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Effects of lead and growth-promoting bacteria on auxin, gibberellin, cytokinin and abscisic acid in barley (*Hordeum vulgare* L.)

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Key words: Heavy metal, lead, plant growth promoting bacteria (PGPB), abscisic acid, auxin, gibberellins, cytokinin, barley.

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Abstract

Contamination of soil by Pb is a major problem in industrialized and developing countries. phytoremediation is a simple and inexpensive method to reduce the environment Contamination. The effects of five levels of PGPB (control, azosperillium, azotobacter, pseudomonas and azotobacter+ azospirillium+ pseudomonas) and four levels of lead (0, 250, 500, 750 mg kg^{-1} of soil) were studied on chl.a, chl.b, chl.a + b auxin, gibberellin, cytokinin and abscisic acid contetns in barley (Hordeum vulgare L). Experiment was carried as a factorial based on completely randomized design with four replications. The results indicated that chl.a, chl.b, chl.a + b auxin, gibberellin, cytokinin and abscisic acid contents were significantly affected by Pb and PGPB. Contens of chl.a, chl.b, chl.a + b auxin, gibberellin, cytokinin and abscisic acid were decreased by increasing Pb concentrations and enhanced by inoculation with PGPB. Significant contents of Pb accumulated in roots and shoot of barley. The present study revealed that barley appears to have great potential for cleaning Pb-contaminated soils.

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Introduction

In recent years, the use of plant species with high biomass production such as corn, peas, onion, sunflower, barley, mustard and rapeseed has been proposed in phytoremediation operation (Blaylock *et al*, 1997: Deram *et al*., 2000). Contamination of soil by lead is a major problem in developing and industrial countries. The sources of lead contamination in soil can be classified in large groups including industrial activities such as mining and refineries, agricultural practices such as pesticide use and application of sewage sludge and municipal activities such as the use of lead in gasoline and paint. Many researches are ongoing regarding lead removal from contaminated soils using physical, chemical and biological processes (Shen *et al.,* 2000).

In general, plants selected in phytoremediation of soils should have the following properties: 1 – a high absorbency 2 – a high biomass production 3 – a high transport of heavy element from root to shoot (Raskin and Ensley, 2000). Soil solubility and therefore plant uptake of heavy metals is different depending on their forms.

Despite high levels of lead in the soil, the amount of lead available for uptake into plants (soluble and exchangeable part in soil) is low due to strong complexes with organic matter, iron and manganese oxides, clay and sedimentation with carbonate, hydroxide and phosphate (Zhang *et al*., 1997).

The average rate of Pb in contaminated soils is 15 mg kg^{-1}, while Pb content in contaminated soil is more than 100 mg kg^{-1}. Toxic effects of lead in plant leaves are generally appeared at greater concentrations than 30 mg kg^{-1} and result in a decrease in chlorophyll synthesis and vegetative growth. Toxicity caused by lead is due to the fact that lead can simulate many aspects of calcium metabolism and suppress the activity of many enzymes (Hutzinger, 1980). Phytoextraction is a technology to remove heavy metal from soils in which the plants absorb and store contaminants in their plant tissues harvested. In this operation, pollutants are removed from the soil with plants harvesting (Mattina *et al*., 2003).

When heavy metals are absorbed by plants and accumulated in their tissues, they often cause toxicity in two forms: 1- indirect, through competition with other essential nutrients, binding in the structure of pigments or enzymes *instead of their position* and degradation of their performance. Cooper (1999) found that substitution of Mg in chlorophyll with Pb and Cd resulted in a significant decrease in photosynthesis rate, 2- directly, through damage to cell structure. Occurrence of heavy metal induces oxidative stress and increases production of reactive oxygen species (ROS), this can in turn result in various toxic effects in plants such as a reduction in plant growth, a decrease in photosynthesis and chlorophyll contents, prevent enzyme activities, damage to biomolecules such as lipids, proteins and in particular, the nucleic acid particularly DNA (Chaoui and Ferjani, 2005; Mishra *et al*., 2006).

Inhibitory effects of Pb on respiration and photosynthesis through the effects on electron transport processes has been reported in the literature (Orcuut and Nilsen, 2000). Pb induces negative effects on plants. Pb adverse effect involve decrease in chlorophyll, carotenoids, proteins contents, increase chlorophyll a/b ratio and increase in mesophile thickness. Therefore, in soils contaminated by Pb, senescence symptoms such as low concentrations of chlorophyll, carotenoids, chlorophyll a/b ratio, increase phosphatase activity were seen in comparison with plants grown in clean soils (Olivares, 2003).

