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## Bio-Remediation of Pb-contaminated Soil Cultivated With Faba Bean Via Application of Rhizobacteria *Bacillus amyloliquefaciens subsp. plantarum UCMB 5033*

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### ABSTRACT

Bio-remediation of Faba bean (*Vicia faba cv. luz deotono*) plants growing under lead (Pb) contaminated soil using the bacteria strain *Bacillus amyloliquefaciens subsp. plantarum UCMB 5033* was tested under three Pb concentrations (2000mg/L 0.5%, 2500mg/L 1.0%, 3000mg/L 5%). Growth parameters (plant height, plant fresh weight, and plant dry weight) of the Pb-treated bacteria inoculated Faba bean plants showed significant increases compared to the Pb-treated non-inoculated Faba bean plants under Pb concentration of 0.5% and 1.0% giving 5.76%, 50.43% increases in height, 42.68%, 253.36% increase in fresh wt., and 237.69%, 325.97% increase in dry wt. Regarding the plant nutrient, NPK contents the non-inoculated Pb-treated plants gave significantly the highest N content under all Pb concentration levels and the highest PK contents under Pb concentrations 0.5 and 1.0% compared to the bio-remediated plants. But the bio-remediated plants dominated the non-inoculated Faba bean plants in absorbing the highest PK contents under a Pb concentration of 5.0%. Regarding Pb absorption, the bio-remediated plants dominated the non-inoculated Faba bean plants and absorbed the highest Pb especially under 1.0% Pb concentration. *Bacillus amyloliquefaciens subsp. plantarum UCMB 5033* may be considered a bio-remediator for Faba bean plants grown under Pb contaminated soils.

**Keywords:** Bioremediation, Pb resistance bacteria, Faba beans

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## 1. INTRODUCTION

Some lead-resistant bacteria have been found to play a specific role in the growth of lead-exposed plants. For example, the endophyte *Bacillus sp. MN3-4* increases Pb(II) accumulation in *Alnus firma*, and *Pseudomonas fluorescens G10* and *Mycobacterium sp. G16* promotes plant growth and reduces Pb toxicity in *Brassica napus* (Sheng et al., 2008; Shin et al., 2012). Additionally, the activity of the lead-resistant bacteria *Streptomyces sp.* and *Ps. Vesicularis* has been noticed on the marble facade of a cathedral, where it was manifested in the form of red stains. These bacteria were capable of producing a red and red-brown pigment, identified as the lead tetroxide, minium (red lead,  $Pb_3O_4$ ) (Zanardiniet al., 1997). Some heavy metals have biological roles and are detrimental to the organisms even at very low concentrations (cadmium, lead, mercury) (Rathnayake et al., 2010). The major threat that heavy metals pose is that they persist in the environment, accumulate in the biota, and thereby enter the food chain causing health problems to higher animals and humans (Zhang et al., 2009). Chen and Wang, (2007), Sun and Shao, (2007) isolated several micro-organisms resistant to lead (Pb) among them is the gram-positive bacteria *Bacillus cereus*, *Arthrobacter sp.*, and *Corynebacterium sp.* And the gram-negative bacteria *Pseudomonas marginalis*, *P. vesicularis* and *Enterobacter sp.*, and fungi *Saccharomyces cereviside* and *Penicillium sp.* Six bacterial strains were found to be most resistant isolates and amongst them, one isolate showed maximum resistance to lead which was identified as *Bacillus cereus*, Scanning electron microscopic (SEM) photographs and Energy-dispersive X-ray spectroscopy (EDS) signature of *Bacillus cereus* revealed that lead was adsorbed to the cell surface, confirming biosorption

capacity of the bacteria (Murthy et al. 2012). This study aims to examine the effect of the bacteria *Bacillus amyloliquefaciens* subsp. *plantarum UCMB 5033* as a bio-remediator to Faba bean plants growing in Pb contaminated soils.

