Biocatalysis and Agricultural Biotechnology 8 (2016) 13–17

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

# Metal resistant and phosphate solubilizing bacterium improves maize (*Zea mays*) growth and mitigates metal accumulation in plant

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ARTICLE INFO

*Article history:*
Received 7 February 2016
Received in revised form
21 April 2016
Accepted 18 July 2016
Available online 19 July 2016

*Keywords:*
Phosphate solubilization
Heavy metal
Plant growth promotion
*Pseudomonas putida* Ws3
*Zea mays*

ABSTRACT

The current study focuses on plant promoting and metal detoxifying properties of a heavy metal resistant bacterium with phosphate (P) solubilizing and Indole-3-acetic acid (IAA) producing capabilities. The bacterium was a strain of *Pseudomonas putida* Ws3 selected from an agricultural land irrigated with sewage water. It could solubilize P up to 300 mg/l and produce IAA up to 200 μg/l in seven days at optimal culture condition, i.e. NaCl (0.5%), KCl (0.01–0.04%), $Mg/Cl_2$ (0.3%) and $NH_4Cl$ (0.5%) and 30 °C. Regarding soluble P and IAA concentrations in Pikovskaya (PVK) liquid media, the activity of the bacterium declined in media containing $CuCl_2$ (500 mg/l), $PbCl_2$ (300 mg/l) or $CdCl_2$ (300 mg/l). The metals at elevated levels limited the bacterial population growth in a soil-like substrate supporting roots of a model plant (maize or *Zea mays*). Weekly bacterial inoculation to the substrate for 40 days alleviated the condition for plant growth, led to increased plant dry weight and decreased the metals accumulated in plant with large effect sizes in the shoot (g = 1.49) and root (g = 1.36).



## 1. Introduction

The heavy metal entry into agricultural lands has posed serious problems for plantation (Jiang et al., 2008). They lead to decreased productivity of the lands. Moreover, elevated heavy metal contents of the crops are also hazardous to human health (McLaughlin et al., 1999). Heavy metals have direct and detrimental effects on plant growth and healthy production (Wani and Khan, 2010). Indirectly, they limit soil microbial community growth, most useful for healthy plant growth promotion (McLaughlin et al., 1999). However, among microbial community, there are some bacteria resistant to highly elevated heavy metal concentrations (Tripathi et al., 2005; Jiang et al., 2008). The activity of such bacteria can ameliorate the soil condition for other bacteria to grow, and taken together, the activity of these bacteria can lead to re-establish soil condition for plant growth and decreased accumulation of the metals in crops (Ahemad, 2012).

Microbial exploitation is an alternative to chemical fertilizers in improving soil condition for plant growth. They offer many benefits to plant, including solubilization of insoluble phosphorous, excretion of siderophores and phytohormones, production of antibiotics and antifungal metabolites (Pérez et al., 2008; Zaidi et al., 2009; Vassileva et al., 2010; Jog et al., 2014). Moreover, they could trap mobile heavy metals in their *exo*polysacharide layer and cell wall (Biosorption). Accordingly, bacteria capable of offering these services could promote plant growth and healthy development (Plant growth promoting bacteria). They are considered as cost effective and environmentally friendly tools for restricting heavy metal movement to plant organs ameliorating conditions for plantation in metal-contaminated lands (Ahemad, 2012; Glick, 2012).

The main aim of this research is to investigate how a heavy metal-adapted phosphate (P) solubilizing bacteria could affect plant growth and if it could alter heavy metal content of plant residing in high metal containing soils. Thus, maize (*Zea mays*) was chosen as model plant and the mentioned features were measured under more controlled conditions by using a soil-like substrate instead of soil. The bacterium was selected from a heavy metal-contaminated agricultural land, around the Esfahan province, Iran; which was cultivated with maize and was being irrigated with high metals containing sewage sludge. The bacterium was an efficient P solubilizing bacterium exhibiting high metal tolerance, selected from the soil samples screened for metal resistance and P solubilizing capability.

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http://dx.doi.org/10.1016/j.bcab.2016.07.005
1878-8181/

## 2. Materials and methods

### 2.1. Isolation and identification of the bacterium

Samples were collected from a cultivated field contaminated with high concentrations of heavy metals from irrigation by sewage. One gram of each soil sample was suspended in sterile tap water, of which serial dilutions were inoculated on Pikovskaya (PVK) agar medium supplemented with tricalcium phosphate (1 g/l) by streak culture method. The plates were incubated at 30 °C for 7 days. The P solubilizing bacteria were identified based on clear halo zones around their colonies. For isolation of metal resistant bacteria, the potential P solubilizing bacteria were cultured on agar plates supplemented with heavy metals, Hg, Pb, Cu and Cd at different concentrations (50–500 mg/l). Finally, the bacterium with the highest P solubilization capacity and resistance to metal was further characterized via morphological observation and biochemical test followed by 16S rDNA analysis to identify the strain.

