Screening of *Pseudomonas sp.* isolated from rhizosphere of pea plant as plant growth promoter and biocontrol agent

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**ABSTRACT**

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that are found in the rhizosphere and rhizoplane which can improve plant growth. *Pseudomonas* spp. is one of the most promising groups of PGPR which can control plant pathogenic microbes in the soil. In this study, an attempt was made to isolate *Pseudomonas* spp., a potent PGPR in the rhizosphere. Through appropriate microbiological and biochemical methods, the study demonstrated the presence of fluorescent and nonflourescent *Pseudomonads* in the rhizosphere of pea. 12 different strains of *Pseudomonas* were isolated from pea rhizosphere and identified by biochemical tests. Out of these strains, five were screened against wilt and root rot pathogens of pea. Antagonistic activity of *Pseudomonas* isolates was evaluated against wilt and root rots pathogens i.e. *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* through dual culture technique. The study exhibited that all *Pseudomonas* strains significantly inhibited the growth of *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* as compared to control. Among all the *Pseudomonas* isolates, Ps5 showed maximum inhibition against *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum*. Augmentation of such PGPR will ensure a healthy micro climate for pea.

**KEYWORDS**

Biochemical Characterization, PGPR, Biocontrol, *Pseudomonas*, Root Rot, Wilt, Pea

**HOW TO CITE THIS ARTICLE**


Wilt and root rot complex disease of peas caused by *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* is widely distributed in many countries and it is a devastating pathogen right from the establishment of the crop. Many effective fungicides have been tested against soil borne pathogens, but are not considered as long term solutions because of concerns about exposure risks, environmental and health hazards, residue persistence, high cost, the development of resistance against pesticides and the elimination of natural enemies. Biological control is a potential non-chemical means for plant disease management by reducing the harmful effects of a parasite or pathogen through the use of other living entities. Use of Plant Growth Promoting Rhizobacteria having biocontrol (BCA) and plant growth promoting activities is a viable alternative to minimize the use of synthetic chemicals and their hazardous effects, and to provide protection to the plants against resident pathogen populations. PGPR affect plant growth in two ways, directly and indirectly.

The direct promotion of plant growth by PGPR entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the soil. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms (Negi, 2008). Among PGPRs, most common are *Azotobacter*, *Bacillus* spp., fluorescent *Pseudomonas* spp., *Rhizobium* spp., etc. During last decade much of the research, however, has focused on organisms belonging to *Pseudomonas* species. These organisms have shown great antagonistic activity against several soil borne pathogens of economically important crops (Fernando et al., 2004, Savchuk and Fernando, 2004 and Negi et al., 2005). In the present investigation, attempts were made to isolate *Pseudomonas* spp. from pea ecosystem and to further identify and characterize the isolate using standard microbiological and biochemical test. Attempts were also made to test the plant growth promotion activities of *Pseudomonas* spp.
isolates and its antagonistic activity against wilt and root rot pathogens of pea.

MATERIALS AND METHODS

Isolation of Pathogen and Pseudomonas Strains

Isolation of Pathogen

The wilt and root rot pathogens were isolated from pea plants showing typical wilt and root rot symptoms and pure cultures of the pathogen were obtained by the single hyphal tip method (Rangaswami, 1972).

Isolation of Pseudomonas Strains

Rhizobacterial Pseudomonas strains were isolated from soil samples obtained from different parts of Aligarh District. One gram of rhizosphere soil adhering to root surface was collected and transferred in 9 ml of sterilized water and shaken thoroughly to get the soil particle uniformly dispersed in the suspension. After thorough shaking for 15 minutes in a shaker, different dilutions were prepared. One ml of suspension from the first dilution (1:10) was aseptically transferred to another tube (10^2) and this procedure further repeated till the dilution 10^6 was obtained. Transfer 0.1 ml of sample from each dilution in King’s B medium and spread it by sterilized glass spreader. The plates were then incubated for 3 days at 30 ±1°C. The growth of rhizobacterial colonies on King’s B medium plates were observed and recorded continuously for 3 days. The selected isolates of rhizobacteria were subjected to further confirmatory biochemical tests.

