

## ALLEVIATION OF ADVERSE IMPACT OF CADMIUM STRESS IN SUNFLOWER (*HELIANTHUS ANNUUS* L.) BY ARBUSCULAR MYCORRHIZAL FUNGI

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### Abstract

Sunflower (*Helianthus annuus* L.) is an important ornamental plant and good source of vegetable oil, widely accepted as potential promising plant for phytoremediation. A pot experiment was conducted to evaluate the impact of cadmium on the growth and some biochemical attributes of sunflower and role of arbuscular mycorrhizal fungi (AMF) in assuaging the cadmium stress induced changes. Cadmium treatment reduced growth, chlorophyll contents and cell membrane stability. AMF inoculated plants showed increased growth, chlorophyll contents and cell membrane stability and also mitigated changes caused due to cadmium. Cadmium caused increase in lipid peroxidation, and hydrogen peroxide production. An increase in antioxidant enzyme activity was observed due to cadmium treatment which was further enhanced by inoculation of AMF. Increase in proline and total phenols due to cadmium stress was obvious. Cadmium stressed plants showed enhanced fatty acid content. AMF inoculated plants showed higher activities of acid and alkaline phosphatases which were reduced by cadmium stress. However palmitoleic acid (C<sub>16:1</sub>), oleic (C<sub>18:1</sub>), linoleic (C<sub>18:2</sub>) and linolenic acid (C<sub>18:3</sub>) reduced in cadmium treated plants and the negative impact of cadmium was mitigated by AMF.

**Keywords:** Antioxidants, Phosphatases, Lipid peroxidation, Fatty acids, Cadmium, AMF *Helianthus annuus*.

### Introduction

Cadmium is one of the toxic heavy metals present in soil in low concentrations. Due to its high mobility between soil-plant systems it is quickly absorbed by the plants and transported to upper sensitive parts (Hart *et al.*, 1998). Increase in cadmium concentration causes retardation of growth leading to necrosis, altered nutrient uptake, reduced enzyme activity and phytotoxicity (Groppa *et al.*, 2012). Both natural as well as anthropogenic processes further contribute to increase cadmium levels in the soil so further aggravating its deleterious effects on crop growth. Smelting, mining, excessive phosphate fertilization, sewage sludge and use of cadmium polluted water for irrigation are the prime sources of cadmium pollution (Zoffoli *et al.*, 2013).

Although cadmium is a non-redox heavy metal but induces oxidative stress by triggering the excess generation of reactive oxygen species [ROS] (Al-Dhaibani *et al.*, 2013). Cadmium alters redox homeostasis by reducing activities of the enzymes involved in the maintenance of the redox homeostasis and hence resulting in the generation of ROS through interfering with the electron transport chain (Yan *et al.*, 2013). ROS cause peroxidation of membrane lipids leading to loss of integrity and hence leakage. Moreover ROS also causes oxidation of nucleic acids, proteins and chlorophylls thereby affect the normal functioning of cell (Ahmad *et al.*, 2010a; Wu *et al.*, 2014). Plants use several indigenous strategies to avert the toxic effects of cadmium stress. Increased synthesis and accumulation of organic solutes for maintaining the cell water content, upregulation of the activities of antioxidants for quick scavenging of ROS

contribute to better adaptation of the plants to stress conditions (Yan *et al.*, 2013). Antioxidant defence includes both enzymatic as well as non enzymatic constituents for example, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR) and phenolics.

Primary effect of heavy metal stress is the damage to the cellular membranes and causing alteration in lipid composition of membranes. Heavy metal stress causes conversion of polyunsaturated fatty acids into small fragments of hydrocarbons (Kappus, 1985). In addition to this metal ions replace calcium at the plasma membrane sites easily. Increased membrane lipid peroxidation results in loss of membrane integrity and consequently affecting nutrient uptake (Quartacci *et al.*, 2000). Stress induced changes in membrane compositions might have both adaptive as well as deleterious effects on plant physiology (Mansour & Salama, 2004).

Several plants such as *Helianthus annuus* (Kavitha & Nelson, 2013); *Ephedra aphylla* (Alqarawi *et al.*, 2014); *Solanum lycopersicum* (Hashem *et al.*, 2015) and *Vicia faba* (Hashem *et al.*, 2014) are in intimate association with arbuscular mycorrhizal fungi. AMF has been widely accepted as one of the biological tool for mitigating the adverse effects of stresses on crop plants (Wu *et al.*, 2014). AMF promotes plant growth by bringing morpho-physiological and biochemical changes in host plants. AMF causes modification in root morphology so as to mediate increased water and mineral uptake (Alqarawi *et al.*, 2014; Hashem *et al.*, 2015). AMF has been reported to enhance uptake of essential mineral nutrients (Aroca *et al.*, 2013; Wu *et al.*, 2014; Hashem *et al.*, 2015).

Sunflower (*Helianthus annuus* L.) is an important ornamental plant and a good source of vegetable oil used for chemical and industrial purposes (Riva & Calzoni, 2004) and is considered as an important component of phytoremediation of inorganic and organic soil contaminants (Adesodun *et al.*, 2010; Fassler *et al.*, 2010). Present experiments were conducted with the aim to evaluate the deleterious impact of cadmium stress on growth of sunflower focusing on the antioxidant components and the fatty acid composition and the role of AMF in mitigating the stress impacts.

