Gibberellins production by fluorescent Pseudomonas isolated from Rhizospheric soil of Malus and Pyrus

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Abstract

Little is known about the gibberellins producing fluorescent Pseudomonas (FP) diversity associated with rhizosphere of Malus (apple) and Pyrus (pear). Therefore, the objective of the present study was to investigate gibberellins production by Pseudomonas sp., a major component of rhizobacteria with multiform and diverse activities, which alter the structure or life processes of plants to improve quality, increase yields and facilitate harvesting. In the present investigations, thirty Pseudomonas isolates were isolated from the rhizosphere of Pyrus and Malus and were screened for gibberellins production (25-60 µg/ml). Four strains viz PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were selected on the basis of their higher gibberellins production. The maximum production of gibberellins was observed at 72 h incubation period in nutrient broth at pH 7.0 under shaken condition at 28°C. Gibberellins were extracted, purified and evaluated by thin layer chromatography and specific bioassay method.

Highlights

- Pseudomonas isolates were obtained from rhizospheric soils of apple and pear.
- Four efficient gibberellins producing isolates were screened.
- Extracted gibberellins were characterized.

Keywords: Gibberellins, PGPR, pseudomonas sp., thin layer chromatography, alpha-amylase

Fluorescent Pseudomonas species have emerged as largest and potentially most promising group of plant growth promoting rhizobacteria. Plant-growth promotion by PGPR attributed to the synthesis of the plant growth hormones including indole-3-acetic acid, cytokinin and gibberellins (Lincoln and Kotasthane 2014; Jha and Saraf 2015). Such microorganisms inhabiting rhizosphere of various plants are likely to synthesize and release gibberellins as secondary metabolite. Ullah et al. (2004) defined gibberellins (GAs) as plant growth hormones specialized for plant growth and development. MacMillan (2002) also showed the effect of gibberellins in several plants developmental processes including seed germination, stem elongation, flowering, fruit setting and delay of senescence in many organs of a range of plant species. Gibberellins have also been implicated in promotion of root growth since they regulate root hair abundance in accordance with Ullah et al. (2014). Many researchers Joo et al. (2009); Pandya and Desai (2014); Saber et al. (2015); Tsakelova et al. (2006) and Sharma et al. (2014) observed that the production of gibberellic acid is wide spread among Pseudomonas sp. Microbial synthesis of gibberellins and other metabolites is an important factor in improving soil fertility as stated by Ambawade and Pathade (2013). However, to date little information exists on the diversity of plant growth promoting rhizobacteria associated with apple and pear trees. Moreover, in apple and pear growing region of Himachal Pradesh, gibberellins
producing *Pseudomonas* population has never been estimated. In present work, gibberellins producing *Pseudomonas* sp. from rhizospheric soil of apple and pear tree were isolated and characterized for production of one of the important plant growth regulator i.e. gibberellins. More specifically, the soil-borne fluorescent *Pseudomonas* has received particular attention because of their capacity to produce a wide range of secondary metabolites.

**Materials and methods**

**Isolation of fluorescent *Pseudomonas* species from rhizosphere of apple and pear**

Soil samples were collected in triplicates to make it one sample from the rhizosphere soils of pear and apple orchards in Mandi district (Himachal Pradesh), India. Total bacterial count viz-a-viz fluorescent *Pseudomonas* sp. were isolated on Nutrient agar and King’s B media (Hi-media), respectively from soil samples. The proposed isolates were characterized on the basis of morphological, biochemical and physiological analyses as prescribed in Bergey’s manual of systematic bacteriology by Kreig and Holf (1984).

**Screening of isolates for gibberellins production**

*Pseudomonas* sp. isolated from the rhizosphere soil of pear and apple orchards were screened out for the production of gibberellins. The gibberellins were estimated colorimetrically by the method of Holbrook et al. (1961) with slight modifications. To 15 ml of supernatant, 2 ml of zinc acetate reagent, 1 ml of glacial acetic acid, 21.9 g zinc acetate was added and volume was made upto 100 ml with distilled water. After 2 minutes, 2 ml of potassium ferrocyanide (10.6% in distilled water) was added and centrifuged at low speed (2000 rpm) for 15 minutes. To 5 ml of supernatant, 5 ml of 30 per cent HCl was added and mixture was incubated at 20°C for 75 min. For blank, 5 ml of 5 per cent HCl was used. Absorbance was read at 254 nm and concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA₃, Hi-media) as standard (100-1000 µg/ml).