Biochemical Characterization

Twelve isolates were characterized according to Bergey’s manual of determinative bacteriology (Holt et al., 1994). Standard microbiological tests were conducted for rapid identification of Pseudomonas colonies on the King’s B plates, which included colony morphology, Gram staining, motility test and fluorescent pigment test. Pure culture of Pseudomonas spp. was obtained following successive selection of fluorescing colonies on King’s B medium under UV light at 365 nm (Rachid and Ahmed, 2005). The isolates were characterized to be identified as P. fluorescens by performing growth at 4 and 41°C and biochemical tests including oxidase, catalase, gelatin hydrolysis, starch hydrolysis, H2S production, Methyl Production and citrate utilization test (Reynolds, 2004).

Plant Growth Promoting Properties

Plant growth promoting traits like siderophore production, phosphate solublization, HCN and ammonia properties were also studied. In order to identify volatile toxicity in the strains HCN production test was conducted by using filter paper pre soaked in picric acid solution (Wei, et al., 1981). In siderophore production evaluation of isolates with universal Chrome- azurol assay (CAS) helps in detection the siderophore by fluorescent Pseudomonas. This assay mainly depends on the colour zone i.e. orange zone against dark blue background, a positive indication for the presence of siderophore. All the isolates were screened by CAS method (Schwyn and Neilands, 1987) for their ability to produce siderophore. In phosphate solublization test was performed by spot inoculation of test organism on Pikovaskay’s medium. Formation of clear zone around the colony is considered positive of phosphate solublization properties. Pseudomonas isolates were also tested for Ammonia production. Pseudomonas isolates were grown in tube containing peptone solution and incubated at 30°C for 4 days. After adding Nesser’s reagent, change of colour to yellow is the indication of presence of ammonia (Cappuccino and Sherman, 1992).

Efficacy of Bacterial Isolates against Wilt and Root Rot Pathogens

The efficacy of rhizospheric Pseudomonas isolates on radial growth inhibition of test pathogens i.e. Fusarium oxysporum f. sp. pisi, Rhizoctonia solani and Pythium ultimum was studied in vitro, through dual culture technique. Four discs of the test fungus were placed in the periphery of petriplate at equal distance there after the blotting paper discs having the diameter of
10mm dipped in bacterial suspension and placed in the centre of petriplates. In check, no blotting paper was placed. All the Petri plates were incubated for five days at 30°C. Each treatment had three replications. Radial growth inhibition of test pathogens was measured at an interval of 24h for five days to record different stages of antagonism. The observations on radial growth inhibition of test pathogens i.e. *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* were recorded after 120 hrs. The percent inhibition over check, noted after 5 days of incubation. The percent inhibition over check, noted after 5 days of incubation was calculated by the following formula (Vincet, 1947).

\[ I = \frac{C - T}{C} \times 100 \]

Where, \( I \) = Percent Inhibition  
\( C \) = Colony diameter in check  
\( T \) = Colony diameter in treated petriplate.

