INTRODUCTION

Heavy metal in the environment such as agriculture soil and water resources have revealed in many environmental problems (Oztürk, 2007). The increasing industrialization and global economy over the last century has led to dramatically elevated reaction of anthropogenic chemicals into the environment. Discharge of heavy metal by metal processing industries into the soil that in long-term promotes their accumulation (Costa and Duta, 2001). The plantation grown on such land is often contaminated, and their consumption is hazardous (Li et al., 2006).

Although immobilization and soil washing are frequently listed among the best demonstrated available technologies for remediation (He et al., 2005), their high cost is preventing developing countries to benefit from these applications. Alternatives such as phytoremediation, vegetative remediation, uses vegetation and associated microbiota, soil amendments and other agronomic techniques can prove it’s economical in developing countries (Helmisaari et al., 2007). Casuarina (common ironwood) studied vastly for its phytoremediation of petroleum hydrocarbons and heavy metals in soils (Sun et al., 2004). As Frankia, ecological interaction between nonendosymbiont bacteria and phytoremediation ability of Casuarina spp. is not fully understood. Further study in this area may lead to better prospect in application of Casuarina as a phytoremediation agent.

KEY WORDS: Heavy metal, rhizosphere, tolerance, Pseudomonas spp, Bacillus spp.

ABSTRACT

There is a lack in research related to association of nonendosymbiont bacteria with Casuarina spp. Plant Growth Promoting Rhizobacteria (PGPR) nonendosymbiont bacteria such as Pseudomonas sp. and Bacillus sp. are long associated with their properties of heavy metal accumulation and plant growth promoting activity. This study focuses on heavy metal tolerance and accumulation potential of nonendosymbiont PGPR isolated from rhizosphere of Casuarina equisetifolia. The plant growth promoting properties were studied by pot culture studies on fast growing Indian moth bean Vigna aconitifolia. In the pot culture study, three bacterial isolates were found to be increasing the root and shoot length by Pseudomonas sp. RS-1 (7.4 ± 0.64 and 21.7 ± 1.42), Bacillus spp. RS-2 (6.5 ± 0.93 and 21.2 ± 2.09), and Bacillus spp. RS-3 (6.4 ± 0.73, 19.1 ± 1.83), respectively. The maximum tolerance concentration of Pseudomonas spp. RS-1 shows 200 mg/L toward Cr (VI), Pb (II) 150 mg/L, whereas tolerance for Cd (II) and Zn (II) were 100 mg/L. Maximum % removal was shown by Bacillus spp. RS-3 towards Cr, Pb, and Zn (42.51%, 26.35%, and 26.65%, respectively), Pseudomonas spp. RS-1 toward Cd (36.52%). As Frankia, ecological interaction between nonendosymbiont bacteria and phytoremediation ability of Casuarina spp. is not fully understood. Further study in this area may lead to better prospect in application of Casuarina as a phytoremediation agent.
PGPR found in the rhizosphere of various crops have been found to assist in root colonization by rhizobia increasing the development of the plant and in suppressing soilborne plant pathogens (Parmar and Dadarwal, 2000; Jeffries et al., 2003). Metal contamination of soil has an important bearing on PGPR functions (Ali and Zulkifi, 2010). Of the various rhizospheric bacteria, Pseudomonas spp. and Bacillus spp. are aggressive colonizers of the rhizosphere of various crop plants (Schroth and Hancock, 1982). Metal homeostasis resistance in these bacteria is often maintained by sequestration, active efflux, reduced uptake, detoxification, and synthesis of binding protein (Jing et al., 2007). Pseudomonas spp. and Bacillus spp. are ubiquitous and associated with vast range of vegetation. They are known for mechanisms by which they were able to resist heavy metal and able to carrying out its PGPR function in soil containing high concentration of metals such as Cd, Al, Zn, and Cr (Abou-Shanab et al., 2008). This study focuses on heavy metal accumulation potential of nonendosymbiont bacteria along with their plant growth promoting potential.

