### 1 Original Research Paper

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Chromium (VI) reducing *Brevibacillus brevis* OZF6 inoculation enhances pea growth and
decreases metal uptake in pea plants

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### 6 Abstract

Aim: Hexavalent chromium (Cr (VI)) is toxic because it is highly soluble in water, permeable through biological memberanes and interacts with proteins and nucleic acids which makes it more toxic and carcinogenic than trivalent. Microbes convert toxic chromium Cr (VI) to the stable and less soluble Cr (III) can be used for detoxification of Cr (VI) from contaminated environment. In this study authors wanted to see the effect of chromium (VI) reducing bacteria on the growth, photosynthestic pigments, nodulation and metal accumulation in pea crop.

Place and Duration of Study: This study was carried out at the Department of Biological
Sciences, Crescent University Abeokuta Ogun State, Nigeria in the Year 2015.

Methodology: Cr (VI) reduction in both free and immobilized cells was by 1,5-Diphenyl
Carbazide method. Pigments, plant growth and metal accumulation were determined as per the
standard methods.

**Results:** *Brevibacillus brevis* OZF6 reduced Cr (VI) significantly when bacteria were immobilized by sodium alginate compared to free cells. When *Brevibacillus brevis* OZF6 was inoculated to pea, bio-inoculant increased seed germination, growth, nodulation, photosynthetic pigments and protein compared to un-inoculated but amended with metal. OZF 6 significantly checked accumulation of chromium in roots and shoots compared to only metal-amended plants.

Conclusions: Due to above properties, OZF6 could therefore be used as bioremediator of Cr
 (VI) in chromium contaminated environment and thus will protect the environment.

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Keywords: Chromium (VI) tolerance, *Brevibacillus brevis*, Chromium (VI) reduction,
Immobilization, Pea growth, Nodulation, Photosynthetic pigments

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### 29 **1. INTRODUCTION**

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The contamination of chromium (VI) is mainly is due to the use of Cr (VI) in leather, tanning, 31 metallurgy, electroplating, textile, and pigment manufacturing industries [1-3]. Chromium occurs 32 either in trivalent or hexavalent which affect growth of microorganisms present in the 33 environment [4]. Solubility of Cr (VI) makes it highly toxic and thus will easily pass through 34 biological membranes and can easily damage proteins and nucleic acids particularly DNA, thus 35 inhibits the number of species of the microbes and also their growth [5,6]. Reduction of Cr (VI) 36 leads to the formation of stables, less soluble and less toxic Cr (III). Hexavalent chromium being 37 the most toxic, trivalent is an essential micronutrient for animals, plants and humans which is 38 involved in glucose metabolism [7], stimulation of enzyme system [8] and stabilization of 39 nucleic acids by increasing the processivity of DNA polymerase [9]. Reduction of toxic Cr (VI) 40 to Cr (III) is thus a useful process for remediation of Cr (VI) affected environments [10] and thus 41 can be readily used to save our soil and water from the toxic effects of these metals. The 42 reduction of Cr (VI) has been reported in Bacillus [11,12], Pseudomonas sp. [13-14], 43 Escherichia Coli [15], Microbacterium [2], Ochrobactrum intermedium [16] and Micrococcus 44 [17]. 45

Mechanism of chromium (VI) reduction may be direct or indirect and is influenced by pH, 46 temperature, concentration of chromium, incubation periods and the microorganisms used. It is 47 48 the chromium reductases whether intracellular or extracelluar which reduce chromium (VI) into chromium (III) in the direct mechanism [18] whereas in case of indirect method, reductants or 49 oxidant, such as H<sub>2</sub>S, reduce chromium [19]. Furthermore, in growing cultures with added 50 carbon sources as electron donors and in cell suspensions, Cr (VI) reduction can be 51 52 predominantly aerobic or anaerobic, but generally not both. Interestingly, chromium reductases can catalyse reduction of Cr (VI) to Cr (III) anaerobically [20], aerobically [21] and also both 53 54 anaerobically and aerobically. The Cr (VI) reductase may be present in the membrane fraction of the cells of PGPR, as found in Pseudomonas fluorescens and Enterobacter cloacae [22]. 55 56 Chromium reductase may also be present intracellularly which will reduce chromium (VI) into hromium (III) [23]. The resultant insoluble precipitate formed by the reduction of the more toxic 57 form of chromium (Cr (VI)) to less toxic form of chromium (Cr (III)) can be easily removed 58 from wastewater [10]. The enzyme chromium reductase found in *P. ambigua* [24] and *Bacillus* 59