## Material and Methods

**The pot experiment:** Seeds of salt tolerant sunflower (*Helianthus annuus* L., cultivar, Sakha 53) were obtained from Agricultural Research Center, Giza, Egypt. The experiment was set-up in a factorial completely randomized design and each treatment replicated thrice. Seeds were surface sterilized with sodium hypochlorite (0.5%) for 3 min and washed thoroughly with distilled water before germination on blotter. The germinated seeds were planted in plastic pots (25 cm in diameter) which containing peat, perlite and sand (1:1:1). Thinning was done by maintaining one plant per pot. The seedlings were allowed to grow for ten weeks under constant temperature  $25\pm 4$  °C and 12 h of photoperiod with a photosynthetic photon flux density of  $1500 \mu\text{mol m}^{-2} \text{S}^{-1}$ . Plants were irrigated with full strength Hoagland's solution (Hoagland & Arnon 1950) for two weeks. After two weeks of germination (four to five leaves) cadmium stress was induced by supplementing Hoagland's solution with  $100 \mu\text{M CdCl}_2$  and 50ml was used for irrigation after every two days. Pots receiving only Hoagland's solution served as control. The arbuscular mycorrhizal fungi used in the present study was previously isolated from salt march soil (Hashem *et al.*, 2014). AMF was added as mixture of *Funneliformis mosseae* (syn. *Glomus mosseae*), *Rhizophagus intraradices* (syn. *Glomus intraradices*) and *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*). The mycorrhizal inoculum was added to the experimental pots as 10 g of trap soil (approximately 100 spores/g trap soil, M= 80%). The control plants were kept free of AMF and only supplied with normal Hoagland's nutrient solution. At the end of pot experiment, the plants were removed from the pots very carefully and the morphological characters were measured. Fresh plant samples were dried at 70°C up to two successive weights and dry mass of all samples measured.

### Determination of arbuscular mycorrhizal colonization:

The mycorrhizal spores from the experimental soil of each treatment were extracted by wet sieving and decanting method as described by Daniels & Skipper (1982) and modified by Utobo *et al.*, (2011). Total population of mycorrhizal spore was calculated per hundred gram soil. The root system was washed carefully in ice-cold water (4°C) and cleaning was done in 10% KOH followed by staining with trypan blue in lactophenol. The stained root segments (100 segment/treatment, approximately 25-30, 1 cm long root) were examined under light microscope at

400x magnification. The intensity of fungal infection (mycelium, vesicles and arbuscules) and development within the infected regions of the roots were calculated according to the following formula:

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

### Determination of acid phosphatase and alkaline phosphatase activity:

The activity of both acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were determined according to Gianinazzi-Pearson & Gianinazzi (1976). The reaction mixture of acid phosphatase contained 0.2 ml of enzyme extract, 1.0 ml of 5.5 mM p-nitrophenol phosphate in 5.5 mM citrate buffer (pH 4.8). In case of alkaline phosphatase, 0.05M tris-citrate (pH 8.5) was used instead of citrate buffer (pH 4.8). The reaction mixture was incubated at 37°C for 30 minutes and the reaction stopped by adding 10 ml of 200 mM NaOH. Absorbance was recorded at 410 nm and the activity was expressed as  $\mu\text{mol p-nitrophenol released min}^{-1} \text{mg protein}^{-1}$ .

**Determination of phosphorus (P):** Phosphorous was estimated by digesting dry plant sample in nitric-perchloric acid and using Vanado-molybdophosphoric colorimetric method (Jackson, 1962). Standard curve ( $10\text{--}100 \mu\text{g/ml}$ ) of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) was used as reference.

**Determination of cadmium (Cd):** Dry plant material (0.5 g) was digested in mixture of nitric acid and perchloric acid (4:1) using the hot block digestion procedure (overnight at 60°C) according to the method of Jackson (1962) and described in details by Burd *et al.* (2000). After cooling, 1.0 ml of hydrogen peroxide (30%, v/v) was added to each sample and incubated for 2 hours. The cadmium concentration was measured by flame atomic absorption spectrophotometer (Perkin Elmer AA700, USA).

**Photosynthetic pigments:** Photosynthetic pigments were extracted from leaf samples (0.5 g) in 80% acetone as described by Arnon (1949). The optical densities of the supernatant were recorded at 480, 645 and 663 nm against a blank containing acetone (80%). Contents of chlorophylls and carotenoids were calculated using the following formulae:

$$\begin{aligned} \text{Chl } a \text{ (mg g f.wt.}^{-1}\text{)} &= [12.7(\text{OD}_{663}) - 2.69(\text{OD}_{645}) \times V / 1000 \times W] \\ \text{Chl } b \text{ (mg g f.wt.}^{-1}\text{)} &= [22.9(\text{OD}_{645}) - 4.68(\text{OD}_{663}) \times V / 1000 \times W] \\ \text{Carotenoids (mg g f.wt.}^{-1}\text{)} &= A^{\text{car}} / E_m \times 100 \end{aligned}$$

where, V represents volume of the aliquot and W, weight of tissue  $A^{\text{car}} = \text{OD}_{480} + 0.114(\text{OD}_{663}) - 0.638(\text{OD}_{645})$  and  $E_m = 2500$ .

**Estimation of proline:** Leaf samples (0.5 g) were extracted using sulfosalicylic acid followed by centrifugation at 3000g for 30 minutes. Known volume (2.0 ml) of supernatant was mixed with same volume of acid ninhydrin solution [1.25 g ninhydrin, with 30 ml glacial acetic acid, and 20 ml of 6 M phosphoric acid] and

glacial acetic acid. The samples were incubated at 100°C for 10 min and reaction was terminated by keeping the tubes in container filled with ice. After cooling, proline was separated with 4 ml toluene and optical density was measured at 520 nm. The toluene was used as blank (Bates *et al.*, 1973).

$\mu\text{mol proline g}^{-1} \text{ FW} = \mu\text{g proline mL}^{-1} \times \text{mL of toluene}/115.5/\text{sample wt (g)}$ .

