. Increased proline accumulation was clearly evident in stressed

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plants, and the calcium application as well as the AMF inoculation further induced proline synthesis, thereby providing efficient protection against cadmium stress by increasing the maintenance of the systemic resistance criteria.

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1. Introduction

Cadmium is one of the most toxic heavy metals present in the soil at low concentrations. The main sources of cadmium pollution include mining, smelting, the weathering of cadmium rich rocks, the overuse of phosphate fertilizers and the use of sewage sludge and metal polluted water for crop irrigation (Zoffoli et al., 2013). Cadmium shows high mobility in the soil-plant system and is quickly absorbed by plants and transported to more sensitive parts of the plant, damaging normal metabolism (Imadi et al., 2016). An increased uptake of cadmium retards growth, and its immediate effects include necrosis and chlorophyll damage, hampered nutrient uptake, reduced enzyme activity and carbon assimilation and increased phytotoxicity (Singh and Prasad, 2014).

Cadmium is considered a non-redox heavy metal, but it can induce oxidative damage by initiating the formation of toxic reactive oxygen species (ROS). Cadmium stress results in alterations in redox homeostasis by affecting the activities of the enzymes that help maintain redox homeostasis, which generates ROS by interfering with the electron transport chain (Ahanger et al., 2014; Wu et al., 2014). ROS causes peroxidation of membrane lipids, leading to a loss of membrane integrity and results in leakage. Moreover, ROS also causes oxidation of nucleic acids, proteins and chlorophylls, thereby affecting the normal functioning of the cell (Alqarawi et al., 2014; Abd_Allah et al., 2015). Several protective strategies are employed by plants to reduce the toxic effects of cadmium stress. Under stressful conditions, compatible osmolytes accumulate in plants to maintain tissue cell water content, in addition to up-regulating the activities of antioxidant enzymes that contribute to a better adaptation of the plants to harsh conditions (Hashem et al., 2014; Abd_Allah et al., 2015). Proline, glycine betaine are included in compatible organic osmolytes, and enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and polyphenol oxidase (PPO) are the important antioxidant enzymes that are efficient in scavenging toxic free radicals generated from stress (Ahanger et al., 2014; Wu et al., 2014; Abd_Allah et al., 2015). In addition to the enzymatic antioxidants, the non-enzymatic antioxidant system also contributes significantly to the reduction of oxidative stress. Ascorbic acid, tocopherols and glutathione are among the important non-enzymatic antioxidants that help scavenge for ROS. Ascorbic acid and glutathione are the components of an important ROS scavenging pathway, the ascorbate-glutathione pathway (Ahanger et al., 2014; Wu et al., 2014).

During plant development, many plants come into symbiotic associations with arbuscular mycorrhizal fungi (AMF). It has been well accepted that AMF bring positive changes to the soil structure and thereby promote the growth of plants under normal and stressed environmental conditions (Ahanger et al., 2014; Wu et al., 2014; Abd_Allah et al., 2015). AMF

promote growth through morpho-physiological and biochemical changes in the host plants. Arbuscular mycorrhizal fungi (AMF) also serve as an important bio-ameliorator of stress and have a role in mitigating stress-triggered damaging effects in plants (Abd_Allah et al., 2015). AMF colonization can induce changes in the morphology, physiology and nutritional status of host plants that can enhance resistance to abiotic stresses. In addition, AMF directly affect plant growth and vigor (Evelin et al., 2009). Mycorrhizal colonization affects root morphology and the physiological status of the host plants. Modifications in the root architecture due to AMF help maintain water status and essential nutrients (Aroca et al., 2013). AMF colonization enhances the uptake of essential mineral nutrients such as nitrogen, phosphorous and potassium (Hart and Forsythe, 2012; Alqarawi et al., 2014). Calcium has the ability to mitigate the negative effect of abiotic stress by maintaining antioxidant potential and cellular water levels (Ahmad et al., 2015). Calcium is involved in controlling basic functions such as photomorphogenesis, cell division, cell elongation, stress responses, and the maintenance of membrane structure and functions (Ahanger et al., 2014; Ahmad et al., 2015). We hypothesized that calcium, when combined with AMF, is capable of alleviating salt stress in plants when plants are exposed to Cd stress.

Bassia indica (Wight; = *Kochia indica*) A.J. Scott, an annual halophyte shrub, belongs to the *Chenopodiaceae* family, which has adapted to abiotic stress and is widely used for rehabilitation of desert ecosystems, salt phytoremediation and as a source for livestock grazing in salt affected lands (Hashem et al., 2015; Shelef et al., 2012; Zhang et al., 2012). *B. indica* is also found in Saudi Arabia in the form of fragmented populations (Cunningham, 2013; Youssef, 2013). The plant has evolved the unique ability to withstand stress (Shelef et al., 2012; Eid and Shaltout, 2016). Smith and Read (2008) reported that plant species belonging to the *Chenopodiaceae* are non-mycorrhizal plants and arbuscules very rarely to observe in their roots. However, many recent investigations including microscopic characterization, demonstrates that many chenopods can be well colonized by AMF natively and artificially in pot experiments (Wilde et al., 2009; Aleman and Tiver, 2010; Zhang et al., 2012). The aim of this study was to evaluate cadmium stress-induced effects on the growth, lipid peroxidation and antioxidant activity in *B. indica* and the ameliorative role of arbuscular mycorrhizal fungi (AMF) and calcium applications.

