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Extraction and evaluation of gibberellic acid from *Pseudomonas* sp.: Plant growth promoting rhizobacteria

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Abstract
In current investigation thirty isolates of Fluorescent *Pseudomonas* sp. were isolated from apple and pear rhizosphere and characterization of 30 native fluorescent *Pseudomonas* strains was done phenotypically (Gram's staining, oxidase, catalase test, gelatin liquification, denitrification test, gelatin liquification, MR/VP, citrate utilization, indole production) and genotypically (16S rRNA). They were investigated for gibberellins production. The range of the production of gibberellins was found 116.1 -485.8µg/ml. Two isolates *viz.*, An-1-kul and An-13-kul were selected on the basis of higher production of gibberellins. The gibberellins produced by best isolates (An-1-kul and An-13-kul) were extracted and partially purified and characterized by TLC (thin layer chromatography) and estimated via high performance liquid chromatography (HPLC) in liquid culture. Extracted gibberellins gave gray coloured spot with Rf value 0.78 and 0.79 of gibberellins for An-1-kul and An-13-kul respectively. Gibberellins extracted from *Pseudomonas* sp. were further evaluated for alpha-amylase activity. The range of gibberellins production from fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul was 485.8µg/ml and 419.2µg/ml culture with a retention time of 2.35 and 2.32 respectively.

Keywords: *Pseudomonas* sp, PGPR, 16S rRNA, gibberellins, chromatography, HPLC

Introduction
Plant growth regulators such as gibberellins and cytokinins are important biotechnological and economical products. They are commonly used in agriculture; viticulture gardens and horticulture (Bandelier and Renaud, 1997) [4]. Gibberellins (GAs) are a large group of important diterpenoid acids among commercial phytohormones (Martin *et al*., 2000) [22]. They are endogenous hormones functioning as plant growth regulators and influencing a range of developmental processes in higher plants including stem elongation, germination, dormancy, sex expression and fruit senescence (Elezar and Escamilla, 2000; Gelmi and perez-Correa, 2000) [9, 10]. The GAs are naturally produced by higher plants, fungi and bacteria and regulate plant growth and development. They are typical secondary metabolites in microorganisms; however, they act as endogenous hormones in higher organisms such as plants. Over the past 20 years, many gibberellins have been defined using modern analytical techniques and 126 GAs have been identified in plants, fungi and bacteria (Gomi and Matsuoka, 2003) [11]. Gibberellic acid (GA3) is the main product of gibberellins in fungi and bacteria (Bruckner and Blecschmidt, 1991) [7]. It is a terpenoid hormone that is an important phytohormone regulating plant growth and development. It is used in agriculture, nurseries, green houses, viticulture, cosmetic sectors and beer industry (Kumar and Lonasane, 1986) [19]. It is a high-value industrially important biochemical selling at $27-36/g on the international market (Avinash *et al*., 2003) [2]. Currently, GA3 is largely produced by submerged fermentation of the fungus *Gibberella fujikuroi* on an industrial scale (Santos *et al*., 2003) [25]. It is also synthesized from several bacteria such as *Azotobacter*, *Pseudomonas* and *Azospirillium* in culture medium and from wild strains of fungi such as *Sphaceloma* sp., *Phaeosphaeria* sp., and *Neurospora* species (Rademacher, 1994) [24]. Production of GA3 is considerably influenced by cultural conditions. Some of the important factors in obtaining high yields of the GA3 include pH, temperature, incubation time and conditions such as optimization of the fermentation media (Basoacok and Aksoz, 2004; Kahlon and Malhotra, 1986) [6, 14]. Therefore, present studies have been aimed at estimation and extraction of gibberellins from *Pseudomonas* strains through High-performance liquid chromatography (HPLC).

Materials and Methods
Isolation of fluorescent *Pseudomonas* species: Isolation of fluorescent *Pseudomonas* sp. was made from apple rhizosphere in Kullu district of Himachal Pradesh (India).

The rhizosphere soil particles loosely adhering to the roots were gently teased out with small root pieces and mixed well. The soil thus obtained was crushed in a sterile mortar and pestle and shaken with 100 ml of sterile distilled water for 10-20 min. to obtain standard soil suspension. Isolation of fluorescent *Pseudomonas* sp was made by following the serial dilutions and pour plate method using the specific King's B medium (King *et al*., 1954) [17]. *Pseudomonas* sp. isolates were identified on the basis of morphological, biochemical and physiological tests viz., Gram's staining, catalase test, oxidase test, gelatin liquification, denitrification test, IMVIC test (Aneja, 2003) [1] growth at optimum temperature i.e. 4^0C and 41^0C, and gibberellins production.