Azospirillum effects on plant depend on bacterial concentration, plant species, incubation time, and environmental conditions (German *et al*., 2000). According to Nezarat and Golami (2009) biomass, nutrients uptake, the rate of tissue N, plant height, leaf size, root length remarkably increase by inoculation with azospirillum. Azotobacter is able *to fix 10 mg* of *nitrogen per gram of carbohydrate (*Teixeira *et al*., 2005).

Pseudomonas has positive effects on plant growth and increase plants tolerance against pathogens and thus the plant yield (Narula and kumar *et al*., 2000). Many studies have showed that increased growth of plant because of inoculation with Azotobacter is mainly attributed to hormone produced by this bacteria and an improvement in root growth to nitrogen fixation (Zaied *et al*., 2003). In a study which set out to assess the effect of compost, azotobacter , phosphate solubilizing bacteria and NPK on Rosemary (Rosmarinus officinalis L.), Bashan *et al*. (1990) reported plant height, number of branches, dry and fresh weight, number of flower, total N, P, carbohydrate contents and oil contents especially in second are significantly increased in seedling treated with combination of compost and biofertilizers compared to untreated control. Increasing plant growth possibly due to improved water and nutrients contents in roots zone or the effect of bacteria on vital enzymes and hormones production. Application of azotobacte, azospirillum and basilus improved vegetative growth, fresh and dry weight and oil content in fennel (Foeniculum vulgare). In this study, the effects of lead and growth-promoting bacteria on the content of chlorophyll hormones activities were studied.

Materials and methods

The present study was carried out during 2012-2013 at t the research greenhouse of Islamic Azad University (35°55' N, 50°54' E, 1313 m above mean sea level), Karaj, Iran, with relative humidity of 57% and minimum and maximum temperature of 15.5 °C and 30 °C, respectively. Physical and chemical properties, fertility conditions and limiting factors of soil, especially the heavy metal lead were characterized with soil sampling at a depth of 0-30 cm (Table 1).

Experiment was carried as a factorial based on completely randomized design with four replications. Treatments were application of growth promoting bacteria (PGPB) at five levels (control, inoculation with azosperillium, inoculation with azotobacter, inoculation with pseudomonas, inoculation with azotobcter + azospirillium+ pseudomonas) and four levels of Pb (PbN03) (0, 250, 500, 750 mg kg^{-1} of soil).

Measurement of Pb uptake

Plant analysis and Pb content in roots and shoots of plants was estimated according to Tessier *et al.* (1979). The Pb level was determined using atomic absorption method, in which concentration of one element is determined regarding the radiation intensity of atoms excitation of each element. Lead standard solution was used to determine the concentration of the unknown sample (Tessier *et al*., 1979).

Measurement of auxin, gibberellin, cytokinin and abscisic acid (ABA)

Extraction, purification and measurement of auxin, gibberellin, cytokinin and abscisic acid of developing leaves in different stages after flowering was done according to Kelen *et al*. (2004). A Lichrospher RP-18 packed stainless steel column was applied for analysis. The mobile phase used was acetic acid 0.1+ methanol 80% 50+50 (v/v), at a flow rate of 0.8 Ml s^{-1}.

Measurement of chlorophyll a, b and a + b

Chlorophyll a and b were estimated based on Lichtenthaler, (1987) method. Accordingly, 0.5 g of fresh leaves and 0.5 g of sodium carbonate were mixed into a porcelain mortar. Then, 10 ml of pure acetone (100%) was gradually added and the contents were immediately transferred into a glass centrifuge tube and centrifuged at 2500 rpm for 2 min. a volume of 0.5 ml of solution and 4.5 ml of 80% acetone was injected into the spectrophotometer and Chlorophyll a and Chlorophyll b were detected at a wavelength of 663 and 647 nm, respectively. values data absorbance were placed in the formula and chlorophyll a and b contents ($mg.l^{-1}$) were separately determined.

In which, chl.a, chl.b and chl.a + b are chlorophyll a and b contents and total chlorophyll in term of mg L^{-1}, respectively. A is the rate of light absorbed by extract at wavelengths corresponding.

Data were subjected to analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute, 2002). Treatment means were separated with Duncan test at P < 0.05. Graphs were drawn using Excel software.