sp. [25] were purified and characterized. More recently, to clone a chromate reductase gene, 60 novel soluble chromate reductase of P. putida was purified to homogeneity and characterized 61 62 [26]. The reductase activity was NADH- or NADPH-dependent. Reduction of Cr (VI) by H<sub>2</sub>S produced by the bacterial cells is found in soil environments which are rich in sulfate under 63 anaerobic conditions [18]. Hydrogen sulfide, produced in acid sulfate soil under reducing 64 conditions, is easily precipitated as FeS in reduced soils [27] and sediments. Fe (II) and H<sub>2</sub>S, 65 66 both microbially produced, are effective reductants of Cr (VI) under reduced conditions as is the FeS [28]. 67

Present study was therefore under taken (1) to check sodium aliginate as an immobilizing matrix for Cr (VI) removal (3) to check the reduction in fed batch experiments (4) See the effect of chromium (VI) reducing *Brevibacillus brevis* OZF6 on the growth, nodulation, photosynthetic pigment and protein content of pea plants (4) To check the effect of chromium (VI) reducing bacteria on the metal accumulation of pea plants.

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### 2. MATERIALS AND METHODS

### 74 2.1 Chromium (VI) reduction in free and immobilized cells

Natural materials like sodium aliginate (SA) at varying concentration were used to immobilize 75 Brevibacillus brevis OZF6 cells to see their effect on Cr (VI) reduction. Sodium aliginate was 76 used in the concentration of 0.5 g, 1.0 g and 1.5 g. Preparations of beads was performed as 77 follows: (1) Sodium alginate was mixed in 20 ml of deionized water, and then solution was 78 heated to  $80^{\circ}$  C in order to dissolve sodium aliginate; (2) when the immobilizing agent got 79 dissolved in deionized water, then solution was cooled to  $40^{\circ}$  C, (3) After cooling solution, about 80 1 g (fresh weight) of bacterial cells (overnight growth) was added and mixed; (3) For the 81 82 preparation of cell beads we mixed the mixture as drops into 50 ml degassed boric acid solution containing 2 % (w/v) calcium chloride, and was immersed for 24 h. The solution was dropped 83 into immobilizing phase with the help of sterile 10 ml disposable plastic syringe with a 21-G 84 needle. Beads (3-5 mm in diameter) were washed three times with 100 ml sterile distilled water 85 and added aseptically to 100 ml NB medium containing 100 µg/ml K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> in a 250 ml flask. 86 The flasks were incubated at  $37^{0}$  C. Samples were taken at regular intervals and Cr (VI) 87 concentration was detected by 1, 5 – diphenyl carbazide method [29] upto 120 h. Briefly, the test 88

samples were acidified (pH 1-2) and 1,5 diphenyl carbazide (50 µg/ml) was added and Cr (VI)
concentration was detected by UV-VIS spectrophotometer at 540 nm.

### 91 **2.2** Chromium reduction by both free and immobilized cells in fed batch experiments

For the fed-batch experiments, bottles containing 100 ml of NB broth amended with 100 µg/ml Cr (VI) and inoculated with and with out immobilized cells (wet weight, 1 g) were used. The bottles were incubated at 30°C. Samples were collected periodically and monitored for Cr (VI). When almost all of the Cr (VI) was removed from the medium, it was replaced with fresh sterile LB broth (100% exchange) and amended with Cr (VI). This procedure was repeated up to three times. The Cr (VI) content of the liquid samples collected at different times during each batch were determined as above.

