Effect of *Brevibacillus brevis* OZF6 on Reduction of Chromium (VI) and ~~pea~~ Pea ~~growth~~Growth

Abstract

Aim: Hexavalent chromium (Cr (VI)) is toxic due to its high solubility in water and permeability through biological membranes and Cr (VI) interacts with proteins and nucleic acids which makes it more toxic and carcinogenic than trivalent. Microbes converting toxic chromium Cr (VI) to stable and less soluble Cr (III) can be used for detoxification of Cr (VI) from contaminated environment. In this study the authors wanted to evaluate the effect of chromium (VI) reducing *Brevibacillus brevis* OZF6 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 2015.

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

Results: *Brevibacillus brevis* OZF6 significantly reduced Cr (VI) when bacteria were immobilized by sodium alginate as compared to the free cells. Seed germination, pea growth, nodulation, photosynthetic pigments, and protein increased in pea plants after inoculation with *Brevibacillus brevis* OZF6 compared to un-inoculated pea plants amended with metal. OZF6 significantly decreased accumulation of chromium in roots and shoots compared to only metal-amended plants.

Conclusions: Due to above properties, *B. brevis* 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: *Brevibacillus brevis*, Chromium (VI) tolerance, Chromium (VI) reduction, Immobilization, Nodulation, Pea growth~~,~~, Photosynthetic pigments

1. INTRODUCTION

Heavy metal pollution is one of the current most troublesome environmental problems due to the ~~widespread~~ widely use of metals for industrial and agricultural purposes [1]. It adversely affects about 12% of the world's agricultural land [2]. Heavy metal pollution has accelerated dramatically since the beginning of industrial revolution. The metals released from these sources accumulate in soil and in turn adversely affect the agro-ecosystem [3]. The toxic metal contamination of soil environment therefore, requires an effective and affordable attention.

Chromium occurs either in the form of trivalent or hexavalent which affects growth of microorganisms present in the environment [4]. Hexavalent chromium being very toxic. However, trivalent is an essential micronutrient for animals, plants, and humans which involves in glucose metabolism [5], stimulation of enzyme system [6], and stabilization of nucleic acids by increasing the processivity of DNA polymerase [7]. Solubility of Cr (VI) makes it highly toxic and easily passing through biological membranes and damaginge proteins and nucleic acids particularly DNA. Thus, Cr (VI) inhibits the number and growth of species [8, 9]. The contamination of chromium (VI) is mainly due to the use of Cr (VI) in leather, tanning, metallurgy, electroplating, textile, and pigment manufacturing industries [10-12]. Reduction of Cr (VI) leads to the formation of stables, less soluble and less toxic Cr (III). Chromium toxicity to plants, however, can be reduced by applying resistant and reducing microorganisms [13] (Wani et al., 2009). Reduction of toxic Cr (VI) to Cr (III) is useful process remediation of Cr (VI) affected environments [14] and thus can be readily used to save our soil and water from the toxic effects of this metal. The reduction of Cr (VI) has been reported in *Bacillus* sp. [15, 16], *Pseudomonas* sp. [17, 18], *Escherichia coli* [19], *Microbacterium* sp. [11], *Ochrobactrum intermedium* [20], and *Micrococcus* sp. [21].

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Mechanism of chromium (VI) reduction may be direct or indirect. The reduction process is influenced by pH, temperature, ~~concentration~~and concentration of chromium, incubation periods, and the microorganisms used. The chromium reductases whether intracellular or extracelluar can reduce chromium (VI) into chromium (III) directly [22]. Whereas reductants or oxidant, such as H_2S, reduce chromium indirectly [23]. In the media with added carbon sources, Cr (VI)

reduction can be predominantly aerobic or anaerobic and chromium reductases can ~~catalyse~~catalyze reduction of Cr (VI) to Cr (III) anaerobically [24], aerobically [25] and also both. The Cr (VI) reductase may be present in the membrane fraction of the cells of plant growth promoting rhizobacteria (PGPR), as found in *Pseudomonas fluorescens* and *Enterobacter cloacae* [26]. Chromium reductase may also be present intracellularly which will reduce chromium (VI) into chromium (III) [27]. The insoluble precipitate formed by the reduction chromium (Cr (VI)) to chromium (Cr (III)) can be easily removed from wastewater [14]. Chromium reductase found in *P. ambigua* [28] and *Bacillus* sp. [29] were purified and characterized. Recently, to clone a chromate reductase gene, novel soluble chromate reductase of *P. putida* was purified to homogeneity and characterized [30]. 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 [22]. Hydrogen sulfide, produced in acid sulfate soil under reducing conditions, is easily precipitated as FeS in reduced soils [31] and sediments. Fe (II) and H_2S, both microbially produced, are effective reductants of Cr (VI) under reduced conditions as FeS [32].