2. Materials and methods

2.1. Plant material, treatments and growth conditions

B. indica [Wight] A.J. Scott [*Kochia indica*] (Indian bassia) seeds were collected from salt marsh vegetation in the Al Rughabah region, Riyadh, in Saudi Arabia (Fig. 1). Healthy

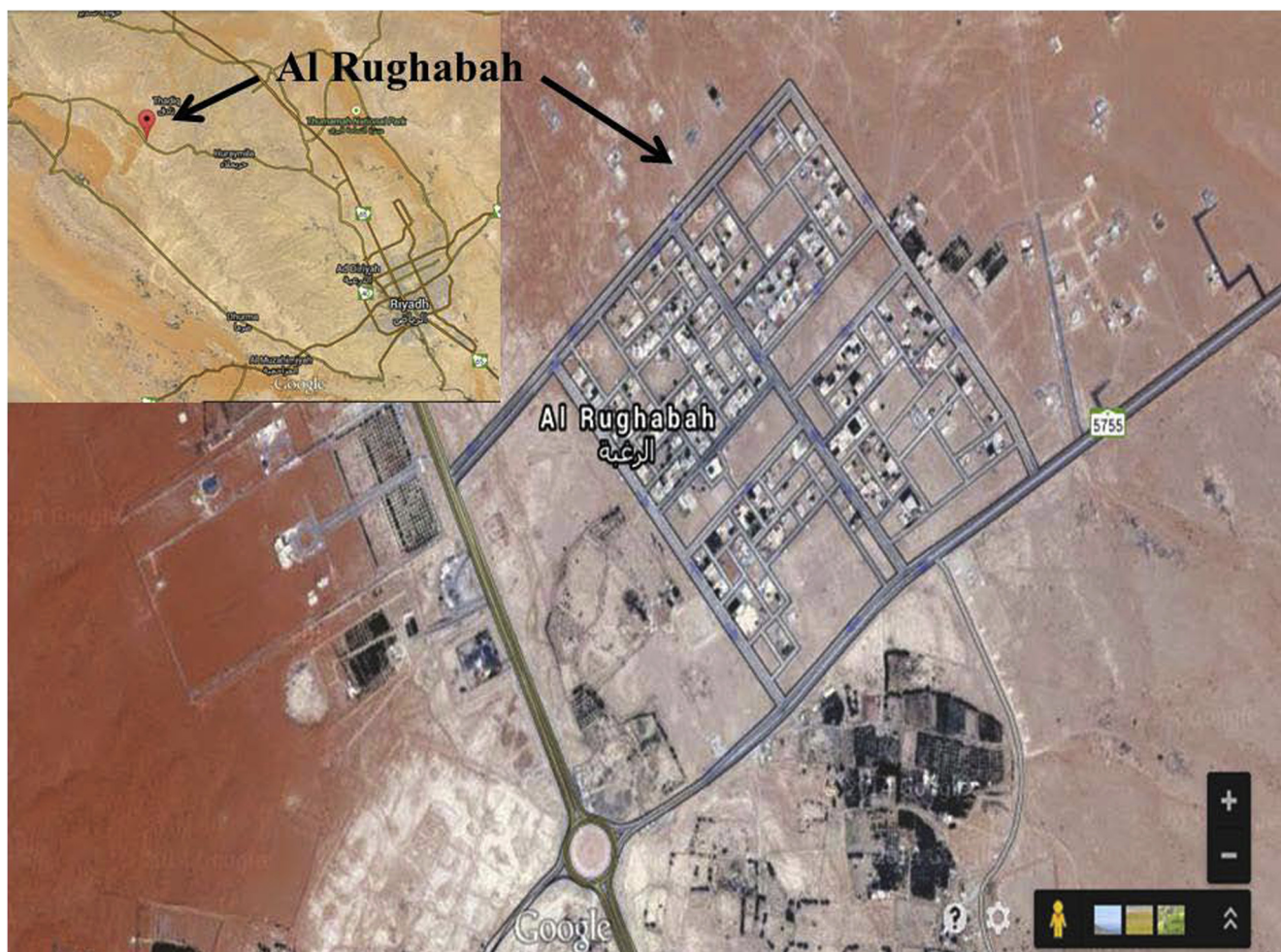


Figure 1 Google map showing the geographical location of seed sampling areas in Al Rughaba, Shakra, Riyadh, Saudi_Arabia.

seeds were germinated in petri dishes lined with blotter paper moistened with full strength Hoagland solution and were kept under controlled growth chamber conditions at 25 °C and a 16/8 h light/dark photoperiod with 1500 $\mu\text{mol m}^{-2} \text{S}^{-1}$ light intensity. After one week, germinated seedlings were transplanted into pots filled with 1.0 kg peat and sand (1:1) and were treated with 50 mL full Hoagland solution. After eight weeks of growth, cadmium stress was induced by adding 150 μM CdCl_2 to the Hoagland solution and pots receiving full strength Hoagland solution alone served as the control. Calcium (50 mM CaCl_2 ; 10 mL plant^{-1}) was applied foliarly using Tween-20 (0.05%) as a surfactant. Treatments of CdCl_2 and CaCl_2 were given every other day. The arbuscular mycorrhizal fungi used were *Funneliformis mosseae* (syn. *Glomus mosseae*), *Rhizophagus intraradices* (syn. *Glomus intraradices*) and *Claroideoglossum etunicatum* (syn. *Glomus etunicatum*) and were inoculated directly in the root medium by adding 10 g of trap soil accounting for approximately 100 spores/g trap soil (Hashem et al., 2014). At the end of pot experiment, the plants were removed from the pots very carefully and both fresh and dry samples of each treatment were collected for further analysis and measurements.

2.2. Determination of arbuscular mycorrhizal colonization

To determine mycorrhizal colonization by spores, wet sieving and decanting methods were adopted (Daniels and Skipper, 1982; Utobo et al., 2011). Roots were washed in ice-cold water (4 °C), cleaned using 10% KOH and were stained using trypan blue. Stained root segments (100 segment/treatment, approximately 25–30, 1 cm long root) were examined under a light microscope at 400 \times magnification. The degree of fungal infection, i.e., mycelium, vesicles and arbuscules, were calculated using the following formula:

$$\% \text{Colonization} = \frac{\text{Total number of AM positive segments}}{\text{Total number of segments studied}} \times 100$$

2.3. Photosynthetic pigments

Photosynthetic pigments were extracted from leaf samples (0.5 g) in 80% acetone and absorbance of the extracts was read at 480, 645 and 663 nm (Arnon, 1949).

