https://doi.org/10.17221/581/2019-PSE

Indole-3-acetic acid synthesizing chromium-resistant bacteria can mitigate chromium toxicity in *Helianthus annuus* L.

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Citation: Fatima H., Ahmed A. (2020): Indole-3-acetic acid synthesizing chromium-resistant bacteria can mitigate chromium toxicity in *Helianthus annuus* L. Plant Soil Environ., 66: 216–221.

Abstract: Use of microorganisms as heavy metal remediators is an effective approach for chromium reduction in plants. Chromium carcinogenicity (Cr^{6+}) beyond the permissible levels elicits environmental and health problems. To reduce chromium toxicity along with the plant growth improvement, a cost-effective and eco-friendly remediation approach is necessary. In the current study, chromium-resistant bacterial species were evaluated for growth improvement of sunflower. Three auxin-producing bacteria able to tolerate hexavalent chromium, i.e., *Sporosarcina saromensis* (EI) and two species of *Bacillus cereus* (AR and 3a) were selected for the proposed study. Growth studies along with auxin synthesis potential of bacterial isolates with and without chromium were conducted. Results revealed a 188% enhancement in plant height under laboratory-grown plants with *B. cereus* (AR) under 500 mg/L chromium stress (Cr^{6+}). *B. cereus* (3a) also showed an 81% increase in leaf number with 400 mg/L chromium stress in laboratory-grown plants. Similarly, 73% increment in the amount of auxin was reported in the case of inoculation with *S. saromensis* isolate (EI) over respective control treatment. These improvements provide an excellent means of reducing chromium (Cr^{6+}) in the contaminated soils naturally by stimulating plant growth along with bioremediation potential.

Keywords: soil pollution; plant hormone; chromium-resistant microbe; hyperaccumulator; bioinoculation; heavy metal contamination

Trivalent chromium (Cr^{3+}) is stable, less toxic and less bioavailable as compared to hexavalent chromium (Cr^{6+}) form which is toxic, non-essential, causes serious illness, e.g., dermatitis, the problem in kidneys and lungs, irritation to eyes and respiratory tract (Oves et al. 2019, Solá et al. 2019, Yahaghi et al. 2019). The unregulated accumulation of chromium (Cr^{6+}) metal ions often leads to biomagnification, which is inimical for all life forms, including humans (Khanna et al. 2019, Levizou et al. 2019). In the present scenario, it is of extreme importance to reduce chromium uptake by natural means to limit the entry of chromium metal into our food chain, which then circulates up to higher living organisms by deteriorating the whole food web (Ferjani et al. 2019).

Auxin being a master plant hormone, has growth stimulatory properties and can be ideal for natural plant growth promotion (Ju et al. 2019). The main hypothesis of the present study is to evaluate the bioremediation potential of chromium-resistant auxin-producing bacterial isolates such as *Sporosarcina saromensis* and *Bacillus cereus*. These bacterial isolates hinder the direct uptake of chromium by converting hexavalent soluble form into the trivalent insoluble form that cannot be taken up by plants. The toxicity caused by chromium to plants is thus minimised. As a result, plant growth is improved in plants growing in chromium-polluted areas. The use of such chromium-resistant microbes limits the use of certain expensive chemicals and equipment to reduce chromium uptake by plants (Chen et al. 2019) and auxin production that helps in growth proliferation (Khanna et al. 2019).

The sunflower is a crop cultivated in wide areas of Pakistan with the potential to meet food and oil requirements. Along with other beneficial uses,

Supported by the University of the Punjab, Lahore, Pakistan.

https://doi.org/10.17221/581/2019-PSE

broad leaves and hyperaccumulator nature of sunflower makes it suitable for bioremediation purpose in chromium-contaminated sites. Bioinoculation of sunflower seeds using these chromium-resistant auxin-producing bacteria results in growth improvement of plants that can be used for oil, seed, and as fodder for cattle. It is a productive effort for healthy agricultural practices, which can be recognised as an economic, feasible, and natural strategy for minimising the chromium toxicity of the polluted soils. The sunflower produced after such practice can be used as a fodder for cattle farming.