### 99 **2.3 Plant growth**

100 The experimental soil was sandy clay loam (organic carbon 0.37%, Kjeldahl N 0.65 g/kg, Olsen 101 P 15.5 mg/kg, pH 7.1 and WHC 0.42 ml/g, Cr (VI) 4.2 µg/g). Seeds of pea var. Arket were surface sterilized (70% ethanol, 3 min; 3% sodium hypochlorite, 3 min), rinsed six times with 102 sterile water and shade dried. The sterilized seeds were coated with Brevibacillus brevis OZF6, 103 grown in nutrient broth, by dipping the seeds in liquid culture medium for two hours using 10% 104 105 gum Arabic as an adhesive to deliver approximately bacterial cells on the seed. The non-coated sterilized seeds soaked in sterile water served as control. The non-inoculated and inoculated 106 107 seeds (10 seeds per pot) were sown in clay pots (30 cm high, 20 cm internal diameter) using three kg sterilized soil with control (without chromium) and one treatment each with 60 mg Cr/ 108 109 kg soil. The concentration of Cr (60 mg Cr/kg) used in this study was comparable to those found in sewage waste water. Six pots used for each treatment were arranged in a complete randomized 110 design. One week after emergence, plants in each pot were thinned to three plants. The pots were 111 watered with tap water when required and were maintained in an open field condition. All plants 112 113 in the pots for each treatment were removed at 90 days after seeding (DAS), and were observed for plant growth. Plants uprooted at 90 days were oven-dried at 80<sup>°</sup> C and the dry matter was 114 measured. Nodule number and nodule dry weight per plant were observed after 90 days of their 115 growth. Total chlorophyll contents in fresh foliage of pea grown in metal stressed and metal free 116 (control) soil was quantified at 90 DAS by the method of Arnon [30]. Protein was measured after 117

90 days of pea growth by the method of Lowery et al. [31]. Caretenoid was measured after 90
days of growth of pea plant amended with and without metal by the method of Sadasivam and
Manikam [32].

The chromium content in roots and shoots of pea plants were measured after 90 DAS. The plant samples were digested in nitric acid and perchloric acid (4:1) following the method of Ouzounidou et al. [33].

### 124 **2.4 Statistical Analysis**

Data of the mean of six replicates of the measured parameters were subjected to two way analysis of variance (ANOVA) to see the main effects and interaction among factors and significant partial difference (LSD) was calculated at 5% probability level. Significant difference among the treatments was calculated using Duncan's multiple range test. Values indicate mean  $\pm$ S.D of the replicates.

130 **3. RESULTS** 

### 131 **3.1 Effect of immobilization on Cr (VI) reduction**

132 In this study we checked the immobilizing effect of sodium aliginate on Cr (VI) reduction by Brevibacillus brevis OFZ6 compared to free cells after 120 hours of incubation (Fig. 1). Among 133 134 different matrices combinations for whole cell immobilization of OZF6, the combination of 1.5g sodium alginate proved to be the best combination for Cr (VI) reduction and reduced chromium 135 136 (VI) significantly as compared to control cells (Fig. 1). Maximum reduction of Cr (VI) was observed in strain OZF6 when immobilized by 1.5 g sodium aliginate compared to the other 137 combinations of 0.5 and 1.0 g SA. Strain OZF 6 reduced Cr (VI) by 87% after 120 hours of 138 incubation when immobilized on 1.5 g sodium aliginate. Concentration of 1.5 g SA showed an 139 140 increase of 13% in Cr (VI) reduction by Brevibacillus brevis OZF 6, compared to free cells after 120 hours of incubation. 141

### 142 **3.2** Fed batch Reduction of Cr (VI) by both free and immobilized *Brevibacillus brevis* OZF6

Fed batch removal of Cr (VI) by both free and immobilized cells of *Brevibacillus brevis* OZF6 is shown in fig. 2. Cr (VI) was repeatedly added after every five days and Cr (VI) reduction was checked after 5, 10 and 15 days of incubation. Reduction approached almost completion in each batch and was sustained in subsequent batches. *Brevibacillus brevis* OZF65 significantly reduced chromium (VI) compared to control cells. *Brevibacillus brevis* OZF65 reduced more than 85%

of Cr (VI) when the strain was immobilized by 1.5 g SA after each batch compared to free cells whose reduction was less than 75% in each batch. In second cycle i.e after ten days of incubation Cr (VI) reduction decreased compared to first cycle but decrease was very less, almost reduction was sustained in the second cycle. Same pattern was observed in the third cycle (after 15 days of incubation) but there was little bit more decrease in reduction. Undoubtedly microbial cells repeatedly can sustain the removal of Cr (VI) in fed batch experiments.