Table 1 Effect of cadmium (150 µM CdCl₂), calcium (50 mM CaCl₂) and AMF on the photosynthetic pigments (mg/g fresh weight) of *Bassia indica* seedlings.

Treatments	Photosynthetic pigments (mg/g fresh weight)																		
	Chlorophyll a			Chlorophyll b			a/b			a + b			Carotenoids			Total pigment			
	AMF +	AMF -	SE	AMF +	AMF -	SE	AMF +	AMF -	SE	AMF +	AMF -	SE	AMF +	AMF -	SE	AMF +	AMF -	SE	
Control	1.75 ± 0.08	2.37 ± 0.14	0.065	0.89 ± 0.02	1.04 ± 0.04	0.031	1.96 ± 0.02	2.29 ± 0.06	0.105	2.65 ± 0.11	3.41 ± 0.12	0.05	0.51 ± 0.03	0.60 ± 0.03	0.019	3.16 ± 0.14	4.02 ± 0.18	0.057	
Cadmium	0.98 ± 0.04	1.33 ± 0.05	0.046	0.41 ± 0.01	0.54 ± 0.02	0.031	2.380.03	2.44 ± 0.04	0.105	1.39 ± 0.07	1.88 ± 0.09	0.05	0.26 ± 0.02	0.37 ± 0.01	0.019	1.65 ± 0.08	2.25 ± 0.12	0.057	
Calcium	1.83 ± 0.08	1.97 ± 0.08	0.046	1.11 ± 0.05	1.16 ± 0.05	0.031	1.64 ± 0.06	1.69 ± 0.03	0.105	2.94 ± 0.13	3.14 ± 0.15	0.05	0.61 ± 0.02	0.69 ± 0.02	0.019	3.56 ± 0.14	3.83 ± 0.16	0.057	
Cadmium + calcium	1.47 ± 0.06	1.55 ± 0.05	0.046	0.61 ± 0.07	0.71 ± 0.03	0.031	2.410.08	2.17 ± 0.05	0.105	2.08 ± 0.07	2.26 ± 0.07	0.05	0.430.01	0.50 ± 0.02	0.019	2.52 ± 0.08	2.77 ± 0.12	0.057	
LSD at: 0.05																			

Data presented are the means ± SE (n = 3), AMF: arbuscular mycorrhizal fungi.

2.4. Estimation of proline

First, 0.5 g of the leaf was extracted using sulfosalicylic acid and followed by centrifugation at 3000×g for 30 min. A known volume (2.0 mL) of supernatant was mixed with an equal volume of acid ninhydrin reagent and glacial acetic acid and samples were incubated at 100 °C for 10 min. The reaction was terminated by keeping the tubes on ice. Proline was separated using toluene and proline content was determined after recording the absorbance at 520 nm (Bates et al., 1973).

2.5. Estimation of lipid peroxidation (malondialdehyde, MDA)

Fresh leaves were macerated in 10% trichloroacetic acid and centrifuged at 1000×g for 10 min. Next, 1 mL of extract reacted with 0.25% thiobarbituric acid (prepared in trichloroacetic acid) at 95 °C for 30 min. After that, samples were ice cooled and centrifuged again at 10,000×g for 10 min and were read at 532 and 600 nm (Heath and Packer, 1968). An extinction coefficient of 155 mM cm⁻¹ was used for the calculation.

2.6. Estimation of hydrogen peroxide (H₂O₂) content

Fresh leaf samples were homogenized in cold acetone and 200 µL aliquots were added to 0.04 mL of TiO₂ (0.1%) and 0.2 mL NH₄OH (20%). The pellet was decollated using acetone and was re-suspended in H₂SO₄ (0.8 mL) and was centrifuged at 6000×g for 15 min. The supernatant was read at 415 nm (Mukherjee and Choudhuri, 1983).

2.7. Extraction and estimation of antioxidant enzymes

First, 500 mg were extracted in 10 mL of cold 50 mM phosphate buffer (100 mM, pH 7.8) and was centrifuged at 15,000×g for 15 min at 4 °C. The supernatant was used as the enzyme source and the proteins in the enzyme extract were estimated according to Lowry et al. (1951).

The activity of catalase (CAT, EC 1.11.1.6) was assayed by monitoring the change in absorbance at 240 nm for 2 min. The assay mixture contained 1.9 mL phosphate buffer (50 mM; pH 7.0), 1 mL H₂O₂, and 100 µL of the enzyme extract in a final volume of 3 mL (Aebi, 1984). The activity was expressed as EU mg⁻¹ protein.

Glutathione reductase (GR; EC 1.6.4.2) activity was assayed by monitoring the decrease in absorbance at 340 nm for 2 min (Foyer and Halliwell, 1976). Activity was expressed as U mg⁻¹ protein and GR activity was calculated using the extinction coefficient for NADPH at 6.2 mM⁻¹ cm⁻¹.

Superoxide dismutase (SOD, EC 1.15.1.1) was measured by following Giannopolitis and Ries (1977). The reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5), 0.1 µM EDTA, 13 mM L-methionine, 75 µM riboflavin, 75 µM NBT and 30 µL of enzyme extract was incubated for 15 min under light and was assayed using the inhibition of the photochemical reduction of nitro blue tetrazolium chloride (NBT) recorded at 560 nm against the non-illuminated blank. A 50% reduction was considered one unit of SOD activity.

For estimation of Ascorbate peroxidase (APX, EC 1.11.1.11) activity, the method by Jebara et al. (2005) was adopted and the formation of oxidized ascorbate was determined by following the change in absorbance at 290 nm. The 1 mL assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM hydrogen peroxide and 100 μ L of enzyme extract. The extinction coefficient of 2.8 $\text{mM}^{-1} \text{cm}^{-1}$ was used for the calculation.

