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EFFECT OF DIAZOTROPHS (*RHIZOBIUM AND AZATEBACTOR*) ON GROWTH OF MAIZE (*ZEA MAYS* L.) AND ACCUMULATION OF LEAD (PB) IN DIFFERENT PLANT PARTS

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Abstract

The effects of diozotrophs which fix atmospheric nitrogen into ammonium ions on lead (Pb) phytoextraction and their subsequent effect on the growth of maize (*Zea mays* L.) was evaluated in a green house study. *Rhizobium leguminosarum* strain TAL-102 and *Azotobacter chroococcum* were used as single culture as well as co-culture. In single culture inoculations, the *Rhizobium* showed better reponse than *Azatobacter*, whilst the co-inoculation treatment showed highly significant increase in growth as well as in dry biomass of plant in Pb polluted soil. Highly significant increase in Pb accumulation was found in plant co-inoculated with *Rhizobium* + *Azatobacter* as compared to control. In single culture treatments, the *Rhizobium* was superior than *Azatobacter* in enhancing Pb uptake by the plant. In roots and stem all the treatments either in single culture or co-culture inoculation showed significant increase in Pb accumulation as compared to control, however the Pb translocation into leaves was significant only in co-inoculated culture. Conclusively the co-inoculation was much better than single culture inoculations in Pb phytoextraction along with increase in plant growth and biomass.

Introduction

The soil contamination with heavy metals is an important environmental issue. One of the concerned heavy metals is lead (Pb). Elevated levels of Pb in the soil result from anthropogenic activities such as mining, metallurgy, industrial wastes, pesticides and urban activities (Marchiol *et al*., 2004). Soil contaminated with Pb may threaten plant health. The restoration of soil contaminated with toxic heavy metals is particularly challenging. Unlike organic compounds, metals cannot be degraded, consequently the clean up usually requires their removal (Lasat, 2002). Most conventional approaches of remediation like excavation and land fill, thermal treatment, acid leaching, and electro-reclamation do not provide reasonable solutions because of the low efficiency have high cost, and cause large destruction of soil fertility (Jing *et al.,* 2007). Remediation of heavy metals contaminated soil is a global challenge and requires new techniques to enhance the process. Biological processes are being used to decontaminate soils. One effective and promising process is phytoremediation, which is the use of plants to extract, sequester and detoxify pollutants (Jing *et al.*, 2007). Phytoremediation includes phytoextraction, phytovolatilization, rhizofiltration and phytostabilization (Chaney *et al.*, 1997). The advantages of phytoremediation include low cost, speed of deployment, preservation of natural soil properties and reliance on solar energy (Zhuang *et al*., 2007). However, plants with exceptionally high metal accumulating capacity often have a slow growth rate and produce limited amounts of biomass on metal contaminated soil (Denton, 2007). Phytoredmediation could be enhanced by relieving the toxicity of heavy metals to

the plant. Plant growth promoting rhizobacteria exert beneficial effect on plant growth and enhance heavy metal stress tolerance by detoxification of metals absorption (Tassi *et al.*, 2008; Han *et al.*, 2005; Glick, 2003; Kloepper & Zablotowicz, 1989).

Maize (*Zea mays* L.) is an important crop plant grown for both grain and green fodder in many areas of Pakistan. It requires significant amounts of nitrogen for rapid growth and biomass production (Shrestha & Ladha, 1998). *Azatobacter* (Pandey & Kumar., 1989) and *Azospirillum* (Riggs *et al*., 2001) could enhance the maize growth and biomass on normal soil. In this study we investigated the role of *Rhizobium* and *Azotobacter* on growth and biomass of plant on Pb polluted soil. We tried to find the ways of decreasing Pb toxicity on plant to enhance plant growth and biomass along with Pb phytoextraction. The maize plant was selected for this study because of its higher biomass. The objective of this study was to evaluate the effect of *Rhizobium* and *Azatobacter* in Pb phytoextraction as well as to verify the relationship of higher biomass with increase in Pb accumulation.