**Relative membrane permeability (RMP):** The method of Yang *et al.* (1996) was used to measure RMP of the leaf tissues. In this method, discs were cut from fully developed 3<sup>rd</sup> leaf from each plant and placed in test tubes filled with 20ml distilled H<sub>2</sub>O and initial electrical conductivity (EC<sub>0</sub>) of each sample was recorded. The samples were stored at 4°C for one day and conductivity (EC<sub>1</sub>) was measured again. Samples were then autoclaved for 20 minutes, cooled to room temp and conductivity (EC<sub>2</sub>) was recorded. The cell membrane permeability was calculated as follow:

Relative membrane permeability (%) =  $[(\text{EC}_1 - \text{EC}_0) / (\text{EC}_2 - \text{EC}_0)] \times 100$

**Estimation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>):** Hydrogen peroxide was estimated according to the method of Velikova *et al.* (2000). Fresh leaf tissue (0.5 g) was macerated with 5 ml of 0.1% tri-chloroacetic acid (TCA). Homogenate was then centrifuged at 12,000g for 15 min. To 0.5 ml of the supernatant, 0.5 ml of potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide were added. After vortexing the mixture its OD was read at 390 nm.

**Estimation of lipid peroxidation (malondialdehyde, MDA):** Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described by Heath & Packer (1968). The absorbance was recorded at 600 nm and 532nm (1% thiobarbituric acid in 20% trichloroacetic acid was used as blank). The concentration of MDA was calculated using an extinction coefficient of 155 mM cm<sup>-1</sup>.

$$\text{MDA} = \Delta (\text{OD}_{532} - \text{OD}_{600}) / 1.56 \times 10^5$$

**Estimation of total phenolics:** The total phenolics were extracted with 80% (v/v) acetone and estimated using sodium carbonate (20%) and Folin and Ciocalteu's phenol reagent following Julkunen-Tiitto (1985). The optical density of the mixture was read at 750 nm. Standard curve of pyrogallol was used as reference.

**Lipid extraction and separation:** The total lipids were extracted according to the method of Folch *et al.* (1957) modified by Bligh & Dyer (1959) using chloroform: methanol (2:1 v/v); 0.05% L<sup>-1</sup> and butylated hydroxytoluene (BHT) was added to all solvents as antioxidant to prevent lipid peroxidation (Cachorro *et al.*, 1993). Fatty acid methyl esters were prepared according

to the method described by Metcalfe *et al.* (1966). Methyl esters of fatty acids were separated and quantified with by gas liquid chromatography (GLC) [Perkin-Elmer Model 910, Perkin Elmer, Shelton, CT, USA] equipped with a flame ionization detector (Johnson & Stocks, 1971). A dual-open recorder and a computing integrator (Perkin-Elmer Model M1) were attached to GLC for recording. Both the injector and detector were maintained at 230 and 250°C, respectively. Nitrogen was used as the carrier gas at 1 ml/min with split injector system (split ratio 1:100). The separation and quantitation of peak fatty acid methyl esters were identified by comparing their retention times with those of an authentic methyl ester standard (Sigma Co., St. Louis, USA).

**Extraction and estimation of antioxidant enzyme:** Fresh leaf samples (500 mg) were homogenized in 10 mL of chilled 50 mM phosphate buffer (pH 7.8) (Mallick & Singh, 1980). The extract was centrifuged at 15,000 x g for 15 min at 4°C. The supernatant was used as enzyme source. Protein in the enzyme extract was estimated according to Lowry *et al.* (1951). Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by the method of Giannopolitis & Ries (1977), by measuring the photoreduction of nitrobluetetrazolium at 560 nm. The reaction mixture contained 500  $\mu\text{L}$  phosphate buffer (pH 7.8), 0.5 mL distilled H<sub>2</sub>O, 100  $\mu\text{L}$  methionine, 50  $\mu\text{L}$  NBT and 50  $\mu\text{L}$  enzyme extract. One unit of SOD was defined as the amount of protein causing a 50% decrease of the SOD-inhibitable NBT reduction. SOD activity was expressed as Unit mg<sup>-1</sup> protein. Peroxidase (POD, EC 1.11.1.7) activity was estimated according to Chance & Maehly (1955). In this method, one ml reaction mixture contained 750  $\mu\text{L}$  50 mM phosphate buffer (pH 5.0), 100  $\mu\text{L}$  guaiacol (20 mM), 100  $\mu\text{L}$  H<sub>2</sub>O<sub>2</sub> (40 mM) and 100  $\mu\text{L}$  enzyme extract. Increase in OD at 470 nm was recorded for 3 min at interval of every 20 sec. The enzyme activity was expressed as EU mg<sup>-1</sup> protein. Method described by Chance & Maehly (1955) was used for estimation of catalase (CAT, EC 1.11.1.6) activity. The reaction mixture contained 1.9 ml phosphate buffer (50 mM; pH 7.0) and 1 mL H<sub>2</sub>O<sub>2</sub> (5.9 mM) and the reaction was initiated by adding 100  $\mu\text{L}$  of the enzyme extract. Decrease in OD at 240 nm was read spectrophotometrically for 2 min and activity was expressed as EU mg<sup>-1</sup> protein. The activity of glutathione reductase (GR, EC 1.6.4.2) was determined according to Carlberg & Mannervik (1985). The decrease in absorbance was read at 340 nm for 2 min. The activity of GR was calculated using the extinction coefficient of NADPH of 6.2 mM<sup>-1</sup> cm<sup>-1</sup> and expressed as EU mg<sup>-1</sup> protein. Guaiacol peroxidase (GPX, EC: 1.11.1.9) activity was measured as described by Elia *et al.* (2003). The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using the extinction coefficient of 6.62 mM<sup>-1</sup>cm<sup>-1</sup>.

**Statistical analysis:** All experiments were repeated three times. Treatment means were statistically analyzed using Least Significant Difference (LSD) analysis of variance for a completely randomized design.

## Results

Sunflower plants exposed to cadmium stress showed reduced growth in terms of both fresh as well dry biomass (Table 1). Cadmium stress reduced fresh and dry weight of shoot by 55.04% and 34.7% respectively and that of root by 34.6% and 45.2%. In AMF inoculated plants increase in fresh and dry weight observed was 26.87% and 52.1% for shoot and 61.5% and 51.1% for root. AMF inoculation also mitigated the adverse impact of cadmium on these attributes (Table 1). Moreover reduction in spore population due to cadmium stress was reported 79.7%. Percent reduction in mycelium, vesicles and arbuscules was 84%, 57.3% and 73.02% respectively (Table 2).