**RESULTS AND DISCUSSION**

The rhizospheric soil collected from different field of pea yielded 40 different bacterial colonies. Among these, 12 isolates (Ps 1 to Ps 12) were identified as *Pseudomonads*. These isolates of *Pseudomonas* were characterized on the basis of their morphological and biochemical characteristics. *Pseudomonas* isolates grown on medium produced pale yellow and mucoid colonies. All the isolates of *Pseudomonas* were found to be gram negative, chemoheterotrophic motile rods with polar flagella. It is evident from Table 1 that all the isolates of *Pseudomonas* designated as PS1 to PS12 were found to be positive for catalase and oxidase test. However, all these isolates exhibited negative test to the production of H₂S, methyl red, gelatine hydrolysis, starch hydrolysis while those isolates produced Fluorescent pigment also showed their growth at 41°C. Some of the *Pseudomonas* isolate showed positive test for growth at 4°C and citrate utilization. *Pseudomonas* isolates (Ps1, Ps2, Ps5, Ps8, Ps9 and Ps10) were also tested for their plant growth promoting activity such as siderophore production, phosphate solubilation, production of HCN and ammonia. All the strains were able to produce siderophore, HCN and phosphate solubilation (table 2). However the isolate Ps5 was strong HCN producer which turned the colour of the filter paper in to complete brown orange. The remaining isolates were moderate HCN. Hydrogen cyanide is produced by many rhizobacteria and has been found to play a very significant role in biological control of soil borne pathogens (Schipper *et al.*, 1987, Weller, 1988 and Voisard *et al.*, 1989). In this study, the *Pseudomonas* isolates were also screened for their capacity to fix nitrogen and excrete ammonia. The observations revealed that all the isolates produce ammonia, though, two of them (PS2 and PS5) exhibited higher production of ammonia (table 2). *Pseudomonas* sp., belonging to plant growth promoting *Rhizobacteria* has received prominent attention because of the dual role of these bacteria in plant growth promotion and diseases control (Pikovskaya, 1948). Cook (1993) reported that certain plant associated bacteria particularly fluorescent pseudomonads have been exploited for suppression of crop diseases. Our work demonstrates the ability of *P. fluorescens* to produce fungistatic metabolites such as HCN. *Pseudomonas* sp. are known to produce volatile compounds. One such metabolite is HCN (Tripathi and Johri, 2002). Afsharmanesh *et al.*, (2010) suggested that fungal growth is mainly inhibited by HCN production and siderophore production. Apart from the biocontrol potential, fluorescent *Pseudomonads* possess other functional properties like, mineral phosphate solubilisation, production of plant growth promoting substances and enzyme activity (Afsharmanesh *et al.*, 2010). Results also revealed that the antifungal activities and other plant beneficial traits appear to be the general and genetically dispersed traits of fluorescent *Pseudomonads*. Knowledge on phenotypic and functional traits of bacteria will help to determine their fitness for successful biofertilization and biological control.
Table 1. Biochemical characterization of *Pseudomonas* isolates

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Gram Staining</th>
<th>Morphology</th>
<th>Motility</th>
<th>Growth at 4°C</th>
<th>Growth at 41°C</th>
<th>Flourescent Pigment</th>
<th>Starch Hydrolysis</th>
<th>Gelatin Hydrolysis</th>
<th>H2S Prod</th>
<th>Methylprod</th>
<th>Citrate Utilization</th>
<th>Catalase Test</th>
<th>Oxidase Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps1</td>
<td>-</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ps2</td>
<td>-</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ps3</td>
<td>-</td>
<td>Rod</td>
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<td>Ps4</td>
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<td>Rod</td>
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<td>Ps5</td>
<td>-</td>
<td>Rod</td>
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<td>Ps6</td>
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<td>Rod</td>
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<tr>
<td>Ps9</td>
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<td>Rod</td>
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<tr>
<td>Ps10</td>
<td>-</td>
<td>Rod</td>
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</tr>
</tbody>
</table>

Table 2. Growth promotion characteristics of *Pseudomonas* isolates

<table>
<thead>
<tr>
<th><em>Pseudomonas</em> isolates</th>
<th>HCN Production</th>
<th>Siderophore Production</th>
<th>Ammonia Production</th>
<th>Phosphate solubilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps1</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ps2</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Ps5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ps8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ps9</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Ps10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Low, ++ Moderate, +++ Strong, - Not detected

Table 3. Efficacy of *Pseudomonas* isolates on radial growth inhibition of wilt and root rot pathogens

