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COMBINED EFFECTS OF CITRIC ACID AND 5-AMINOLEVULINIC ACID IN MITIGATING CHROMIUM TOXICITY IN SUNFLOWER (*Helianthus annuus L.*) GROWN IN Cr SPIKED SOIL

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Phytoremediation, assisted with different amendments, is receiving much attention around the world due to its high efficiency, low cost and ease of handling. The current study was constructed to determine the combined performance of the citric acid (CA) and 5-aminolevulinic acid (5-AA) to enhance chromium (Cr) extraction through sunflower. Healthy seeds of sunflower were grownup in Cr contaminated soil (0, 5, 10 & 20 $mgkg^{-1}$) and externally provided with CA (0, 2.5 &5 mM) and 5-AA (0, 10 & 20 mg L^{-1}) at juvenile stage. The results showed a significant reduction in sunflower agronomic traits and biomass under Cr stress. In response to Cr stress, higher production of the reactive oxygen species (ROS) significantly reduced soluble proteins, photosynthetic pigments and activities of anti-oxidant enzymes. The combined addition of CA and 5-AA considerably mitigated the Cr-induced toxic effects on sunflower. Citric acid (CA) and 5-AA application enhanced plants' agronomic and physiological attributes by lowering electrolyte leakage and ROS production. Furthermore, the introduction of 5-AA and CA significantly up-regulated the actions of antioxidants enzymes in leaves and root of sunflower. With increasing soil Cr concentration, more Cr uptake and accumulation was observed which was further boosted with mutual applications of CA and 5-AA. Results of our work summarize that, CA and 5-AA combined application can effectively mitigate Cr stress and enhance Cr extraction capability of sunflower.

Keywords: Chromium accumulation, chelator, oxidative stress, sunflower, stress mitigation.

INTRODUCTION

Chromium is a non-essential and potentially dangerous heavy metal without any metabolic functions in plants (Hussain *et al.,* 2018a). Chromium has been characterized as a potentially toxic metal for plants with significant concentration present in earth crust (Economou-Eliopoulos *et al.,* 2013). International Agency for Research on cancer reportedly placed Cr as number one carcinogenic heavy metal (IARC, 1987).There are many states of Cr range from -2 to +6, but maximum constant and ordinary states of Cr found in biosphere are trivalent (Cr^{+3}) and hexavalent chromium (Cr^{+6}) (Ashraf *et al.,* 2016). These trivalent and hexavalent states of Cr show different behavior according to their assimilation in soil, translocation, bioavailability as well as absorption in above ground biomass and toxicity in the plant (Choppala *et al.,* 2018). Minute quantity of Cr (III) is essential for sugar and lipid metabolisms in animals and humans, but non-essential for the plants (Shanker *et al.,* 2005).

Many studies have described the Cr toxicity on biochemical and morpho-physiological processes of plants (Jabeen *et al.,* 2016; Hussain *et al.,* 2018a). Chromium toxicity badly influences plant agronomic growth, physiology, proteomics and miRNA expression (Afshan *et al.,* 2015; Ali *et al.,* 2015).

Many soil and plant relevant factors effect transfer of Cr from soil to plant tissues like; plant types, root surface area, gas exchange attributes, soil texture, soil electrical conductivity (EC) and pH (Islam *et al.,* 2016). Plants having least tendency of metal hyper-accumulation tend to accrue more metals in their roots than aerial parts (Rehman *et al.,* 2016). A variety of Cr-hyperaccumulators have been reported in literature like, *Brassica napus* L (Gill *et al.,* 2015a, b), sunflower (Fozia *et al.,* 2008; Rizwan *et al.,* 2016b; Farid *et al.,* 2017), rice (Mantry and Patra, 2017), spleen amaranth (*Amaranthus dubius*) atlantic cord grass (*Spartina argentinensis*) and many others. Among different hyperaccumulator plants, sunflower has the properties to grow easily under the contamination of metals (Tassi *et al.,* 2017). Sunflower can accumulate different heavy metals

(HMs) like As (Imran *et al.*, 2013), Cr (Fozia *et al.*, 2008; Atta *et al.*, 2013), Zn (Nehnevajova *et al.*, 2012; Hao *et al.*, 2012), Ni (Ahmad *et al.*, 2011), Cd (Júnior *et al.*, 2014), Cu (Lin *et al.*, 2003) and Pb (Adesodun *et al.*, 2010).

Extensive range of different amendments has been applied to improve the metal translocation from soil to aerial parts of plants. It includes the use of natural or synthetic organic and inorganic acids and bacterial species having ability to reduce metal oxidation state and promote plant growth (Islam *et al.*, 2016; Khaliq *et al.*, 2016). Organic acids like Citric acid, gibberellic acid, glutamic acid, salicylic acid, EDTA, EDDS etc. can also serve this purpose (Adrees *et al.*, 2015a, b). The CA application is well documented because of its high biodegradability and low cost in comparison with persistent chelators and expensive synthetic compounds (Habiba *et al.*, 2015).

In recent years, plants growth regulators (PGR) have gained much attention because of their ability to support plants' normal functioning under biotic and abiotic stress. Work published by Gill *et al.* (2015a) and Rizwan *et al.* (2016b) has documented a significant decrease in endogenous secretion of aminolevulinic acid (AA) under heavy metal treatment which can also be applied exogenously to support the plants against stress. Gill *et al.* (2015a) and Ali *et al.* (2015) stated that AA is very helpful for the reduction of heavy metals toxicity in oilseed crops such as *Brassica napus* L. like the results of Feng *et al.* (2015) and Liu *et al.* (2016) for *Litchi Chinensis* Sonn.cv., Nunkaew *et al.* (2014) for rice and Akram *et al.* (2012, 2013) for sunflower.

The current research was directed to observe the toxicity of Cr on biochemical, morphological and physiological attributes of the sunflower with the possible ameliorative role of 5-aminolevulinic acid (5-AA) and chelating ability of citric acid (CA) separately as well as in combined form.