Results

The effects of Pb and PGPB on ABA activity

The activity of ABA was affected by Pb and PGPB and Pb * PGPB interactions (at the p = 0.01 level). The activity of ABA decreased with increasing Pb concentrations. In contrast, the activity and production of ABA increased by the application of PGPB. ABA activity was found to be 33.9 at a 750 mg of Pb and control treatment. Whereas, ABA activity improved by 110.8 in plots inoculated with PGPB and 750 mg of Pb. Application of Pb at a concentration of 250 mg caused a enzymatic activity by 44.4 unit, while the activity of ABA increased by 167.72 with 250 mg and the presence of PGPB (Figure 1).

Table 1. Experimental soil properties: chemical and physical characteristics.

%Total N	%O.C	%T.N.V	pH	ds/m	texture	Depth
57	0.082	10.8	7.5	5.58	Loam-sand	0-30
Pb(mg/Kg)	Mn(mg/Kg)	Cu(mg/Kg)	Zn(mg/Kg)	Fe(mg/Kg)	K(p.p.m)	P(p.p.m)
4.2	7.3	0.89	1.7	8	315	32.3

*The effects of Pb and PGPB on **auxin** production*

The effects of Pb , PGPB and Pb * PGPB interactions on auxin production was significant (P < 0.01) (Table 2). The auxin production was reduced with increasing Pb concentration and increased by the application of PGPB. The highest (277.25) and lowest (212.75) auxin production were detected in control and plots with 750 mg kg^{-1}, respectively. These findings highlighted the role of Pb on auxin reduction, the effect of PGPB and combination of these two factors on the increase of auxin. The application of PGPB under different levels of Pb resulted in an increase in the production auxin (Figure 2).

Table 2. Analysis of variance was measured by Rapeseed (mean-square).

		MS			
S.O.V	d.f	ABA	Auxin	Gibberelin	Cytokinin
A(PGPR Bacteria)	4	31962.32**	35036.2**	40853.66**	32526.31**
B(Lead)	3	18331.56**	27061.8**	22702.95**	19548.67**
A*B	12	1778.21**	1171.82**	1109.71**	1774.54**
Error	57	1115.23	1097.32	903.87	921.94
c.v.(%)	-	17.56	13.13	13.92	16.26

ns, * and **, respectively, non significant, significant at the five and one percent.

The effects of Pb and PGPB on cytokinin production

The ABA activity was affected by Pb , PGPB and Pb * PGPB interactions (at the p = 0.01 level) (Table 2). Figure 3 shows the changes in cytokinin rates under Pb * PGPB interactions. From this table it is apparent that cytokinin production decreased with increasing Pb concentration and enhanced by PGPB application. The maximum cytokinin was found in control plots. Conversely, the minimum cytokinin was related to plots exposed to 250, 500 and 750 mg kg^{-1}, 87.25, 83.5, 83.5 ng/g.fw. The results indicated the role of Pb in decrease of cytokinin content and the role of PGPB in cytokinin increasing. Surprisingly, unlike auxin and gibberellin which showed the largest increase in plots inoculated with azotobcter+ azospirillium+ pseudomonas, cytokinin production enhanced more under pseudomonas treatment. The highest level of cytokinin (218.7) was found under

azotobcter+ azospirillium+ pseudomonas and 500 mg kg treatments and the lowest level of cytokinin was observed in plots inoculated with pseudomonas and without Pb.

The effects of Pb and PGPB on gibberellin production

Gibberellins content was affected by Pb, SSB and Pb* SSB interactions (Table 2). Gibberellin content decreased with increasing Pb rates and increased by PGPB. The changes trend in gibberellin content at different levels of treatments was similar to that of auxin. The greatest content of gibberellin (207.25 ng/g.fw) was found in plots without Pb. on the other hand, the minimum gibberellin content (150.25 ng/g.fw) was observed in plots exposed with 750 mg kg^{-1}. As observed in Figure 4, application of PGPB except azotobacter (0 and 250 mg kg^{-1}) could enhance gibberellin content. among the bacterial treatments, pseudomonas had the maximum effect on gibberellin content. In general, the most gibberellin content (331.5 ng/g.fw) was detected in plots inoculated with azotobcter+ azospirillium+ pseudomonas and with 250 m kg-1 of Pb. However, no significant difference was found between abovementioned treatment and control treatment (Table 3).

Table 3. Comparison of measured average yield barley.