### 2.2. Media optimization in terms of P solubilization and IAA production

P solubilization activity and IAA production were determined in the presence of chloride salts of Na (0.5%, 1.5%, 3.5%, 4.5% and 5.5%), Mg (0.1%, 0.3%, 0.5%, 0.7% and 1%), K (0.01%, 0.02%, 0.04%, 0.06% and 0.08%) and $NH_4$ (0.1%, 0.3%, 0.5%, 0.7% and 1%) in PVK liquid medium supplemented with tricalcium phosphate (1 g/l) as the sole P source and L-tryptophan (200 mg/l) as the precursor of IAA. These experiments were conducted in 250 ml flasks containing 100 ml the media inoculated with 1 ml fresh bacterial cells ($10^6$ cells /ml). The flasks were incubated at 30 °C for 7 days in a rotary shaker (100 rpm). The control flask remained uninoculated was incubated at the same salinities and conditions whose dissolved P content value was subtracted from the experimental values in calculation of soluble P mediated by the bacteria.

### 2.3. Phosphate solubilization and IAA production

To assay P solubilization activity, soluble orthophosphate released in medium was measured by the molybdenum blue method (Murphy and Riley, 1962). The growth rate of the isolate was indirectly assessed by a spectrophotometric based turbidity measurement as optical density (OD) at 600 nm. Indole-3-acetic acid (IAA) production was quantified spectrophotometrically at 535 nm in the bacterial cell filtrates using Salkowaski reagent (0.4 M $FeCl_3$ n 35% perchloric acid) as described by Chrastil (1976). The experimental groups were inoculated PVK media supplemented with 200 mg/l L-tryptophan that contained either of the heavy metals, i.e. $CuCl_2$ (500 mg/l), $CdCl_2$ (300 mg/l) or $PbCl_2$ (300 mg/l) as well as a control flask without metals. The heavy metals were chosen from a larger group of toxic metals including Hg, As and Co, to which the strain was highly sensitive. the concentrations of heavy metal chloride salts was attained by agar diffusion method (Saurav and Kannabiran, 2009), at which no clear halo zone was detectable around the wells.

### 2.4. Pot experiment

The pot experiment was conducted with maize, Zea mays, as a model crop under initial axenic condition. The main part of the soil-like substrate used for the experiment was made from saw dust powder and sand (1:1 w/w) that had been previously subjected to pretreatment processes before usage. It had been previously suspended in distilled water, boiled for an hour, washed three times with sterilized distilled water, oven-dried at 100 °C and finally grounded and passed through a 2 mm sieve. The substrate was supplemented with 5% by weight of individually pre-autoclaved $NH_4Cl$, $Ca_3PO_4$ and KCl in the ratio of 6:3:1.5 and was further supplemented with heavy metal chlorides of Pb, Cd and Cu (each 300 mg/kg substrate) and transferred to pots (1 kg per pot). For surface sterilization of the seeds, they were treated with ethanol 70% (v/v) for 30 s followed by 10% bleaching for 10 min and washed 5 times with sterilized distilled water. Thereafter, eight uniform maize seeds were sown in every pot, watered immediately afterwards and when required by distilled water. The soil-like substrate was inoculated with the bacterial suspension ($10^8$ cell/mg substrate) at weekly intervals for 40 days. The experimental groups included the pots that were subjected to heavy metal supplementation and/or bacterial inoculation and ones that were neither inoculated with bacteria nor amended with heavy metals. The pots were allowed to grow indoors under artificial light (6 h light/day) and at 30 °C.

### 2.5. Plant growth promotion and metal accumulation study

The probable beneficial effect of the bacteria on plant growth and their plausible detoxifying impact was assessed in maize. At the end of cultivation period, the crops were harvested carefully, washed firstly with tap water and then with distilled water, and their root and shoot length was recorded. In addition, total, root and shoot dry mass was determined based on weight after oven drying of plant at 70 °C for 10 h. Subsequently the levels of the heavy metals were analyzed in dried and milled root and shoot samples with inductively coupled plasma –mass spectrometry (Perkin Elmer Model Elan6000 DRC) after hot digestion with concentrated $HNO_3$. These parameters were calculated as a percentage of the corresponding control groups' values.

### 2.6. Bacterial count in soil-like substrate

The number of viable bacterial cells was estimated in the soil-like substrate around the plant roots. Five hundred milligram of the substrate near the roots was dissolved in 10 ml sterile phosphate buffered saline (PBS), pH 7.4 in a rotary shaker to detach bacterial cells from the substrate particles. The total number of viable bacteria was then estimated by the standard plate count method using colony forming units (SFU) on agar plates. Hence, the supernatant was serially diluted by a factor of ten with PBS, from which aliquots of 0.1 ml were streaked on a nutrient agar plate. After three days of incubation at room temperature, the colonies were counted on the plate with discrete colonies, and the total was multiplied by the dilution factor of the original supernatant.

### 2.7. Statistical analysis

The data were analyzed using Microsoft Excel Package (version 2007) and GraphPad Prism (version 5). One-way analysis of variance (ANOVA) and Newman–Keuls multiple comparisons ($p < 0.05$) were employed to evaluate differences among groups. Finally the total effect sizes, including Pearson's correlation and Hedges' g estimation was calculated to denote the magnitude of differences. The data were presented as mean $\pm$ standard error of eight replicates.

## 3. Results and discussion

On screening soil samples, there was a little chance to find bacterial cells capable of both tolerating metal toxicity and dissolving P even in an environment exposed to heavy metals for a long time. However, the soil seemed still appreciably rich in