**Effect of different media on gibberellins production by *Pseudomonas* sp. at different incubation period**

The test organisms were grown in five different media; succinate media, king's media, nutrient media, peptone water and trypticase soyabroth. Flasks were incubated at 28°C for 24, 48, 72 h under shaken conditions (90rpm). Supernatant were harvested by centrifugation in multi-fuge XIR (Thermoscientific Germany) at 10,000 rpm for 30 minute at 4°C and were further used for estimation of gibberellins.

**Extraction and separation of gibberellins**

Gibberellins like substance were extracted by the method of Borrow et al. (1955) with slight modifications. 100 ml of cell free extract of bacterial culture were mixed with 250 ml of saturated NaHCO₃ solution in separatory funnel and extracted with 300ml ethyl acetate (2X). Aqueous layer acidified to pH 2.5 with 5 N HCl. Equal volume of ethyl acetate added and shaken vigorously for 5 minutes. Separated the ethyl acetate fraction; and re-extracted the aqueous layer (2X) with 300 ml of ethyl acetate solvent. All ethyl acetate fractions pooled and dried over Na₂SO₄. Evaporated the ethyl acetate extract on a rotary evaporator at 40°C. Residue dissolved in 2 ml of ethanol for thin layer chromatography and in 2 ml of water with Tween 20 for bioassay. Gibberellins were further separated by thin layer chromatography using silica gel-G plates, developed in solvent isopropanol; ammonium hydroxide; water (10:1:1 v/v/v) and silica gel-G plates were heated at 120°C for 10 minutes in oven and sprayed with water: concentrated sulphuric acid (30: 70 v/v).

**Evaluation of gibberellins by alpha-amylase release test**

Cut apical halves of rice seeds without embryo and sterilized with 1% sodium hypochloride solution for 10-15 minutes and rinsed with sterilized water (3X). They were weighed in group of five. Added 1ml of sterilized distilled water (blank), test solution (supernatant or extracted sample) and standard solution 10-100 µg. Incubated at 30°C for 4 days in dark as described by Jones and Varner (1967). Test solution was discarded and the endosperm rinsed with small volume of sterilized distilled water were further grinded in 5ml of 0.2% of Cacl₂ (200 ml). Mixture was centrifuged at 2000 rpm for 15minutes. Added 4ml of acetate buffer containing 0.25% starch solution to 1ml of filtrate and incubated at 40°C for 30 minutes. 5ml of 0.03N iodine solution was added to reaction mixture. Absorbance was measured at
Gibberellins production by fluorescent *Pseudomonas* isolated from Rhizospheric soil of *Malus* and *Pyrus*

700nm. Zero hour control was maintained i.e. 5 ml of acetic acid was added to first step.

**Observations:** OD at 700nm using red filter.

\[ \alpha \text{- amylase activity: } \% \text{unit} = \left( \frac{C - E}{C} \right) \times 100 \]

C = OD at initial starch solution

E = OD at the end of the reaction

**Results and Discussion**

All the collected samples of rhizospheric soil were evaluated for the status of total bacterial population viz a viz *Pseudomans* sp. Total bacterial count was found to be higher as compared to *Pseudomonas* sp. Seventeen *Pseudomonas* isolates were obtained from pear rhizosphere soil and thirteen were obtained from apple rhizosphere soil (Table 1).

**Table 1:** Survey and collection of rhizosphere soil samples of pear and apple trees of Mandi district viz Nagwain and Sanyardi (H.P.)

<table>
<thead>
<tr>
<th>Trees</th>
<th>Site name</th>
<th>Total no of composite soil sample</th>
<th>Total bacterial count (log cfu/g soil)</th>
<th>Total number of <em>Pseudomonas</em> isolates</th>
<th>Symbolic designation to isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>Nagwain</td>
<td>5</td>
<td>6.1</td>
<td>13</td>
<td>AN-Nag</td>
</tr>
<tr>
<td>Pear</td>
<td>Sanyardi</td>
<td>4</td>
<td>6.3</td>
<td>17</td>
<td>PN-San</td>
</tr>
</tbody>
</table>

#Composite soil samples made by mixing the soil sample that were collected randomly from different plant rhizosphere in the same site

On the basis of morphology and biochemical analyses, these isolates were identified as Gram negative, rod shaped bacteria which produce fluorescence under ultra violet radiations and showed positive test for catalase and oxidase. In our studies it was found that the all *Pseudomonas* isolates produced gibberellins in the range of 19-60 µg/ml (Figure 1).