Treatment	Cytokinin (ng/g.fw)	Gibberelin (ng/g.fw)	Auxin (ng/g.fw)	ABA (ng/g.fw)
Bacteria levels				
Control	91.43e	178.31e	245.18e	44.88e
Azospirilum	124.56c	217.56c	283.12c	84.00c
Azotobacter	108.62d	188.00d	255.875d	56.6d
Pseudomonas	164.68d	256.87b	321.93b	117.1b
Integerated bacteria	202.87a	300.12a	357.50a	153.8a
Lead Levels				
Pb(No3)2=0 mg/kg	175.25a	268.85a	334.25a	126.52a
Pb(No3)2=250mg/kg	150.45b	247.85b	310.30b	102.50b
Pb(No3)2=500mg/kg	125.00c	210.10c	275.00c	80.43c
Pb(No3)2=750mg/kg	103.05d	185.90d	251.35d	55.73d

Mean with the same letters in each column are not statistically significant at the 5% level.

Table 4. Analysis of variance was measured by Rapeseed (mean-square) .

		MS				
S.O.V	d.f.	Shoot lead	Root lead	Chl a	Chl b	Chl total
A(PGPR Bacteria)	4	2.85^{ns}	8.66^{**}	$0/126^{**}$	$0/024^{**}$	$0/263^{**}$
B(Lead)	3	613.67^{**}	2301.12^{**}	$0/052^{**}$	$0/011^{**}$	$0/111^{**}$
A*B	12	2.06^{ns}	2.2^{ns}	$0/0004^{**}$	$0/002^{**}$	$0/002^{**}$
Error	57	1.01	1.78	0.0003	0.001	0.001
c.v.(%)	-	13.15	10.66	2/46	5/27	3/54

ns, * and **, respectively, non significant, significant at the five and one percent.

The effects of Pb and PGPB on chl.a content

The effects of Pb and PGPB on chl.a content was significant ($p<0.01$). chl.a content decreased by increasing Pb concentration and increased by PGPB application(Figure 5). Chl.a content was high when Pb concentration was low and plots were inoculated with PGPB, so that chl.a content was 0.61 ng/g.fw under maximum level of Pb and without PGPB application (control). Under minimum level of Pb, chl.a contents were 0.77, 0.94, 0.83, and 0.93 ng/g.fw in plots inoculated with azospirillium, azotobcter, pseudomonas and azotobcter+ azospirillium+ pseudomonas, respectively (Table 5).

Table 5. Comparison of measured average yield barley.

Treatment	chl total	chl b	chl a	Root lead	Shoot lead
Bacteria					
Control	1.038d	0.348bc	0.692d	11.67b	94.77b
Azospirilum	1.065c	0.346c	0.723c	12.31ab	113.63a
Azotobacter	1.302a	0.418a	0.885a	12.53ab	114.19a
Pseudomonas	1.136b	0.361b	0.775b	13.21a	114.91a
Integerated bacteria	1.307a	0.426a	0.882a	13.52a	118.37a
Lead					
Pb(No3)2=0 mg/kg	1.250a	0.407a	0.848a	0.647d	5.07d
Pb(No3)2=250mg/kg	1.202b	0.392b	0.810b	8.664c	74.59c
Pb(No3)2=500mg/kg	1.153c	0.370c	0.783c	15.29b	115.31b
Pb(No3)2=750mg/kg	1.075d	0.352d	0.726d	26.00a	249a

Mean with the same letters in each column are not statistically significant at the 5% level..

The effects of Pb and PGPB on chl.b content

Analysis of variance indicated that chl.b content was significantly affected by Pb, SSB and Pb* SSB interactions (Table 4). chl.b content decreased under high level of Pb and improved by the application of PGPB. The greatest the chl.b content was achieve when plots were inoculated with the integrated treatment of PGPB and azospirillium, 0.462 and 0.418 ng/g.fw, respectively. chl.b content was 0.348 ng/g.fw in control plots. Results also demonstrated that chl.b content was 0.352 ng/g.fw by Pb application at 750 mg kg $^{-1}$, while the chl.b content was 0.407 in control plots (Table 5). Different levels of PGPB could prevent of excessive loss of chlorophyll b content under high level of Pb (Figure 6).

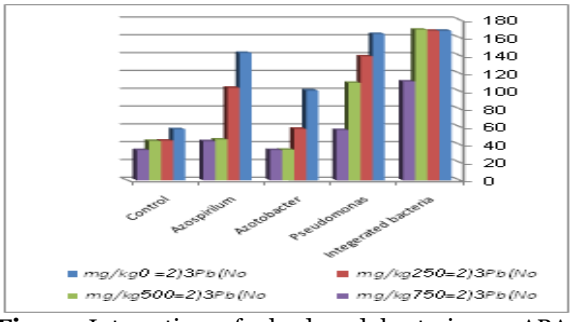

Fig. 1. Interaction of lead and bacteria on ABA hormone activity in barley.