Molecular characterization of fluorescent Pseudomonas sp. by 16S rRNA technique Genomic Deoxyribonucleic acid (DNA) was extracted with DNA isolation kit (Bangalore GeNei), and the 16S rDNA gene was amplified by PCR using the set of primers FP-1(GGTCTGAGAGGATGATCAGT) and RP-1(TTAGCTCCACCTCGCGGC) in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100). The PCR amplification was carried out in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100) with a total of 35 cycles. Amplification was as follows: denaturation at 94^0C for 1 min, annealing at 55^0C for 2 min, and elongation at 72^0C for 2 min (Widmer *et al*., 1998; Weisburg *et al*., 1991) [33, 32]. For DNA sequencing, eluted amplified DNA products of two selected *Pseudomonas* sp. (An-1-kul, An-13-kul) was first purified followed by sequencing in Bioserve Private limited (Hyderabad, India). Similarity searches of the Genbank database were performed with BLAST.

Estimation of gibberellins: The gibberellins were estimated calorimetrically by standard method (Holbrook *et al*., 1961) [12] with slight modifications. To 15 ml of supernatant, 2 ml of zinc acetate reagent (21.9g zinc acetate + 1 ml of glacial acetic acid and volume was made upto 100 ml with distilled water) was added. After 2 minutes, 2 ml of potassium ferrocyanide (10.6% in distilled water) was added and was 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 20^0 C for 75 min. For blank 5 ml of 5 per cent HCL was used. Absorbance was read at 254 nm concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA3, Hi-media) as standard (100-1000 µg/ml).

Extraction of gibberellins: Gibberellins were extracted and separated from supernatant with diethyl ether and detected for homogeneity by thin layer chromatography (TLC). The isolates viz., An-1-kul and An-13-kul were grown in nutrient broth for 72 h at 28 $\pm$ 2^0C under shake conditions (90rpm) and supernatants were harvested by centrifugation at 10,000 rpm for 20 minutes (Mahadvan and Sridhar, 1986) [21].

100 ml of supernatant mixed with 250 ml of saturated $NaHCO_3$ solution in separatory funnel

↓

Extracted with 300ml ethyl acetate (2X) and organic phase was discarded

↓

Aqueous layer Acidified to pH 2.5 with 5 N HCl

↓

Equal volume of ethyl acetate added and was 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 anhydrous Na_2SO_4

↓

Evaporated the ethyl acetate extract on a vacuum rotary evaporator at 40^0 C to dryness

↓

Residue dissolved in 2 ml of ethanol for thin layer chromatography and in 2 ml of water

↓

with Tween 20 for bioassay

Thin layer Chromatography: TLC of auxins extracts was carried out on silica gel G pre-coated aluminium plates of Merck brand. 100 µl of the extracted gibberellins dissolved in methanol were spotted on silica gel G along with gibberellins (GA3) standard. Plates were kept in solvent 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). Plates were observed for presence of gray colour spot and R_f value was also calculated using following equation:

$$R_f = \frac{\text{Distance covered by solute}}{\text{Distance run by the solvent}}$$

Bioassay of Gibberellins: Bioassay of gibberellins was done by α- amylase release test (Lindow *et al*., 1998) [20]. Enzyme activity expressed per unit of reducing starch and dose response curve prepared with GA3 to calculated gibberellins in test solution.

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

Where;
C = OD at initial starch solution
E = OD at the end of the reaction

Partial purification of extracted gibberellins by column Chromatography: Ethyl acetate extracted sample from *Pseudomonas aeruginosa* An-1-kul and An-13-kul were used for Sephadex G-25 column chromatography for gibberellins.10g of Sephadex G-25 was soaked in distilled water for overnight and was boiled for 4 h. for complete swelling of the gel. Gel was washed and equilibrated with (0.2M, pH 7.2) tris-HCl buffer and column (50×1.5cm) was packed and equilibrated with tris-HCl buffer. 5ml sample was applied to column and eluted with tris-HCl buffer with flow rate 3ml/12minute. 3ml fractions were collected and observance was detected at different wavelengths (220nm, 240nm, 260nm, 280nm, 300nm, 320nm) for gibberellins.

Estimation of plant growth regulators were done in each fraction and fraction showing production of plant growth regulators were pooled and lyophilized and stored at -20°C for further use.