## 2. MATERIALS AND METHODS

### The pot experiments

*Bacillus amyloliquefaciens* subsp. *plantarum UCMB 5033*, isolated from a lead concentration of i.e., 2500 mg/l  $PbCl_2$  (lead chloride) was selected for evaluating its abilities in absorbing lead in the liquid medium. A pot experiment was carried out in the greenhouse of the biological science department, faculty of science, King Abdul-Aziz University to study the bioremediation process of Pb in sandy soil cultivated with faba bean. Plastic pots of 25 ml diameter were filled with 8 kg per each sandy soil of pH 7.8 and E.C.  $1.1 \text{ dsm}^{-1}$ . The soil in each pot was artificially contaminated with concentrations of lead chloride ( $PbCl_2$ ) by 0.5, 1, and 5% according to the proposed treatments. Three pre-germinated faba bean seeds were cultivated in each pot and immediately inoculated with the bio-remediating bacteria or each heavy metal concentration where each pot received 30 ml inoculum of the bacteria and irrigated with tap water to the field capacity (without excess water leaking outside the pot), each treatment was triplicated. During the experiment, irrigation was carried out according to the plant needs with half-strength solutions, where both compounds  $Ca(NO_3)_2 \cdot 4H_2O$  and  $NH_4NO_3$  were eliminated, Hoagland nutrient solution (Hoagland and Arnon, 1950) and tap water interchangeably. As a leguminous plant, the whole treatments of faba bean were inoculated with a liquid culture of *Rhizobium leguminosarum* previously isolated from faba bean root nodules. The following 7 treatments were applied: control,

0.5% (2000mg/L) Pb, 0.5% (2000mg/L) Pb + Pb-remediating bacterium, 1.00% (2500mg/L) Pb, 1.00% (2500mg/L) Pb + Pb-remediating bacterium, 5.00% (3000mg/L) Pb, 5.00% (3000mg/L) Pb + Pb-remediating bacterium.

### 3.9. Growth parameters determination

At the flowering stage, the faba bean plants were carefully uprooted and washed several times to remove the soil detritus. The plants were put to dry and the following parameters were estimated, plant fresh weight, plant height (cm), plant dry weight (gm/plant). The plants were oven-dried at 75°C for 48 hr. to constant dry weight.

#### Determination of mineral elements

The determination of mineral elements of each treatment was performed according to Humphries (1956) method. The fine powder of plant 0.25 gm was placed in digestion tube with 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> and placed on a sand heater inside the hood for 15-20 minutes till it became dark in color then left to cool and 1 ml of a mixture of perchloric acid and concentrated H<sub>2</sub>SO<sub>4</sub> was added and heated again for 30-50 minutes until the sample's color became transparent as water. The tubes were lifted out and distilled water was added up to 100 ml. The nutrient elements N, P, K, and Pb were determined using spectrophotometer-ICP-DES-Oplmia 8000.

#### Statistical Analysis

The obtained results were statistically analyzed using the computer program SPSS 17(2007). ANOVA test (one way) was applied to differentiate between the various means of treatments. The experimental design was a complete randomized block design. Differences among means were compared using the least significant difference (LSD) at  $p < 0.05$ .

## 3. RESULTS

Bio-remediation of faba bean by a Pb-tolerant bacteria:

### A - Influence of inoculation of faba bean plants by the bacteria *Bacillus amyloliquefaciens* on the plant growth parameters:

Regarding studying the bio-remediating efficiency of the bacteria *Bacillus amyloliquefaciens* on faba bean plant growth parameters and nutrient content, the results are presented in (table1).

Concerning faba bean plant height inoculation of faba bean plants with *B. amyloliquefaciens* increased plant height under Pb concentrations 0.5% and 1.0% without a significant difference at 0.5% but with a significant difference at 1.0% Pb concentration giving 55.83 and 55.66 cm in comparison with the same non-inoculated plants at 0.5% and 1.0% Pb concentration with 52.83 and 37.00 cm, but at 5% PbCl<sub>2</sub> concentration the non-inoculated treatment significantly exceeded the inoculated treatment giving the highest plant height 32.00 cm compared to 16.00 cm. It is worthy to mention that the control treatment-induced plant height reached 60 cm which is higher than all other treatments. Also for the plant fresh weight the inoculated faba bean plants gave the highest plant fresh weight at 0.5% and 1.0% Pb concentrations compared to the non-inoculated plants with no significant difference at 0.5% Pb concentration but with a significant difference at 1.0% reaching 36.63 (41.1% increase) and 34.33 (250.3% increase) gm/plant under inoculated plants compared to 25.96 and 9.80 gm/plant under non-inoculated plants.

At 5% PbCl<sub>2</sub> concentration the non-inoculated plants dominated the inoculated plants giving the highest plant fresh weight 13.4 gm/plant compared to 3.4 gm/plant by the inoculated plants without significant difference between them. The bio-

remediated treatments with *B. amyloliquefaciens* produced faba bean fresh weight comparable to that obtained with the control treatment without significant variations at 0.5 and 1% PbCl<sub>2</sub> concentrations.

