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Influence of Residual Pesticide on Plant Growth Promoting Bacteria Isolated from Agriculture Field

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Abstract

Continuous use of pesticides in the agriculture field invites many problems including loss of soil fertility by altering biogeochemical cycle of soil. Presence of residual pesticide in the soil influence negatively the soil microbial community shift thereby enriches populations which are able to survive in the altered microenvironment. Hence, present study was planned to evaluate the impact of residual pesticide, heavy metals and other components on the plant growth promoting bacteria and soil fertility. A total of 20 isolates were obtained from five different agriculture soils of cassava fields (S-1, S-2, S-3, S-4 and S-5). All isolates were tested for their resistance against different heavy metal (Mg⁺, Mn⁺, Pb⁺, Zn⁺ and CuSO₄⁺) concentrations (100–1000 mg L⁻¹) and salinity (1–10%). Antibiotic resistance was determined by using different antibiotic discs [Gentamicin (30 μg), Ampicillin (10 μg), Erythromycin (10 μg), Kanamycin (5 μg), Tetracyclin (10 μg), Vancomycin (25 μg), and Chloramphenicol (10 μg)]. Majority percent wise determination of Plant Growth Promoting Bacteria (PGPR) resistant isolates were found plant growth promoting properties. Hence, the present study is an insight to show impact of pesticides exposed soil among multi-resistant PGPR population in the agriculture soil.

Keywords: Pesticides; Exposed soil; PGPR; Multidrug resistance

Introduction

Abundant use of the pesticides particularly cholorianted pesticides, carbamates, cadycylic acid, paris green etc and other agricultural practices that release heavy metals into the agricultural soil. The accumulation of these metal ions over a period results in the toxicity through biomagnification and thereby causes malfunctioning of organs, chronic syndromes, and carcinogenic effects. It also affects soil fertility, diversity of plants and microbes widely [1,2]. Metal polluted field exhibits poor water holding capacity and high evaporation rates, which affect the plant growth. In order to overcome this problem, phytoremediation is considered as a promising energy and cost-effective way to rehabilitate metal contaminated environments in opposition to conventional remediation technologies [3]. In this case, Plant Growth Promoting Rhizobacteria (PGPR) which has the capability of adsorbing heavy metal ions and converting them into eco-friendly alternative for chemical delators are exploited [4]. Thus, the applications of heavy metal tolerant bacteria have both the advantages of phytoremediation and also the plant growth enhancement [5,6]. There are the wide ranges of stress tolerant bacteria that can confer adaptive benefit to their host plant against various stress, including heavy metals [7].

Heavy metal resistant bacteria from rhizosphere can promote plant survival and production of plant growth promoting (PGP) substances as well as the biocontrol of plant diseases. They also alleviate phytotoxic effects by producing siderophores, organic acids, biosurfactants and extra cellular polymeric substances [8]. Recently, many metal resistant bacteria have been characterized that were able to promote plant growth phytoremediation process in metal polluted soils [9,10]. Many genera of microbes like Bacillus, Enterobacter, Escherichia, Pseudomonas and also some yeasts and molds help in bioremediation of metal and chromium contaminated soil and water by bio-absorption and bioaccumulation of chromium [11]. The heavy metal removal by the Gram positive bacteria belonging to the genus Bacillus, Arthrobacter and Corynebacterium as well as Gram negative bacteria such as Pseudomonas, Alcaligenes, Ralstonia and Burkholderia was attributed to the cellular growth of these organisms [12]. Hence the current study aimed to find the multi-resistance capacity of plant growth promoting bacteria from cassava field soil and to know the symbiotic relationship with plant and microbes.

Material and Methods

Sample Collection

The samples were collected from five different cassava agricultural fields (S-1, S-2, S-3, S-4 and S-5) of Salem (11.80°N 77.80°E) and Namakkal (11.08°N 78.17°E) Districts, Tamilnadu, India. The samples were collected in sterile bags and transported to the laboratory.