Materials and Methods

This work was conducted in the Plant Sciences Department of Quaid-i-Azam University, Islamabad, Pakistan. The seeds of *Zea mays* L. *cv*. Pioneer-33556 were obtained from the National Agriculture Research Centre (NARC) Islamabad. Soil was prepared by mixing clay, sand and humus in the ratio of 2:1:1 respectively and dried in the sun light. The dry soil was artificially polluted by higher quantity of lead 800mg lead nitrate Pb $(NO_3)_2$ kg $^{-1}$ soil (Sonmez *et al*., 2008) and mixed well for three days. Each plastic pot (18cm x 24cm) was filled with 2.5kg soil (soil pH 6.2 ±0.3 n=5). Pots were watered 24hours before sowing the seeds. Ten seeds were sown in each pot. Three replicate pots were used for each treatment and arranged in completely randomted design. After germination three healthy seedlings were selected in each pot for experiment and the rest were removed. All the plants were grown in green house at 30/15^{o}C in natural light, no additional fertilizers were added and the pots were watered twice a week according to water holding capacity of soil.

The following treatments were made during experiment. C (control without Pb), C1 (Pb only), T1 (*Rhizobim* + *Azatobacter* + Pb), T2 (*Rhizobium* + Pb), T3 (*Azatobacter* + Pb). C was compared with C1 for the Pb effect on plant growth and biomass. For the effect of microbial treatments, C1 was compared with all treatmnets. bacterial cultures were obtained from the Agriculture Biotechnology Institute, National Agriculture Research Centre, Islamabad, Pakistan. A stock culture of *Rhizobium leguminosarum* strain TAL-102 was sub-cultured in Yeast Mannitol Broth (Vincent, 1980) and *Azotobacter chroococum* in Jensen's media (Jensen, 1951), incubated for 72 hours on an orbital shaker at 120 rpm at 25^{o}C. The incubated broth cultures were centrifuged at 3000 rpm for 15 mins. Pelleted cells were re-suspended in sterile tap water and adjusted to about 10^8 cells ml^{-1} based on an optical density (OD_{660} = 0.08) (Bhuvaneawari *et al*., 1980). Inoculums were applied both as single cultures and as a co-inucluation where they were mixed at a 1:1 ratio on volume basis. Two millileters of either the single culture or the co-culture were injected into root zone when the young seedlings were at the early 1st leaf stage.

Plants were harveted after sixty days of treatments and washed with tap water, Plant height and root length were measured with a centimeter rule from the base of stem to the top of the apical leaf and the root length from base to tip. Plants were separated into roots, stem and leaves. The roots were further washed with a solution containing 5 *mM*

Tris *HCl* pH 6.0 and 5 mM EDTA and then rinsed with distilled water in order to remove surface bound metal ions (Genrich *et al*., 2000). Roots and shoots were dried in oven at 80ºC for 48 hrs and then dry weight was noted. The dried samples were digested using Perchloric-acid digestion method of Allen (1974) for Pb analysis. All replicate plants were analysed in the same way and 0.25g roots, stem and leaves samples were taken in 50ml flask and 6.5 ml of mixed acid solution i.e. Nitric acid, Sulfuric acid and Perchloric acid in the ratio of 5:1:0.5 was added and digested on hot plat until the white fumes came out from the flasks. Thereafter the digested samples were transferred in 50ml volumetric flasks and the volume was made up to 50ml with distilled water. These samples were filtered and the filtrates were analyzed for Pb contents (μgg^{-1} d.w) in roots, stem and leaves samples by Shimadzu AA-670 Atomic Absorption/flame spectrophotometer. The total Pb content in plants were calculated as, Total Pb accumulated = total dry weight x Pb µgg-1 d.w. Data analysis was done with Minitab15 Statistical Software (Minitab Inc. USA). One way analysis of variance (ANOVA) was carried out for confirmation of data variability ($p \leq 0.05$) and mean values of treatments were compared by Tukey's Honestly Significant Difference test at $p \leq 0.05$.