MATERIAL AND METHODS

Characterisation of chromium-tolerant isolates. Three selected chromium-tolerant bacterial isolates (i.e., EI, AR, and 3a) previously isolated by Fatima and Ahmed (2016) from plant rhizosphere were checked for their auxin production potential, characterised by Cappuccino and Sherman (2007) and identified using 16S rDNA. Bacterial auxin was optimised by growing the plants under various physiological conditions, i.e., varying temperatures, different concentrations of chromium, and addition of a precursor.

Soil analysis. The used soil was loamy soil with 0 mg/L of chromium content. The soil temperature (i.e., 29 °C), with pH 8 and 0.71 m/S electrical conductivity of the used loamy soil were recorded, respectively. The NPK and carbon content were estimated following Motsara and Roy (2008), i.e., N (Kjeldahl) 485 mg/kg, P (Olsen) 3 mg/kg, K (Jenway PFP7 Flame Photometer) 243 mg/kg and carbon content (Walkley and Black chromic acid wet oxidation method) 9 700 mg/kg, respectively.

Inoculation experiment with *Helianthus annuus* L. The inoculation experiment was performed using selected isolates under chromium stress, i.e., 0, 100, 200, 300, 400, and 500 mg/L following Fatima and Ahmed (2016). For wirehouse and laboratory experiments, 7.3 kg and 181.9 g soil was used in each pot, respectively, with no added fertilisers. The wirehouse temperature was 27 + 3 °C with 50–60% humidity and 11 h photoperiod. Five seeds per pot (var. 6741) procured from Punjab Seed Corporation, Pakistan, were sown in triplicates for each treatment. In the experimental setup, plants were grown as control, with chromium stress (0–500 mg/L), with bacterial inoculation only and with both bacterial inoculation and chromium stress simultaneously. In the case of laboratory-grown plants, seedlings were harvested after 25–30 days while wire house plants were harvested at maturity. Various growth and biochemical parameters including plant height (i.e., root and shoot in cm), leaf number, auxin estimation (Mahadevan 1984), pigment analysis (Lichtenthaler and Wellburn 1983) and protein (Lowry et al. 1995) and proline estimation (Bates et al. 1973) were recorded.

Statistical analysis. Data analysis was conducted using the SPSS 16.0 software (Chicago, USA) by applying Duncan's multiple range test.

RESULTS

Characterisation of chromium-tolerant isolates. Morphological characterisation demonstrated that all the isolates were non-motile, gram-positive rods with spore-forming ability. Isolates EI and 3a produced 8 mg/L auxin, while isolate AR showed 6 µg/mL auxin when evaluated for indole-3-acetic acid (IAA) production potential. Maximum bacterial growth was noted at 37 °C after overnight incubation with chromium (500 mg/L). Better growth was recorded in liquid broth growth medium at pH 6 and 8, but the maximum bacterial growth was noted at pH 7 with no chromium. The isolate EI showed homology to *Sporosarcina saromensis* (accession No. KT321457), while the isolates 3a and AR have similarity with *Bacillus cereus* (accession No. KM409709 and KT321456, respectively). Auxin quantification at isolates grown with and without chromium was also noted. Higher levels of heavy metal stress lowered the bacterial IAA synthesis due to the destructive effects of hexavalent chromium as compared to isolates grown with no chromium where IAA synthesis was more pronounced.

Inoculation experiment with *Helianthus annuus* L. Under chromium stress, non-inoculated plants showed a remarkable decrease in sunflower growth. However, the inoculation with *Bacillus cereus* (3a) showed a 131% increment in shoot length at 500 mg/L chromium over control (Figure 1). Maximum phytotoxicity was observed at 500 mg/L, which exerted adverse effects on root growth, causing root damage and reduced mineral uptake. Treatment with *Bacillus cereus* (3a) with 0, 300, 400, and 500 mg/L of chromium (Cr^{6+}) caused improvement in root length up to 12, 10, 54, and 51%, respectively. Similarly, the bacterial treatment also exerted positive effects on leaf number as 14, 20, 32, 45, 81 and 57% increase was noted with 0–500 mg/L chromium with *Bacillus cereus* (3a) in laboratory-grown plants. At maturity, large flowers with a higher number of ray florets were

