Ecological Engineering 116 (2018) 163–173

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Effect of inoculation of zinc-resistant bacterium *Enterobacter ludwigii* CDP-14 on growth, biochemical parameters and zinc uptake in wheat (*Triticum aestivum* L.) plant

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ARTICLE INFO

Keywords:
ACC deaminase
GC–MS
Metal stress
Osmolytes
Plant growth

ABSTRACT

A metal resistant bacterium was isolated from the rhizosphere of Kair '*Capparis decidua*' and screened for its phytoextraction ability under gradient metal stress conditions. Based on 16S rDNA analysis, the strain was identified as *Enterobacter ludwigii*. Among the plant growth promoting traits, isolate showed the ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, production of indole-3-acetic acid in tryptophan supplemented medium and solubilize the inorganic phosphate. The isolate was resistant to heavy metals like zinc (Zn), nickel (Ni), copper (Cu), and cadmium (Cd). The fatty acid adaptation of isolate growing at different concentration of Zn (100–300 mg kg^{-1}) was also studied, which indicated that metal concentration strongly influenced the fatty acid composition of bacterium, particularly by increasing the unsaturated fatty acids. Furthermore, inoculation with the test isolate was found to significantly ($p < 0.01$) increase the various growth parameters of wheat plants and also improve the photosynthetic pigments. In addition, inoculation with isolate resulted in significant ($p < 0.01$) increase in the Zn content in wheat plant under metal stress. Moreover, bacterial application significantly ($p < 0.01$) increased the various compatible solutes such as proline content (30–65%), total soluble sugar (9–49%), and decreased the malondialdehyde (MDA) content (38–47%) as compared to control, illustrating its protective effect under metal induced oxidative stress. Inoculation with test isolate also increased the total protein content in range of 16–52%. Our work revealed that metal resistant plant growth promoting rhizobacterium could be exploited as microbial mediated phytoremediation of metal polluted soils.

1. Introduction

Various agricultural and industrial activities such as use of agrochemicals, steel processing, vehicle exhausts, waste disposal, and waste incineration lead to accumulation of heavy metals in soil which pose a serious threat to food safety and potential health risks (Giller et al., 1998). Contamination of soils with toxic heavy metals is of great concern due to its detrimental effects on soil biological systems as well as its considerable persistence in the environment. Previous studies also illustrated that various agricultural crops growing under metal contaminated soil causes severe environmental problems and serious health issues (Zhuang et al., 2007; Velma and Tchounwou, 2010; Lockhart et al., 2013; Wu and Ma, 2015). Zinc (Zn) toxicity in plants is one of the major concerns these days, due to its interference in plant metabolism. At relatively low concentrations, zinc is required by plant as cofactor and activator of enzymes and proteins (Saravanan et al., 2007). In addition, it also exerts a catalytic property as a prosthetic group in metalloproteins. However, excess zinc may cause serious manifestations such as induction of oxidative damages and interferes with cellular metabolic process that causes the cellular damages etc. Previous studies illustrated that excess zinc accumulation leads to deficiency in necessary micronutrients such as iron magnesium, and phosphate in agricultural crops (Marschner, 1995; Ebbs and Kochian, 1997). Such deficiencies arise due to obstructed supply of these necessary micronutrients from roots to aerial parts of plant. For example, presence of excess zinc reduced the photosynthetic activity in common bean (*Phaseolus vulgaris*) by competing with or replacing magnesium in both Rubisco and photosystem II (Van Assche and Clijsters, 1986). Excess of metal stress like zinc also adversely affects the availability of phosphate to plants that results in reduced growth, chlorosis,

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https://doi.org/10.1016/j.ecoleng.2017.12.033
Received 3 March 2016; Received in revised form 24 May 2016; Accepted 23 December 2017

promotion of senescence, appearance of purplish-red color in leaves etc. (Yadav, 2010).

Therefore, the development of a cost-effective and eco-friendly strategy to remediate the metal contaminated soil that does not affect the neighbour environment is necessary. Conventional remediation such as soil excavation, electrokinetic treatment, leaching and immobilization are expensive and even harmful to biological systems. However, phytoremediation, which refers to use of plants and associated microflora, has gained more attention for cleaning up heavy metal contaminated soil (Glick, 2003). Application of metal-resistant plant growth promoting bacteria (PGPB) to ameliorate plant stress has emerged as one of the effective strategies in improving plant growth in metal polluted soils (Glick, 2010; Ma et al., 2011; Rajkumar et al., 2012, 2013). PGPB belonging to various genera such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas* and *Serratia* sp. have been known to improve plant growth through various properties including phytohormone production, phosphate solubilization, siderophore production, and ACC deaminase activity etc. (Sheng et al., 2008a,b; Ma et al., 2009). Bacterial strains producing ACC deaminase (ACCD) can substantially minimize plant growth inhibition arose from metal stress by lowering the level of stress ethylene (Glick, 2005; Rajkumar et al., 2012). Bacterial genera equipped with ACC deaminase activity can act as plant growth promoting agents. It is assumed that even low level of ACCD activity ($>$ 20 nmol-ketobutyrate mg^{-1} protein h^{-1}) is enough for alleviating stress and thus enhances plant growth (Penrose and Glick, 2003). The potential of ACCD producing rhizobacteria to promote plant growth and to alleviate the toxicity of metal stress ($NiCl_2$) on canola (*Brassica napus*) and tomato (*Lycopersicon esculentum*) has been documented (Stearns et al., 2005; Farwell et al., 2006). Similarly, augmentation of root and shoot growth of Indian mustard (*Brassica juncea*) growing in $CdCl_2$ by ACCD producing bacteria has also been monitored (Belimov et al., 2005). Moreover, transgenic canola plant expressing foreign ACC deaminase gene accumulated more arsenate from the metal contaminated soil than non-transformed plants. These reports indicates importance of ACCD in alleviating effects of metal stressors, Further, plant growth-promoting bacterial strains capable of solubilising inorganic phosphate have also been reported to be associated with the metal uptake and increased availability of phosphates to the plants (Sheng et al., 2008a,b; Ma et al., 2009). Therefore, metal tolerant rhizobacterium with plant growth promoting ability are of significant importance to facilitate remediation of metal polluted soil.

