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Characterization of a phosphate solubilizing and pesticide tolerant endophytic bacterium (*Pseudomonas* sp.) isolated from apple orchards of Himachal Pradesh

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ABSTRACT

Jammu-Kashmir, Himachal Pradesh and Uttarakhand are the core areas of apple production in India. Synthetic chemicals, fertilizers, and pesticides are used for improving the yield in apple orchards. Overuse of phosphate fertilizers, untreated/ treated wastewater, sewage sludge, composts, etc leads to serious conditions in apple orchards. In the present study, enrichment culture technique was used to isolate and screen the pesticide tolerant bacterial strain from apple roots. A bacterial culture, identified as *Pseudomonas* sp., was obtained to possess phosphate solubilizing and pesticide tolerance properties. It tolerated Ridomil 80%, Meera 71, carbendazim and sulphur 50% up to 1000 µg/ml. The bacterium also showed tolerance to streptomycin up to 50 µg/ml.

Keywords: Endophytic bacterium; *Pseudomonas*; apple roots; phosphate solubilization; pesticide tolerance; Himachal Pradesh.

1. INTRODUCTION

Apple (*Malus domestica* Borkh.) is cultivated worldwide and is the most widely grown species in the genus *Malus* family *(Rosaseae)*. They are also packed with vitamins and minerals that promote healthy bones, teeth and skin. Apples are loaded with vitamin C that boosts immunity, keeping any ailment at bay (Simon *et al*., 2020). High in pectin fibers, apples boost metabolic levels, help in improving heart and regulate body's blood sugar levels by regulating the release of sugar (Sharma *et al*., 2014). The Indian Himalayan region (IHR) is characterized by the presence of extreme environments and thus provides unique opportunity to study rhizosphere microorganisms (Khatri *et al*., 2019). Climate changes as well as extreme conditions of Indian Himalaya are the key factors for changes in the soil ecosystem (Trivedi *et al*., 2012). Organic and biotechnological significance of microbial communities are sturdy admirer of soil ecosystems due to variation in climatic conditions as well as extreme environment of mountains (Rinu *et al.,* 2012; Pandey & Yarzabal, 2019). The geographies of soils vary in the mountain concerning the altitudinal gradient and the climatic conditions; these multiple factors lead to a challenge to study in Upper Himalayan Region (Massaccesi *et al*., 2015).

The use of synthetic chemicals has been leading to complicated link between soil and microbiota (Thakur & Pathania, 2020). Excess use of synthetic chemicals decreased crop quality, soil health and increased health risk and also leaded to produce bad quality fruits and crops (Mwakalapa *et al*. 2018). Microorganisms can transform various toxic hazardous wastes from various types of industries (such as fabric industries, pharmaceutical industries etc.) into nontoxic ones (Dangi *et al*. 2018).

The experiments planned under present study generated the basic knowledge on the soil-microbial interactions and pesticide tolerance potential of rhizospheric microorganisms. From literature, it is clear that bio-inoculants can improve remediation technology through increasing soil availability of metal-based pesticides and can promote growth. Data on the applications and role of bio-inoculants from extreme environmental conditions (dry cold zone) of Indian Himalayan region for soil and root availability of metal-based pesticides and their accumulation in crops are still in scare. Therefore, the present study aims to isolate and characterize bacteria from the apple rhizosphere with a view to raise the potential strains in form of inoculant with particular reference to plant growth promotion and pesticide resistance.

2. METHODS

The present study was carried out in apple orchards of Palchan (Kullu- Manali) Lat N32°18.624' Long E077°10.369' 2400 m amsl, Himachal Pradesh, India. The site was selected after conducting the survey with local farmers as well as in consultation with subject experts of Kullu Valley. The site came under the dry cold zone of Kullu-Manali, Himachal Pradesh.

Samples of root were collected from the selected apple trees. Samples of root were collected in triplicates from selected apple orchards. At each site, a monolith of 10 cm×10 cm×20 cm size was obtained by digging out, and fine root samples were taken. The samples were stored in the refrigerator at a temperature of 4 ^{o}C for general inumeration of microflora.

The root samples of the apple plants were taken and processed separately. Healthy and undamaged fine roots were collected from root region of the apple plant. Collected roots were crushed using a mortar pestle. These were than washed under running water and dried followed by surface sterilization. Surface sterilization was done first washing in 1% soap solution for 5 min. Subsequently these roots were treated with 0.1 % mercuric chloride for surface sterilization.