# 3.3 Effect of chromium reducing *Brevibacillus brevis* OZF6 inoculation on the growth and nodulation of pea crop under the influence of the metal

- Seed germination of pea crop decreased in the presence of the metal. But when the crop was inoculated with the *Brevibacillus brevis* OZF6 amended with and without metal, seed germination of pea increased significantly as compared to the control plant (Table 1).
- Pea plants grown in soil amended with chromium (VI) showed variable growth and nodulation (Table 1 and 2). Generally, length, total dry weight and nodulation at 90 days, decreased significantly when pea was exposed to the metal. In contrast, plants inoculated with *Brevibacillus brevis* OZF6 significantly increased the measured parameters, even in the presence of the metal (Table 1 and 2). The two way ANOVA revealed that individual effects of inoculation and Cr (VI) and their interaction (inoculation x Cr (VI)) were significant (pB  $\leq$  0.05) for measured parameters at 90 DAS.

# 3.4 Effect of *Brevibacillus brevis* OZF6 inoculation on photosynthetic pigments and seed protein

- Photosynthetic pigments like chlorophyll and carotenoid and seed protein decreased significantly 168 at 60 mg Cr/kg of soil compared to the control plants (Table 1 and 2). But when the pea crop was 169 inoculated with the Brevibacillus brevis OZF6, increased the measured parameters significantly 170 171 compared to the control plants. Even when metal was amended with the bacterial strains, bacterial strains increased chlorophyll, carotenoid and seed protein significantly compared to the 172 173 control plants (Table 1 and 2). The two way ANOVA revealed that the individual effects of inoculation and Cr (VI) and their interaction (inoculation x Cr (VI)) were significant (p B  $\leq 0.05$ ) 174 for the measured parameters at 90 DAS. 175
- 176 **3.5** Accumulation of metal in plant tissues

177 The accumulation of chromium in plant tissues differed among treatments (Table 2). The uptake 178 of chromium by the roots and shoots of pea plants was higher in the presence of the metal. In

179 contrast, the bioinoculant significantly ( $P \le 0.05$ ) decreased the concentration of the metal in tissues, compared to the un-inoculated but metal amended plants. 180

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### 4. **DISCUSSION**

Hexavalent chromium being the most toxic, trivalent is an essential micronutrient for animals, 182 plants and humans which is involved in glucose metabolism [7], stimulation of enzyme system 183 [8] and stabilization of nucleic acids by increasing the processivity of DNA polymerase [9]. 184 185 Reduction of toxic Cr (VI) to Cr (III) is thus a useful process for remediation of Cr (VI) affected environments [10] and thus can be readily used to save our soil and water from the toxic effects 186 of these metals. Brevibacillus brevis OFZ6 was isolated and identified as described previously 187 [34]. Maximum reduction of Cr (VI) was observed in strain OZF6 when immobilized by 1.5 g 188 sodium aliginate compared to the other combinations of 0.5 and 1.0 g SA after 120 hours of 189 incubation. Our study is in correlation with the study of Humphries et al. [35]; Poopal and 190 Laxman [36]. They also observed that when Desulfovibrio vulgaris was immobilized by agar 191 reduced 0.5 mM (VI) in 22 hours whereas Microbacterium sp. NCIMB 13776 when immobilized 192 by agar reduced 0.5 mM Cr (VI) within 65 hours of incubation [35] while the PVA-alginate 193 immobilized Streptomyces griseus cells removed 0.48 mM Cr(VI) within 24 h [36]. In another 194 study Pang et al., [37] also observed 50% Cr (VI) reduction in 84 hours when Pseudomonas 195 aeruginosa was immobilized in polyvinyl alcohol/sodium aliginate matrix. 196

197 Brevibacillus brevis OZF65 reduced more than 85% of Cr (VI) when the strain was immobilized by 1.5 g SA after each batch compared to free cells whose reduction was less than 75% in each 198 199 batch. Undoubtedly microbial cells repeatedly can sustain the removal of Cr (VI) in fed batch experiments. This study has demonstrated that Cr (VI) reduction was dependent on the initial 200 201 content of bacterial biomass, as it was also observed by others [38]. Furthermore, the negative impact of the metal is avoided if we will use already grown bacteria for the reduction of Cr (VI). 202 The lack of a delay demonstrates that the necessary enzymes are constitutively expressed. This 203 study has concluded that for the successful bioremediation it is not necessary to previously 204 expose the bacterial cells to chromium and subsequent microbial enrichment. This could be 205 mainly due to the involvement of constitutive chromate reductases, thus corroborating the earlier 206 observation of the rapid reduction of Cr (VI) by *Pseudomonas putida* unsaturated biofilms [39]. 207

Seed germination of pea crop decreased in the presence of the metal. But when the crop was inoculated with the *Brevibacillus brevis* OZF6 amended with and without metal, seed germination of pea increased significantly as compared to the control plant (Table 2).