Metal tolerant microbes play significant roles in mobilization of heavy metals by excreting various metabolites including organic acids or extracellular polymeric substances. Improving the association between metal tolerant beneficial microbe and their compatible host plants is an important aspect of phytoremediation technology for enhanced heavy metal tolerance and biomass production in plants (Wenzel and Jockwer, 1999; Glick, 2003). Similarly, recent study has also indicated that under heavy metal stress conditions, inoculation with PGPR possessing the ability to produce siderophores and solubilize phosphate, increased growth of the inoculated plants primarily through enhancing the nutrient uptake (Ma et al., 2011). Study of Ramesh et al. (2014) reported role of Zn solubilizing strain *Bacillus saryabhattai* in improving mobilization of Zn in soybean and wheat. Similarly, few strains of *Serratia* sp., *Pseudomonas* sp., and *Bacillus* sp., enhanced 7–12% translocation of Zn in wheat grains (Abaid-Ullah et al., 2015). Another study by Rajkumar and Freitas (2008) reported that inoculation of *Ricinus communis* with *Pseudomonas jessenii* increased Zn concentrations in shoot tissues compared with uninoculated controls. Recent study by Islam et al. (2015) demonstrated that PGPR *Pseudomonas aeruginosa* improved the oxidative stress tolerance in wheat plants at high Zn stress. Therefore, the use of microbial application has been gaining wide acceptance for sustainable agricultural practices across the globe (Shen et al., 2015).

Members of *Enterobacteriacae* family have long known for their potential pathogenicity in humans causing different disease. However, several recent reports have indicated that members of genera as *Enterobacter* establish close plant-microbe associations helping in plant growth promotion, biological control against phytopathogens and hydrocarbon degradation (Taghavi et al., 2009; Madhaiyan et al., 2010; Yousaf et al., 2011). *Enterbacter ludwigii* is now known to be isolated from various environmental samples as endophytes and rhizosphere soil exhibiting plant growth promotion and hydrocarbon degradation potential by producing degradative genes thereby reducing toxicity levels in plant environment (Shoebitz et al., 2009; Yousaf et al., 2011). In a recent study, three different endophytic strains of *E. ludwigii* were isolated from birdsfoot trefoil (BRI10-9), alfalfa and Italian ryegrass (IRI10-4) plant species. All three strains were capable of plant interior colonization, hydrocarbon degradation, expressing CYP153 gene and accelerating plant growth under artificially diesel spiked (1% diesel) conditions. Strains ISI10-3 and BRI10-9 were capable of reducing hydrocarbon contamination up to 68% and showed best results when inoculated in combination to Italian ryegrass and alfalfa (Yousaf et al., 2011)

The mobilization of heavy metal as well as its solubility in the rhizospheric soils caused by chemical methods can have dramatic effect on heavy metal uptake and its accumulation in plants (Sessitsch et al., 2013). Therefore, the present study was an attempt to study the role of a rhizospheric microbe on metal accumulation in roots and shoots of wheat plants. Wheat is the second most produced cereal grain, commonly used as staple crop, and widely grown across the world.While growing under field conditions it is exposed to various abiotic stressors particularly metal stressors. Moreover, effects of interactions of metal mobilizing microbe *Enterobacter ludwigii* on the heavy metal phytoremediation to our knowledge have not been investigated. The objective of our study was (i) to isolate and characterize zinc (Zn) resistant PGPR from the rhizosphere of '*Capparis deciduas*' (ii) to investigate its effects on wheat plant growth, and metal uptake for improving the efficiency of phyoremediation of Zn-contaminated soil (iii) to assess the influence of metal resistant microbe on biochemical parameters of wheat plant under metal stress.

2. Materials and methods

2.1. Isolation of Zn resistant ACCD containing bacteria

The rhizospheric soil of '*Capparis decidua*' growing around the shekhawati region of Rajasthan, India (28.13°N, 75.4^0 East) was collected, stored in ice and brought to the laboratory for study. '*Capparis decidua*' locally known as 'Kair', is a shrub and grows primarily in arid region. This plant was selected for the isolation of bacteria due to its ability to grow luxuriantly in stressed environment. For the isolation of bacteria, 1 g of soil sample was dissolved in 10 mL sterile water and serially diluted decimally up to 10^{-9}, using 25 mM phosphate buffer (pH 7.4) and spread over on sucrose minimal salts low-phosphate (SLP) medium amended with different concentrations of Zn ($ZnSO_4$, 100–300 mg L^{-1}) (Jiang et al., 2008). The plates were incubated at 30 °C for 48 h in an incubator. Appearance of metal-resistant colonies was further streaked separately on the SLP-agar medium supplemented with above mentioned concentrations of Zn. Altogether, twelve different morphotypes of bacterial colonies were selected and tested for its/their ACC utilization ability by subculturing on solid DF salt minimal medium amended with 3 mM ACC (Sigma-Aldrich, USA) and incubated for 48–72 h at 30 °C. Based on rich growth on selective medium, bacterial isolate CDP-14 was selected and subcultured several times on DF-ACC agar plate to ensure its ability to use ACC as carbon and nitrogen source. ACCD activity of selected test isolate was quantified by standard protocol (Penrose and Glick, 2003). Glycerol stock (15% w/v) of the isolate was prepared and stored at −70 °C until further use.