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Heavy metal induced oxidative damage and root morphology alterations of maize (*Zea mays* L.) plants and stress mitigation by metal tolerant nitrogen fixing *Azotobacter chroococcum*

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ABSTRACT

Heavy metals are one of the major abiotic stresses that adversely affect the quantity and nutritive value of maize. Microbial management involving the use of plant growth promoting rhizobacteria (PGPR) is a promising inexpensive strategy for metal clean up from polluted soils. Considering these, metal tolerant plant growth promoting nitrogen fixing rhizobacterial strain CAZ3 identified by 16SrRNA gene sequence analysis as *Azotobacter chroococcum* was recovered from metal polluted chilli rhizosphere. When exposed to varying levels of metals, *A. chroococcum* survived up to 1400 and 2000 μg mL^{-1} of Cu and Pb, respectively and expressed numerous plant growth promoting activities even under metal stress. Strain CAZ3 secreted 65.5 and 60.8 μg mL^{-1} IAA at 400 μg mL^{-1} each of Cu and Pb, respectively and produced siderophores, ammonia and ACC deaminase under metal pressure. The melanin extracted from *A. chroococcum* revealed metal chelating ability under EDX. Following application, strain CAZ3 enhanced growth and yield of maize grown both in the presence of Cu and Pb. The dry biomass of roots of inoculated plants grown with 2007 mg Cu kg^{-1} and 585 mg Pb kg^{-1} was increased by 28% and 20%, respectively. At 585 mg Pb kg^{-1}, the bioinoculant also increased the kernel attributes. At 2007 mg Cu kg^{-1} strain CAZ3 enhanced the number, yield and protein of kernels by 10%, 45% and 6%, respectively. Interestingly, strain CAZ3 significantly reduced the levels of proline, malondialdehyde and antioxidant enzymes in foliage. The roots of inoculated plants accumulated greatest amounts of metals compared to other organs. In kernels, the concentration of Pb was more as compared to Cu. The metal concentrations in roots, shoots and kernels, however, declined following CAZ3 inoculation. Copper and lead had substantial distortive impact on root and leaf morphology while cell death were visible under CLSM and SEM. Conclusively, *A. chroococcum* CAZ3 could be a most suitable and promising option to increase maize production in metal polluted soils despite the soils being contaminated with heavy metals.

1. Introduction

Maize (*Zea mays* L.), an edible flowering plant is cultivated preferably during spring and summer. Globally, maize, ranked as the third most important cereal crop after wheat and rice (Akongwubel et al., 2012) is used largely as food both by humans and animals (Lu et al., 2015). Maize provides carbohydrates, protein, minerals, vitamin B and iron in human diet (Olaniyan, 2015). Maize, like many other plants, however, suffers heavily by metals and eventually the growth and grain yield is greatly reduced (Aliu et al., 2013). Among metals, Pb for instance, is highly toxic and at excessive levels, slows down the germination rate and obstructs the height and biomass of maize plants (Ghani et al., 2016). Also, at elevated levels, it decreases cell division with brittle leaves and dark purple colour spots on plants (Imdad et al., 2017). Other toxic impact of Pb includes water imbalance, disturbances in nutrient uptake, disruption in photosynthetic process, altered enzymatic activities and overall hormonal imbalance (Sędzik et al., 2015; Ashraf et al., 2015). Copper (Cu) is another important metal which at lower concentration acts as essential micronutrient but at higher concentration it becomes highly toxic and inhibits photosynthesis (Dey et al., 2014), nutrient absorption, plant growth (Adrees et al., 2015) and ultimately cause cell death (Printz et al., 2016). Also, excessive amounts of Cu generates reactive oxygen species (ROS) leading to the oxidative damage of the plant cell (Liu et al., 2014). Due to these, there is urgent need to find solutions to the metal toxicity problems so that maize production continues even in soils contaminated with heavy metals. In this regard, certain beneficial bacteria aggressively colonizing the plant roots termed as plant growth promoting rhizobacteria