Chlorophyll pigments also showed a decline due to cadmium stress and AMF inoculated plants showed higher content as compared to control (Table 3).

Percent increase in chlorophyll a, chlorophyll b total chlorophyll and carotenoid contents in AMF inoculated plants was 34.8%, 16.2%, 37.4% and 24.4% respectively. Cadmium stress reduced chlorophyll a, chlorophyll b total chlorophyll and carotenoid by 46.9%, 47.5%, 51.5% and 61.2% respectively. However reduction in AMF inoculated cadmium stressed (100  $\mu$ M Cd +AMF) plants was only 27.4%, 24.7%, 26.7% and 26.53% respectively (Table 3).

Increase in MDA, H<sub>2</sub>O<sub>2</sub> and RMP was observed in cadmium treated plants. In cadmium stressed plants, relative to control, MDA, H<sub>2</sub>O<sub>2</sub> and RMP increased by 24.7%, 56.3% and 24.9% respectively (Table 4). Under cadmium stress inoculation of AMF (100  $\mu$ M Cd +AMF) mitigated the negative impact of cadmium and increase in H<sub>2</sub>O<sub>2</sub> and RMP was only 10.3% and 6.6% respectively.

**Table 1. Fresh weight (FW, g/ plant), dry weight (DW, g plant<sup>-1</sup>) of both shoot and root systems of *H. annuus*, subjected to cadmium stress (100  $\mu$ M) in presence and absence of Arbuscular mycorrhizal fungi (AMF). Data presented are the means  $\pm$  SE (n = 5).**

Treatment	Growth criteria			
	Shoot		Root	
	FW	DW	FW	DW
Control	17.04 $\pm$ 2.01	2.39 $\pm$ 0.08	1.39 $\pm$ 0.07	0.17 $\pm$ 0.02
AMF	21.62 $\pm$ 2.24	3.59 $\pm$ 0.11	2.16 $\pm$ 0.9	0.26 $\pm$ 0.03
Cadmium	7.66 $\pm$ 0.78	1.59 $\pm$ 0.07	0.85 $\pm$ 0.02	0.09 $\pm$ 0.01
Cadmium + AMF	10.75 $\pm$ 1.16	2.02 $\pm$ 0.9	1.10 $\pm$ 0.04	0.12 $\pm$ 0.01
LSD at: 0.05	0.6892	0.2223	0.0813	0.0217

**Table 2. Mycorrhizal spore count (MSC, number/ 100 g soil) and mycorrhizal colonization (myclium, vesicles, arbuscules, %) of *H. annuus*, subjected to cadmium stress (100  $\mu$ M) in presence and absence of Arbuscular mycorrhizal fungi (AMF). Data presented are the means  $\pm$  SE (n = 5).**

Treatment	MSC	Mycorrhizal colonization (%)		
		Myclium	Vesicles	Arbuscules
AMF	408.32 $\pm$ 15.01	63.47 $\pm$ 3.12	47.66 $\pm$ 2.35	84.54 $\pm$ 5.11
Cadmium	82.706 $\pm$ 6.32	10.11 $\pm$ 1.78	20.33 $\pm$ 2.41	22.66 $\pm$ 2.01
LSD at: 0.05	42.11	4.21	3.15	7.35

**Table 3. Pigments system (chlorophyll a, chlorophyll b, total chlorophylls, and carotenoid, as mg g fresh weight<sup>-1</sup>) of *H. annuus*, subjected to cadmium stress (100  $\mu$ M) in presence and absence of Arbuscular mycorrhizal fungi (AMF). Data presented are the means  $\pm$  SE (n = 5).**

Treatment	Pigments system (mg g fresh weight <sup>-1</sup> )				
	Chlorophyll a	Chlorophyll b	Chlorophyll a/b	Carotenoids	Total pigments
Control	0.681 $\pm$ 0.08	0.198 $\pm$ 0.02	3.435 $\pm$ 0.76	0.049 $\pm$ 0.01	0.928 $\pm$ 0.13
AMF	0.918 $\pm$ 0.13	0.230 $\pm$ 0.07	3.982 $\pm$ 0.76	0.061 $\pm$ 0.01	1.209 $\pm$ 0.63
Cadmium	0.361 $\pm$ 0.08	0.104 $\pm$ 0.02	3.515 $\pm$ 0.72	0.019 $\pm$ 0.01	0.485 $\pm$ 0.07
Cadmium + AMF	0.494 $\pm$ 0.06	0.149 $\pm$ 0.03	3.302 $\pm$ 0.71	0.036 $\pm$ 0.01	0.680 $\pm$ 0.06
LSD at: 0.05	0.02	0.01	0.43	0.01	0.02

**Table 4. Malondialdehyde (MDA, nm g fresh weight<sup>-1</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, μM g fresh weight<sup>-1</sup>), relative membrane permeability (RMP, %), free proline (FP, μM g fresh weight<sup>-1</sup>) and total phenols (TP, mg g fresh weight<sup>-1</sup>) of *H. annuus* subjected to cadmium stress (100 μM) in presence and absence of Arbuscular mycorrhizal fungi (AMF). Data presented are the means ± SE (n = 5).**

Treatment	Systemic resistance attributes				
	MDA	H <sub>2</sub> O <sub>2</sub>	RMP	FP	TP
Control	54.17	240.27	50.56	0.658	3.47
AMF	56.32	264.28	43.11	0.512	3.78
Cadmium	67.55	375.63	63.15	0.869	4.63
Cadmium + AMF	59.77	298.00	53.91	0.718	3.93
LSD at: 0.05	1.28	4.01	1.63	0.01	0.10