Isolation of Multitolerant Bacteria

The bacterial isolates were obtained from agriculture field which were checked for their tolerance against heavy metal ions by dilution method. For this purpose, freshly prepared nutrient agar plate with various heavy metal salts like Mg⁺, Mn⁺, Pb⁺, Zn⁺ and CuSO₄ (3 mg/ml) were inoculated with log phase cultures and incubated at 37°C for 24 hours. The samples were collected from S1, S2, S3, S4 and S5. The bacterial colonies that are heavy metal tolerant were isolated and the distribution of such bacteria was identified.

Bacteria Stress Tolerance Test

Bacterial metal tolerance levels were assessed in nutrient broth containing different concentrations of heavy metals (Mg⁺, Mn⁺, Pb⁺, Zn⁺ and CuSO₄; 0.3–2 mg/ml) and incubated at 37°C for 24 hours. The high metal concentration allowing bacterial growth was identified as the maximum resistance level.

Biochemical characterization of isolates including cytochrome oxidase, oxidative fermentation, catalase and motility tests were conducted and utilisation of other carbon and substrate tests were conducted for identification of isolates [13].

Genetic Identification and Phylogenetic Analysis

Identification of isolated bacterial strain was done by 16S rRNA gene sequencing. Genomic DNA of bacterial isolate was amplified with universal primer 27-F (5‘- AGAGTTTGATCMTGGCTCAG-3’) and 1494-R (5‘-TACGCTACCTTGTTACGAC-3’) in a 25 µl reaction

Keywords: Pesticides; Exposed soil; PGPR; Multidrug resistance
mixture containing 10X buffer (with 2.5 mMol l⁻¹ MgCl₂, 2.5 µl, 20 pmol forward and reverse primer each 2 µl, dNTP mixture (2.5 mM) 3.0 µl, 0.5 µl of Taq DNA polymerase (2.5 U), nuclease free water and 50 ng of DNA template. The thermocycling conditions consisted of a denaturation step at 94°C for 3 min, 34 amplification cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for one min and final polymerization for 8 min with thermal cycler (Eppendorf AG 22331). Electrophoresis was continued for 30 min at 100 V (Taron electrophoresis unit). The size of fragments was determined by comparison with 1 kb marker (Fermentas) 1500 bp. PCR products were visualized on 1.0% agarose gel with gel documentation (Medicare H6ZO11). The purified PCR products were then sequenced in Eurofins Pvt Ltd, Bangalore, India. Nucleotide sequences obtained were analyzed using Blast-n (National Center for Biotechnology Information Databases). Phylogenetic and molecular evolutionary analysis was done by using software MEGA 7.0 [14].

3. Determination of Optimum Growth Temperature

The optimum temperature for bacterial growth was determined by growing bacteria at various temperatures (4°C, 28°C, 37°C and 40°C). Isolates were grown in TYEG medium for 24 hrs and the growth was observed at 600 nm using Spectrophotometer.

4. Salt Tolerance

The bacteria were further analyzed for their ability to resist different concentrations of salt (5–9%). TYEG medium impregnated with different salt concentrations was inoculated with different isolates and incubated at 37°C for 24 hours. The growth was analyzed using the spectrophotometer at 600 nm.

5. Antibiotic Sensitivity Test

Isolated bacterial strain was tested for its resistance against standard antibiotics (Himedia) [gentamicin (30 µg), ampicillin (10 µg), erythromycin (10 µg), kanamycin (5 µg), tetracyclin (10 µg), vancomycin (25 µg), and chloramphenicol (10 µg)] by the antibiotic sensitivity assay. Briefly, the bacterial culture was swabbed onto NA media plates. The standard antibiotic disc (6 mm) was placed over the media surface and the plates were incubated at 37°C for 24 h. The experiment was done in triplicate. The results were interpreted on the basis of the diameter of inhibition zone using the zone size interpretative chart supplied by the manufacturer (Himedia, India).