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(PGPR), when applied as soil/seed inoculant, have shown positive impact on crop production under abiotic stress (Khan et al., 2009; Hassan et al., 2017) The metal tolerant bacteria abolishes the toxicity of metals by one or the simultaneous mechanisms of exclusion, extracellular and intracellular sequestration, or transformation (Gupta et al., 2016). Maize crops can therefore, be protected from the toxic effects of metals by using metal tolerant PGPR. When firmLy stabilized in the rhizosphere, the PGPR facilitates the growth and yields of cereals by supplying important plant nutrients like P (Sarker et al., 2014) and N (Kuan et al., 2016) besides protecting the crops from damaging effect of phytopathogens through antimicrobial compounds (Gad et al., 2014) and siderophores (Vejan et al., 2016). Such metal tolerant PGPR expressing manifold plant growth promoting activities therefore, can play an important role in metal clean up *vis-a-vis* plant growth promotion under stressed environment. However, there is little information available on the effects of metal tolerant PGPR on the performance of maize grown in soils enriched with metals. Also, there is hypothesis that the ability of PGPR to secrete plant growth regulators continues even under metal pressure. Realizing the distinct food value of maize on one hand and the destructive impact of metals onto both PGPR and maize on the other hand, the present study was conducted to test such hypotheses and aimed at-(i) identifying metal tolerant PGPR endowed with multiple plant growth promoting activities (ii) determining the toxic impact of copper and lead on maize plants (iii) evaluating the bioremediation potentials of PGPR using maize as a test crop and (iv) examine cellular damage induced by copper and lead and (v) assessment of metal distribution in maize organs employing SEM, EDX and CLSM.

2. Materials and methods

2.1. Total heavy metal concentrations and microbial composition

The soil samples were collected from the agricultural fields receiving consistent sewage waters originating from different industries located near Grand Trunk Road, Aligarh (27°53 ´N 78°05 ´E 27.88°N 78.08°E), Uttar Pradesh, India (S1) and heavy metals were determined (Rizvi and Khan, 2017). The rhizobacterial strains were isolated from rhizospheric soils of chilli (*Capsicum annum*), grown in metal contaminated agricultural fields. For bacterial recovery, a 100 µl suspension of serially diluted soil sample was spread plated on Ashby's mannitol agar plates (g/l: mannitol 20.0; dipotassium phosphate 0.2; magnesium sulphate 0.2; sodium chloride 0.2; potassium sulphate 0.1; calcium carbonate 5.0; agar 15.0; pH 7.4) and incubated at 28 ± 2 °C for 6–7 days. Following incubation, the bacterial colonies appearing on the medium were picked and re-streaked three times on Ashby's mannitol agar medium so as to validate the purity of cultures.

2.2. Selection, characterization and identification of metal tolerant PGPR

Bacterial strains were grown on nutrient agar plates amended with increasing concentrations (0–2400 µg mL^{-1}; at a two-fold dilution interval) of Cu ($CuSO_4{\cdot}5H_2O$) and Pb [$Pb(CH_3COO)_2{\cdot}3H_2O$]. Spot inoculated plates (10 µl of 10^7 cells mL^{-1}) were maintained at 28 ± 2 °C for 48 h and scored for growth. The bacterial strain surviving well at the highest concentration of metals was considered as the metal tolerant bacterial strain (MTBS). Of the total 20 bacterial strains, strain CAZ3 showing maximum tolerance to Cu and Pb was selected and maintained on Ashby's mannitol agar medium for further use. The selected bacterial strain was characterized morphologically and biochemically (Holt et al., 1994). The bacterial strain (CAZ3) was further identified by 16 S rRNA partial gene sequencing services of Macrogen, Seoul, South Korea, using universal primers, 785 F (5′-GGATTAGATACCCTGGTA-3′) and 907 R (5′-CCGTCAATTCMTTTRAGTTT-3′). The processed and trimmed nucleotide sequence was deposited to GenBank sequence database. The BLASTn online programme was used to identify strain CAZ3 to species level. Moreover, sequence alignment was done using maximum likelihood method and a phylogenetic tree was constructed using MEGA 6.0 software.