The bacteria inoculated plants produced significantly high dry weight reached 8.78 and 6.56 g/plant against 2.60 and 1.54 g/plant at 0.5 and 1% PbCl<sub>2</sub> concentrations by non-inoculated plants

respectively. But at 5% PbCl<sub>2</sub> concentration the non-inoculated faba bean plants produced a higher dry weight compared to the inoculated plants without significant differences between them. Furthermore, the bio-treated plants of faba bean produced dry weight with no significant variations with the control treatment at 0.5 and 1% PbCl<sub>2</sub> concentration.

**Table (1): Bioremediation influence of *B. amyloliquefaciens* on some growth parameters of Faba bean plants**

Parameters		Plant height (cm)	Fresh weight (g/plant)	Dry weight (g/plant)
<b>Treatments</b>				
<b>Lead concentration (%)</b>	<b>Control</b>	60.00a	38.93a	10.046a
	<b>0.5</b>	52.83 a	25.96 ab	2.603 bc
	<b>1.0</b>	37.00b	9.80c	1.540c
	<b>5.0</b>	32.00b	13.40bc	2.780bc
<b>Lead concentration (%) + <i>B. amyloliquefaciens</i></b>	<b>0.5</b>	55.83a	36.63a	8.783a
	<b>1.0</b>	55.66a	34.33a	6.560ab
	<b>5.0</b>	16.00c	3.50c	0.380c
<b>LSD</b>		<b>9.32</b>	<b>14.41</b>	<b>4.83</b>

Values of the same letters are not statistically significant; values are the means of three replicates.

### **B – Effect of *B. amyloliquefaciens* inoculation on faba bean plant content of nutrients:**

#### **1 – N- content:**

The impact of various lead concentrations either alone or in combination with the bio-remediator *Bacillus amyloliquefaciens* on the macro-nutrients (N, P, and K) in addition to Pb-content in faba bean plants is shown in Table (2).

Regarding the N-content of faba bean plants, the results reveal that the non-inoculated plants gave significantly the highest N-content at Pb concentrations 0.5% and 1.0% giving 20.57 and 21.95 mg/g N dry weight respectively compared to

the inoculated plants with 11.62 and 13.11 mg/g N dry weight respectively. No significant variations were recorded between the plant N content under the *B. amyloliquefaciens* inoculated plants and the non-inoculated faba bean plants treated with 5% PbCl<sub>2</sub> concentration as the results were 21.95 and 21.98 mg/g dry weight respectively. Plant Nitrogen content under inoculation at lead concentrations 0.5 and 1% induced N-content comparable to that obtained with the control treatments with no significant differences.

#### **2 – P-content**

As for the P-content of faba bean plants, the results (Table 2) indicate that P-content in the plants inoculated with *B. amyloliquefaciens* ranged between 0.12 and 2.24 mg/g dry weight, the highest significant value was found with the treatment of 5% PbCl<sub>2</sub> (2.24 mg/g dry weight). The non-inoculated plants gave significantly the highest P-content at Pb concentrations 0.5% and 1.0% giving 0.37 and 0.55 mg/g P dry weight respectively compared to the inoculated plants with 0.13 and 0.23 mg/g P dry weight respectively. While the *B. amyloliquefaciens* inoculated plants significantly dominated the non-inoculated faba bean recording the highest P content 2.24 compared to 0.35 mg/g P dry weight under treatment with 5% PbCl<sub>2</sub> concentration. Faba bean plant P content under inoculation at lead concentrations 0.5 and 1% induced P-content comparable to that obtained with the control treatments with no significant differences between them.

### 3 – K-content

Regarding faba bean plants content of potassium (K), the results (Table 2) indicate that K-content in the plants inoculated with *B. amyloliquefaciens* ranged between 1.72 and 15.32 mg/g dry weight, the highest significant value was found with the

treatment of 5% PbCl<sub>2</sub> (15.32 mg/g dry weight). The non-inoculated plants gave significantly the highest K-content at Pb concentrations 0.5% and 1.0% giving 18.17 and 14.11 mg/g K dry weight respectively compared to the *B. amyloliquefaciens* inoculated plants with 10.55 and 1.72 mg/g K dry weight respectively. But the *B. amyloliquefaciens* inoculated plants significantly dominated the non-inoculated faba bean recording the highest K content 15.32 compared to 12.58 mg/g K dry weight under treatment with 5% PbCl<sub>2</sub> concentration.

