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Evaluation of Fluorescent Pseudomonas sp. for their Activity against Plant Pathogenic Fungi in Replant Sites of Apple and Pear

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Abstract

The aim of the study was to assess Pseudomonas sp. for their plant growth and antagonistic activity against most probable fungal pathogens associated with apple and pear plants viz., Dematophora sp., Fusarium sp., Pythium sp. and Sclerotium sp. These isolates were phenotypically and biochemically characterized from the rhizosphere of normal and replant sites of apple and pear from different locations of two districts of H.P i.e. Chamba and Mandi. The isolates of Pseudomonas sp. were screened out for the direct and indirect plant growth promoting activities viz., siderophores, phosphate solubilizing, HCN, ammonia, lytic enzymes and plant growth regulators viz., auxins, cytokinins and gibberellins so as to screen potential strains to be further developed and used as inoculants for management of replant problem of apple and pear. Out of twenty nine Pseudomonas sp. isolates, seven isolates were selected on the basis of overall best PGPR activities including antifungal activity for the extraction and isolation of antifungal metabolite DAPG (2,4-diacetylphloroglucinol) from them.

Keywords

Pseudomonas sp., PGPR, Replant problem, 2, 4-diacetylphloroglucinol, Antifungal activity

Introduction

Biocontrol with antagonistic bacteria (such as fluorescent Pseudomonads) is deemed to be an alternate approach to agrochemicals which are detrimental to human health and environment. Fluorescent Pseudomonad species such as Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas aureofaciens, Pseudomonas putida and Pseudomonas pyrrocinia have shown varying degrees of antagonism to demonstrate antifungal activity (Huet et al., 2005) because of the production of indole-3-acetic acid, siderophores and antibiotics such as phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, PCN, 2,4 diacetylphloroglucinol, viscosinamide and tensin (Nielsen et al., 2000, Hammer et al., 1999). The incorporation of their important features such as antifungal antibiotics production,
siderophore iron chelation, lytic enzymes, plant growth regulators, phosphate solubilization, ammonia and HCN production with ecological strain fitness will become essential for the design of effective, safe and reliable novel bioagents (Rana et al., 2016).

The development of different strategies using a mixture of PGPR strain especially fluorescent *Pseudomonas* sp. would be an important emerging area in crop growth promotion, protection and also in establishment of newly planted trees on old sites of apple and pear. It may reduce the losses caused by replant problem in economically important fruit crops especially apple.

The large scale applications of PGPR to crops as inoculants would be attractive. So isolation, preliminary and genetic characterization of indigenous fluorescent *Pseudomonas* sp. is very important. Current work has therefore been carried out to find best bacterial isolates effectively control the phytopathogens and promote plant growth in replant site of apple and pear.

**Materials and Methods**

**Sample collection**

Isolation of fluorescent *Pseudomonas* sp. was done from soil samples collected from the rhizosphere of apple and pear in normal and replant sites of Chamba and Mandi districts of H.P.

The media employed for the isolation of *Pseudomonas* sp. were nutrient agar (NA) and selective King’s B medium supplemented with 3 antibiotics i.e., Penicillin-G (75,000 units/l), Cycloheximide (75 mg/l) and Novobiocin (45 mg/l). Plates were incubated at 28 ± 2°C for 24 – 48 h (Rana et al., 2014).

**Screening of Pseudomonas** sp. **Isolates for in vitro production of plant growth promoting activities**

All isolates were screened for the production of siderophores, phosphate solubilization, HCN, ammonia, lytic enzymes and plant growth regulators. Quantitative detection of siderophore production in liquid medium of Chrome-azurol-S (CAS) was carried out and change in the colour of reaction mixture was observed from dark blue to orange or pink (Schwyn and Neilands, 1987). Phosphorous estimation (Sharma et al., 2014) was done quantitatively in PVK broth supplemented with 5.0 g/l tricalcium phosphate (TCP). Estimation of hydrocyanic acid (HCN) production by *Pseudomonas* sp., a color change was observed from yellow to orange brown to dark brown in the sodium picrate containing filter paper strip (Bakker and Schippers, 1987). The method of Lata and Saxena, (2003) was used for the estimation of ammonia production. All *Pseudomonas* sp. isolates were screened out for proteolytic activity by well plate assay (Kaur et al., 1988) on skim milk agar plates. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well. Quantitative measurement of auxins was done by colorimetric method (Gorden and Paleg, 1957) with slight modification. The gibberellins were estimated colorimetrically by the method of Holbrook et al., (1961). For bioassay of cytokinins, the radish cotyledon expansion test was employed (Letham, 1971). The bioassay response (final weight - initial weight) was expressed as increase in weight of cotyledons. Antifungal activity was checked by well plate assay method (Vincent, 1947) using dual culture technique. Plates were incubated at 28 ± 2°C for 4 days and observed for inhibition of mycelial growth produced around the well. For control, culture bit of indicator fungus was kept in the centre of MEA plate and incubated at 28 ± 2°C for 4 days.
**Extraction of antifungal metabolite from selected *Pseudomonas* sp. Isolates**

