Original Research Paper

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

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.

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

1. INTRODUCTION

The contamination of chromium (VI) is mainly ~~is~~ due to the use of Cr (VI) in leather, tanning, metallurgy, electroplating, textile, and pigment manufacturing industries [1-3]. Chromium occurs either in trivalent or hexavalent which affect growth of microorganisms present in the environment [4]. Solubility of Cr (VI) makes it highly toxic and ~~thus will~~ easily pass through biological membranes and ~~can easily~~ damage proteins and nucleic acids particularly DNA, thus inhibits the number of species ~~of the microbes and also their growth~~ [5,6]. Reduction of Cr (VI) leads to the formation of stables, less soluble and less toxic Cr (III). Hexavalent chromium being ~~the most~~ toxic, trivalent is an essential micronutrient for animals, plants and humans which ~~is~~ involved in glucose metabolism [7], stimulation of enzyme system [8] and stabilization of nucleic acids by increasing the processivity of DNA polymerase [9]. 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 of ~~these~~ metal~~s~~. The reduction of Cr (VI) has been reported in *Bacillus* [11,12], *Pseudomonas* sp. [13-14], *Escherichia Coli* [15], *Microbacterium* [2], *Ochrobactrum intermedium* [16] and *Micrococcus* [17].

Mechanism of chromium (VI) reduction may be direct or indirect ~~and~~ is influenced by pH, temperature, concentration of chromium, incubation periods and the microorganisms used. ~~It is~~ 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 oxidant, such as H_2S, reduce chromium [19]. Furthermore, in growing cultures ~~with~~ added carbon sources as electron donors and in cell suspensions, Cr (VI) reduction can be 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 ~~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]. 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 form of~~ chromium (Cr (VI)) to ~~less toxic form of~~ chromium (Cr (III)) can be easily removed from wastewater [10]. The ~~enzyme~~ chromium reductase found in *P. ambigua* [24] and *Bacillus*

sp. [25] were purified and characterized. More recently, to clone a chromate reductase gene, novel soluble chromate reductase of *P. putida* was purified to homogeneity and characterized [26]. The reductase activity was NADH- or NADPH-dependent. Reduction of Cr (VI) by H_2S produced by the bacterial cells is found in soil environments which are rich in sulfate under anaerobic conditions [18]. Hydrogen sulfide, produced in acid sulfate soil under reducing conditions, is easily precipitated as FeS in reduced soils [27] and sediments. Fe (II) and H_2S, both microbially produced, are effective reductants of Cr (VI) under reduced conditions as is the FeS [28].

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.

2. MATERIALS AND METHODS

2.1 Chromium (VI) reduction in free and immobilized cells

Natural materials like sodium aliginate (SA) at varying concentration were used to immobilize *Brevibacillus brevis* OZF6 cells to see their effect on Cr (VI) reduction. Sodium aliginate was used in the concentration of 0.5 g, 1.0 g and 1.5 g. Preparations of beads was performed as follows: (1) Sodium alginate was mixed in 20 ml of deionized water, and then solution was heated to 80^0 C in order to dissolve sodium aliginate; (2) when the immobilizing agent got dissolved in deionized water, then solution was cooled to 40^0 C, (3) After cooling solution, about 1 g (fresh weight) of bacterial cells (overnight growth) was added and mixed; (3) For the 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 into immobilizing phase with the help of sterile 10 ml disposable plastic syringe with a 21-G needle. Beads (3–5 mm in diameter) were washed three times with 100 ml sterile distilled water and added aseptically to 100 ml NB medium containing 100 µg/ml $K_2Cr_2O_7$ in a 250 ml flask. The flasks were incubated at 37^0 C, Samples were taken at regular intervals and Cr (VI) concentration was detected by 1, 5 – diphenyl carbazide method [29] upto 120 h. Briefly, the test

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.

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.

2.3 Plant growth

The experimental soil was sandy clay loam (organic carbon 0.37%, Kjeldahl N 0.65 g/kg, Olsen 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 sterile water and shade dried. The sterilized seeds were coated with *Brevibacillus brevis* OZF6, grown in nutrient broth, by dipping the seeds in liquid culture medium for two hours using 10% 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 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/ 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 design. One week after emergence, plants in each pot were thinned to three plants. The pots were watered with tap water when required and were maintained in an open field condition. All plants 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^{0} C and the dry matter was measured. Nodule number and nodule dry weight per plant were observed after 90 days of their growth. Total chlorophyll contents in fresh foliage of pea grown in metal stressed and metal free (control) soil was quantified at 90 DAS by the method of Arnon [30]. Protein was measured after

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].

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 ± S.D of the replicates.