### 4 – Pb-content

The Pb-content of faba bean plants ranged between 0.5 under the control and 11.0 ppm, under 5% PbCl<sub>2</sub> concentration. No significant differences between faba bean plant Pb content under non-inoculation and inoculation with the bacteria *B. amyloliquefaciens* Pb concentration of 0.5% and 5% where the results were significantly 1.6 and 11.0 ppm under non-inoculation and 1.0 and 10.0 ppm under inoculation with the bacteria respectively. But at a Pb concentration of 1.0% the bacterial inoculated plants dominated giving significantly the highest faba bean plant Pb content 4.0 ppm compared to the non-inoculated faba bean plants with only 2.2 ppm.

**Table 2: Faba bean plants content of N, P, K, and Pb as affected by *B. amyloliquefaciens* inoculation at different Pb concentrations**

Parameters		N-content (mg/g)	P-content (mg/g)	K-content (mg/g)	Pb-content (ppm)
Treatments					
Lead concentration (%)	Control	13.67b	0.12d	6.73d	0.5e
	0.5	20.57a	0.37c	18.17a	1.6d
	1.0	21.95a	0.55b	14.11b	2.2d
	5.0	22.19a	0.35c	12.58bc	11.00a
Lead concentration (%) + <i>B. amyloliquefaciens</i>	0.5	11.62b	0.13d	10.55c	1.0d
	1.0	13.11b	0.23cd	1.72bc	4.0c
	5.0	21.98a	2.24a	15.32ab	10.0 b
LSD		2.86	0.19	3.49	0.9

Values of the same letters are not statistically significant; values are the means of three replicates.

#### 4. DISCUSSION

##### Bio fertilization of faba bean with Pb-remediation bacteria

Faba bean plants when inoculated with *Bacillus amyloliquefaciens* and planted in Pb polluted soil have shown a significant increase in plant height, plant fresh weight, and plant dry weight compared to those planted without bacterial inoculation, especially under Pb concentration 0.5% and 1.0%. These findings are in agreement with many other results found by other researchers. Wu *et al.* (2006) found the inoculation of *Azotobacter chroococcum* stimulated the growth of *Brassica juncea* under Pb concentration. Gomaa *et al.* (2012) stated that the application of *Azospirillum* and *Rhizobium* in the presence of 300 ppm Pb induced a highly significant increase in wheat shoot dry weight over the positive control.

Jing *et al.* (2013) found that inoculation of rapeseed with bacterial strain *Enterobacter* sp. (JYX7) and *Klebsiella* sp. (JYX10) had significantly higher dry weights compared to those without inoculation grown in soils amended with Pb (200 mg/kg). Ahmed and Khan, (2012) found that in the soil amended with Pb, the *Rhizobium* strain RL9 isolated from the nodules of lentil grown in metal contaminated soil, significantly increased growth and nodulation of lentil compared to plants grown in absence of this *Rhizobium* strain.

But the Pb contaminated soil without bacterial inoculation dominated those with inoculated bacteria only under 5% Pb concentration given the highest plant height, fresh and dry weight. This may be attributed to the fact that the inoculated bacteria itself was affected by the accumulation of high Pb concentration, thus resulting in unavailability of nutrient to the plant and thus causing retardation in the growth parameters of Faba bean (height, fresh,

dry weight) as was reported by (Henry 2000) that oxyhydroxides, organic, carbonate, and precipitated forms of lead are the most strongly bound to the soil. The capacity of the soil to adsorb lead increases with increasing pH, cation exchange capacity (CEC), organic carbon content, soil/water Eh (redox potential), and phosphate levels.

Mangkoediharjo and Surahmaida (2008) planted *Jatropha curcas* phytoremediation in pots for 30 days in the soil polluted with different Pb concentrations and found that beyond 50 mg/kg soil Pb was more toxic to plants. Gupta *et al.* (2012) mentioned that heavy metals generally when present in soil show

##### Role of the rhizosphere bacterium *B. amyloliquefaciens* in faba bean content N, P, K, and Pb:

Inoculation of faba bean plants with *B. amyloliquefaciens* significantly increased the plant content of nutrients N, P, K, and Pb at Pb concentration of 5.0% concentration compared to the control. On the other hand, the non-inoculated faba bean plants contained significantly higher N, P, and K at the Pb concentration of 0.5% and 1.0% compared to the bacteria inoculated plants, but at 5.0% Pb concentration the *B. amyloliquefaciens* inoculated plants gave significantly the highest N, P, K, and Pb content compared to the non-inoculated plants. It seems that when Pb concentration increases up to 5.0% the inoculated plants absorb more N, P, K and Pb compared to non-inoculated plants. This finding agrees with the results obtained by many other researchers. Panwichian *et al.* (2010) inoculated two metal-resistant bacteria *Rhodobiummarinum* (NW16) and *Rhodobactersphaeroides* (KM524) with 62.63 mg/kg dry weight Pb, and they were able to reduce the level of Pb in the soil by 39%.