6. Determination of PGPR Characteristics

Phosphate solubilizing activity: The bacteria were inoculated onto Pikovskaya’s agar medium with 0.5% of tricalcium phosphate as an inorganic phosphate source. This plate was incubated at 30°C for 72 hrs and a clear zone around the bacterial colony indicated the capacity to solubilize the phosphate content by the bacteria.

Siderophore production: Assay for siderophore production of the isolate was carried out by spot inoculating test organism (1 µl) on chrome azurole S agar plates and in cubing at 30°C for 4–5 days in dark. Appearance of orange halo zone around the colony was considered as positive for siderophore production [15].

ACC deaminase activity: For ACC deaminase activity, isolated strain was grown in tryptic soy broth up to late log phase at 30°C in an orbital shaker at 150 rpm for 24 hrs. Then the liquid broth was centrifuged at 10,000 rpm, the cell pellets washed with 0.1M Tris - HCl (pH 7.6) and finally suspended in DF (Dworlanski and Foster) minimal salt medium with 3 mM ACC as sole nitrogen source. The enzyme activity was determined by measuring the amount of α-ketobutyrate generated by enzymatic hydrolysis of ACC [16]. This keta-tobutyrate was determined at 540 nm spectrophotometrically.

7. Statistical Analysis

Data was statistically analyzed using one way ANOVA model [17] to determine the comparison of the mean values of CFU/g of metal tolerant bacteria from S1, S2, S3, S4 and S5.

8. Results

Sample Collection and Isolation of Bacteria

The soil samples that were collected from different locations of cassava agricultural and non-agricultural fields were occupied by heavy metal load which caused a great damage to the plant growth. The maximum bacterial isolates, 6.7×10⁶ CFU/g soil were obtained from S-3 soil sample that was collected from an agriculture field (Table 1).

Distribution of Multi-Resistant Bacteria

Distribution pattern of multi resistant bacteria from different fields indicated that agricultural fields contained higher number of resistant isolates as compared to waste land. Nearly, 14 bacterial isolate which are tolerant to multi were obtained from agricultural field soil samples (S-1, S-2 and S-3) (Table 2 and Figure 1).

Metal Stress Tolerance

All strains showed tolerance to some specific metals whereas Pu11 was resistant to all heavy metals (Mg²⁺, Mn²⁺, Pb²⁺, Zn²⁺ and Cu²⁺) observed via spectrophotometer using standards as Magnesium (400 mg/L), Manganese (500 mg/L), Lead (300 mg/L), Zinc (300 mg/L) and Copper Sulphate (400 gm/L) (Table 3).

Bacterial Identification

Preliminary phenotypic characterization showed that the heavy metal resistant isolate was Gram negative straight, motile rod, capable of glucose, L-arabinose, mannose, and ribose fermentation and citrate positive. The selected strain was capable of producing fluorescent pigment on nutrient agar. The comparison of BLAST search of 16S rRNA gene sequences of strain with 16S rRNA gene sequences of NCBI Gene Bank database showed the highest identity to Pseudomonas sp. The sequence has been submitted in Gen Bank under the accession numbers KP299294.1 (Figure 2).

Physicochemical Tests

Pseudomonas sp. showed optimum growth at 28°C and was capable of resisting salt concentration up to 6%. Pseudomonas was resistant to the antibiotics such as gentamycin, ampicillin (3 mm),