MATERIALS AND METHODS

***Soil treatments and plant growth*:** For current study, Sandy loam soil (17.2% clay, 15.1% silt, 67.7% sand) was used, obtained at the depth of 0-15 cm, from farm area Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad (ISES-UAF), Pakistan. Soil was air dried and sieved to get rid of debris and crop remaining. Healthy and uniform seeds of the sunflower (FH-614) were obtained from Ayub Agriculture Research Institute (AARI), Faisalabad. Before sowing in plastic pots with 5 kg soil, the seeds were soaked in 10% H_2O_2 solution and rinsed with distilled water. The soil was concentrated with 4 levels of Chromium (0, 5, 10, 20 $mgkg^{-1}$) using Potassium dichromate ($K_2Cr_2O_7$). Ten healthy seeds of sunflower per pot were sown and the population of 5 healthy plants per pot was maintained via thinning after the 15th day of emergence. Uprooted plants were crushed and mixed with soil of the same pots after thinning. Fertilizer was applied in solution form to fulfill recommended (N: P_2O_5: K_2O) doses equivalent to 120:90:60 Kg ha^{-1} requirement of crop.

***Treatments*:** Soon after the seed emergence, 6 weeks old seedlings were applied with 3 concentrations of CA (0, 2.5 and 5.0 mM) via soil application and 5-AA (0, 10, and 20 mgL^{-1}) via foliar application. For this study, a total 36 treatments were designed, each treatment was applied in triplicate while moving randomly. Soil was artificially concentrated with chromium (0, 5, 10, 20 $mgkg^{-1}$). The citric acid and 5-AA solutions were made in ultra-pure distilled water for easy application. Citric acid was provided to the soil in solution form while 5-AA was foliar applied with shower on leaves till completely wetted, and both practices were applied to each pot after four days intervals for eight weeks.

***Plant sampling and analysis*:** After the application of treatments for 8 weeks, plants were separated from the soil, washed with the help of distilled water and separated carefully into stem, leaves and root. Agronomic traits like root length, plant height, root fresh weight, leaves and stem weights were measured. Scale and electrical balance were used to measure the weight of crop tissues.

***Chromium analysis*:** The 0.5 g grinded plant (root, leaf, stem) sample from each plant tissue was put in the muffle furnace for 6 hr at 650 ^oC for ash development which was dissolved in 4 mL, 3:1 mixture of conc. HNO_3 and conc. HCL. Distilled water was used to dilute solution up to 50 mL and was analyzed by AAS (Model novA A400, Analytik Jena, Germany) following method explained by Ryan *et al.* (2001) as followed by Farid *et al.* (2018).

***Electrolyte leakage, MDA and H_2O_2*:** Electrolyte leakage was recorded following Dionisio-Sese and Tobita (1998), for which 100 mg of fresh leaf sample was kept in topped test-tube having 10 mL DI water. These tubes were placed in water bath at 32^oC for 2 hr and 1st EC reading (EC1) was recorded. Then, test tubes were placed in autoclave at 121^oC for 20 min to destroy plant tissue and release electrolyte in test tube solution. Test tubes were removed from autoclave and once their temperature normalized (reached 25 ^oC), the final EC (EC2) was recorded. Electrolyte leakage was recorded using following formula;

$$Electrolite\ Leakage = \frac{EC1}{EC2} \times 100$$

Malondialdehyde concentration was recorded by given method of Dhindsa *et al.* (1981) which was adapted from Heath and Packer (1968) using extinction coefficient of 155 mM^{-1} cm^{-1}. The concentration of H_2O_2 was measured by procedure reported by Jana and Choudhuri (1981).

***SPAD value*:** One week before plant harvest, Leaf greenness (chlorophyll) was measured on 2nd uppermost fully extended leaf by using SPAD meter (SPAD-502).

***Antioxidants enzymes and the protein contents*:** The POD and SOD contents were recorded by following Zhang (1992) procedure. The soluble protein contents were also recorded following the protocol suggested by Bradford (1976). The

CAT concentrations were estimated by following the procedure of Aebi (1984). The APX activity was assessed by the procedure given by Nakano and Asada (1981).

***Statistical Analysis*:** There were three replicates in the data presented in our study. Data were statistically analyzed by SPSS (statistical software). To see the significant variations, the ANOVA was applied and Tukey post-hoc test was used for pair wise comparison of data (Steel *et al.,* 1997).

RESULTS

***Plant growth in prospect of 5-AA and citric acid exposure*:** Root length and plant height was measured to evaluate effect of CA and 5-AA application on the growth of Cr stressed plant (Table 1). The reduction in growth was recorded in plants experiencing Cr stress compared to control. Application of Citric acid (2.5 and 5mM solution) and 5-AA (10 and 20 mg L^{-1}) supported the growth of plant in control and stress condition separately as well as when applied in combination at higher doses. The highest growth decrease was noticed for plants treated with 20 mg, followed by 10 and 5 mg Cr kg^{-1} soil without any amendments. Growth encouraging ability of acids in plants which are Cr stressed became even more developed when applied in combine form, in contrast to their separate application. The encouraging trend of growth can be described as follow;

$Cr_{(control)}$ without amendment< Cr-CA or AA < Cr-CA-AA

***Plant Biomass*:** Growth and biomass production of Cr stressed plants treated or untreated with citric acid, 5-AA individually or in combination were observed by measuring their root, leaf and stem fresh weights (Table 1). Lowest reduction in biomass was seen in plants treated with maximum concentrations of both 5-AA (20 mgL^{-1}) and CA (5 mM) in the absence of Cr stress, as compared with the control treatment. While only Cr treated plants showed maximum reduction, which was directly proportional to increasing Cr concentration.

***Soluble protein and SPAD content*:** The acid amendments role in Cr stressed and non-stressed plants, related to reduced soluble protein contents and SPAD value, is demonstrated in

Table 1. Effects of different concentrations of chromium, citric acid and 5-AA on plant growth of sunflower.