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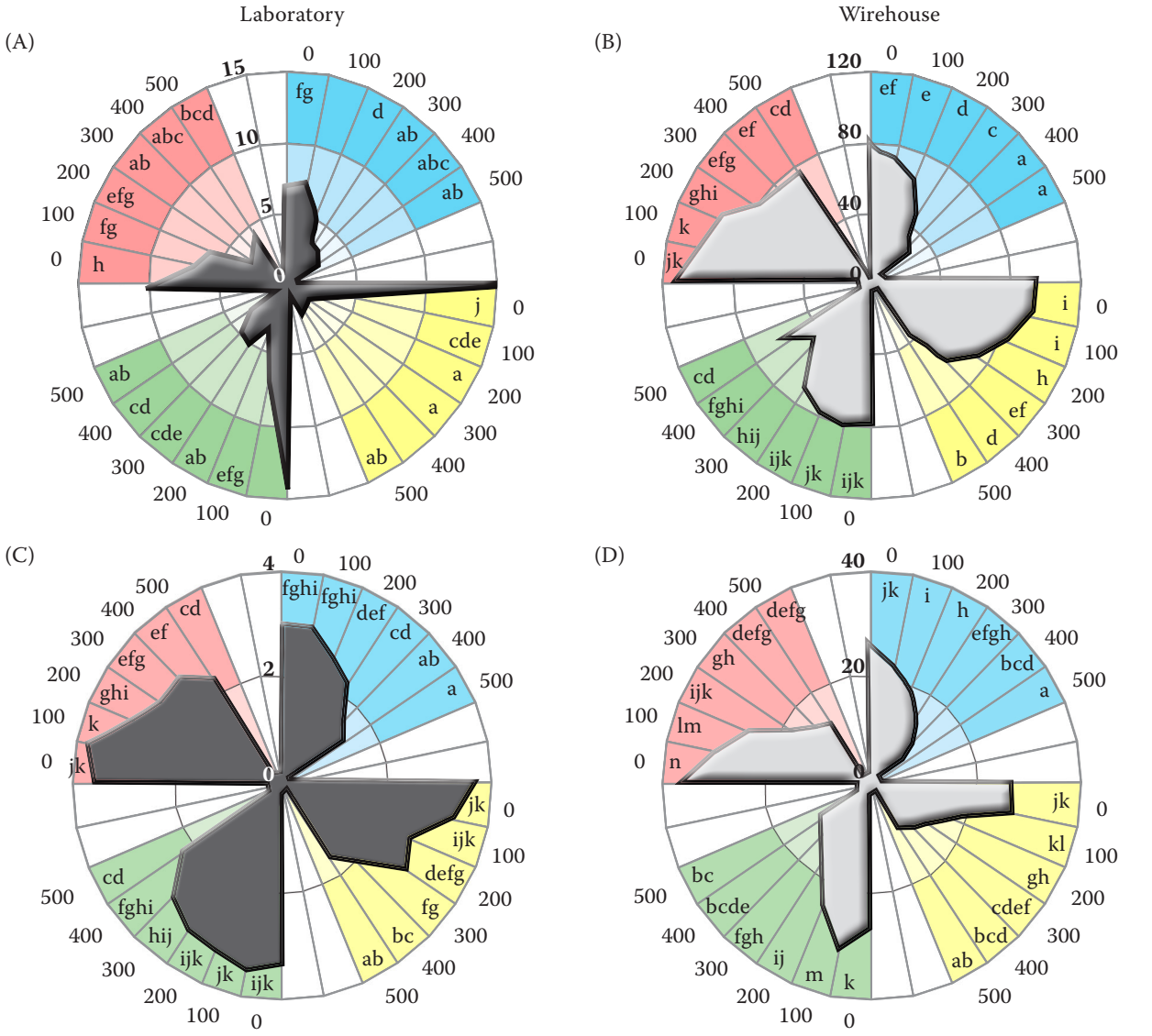
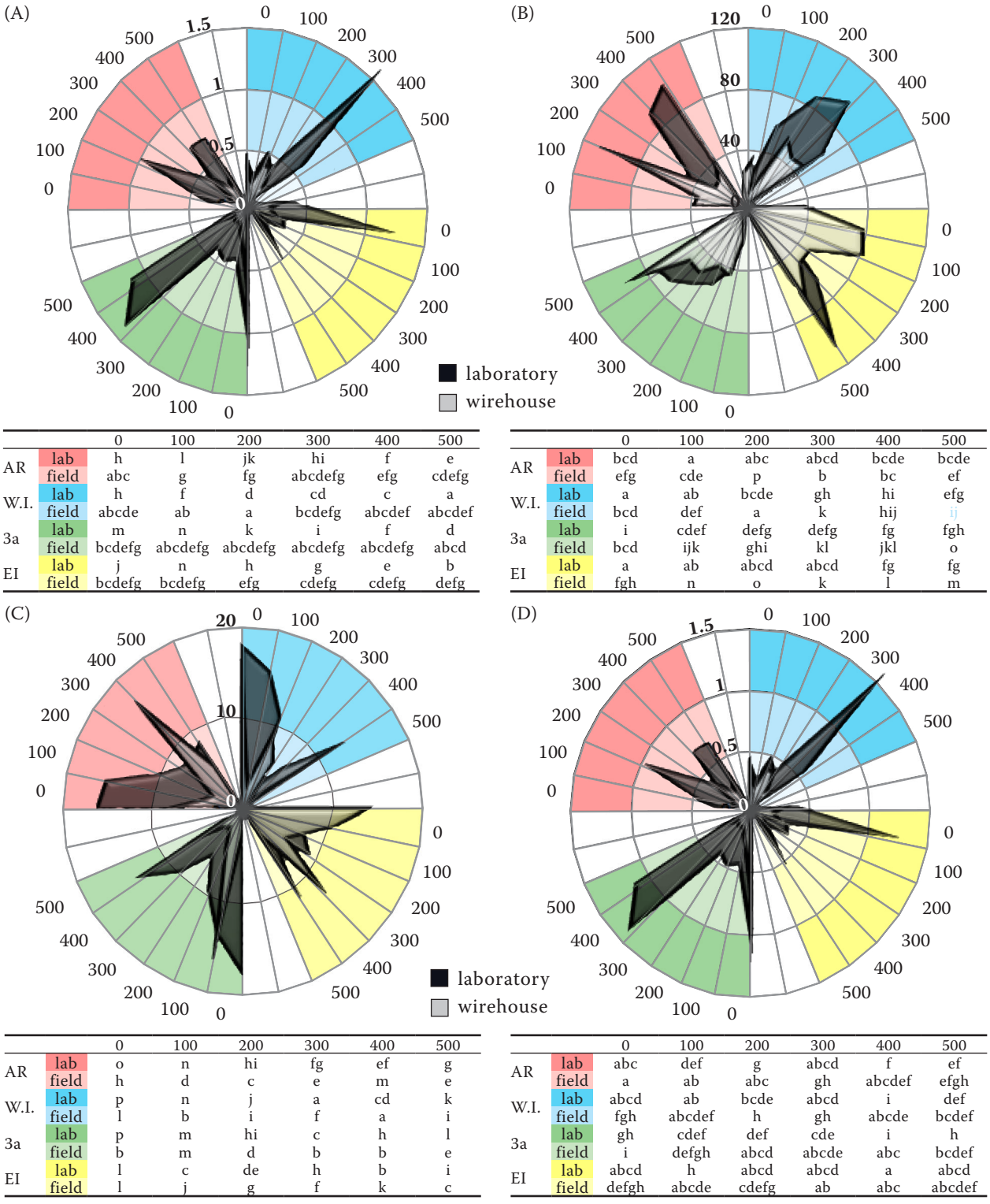