Results and Discussion

Effect on plant growth and dry biomass: The result revealed that the plant height was significantly decreased by the addition of lead (Pb) in the growing media (Table 1). However inoculation with *Rhizobium* and *Azotobacter* significantly increased the Plant height over C1 (Plants grown in Pb added soil); although the plant height was still less than that of control (uninoculated untreated plants). Co-inoculation treatment with *Rhizobium* and *Azotobacter* resulted in significant stimulation in plant height over control such that no significant difference existed between the control (untreated) and Pb treated plants. There was reduction in plant growth and biomass on Pb polluted soil (Xiong, 1998; Martha *et al*., 2009; Devkota & Schmidt, 2000; Ouzounidou *et al*., 1997; Dudka *et al*., 1996).

Root length was significantly decreased due to Pb treatment but inoculation treatment with *Rhizobium* and co-inoculation treatments with both *Rhizobium* and *Azotobacter* resulted in significant stimulation of root length over Pb treated plants such that the values did not differ significantly than the untreated uninoculated control. *Azotobacter* inoculation has no effect on mitigation of adverse effects of Pb on root length (Table 1).

Both the root and shoot dry biomass was significantly inhibited by Pb treatment. The magnitude of inhibition was greater in root dry biomass. However, inoculation with both the microbes had not only overcome the inhibitory effect of the Pb treatment but also stimulated the shoot dry biomass over control (untreated).

The microbes in co-inoculation interact synergistically and the mechanism involves direct stimulation of growth by enlargement of root system for enhanced nutrient uptake (Camacho *et al*., 2001). Diazotrophic rhizobacteria interact with crop plants and increase their vegetative growth (Ivan *et al*., 2004) on heavy metal contaminated soils by assisting root growth and branching (Jing *et al*., 2007) and can promote tolerance to contaminants to reduce heavy metals toxicity (Burd *et al*., 2000; Glick, 1995; Shah *et al*., 1998). These findings suggest that co-inoculation of *Rhizobium* and *Azatobactor* responded better than single inoculation with *Rhizobium* or *Azatobacter*. Previous researchers have demonstrated that microbial inoculation effects were greater on growth when seedlings were inoculated with combination of microbes rather than individually (Muthukumar *et al*., 2001; Jetiyanon & Kloepper, 2002).

Phytoaccumulation of Pb in different parts: All the microbial treatments either alone or in combination showed significant increase in Pb accumulation in roots (Fig. 1a) as well as in stem (Fig. 1b). While in the leaves the Pb accumulation was significant only in coinoculated plants(T_1) but the inoculation with either *Rhizobium* or *Azatobactor* treatments exhibited no significant difference when compared with control C_1 (Fig. 1c). The total Pb accumulated in roots as well as in shoot was presented in Fig. 1d, where the maximum total Pb accumulation was found in co-inoculated plant (T_1) while the Pb concentration was higher in roots than in shoot. This is because that Pb accumulation in leaves was less than that of stem and roots following coinoculation or single inoculation treatments with *Rhizobium* and *Azotobacter*.

This finding was further strengthened by the data presented in Fig. 2 indicating the inoculation induced root proliferation; coinoculation with both these microbes further augmented the root growth.

Vivas *et al*., (2003) showed that bacterial strain A belonging to genus *Brevibacillus* decreased the amount of Pb absorbed by plants, when expressed on a root weight basis. The bacterium also increased the production of IAA which is turn stimulate the root biomass and enhanced plant growth nitrogen, P accumulation and nodule formation.

Denton (2007) reported that Plant growth promoting rhizobacteria and AM fungi stimulated plant growth in heavy metal contaminated soil and mitigated the toxic effects of heavy metals through secretions of acids, proteins, phytoantibiotics and other chemicals.

Metals are mostly found in insoluble phase (Lasat, 2002), rhizobacteria release the metal to soluble phase by decreasing the pH of the environment that facilitate plant uptake (Zhuang *et al*., 2007). It has been demonstrated by many workers that bacteria might enhance the process of metals accumulation (Carlot *et al*., 2002; Whiting *et al.*, 2001; Abou-Shanab *et al*., 2006). The *Rhizobium* and *Azatobactor* in combination (co-inoculation) highly increased the Pb uptake into roots and translocation into stem and leaves . It might be due to the synergistic effect of microbes on enhanced nutrient and ions uptake and translocation (Kumar *et al*., 1999; Creus *et al*., 1996).