The samples of roots were cut with sterilized blade into small pieces and macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. Tissue extract were then prepared for tenfold dilution in sterile saline. Serial dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were prepared from this extract. For inoculations 0.1 mL of the aliquot was used on a Nutrient Agar medium. The inoculations were done in triplicates separately for roots extract. These plates were then incubated at 37 ^{o}C. Observations were taken after 48 to 72 h. Bacterial colonies were differentiated on the basis of morphological colony characters. Bacterial isolates were picked from plates and purified by streaking techniques and incubated at 37 ^{o}C. The isolation process repeated till monocultures were obtained for further experimentations. Pseudomonas Isolation Agar was used for isolation of *Pseudomonas* sp.

The ability to solve phosphate was tested on a Pikovskaya medium. Potential bacterial isolate was checked for phosphate solubilization (one of the plant growth promoting activity) on prepared Pikovskaya's (1948) medium agar plate. The appropriate medium was autoclaved by adding 0.3 g/mL bacteriological agar at 121°C at a pressure of 15 lbs for 15 min. After solidifying, the agar plates were spot inoculated with a 24 h broth culture in triplicates using sterilized stainless-steel loop and incubated at 30 °C for 5-7 days. After incubation at appropriate temperature the halo and colony diameters were measured. Clear zone development around the spot after incubation was an index of phosphate solubilization.

The pesticide tolerance potential of selected bacterial isolate was determined using maximum tolerable concentration (MTC) approach. Selected bacterial strain was tested to determine the maximum tolerable concentrations (MTCs) of the selected pesticides by a poison food technique method. Five pesticides were selected for the detailed study, i.e., ridomil 80% (pesticide basically used for root rot disease of apple), carbandazim (used for the control of a wide range of fungal diseases such as mold, spot, mildew, scorch, rot and blight in a variety of crops), Meera 71 (ammonium salt of glyphosate 71% and used as weedicide), streptomycin (antibiotic used as fungicide for plant disease management), and sulphur 50% (micronutrient and used to repel both chiggers and mites). Selected chemical fertilizers were treated at different concentration against selected bacterial isolates to determine the tolerance potential of that rhizospheric microbial isolate.

Production of ammonia was estimated in the selected bacteria. Selected bacterial isolate was grown in peptone agar slant tubes. Tubes were incubated at 30 ^{o}C for 4 days. After 4 days, 1 mL of Neissler's reagent was added to each tube. The presence of faint yellow colour (+) indicated small amount of ammonia and deep yellow (++) indicated good amount of ammonia production.

The Citrimide Agar was prepared and streaked slant back and forth with inoculum picked from the center of well-isolated colony. The agar was incubates aerobically at 35-37 °C for up to 7 days. The growth and pigment were observed. If no pigment was visible, The growth was examined under UV light for the presence of fluorescein. If negative for pigment at 24 h, the agar was then incubated for another days at 25°C in the dark to enhance the pigment production.

Cultural characteristics including growth, color, shape, and colony appearance were examined. Gram's staining was performed to identify the gram + and – bacterial strain.

3. RESULTS AND DISCUSSION

3.1 Phosphate solubilization (Beneficial characterization of isolated endophytic bacterium

The results of physiological parameters of selected bacterial isolate showed that the strain was solubilizing phosphate as a clear zone of inhibition has seen in the pikovaskya's agar. The results are shown in Figure 1 and Table 1. A higher altitudinal soil ecosystem has been successfully colonized by cold adapted microorganisms that were able to thrive, better survive and even maintain metabolic activity at sub-zero temperatures due to low temperature and extreme enzymes production (Rincon-Molina *et al*., 2019; Rinu *et al*., 2012; Pandey & Yarzabal, 2019). Microorganism from higher altitude were isolated by no of researchers and their finding also showed that rhizospheric ratio of microorganisms was found with their outstanding characteristics (Simon *et al*., 2020; Lemanceau *et al*., 2017; Trivedi *et al*., 2012). Several researchers found that rhizospheric as well as endophytic bacteria were able to colonize bacterial root region directly or indirectly which in turns promote growth, control various biotic and abiotic stresses such as heavy metals, pesticides and other stresses (Guerrero-Zuniga *et al*., 2019; Singh *et al*., 2020).

Table 1. Physiological and morphological parameters of the selected bacterial isolates.