Muneer (2005) isolated 8 Pb-resistant bacteria from industrial wastewater, and they showed maximum resistance against  $Pb^{+2}$  (2.9 mg/L) and removed 65-82% of the Pb from the medium after 72 hours of the inoculation. Jing *et al.* (2013) inoculated Rape with bacterial strains *Enterobacter* sp. (JYX7) and *Klebsiella* sp. (JYX10) and got significant concentrations and uptake of Pb by plants both in roots and shoots compared to those without inoculation growth in soils amended with Pb (200 mg/kg).

The results of the contents of the nutrients, N, P, K in Faba bean plants with the different Pb concentrations showed that the plants inoculated with the bacteria *B. amyloliquefaciens* attained the highest N, P, and K plant content when planted under Pb concentration of 5.0% Pb  $Cl_2$  compared to both other treatments, control and without bacterial inoculation. Work by other researchers may augment these results obtained in this study. Mamaril *et al.* (1997) mentioned that the bacteria can protect the plant against the toxic effects of the Pb through adsorption/desorption mechanisms and Ahmed and Khan (2012) found that in a soil amended with Cr iii, the Rhizobium strain RL9 isolated from Pb, the contaminated soil, significantly increased nodulation of the lentil plants due mostly to increase of the nutrient uptake compared to those plant grown in the absence of this Rhizobium strain.

As for the effects of bio-remediation using the bacteria *B. amyloliquefaciens* in sequestering of Pb from soil contaminated with Pb, the results of this study showed that at 1.0% of lead concentration, in faba bean inoculated with bacteria, there is a high degree of tendency to accumulate and concentrate lead more than those treated with lead alone. This may be attributed to that at high Pb concentrations

in the soil the associates faba bean shoot itself might be affected.

On the other hand, at the highest concentration of lead 5%, the fraction of lead absorbed from contaminated soil by faba bean-free from remediating bacteria has no significant difference from that absorbed by faba bean remediated by bacteria, and this can be postulated as reported by Meagher (2000) that not all contaminants are easily treated by bioremediation using microorganisms, like Pb and Cd are not easily absorbed or captured by microorganisms.

Yadav *et al.* (2012) observed that the absorption capacity of *Bacillus subtilis* for Cd increases by increasing initial metals concentration, while there's some kind of changeable for sorption of (Pb) with the increasing of metal ion concentration, at the different initial concentration of the lead solution, it was slow when the concentration of lead increased from 5 to 10 mg.  $l^{-1}$ , but it increased lead absorption via *Bacillus subtilis* with concentration 25 mg/l and then reduced with concentration 50 mg/l. The differences between these two results of removing ions by the same bacterium may be attributed to an alteration in a capability of the active site suitable for both metal ions on the surface of the cell and this agrees with the finding of this study that the highest Pb absorbed by the plants was with the concentration 1.0% and not with the highest concentration 5.0 %.

## CONCLUSION

The bacteria strain *Bacillus amyloliquefaciens* subsp. *plantarum* UCMB 5033 was found good bio-remediator of Faba bean plants growing under lead (Pb) contaminated soil up to 2500mg/L 1.0% mg/L 5%. Growth parameters (plant height, plant fresh weight, and plant dry weight) of the bacteria inoculated Faba bean plants were significantly

higher compared to the non-inoculated Fabia bean plants under Pb concentration of 0.5% and 1.0%. Regarding the plant nutrient NPK contents the non-inoculated plants gave significantly higher contents compared to the bio-remediated plants at the Pb concentrations of 0.5 and 1.0%, but the bio-remediated plants dominated the non-inoculated faba bean plants in absorbing the highest Pb especially under 1.0% Pb concentration. *Bacillus amyloliquefaciens* subsp. *plantarum* UCMB 5033 may be considered a bio-remediator for Fabia bean plants grown under Pb contaminated soils.

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