Present study was conducted (1) to evaluate sodium aliginate as an immobilizing matrix for bacterial strain ~~*Brevibacillus*~~ *B. brevis* OZF6 to remove Cr (VI), (2) to evaluate the effect of ~~*Brevibacillus*~~ *B. brevis* OZF6 on the reduction of Cr (VI), plant growth, nodulation, photosynthetic pigment and protein content in pea.

2. MATERIALS AND METHODS

2.1 Chromium (VI) reduction in free and immobilized cells

The ~~*Brevibacillus*~~ *B. brevis* OZF6 from our own culture collection was chromium tolerant and chromium (VI) reducing which were isolated from industrial waste water of Abeokuta, Ogun State Nigeria and was identified as identified as described previously [33]. The strain was cultured on nutrient agar plates. Natural materials like sodium alginate (SA) at varied concentration was used to immobilize ~~*Brevibacillus*~~ *B. brevis* OZF6 cells to evaluate their effect on Cr (VI) reduction. Sodium alginate in concentrations of (g/20 ml) 0.5, 1.0 and 1.5 were used in the experiment. Preparations of beads was performed as follows: (1) SA was mixed in 20 ml of deionized water, and then solution was heated to 80 °C ~~°C~~ in order to dissolve SA; (2) when the immobilizing agent dissolved in deionized water, the solution was cooled to 40 °C ~~°C~~, (3)

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after cooling, about 1 g (fresh weight) of bacterial cells (grown overnight in nutrient broth at a temperature of 30 ± 2 °C ~~°C~~) was added and mixed with the solution; (3) for the preparation of cell beads, the mixture was prepared as 50 ml degassed boric acid solution (100 %) containing 2 % (w/v) calcium chloride was mixed, and immersed for 24 hours. The solution was put into immobilizing phase using 10 ml sterile 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 flask was incubated at 37 °C ~~°C~~. Samples were taken at 60 and 120 hours and Cr (VI) concentration was detected using 1, 5 – diphenyl carbazide method [34] upto 120 h. Briefly, the test samples were acidified (pH 1-2) and added 1,5 diphenyl carbazide (50 µg/ml). Cr (VI) concentration was detected by using UV-VIS spectrophotometer at 540 nm. Experiments were repeated three times.

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

For the fed-batch experiments, 100 ml of NB broth in the bottle was amended with 100 µg/ml Cr (VI) and inoculated with and with–out immobilized cells (wet weight, 1 g). The bottles was incubated at 30 °C ~~°C~~. Samples were collected at 60 and 120 hours and monitored for Cr (VI) remaining. When almost all of the Cr (VI) was removed from the medium, medium 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 from the liquid samples collected at different times during each batch were determined as above. Experiments were repeated three times.

2.3 Plant growth

The autoclaved soil used in the experiment was sandy clay loam (organic carbon 0.37%, Kjeldahl N 0.65 g/kg, Olsen P 15.5 mg/kg, pH 7.1, WHC 0.42 ml/g, and Cr (VI) 4.2 µg/ g). Seeds of pea var. Arket were surface sterilized with 70% ethanol for 3 min then 3% sodium hypochlorite for 3 min), rinsed six times with sterile water, and shade dried. The sterilized seeds were coated with *Brevibacillus brevis* OZF6 which was grown in nutrient broth. Seeds were dipped in liquid culture medium for 2 h using 10% gum Arabic as an adhesive to help 10^8 cells $seed^{-1}$ attach on the seeds. 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 and 20 cm internal diameter) filled with 3 kg sterilized soil without chromium (VI) as

control and 60 mg Cr (VI)/ kg soil as treatment in a completely randomized design in an open condition. 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 planting (DAP), and were observed for plant growth in terms of their root length and shoot length. Plants uprooted at 90 DAP were oven-dried at 80 ^{0}C and the dry matter was measured. Nodule number and nodule dry weight per plant were observed at 90 DAP. Total chlorophyll contents in fresh foliage of pea grown in metal stressed and metal free (control) soil was quantified at 90 DAP using the method described by Arnon [35]. Protein was measured after 90 DAP by the method of Lowery et al. [36]. Caretenoid was measured after 90 days of growth of pea plant amended with and without metal by the method of Sadasivam and Manikam [37].