**Table 5. Phosphorus (mg/ g dry weight) and cadmium (μg g dry weight<sup>-1</sup>) contents of both shoot and root systems of *H. annuus*, subjected to cadmium stress (100 μM) in presence and absence of Arbuscular mycorrhizal fungi (AMF). Data presented are the means ± SE (n = 5).**

Treatment	Phosphorous		Cadmium	
	Shoot	Root	Shoot	Root
Control	1.87 ±0.14	0.81 ±0.11	ND	ND
AMF	2.21 ±0.19	1.04 ±0.15	ND	ND
Cadmium	0.52 ±0.07	0.37 ±0.03	53.727 ±3.75	2436.8 ±78.12
Cadmium + AMF	0.64 ±0.09	0.62 ±0.05	14.627 ±2.04	709.73 ±10.41
LSD at: 0.05	0.08	0.14	5.33	80.17

ND: Not detected under the experimental conditions.

Proline accumulation increased in cadmium stressed sunflower plants (Table 4). Inoculation of AMF caused a slight decrease in proline content. In cadmium treated plants proline content increased by 32.4% while as in AMF inoculated cadmium stressed (100 μM Cd +AMF) proline increased by 9.1% as compared to control (Table 4). Phenols also increased as a result of cadmium stress as well as AMF inoculation (Table 4). AMF alone increased phenols by 8.9% as compared to control uninoculated plants. Relative to control, percent increase in phenols due to cadmium stress without (100 μM Cd) and with AMF (100 μM Cd +AMF) was 33.5% and 13.2% respectively (Table 4).

AMF inoculation caused a considerable increase in the activities of antioxidant enzymes (Fig. 1A-E). Cadmium stress (100 μM Cd) increased activity of SOD, POD, CAT, GPX and GR by 178.9%, 36.07%, 73.4%, 53.9%, and 42.1% respectively. In combination with cadmium, AMF (100 μM Cd+AMF) increased activity of antioxidants. Relative to control, AMF inoculated plants showed higher activities of antioxidant enzymes and the percent increase being 46.8%, 4.2%, and 4.8% for SOD, POD, and CAT respectively. However a slight decrease in APX and GR was observed in AMF inoculated plants.

Under cadmium stress and AMF inoculation activity of acid phosphatase and alkaline phosphatase increased (Fig. 2). However the impact was more obvious in AMF inoculated plants. Relative to control, percent increase in activity of acid phosphatase and alkaline phosphatase was 131.2% and 95.5% in AMF inoculated plants as compared to cadmium stressed which showed 89.1% and 69.3% increase.

Cadmium stressed plants showed reduced uptake of phosphorous while as AMF not only increased the phosphorous uptake but also ameliorated the deleterious effect of cadmium on phosphorous uptake (Table 5). More phosphorous content was observed in shoot as compared to root. In shoot and root percent increase in phosphorous due to AMF was 17.6% and 26.4%, respectively (Table 5). In shoot cadmium stress induced percent reduction under non AMF (100 μM Cd) and AMF (100 μM Cd +AMF) conditions was 72.1% and 54.6% respectively (Table 5). In control and AMF inoculated plants cadmium content was very low. Cadmium stressed plants accumulated more cadmium in shoot as compared to root. However AMF inoculated plants accumulated lesser quantity of cadmium (Table 5).

In our study sunflower plant subjected to cadmium stress showed a marked increase in total fatty acid content (Table 6). Percent reduction in total fatty acid content due to AMF was 55.1% and cadmium stressed plant showed 58.84% increase as compared to control. Individually, among the different lipid classes only few including palmitoleic acid (C<sub>16:1</sub>), oleic (C<sub>18:1</sub>), linoleic (C<sub>18:2</sub>) and linolenic acid (C<sub>18:3</sub>) showed a decline with cadmium treatment. A marked increase in these four lipids was observed in AMF inoculated plants and AMF inoculation also ameliorated cadmium effects on these lipid classes (Table 6). Increase in palmitoleic acid (C<sub>16:1</sub>), oleic (C<sub>18:1</sub>), linoleic (C<sub>18:2</sub>) and linolenic acid (C<sub>18:3</sub>) due to AMF was 160.3%, 23.1%, 30.1% and 15% respectively.

**Table 6. Free fatty acids (%) of *H. annuus*, subjected to cadmium stress (100  $\mu$ M) in presence and absence of Arbuscular mycorrhizal fungi (AMF).**

Treatment	C <sub>6</sub>	C <sub>8</sub>	C <sub>10</sub>	C <sub>12</sub>	C <sub>14</sub>	C <sub>15</sub>	C <sub>15:1</sub>	C <sub>16</sub>	C <sub>16:1</sub>	C <sub>17</sub>	C <sub>18</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	C <sub>20</sub>	TS
Control	4.42	2.48	1.17	0.18	0.94	0.43	1.76	7.72	1.84	2.91	1.75	27.17	17.15	21.72	8.36	32.12
AMF	2.11	1.17	0.52	0.09	0.63	0.12	0.85	3.45	4.79	1.32	1.03	33.45	22.32	25.04	3.11	14.40
Cadmium	6.01	5.01	3.27	0.34	2.17	1.95	3.04	9.88	0.62	4.22	3.05	20.45	11.81	16.02	12.16	51.1
Cadmium + AMF	4.93	3.77	2.13	0.27	1.63	1.04	2.15	8.11	2.67	3.42	2.03	24.11	15.07	18.44	10.23	39.71
LSD at: 0.05	0.65	0.13	0.07	0.03	0.06	0.03	0.14	0.28	0.37	0.23	0.14	2.06	1.78	1.33	1.05	3.46

C<sub>6</sub>: Caproic acid; C<sub>8</sub>: Caprylic; C<sub>10</sub>: Capric; C<sub>12</sub>: Lauric; C<sub>14</sub>: Myristic; C<sub>15</sub>: Pentadecanoic; C<sub>15:1</sub>: Cis-10-Pentadecanoic acid; C<sub>16</sub>: Palmitic; C<sub>16:1</sub>: Palmitoleic; C<sub>17</sub>: Heptadecanoic; C<sub>18</sub>: Stearic; C<sub>18:1</sub>: Oleic; C<sub>18:2</sub>: Linolenic; C<sub>18:3</sub>: Linolenic; C<sub>20</sub>: Arachidic; TS: Total saturation percent.