Out of twenty-nine isolates seven *Pseudomonas* sp. isolates were selected on the basis of overall best PGPR activities for the extraction of antifungal metabolite. Isolates were grown in 25 ml of pigment production media broth, incubated for five days at 30°C and centrifuged at 3500 rpm for 5 minutes. The collected supernatant of each *Pseudomonas* sp. was acidified to pH 2.0 with 1 N HCL.

Extraction was done with equal volume of ethyl acetate (Rana S. et al., 2014). Ethyl acetate fraction of each *Pseudomonas* isolates was reduced to dryness in Vacuum. Residue were redissolved in methanol separately. 10µl samples was chromatographed on Silica-G TLC plates and Chloroform:methanol (9:1) solvent system was used. TLC was visualized under UV and sprayed with Para-anisaldehyde and compared bands with reference compound. Antifungal activity of each crude extract was checked by well plate assay method.

**Results and Discussion**

*Pseudomonas* and many soil microorganisms developed antifungal and antibacterial compounds such as antibiotics, iron-chelating siderophores, cyanides and enzymes such as gluconase, cellulite and chitinolytic enzymes which inhibit fungal growth by damaging cell walls (Voisard et al., 1989, Sindhu and Dadarwal, 2001).

Variable compounds with antifungal and antibacterial nature were produced by *Pseudomonas* sp. and many soil microorganisms such as antibiotics, iron chelating siderophores, cyanide and enzymes such as gluconase, cellulolytic and chitinolytic enzymes (Voisard et al., 1989). The activity of these compounds was reported among antifungal mechanisms by which *Pseudomonas* strains inhibited the fungal growth through damaging of cell walls (Sindhu and Dadarwal, 2001).

In the present study, bacterial isolates from the apple and pear rhizosphere were found to be fluorescent, pigmented (greenish, yellowish green and some also produced dark brownish pigment along with fluorescence), transparent to translucent, with maximum isolates being coccobacillus and irregular in shape while few were rods. All the isolates were gram-negative, negative spore, and motile (Table 1). Almost all the isolates were positive for catalase, oxidase, gelatin liquefaction, denitrification (Table 2). Maximum % of siderophore production which was estimated by CAS assay was showed by PN-11-San i.e. 47% siderophore unit followed by PN-12-San i.e. 45% siderophore unit. Phosphorus (P) is an essential macronutrient for biological growth and development (Fernandez et al., 2007).

Quantitative estimation of phosphate solubilization revealed that the *Pseudomonas* sp. isolates PR-2-San followed by PN-7-Cha have a higher capacity to release 355 µg P ml⁻¹ and 345µg P ml⁻¹, in PVK-TCP culture media, respectively.

Bacteria from the rhizosphere protect crops by producing secondary metabolites or extracellular lytic enzymes. Several studies have shown that exposure to lytic enzymes such as chitinases, proteases or glucanases of selected phytopathogenic fungi can lead to degradation of the structural matrix of fungal cell walls (Dunne et al., 1997b). *Pseudomonas* sp. are also able to synthesize HCN to which these *Pseudomonas* sp. are themselves resistant that may be linked to the ability of these strains to inhibit some pathogenic fungi.
Table 1: Morphological characterization of fluorescent *Pseudomonas sp.* isolates from pear rhizosphere

| Site     | Isolates | Colony Morphology | Gram reaction | Spore staining | Pigment | Levan /Slime Production |
|----------|----------|-------------------|---------------|---------------|---------|-------------------------|
|          |          | Shape             | Elevation     | Edge          | Opacity |                        |
| Chamba   | AN-1-Cha | Irregular         | Flat           | Entire        | Transparent | -                       | - | Yellowish green | - |
|          | AR-5-Cha | Irregular         | Flat           | Entire        | Transparent | -                       | - | Yellowish green | - |
|          | PN-7-Cha | Irregular         | Flat           | Entire        | Transparent | -                       | - | Yellowish green | - |
|          | PN-8-Cha | Irregular         | Flat           | Entire        | Transparent | -                       | - | Yellowish green | - |
| Mandi    | PN-11-San| Irregular         | Raised         | Entire        | Translucent | -                       | - | Dark green      | Mucoid |
|          | PN-12-San| Rods              | Flat           | Entire        | Translucent | -                       | - | Grayish         | - |
|          | PR-2-San | Coccobacillus     | Raised         | Entire        | Translucent | -                       | - | Grayish         | - |