The chromium content in roots and shoots of pea plants were measured after 90 DAP. The plant samples were digested in nitric acid and perchloric acid (4:1) following the method of Ouzounidou et al. [38].

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 indicated mean ± S.D of the replicates.

3. RESULTS

3.1 Effect of immobilization on Cr (VI) reduction

In this study we evaluated the immobilizing effect of sodium alginate on Cr (VI) reduction by *B. brevis* OFZ6 compared with free cells after 120 h of incubation (Fig. 1). Among different matrices combinations for whole cell immobilization of OZF6, the combination of 1.5 g sodium alginate proved to be the best combination for Cr (VI) reduction and reduced highest concentration of chromium (VI) compared to control cells (Fig. 1). Maximum reduction of Cr (VI) was observed in strain OZF6 when immobilized by 1.5 g sodium alginate compared to the other combinations of 0.5 and 1.0 g SA. Strain OZF 6 reduced Cr (VI) significantly by 87% after

120 h of incubation when immobilized on 1.5 g sodium alginate. Concentration of 1.5 g SA showed a significant increase of 13% in Cr (VI) reduction by *Brevibacillus brevis* OZF 6, compared to free cells after 120 h of incubation.

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

Reduction approached almost completion in each batch and was sustained in subsequent batches (Fig. 2). *Brevibacillus brevis* OZF65 significantly reduced chromium (VI) compared to control cells (Fig. 2). *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. Same pattern was observed in the second and third cycle (after 15 days of incubation). Undoubtedly microbial cells repeatedly can sustain the removal of Cr (VI) in fed batch experiments.

3.3 Effect of *Brevibacillus brevis* OZF6 inoculation on chromium (VI) reduction and plant growth and nodulation of pea under the influence of the metal

Seed germination of pea was decreased in the presence of the metal. However, when the plant was inoculated with the *B. brevis* OZF6 amended with and without metal, seed germination of pea was increased significantly as compared to the un-inoculated 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 including chlorophyll, carotenoid, and seed protein were decreased significantly in plants grown at 60 mg Cr/kg soil compared to the un-inoculated control plants (Table 1 and 2). But when the pea crop was inoculated with the *B. brevis* OZF6, the measured parameters were increased significantly compared to the control plants. Even when metal was amended with the bacterial strains, chlorophyll, carotenoid and seed protein were increased

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 ≤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). Chromium accumulation in 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 root and shoot tissues, compared to the un-inoculated but metal amended plants.

4. DISCUSSION

Hexavalent chromium is a carcinogen which is found in large amounts in soil whereas trivalent is an essential micronutrient found in small amounts in the soil. Trivalent is responsible for the metabolism of glucose and also increases different enzymes [5, 6]. Reduction of toxic Cr (VI) to Cr (III) is thus a useful process for remediation of Cr (VI) affected environments [14] and thus can be readily used to save our soil and water from the toxic effects of these metals. 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 hours of incubation. Strain OZF 6 reduced Cr (VI) by 87% after 120 hours of incubation when immobilized on 1.5 g sodium alginate compared to free cells. Concentration of 1.5 g SA showed an increase of 13% in Cr (VI) reduction by strain OZF 6, compared to free cells. Our study agree with the study of Humphries et al. [39]; Poopal and Laxman [40]. They also observed that when *Desulfovibrio vulgaris* was immobilized by agar reduced 0.5 mM (VI) in 22 h whereas *Microbacterium* sp. NCIMB 13776 was immobilized by agar was 0.5 mM Cr (VI) was reduced within 65 h of incubation [39], while the PVA-alginate immobilized *Streptomyces griseus* cells removed 0.48 mM Cr(VI) within 24 h [40]. In another study Pang et al., [41] also observed 50% Cr (VI) reduction in 84 hours when *Pseudomonas aeruginosa* was immobilized in polyvinyl alcohol/sodium alginate 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 removed 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 [42]. 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 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 [43].