![Fig. 1: Gibberellins production by fluorescent *Pseudomonas* isolated from (a) Apple and (b) Pear](image)

Four strains viz PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were selected on the basis of their higher gibberellins production. The efficient isolates produced gibberellins like substances in the stationary growth at 28°C (Table 2). Besides that, statistically higher concentrations of gibberellins in all the efficient isolates were obtained in nutrient broth i.e. 336, 300, 235, 220 µg/ml, respectively after 72 h of incubation.
Table 2: Effect of media on the production of plant growth regulator gibberellins (µg/ml) by fluorescent Pseudomonas at different incubation period.

<table>
<thead>
<tr>
<th>Media</th>
<th>Incubation period (h)</th>
<th>PN-4-SAN</th>
<th>PN-10-SAN</th>
<th>AN-10-NAG</th>
<th>AN-10-NAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
<td>72</td>
<td>Mean</td>
<td>24</td>
</tr>
<tr>
<td>Succinate media</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>King’s media</td>
<td>90</td>
<td>128</td>
<td>150</td>
<td>122.7</td>
<td>74</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>154</td>
<td>211</td>
<td>336</td>
<td>233.7</td>
<td>177</td>
</tr>
<tr>
<td>Peptone water</td>
<td>120</td>
<td>147</td>
<td>200</td>
<td>155.7</td>
<td>74</td>
</tr>
<tr>
<td>Trypticane soya broth</td>
<td>96</td>
<td>117</td>
<td>151</td>
<td>121.3</td>
<td>115</td>
</tr>
<tr>
<td>Mean</td>
<td>117.6</td>
<td>149.8</td>
<td>205</td>
<td>112.7</td>
<td>154</td>
</tr>
</tbody>
</table>

The homogeneity of the partially purified gibberellins along with GA₃ as standard was checked by thin layer chromatography. Gibberellins gave the maximum Rf value of 0.80. Grey spots corresponding to gibberellins or gibberellins like substances were visible when sprayed with reagent (Figure 2, Table 3).

Table 3: Thin layer chromatographic analysis on silica gel-G of partially purified gibberellins from Pseudomonas sp.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Solvent system</th>
<th>Spraying reagent</th>
<th>Color of spots</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN-4-SAN</td>
<td>Isopropanol: NH₄OH: Water (10:1:1)</td>
<td>Water: H₂SO₄ (30:70)</td>
<td>Grey</td>
<td>0.80</td>
</tr>
<tr>
<td>PN-10-SAN</td>
<td>Isopropanol: NH₄OH: Water (10:1:1)</td>
<td>Water: H₂SO₄ (30:70)</td>
<td>Grey</td>
<td>0.79</td>
</tr>
<tr>
<td>AN-2-NAG</td>
<td>Isopropanol: NH₄OH: Water (10:1:1)</td>
<td>Water: H₂SO₄ (30:70)</td>
<td>Grey</td>
<td>0.79</td>
</tr>
<tr>
<td>AN-4-NAG</td>
<td>Isopropanol: NH₄OH: Water (10:1:1)</td>
<td>Water: H₂SO₄ (30:70)</td>
<td>Grey</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Rf value calculated as = Distance run by the compound / Distance run by the solvent front

As the result showed (Table 4), partially purified gibberellins like substances from 72 h old culture supernatants of PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG has increased the alpha-amylase
activity. They released 45 to 51 % units of enzyme alpha-amylase from seeds half that correspondent to 35 to 40 µg/ml concentration of GA₃ as calculated from the standard curve.

Table 4: Effect of partially purified gibberellins like substances from fluorescent Pseudomonas on α-amylase release activity from rice seed without embryo.

<table>
<thead>
<tr>
<th>Partially purified gibberellins</th>
<th>Gibberellins* α-amylase activity (µg/ml)</th>
<th>(%) units</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN-4-SAN</td>
<td>40.00</td>
<td>45.00</td>
</tr>
<tr>
<td>PN-10-SAN</td>
<td>37.00</td>
<td>48.00</td>
</tr>
<tr>
<td>AN-2-NAG</td>
<td>35.00</td>
<td>51.00</td>
</tr>
<tr>
<td>AN-4-NAG</td>
<td>35.00</td>
<td>51.00</td>
</tr>
<tr>
<td>O.D. of initial starch</td>
<td>0.386</td>
<td></td>
</tr>
</tbody>
</table>

*Gibberellins of (µg/ml) of gibberellic (GA₃) as calibred from standard curve (100-1000 µg/ml).