Treatments	Cr Concentration (mg kg^{-1})				Cr Concentration (mg kg^{-1})			
	Cr 0	Cr 5	Cr 10	Cr 20	Cr 0	Cr 5	Cr 10	Cr 20
	Leaf fresh weight (g)				Plant height (cm)			
CA0, AA0	25.7±0.51f	21.9±1.00e	16.3±0.56g	13.3±0.17f	91.1±1.60f	75.4±1.36g	60.1±1.04f	42.3±2.51h
CA2.5	28.9±0.43e	24.3±1.15de	18.1±0.29fg	16.0±0.50e	97.5±1.80cde	84.5±1.36e	64.6±2.51ef	51.7±1.47ef
CA5	29.9±1.41de	26.8±.95cd	20.9±1.01de	17.7±0.25cd	100.6±1.52c	88.9±1.24d	71.0±2.00cd	55.4±1.20de
AA10	28.7±0.51e	24.6±0.60d	19.6±0.65ef	15.5±0.40e	96.8±1.60e	80.1±2.20f	64.1±1.25ef	46.6±1.52g
CA2.5+AA10	33.5±0.97bc	28.9±1.02bc	21.8±0.58cd	18.3±0.76c	102.1±1.25bc	89.9±1.68cd	72.1±2.00bcd	57.4±0.91cd
CA5+AA10	35.5±0.72ab	31.8±0.95a	24.9±0.44b	20.5±0.50b	104.5±0.45ab	94.3±1.05b	76.3±1.52ab	61.4±1.11bc
AA20	31.4±0.67cd	26.9±0.98cd	21.7±0.88de	16.7±0.25de	100.5±1.32cd	86.1±1.20de	67.1±2.25de	51.3±1.00f
CA2.5+AA20	34.5±0.97b	30.9±1.00ab	23.8±0.58bc	20.1±0.50b	105.1±1.04ab	93.2±1.12bc	76.2±2.20labc	64.1±1.72b
CA5+AA20	37.9±0.85a	33.4±0.50a	27.3±1.15a	22.3±0.76a	107.2±1.21a	99.3±1.05a	80.6±1.52a	69.7±1.40a
	Stem fresh weight (g)				Root length (cm)			
Cr0, AA0	47.9±1.72e	35.6±0.89e	31.1±1.52f	25.5±1.00f	29.3±0.50f	24.6±0.65f	20.8±0.32f	16.1±0.94f
CA2.5	51.9±1.10d	39.2±1.81d	34.6±1.08e	29.4±1.05de	31.4±0.90e	28.7±0.50d	23.1±0.45e	18.7±0.32e
CA5	54.4±0.79cd	44.4±0.89c	38.1±0.76cd	31.7±0.72d	33.9±0.45cd	31.9±0.55bc	24.7±0.25de	21.2±0.60c
AA10	52.3±0.50d	38.6±0.88de	35.1±0.83de	28.5±1.00e	32.2±0.55de	27.2±0.73e	24.0±1.01e	19.3±0.61de
CA2.5+AA10	55.6±1.31c	43.2±0.10c	38.6±1.25c	34.4±1.10c	35.2±0.65c	31.3±0.30c	26.7±0.79c	22.5±0.66c
CA5+AA10	60.7±1.14b	49.1±1.11b	42.8±1.26ab	37.7±0.30b	37.6±0.75b	33.3±0.26ab	30.1±0.40ab	25.1±0.45ab
AA20	54.9±0.99cd	41.6±0.90cd	37.4±1.00cde	30.5±0.75de	33.7±0.40cd	29.6±0.37d	26.0±0.50cd	21.1±0.70cd
CA2.5+AA20	58.6±1.24b	47.5±1.30b	42.2±0.77b	37.4±0.0b	37.2±0.66b	32.8±0.55abc	28.7±0.77b	24.7±0.32b
CA5+AA20	64.4±0.73a	52.7±0.40a	45.8±1.24a	40.7±0.72a	39.9±0.45a	34.2±0.58a	31.4±0.65a	26.9±0.91a
	Root fresh weight (g)							
Cr0, AA0	22.5±1.10f	19.6±0.77h	16.5±0.50e	12.9±0.55g				
CA2.5	24.8±0.66ef	21.9±0.72g	18.2±0.50d	14.1±0.28fg				
CA5	27.3±1.00cd	24.4±1.28ef	19.8±0.76c	15.3±0.56ef				
AA10	25.2±0.67fde	22.6±0.70fg	19.0±0.20cd	14.9±0.28f				
CA2.5+AA10	27.8±0.60c	26.6±0.62cd	22.0±0.30b	17.8±0.54cd				
CA5+AA10	30.3±0.45ab	28.6±0.26ab	23.5±0.76ab	19.3±0.50b				
AA20	27.2±0.45cd	25.3±0.25de	20.1±0.75c	16.5±0.51de				
CA2.5+AA20	29.3±0.60bc	28.2±0.15bc	23.2±0.50b	19.1±0.76bc				
CA5+AA20	32.3±1.01a	30.4±0.40a	25.1±0.50a	21.3±0.55a				

Sunflower plants were grown in Cr spiked soil (0, 5, 10 and 20 mg/kg) and exogenously supplied with increasing citric acid concentrations (0, 2.5 and 5 mM) and 5-AA (0, 10 and 20 mg/L). Values are the means of three replications ±SD. Values possessing the different small letters are statistically significant at $P>0.05$.

Fig. 1. Control plants applied with the highest dose of CA and 5-AA showed the highest SPAD value and soluble protein in plants leaves and the root. The plants treated with Cr only, showed reduced SPAD value and soluble protein contents and highest reduction was recorded in plants treated with 20, followed by 10 and 5 mg kg^{-1} Cr contamination, without acid amendments. However, both acids played a promoting role in enhancing soluble protein and SPAD values in Cr-treated plants even at the highest stress level i.e. 20 mg kg^{-1}Cr spiked soil. Combined application of acids caused more pronounced stress-ameliorative effects, as compared to their individual application effects.