Seed germination of pea decreased in the presence of the metal. But when the crop was inoculated with the *B. brevis* OZF6 amended with and without metal, seed germination was 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 [44]. Similar results were also reported by Karthak et al. [45] who also studied decrease in seed germination of the legume crop when the plant was grown under heavy metal stress. Karthak et al. [45] reported that when inoculated the crop with bio-inoculant amended with metal, significant increase in the seed germination was observed 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 DAP, decreased significantly when pea was exposed to the metal. In contrast, plants inoculated with ~~*Brevibacillus*~~ *B. 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 root fresh weight, cell division, and root elongation and reduced the uptake of water and nutrients [46]. Moreover, accumulation of heavy metals in plant tissues may trigger water deficit, resulting in reduced growth and development of plants [45]. But when the seed were inoculated with the bio-inoculants, root length, dry weight and nodulation of the pea were significantly increased. These bacteria can increase the growth of the plant due to the reduction of chromium (VI) to chromium (III) [45]. Trivalent is an important micronutrient used by animals, plants and humans which triggers glucose metabolism [5], stimulates enzymes [6] and stabilizes nucleic acids by increasing the processivity of DNA polymerase [7].

Photosynthetic pigments including chlorophyll and carotenoid and seed protein were decreased significantly at 60 mg Cr/kg of soil compared to the control plants. But when the pea crop was inoculated with the *B. brevis* OZF6, the measured parameters were increased significantly like chlorophyll, carotenoid and seed protein 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 [45]. Wani and Khan [47] also observed increase in the

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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. [48], Wani et al. [49] and Wani and Khan [47]. Karthik et al. [45] 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 bacteria was inoculated to pea crop and amended with the metal, the germination, growth, nodulation, photosynthetic pigments and protein were significantly increased as 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.

Competing Interest

Authors do not have any competing interests

Submission Declaration

This work has not been published neither is under consideration for publication

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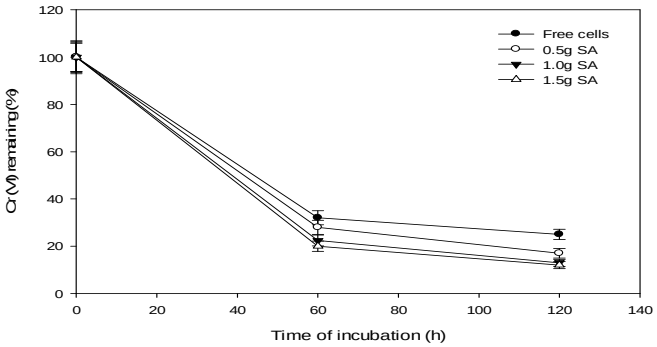

Fig.1. Chromium (VI) reduction by free and immobilized cells of *Brevibacillus brevis* OZF6 in nutrient broth (pH 7.0) amended with 100 µg /ml Cr (VI).

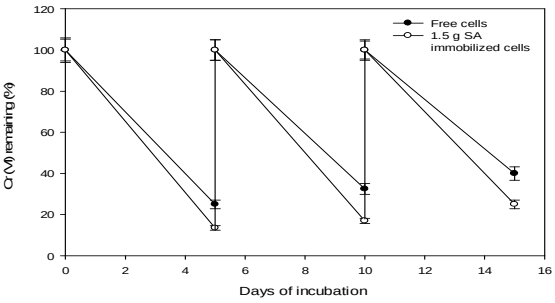

Fig.2. Chromium (VI) reduction by free and immobilized cells of *Brevibacillus brevis* OZF6 using repeated spiking of 100 µg /ml Cr (VI) in nutrient broth (pH 7.0) after every five days.

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 $*p \leq 0.05$. Within columns, means followed by the different letter are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 2. Effect of inoculation of 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 (df= 1)	98.1*	62.2*	654.3*	89.43*	62.2*
	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 ($p \leq 0.05$).