Table 2: Physiological and biochemical characteristics of fluorescent *Pseudomonas* isolates

| Site     | *Pseudomonas* Isolates | Catalase test | Oxidase test | Aerobic/Anaerobic growth | Growth at 4°C, 25°C and 41°C | Gelatin liquification | Denitrification test |
|----------|------------------------|---------------|--------------|--------------------------|-------------------------------|----------------------|----------------------|
|          |                        |               |              |                          | 4°C  | 25°C  | 42°C |                           |                     |                       |
| Chamba   | AN-1-Cha               | +             | +            | Aerobic                  | -   | +     | +    | +                          | +                   |                       |
|          | AR-5-Cha               | +             | +            | Aerobic                  | -   | +     | +    | +                          | +                   |                       |
|          | PN-7-Cha               | +             | +            | Aerobic                  | +   | +     | +    | +                          | +                   |                       |
|          | PN-8-Cha               | +             | +            | Aerobic                  | -   | +     | +    | -                          | -                   |                       |
| Mandi    | PN-11-San              | +             | +            | Aerobic                  | -   | +     | +    | +                          | +                   |                       |
|          | PN-12-San              | +             | +            | Aerobic                  | +   | +     | +    | -                          | -                   |                       |
|          | PR-2-San               | +             | +            | Aerobic                  | -   | +     | -    | +                          | +                   |                       |

Table 3: Plant growth promoting activities of fluorescent *Pseudomonas* isolates

| *Pseudomonas* isolates | Site     | Siderophores (%SU) | Phosphate Solubilization (µg/ml) | Proteolytic (mm) | HCN | Ammonia | Auxins (µg/ml) | Gibberrellins (µg/ml) | Cytokinins (µg/ml) |
|------------------------|----------|--------------------|----------------------------------|------------------|-----|---------|---------------|----------------------|---------------------|
| AN-1-Cha               | Chamba   | 28.39              | 290                              | 25               | +   | +++     | 7             | 510                  | 394                 |
| AR-5-Cha               | Chamba   | 32.14              | 115                              | 28               | +   | +++     | 10            | 490                  | 391                 |
| PN-7-Cha               | Chamba   | 40.71              | 345                              | 28               | +   | +++     | 7             | 550                  | 450                 |
| PN-8-Cha               | Chamba   | 35.35              | 225                              | 25               | +++ | +++     | 4             | 550                  | 502                 |
| PN-11-San              | Mandi    | 47                 | 285                              | 24               | +++ | +++     | 3             | 365                  | 232                 |
| PN-12-San              | Mandi    | 45                 | 300                              | 23               | +++ | +++     | 12            | 470                  | 240                 |
| PR-2-San               | Mandi    | 39                 | 355                              | 22               | +++ | +++     | 3             | 490                  | 570                 |
Table.4 Antifungal activity shown by fluorescent *Pseudomonas* isolates against different plant pathogenic fungi in terms of per cent inhibition

| Pseudomonas isolates | Site        | Dematophora sp.(% I) | Fusarium sp. (%I) | Pythium sp. (%I) | Sclerotium sp. (%I) |
|----------------------|-------------|-----------------------|-------------------|------------------|----------------------|
| AN-1-Cha             | Chamba      | 45.83                 | 28.57             | 22.22            | 37.14                |
| AR-5-Cha             | Chamba      | 27.08                 | 0.00              | 22.22            | 28.57                |
| PN-7-Cha             | Chamba      | 29.16                 | 22.85             | 22.22            | 34.28                |
| PN-8-Cha             | Chamba      | 27.08                 | 28.57             | 26.66            | 28.57                |
| PN-11-San            | Mandi       | 0.00                  | 34.28             | 22.22            | 28.57                |
| PN-12-San            | Mandi       | 33.33                 | 22.85             | 33.33            | 37.14                |
| PR-2-San             | Mandi       | 37.5                  | 40                | 33.33            | 31.42                |