The assay of polyphenol oxidase (PPO, EC 1.10.3.1) was done following the method proposed by Raymond et al. (1993). The oxidation of pyrogallol was observed at 430 nm and the activity was expressed in μM of pyrogallol oxidized per min per mg protein [unit mg^{-1} protein].

The method by Abeles and Biles (1991) was used to determine the peroxidase (POX, EC 1.11.1.7) activity. The 4 mL reaction mixture contained 0.2 M acetate buffer (pH 4.8), 3% H_2O_2 (0.4 mL), 20 mM benzidine (0.2 mL) and 0.03 mL enzyme extract. The change in absorbance was measured at

530 nm and activity was determined as 1 μM of benzidine oxidized per min per mg protein [unit mg^{-1} protein].

2.8. Estimation of ascorbate, reduced glutathione, oxidized glutathione and S-nitrosoglutathione by Liquid chromatography-electrospray/mass spectrometry

First, 0.5 g of plant samples were extracted in 0.1 M HCl (1 mL) using a mortar and pestle and centrifuged at 15,000 \times g for 20 min at 4 $^{\circ}\text{C}$. The supernatant was filtered through polyvinylidene fluoride filters (0.22 μm) and were analyzed. GSH (reduced glutathione), GSSG (oxidized glutathione) and GSNO (S-nitrosoglutathione) were quantified using liquid chromatography-electrospray/mass spectrometry (LC-ES/MS) connected to a Micromass Quattro Micro API triple quadrupole mass spectrometer GSNO (Airaki et al., 2011).

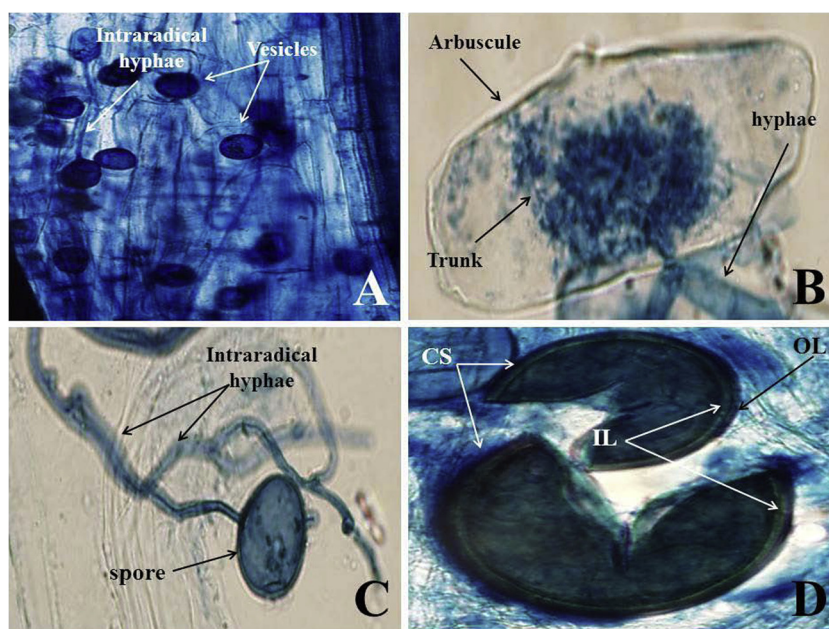


Figure 2 (A–D) (A, 20X): The structural colonization of AMF in roots of *Kochia* (*Bassia indica*). (B, 40X): Trunk, arbuscule and hyphae of AMF (arrow). (C, 40X): Intact mycorrhizal spore and intraradical hyphae (arrow). (D, 40X): Crushed spores (CS) showing the outer thin layer (OL) and inner thick laminated layer (IL).

Table 2 Effect of cadmium (150 μM CdCl_2) and calcium (50 mM CaCl_2) on the mycorrhizal status of *Bassia indica* seedlings.

Treatments	Mycorrhizal status			
	Total spores count/100 g soil	Structural colonization (%)		
		Mycelium	Vesicles	Arbuscules
Control	160.3 \pm 6.12	94.9 \pm 3.15	62.6 \pm 3.55	66.9 \pm 3.75
Cadmium	47.6 \pm 3.71	53.1 \pm 2.79	41.6 \pm 1.57	17.1 \pm 2.47
Calcium	214.5 \pm 7.36	109.3 \pm 5.01	71.2 \pm 3.82	36.9 \pm 3.05
Cadmium + calcium	102.4 \pm 4.85	83.0 \pm 2.93	53.9 \pm 2.34	24.7 \pm 1.44
LSD at: 0.05	12.4	7.07	6.37	3.12

Data presented are the means \pm SE ($n = 3$).

Table 3 Effect of cadmium (150 μM CdCl_2), calcium (50 mM CaCl_2) and AMF on hydrogen peroxide (H_2O_2) as mM/g fresh weight, lipid peroxidation as malondialdehyde (MDA, $\mu\text{g g}^{-1}$ fresh wt) and proline ($\mu\text{mol proline g}^{-1}$ FW) of *Bassia indica* seedlings. Data presented are the means \pm SE ($n = 3$).