2.3. Plant growth regulators of A. chroococcum CAZ3 under metal stress

2.3.1. Indole acetic acid and siderophores

The IAA production by *A. chroococcum* CAZ3 was quantified by the modified method of Brick et al. (1991). Briefly, a-100 µl of overnight grown CAZ3 culture was inoculated in 25 mL of Luria Bertani (LB) broth amended with 200 µg tryptophan mL^{-1} and treated with 0, 25, 50, 100, 200 and 400 µg mL^{-1} of Cu and Pb. The cultures were incubated for 48 h at 28 ± 2 °C with shaking at 125 rpm. After incubation, two mL culture from each set was spun (10,000 g) for 15 min and 2–3 drops of orthophosphoric acid and four mL of Salkowsky reagent (2% 0.5 M $FeCl_3$ prepared in 35% $HClO_4$) were added to supernatant. Samples were kept for 1 h in dark at 28 ± 2 °C. The IAA released in supernatant was assayed at λ 530 nm by recording the absorbance of pink colour developed during reaction against a standard curve of pure IAA. For detection of siderophores, the method of Alexander and Zuberer (1991) was followed wherein the chrome azurol S (CAS) agar plates containing 0, 25, 50, 100, 200 and 400 µg mL^{-1} of Cu and Pb were spot inoculated with 10^7 cells mL^{-1} of CAZ3. Formation of yellow to orange zone (halo) around bacterial growth signified the secretion of siderophore. Also, the siderophores was quantitatively assayed using Modi medium (K_2HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1.0%; glutamine 0.1%; NH_4NO_3 0.1%). For this, a 100 µl of bacterial suspensions containing approximately 10^7 cells mL^{-1} was inoculated into Modi medium supplemented with 0, 25, 50, 100, 200 and 400 µg mL^{-1} of Cu and Pb and incubated at 28 ± 2 °C for 5 days. The cultures were centrifuged at 6000 rpm and the catechol type phenolates [salicylic acid (SA) and 2,3-dihydroxybenzoic acid (DHBA)] in the supernatant were quantified (Reeves et al., 1983).

2.3.2. Assay of hydrogen cyanide, ammonia and ACC deaminase

Hydrogen cyanide (HCN) secreted by strain CAZ3 was detected by the method of Bakker and Schipper (1987) using King's B agar plates treated with 4.4 g/l glycine and 0, 25, 50, 100, 200 and 400 µg mL^{-1} each of Cu and Pb. The top side of petri plates were wrapped with filter paper disc dipped in 0.5% picric acid prepared in 2% sodium carbonate. Each experimental plate was sealed with parafilm and incubated for four days at 28 ± 2 °C. The change in colour of filter paper was monitored at the end of incubation periods. Variation in colour from yellow to orange was considered a positive reaction. Similarly, the method of Dye (1962) was used to detect ammonia.

The ACC deaminase secreted by CAZ3 was qualitatively detected by spot inoculation method using Dworkin and Foster (DF) salts minimal medium (Dworkin and Foster, 1958) enriched with 3 mM ACC as the principal N source. The DF plates without ACC but containing $(NH_4)_2SO_4$ (0.2% w/v) served as negative and positive control plates, respectively. All plates were incubated for 72 h at 28 ± 2 °C and monitored on daily basis for bacterial growth. *Mesorhizobium* LMS-1 containing pRKACC plasmid (Shah et al., 1998) served as positive control. The ACC deaminase was also quantified by the modified method of Penrose and Glick (2003). The concentration of α -ketobutyrate released due to breakdown of ACC was measured by comparing the absorbance of samples against a standard curve of pure α-ketobutyrate. The ACC deaminase activity was expressed as the amount of α-ketobutyrate/mg protein/h.

2.4. Heavy metal toxicity to A. chroococcum observed under SEM and EDX

Cellular damage to *A. chroococcum* strain CAZ3 after growing in nutrient broth amended with 100 µg Cu mL^{-1} and 400 µg Pb mL^{-1} was observed under SEM (model JSM 6510 LV; JEOL, Japan). The cellular location of heavy metals was also detected by EDX. Along this