***Electrolyte leakage and oxidative stress in plants*:** Electrolyte leakage was noticed in the term of electrical conductivity in the leaves and the root of Cr stressed plants and amended by CA and 5-AA (Fig. 2**)**. Chromium-treated

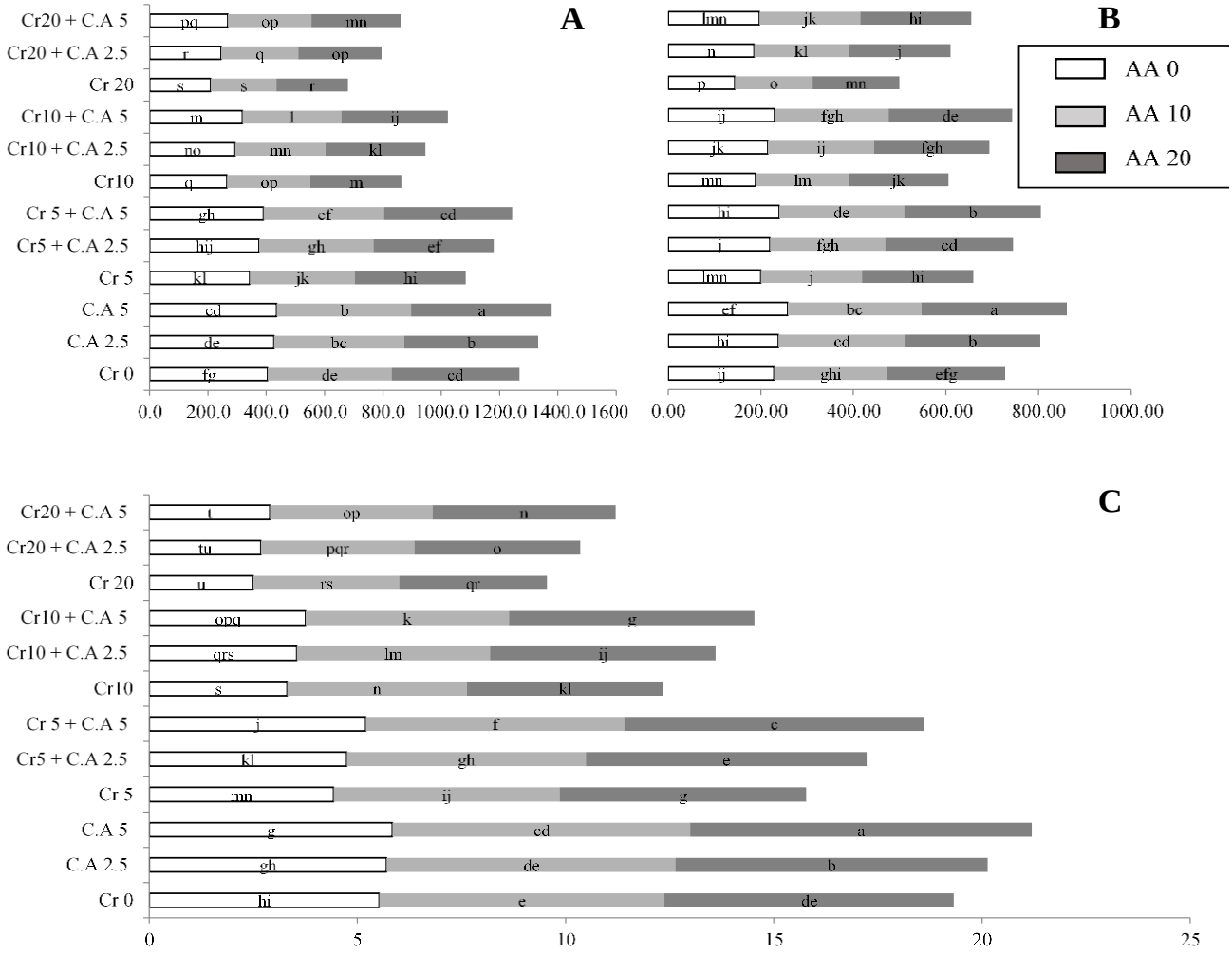

Figure 1. Combined effects of citric acid (0, 2.5 and 5mM) and 5-Aminolevulinic acid (0, 10 and 20 mg/L) on soluble protein (Units g^{-1} Fresh Weight)in leaves (A) and roots (B), and the SPAD values (C) of sunflower grown in Cr concentration (0, 5,10 and 20 mg/kg). Values represented the means of three different replicates along-with the standard deviations. Different letter bars indicated that the values are significantly different at *P<0.05*.