Treatments	Aspect of acquired systemic resistance					
	H_2O_2		MDA		Proline	
	AMF -	AMF +	AMF -	AMF +	AMF -	AMF +
Control	12.6 \pm 1.04	15.4 \pm 0.71	16.5 \pm 0.82	17.6 \pm 0.94	58.1 \pm 1.83	62.7 \pm 2.04
Cadmium	32.6 \pm 2.33	26.9 \pm 1.53	41.8 \pm 1.59	30.8 \pm 1.76	134.7 \pm 4.25	142.1 \pm 4.34
Calcium	17.7 \pm 1.12	16.6 \pm 0.78	18.0 \pm 0.98	17.3 \pm 0.94	70.2 \pm 2.56	75.5 \pm 2.75
Cadmium + calcium	24.7 \pm 1.75	21.8 \pm 1.23	27.7 \pm 1.69	24.8 \pm 1.71	146.0 \pm 6.94	154.4 \pm 7.11
LSD at: 0.05	0.32	0.23	0.40	0.28	0.39	0.28

2.9. Determination of nicotinamide adenine dinucleotide phosphate (NADPH and NADP^+)

Frozen plant samples were powdered and subsequently suspended in separate preheated 0.1 N NaOH or HCl solutions. After incubating at 100 $^\circ\text{C}$ for 2 min, samples were cooled on followed by centrifugation for 6 min at 12,000 $\times g$. The supernatants were used to quantify NADPH and NADP following the enzyme cycling method (Matsumura and Miyachi, 1980).

2.10. Statistical analysis

Experiments were performed three times and data were statistically analyzed using SPSS 20 software. Least Significant Difference (LSD, $P = 0.05$) was calculated at $P < 0.05$ in a completely randomized design.

3. Results

The results related to the effects of cadmium stress on chlorophyll pigments are shown in Table 1. Cadmium stress resulted in a drastic decline in chlorophyll *a*, chlorophyll *b*, total chlorophylls and carotenoids by 44.1%, 53.9%, 47.7% and 49.3%, respectively. Plants treated with calcium and inoculated with AMF alone showed enhanced synthesis of these pigments under non-stress conditions. The increase in chlorophyll *a*, chlorophyll *b*, total chlorophylls and carotenoids due to the calcium treatment was 4.5%, 24.7%, 12.6% and 20%, respectively, while the AMF increased the pigments 35.3%, 16.8%, 21.3% and 16.9%, respectively. Reduced chlorophyll *a* (16.1%), chlorophyll *b* (31.1%), total chlorophylls (20.2%) and carotenoids (15%) were observed in the calcium-treated plants under Cd stress. The stressed plants treated with calcium combined with AMF reduced chlorophyll *a*, chlorophyll *b*, total chlorophylls and carotenoids by 11.5%, 19.8%, 112.3%, and 2.5%, respectively, (Table 1).

Different structural colonizations of AMF as mycelium, vesicles and arbuscules were observed in the roots of *B. indica* as described in Fig. 2(A–H). Mycorrhizal colonization status measured in terms of percent mycelium, vesicles and arbuscules were reduced drastically due to cadmium stress (Table 2). The percent reduction in mycelium, vesicles and arbuscules due to cadmium stress was 44%, 33.4% and 74.4%, respectively. Calcium alone increased the AMF colonizing potential with a 15.17%, 13.7% and 44.83% increase in the mycelium, vesicles and arbuscules, respectively. Moreover, the calcium treat-

ment also mitigated the adverse effects of cadmium on such parameters. Relative to the control, calcium-treated plants showed only a 12.5%, 13.8% and 63% reduction in mycelium, vesicles and arbuscules, respectively, under Cd stress (Table 2).

Results pertaining to the levels of oxidative damage measured in terms of H_2O_2 production and malondialdehyde (MDA) content are shown in Table 3. The stressed plants showed a 158.4% and 152.1% increase in H_2O_2 and MDA, respectively. However, relative to the control, calcium-treated stressed plants showed a 48.79% and 67.15% increase, while the AMF-inoculated cadmium-stressed (AMF + Cd) plants showed only a% and% increase in H_2O_2 and MDA, respectively (Table 3). Calcium combined with AMF resulted in an increase in H_2O_2 and MDA under Cd stress by 72.8% and 49.9%, respectively. Cadmium stress increased proline accumulation by 131.6%, and increased in AMF-inoculated plants by 7.9% relative to the control (Table 3). Cadmium-stressed AMF-inoculated (Cd + AMF) seedlings showed a 144.4% increase in proline accumulation. Calcium alone and in combination with AMF enhanced proline accumulation by 20% and 29.8%, respectively (Table 3). Relative to the control, calcium-treated cadmium-stressed (Ca + Cd) and calcium-treated AMF-inoculated cadmium-stressed (Ca + AMF + Cd) plants increased plant proline by 151.2% and 165.6%, respectively (Table 3).

Cadmium stress reduced ascorbate, oxidized glutathione (GSSG), and reduced glutathione (GSH) and S-nitrosoglutathione (GSNO) by 72.3%, 26%, 57.4% and 62.33%, respectively (Fig. 1a–d). However, in plants treated with calcium alone, ascorbate, GSSG, GSH, and GSNO increased by 14.4%, 32.9%, 13.6% and 19.6%, respectively, while AMF inoculation alone caused a 10.2%, 19.57%, 7% and 9.7% increase in ascorbate, GSSG, GSH, and GSNO, respectively. Relative to the control, the reduction in cadmium-stressed AMF-inoculated (Cd + AMF) and cadmium-stressed calcium-treated (Cd + Ca) plants was only 39.9%, 15.6%, 38.5%, 41.4% and 21.5%, 8%, 32.1%, and 30.4% for ascorbate, GSSG, GSH, and GSNO, respectively (Fig. 1A–D).