3. RESULTS

3.1 Effect of immobilization on Cr (VI) reduction

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 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 (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 combinations of 0.5 and 1.0 g SA. Strain OZF 6 reduced Cr (VI) by 87% after 120 hours of incubation when immobilized on 1.5 g sodium aliginate. Concentration of 1.5 g SA showed an increase of 13% in Cr (VI) reduction by *Brevibacillus brevis* OZF 6, compared to free cells after 120 hours of incubation.

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 1and 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 at 60 mg Cr/kg of soil compared to the control plants (Table 1 and 2). But when the pea crop was inoculated with the *Brevibacillus brevis* OZF6, increased the measured parameters significantly 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 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) for the measured parameters at 90 DAS.

3.5 Accumulation of metal in plant tissues

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

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

4. DISCUSSION

~~Hexavalent chromium being the most toxic, trivalent is an essential micronutrient for animals, plants and humans which is involved in glucose metabolism [7], stimulation of enzyme system [8] and stabilization of nucleic acids by increasing the processivity of DNA polymerase [9]. 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 of these metals.~~ ~~*Brevibacillus brevis* OFZ6 was isolated and identified as described previously~~ [34]. Maximum reduction of Cr (VI) was observed in strain OZF6 when immobilized by 1.5 g sodium aliginate compared to the other combinations of 0.5 and 1.0 g SA after 120 h~~ours~~ of incubation. Our study ~~is in correlation with~~ the study of Humphries et al. [35]; Poopal and Laxman [36]. They also observed that when *Desulfovibrio vulgaris* was immobilized by agar reduced 0.5 mM (VI) in 22 h~~ours~~ whereas *Microbacterium* sp. NCIMB 13776 ~~when~~ immobilized by agar ~~reduced~~ 0.5 mM Cr (VI) within 65 h~~ours~~ of incubation [35] while the PVA-alginate immobilized *Streptomyces griseus* cells ~~removed~~ 0.48 mM Cr(VI) within 24 h [36]. In another study Pang et al., [37] also observed 50% Cr (VI) reduction in 84 h~~ours~~ when *Pseudomonas aeruginosa* was immobilized in polyvinyl alcohol/sodium aliginate matrix.

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. 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 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). The lack of a delay demonstrates that the necessary enzymes are constitutively expressed. ~~This study has concluded that for the successful bioremediation it is not necessary to previously expose the bacterial cells to chromium and subsequent microbial enrichment.~~ This could be mainly due to the involvement of constitutive chromate reductases, thus corroborating the earlier observation of the rapid reduction of Cr (VI) by *Pseudomonas putida* unsaturated biofilms [39].

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. 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. Chromium (VI) toxicity exerted severe effects on root growth and function, resulting in root damage, reduction in fresh weight, cell division, root elongation and reduced the uptake of water and nutrients [42]. Moreover, accumulation of heavy metals in plant tissues may trigger water deficit, resulting in reduced growth and development of plants [41]. But when the seed was inoculated with the bio-inoculants, significantly increased the length, dry weight and nodulation of the pea. These bacteria can increase the growth of the plant due to the reduction of chromium (VI) to 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 glucose metabolism [7], stimulates enzymes [8] and stabilizes nucleic acids by increasing the processivity of DNA polymerase [9].

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 the *Brevibacillus brevis* OZF6, increased the measured parameters like chlorophyll, carotenoid and seed protein significantly compared to the control plants. Similar increase in the 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 increase in the photosynthetic pigments and seed protein upon inoculation of the bacterial strain in metal amended soil.

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 contrast, the bioinoculant significantly ($P \leq 0.05$) decreased the concentration of the metal in tissues, compared to the un-inoculated but metal amended plants. The decreased concentration of chromium in plant organs could be due to the reduction, adsorption/desorption of metal by the OZF6 strain, as reported by Mamaril et al. [44], Wani et al. [45] and Wani and Khan [43]. Karthik et al. [41] also repotted significant decrease in metal accumulation in the plant tissue when bio-inoculant was inoculated to the crop amended with the metal.

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.