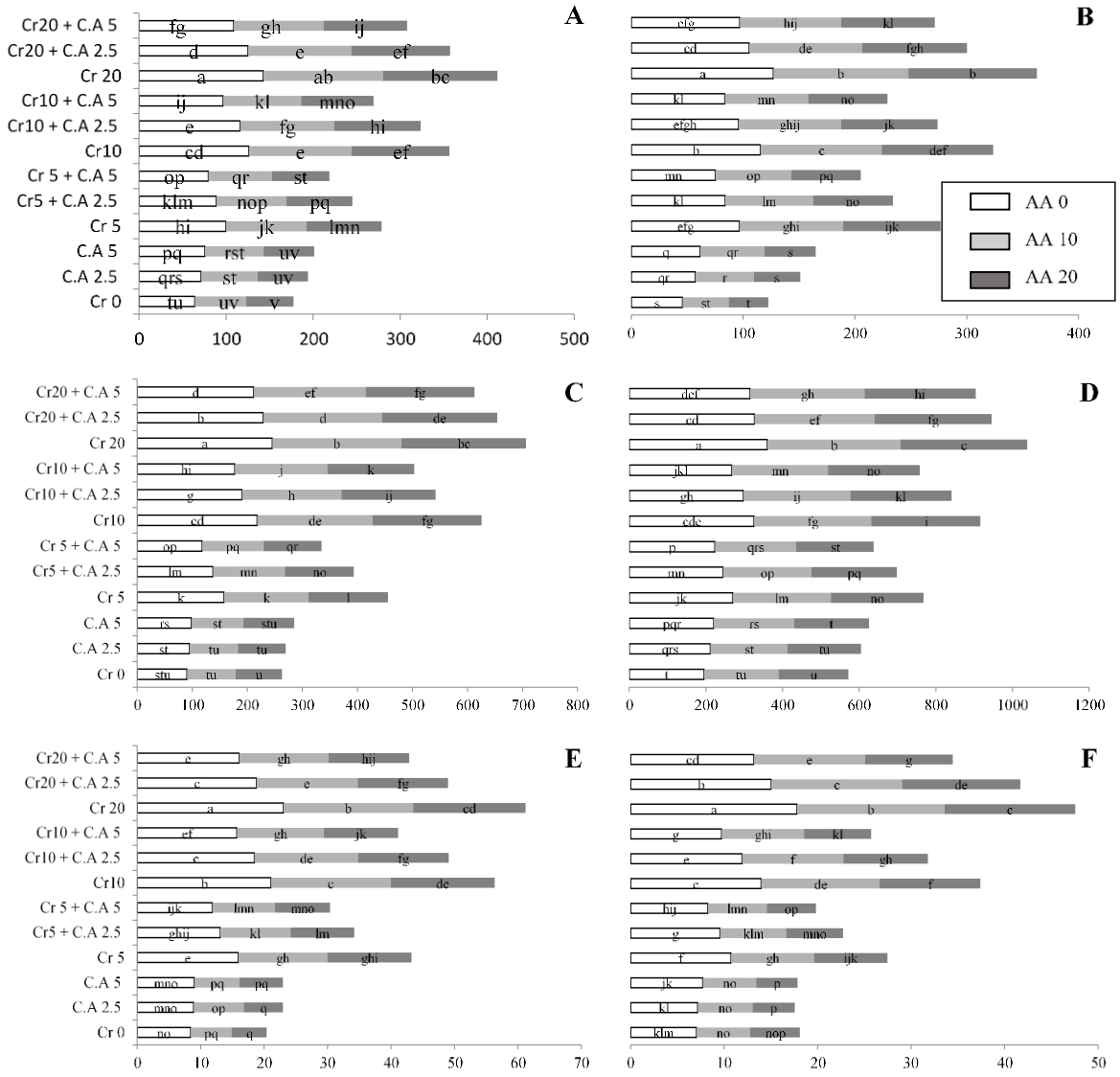

Figure 2. Combined effects of citric acid (0, 2.5 and 5mM) and 5-Aminolevulinic acid (0, 10 and 20 mg/L) on electrolite leakage (%)in leaves (A) and roots (B), H_2O_2(Units g^{-1} Fresh Weight)in leaves (C) and roots(D), MDA(Units g^{-1} Fresh Weight) in leaves (E) and roots (F) of sunflower grown in different Cr concentration (0, 5, 10 and 20 mg/kg).Values represented means of three different replicates along-with the standard deviations. Different small letter bars indicated that the values are significantly different at *P<0.05*.

plants, without acid amendments, showed significant electrolyte leakage at all stress levels. Highest electrolyte leakage was measured on plants grown under 20, trailed by 10 and 5 mg kg^{-1}Cr spiked soil. Same was the case with hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents which were measured to get an idea about oxidative stress in plants bearing Cr toxicity. The application of citric acid (2.5 and 5 mM) and 5-AA (10 and 20 mgL^{-1}), separately or in combination, reduced the electric conductivity and oxidative stress in Cr-treated plants. The combined effect of both acids was higher than the individual ones but, the effect of increasing concentration of acids was also considerable. Citric acid (5 mM) and 5-AA (20 mgL^{-1}) in combination showed supportive role in reducing electrolyte leakage and the oxidative damage in plants grown under Cr toxicity.

***Antioxidant defense system*:** Antioxidant enzymatic activity in plants leaves and the root, against Cr (5, 10 and 20 $mgkg^{-1}$) and the acid amendments, is presented in Fig. 3. SOD, POD,

APX and CAT enzymes were normally active in control plants, but their activities were further amplified with the increasing soil Cr concentration. Highest enzymatic activity was measured at 10 mg, followed by 5 mg Cr/kg soil which was further enhanced by the acid amendments. Suppressed enzymatic activity was observed at highest concentration level of Cr i.e. 20 mg/kg soil. Citric acid (2.5 & 5 mM) and 5-AA (10 & 20 mgL^{-1}) enhanced enzymes activities in Cr-stressed plants even at its higher level. Highest concentrations of both acids when applied in combination, showed the greater promoting role as compared to their individual effects.

Only Cr (initial) ↑<Cr (initial) + CA/5-AA↑
< Cr (initial) + CA + 5-AA > Cr (highest) ↓