Conclusions

The finding showed that diazotrophic microbes (*Rhizobium* and *Azatobactor*) increased maize growth on heavy metal polluted soil, which might be due to reduction in metal toxicity on plant, because the growth on Pb added soil in the absense of these microbes was reduced significantly. Burd *et al*., (2000) and Guan *et al*., (2001) demonstrated that soil microorganisms affect the metal mobility and availability to plants through acidification ,redox changes and siderophore production as well as mobilizing the metal phosphates The Pb accumulation in plant tissues was associated with increase in dry biomass by these microbes; the co-inocultaion treatment showed higher Pb accumulation along with higher biomass. These results suggest the use of *Rhizobium* and *Azatobactor* in combination (co-inoculation) either for increase in growth and biomass on metal polluted soil, or to use for phytoremediation purposes. Moreover this association of plant and microbes is environmentally friendly as compared to other chemical based extraction of heavy metals which causes severe soil and ground water pollution.

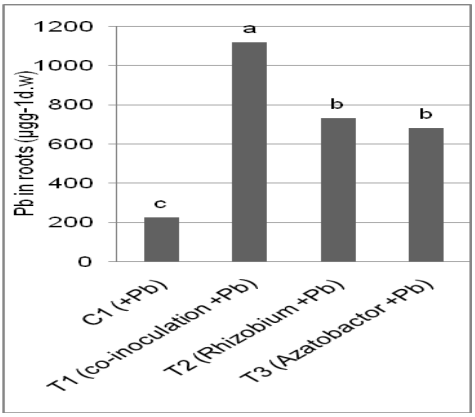

Fig. 1a.

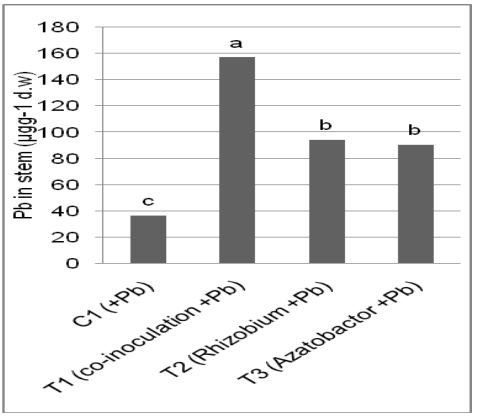

Fig. 1b.

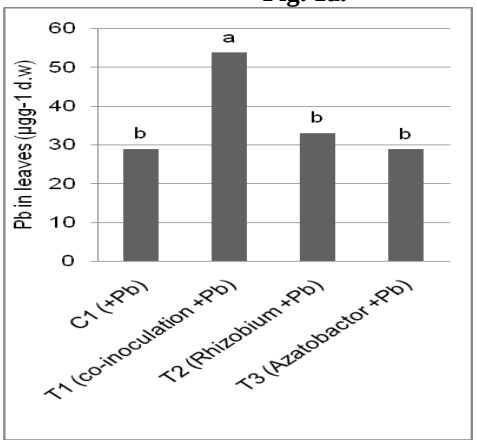

Fig. 1c.

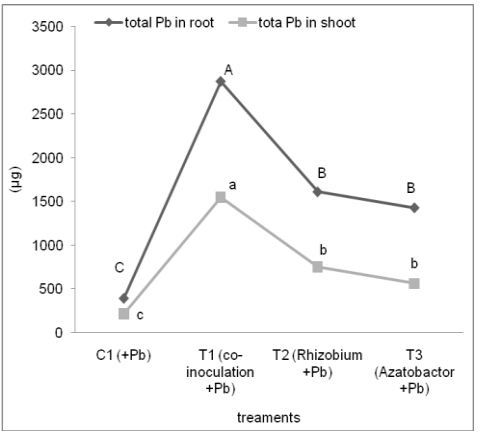

Fig. 1.d

Fig. 1a-d. Effect of treatments on lead (Pb) accumulation in plant; (a) Pb in roots, (b) Pb in stem, (c) Pb in leaves. (d) Total Pb accumulation in plant. Total Pb accumulated = total dry weight x Pb µgg^{-1} d.w.

Fig. 2. Effect of treatments on root growth.

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