Heavy metals toxicity results in change in the cell permeability. Additionally, heavy metals inhibited the expression of specific enzymes for germination, which are involved in the seed coat breakdown [40]. Similar results were also reported by Karthak et al. [41] who also studied decrease in seed germination of the legume crop when the plant was grown under heavy metal stress. Karthak et al. [41] reported that on inoculation of the crop with the bioinoculant amended with metal, there was significant increase in the seed germination compared to control plants.

Pea plants grown in soil amended with chromium (VI) showed variable growth and nodulation. 217 Generally, length, total dry weight and nodulation at 90 days, decreased significantly when pea 218 was exposed to the metal. In contrast, plants inoculated with Brevibacillus brevis OZF6 219 significantly increased the measured parameters, even in the presence of the metal. Chromium 220 (VI) toxicity exerted severe effects on root growth and function, resulting in root damage, 221 reduction in fresh weight, cell division, root elongation and reduced the uptake of water and 222 nutrients [42]. Moreover, accumulation of heavy metals in plant tissues may trigger water deficit, 223 resulting in reduced growth and development of plants [41]. But when the seed was inoculated 224 with the bio-inoculants, significantly increased the length, dry weight and nodulation of the pea. 225 These bacteria can increase the growth of the plant due to the reduction of chromium (VI) to 226 227 chromium (III) which may have increased the growth and nodulation of the pea plant [41]. Trivalent is an important micronutrient used by animals, plants and humans which triggers 228 glucose metabolism [7], stimulates enzymes [8] and stabilizes nucleic acids by increasing the 229 processivity of DNA polymerase [9]. 230

231 Photosynthetic pigments like chlorophyll and carotenoid and seed protein decreased significantly at 60 mg Cr/kg of soil compared to the control plants. But when the pea crop was inoculated with 232 the Brevibacillus brevis OZF6, increased the measured parameters like chlorophyll, carotenoid 233 and seed protein significantly compared to the control plants. Similar increase in the 234 235 photosynthetic pigments was observed when plant was inoculated with the bacterial strains amended with or without metal [41]. In another study Wani and Khan [43] also observed 236 increase in the photosynthetic pigments and seed protein upon inoculation of the bacterial strain 237 in metal amended soil. 238

239 The accumulation of chromium in plant tissues differed among treatments. The uptake of chromium by the roots and shoots of pea plants was higher in the presence of the metal. In 240 241 contrast, the bioinoculant significantly ( $P \le 0.05$ ) decreased the concentration of the metal in tissues, compared to the un-inoculated but metal amended plants. The decreased concentration of 242 chromium in plant organs could be due to the reduction, adsorption/desorption of metal by the 243 OZF6 strain, as reported by Mamaril et al. [44], Wani et al. [45] and Wani and Khan [43]. 244 Karthik et al. [41] also repotted significant decrease in metal accumulation in the plant tissue 245 when bio-inoculant was inoculated to the crop amended with the metal. 246

### 247 **5. CONCLUSIONS**

This study concluded that sodium alginate immobilized cells can remove chromium (VI) more efficiently and in high concentration than free cells. When chromium reducing bacteria is inoculated to pea crop amended with the metal, significantly increased the germination, growth, nodulation, photosynthetic pigments and protein compared to un-inoculated but metal amended plant. Bacteria also reduced the accumulation of metal in the pea plant, thus can be used for bioremediation of chromium (VI) in the environment.

### 254 **References**

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nutrient broth (pH 7.0) amended with 100  $\mu$ g /ml Cr (VI).



371 Days of incubation
 372 Fig.2. Chromium (VI) reduction by free and immobilized cells of *Brevibacillus brevis* OZF6

using repeated spiking of  $100 \ \mu g \ /ml \ Cr \ (VI)$  in nutrient broth (pH 7.0) after every five days.

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## Table 1. Effect of chromium (VI) reducing bacterial inoculation (OZF6) on the plant growth and photosynthetic pigments of pea plants grown in the presence and absence of metal.