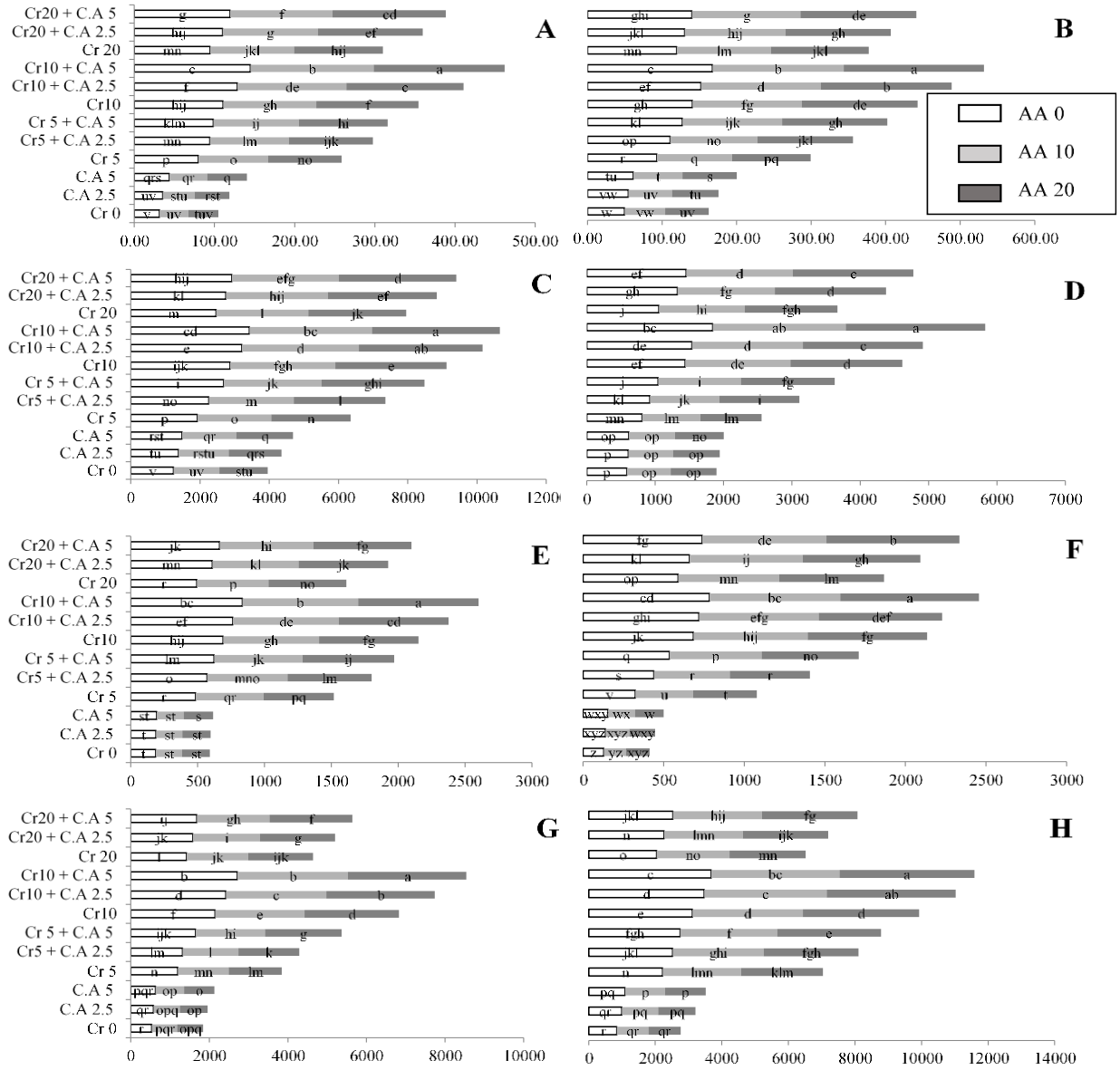

Figure 3. Combined effects of citric acid (0, 2.5 and 5mM) and 5-Aminolevulinic acid (0, 10 and 20 mg/L) on SOD (Units g^{-1} Fresh Weight)in leaves (A) and roots (B), POD(Units g^{-1} Fresh Weight) in leaves (C) and roots (D), APX (Units g^{-1} Fresh Weight) in leaves (E) and roots (F), CAT (Units g^{-1} Fresh Weight) in leaves (G) and roots (H) of sunflower grown in increasing Cr concentration (0, 5,10 and 20 mg/kg). Values represented the means of three different replicates long-with the standard deviations. Different small letter bars indicated that the values are significantly different at *P<0.05*.

Table 2. Chromium accumulation in leaves, stem and roots of sunflower plants under different concentrations of Cr, citric acid and 5-AA.

Treatments	Cr Accumulation (µg plant^{-1})			
	Cr 0 (mg kg^{-1})	Cr 5 (mg kg^{-1})	Cr 10 (mg kg^{-1})	Cr 20 (mg kg^{-1})
		Leaf		
Cr 0, AA 0	2.9±0.47f	92.7±3.8f	138.1±7.66g	238.3±12.1f
CA 2.5	4.7±0.44f	131.4±6.7de	198.9±11.1lf	358.6±24.1d
CA 5	8.1±0.77e	167.7±5.0c	309.0±10.1cd	468.7±13.6c
AA 10	11.2±1.26de	124.3±12.0ef	201.4±12.9lf	303.6±8.4he
CA 2.5 + AA 10	17.8±1.22c	180.4±13.2c	282.8±13.9de	500.5±23.9c
CA 5 + AA 10	22.5±1.35b	263.4±11.1b	413.5±25.3b	632.4±28.1b
AA 20	12.6±0.85d	157.9±5.4cd	259.6±10.7e	390.8±10.6d
CA 2.5 + AA 20	21.5±0.77b	266.9±9.9b	348.01±11.1c	593.9±16.0b
CA 5 + AA 20	48.3±2.10a	354.7±23.5a	483.4±20.0a	746.7±15.9a
		Stem		
Cr 0, AA 0	5.0±0.4f	241.5±13.6e	546.3±14.4f	742.7±45.7e
CA 2.5	11.8±0.5e	391.5±35.4d	739.4±51.6e	920.0±27.3d
CA 5	15.3±1.5de	567.8±29.6c	957.3±71.1c	1156.7±65.4c
AA 10	11.7±1.74e	289.0±14.7e	674.2±39.6ef	868.5±52.4de
CA 2.5 + AA 10	18.7±2.2d	523.2±36.5lc	914.0±27.7cd	1099.4±32.9c
CA 5 + AA 10	28.2±1.9c	709.9±46.18b	1121.1±32.2b	1459.0±97.2b
AA 20	16.0±0.8de	333.2±26.51de	797.5±47.1de	1018.2±60.0cd
CA 2.5 + AA 20	46.3±1.8b	691.5±49.0b	1124.7±53.6b	1382.6±89.5b
CA 5 + AA 20	79.7±2.1a	956.3±25.2a	1364.1±43.8a	1768.7±42.7a
		Root		
Cr 0, AA 0	4.6±0.5f	173.6±24.0f	300.1±20.6lf	384.6±17.1e
CA 2.5	9.6±1.0ef	277.6±9.8de	406.3±32.3de	498.6±28.6d
CA 5	14.3±2.3de	350.0±29.8c	540.4±37.9c	597.7±24.1c
AA 10	6.5±0.9f	241.0±8.8e	382.8±22.2e	490.4±19.6d
CA 2.5 + AA 10	16.3+5.1d	347.4±19.2c	557.1±17.3fc	641.4±28.5c
CA 5 + AA 10	33.1±2.02b	459.2±24.4b	758.3±31.1b	757.2±33.1b
AA 20	7.6±0.7f	313.4±17.1cd	455.5±10.2d	600.1±23.7c
CA 2.5 + AA 20	24.8±1.45c	469.8±26.06b	692.1±23.4b	827.9±43.6b
CA 5 + AA 20	41.6±1.85a	624.6±14.3da	947.4±32.1a	1000.2±42.1a

Sunflower plants were grown in Cr spiked soil (0, 5, 10 and 20 mg kg^{-1}) and exogenously supplied with increasing citric acid concentrations (0, 2.5 and 5 mM) and 5-AA (0, 10 and 20 mg/L). Values are the means of three replications ±SD. Values possessing the different small letters are statistically significant at *P>0.05*.