The effects of Pb and PGPB on chl.a+b content

Pb, SSB and Pb* SSB interactions had significant effects on chl.a+b content (Table 4). Chl.a+b content decreased by increasing Pb concentration and increased by PGPB application. The maximum total chlorophyll was found in plots inoculated with the integrated treatment of PGPB and without Pb (1.38 ng/g.fw). In contrast, the least total chlorophyll was related to plots with no PGPB at 750 mg kg $^{-1}$ of Pb (0.93 ng/g.fw). These results also showed that Pb had a decrease role on chlorophyll content and PGPB application especially the integrated treatment of PGPB could mitigate negative and destructive effects of Pb (Table 5).

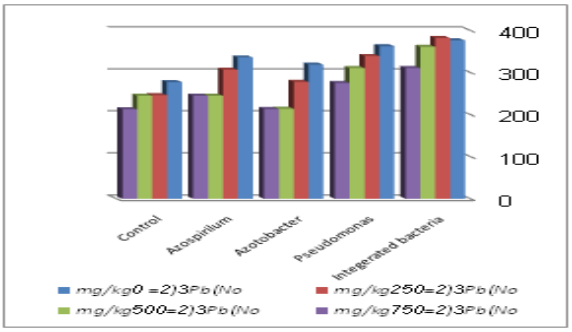

Fig. 2. Interaction of lead and bacteria levels of the auxin hormone content in barley.

Discussion

The chl.a content in barley was affected by Pb and PGPB with opposite effects. Chl.a content in barley reduced remarkably at different levels of stress. The highest chl.a loss occurred in plots with maximum Pb. On the other hand, application of PGPBs at all levels induced opposing effects on Chl.a contents and resulted in an increase in Chl.a contents compared to control. This is may be due to phytoremediation effectiveness of barley and the greater transfer of Pb to shoots (leaves), where chlorophylls are accumulated. One of the main reasons for the decrease in chlorophyll content is the *destructive* and harmful effects of *reactive oxygen species* (ROS).

Thus, reduction in chlorophyll contents occurred with increased production of ROS. The production rate of ROS depends on species, stress period, plant age and particularly stress intensity (Navari-Izzo *et al*., 1998).

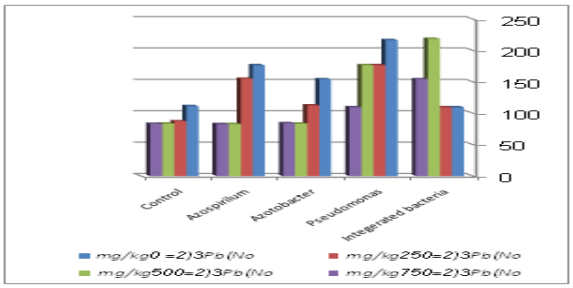

Fig. 3. Interaction of lead and bacteria levels of the hormone cytokinin content in barley.

The toxic effects of Pb on plants include inhibition of growth, reduction of photosynthesis and chlorophyll content, inhibition of enzyme activity, and damage to biomolecules such as lipids, proteins and nucleic acids, especially DNA (Chaoui and Ferjani, 2005; Mishra *et al*., 2006). Decrease of chlorophyll content in plants exposed to high level of copper has been frequently reported in the literature (Devi and Prassad, 1998). According to Prasad *et al.* (2001) Cu-induced chlorophyll content decrease attributed to chlorophyll degradation and structural damage of Cu. Pb adverse effects on chlorophyll synthesis is because of inhibition of Mg and Fe uptake. Under significant Pb accumulation, the increase in chlorophyllase activity increase chlorophyll degradation (Sharma and Dubey, 2005), in such manner that chlorophyllase with separation of phytole from chlorophyll, Mg from chlorophyllide and phaophorbids formation and finally decomposition of the four pyrovile rings cause chlorophyll degradation (Boyer *et al*., 1987). The results also indicated the link between lipid peroxidation and chlorophyll content in barley atmosphere which are in agreement with Ali *et al.* (2003) findings. The toxic effects of lipid peroxidation under heavy metal stress on chlorophyll content and decrease of chlorophyll synthesis in plant species is owe to interaction between heavy metal with SH-groups of necessary enzymes (Ali *et al*., 2003). The result of present study indicated an increase in Chl.a/ Chl.b ratio at all levels of the stress. These results are consistent with results of Olivares (2003) who reported that Pb had a negative sffects on plants including decrease in chlorophyll content and increase in Chl.a/Chl.b ratio. Kupper *et al.* (1996) found that chlorophyll content in Ceratophyllum demersum, Lemna trisulca and Myriophyllum spicatum species decreased by nickel chloride and cadmium sulfate treatments *due* to *replacement* of heavy metals with Mg at the center of the porphyrin ring.