MATERIALS AND METHODS

Sample Collection

A month old healthy Casuarina equisetifolia seedlings were grown in soil, which was treated with salts of chromium (Cr(VI)), lead (Pb(II)), cadmium (Cd(II)), and zinc (Zn(II)) at concentration of 100, 100, 50, and 50 mg/kg, respectively, as per the methodology followed by Stuczynski et al. (2003). After 1 year rhizosphere soil samples were collected from five different metals treated C. equisetifolia trees, respectively, for the study. The samples collected were about 250 g surrounding the root area at a depth of 10-15 cm in separate sterile 1 kg polyethylene bags. The five rhizosphere samples were uniformly mixed to get 1.25 kg of composite sample. From this 500 g of the soil was dried sieved and stored in a sterile polyethylene bag at 4°C for further studies (Bhat and Kaveriappa, 2009).

Isolation of Heavy Metal Tolerant Bacteria

Chromium, lead, cadmium, and zinc resistant bacterial strains were isolated from the rhizosphere soil samples using enrichment media supplemented with heavy metals. 100 ml nutrient broth (NB) was supplemented with Cr(VI), Pb(II), Cd(II), and Zn(II) by addition of K2Cr2O7, PbCl2, CdCl2, and ZnSO4 at 100 mg/L concentration and pH was adjusted to 6; the medium was then inoculated with 1 g of the rhizosphere soil under aseptic condition and incubated at 30°C in the rotary shaker incubator at 120 rpm for 3 days till the medium appeared turbid. The medium was serially diluted by standard spread plate technique onto agar medium supplemented with K2Cr2O7, PbCl2, CdCl2, and ZnSO4 each at 100 and 50 mg/L conc. The plates were incubated at 30 ± 2°C for 48 h. After 48 h incubation, larger identical colonies from each plate were isolated.

Seed Germination Bioassay

Seed germination bioassay was carried out for the growth promoting activity of isolates. A total of nine isolates were grown in nutrient medium agar plates at 30 ± 2°C for 24 h. The inoculants for treating Vigna aconitifolia seeds were prepared by suspending cells from agar plates in a standard NB as described earlier (Dey et al., 2004; Gerhardson et al., 1985; Pal et al., 1999). Four pregerminated seeds per boiling tube with three replications for each treatment were used and incubated at 28°C. The length of the each seedling was measured after 7 days and expressed in cm and compared with control on day 7 with sterile NB. A total of three isolates were found to enhance the root length significantly. The isolates were identified by morphological, physiological, and biochemical characteristics following Bergey’s Manual of Systematic Bacteriology (8th Edition).

Pot Trials

A total of three cultures were selected to evaluate their effects on the growth in pot trials. The pots containing soil (medium black and calcareous, pH 7.85, organic matter 2.16%, total nitrogen content 287 mg/kg, available phosphorus 200 mg/kg, and potassium exchangeable K 338 mg/kg data shown in Table 1. The sterile soil was used for the experiments. There were a total of four treatments, each having six replications. Each isolate of PGPR was grown overnight in Kings’ B broth and NB. The seeds for each treatment were soaked for an hour broth containing the suspension of the PGPR isolates, six to eight seeds (95% germination) were sown at a depth of 5 cm. After germination, five seedlings were maintained in each pot (Dey et al., 2004). Dry weight, shoot length, and root length were recorded at 15 days after sowing.

Heavy Metal Solutions

The heavy metal solutions of Cr(VI), Cd(II), Pb(II), and Zn(II) of 1000 mg/L concentration were used as stock solutions, slightly acidified with HNO3, 2-3 drops of concentrated HNO3, and were sterilized at 121°C for 15 min. These solutions, in various concentrations according to the metal tested, were kept at 25°C.
Maximum Tolerance Concentration (MTC)

To check MTC, to each plate of Mueller-Hinton agar medium, 100 μL of the appropriate metal salt solutions were added in four different wells (6 mm diameter). Plates were incubated at 37°C for 24 h to allow diffusion of the metal into the agar, it was supposed by that time, that a concentration gradient of the metal was formed. On each metal plate isolates was swabbed using 24 h broths of RS 1, RS 2, and RS 3. The plates were incubated at 37°C for 48 h, after incubation the plates were observed for the zone of inhibition to determine the tolerance (Hassen et al., 1998).