### Table 1: Enumeration of viable count from different soil samples. (Total count value is mean of 10 samples).

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Total Viable Number (CFU/g of soil)</th>
<th>Multi resistance populations (mg L⁻¹)</th>
<th>Pseudomonas sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhizosphere</td>
<td>Non rhizosphere</td>
<td>Waste land</td>
</tr>
<tr>
<td>S-1</td>
<td>6.0 x 10⁵</td>
<td>4.0 x 10⁴</td>
<td>3.0 x 10⁵</td>
</tr>
<tr>
<td>S-2</td>
<td>5.3 x 10⁵</td>
<td>3.0 x 10⁴</td>
<td>3.0 x 10⁵</td>
</tr>
<tr>
<td>S-3</td>
<td>6.7 x 10⁵</td>
<td>3.3 x 10⁴</td>
<td>2.7 x 10⁵</td>
</tr>
<tr>
<td>S-4</td>
<td>5.3 x 10⁵</td>
<td>4.0 x 10⁴</td>
<td>2.7 x 10⁵</td>
</tr>
<tr>
<td>S-5</td>
<td>5.0 x 10⁵</td>
<td>3.0 x 10⁴</td>
<td>3.3 x 10⁵</td>
</tr>
<tr>
<td>S. No</td>
<td>Agriculture Soil</td>
<td>Non-Rhizosphere soil</td>
<td>Waste Land</td>
</tr>
<tr>
<td>-------</td>
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<td>------------</td>
</tr>
<tr>
<td>1</td>
<td>Escherichia coli (PU-01)</td>
<td>Bacillus sphericus (PU-12)</td>
<td>Enterobacter agglomerans (PU-15)</td>
</tr>
<tr>
<td>2</td>
<td>Bacillus cereus (PU-02)</td>
<td>Bacillus subtilis (PU-13)</td>
<td>Elocridiumbutyricum (PU-16)</td>
</tr>
<tr>
<td>3</td>
<td>Bacillus pasteur (PU-03)</td>
<td>Micrococi (PU-14)</td>
<td>Pseudotogamvien (PU-17)</td>
</tr>
<tr>
<td>4</td>
<td>Bacillus azotoformens (PU-04)</td>
<td></td>
<td>Quercus petraea (PU-18)</td>
</tr>
<tr>
<td>5</td>
<td>Bacillus mycoides (PU-05)</td>
<td></td>
<td>Bacillus subtilis (PU-19)</td>
</tr>
<tr>
<td>6</td>
<td>Klebsiella (PU-06)</td>
<td></td>
<td>Escherichia coli (PU-20)</td>
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<tr>
<td>7</td>
<td>Staphylococcus arues (PU-07)</td>
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<tr>
<td>8</td>
<td>Vibrio (PU-08)</td>
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<tr>
<td>9</td>
<td>Bacillus polymyx (PU-09)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Bacillus sphericus (PU-10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Pseudomonas sp (PU-11)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Distribution of the bacterial isolates in agricultural soil and waste land.

Mg$^{2+}$ 400
Mn$^{2+}$ 500
Pb$^{2+}$ 300
Zn$^{2+}$ 300
CuSO$_4$ 400
Salt tolerance (%) 6.0
Temperature (°C) 5–40°C
Antibiotic Resistance (mm)  
Gentamicin (30 μg) 0 (R)  
Ampicillin (10 μg) 3 (R)  
Erythomycin (10 μg) 5 (R)  
Kanamycin (5 μg) 4 (R)  
Tetracyclin (10 μg) 0 (R)  
Vancomycin (25 μg) 0 (R)  
Chloramphenicol (10 μg) 4 (R)  
Siderophore (CAS: mm) 2.0 ± 0.6  
PO$_4$ solubilization (mg L$^{-1}$) 0.6 ± 0  
ACC deaminase production 20.0 ± 3.0

Table 3: PGPR activity multi resistant bacteria. (*R: Resistant (< 10 mm); S: Susceptible (> 15 mm); ACC: 1-aminocyclopropane-1-carboxylate; α-KB: α-ketobutyrate; P: Phosphate; CAS: Chrome Azurol S; −: negative).
kanamycin (4 mm), tetracycline, vancomycin and chloramphenicol (4 mm) whereas other bacteria were sensitive to some of these antibiotics.

**PGPR Activity**

**Phosphate solubilization:** Phosphate solubilization was positive for the *Pseudomonas* sp. It utilizes the inorganic phosphate source by 0.6 mg/L which indicates the phosphate solubilization capacity of the bacterial strains.

**Siderophore production:** Siderophore production was positive for the *Pseudomonas* sp. It is indicated by the orange halo zone around the culture of about 2.0 ± 0.6 mm zone which can be identified by the dye chrome azurul S.

