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MICROBIOLOGY

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, **a**lpha-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 HCI was added and mixture was incubated at 20^0C for 75 min. For blank, 5 ml of 5 per cent HCI was used. Absorbance was read at 254 nm and concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA_3, 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 28^0C for 24, 48, 72 h under shaken conditions (90rpm). Supernatant were harvested by centrifugation in multifuge XIR (Thermoscientific Germany) at 10,000 rpm for 30 minute at 4^0C 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 $NaHCO_3$ 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 shaked 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 Na_2SO_4. Evaporated the ethyl acetate extract on a rotary evaporator at 40^0C. 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 120^0C 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 30^0C 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 $Cacl_2$ (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 40^0C for 30 minutes. 5ml of 0.03N iodine solution was added to reaction mixture. Absorbance was measured at

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.

$$\alpha\text{- amylase activity: \%unit} = \frac{C - E}{C} \times 100$$

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.).

Trees	Site name	Total no of #composite soil sample	Total bacterial count (log cfu/g soil)	Total number of *Pseudomonas* isolates	Symbolic designation to isolates
Apple	Nagwain	5	6.1	13	AN-Nag
Pear	Sanyardi	4	6.3	17	PN-San

#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).

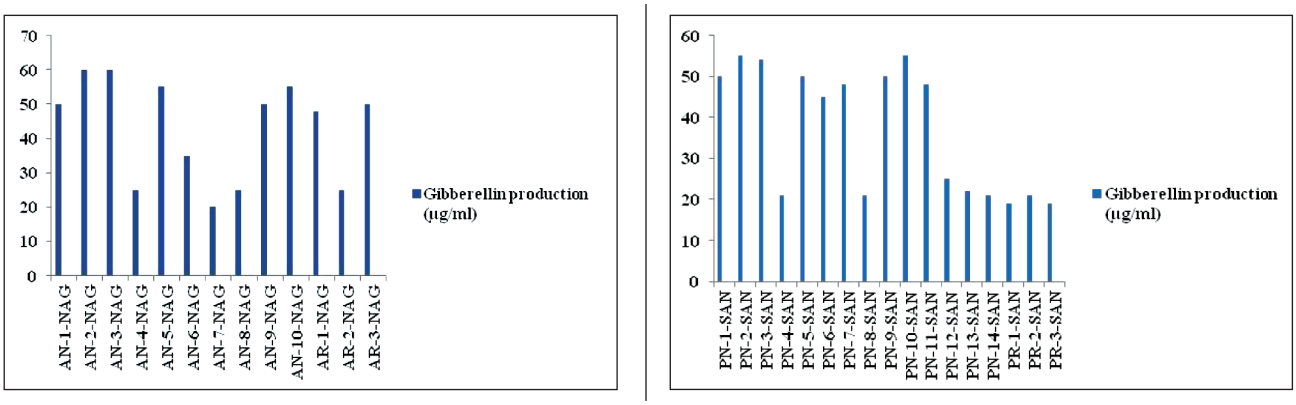

Fig. 1: Gibberellins production by fluorescent *Pseudomonas* isolated from (a) Apple and (b) Pear

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.

Media	Incubation period (h)															
	PN-4-SAN				PN-10-SAN				AN-10-NAG				AN-10-NAG			
	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean
Succinate media	128	146	189	154.3	124	179	217	173.3	145	169	190	168	120	147	189	152
King's media	90	128	150	122.7	74	117	140	110.3	60	90	120	90	70	120	170	120
Nutrient broth	154	211	336	233.7	177	250	300	242.7	150	171	235	185.5	134	196	220	183.3
Peptone water	120	147	200	155.7	74	103	130	102.3	70	120	147	112.3	96	131	169	132
Trypticane soya broth	96	117	151	121.3	115	124	176	138.3	84	120	169	124.3	96	147	184	142.3
Mean	117.6	149.8	205		112.7	154	192.6		101.8	134	172.2		103.2	148.2	186.4	
Effects CD 0.05					Effects CD 0.05				Effects CD 0.05				Effects CD 0.05			
Media 0.96					Media 0.96				Media 0.96				Media 0.96			
Interval 0.7					Interval 0.75				Interval 0.7				Interval 0.75			
Media×Interval 1.63					Media×Interval 1.63				Media×Interval 1.63				Media×Interval 1.63			

The homogeneity of the partially purified gibberellins along with GA_3 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).

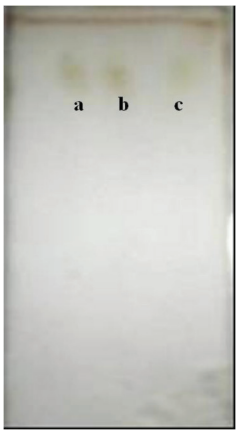

Fig. 2: Thin layer chromatographic pattern on silica gel-G of partially purified gibberellins of *Pseudomonas sp*. PN-4-SAN (a), PN-10-SAN (b) and AN-4-NAG (c)

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

Isolates	Solvent system	Spraying reagent	Color of spots	Rf value
PN-4-SAN	Isopropanol: NH_4OH: Water (10:1:1)	Water: H_2SO_4 (30:70)	Grey	0.80
PN-10-SAN	Isopropanol: NH_4OH: Water (10:1:1)	Water: H_2SO_4 (30:70)	Grey	0.79
AN-2-NAG	Isopropanol: NH_4OH: Water (10:1:1)	Water: H_2SO_4 (30:70)	Grey	0.79
AN-4-NAG	Isopropanol: NH_4OH: Water (10:1:1)	Water: H_2SO_4 (30:70)	Grey	0.80

$$\text{Rf value calculated as} = \frac{\text{Distance run by the compound}}{\text{Distance run by the solvent front}}$$

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 GA_3 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.

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

***Gibberellins of (µg/ml) of gibberellic (GA_3) as calibrated from standard curve (100-1000 µg/ml).**

$$\text{alpha amylase activity (\% units)} = \frac{C - E}{C} \times 100\ (\%)$$

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 (GA_3) 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 GA_3 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 GA_3 production.

In our study, the GA_3 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 GA_3 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 GA_3 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 acitivity. 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 R_f value 0.79-0.80. However, Streelczyk 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 GA_3 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.

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