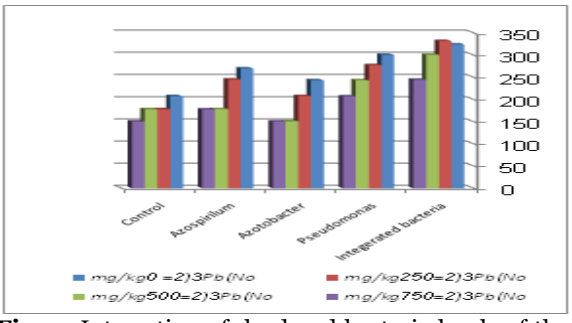

Fig. 4. Interaction of lead and bacteria levels of the hormone gibberellin content in barley.

PGPB by creating more favorable conditions for the development, increase in chlorophyll production and water and nutrient uptake and the ability in *breaking down toxic compounds can* have positive effects on chlorophyll content and prevent its chlorophyll degradation (Bashan and Holguin, 1997).

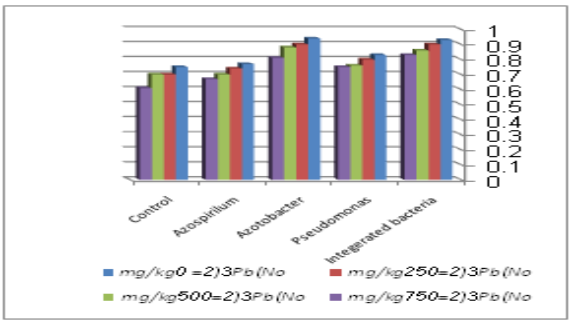

Fig. 5. Interaction of lead and bacteria levels on a chlorophyll content in barley.

The rate of auxin, gibberellins and cytokinin was greatest in control treatment. While, the rates of hormones was maximum by combined inoculation of azotobcter+ azospirillium+ pseudomonas. The efflux of maize roots added to the culture medium of Azotobacter remarkably increased auxin, gibberellins and cytokinin production. This is because of suitability of root efflux as a carbon source for growth of Azotobacter (Martinez-Toledo *et al*., 1988).

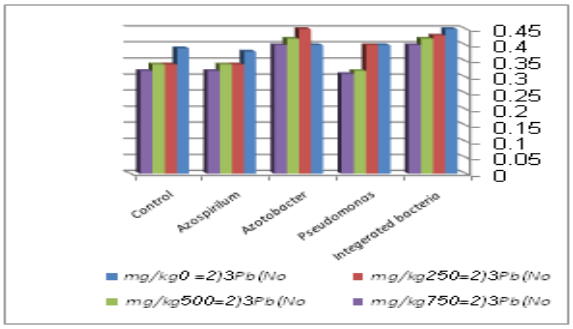

Fig. 6. Interaction of lead and bacteria levels on b chlorophyll content in barley.

Among different PGPB, pseudomonas has considerable importance due to the ability to produce a wide range of plant growth regulators, combination of Fe and chelated –Fe uptake, production of organic acids such as succinic acid and lactic acid, and the biological control of plant pathogens. With regard to results, the role of PGPBs on hormones increasing especially auxin is obvious. This is owing to the development of the root system of in PGPBs -treated seedlings (Bashan and Levanony, 1990). *Evidence suggests* that abscisic acid is also produced from oxidation of xanthophyll such as violaxanthine. There is a relationship between ABA accumulation and stomatal closure in leaves under stress conditions. In fact, ABA *is responsible for stomatal opening and closing (*Dorffling, 1972). ABA can prevent proton exit and K uptake on the cell surface (Cocucci and cocussi, 1977).

As the results demonstrated, the increase in Pb concentration had antagonist effects and inoculation by the application of growth-promoting bacteria had synergist effects on ABA accumulation in plants. The results also highlighted that the integrated treatment of PGPB showed a higher efficacy with changes in plant growth, increase in chloroplast production and in increase in abscisic acid production. The present study indicated that barley appear to have great potential for cleaning Pb-contaminated soils.

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