**ACC Deaminase test:** ACC deaminase activity was measured the production of α-ketobutyrate by spectrophotometer which is about 20.0 mg/h protein and was positive for *Pseudomonas* sp.

**Discussion**

The pollution of heavy metal is a major worldwide environmental concern that motivated researchers to develop novel biological tools as an alternative to physical and chemical methods of environmental cleanup. In this study, number of bacterial isolates were isolated from this soils which are tolerant to heavy metals. Microorganisms that are able to metabolize heavy metals would be helpful in the waste water treatment [18].

These organisms which have the beneficial traits increases the productivity of agricultural crops growing under stress conditions and which is used as a strong biotechnological alternative of chemical fertilizer and pesticides that have a negative effect on the environment [19]. Stress conditions generated by salinity, drought, water logging, heavy metals and pathogenicity, the endogenous production of toxins which adversely affects the root growth and consequently the plant as a whole.

A wide range of stress tolerant bacteria can confer adaptive benefit to their host plant against various stress, including heavy metals, drought, salt and phytopathogens [20–22] etc., making the entire phytoremediation process much more efficient. In addition, certain bacteria can potentially alleviate phytotoxic effects by producing siderophores, organic acids, biosurfactants and extracellular polymeric substances [8,9]. Therefore, such microorganisms with a biostatic stress resistant and PGP activities are of practical importance for efficient phytoremediation process. Recently, we have characterized several metal resistant bacteria that were able to promote plant growth and phytoremediation process in metal polluted soils [9,10]. Microbes having bio-control ability to generate induce systemic resistance (ISR) in several plant species thereby protecting plants against various diseases [23]. PGP able to produce IAA have a significant advantage to enhance root growth and development which helps in nutrient uptake [24]. Isolated plant growth promoting rhizobacteria (PGPR) capable of promoting plant growth by colonizing the plant root [25]. One possible explanation of such improvement of antibiotic resistance is that the presence of heavy metals enhanced the enrichment and growth of indigenous bacteria in the microbial community, which are already bearing antibiotic resistance genes; another possibility is that the resistance in bacteria which is sensitive to antibiotic scold be induced due to the co-existence of heavy metals and antibiotics in the environment. Some investigations have demonstrated the positive correlation between the abundance of antibiotic resistance genes and the elevated concentrations of antibiotic and heavy metals in environments [26,27]. As a matter of fact, the rhizobacteria secretions, such as phosphate solubilization, IAA, and siderophores, were evidenced to be involved in the increasing bioavailability and facilitating root absorption of heavy metals [28]. Rhizosphere bacteria have an exceptional ability to promote the growth of the host plant by various mechanisms such as nitrogen fixation, production of siderophore, phosphate solubilization and ACC deaminase activity pathway responsible for a plant growth promotion bioremediation properties adopting depend on condition and exposed [29,30].

Previously, Glick BR [31] also reported that the decrease in stress-induced ethylene production in *Pisum sativum* after inoculation with *Pseudomonas* spp. containing ACC deaminase could enhance plant growth under DS conditions. Strain TR1 and Ph3R3 exhibited high tolerance to extreme temperature. According to previous reports IAA and ACCCD could work synergistically and promote plant growth, especially root elongation [32,33].

**Conclusions**

These findings suggest that the agriculture soils are pesticide exposed developed multi-resistant bacteria with heavy metals, salinity and antibiotic. This study has taken many risk assessment of modern agricultural practices that have spread worldwide which adversely affects biogeoecycle and altering genome metabolic pathway in the soil microbes. These may increase multi-resistance properties among soil microbes, which might occur during many steps in the food chain and ecological cycle, may reduce the susceptibility of the cultures to frequently used pesticides. Many countries that continuously using pesticides have acquired high multi-resistance (heavy metal, salinity and antibiotic) accumulation the agricultural fields.

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**Conflict of interest**

Authors declared that they have no conflict of interest.

**References**


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