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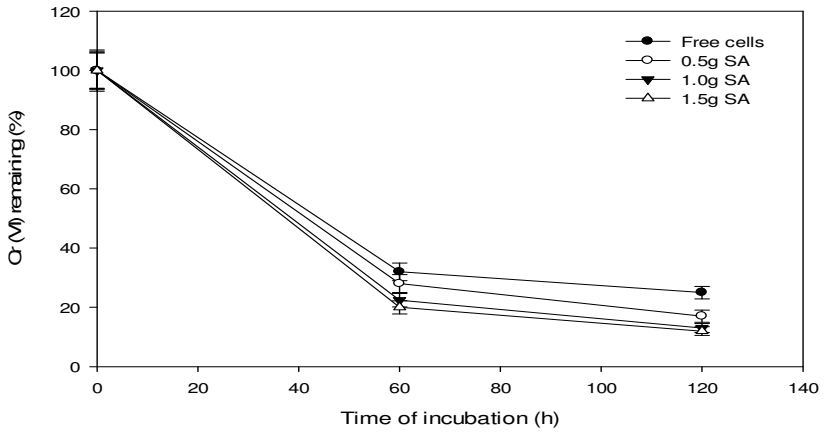

Fig.1. Chromium (VI) reduction by free and immobilized cells of *Brevibacillus brevis* OZF6 in nutrient broth (pH 7.0) amended with 100 µg /ml Cr (VI).

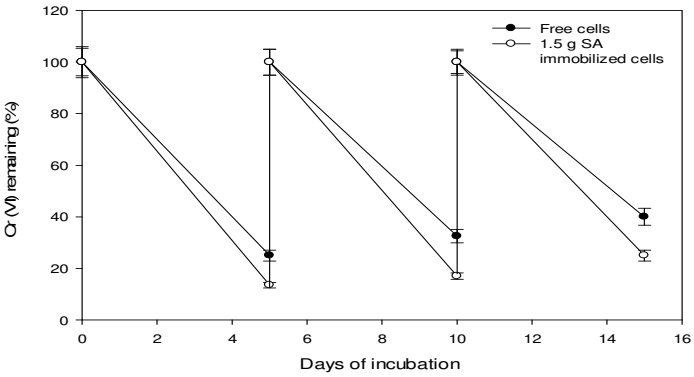

Fig.2. Chromium (VI) reduction by free and immobilized cells of *Brevibacillus brevis* OZF6 using repeated spiking of 100 µg /ml Cr (VI) in nutrient broth (pH 7.0) after every five days.

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.

Treatment	Dose Rate of Cr (VI) (mg/kg of soil)	Seed Germination (%)	Root Length (cm)	Shoot Length (cm)	Total dry weight (g)	Total chlorophyll (mg/g)	Carotenoid (mg/g)
Un-inoculated	Control	82^{a}±3.2	31^{b}±1.6	45^{b}±2.5	23^{b}±1.4	0.28^{b}±0.6	1.02^{b}±0.4
	60	60^{b}±2.7	22^{c}±1.4	33^{c}±2.1	18.5^{c}±1.3	0.19^{c}±0.5	0.82^{c}±0.3
Inoculated (OZF6)	Control	90^{a}±3.5	43^{a}±1.8	51^{a}±2.7	29^{a}±1.8	0.35^{a}±0.7	1.25^{a}±0.5
OZF6+ Cr (VI)	60	88^{a}±3.4	41^{a}±1.7	49^{a}±2.5	26^{a}±1.6	0.33^{a}±0.6	1.23^{a}±0.6
LSD		8.6	5.1	5.0	3.3	0.17	0.61
F Value	Inoculation (df= 1)	1121.1^{*}	211.6^{*}	203^{*}	214.4^{*}	224.7^{*}	207.2^{*}
	Metals (df= 1)	91^{*}	420^{*}	91.7^{*}	98.4^{*}	338^{*}	170.1^{*}
	Interaction (df = 1)	101.4^{*}	173.2^{*}	505.4^{*}	408.2^{*}	209.2^{*}	233.3^{*}

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

Table 2. Effect of ~~bacterial~~ inoculation of ~~the~~ strain OZF6 on nodulation, protein content and metal accumulation in pea plants

Treatment	Dose Rate of Cr (VI) (mg/kg of soil)	Nodule no./plant	Nodule Dry weight (mg/plant)	Seed Protein (mg/g)	Cr (VI) accumulation (µg/g)	
					Root	Shoot
Un-inoculated	Control	14^{b}±1.1	10^{b}±0.7	264^{c}±12.4	ND	ND
	60	08^{c}±0.6	7^{c}±0.5	233^{d}±11.2	14.7^{a}±1.0	5.5^{a}±0.7
Inoculated (OZF6)	Control	19^{a}±1.4	15^{a}±1.0	293^{a}±15.5	ND	ND
OZF6+ Cr (VI)	60	16^{b}±1.3	14^{a}±1.2	269^{b}±12.0	4.12^{b}±0.6	1.87^{b}±0.3
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 (df= 1)	154.2*	212.4*	232.1*	164.5*	129.7*
	Interaction (df = 1)	71.7*	502.1*	435.2*	46.4*	120.3*

df indicates degree of freedom. Each value is a mean of six independent experiments ± 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 ($p \leq 0.05$).