AMF inoculation enhanced NADPH and NADP by 5.78% and 5.78%, respectively, while cadmium stress reduced NADPH by 32.3% and increased NADP by 5.7% (Fig. 2E and F). The calcium treatment increased NADPH by 7.9% and calcium along with AMF (Ca + AMF) caused a 9.5% increase (Fig. 1E). Cadmium-stressed calcium-treated (Cd + Ca) and cadmium-stressed AMF-inoculated (Cd + AMF) seedlings showed only a 9.5% and 17.8% reduction in

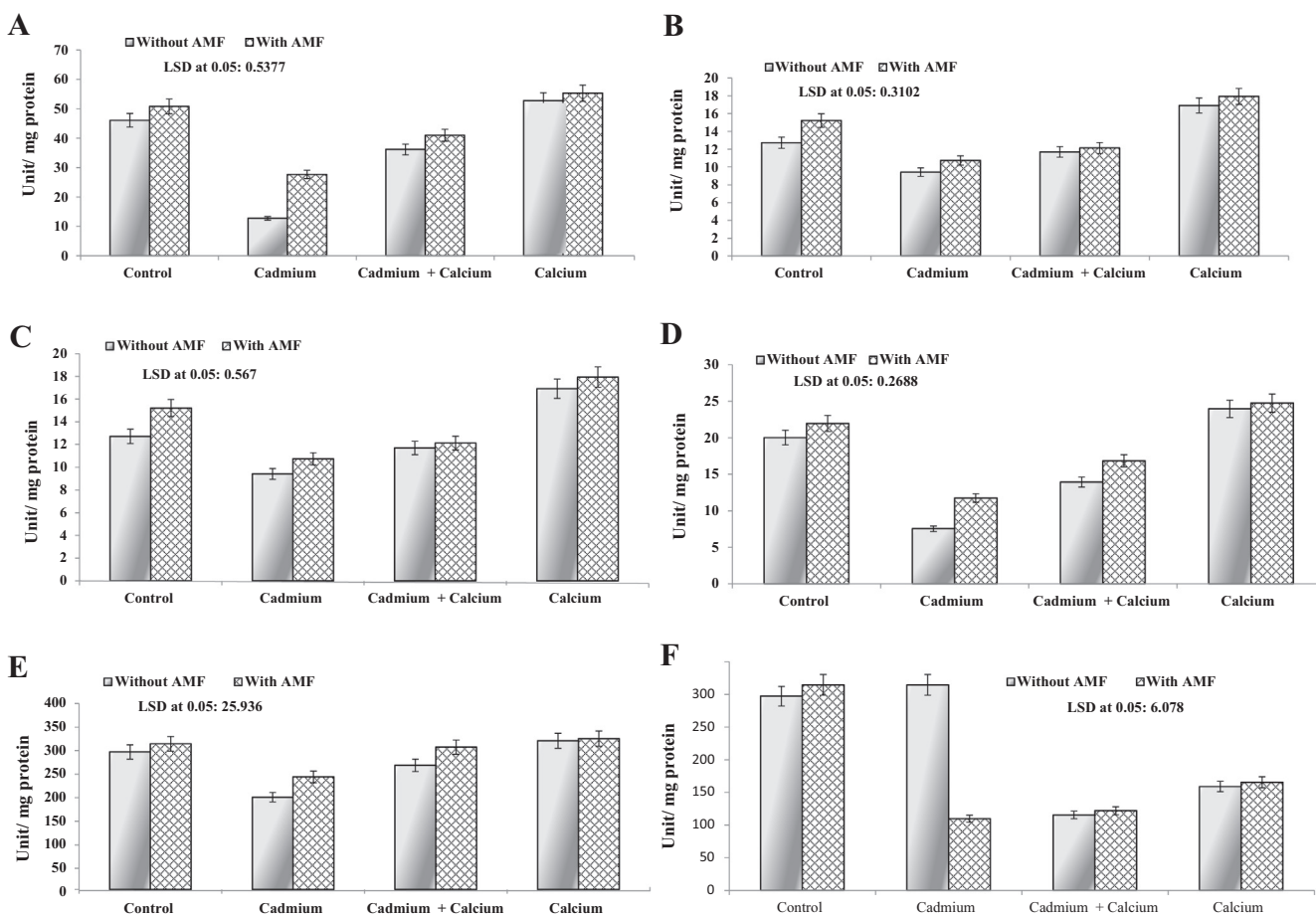


Figure 3 (A–F) Effect of cadmium (150 μM CdCl_2) in the presence and absence of calcium (50 mM) and AMF on (A) ascorbate, (B) oxidized glutathione (GSSG), (C) reduced glutathione (GSH), (D) S-nitrosoglutathione (GSNO), (E) NADPH and (F) NADP of *Bassia indica* seedlings. Data presented are the means \pm SE ($n = 3$).

NADPH, which is lower than cadmium stress alone (Fig. 1E). Cadmium-stressed calcium-treated AMF-inoculated (Cd + Ca + AMF) plants showed a 3.3% increase in NADPH (Fig. 1E).

The antioxidant enzyme activities are shown in Fig. 2A–F. Cadmium stress enhanced the activity of all the antioxidant enzymes studied, and the percent increase in APX, SOD, PPO, CAT, POX and GR due to cadmium stress was 401.3%, 86.6%, 38.7%, 141.4%, 100% and 113.6%, respectively. The application of calcium and AMF inoculation to cadmium-stressed plants further increased the activity of all the enzymes. The application of calcium alone caused an increase of 51.7%, 18.3%, 15.4%, 24.34%, 17.2% and 56.8% in APX, SOD, PPO, CAT, POX and GR, respectively, (Fig. 2a–f). The application of calcium to stressed plants increased the activity of APX, SOD, PPO, CAT, POX and GR by 491.1%, 105.7%, 77.1%, 186.4% 133.7% and 232.9%, respectively (Fig. 2a–f). The inoculation of AMF increased the activities of APX, SOD, PPO, CAT, POX and GR by 28.5%, 11.24%, 7%, 11.06%, 10.6% and 39.7%, respectively. The AMF inoculation to cadmium-stressed plants (Cd + AMF) caused an increase of 442%, 97.4%, 57.4%, 158.8%, 113.1% and 182.9% in the activity of APX, SOD, PPO, CAT, POX and GR, respectively (Fig. 2A–F). More-

over, cadmium-stressed calcium-treated and AMF-inoculated (Cd + Ca + AMF) seedlings showed a 536.8%, 133.5%, 93.4%, 201.4%, 147.5% and 269.3% increase in APX, SOD, PPO, CAT, POX and GR, respectively (see Figs. 3 and 4).