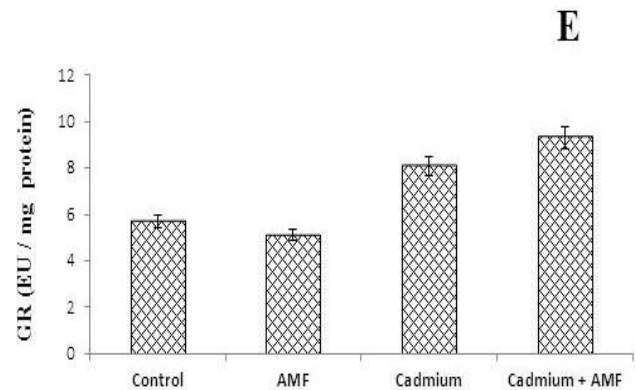
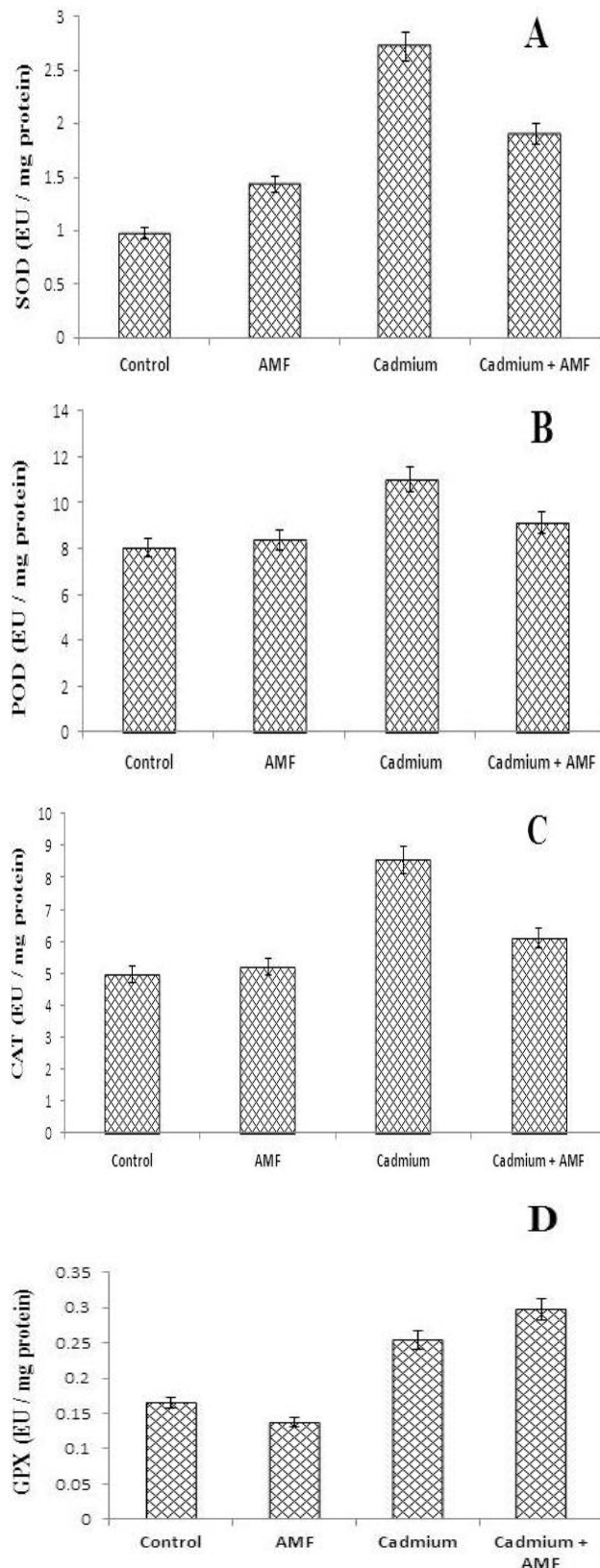


Fig. 1. A-E: Effect of cadmium (100  $\mu$ M) on antioxidant enzymes activity of *H. annuus* in presence and absence of AMF. Columns represent means for five plants (N=5) with error bars showing standard deviation.

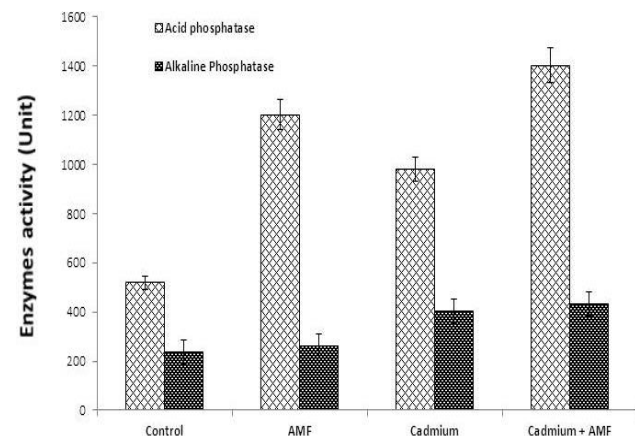


Fig. 2. Effect of cadmium (100  $\mu$ M) on acid and alkaline phosphatases activity of *Helianthus annuus* L., in presence and absence of AMF. Columns represent means for five plants (N=5) with error bars showing standard deviation.