C - E

α-amylase activity ( % units) =  × 100 (%)

C

Where, C is O.D. of initial starch solution and E is O.D. at end of reaction

In this study significant difference in total bacterial and total Pseudomonas communities of apple and pear orchards was observed. Over all it was found that the Pseudomonas population was far less as compared to total population present in rhizosphere soils of both apple and pear trees. According to Yao et al. (2006), population shift may be related to change in soil moisture; soil temperature, rhizodeposition and root turn over. Gibberellins are one of the major groups of growth hormones which play an essential role in the growth and development of plants. Gibberellin production by PGPR promote the growth and yield of many crop plants by deconjugation of gibberellins glucosyl in the root zone, causing 3β-hydroxylation of inactive 3-deoxy GAs to active forms such as GA1, GA3, and GA4 bacterial enzymes (Kang et al. 2014). GAs have been identified and isolated from higher plants, fungi, and bacteria. Gibberellins producing ability is inherent in all groups of microorganisms including epiphytic and rhizospheric bacteria (Pandya and Desai 2014). Lenin and Jayanthi (2012) have also isolated and purified gibberellic acid (GA₃) and gibberellins like compounds from genera Azotobacter, Bacillus and Pseudomonas isolated from soil in the range of 6.45 to 7.10 µg 25 ml⁻¹ broth whereas, in our findings gibberellins production was in the range of 25-60 µg ml⁻¹. So the selected Pseudomonas isolates were quite efficient for the production of gibberellins like substances. Karakoc and Aksoz (2006) stated that among G. fujikuroi and Pseudomonas sp. best yields of GA₃ was achieved after 12 or 18 days of incubation and Pseudomonas sp. has a shorter incubation period and simple cultural conditions compared to G. fujikuroi for GA₃ production.

In our study, the GA₃ yield was maximized (60 µg/ml) at 72 h of incubation at pH 7 at 28°C on a rotary shaker. It might be due to the reason that cultural conditions play an important role in cellular growth and production of biological activities by microorganisms as stated by Sanchez et al. 2010. The results showed that production of GA₃ is highly dependent on the optimization of some cultural parameters. The selection of suitable medium for the cultivation of microorganisms is very important to reveal their potential ability to produce secondary metabolites with biological activities. In our study all the selected isolates showed maximum production of gibberellins in nutrient broth after 72 h of incubation at 28°C which corroborate with earlier report of Karakoc and Aksoz (2006) on the production of GA₃ by Pseudomonas sp.

Many workers have been reported that gibberellins induced the synthesis of enzyme alpha-amylase and measurement of alpha-amylase activity is a direct method for assaying gibberellins because primary role of it to induce the synthesis of enzyme activity (Kaneko et al. 2002). In our study the efficient Pseudomonas isolates showed 45-51% alpha-amylase activity. Gibberellins are involved in the natural process of breaking dormancy and various other aspects of germination. Gibberellins in the seed embryo are believed to signal starch hydrolysis through inducing the synthesis of the enzyme α-amylase in the aleurone cells. α-Amylase then hydrolyses starch into glucose, that can be used in cellular respiration to produce energy for the seed embryo. Partially purified gibberellins like substance has been checked by thin layer chromatography having Rf value 0.79-0.80. However, Strelczzyk and Burdziej (1984) studied the production of gibberellins like substances by
mycorrhizal fungi, bacteria and actinomycetes by using chromatography and bioassays methods and they found highest biological activity exhibited in substances located at the Rf 0.2-0.4, as gibberellins production was found to be less in comparison of our findings.

Conclusion
It can be concluded that Pseudomonas sp. can be used as a potent bacterial source to obtain high yields and provide a good alternative for GA₃ biosynthesis. As compared to gibberellins obtained from G. fujikuroi or other alternatives, they are less expensive and less time consuming. These isolates can be used for the commercial production of gibberellins and to improve the growth and quality of plants.

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Conflict of Interest
There is no any conflict of interest in the submission of this manuscript. The manuscript does not contain experiments using animals or human studies.

References