Table.5 Antifungal activity of extracted metabolite against *Pythium* sp

| Pseudomonas isolates | Site | % Growth inhibition |
|----------------------|------|---------------------|
| AN-1-Cha             | Chamba | 22.87 |
| AR-5-Cha             | Chamba | 34.37 |
| PN-7-Cha             | Chamba | 23.75 |
| PN-8-Cha             | Chamba | 28.12 |
| PN-11-San            | Mandi  | 27.27 |
| PN-12-San            | Mandi  | 37.27 |
| PR-2-San             | Mandi  | 33.36 |

Fig.1 Thin layer chromatographic analysis (Under UV) of extracted antifungal metabolite 2,4-diacetylphloroglucinol (Rf 0.86) from fluorescent *Pseudomonas* sp. Lane (R) Reference, (1) AN-1-Cha, (2) AR-5-Cha, (3) PN-7-Cha, (4) PN-8-Cha, (5) PN-11-San, (6) PN-12-San, (7) PR-2-San, on Silica gel G by using solvent system chloroform: methanol (9:1)
Maximum Proteolytic activity was observed in case of *Pseudomonas* sp. isolates AR-5-Cha and PN-7-Cha (28mm dia.). *Pseudomonas* sp. isolate PN-11-San showed maximum HCN production followed by PN-12-San, PR-2-San and PN-8-Cha. Similarly, AR-5-Cha, PN-11-San and PN-12-San showed maximum amount of ammonia production.

Different micro-organisms have been recognized to exude different plant growth promoters. Quantitative estimation of plant growth regulators revealed that PN-12-San showed maximum auxin production i.e. 12 µg/ml. PN-7-Cha and PN-8-Cha showed maximum production of gibberellins i.e. 550 µg/ml. Maximum cytokinin production was observed in case of PN-8-Cha i.e. 502 µg/ml (Table 3). Dual culture studies revealed that all the bacterial isolates effectively control the phytopathogens (Table 4). Against *Dematophora* sp. AN-1-Cha showed maximum % inhibition i.e. 45.83 %I, similarly PR-2-San, PN-12-San and PR-2-San; AN-1-Cha and PN-12-San showed maximum % inhibition against *Fusarium* sp. i.e. 40%I, *Pythium* sp. i.e. 33.33%I and *Sclerotium* sp. i.e. 37.14%I respectively. In case of PN-11-San and AR-5-Cha no inhibition was observed against *Dematophora* sp. and *Fusarium* sp. respectively.

Bacteria that produce DAPG play a key role in agriculture, and their potential for use in sustainable agriculture is promising. Production of antibiotics is a primary mechanism of pathogen inhibition (Weller, 1988). DAPG is produced by fluorescent *Pseudomonas* sp. of diverse geographic origin that has common ability to suppress one or more root and seedling diseases of crop plants caused by soil-borne pathogens. Seven *Pseudomonas* isolates were chosen overall on the basis of best plant growth promoting activities for the extraction of active antifungal metabolite i.e. may belong to 2,4-diacyethylphloroglucinol. Thin layer chromatography showed orange coloured amount of spots that is the indication of antifungal activity. The Rf value shown by the ethyl acetate extracted metabolite of *Pseudomonas* isolates was 0.86 (Fig. 1). These results gave an idea that extracted antifungal metabolites may be related to the group of antibiotic i.e. 2, 4-diacyetylphloroglucinol. Crude extract of all seven *Pseudomonas* isolates showed percent growth inhibition against *Pythium* sp. Maximum percent growth inhibition was shown by PN-12-San (37.37%I) and AR-5-Cha (34.37%I) (Table 5). This research describes the multifarious role played by *Pseudomonas* sp. with potentials encouraging plant growth. The option of such bacteria will increase their utility in sustainable organic farming as bio-inoculations further.

In conclusion the fluorescent *Pseudomonas* are effective root colonizer along with other direct and indirect plant growth promoting activities. They control deleterious fungi and bacteria due to production of many indirect PGP activities like broad-spectrum antifungal antibiotics, iron chelating siderophores, HCN, ammonia, supply of nutrients like available phosphorus, iron ions and other small molecules through P-solubilizing and lytic enzymes. Therefore, these isolates can be further used as inoculants for management of replant problem of apple and pear and can be considered as an alternative strategy to agrochemicals as they are environment friendly.

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