## Discussion

Cadmium stress reduced the biomass (fresh as well as dry) of sunflower considerably and the affect was subsequently ameliorated by the inoculation of AMF. Cadmium stress perturbs the activities of key metabolic enzymes and promotes leaf necrosis leading to growth retardation (Arduini *et al.*, 1996; Amaya-Carpio *et al.*, 2009). Cadmium has been reported to reduce cell division and elongation rates through its irreversible impact on membrane potential and the proton pump responsible for such growth promoting functions (Karcz & Kurtyka, 2007). Al-Dhaibani *et al.* (2013) also demonstrated reduction in biomass accumulation in sunflower due to cadmium application. Cadmium stress reduces hydraulic conductivity and hamper cell wall extensibility resulting

in deleterious impact on the morphological attributes of plants (Ehlert *et al.*, 2009). Our results of enhanced growth due to AMF are in concurrence with the findings of Zolfaghari *et al.* (2012) for *Ocimum basilicum* and Tang *et al.* (2009) for *Zea mays*. Tang *et al.* (2009) observed that inoculation of AMF mitigated the deleterious impact of diesel stress by enhancing the uptake of essential nutrients and maintaining the cell water content. Moreover reduction in AMF colonization due to stress in our results corroborate with the findings of Pawlowska & Charvat (2004) and Aroca *et al.* (2013). Pawlowska & Charvat (2004) have also reported perturbed spore germination and reduced hyphal density due to heavy metal stress.

Drastic decline in chlorophyll contents depicted in our results due to cadmium application support the findings of Rivelli *et al.* (2012) for *Helianthus annuus* and Mangal *et al.* (2013) for *Abelmoschus esculentus* L. and *Cyamopsis tetragonoloba* L. Stress enhances activity of chlorophyllase enzyme resulting in impeded biosynthesis of chlorophyll pigments. AMF induced enhancement in chlorophyll pigment has earlier been reported by Hajiboland *et al.* (2010) and Malekzadeh *et al.* (2012). AMF enhances de novo synthesis of proteins and chlorophyll because of its direct influence on the uptake of magnesium which forms an important part of chlorophyll molecule (Sheng *et al.*, 2008).

Cadmium stress caused considerable increase in H<sub>2</sub>O<sub>2</sub> production and lipid peroxidation in sunflower plants which was mitigated by AMF inoculation. Our results of increased H<sub>2</sub>O<sub>2</sub> and MDA accumulation due to exposure of cadmium is in corroboration with the results of Ouariti *et al.* (1997) for tomato, Ahmad *et al.* (2011) for mustard and Hossain *et al.* (2006) for *Chrysanthemum morifolium*. Stress induced peroxidation of lipids is widely accepted as one of the important parameter for measuring the magnitude of environmental stress. Membrane lipids are very sensitive to ROS and undergo oxidation resulting in the formation of peroxide radicals. Increased peroxidation of membrane lipids result in loss of membrane integrity and hence leakage of essential elements occurs (Macri *et al.*, 1994; Djebali *et al.*, 2005). Moreover under stress conditions lipoxygenase activity also goes up resulting in rapid peroxidation of lipids and increased production of peroxides and hydroxyl radicals which further deteriorate the membranes and other cellular components (Clijsters *et al.*, 1991; Macri *et al.*, 1994; Djebali *et al.*, 2005). In rice, Kuo & Kao (2004) also demonstrated an increase in the production of H<sub>2</sub>O<sub>2</sub> resulting in oxidative stress and reduced growth. AMF ameliorated the negative impact of cadmium stress by causing considerable reduction in H<sub>2</sub>O<sub>2</sub> production and MDA content. Reduced content of MDA in AMF inoculated plants justifies the ameliorative role of AMF. This AMF induced reduction in H<sub>2</sub>O<sub>2</sub> and MDA may be ascribed to increased phosphorous assimilation and antioxidant activity. H<sub>2</sub>O<sub>2</sub> is a deleterious ROS continuously produced during plant metabolism and reduced H<sub>2</sub>O<sub>2</sub> in AMF treated plants in our results support the role of AMF in protecting plant cells from deleterious impact of ROS. Ling-Zhi *et al.* (2011) also observed ameliorative role of AMF under cadmium stress.

Proline is one of the important osmotic constituent actively involved in maintenance of cell water content. Increase in proline content under cadmium stress was obvious and further increase caused by AMF inoculation infers the important role of AMF in enhancing stress tolerance of plants. Under stress conditions activity of proline synthesizing enzymes is upregulated while as its catabolism is lowered (Ahmad *et al.*, 2010b; 2014). Proline protects membranes and other cellular structures. Moreover it protects enzymes and mediates neutralization of toxic ROS hence contributes to better growth under stress conditions (Hare & Cress, 1997; Irfan *et al.*, 2014). Our findings of increased proline accumulation due to cadmium stress is in support of the findings of Zengin & Munzuroglu (2006) and Irfan *et al.* (2014). In sunflower, Zengin & Munzuroglu (2006) demonstrated increased accumulation of proline in cadmium stressed plants leads to enhanced tolerance of plants. Accumulation of higher contents of proline helps to maintain osmotic balance of cells so that cell water content can be maintained. Proline do not interfere with the metabolic pathways rather it replaces water in these processes (Zhifang & Loescher, 2003). Jindal *et al.* (1993) and Ruiz-Lozano *et al.* (1995) also reported enhanced proline accumulation in AMF inoculated plant under normal as well as stressed conditions.