Figure 1. Effect of bacterial inoculations under chromium stress [K_2CrO_4 (0–500 mg/L)] on shoot length (cm) and leaf number of *Helianthus annuus* L. under laboratory and wirehouse conditions. Data represent mean of fifteen replicates. W.I. (□) – without bacterial inoculation; *Sporosarcina saromensis* (EI, □) and *Bacillus cereus* (3a, □) and *B. cereus* (AR, □) – bacterial isolates; (A) shoot length under laboratory conditions; (B) shoot length under wirehouse conditions; (C) leaf number under laboratory conditions; (D) number of ray florets under wirehouse conditions. Different letters indicate significant differences between treatments using the Duncan's multiple range test ($P \leq 0.05$)

recorded under stress conditions (Figure 1). In treated pots, 22% and 80% increment in the number of ray florets was noted with 400 and 500 mg/L chromium by *Bacillus cereus* (AR).

Auxin content also showed an increase up to 500 mg/L chromium. For instance, *Bacillus cereus* (AR) caused 68, 121, 93, 70 and 130% increment under laboratory conditions and 164, 135, 92, 115 and 108% increase under field-grown plants with 100–500 mg/L chromium stress, respectively which is statistically analysed at $P \leq 0.05$ (Figure 2). Similar enhancement was also recorded in other parameters such as proline content, amount of chlorophyll *a*, *b* and total chlorophyll and protein content in laboratory and wirehouse conditions. In the current experiment, the toxicity of chromium caused a significant reduction in the overall yield of the plants, which is statistically analysed at $P \leq 0.05$ (Figure 2).

https://doi.org/10.17221/581/2019-PSE



(A)

		0	100	200	300	400	500
AR	lab	h	l	jk	hi	f	e
	field	abc	g	fg	abcdefg	efg	cdefg
W.I.	lab	h	f	d	cd	c	a
	field	abcde	ab	a	bcdefg	abcdef	abcdef
3a	lab	m	n	k	i	f	d
	field	bcdefg	abcdefg	abcdefg	abcdefg	abcdefg	abcd
EI	lab	j	n	h	g	e	b
	field	bcdefg	bcdefg	efg	cdefg	cdefg	defg

(B)

		0	100	200	300	400	500
AR	lab	bcd	a	abc	abcd	bcde	bcde
	field	efg	cde	p	b	bc	ef
W.I.	lab	a	ab	bcde	gh	hi	efg
	field	bcd	def	a	k	hij	ij
3a	lab	i	cdef	defg	defg	fg	fgh
	field	bcd	ijk	ghi	kl	jkl	o
EI	lab	a	ab	abcd	abcd	fg	fg
	field	fgh	n	o	k	l	m

(C)

		0	100	200	300	400	500
AR	lab	o	n	hi	fg	ef	g
	field	h	d	c	e	m	e
W.I.	lab	p	n	j	a	cd	k
	field	l	b	i	f	a	i
3a	lab	p	m	hi	c	h	l
	field	b	m	d	b	b	e
EI	lab	l	c	de	h	b	i
	field	l	j	g	f	k	c

(D)

		0	100	200	300	400	500
AR	lab	abc	def	g	abcd	f	ef
	field	a	ab	abc	gh	abcdef	efgh
W.I.	lab	abcd	ab	bcde	abcd	i	def
	field	fgh	abcdef	h	gh	abcde	bcdef
3a	lab	gh	cdef	def	cde	i	h
	field	i	defgh	abcd	abcde	abc	bcdef
EI	lab	abcd	h	abcd	abcd	a	abcd
	field	defgh	abcde	cdefg	ab	abc	abcdef

Figure 2. Effect of bacterial inoculations under chromium stress [K_2CrO_4 (0–500 mg/L)] on the auxin, proline, total chlorophyll and protein content (μg/g) of *Helianthus annuus* L. under laboratory and wirehouse conditions. Data represent mean of fifteen replicates. W.I. (□) – without bacterial inoculation; *Sporosarcina saromensis* (EI, □) and *Bacillus cereus* (3a, □) and *B. cereus* (AR, □) – bacterial isolates; (A) auxin content under laboratory and wirehouse conditions; (B) proline content under laboratory and wirehouse conditions; (C) total chlorophyll content under laboratory and wirehouse conditions; (D) protein content under laboratory and wirehouse conditions. Different letters indicate significant differences between treatments using the Duncan's multiple range test ($P \leq 0.05$)