4. Discussion

In our study, we observed that cadmium negatively affects plant growth and the physio biochemical attributes of *B. indica*. The inhibited chlorophyll content in plants due to cadmium stress was also observed by Chen et al. (2011) for mustard, by Liu et al. (2014) and for cotton and for sunflower (Abd_Allah et al., 2015). Plants exposed to stressful conditions have enhanced chlorophyllase activity, thereby exhibiting increased chlorophyll pigment degradation. AMF inoculation not only increased the chlorophyll pigments but also mitigated the harmful impact of cadmium stress. Our results showing enhanced pigment synthesis and the subsequent amelioration by AMF are in corroboration with the results of Malekzadeh et al. (2012) and Abd_Allah et al. (2015). Our results of increased chlorophyll content due to calcium applications supports the findings of Ahmad et al. (2015) for *Brassica juncea* and Khayyat et al. (2009) for strawberry, who

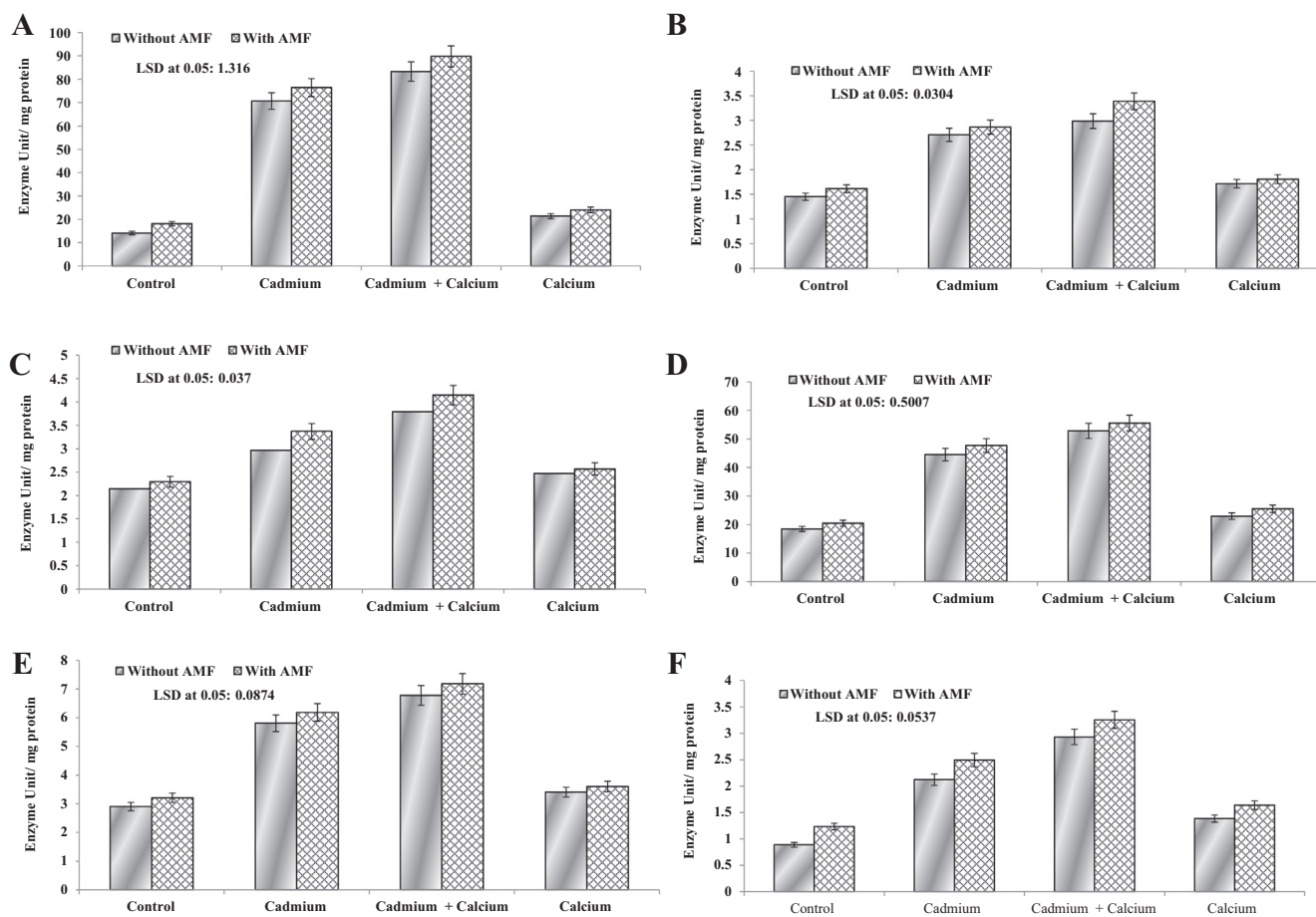


Figure 4 (A–F) Effect of cadmium (150 μM CdCl_2) in the presence and absence of calcium (50 mM CaCl_2) and AM fungi on (A) APX, (B) SOD, (C) PPO, (D) CAT, (E) POD and (F) GR of *Bassia indica* seedlings. Data presented are the means \pm SE ($n = 3$).

observed an increase in chlorophyll pigments due to calcium applications, even under cadmium stress. AMF inoculation had a direct effect on the uptake of mineral elements, such as magnesium, which forms an important part of the chlorophyll pigment. Sheng et al. (2008) also showed a close relation between chlorophyll pigments and magnesium uptake. An increase in chlorophyll synthesis and the subsequent recovery from cadmium stress due to AMF may be because of the increased uptake of magnesium, as well as increased de novo synthesis of proteins and chlorophylls. Moreover, cadmium stress exerted a great reduction in AMF colonization potential, and our results corroborate with the findings of Aroca et al. (2013) and Abd-Allah et al. (2015).