Plant phenolics are the broad group of secondary metabolites having immense importance for plants. Phenols are important non enzymatic antioxidants and are involved in the interactions with several biotic and abiotic factors (Reddy *et al.*, 2004; Tomar & Agarwal, 2013). Phenols mitigate stress by protecting plant cells from oxidative stress and increasing membrane stability (Burguieres *et al.*, 2006; Khattab, 2007). Under stress condition phenolic content of plants is enhanced considerably so that the stress induced changes can be averted. Increased accumulation of plant phenolics is ascribed to the upregulation of the enzymes involved in their synthesis (Scott *et al.*, 2004; Wada *et al.*, 2014). In our study increased phenol content in stressed plants corroborate with the findings of Tomar & Agarwal (2013) for wheat, Wada *et al.* (2014) for pharbitis, Dawood *et al.* (2014) for *Vicia faba*. AMF inoculated plants accumulated slightly higher contents of phenols contributing to better growth under normal as well as stress conditions. In *Salvia officinalis* L., Nell *et al.* (2009) also demonstrated that AMF colonized plants showed enhanced phenol synthesis as compared to the uninoculated counterparts.

Increase in activities of antioxidant enzymes due to cadmium stress is in corroboration with the findings of Milone *et al.* (2003) for wheat, Shan *et al.* (2012) *Arachis hypogaea* L. and Irfan *et al.* (2014) for mustard. Increased activities of antioxidant enzymes results in quick scavenging of toxic ROS therefore avoiding the oxidative stress. AMF inoculation induced increment in antioxidant activities is in support of the findings of Ling-Zhi *et al.* (2011), Bhaduri & Fulekar (2012) and Malekzadeh *et al.* (2012). SOD, POD, CAT, GPX and GR are important antioxidant defence enzymes having active roles in ROS detoxification. SOD is one of the key enzymes involved in detoxification of superoxide radicals and hence preventing cellular damage. H<sub>2</sub>O<sub>2</sub> produced is further

converted into water by the activity of either catalase or peroxidase (Ashraf, 2009). GR is the important enzyme of ascorbate-glutathione cycle and catalyses conversion of oxidized glutathione (GSSG) into reduced glutathione (GSH) and hence leading to maintenance of higher GSH/GSSG ratio (Noctor & Foyer, 1998; Rausch *et al.*, 2007). In *Ipomoea aquatica*, Bhaduri & Fulekar (2012) also demonstrated that cadmium stress increased the activities of antioxidant enzymes and AMF inoculation resulted in further enhancement of their activities resulting in better stress adaptation and growth through averting the ROS induced oxidative stress. Higher activities of antioxidant enzymes in sunflower suggest their importance in stress mitigation and enhancement in the activities of antioxidants due to AMF inoculation suggest the use of AMF as a potential prospective strategy for enhancing tolerance level of sunflower.

In our results Cd concentrations decreased by AMF inoculation to great degrees. Reduction in cadmium may be because of the ability of fungal hyphae to bind heavy metals outside and inside the roots and restrict their uptake to upper parts (Hua *et al.*, 2010). Decreasing the uptake of cadmium to upper plant parts results in maintained growth and metabolism through its effect on the biomass dilution (Janouskova *et al.*, 2007). Our results are in corroboration with the findings of Toler *et al.* (2005) for sorghum and Ling-Zhi *et al.* (2011) for *Tagetes erecta* L. In *Tagetes erecta* L., it has been observed that AMF inoculation reduced the uptake of cadmium to shoot resulting in better adaptation to heavy metal polluted soils (Ling-Zhi *et al.*, 2011). AMF induced enhancement in phosphorous nutrition has direct relation with growth, antioxidant and nitrogen metabolism (Garg & Manchanda, 2008; Aziz *et al.*, 2011). In plants improved uptake of phosphorous help to maintain vacuolar membrane integrity and hence facilitating efficient compartmentalization within vacuole and as well as selective ion uptake so protecting cells from toxic ion induced interference in metabolic pathways (Cantrell & Lindermann, 2001). Our results are support the findings of Ortas, (2010) and Hart & Forsythe (2012). AMF increases phosphorous uptake because of its effect on the root morphology and the enzymes of phosphate metabolism. Our results of increased phosphorous content in AMF treated plants is in confirmation with the results of Amaya-Carpio *et al.* (2009) for *Ipomoea carnea* and Prasad *et al.* (2012) for *Chrysanthemum indicum* L.

Our results of increased activities of alkaline and acidic phosphatases in AMF inoculated plants are in confirmation with the results of Amaya-Carpio *et al.* (2009), Prasad *et al.* (2012) and Kebradadi *et al.* (2014). Acid phosphatases (both alkaline and acidic) are the active players in the processes of phosphorous nutrition mediating efficient absorption, assimilation and metabolism of phosphorous. AMF induced enhancement in phosphatase activity could possibly mediate the release of organically bound phosphorous and hence increasing transport and uptake of phosphorous in AMF inoculated plants. Increased activity of phosphatases has direct bearing with the phosphorous metabolism. Bhadraiah *et al.*

(1999) observed a high degree of correlation between phosphorous uptake and activity of phosphatases.

An obvious increase in total fatty acid content in cadmium stressed plants was observed in our study. Among the different fatty acids classes demonstrated palmitoleic acid (C<sub>16:1</sub>), oleic (C<sub>18:1</sub>), linoleic (C<sub>18:2</sub>) and linolenic acid (C<sub>18:3</sub>) decreased with cadmium stress and AMF inoculation resulted in subsequent amelioration of the negative effect. Our findings of reduced fatty acid component under cadmium stress support the findings of Ouariti *et al.* (1997), Nouairi *et al.* (2006), Elloumi *et al.* (2014). In *Prunus dulcis*, Elloumi *et al.* (2014) also observed alteration in fatty acid content due to cadmium stress. Similar to our findings they also reported an increase in certain classes including C<sub>16:0</sub>, and C<sub>18:0</sub>. Cadmium stress induced reduction in palmitoleic acid (C<sub>16:1</sub>) and linolenic acid (C<sub>18:3</sub>) observed in our study support the finding of Nouairi *et al.* (2006) who have proposed cadmium stress preferentially affects chloroplastic membrane lipids. In leaves C18:3 fatty acid class is usually associated with galactolipids that form more than 85% of total lipid component of thylakoid and are ubiquitous for the photosynthetic functions (Somerville & Browse, 1991; Mizusawa & Wada, 2012).

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