***Cr content in plant*:** Cr accumulation in the plant's roots, leaves and stem grown on Cr-concentrated soil, along with the amendments of acids, is shown in Table 2. Increased uptake and accumulation of Cr was detected in plants provided with CA (2.5 mM, 5 mM) and 5-AA (10 mgL^{-1} and 20 mgL^{-1}) alone and in combination along with Cr. CA and 5-AA separately increased Cr uptake and accumulation and in collective application they presented additive impact for Cr uptake and accumulation and played vital role in supporting plants under Cr stress. Chromium stressed plants only showed fewer contents compared to those which are treated with Cr along with acid. Greater content of Cr was detected in different parts of plant under 20 mg Cr kg^{-1} of soil and collective supplementation of CA (5 mM) and 5-AA (20 mgL^{-1}).

DISCUSSION

***Agronomic traits*:** Growth and biomass characteristics of sunflower plants experiencing Cr toxicity and impact of CA and 5-AA combined as well as alone addition are presented in Table 1. Increased chromium dose (0, 5, 10 and 20 mgkg^{-1}) in soil steeply decreased plant growth parameters. Farid *et al.* (2015) and Adrees *et al*. (2015a) also identified reduced biomass and growth of plants under metal toxicity in *Brassica napus* L. and wheat respectively. In present study, toxicity of Cr minimized plant agronomic traits (Table 1) which is relevant to previous results of Afshan *et al.* (2015). According to Rizwan *et al*. (2016a), the growth reduction, due to substantial Cr uptake is due to suppressed uptake of important minerals required for optimal plants growth. Physiological

traits suppression and symptoms of toxicity were clear signs of Cr toxicity in the plant's species (Júnior *et al*., 2014). Plants supplemented with CA and 5-AA showed tolerance to the toxicity of Cr in comparison to plants grown only in Cr treated soil without any acid application (Table 1). The CA can enhance metal extraction and has played a role in different research works as reviewed by Rizwan *et al.* (2016b). Application of CA significantly increased the agronomic traits of *Brassica napus* L under Cr stress (Shakoor *et al.* 2014). Modern research recommended that successful supplementation of CA is proficient in effectual removal of metals through the plant (Ehsan *et al.,* 2014). The results described better Cr extraction in the occurrence of CA (2.5 mM and 5 mM) by increasing availability of metals in the soil. The CA helpful role in extraction of metals and health of sunflower is might be due to improved nutrients (macro and micro) supply (like Magnesium, Zinc, Copper and Iron) in the growing media. Application of 5-AA has reported to have antioxidant enzymes enhancement impact resulting growth enhancement under stress conditions (Naeem *et al.,* 2011). Greater 5-AA concentrations showed to be herbicidal (Sasikala *et al.,* 1994) and in current study 10 and 20 mgL^{-1} of 5-AA minimize the harmful effects of metals in plant. It might be due to regulation of the K^+ flux and the electrons transport chain which additionally encouraged antioxidants defensive system in plants (Gill *et al.,* 2014). During metal stress, the helpful role of both acids is greater than the separate application. Plants growth under Cr stress, is significantly controlled by the preserving effects of CA and 5-AA application.

***Soluble protein and SPAD value*:** Plants along with or/and without acid amendments appeared to have reduced chlorophyll and proteins content in leaves and the root of sunflower respectively under Cr stress (Fig. 1). The highest decrease was noticed in plants grownup under the Cr treatment only (5, 10 and 20 mg/kg) while the plants treated with acid amendments showed enhanced contents of soluble protein and SPAD value. Kotapati *et al.* (2016) and Khaliq *et al.* (2016) revealed that chloroplast distortion, which triggered the disturbance among gas exchange attributes including stomatal conductance and photosynthetic rate, might be the reason for suppressed SPAD value and soluble proteins of metal stressed plants. Afshan *et al.* (2015) and Jabeen *et al.* (2016) found the same type of decrease in chlorophyll value and protein contents in *Vigna radiata* and *Brassica napus* under Cr stress. Our findings are supported by previous results of Ali *et al.* (2013) and Gill *et al.* (2015a) on Cadmium and Chromium induced toxicity in *Brassica napus* who found the same declining trend of both contents. Maximum reduction in protein content and SPAD value was detected in plants grown in soil treated with 20 mg Cr/kg without any amendment. According to Ehsan *et al.* (2014) and Shakoor *et al.* (2014), exogenously applied citric acid (2.5 mM) significantly enhanced the nutrient uptake and chlorophyll contents of plants which further regulate the soluble protein and SPAD value. Citric acid can make complexes with micro and macronutrients, thereby facilitating their translocation from roots to shoots through xylem (Ramzan *et al*., 2016a). Improved antioxidant defense system might be another reason for the maintained soluble protein and SPAD value, as revealed by Sinhal *et al.* (2010) and Bareen *et al.* (2012). The highest amounts of SPAD value and soluble protein were found in plants under collective treatment of CA and 5-AA, with or without Cr stress. Highest concentrations of both acids i.e., 5 mM of CA and 20 mgL^{-1} of 5-AA, showed better performance in maintaining chlorophyll and protein content of plants. The 5-AA foliar application remarkably regulated the soluble proteins contents as well as SPAD value via limiting the generation of ROS and reduction in electrolyte leakage from plant cell (Fozia *et al.,* 2008; Nunkaew *et al.,* 2014). The combined role of both acids helped the plants grow normally under Cr toxicity. Even at higher Cr toxicity (20 mg/kg soil), the additive effect of both acids supported the plant to combat toxicity by maintaining protein and SPAD value.