Heavy Metal Removal Assay

For metal removal studies, 100 ml of sterile media was prepared with 10 mg/L of Cr6+ in four different 250 ml conical flasks. Bacterial cultures RS 1, RS 2, and RS 3 were inoculated, respectively. This process was repeated for the other metals, namely, Cd2+, Zn2+, and Pb2+, respectively. The culture was incubated at 35°C in an incubator shaker at 120 rpm, and then, aliquots were taken at regular intervals of 2, 4, 6, 8, 10, and 12 and it were centrifuged at 8000 rpm for 15 min. The residual heavy metal in the supernatant was estimated using Atomic Absorption Spectrophotometer (AAS Varian AA240).

The amount of heavy metal removed by the cell was calculated by the formulae: % Removal = (C0 − Cf/C0) × 100

Where, C0 = Initial metal concentration (mg/L), Cf = Final metal concentration (mg/L) (Velásquez and Dussan, 2009; Ozdemir et al., 2009).

RESULTS

Isolation of Heavy Metal Tolerant Bacteria

A total of nine types of heavy metal bacteria colonies were isolated, which were able to grow on nutrient agar medium supplemented with K2Cr2O7, PbCl2, CdCl2, and ZnSO4 each at 100 and 50 mg/L concentrated.

Seed Germination Bioassay

In germinating seed bioassay, a total of three isolates were found to enhance the length of the seedlings of Vaconitifolia significantly. The three isolates were identified based morphological, physiological, and biochemical characteristics and found belonging to the genera Pseudomonas spp. and Bacillus spp. and further referred as Pseudomonas spp. RS-1, Bacillus spp. RS-2, and Bacillus spp. RS-3. In comparison with the control 5.30 ± 0.76 cm, Pseudomonas spp. RS-1, Bacillus sp. RS-2 and Bacillus sp. RS-3 were found increase the seed length to 8.08 ± 0.6, 7.29 ± 0.39, and 6.37 ± 0.48, respectively. The results show that all the three isolates enhance the length of the seedling, in which Pseudomonas spp. RS-1 shows more significant in promotion of growth (Table 2).

Pot Trials

In pot culture study, three isolates when inoculated with the soil the plant showed a significant growth in terms of root and shoot length when compared to the control. The root and shoot length for control are 4.5 ± 0.75 and 16.2 ± 1.02 for pots with PGPR are Pseudomonas spp. RS-1 (7.4 ± 0.64 and 21.7 ± 1.42), Bacillus spp. RS-2 (6.5 ± 0.93 and 21.2 ± 2.09), and Bacillus sp. RS-3 were found increase the seed length to 8.08 ± 0.6, 7.29 ± 0.39, and 6.37 ± 0.48, respectively. The results show that all the three isolates enhance the length of the seedling, in which Pseudomonas spp. RS-1 shows more significant in promotion of growth (Table 2).

MTC

The heavy metal tolerance toward Cr (VI), Pb (II), Cd (II), and Zn (II) were determined by well diffusion method. The MTC of Pseudomonas sp. RS-1 shows maximum of 200 mg/L toward Cr (VI), Pb (II) 150 mg/L whereas tolerance for Cd (II), and Zn (II) were 100 mg/L (Figure 2).
Kumar, et al.: Heavy metal tolerant nonendosymbiont PGPR’s; Pseudomonas spp. and Bacillus spp.

Table 2: Germinating seed bioassay

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Growth of the seedlings (Length (cm))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.30±0.76</td>
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<tr>
<td>RS 1</td>
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Length of the seedling plant is reported as mean±standard deviation, (n=12). RS-1: Pseudomonas spp., RS-2: Bacillus spp., RS-3: Bacillus spp.

Table 3: Pot culture assay

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<thead>
<tr>
<th>Test organisms</th>
<th>Growth of plant (cm)</th>
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<tbody>
<tr>
<td></td>
<td>Total length Root shoot</td>
</tr>
<tr>
<td>Control</td>
<td>20.8±1.64 4.5±0.75 16.2±1.02</td>
</tr>
<tr>
<td>RS 1</td>
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<tr>
<td>RS 2</td>
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Length of the plant is reported as mean±standard deviation (n=24)

(Figure 1), whereas maximum % removal was shown by RS 3 toward Cr, Pb, and Zn (42.51%, 26.35%, and 26.65%, respectively) and RS1 toward Cd (36.52%) shown in Figure 3.