<table>
<thead>
<tr>
<th>Isolates</th>
<th><em>F. oxysporum f. sp. pisi</em></th>
<th><em>R. solani</em></th>
<th><em>P. ultimum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radial growth* (cm)</td>
<td>Inhibition (%)</td>
<td>Radial growth* (cm)</td>
</tr>
<tr>
<td>Ps1</td>
<td>2.0</td>
<td>70.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Ps2</td>
<td>1.9</td>
<td>71.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Ps5</td>
<td>1.8</td>
<td>72.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Ps9</td>
<td>3.0</td>
<td>55.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Ps10</td>
<td>3.1</td>
<td>53.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Control</td>
<td>6.6</td>
<td>00.0</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Mean of three replicate
Efficacy of Bacterial Isolates against Wilt and Root Rot Pathogens

Among the 12 Pseudomonas isolates, 5 isolates were evaluated for their antagonistic potential against wilt and root rot pathogens. The results, thus obtained and presented in Table 3 reveal that all Pseudomonas isolates significantly inhibited the growth of test pathogens in comparison to control. However the maximum growth inhibition of Fusarium oxysporum (1.8), Rhizoctonia solani (2.0) and Pythium ultimum (1.8) was resulted due to isolate Ps5 followed by Ps2 and Ps1. The percent inhibition in growth of test pathogens corresponding to the isolates Ps5, was recorded 72.50, 70.24 and 70.27 percent for Fusarium oxysporum, Rhizoctonia solani and Pythium ultimum, respectively, the differences in the radial growth inhibition of test pathogens due to isolates Ps5, Ps2 and Ps1 were found to be statistically non significant when compared from one another. On the other hand isolates Ps9 and Ps10 were found to be least effective and exhibited insignificant difference in their efficacy when compared from each other (table 3). The study also indicates that in general, there was a proportionate increase in the antagonistic potential of all Pseudomonas isolates at different interval after inoculation. It was worthy to note that isolates Ps1, Ps2 and Ps5 exhibited more or less a similar trend having a close pace in inhibiting the growth of test pathogens at each interval of observation. Yuan et al, (2012) noted that volatile compound produced by the bacteria reduced the mycelia growth and inhibited spore germination of F. oxysporum. The P. fluorescens is very effective antibiotic producer. Many secondary metabolites of P. fluorescens acts as antibiotics against plant pathogens. The P. fluorescens produces antifungal compounds like phenazine-1-carboxylic acid (PCA), 2, 4 – diacetylphloroglucinol (DAPG), pyococine, pyrrolnitrin, pyoluteorin and oomycin-A which are fungistatic, inhibiting spore germination and lysis of fungal mycelia (Kell et al. 1992; Hass and Defago, 2005). Urkade (2010) studied invtro antibiosis of Pseudomonas fluorescens against Rhizoctonia bataticola and reported that Pseudomonas fluorescens isolates Pf2 and Pf5 were most effective against R. bataticola which recorded 30.28 % and 28.12 % growth inhibition, respectively. Suppression of Rhizoctonia bataticola by Pseudomonas fluorescens in agar plate might be due to the production of siderophores (Laha et al., 1992). Gupta et al., (1999) successfully used Pseudomonas florescens in vitro against Macrophomina phaseolina and Fusarium oxysporum and found antifungal activity of the strain. The production of hydrogen cyanic acid and indole acetic acid was also recorded under normal growth condition. The P. fluorescens produces HCN which can check growth of phytopathogen. In another experiment, Velzhahan et al., (1999) isolated several strain of Pseudomonas florescens from the rhizosphere of rice plants and tested against Rhizoctonia solani causing sheath blight in rice and were found to be effective in inhibiting the mycelial growth of the pathogens. Therefore, it is concluded from the study that Pseudomonas strain can be exploited as biofertilizer and as biocontrol agents to replace the chemical fertilizer and fungicides that impair to human health. Pseudomonas could be one of the potential candidates in the development of microbial pesticides to manage wilt and root rot complex in pea diseases, for sustained crop productivity.

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