Physiological Characteristics			Morphological Characteristics		
S.No	**Parameters**	**Observation**	**S.No**	**Parameters**	**Observation**
1	Phosphate Solubilization	+ve	1	Growth on NAM	Off white
2	Ammonia Test	+ve	2	Growth on King's medium	Greenish
3	Urease Production	-ve	3	Color of pigment	Green
4	Citrimide Test	+ve	4	Shape	Curve rod
-	-	-	5	Colony appearance	Circular, smooth
-	-	-	6	Gram's stain	Gram-negative rods

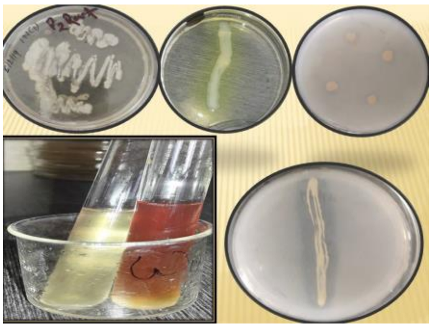

Figure 1. *Pseudomonas sp.* in (A) a plate showing off white colony, (B) nutrient agar media showing greenish appearance colony, (C) showing phosphate solubilization, (D) showing control tube and tube with production of ammonia, and (E) showing a clear zone of solubilization of phosphate in Pikovaskya's agar.

3.2 Pesticide tolerance potential of the selected bacterial isolates

The results of pesticide tolerance potential of the selected bacterial isolate showed that the strain was found tolerable up to 1000 mg.kg^{-1} against the selected pesticides such as Ridomil 80%, Meera 71, carbendazim and sulphur 50%. While for streptomycin, it was tolerating only up to the 50 mg.kg^{-1} (Figure 2). Streptomycin is considered as antibiotics while in field it is used as fungicides. Many of bacterial sp. resists the growth in streptomycin that's why here also, the selected isolate was inhibiting growth in streptomycin after 50 µg/mL. *Bacillus* sp., *Mycobacterium* sp., *Pseudomonas* sp., *Klebsiella* sp. microbes played a vital role in pesticidal remediation (Jaivel *et al*., 2017; Bhat *et al*., 2019). Alam (2018) explained that the bacterial strain was tolerating up to 50 WP (5000 mg/L) of carbendazim.

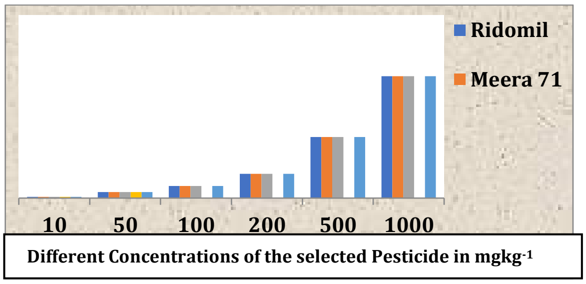

Figure 2. Pesticide tolerance potential of the selected bacterial isolate against the selected concentrations of different pesticides.

3.3 Physiological and morphological parameters of selected endophytic bacteria (Pseudomonas sp.)

3.3.1 Physiological characteristics of selected isolates

The results further showed that the strain was able to produce ammonia and citrimide while it was found negative for urease production. Production of ammonia was found higher in the selected strain as compared to others (Table 1). Plant growth promoting rhizobacteria were applicable in biocontrol of plant diseases and also as growth promoter of plants (Bonilla *et al*., 2019). The mechanism behind growth promoting and metal tolerance of PGPR included production of phytohormones, diazotrophic fixation of nitrogen, and solubilization of phosphate as well as production of inhibitory compounds and hydrolytic enzymes that were often active against a broad spectrum of phytopathogens (Arora *et al*., 2019; Damalas *et al*., 2018).

3.3.2 Morphological characteristics of selected isolate

On nutrient agar, colony of the selected isolate formed off white in color with smooth edge. When the colony was observed under sun light, small fluorescent color greenish yellow was appeared around colony growth. On Pseudomonas isolation medium, greenish color was highly appeared around the cream colony with smooth edge, fluorescent under the sun light that was wider than colony on nutrient agar (Table 1). The results of physiology and morphology of the selected isolate showed that the selected bacterial isolate was belongs from the genera *Pseudomonas* sp. as it was clearly growing on the pseudomonas isolation agar. Furthermore, colony wise it was gram-ve rod shaped and also solubilizing phosphate, producing ammonia, inhibiting urease production (Garg *et al*., 2018; Cerezo *et al*., 2018). Some study showed that the bacteria such as *Pseudomonas* sp., *Stenotrophomonas* sp. and *Bacillus* sp. played key role in controlling plant pathogens, metal tolerance and tends to increase plant growth (Khatri *et al*., 2019; Bhat *et al.,* 2019; Bonilla *et al*., 2019)).

4. CONSLUSIONS

Overall conclusion revealed that the isolated microbial strain have been found as more effective pesticide-tolerant plant growth-promoting microorganism as microbial consortia for enhancing sustainable fruit production in Dry Cold Zone of Himachal Pradesh. It was environmentally friendly, economically viable and socially acceptable to use as bio-fertilizer in the soil ecosystem. It was also alternate to chemical fertilizer for enhancing crop production and soil fertility.

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