**DISCUSSION**

Plants are sessile thus releasing an array of chemical signals to interact with other organisms. The rhizosphere interaction is not solely driven by root but are highly integrated and influenced by residing organisms and local edaphic factor (Badri et al., 2009). Casuarina crop has been recently exploited for it bioremediation properties in heavy metal contaminated sites (Sun et al., 2004). Our study highlights, the importance of PGPR’s as nonendosymbiont microbes that play an efficient role bioremediation of heavy metal. Previous studies done on Casuarina are limited to the endosymbiont, especially Frankia in their ability to accumulate metallic ions (Gollop et al., 2011).

PGPR are rhizosphere bacteria that exert a positive influence on the plant growth especially under stress condition (Kloepper et al., 1980); they can influence plant growth directly either by providing specific compounds that help plant growth or by providing facilitating uptake of nutrients from the soil and indirectly by suppressing the phytopathogenic organisms in the rhizosphere (Glick, 1985). Not all isolates which are found tolerant to heavy metals and able to influence the plant growth only three out of nine were potential growth promoters. Pseudomonas spp. RS-1, Bacillus spp. RS-2, and Bacillus spp. RS-3 in this study showed plant growth promotion in corresponding genera

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(a number other of PGPR, e.g., Pseudomonas putida GR12-2 (Jacobson et al., 1994), Bacillus subtilis A13 (Turner and Backman, 1991), B. licheniformis CECT5106 (Probanza et al., 2002), B. pumilus CECT5105 (Probanza et al., 2002), and others such as Pseudomonas fluorescens PF-5, P. fluorescens 2-79, and P. fluorescens CHA0 (Wang et al., 2000) have been identified to have similar properties. The high resistance of isolates toward Cr, Pb, Cd, and Zn due to the adaptation of microbes to the toxicity of high concentration of these metals in the rhizosphere of the plant (Gadd and Griffiths, 1997) by strategies such as oxidative stress and multiple efflux pump. The metal uptake properties of the isolates explained by Bae et al. (2001) as microbes synthesize compounds which bind to metals and reduce toxicity, compounds which accumulate heavy metal by metal binding peptides (Schroth and Hancock, 1982).
In this study, it was found even under stressed condition, i.e. in the presence of toxic heavy metal, it was observed that all three organisms showed rapid uptake within few hours. The efficiency of absorption slowly increased as the bacterial growth increased, later it was found that the rate of removal was proportional to concentration of the cells (Chatterjee et al., 2011). *Pseudomonas* spp. and *Bacillus* spp. complex genomic machineries by are quick to response to the environmental stress (Nelson et al., 2002). Although there are different mechanisms for coping different toxic heavy metals (Canovas et al., 2001), this study shows they respond with equal efficiency without disturbance in its normal activity PGPR.

In this study, we isolated PGPR from *Casuarina* (common ironwood) and demonstrated their heavy metal removal property. This suggests not only endosymbiotic bacteria such as *Frankia*, the nonendosymbiont bacteria can also help plant to better respond metal contamination stress, further the plant growth-promoting rhizobacteria can be used in the development of phytoremediation strategies to treat plants for better yield and stabilize and remediate metal-contaminated soils. This study must be carried out in field condition to validate the efficacy of these PGPR’s in the environment, in this context, the optimization of PGPR inoculums must be tested in the presence of diverse environmental factors. In addition, to maintain the maximum viability and activities of PGPR, an appropriate carrier should be developed.

**ACKNOWLEDGMENT**

The authors are thankful to VIT University for providing laboratory support to carry out this work. The authors are also thankful to Dr. R Vidya for her guidance and support in the study.

**REFERENCES**


Canovas D, Cases I, de Lorenzo V. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Environ Microbiol 2001;5:1242-56.


Costa AC, Dutta FP. Bioaccumulation of copper, zinc, cadmium, and lead by *Bacillus* sp., *Bacillus cereus*, *Bacillus sphaericus*, and *Bacillus subtilis*. Braz J Microbiol 2011;2:876-87.


Gollop N, Shores M, Guetsky R, Rindner M, Zakin V, Bernstein N. Differential response of *Frankia* strains to

![Figure 3](image-url)