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DISCUSSION

Presently, chromium toxicity causing seed germination inhibition, poor flower quality, chlorosis, and overall reduced plant growth is a major environmental concern (Gupta et al. 2017). Furthermore, plant height reduction with a gradual increase in chromium carcinogenicity (0–500 mg/L) is another drawback resulting in poor yield (Stambulska et al. 2018). Rhizoshpheric bacteria used in the current studies, i.e., *Sporosarcina saromensis* and *Bacillus cereus*, are beneficial, causing growth-promoting impact by increasing root permeability and root metabolites absorption. Auxin being a master hormone has growth stimulatory properties and can be ideal for plant growth promotion naturally. The growth stimulation impact of these auxin-producing bacteria was utilised in Cr^{6+} reduction into Cr^{3+} form along with growth improvement simultaneously. These microorganisms are helpful in reducing the toxic effects of chromium and ultimately control environmental pollution (Nafees et al. 2018). Soil analysis provides an insight to the subsequent soil elements by determining nutrient quantity, which is crucial for determining overall plant growth.

Due to defense mechanisms, the production of reactive oxygen species (ROS) by plants provides another hallmark modifying protein conformation, nucleic acids, and lipids during survival strategies (Ali et al. 2018, Rizvi et al. 2019). Higher chromium concentrations reduced the growth of sunflower leaves, the main photosynthetic plant organ. The leaf biomass and leaf area were significantly reduced, which was accompanied by decreased photosynthesis, chlorosis, and necrosis (Zunji et al. 2019). Chromium toxicity also affects the plant cell metabolism by declining the number of active reaction centres of photosystem II, which then affects the rate of electron transport and also changes the overall photosynthetic activity of the plant (Habib et al. 2019). Low photosynthesis results in shoot length reduction in plants growing under stress environments. During the wirehouse trial, inoculated plants showed less chronic conditions and better growth as compared to non-inoculated plants grown in stress environment (400 and 500 mg/L) with pale-yellow colour, necrotic appearance and tip burns as the visible symptoms due to chromium toxicity. The plants possess certain regulatory mechanisms for chromium detoxification, including reduction of Cr^{6+} to Cr^{3+} in the thin lateral roots, immobilisation of Cr ions by a root cell wall, the formation of highly stable complexes including peptides, carbohydrates, nicotinamide adenine dinucleotide (NADH) and organic acids. The storage of these complexes in root vascular cells caused morphological and physiological changes (Zhou et al. 2019, Ripa et al. 2019). The defense mechanisms act as a shelter for the growing plants from adverse effects of chromium and also protect them from inhibition of seed germination, protein inactivation, modification in enzyme activity, DNA damage, inhibition of electron transport systems and the overall reduction of photosynthesis leading to leaf chlorosis in extreme situation (Francisco et al. 2018).

In this experiment, hexavalent chromium at low concentrations (100–400 mg/L) caused an increase in proline content while at high concentrations, reduction in proline was noted during wirehouse trials. This trend has been reported by many scientists indicating the induction of adaptive response in plant tissues at low concentrations allowing plants to tolerate metal toxicity without substantial negative effects, which fails as toxicity increases (500 mg/L), resulting in low yield (Rocha et al. 2019). Under wirehouse conditions, the gradual increase in plant growth up to maturity exhibited various biochemical changes, including lowering of auxin and protein contents both under chromium and without chromium conditions. A similar trend was reported in the photosynthetic pigments of sunflowers due to chromium toxicity (Figure 2).

Acknowledgment. We thank University of the Punjab, Lahore, Pakistan for providing opportunity to do research.

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Received: October 28, 2019
Accepted: April 29, 2020
Published online: May 19, 2020