The exposure of *B. indica* to cadmium stress caused an increased production of free radicals, such as H_2O_2 , and enhanced the rate of membrane lipid peroxidation. Membrane lipids are very sensitive to toxic ROS and undergo oxidation, resulting in the formation of peroxide radicals. Increased lipid peroxidation under stressful conditions is ascribed to increased lipoxygenase activity, resulting in rapid peroxidation of membrane lipids and an increased production of toxic peroxides and hydroxyl radicals that cause further deterioration to the membranes and other important cellular components (Djebali et al., 2005; Tuteja et al., 2009). In rice, Kuo and Kao (2004) demonstrated that an increase in the production of H_2O_2 resulted in oxidative stress and reduced growth. Our

study showed that the calcium and AMF treatments ameliorated the negative impact of cadmium stress by causing a considerable reduction in H_2O_2 production and MDA content. Our results showing reduced lipid peroxidation and H_2O_2 production in calcium-treated, as well as AMF-inoculated plants, confirms the role of calcium and AMF in the mitigation of cadmium stress-induced harmful changes. This may be due to increased uptake and assimilation of phosphorous, as well as enhanced antioxidant activity in calcium-treated and AMF-inoculated plants. Ling-Zhi et al. (2011) have also observed the ameliorative role of AMF against cadmium stress. Blasco et al. (2015) have reported reduced lipid peroxidation due to a calcium treatment in *Brassica rapa*. The reduced H_2O_2 and MDA production in AMF-inoculated and calcium-treated plants suggest the mitigation of cadmium induced oxidative damage.

Under stress condition, plants try to up-regulate synthesis and the accumulation of several organic osmolytes that have an important role in growth maintenance under such conditions (Tomar et al., 2015; Abd-Allah et al., 2015). Among such osmolytes, proline, glycine, betaine and sugar alcohols and their accumulation help plants to maintain cellular water potential well below that of the external soil solution. Proline protects cellular membranes, enzymes and other important cellular structures by neutralizing ROS, thereby contributing to growth under stress conditions (Hare and Cress, 1997). Higher

contents of proline do not interfere with the metabolic pathways; rather, it replaces water in these processes (Zhifang and Loescher, 2003). In our results, a clear increase in proline accumulation was reported in cadmium-stressed plants, which was further increased by the AMF inoculation and calcium treatments. Similar observations were reported for *Lycopersicon esculentum* (Hayat et al., 2011) and *Helianthus annuus* (Abd_Allah et al., 2015). The enhancement in the accumulation of proline in our study due to the calcium treatment and AMF inoculation support their role in maintenance of growth under stress conditions through maintaining tissue water content. In cadmium-stressed sunflower plants, Abd_Allah et al. (2015) also demonstrated an AMF induced increase in proline accumulation and a subsequent amelioration of cadmium stress. In drought-stressed *Zoysia japonica*, Xu et al. (2013) observed an increase in proline accumulation due to an exogenous application of calcium.

The exposure of plants to stress conditions can cause an abrupt increase in ROS production, resulting in impeded growth and metabolism. To mitigate the adverse impact of stress, plants up-regulate the activity of the antioxidant defense system that comprises both enzymatic as well as non-enzymatic systems, working in coordination to scavenge toxic ROS. An increase in antioxidant enzyme activity due to cadmium stress is in corroboration with the findings of Irfan et al. (2014) for mustard and Abd_Allah et al. (2015) for sunflower. This increased antioxidant activity to stress exposure mediates the quick removal of toxic ROS, thereby reducing the oxidative damage induced harmful impact to some extent and helping plants to recover from the stress (Ahanger et al., 2014; Wu et al., 2014). The antioxidant enzymes assayed (SOD, CAT, POD, GR, PPO and APX) in our study showed increased activity due to AMF inoculation and the calcium treatment, which strongly supports the role of calcium and AMF in enhancing cadmium stress tolerance by mediating the scavenging of ROS. In cadmium-stressed *Ipomoea aquatica* (Bhaduri and Fulekar, 2012) and sunflower (Abd_Allah et al., 2015), an increase in the activities of antioxidants had been demonstrated, where there was more of an increase due to AMF inoculation, resulting in maintained growth to some extent. In addition to the enzymatic components, ascorbate peroxidase (APX) and glutathione reductase (GR), the non-enzymatic components ascorbate, reduced glutathione (GSH) and oxidized glutathione (GSSH), form the important components of the ascorbate–glutathione cycle. The increased synthesis of ascorbate, GSH and GSSH in AMF-inoculated and calcium-treated plants was clear in our results. AMF and calcium increased the content of these non-enzymatic components that helped maintain the optimal functioning of the important ROS scavenging pathway, the ascorbate–glutathione cycle, which mediates the efficient scavenging of H₂O₂. The NADPH dependent conversion of GSSH to GSH is carried out by GR to maintain the higher ratio of GSH/GSSH (Noctor and Foyer, 1998). In our results, the increased contents of NADPH in AMF-inoculated and the calcium-treated plants suggest the role of AMF and calcium in maintaining the optimal activity of GR by keeping sufficient levels of electron donor NADPH available for its activity. In calcium-treated *B. rapa*, Blasco et al. (2015) demonstrated an enhanced activity of enzymatic antioxidants as well as the contents of non-enzymatic antioxidants.

5. Conclusion

Cadmium stress causes problems in the normal metabolism of *B. indica*, which was clear in the evaluated physiological and biochemical attributes. However, AMF inoculation combined with calcium not only ameliorated the adverse impacts of cadmium but also strengthened the antioxidant system. The enhanced activities of antioxidant enzymes due to AMF and calcium applications support the role of calcium in enhancing the growth of *B. indica* in AMF symbioses under Cd stress and suggest the treatment as a potential strategy for enhancing stress tolerance levels.

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