Traatmant	Dasa Data of	Saad	Deet	Shoot	Total day	Total	Constansid
Treatment	Dose Rate of	Seed	KOOL	Shoot	Total dry		Carolenoid
	Cr(VI) (mg/kg)	Germination	Length	Length	weight	chlorophyll	
	of soil)	(%)	(cm)	(cm)	(g)		(mg/g)
						(mg/g)	
Un-inoculated	Control	$82^{a}+32$	$31^{b}+1.6$	$45^{b}+25$	$23^{b}+1.4$	$0.28^{b} + 0.6$	$1.02^{b}+0.4$
On-moculated	Control	02 ±3.2	51 ±1.0	4J 12.J	25 ±1.4	0.20 ±0.0	1.02 ±0.4
	60	$60^{b} \pm 2.7$	22 <sup><u>c</u></sup> ±1.4	33 <sup>c</sup> ±2.1	$18.5^{\circ} \pm 1.3$	$0.19^{\circ}\pm0.5$	0.82°±0.3
Inoculated	Control	$90^{a} \pm 3.5$	$43^{a} \pm 1.8$	$51^{a}\pm 2.7$	$29^{a} \pm 1.8$	$0.35^{a}\pm0.7$	$1.25^{a}\pm0.5$
(OZF6)							
OZF6+ Cr (VI)	60	88ª±3.4	41ª±1.7	$49^{a}\pm 2.5$	$26^{a} \pm 1.6$	$0.33^{\circ}\pm0.6$	$1.23^{a}\pm0.6$
LOD		0.6	<b>5</b> 1	5.0	2.2	0.17	0.(1
LSD		8.6	5.1	5.0	3.3	0.17	0.61
E Value	Inoculation	1121.1*	211.6*	203*	214.4*	224 7*	207.2*
1° value	(df-1)	1121.1	211.0	203	214.4	224.7	207.2
	(ui= 1)						
	Metals	91 <sup>*</sup>	420*	91.7 <sup>*</sup>	98.4*	338*	170.1*
	(df= 1)						
	Interaction	$101.4^{*}$	173.2*	505.4*	$408.2^{*}$	$209.2^{*}$	233.3*
	(df = 1)						
1	1		1		1	1	1

df indicates degree of freedom. Each value is a mean of six independent experiments  $\pm$ S.D. Mean values are significant at \*p  $\leq$  0.05. Within columns, means followed by the different letter are significantly different according to Duncan's multiple range test (p  $\leq$  0.05).

381Table 2. Effect of bacterial inoculation of the strain OZF6 on nodulation, protein content and

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Treatment	Dose Rate of Cr (VI) (mg/kg of	Nodule no./plant	Nodule Dry weight	Seed Protein (mg/g)	Cr (VI) accumulation (µg/g)	
	soil)		(mg/plant)	(8-8)	Root	Shoot
Un-inoculated	Control	14 <sup>b</sup> ±1.1	$10^{b} \pm 0.7$	264 <sup>c</sup> ±12.4	ND	ND
	60	08°±0.6	7°±0.5	233 <sup>d</sup> ±11.2	$14.7^{a}\pm1.0$	5.5 <sup>a</sup> ±0.7
Inoculated (OZF6)	Control	$19^{a}\pm1.4$	$15^{a} \pm 1.0$	293 <sup>a</sup> ±15.5	ND	ND
OZF6+ Cr (VI)	60	16 <sup>b</sup> ±1.3	14 <sup>a</sup> ±1.2	269 <sup>b</sup> ±12.0	$4.12^{b}\pm0.6$	$1.87^{b}\pm0.3$
LSD		2.6	1.3	16.5±	2.56	2.21
F Value	Inoculation	98.1*	62.2*	654.3*	89.43*	62.2*

(df=1)					
Metals	154.2*	212.4*	232.1*	164.5 <sup>*</sup>	129.7 <sup>*</sup>
(df=1)					
Interaction	71.7*	502.1*	435.2*	46.4*	120.3*
(df = 1)					

df indicates degree of freedom. Each value is a mean of six independent experiments  $\pm$  S.D. Mean values are significant at \*p  $\leq$  0.05. Within columns, means followed by the different letter are significantly different according to Duncan's multiple range test (p  $\leq$  0.05).

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