***Electrolyte leakage, H_2O_2 and MDA*:** Heavy metal-stressed plants produced more ROS as compared to non-stressed ones (Gill and Tuteja, 2010). Rise in Cr level in soil upgraded the generation of ROS, electrolyte leakage and MDA in sunflower tissues (Fig. 2**).** Cr has been studied previously for increasing oxidative stress and lipid peroxidation in sunflower and *Brassica napus* by Fozia *et al.* (2008), Afshan *et al.* (2015) and Gill *et al.* (2015a). Electrolyte leakage indicated that the plants were facing severe stress in maintaining its normal electron transportation and absorption of enough micro and macronutrients (Adrees *et al.,* 2015b). The enhanced oxidative stress further resisted plant growth and inhibited production of photosynthetic pigments (Ali *et al.,* 2015). The acid-amended plants showed tolerance to toxicity of Cr and reduced oxidative stress as compared to non-amended plants (Ali *et al.,* 2013, 2015). Citric acid and 5-AA addition lessened the Cr induced oxidative damage in both control and treated plants, which promised the defensive characteristic of Citric acid (Shakoor *et al.,* 2014; Ehsan *et al.,* 2014). Rizwan *et al.* (2016b) studied the protective role of CA in different plant species against heavy metals. Plants' defense system, strengthened by the application of CA, combats oxidative stress and supports normal functioning of plants (Afshan *et al.,* 2015). The 5-AA foliar application scavenged ROS under the Cr stress by enhancing heme-based molecules activities which further increased the antioxidant enzymes contents (Naeem *et al.,* 2011; Gill *et al.,* 2015). Previous studies also stated the potential of 5-AA scavenging of ROS along with reduction in electrolyte leakage under salinity and drought (Li *et al.,* 2011; Freije and Alkhuzai, 2015).

***Antioxidant defense system*:** Plants naturally possess self-protection mechanism against abiotic and biotic stresses (Xu

et al., 2015) which consists of various antioxidants enzymes (SOD, POD, APX and CAT). The antioxidants enzymes respond differently in the leaves and roots of the sunflower. Effect of exogenously supplied CA and 5-AA in plants with and without Cr stress are given in Fig. 3**.** These enzymatic activities tend to boost up when plant bears oxidative stress caused by the H_2O_2 under metal toxicity as described by Farid *et al.* (2015), Arshad *et al.* (2016) and Jabeen *et al.* (2016). Zeiner *et al.* (2015) and Anjum *et al.* (2016) described the relation between the ROS scavenging and enzymatic activities. According to present study, the Cr treatment from 5 to 10 $mgkg^{-1}$ boosted up the enzymatic activities at their peak while at highest Cr concentration i.e. 20 $mgkg^{-1}$ the enzymatic activities and their effectiveness dropped down. This sudden decline in enzymatic activities with increasing Cr level indicates the severity of stress at higher Cr concentration i.e. 20 mg per kg soil (Adrees *et al.,* 2015b). Citric acid and 5-AA-amended plants possessed higher defensive activities in contrast to non-amended plant exposed to Cr toxicity. Citric acid addition, individually and in combination with 5-AA, to Cr stressed plants at any level of Cr, enhanced the defensive activities of enzymes. The sudden drop in enzymatic activity in the plants, treated with the greatest Cr concentration (20 $mgkg^{-1}$), was also restored under CA and 5-AA application. The collective impact of both acids was found to be additive and their protective role in stress bearing plant have already been studied under various heavy metals such as chromium, lead, copper, cadmium, arsenic (Shakoor *et al.,* 2014; Afshan *et al.,* 2015; Farid *et al.,* 2016). The protectiveness of CA towards reducing oxidative damage in plants, by increasing and strengthening defensive system, has also been reported by Mirzajani *et al.* (2015). Similar effect was observed in this study where application of CA (5mM) and 5-AA (20mg L^{-1}) has shown higher enzymatic activity than other sets of experiment (Fig. 3).

***Chromium uptake and accumulation*:** Exogenous application of citric acid (CA) and 5-AA significantly affected sunflower plant Cr accumulation. Exogenous supplementation of both CA and 5-AA acted differently for accumulation of Cr in the sunflower leaves, stem and the root (Table 2). Previous studies also reported the similar accumulation and uptake of Cr in various plants (Ali *et al.,* 2013, 2015). The plants accumulating great quantity of Cr can't absorb desire quantity of essential nutrients, which eventually disturb normal development and growth (Ali *et al.,* 2013), transpiration (Maqbool *et al.,* 2015), photosynthesis (Afshan *et al.,* 2015) and alters ultra-cellular structure (Gill *et al.,* 2014). As per this study results, concentration of Cr in the plant tissues was proportionally equivalent to level of Cr in soil (5, 10 and 20 $mgkg^{-1}$). Fozia *et al.* (2008) reported that the Cr in soil was significantly accumulated by sunflower. Heavy metal availability and solubility is improved by the addition of organic chelates like Citric acid (CA) (Sinhal *et al.,* 2010; Shakoor *et al.,* 2014). Afshan *et al.* (2015) used CA application approach for enhancing Cr phytoextraction from soil. Our results showed that the CA (2.5 & 5mM) treated plants possessed greater concentration and accumulation of Cr with maintained growth and strength of plant as compared to controls. In similar way, according to Hotta *et al.* (1997) and Ali *et al.* (2018), a little concentration of AA was also liable for improving the growth and increasing the crop yield under heavy metals stress. Foliar shower application of AA (10 and 20 mgL^{-1}) to sunflower grown under Cr stress (at 5, 10 and 20 $mgkg^{-1}$) level showed increased accumulation of Cr in contrast to the plants not applied with 5-AA. The combined effect of CA and 5-AA on Cr uptake and accumulation under Cr stress is higher than their individual effects.

***Conclusion*:** Present study determined that collective addition of CA and the AA remarkably mitigated sunflower from Cr-induced biochemical, morphological and physiological toxicity. Chromium 20 mg kg^{-1} spiked sets showed the highest reduction of growth in different sunflower tissues. Effective addition of CA and 5-AA, separately and in combination substantially improved the content of Cr in sunflower by increasing uptake of Cr and improved the antioxidant defense system which additionally supported plant normal functioning and metabolism. The maximum beneficial impact in terms of much improved growth and elevated Cr content was noticed with joint supplementation of CA (2.5 mM and 5 mM) and 5-AA 20 mgL^{-1} in control and 20 mg/kg Cr stress respectively.

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