HPLC Analysis: Analytical method for estimation of gibberellins was standardized on binary Waters HPLC Unit using Waters HPLC pump 515 with Waters spherisorb 5µm ODS 2 analytical column (4.6 x 250mm) and dual lambda absorbance detector 2487. HPLC system was initially run for overnight with properly cleansed HPLC grade methanol: water (80:20 v/v). HPLC chromatograms were produced by injecting 10µl of filtered extracts of gibberellins onto Water made (C18 silica column 25 X 4.6mm) in a chromatograph equipped with a differential UV detector absorbing at 270nm. Mobile phase was methanol and water (80:20 v/v) flow rate (1ml/min) for 15 minutes. RT for peaks was compared to those of authentic standards of gibberellins (GA3, CDH) and quantification was done by comparison of peak heights (Khalifah *et al*., 1966) [16].

Results

Morphological characterization fluorescent *Pseudomonas* sp.: In the present study, thirty isolates of Fluorescent *Pseudomonas* sp**.** were isolated from the rhizosphere soil of apple. The results of the biochemical tests performed for the identification of the effective native isolates of Fluorescent *Pseudomonas* sp. showed that all the isolates produced similar results with regard to gram staining (negative), gelatin liquefaction (positive), catalase test (positive), oxidase test (positive) and fluorescent pigmentation (positive). In this study, all the ten identified gram-negative *Pseudomonas* isolates were found to be green fluorescent on King's B medium under ultraviolet light. The biochemical tests i.e. gelatin liquefaction, denitrification, catalase test, oxidase test, gibberellins production, further confirmed that these isolates belong to genus *Pseudomonas* (Table 1).All the isolates showed positive results in gibberellins production. Among the isolates An-1-kul produced more quantity (485.8µg/ml) of gibberellins followed by An-13-kul (419.8µg/ml) and therefore selected for further study.

Molecular characterization: Using the universal primer set, DNA fragment of the 16S rDNA gene was amplified by PCR. The PCR amplified 16S rDNA region was sequenced and sequence data were analyzed by BLAST and the nearest match from the GenBank data was reported. DNA sequencing and phylogenetic analysis revealed that strain An-1-kul and An-13-kul was shown to have a 98% similarity with *Pseudomonas aeruginosa* DK2 (Accession number NC_018080.1) and 99 % similarity with *Pseudomonas aeruginosa* PA7 (Accession number NC_009656.1) respectively to the sequences within GenBank.

Accession number: Sequences were submitted to Gen Bank and accession numbers were assigned for isolate KF682468, KF682469 to An-1-kul and An-13-kul respectively.

Thin layer chromatography and bioassay analysis of gibberellins: Gibberellin was extracted from the culture of fluorescent *Pseudomonas* using ethyl acetate. TLC analysis of gibberellins compounds obtained from both the culture (An-1-kul and An-13-kul) confirmed the presence of gibberellins with Rf value 0.78 and 0.79 respectively identical to the Rf of the standard (0.85 Partially purified extracts of supernatants of *Pseudomonas aeruginosa* An-1-kul and An-13-kul concentration of gibberellins is about upto 34.02-38.28% respectively and it has α – amylase activity (Table 2). This indicates that gibberellins are present in the partially purified samples.

Table 1: Physiological and biochemical characteristics of selected isolates of fluorescent *Pseudomonas* isolate

Isolate	-Characteristics - Growth (on King's B agar)												
	Gram's staining	Denitrification	Oxidase	Catalase	Gelatin liquification	*MR /VP	Citrate Utilization	Indole	4^0C	25^0C	37^0C	41^0C	Production of Gibberellins**
An-1-Kul	-	+	+	+	+	-	-	-	-	+	+	+	485.8
An-2-Kul	-	+	+	+	+	-	-	-	-	+	+	+	252.2
An-3-Kul	-	+	+	+	+	-	-	-	-	+	+	+	203.5
An-4-Kul	-	+	+	+	+	-	-	-	-	+	+	+	196.4
An-5-Kul	-	+	+	+	+	-	-	-	-	+	+	+	303.5
An-6-Kul	-	+	+	+	+	-	-	-	-	+	+	+	205.8
An-7-Kul	-	+	+	+	+	-	-	-	-	+	+	+	316.1
An-8-Kul	-	+	+	+	+	-	-	-	-	+	+	+	160.0
An-9-Kul	-	+	+	+	+	-	-	-	-	+	+	+	143.5
An-10-Kul	-	+	+	+	+	-	-	-	-	+	+	+	165.4
An-11-Kul	-	+	+	+	+	-	-	-	-	+	+	+	261.2
An-12-Kul	-	+	+	+	+	-	-	-	-	+	+	+	174.8
An-13-Kul	-	+	+	+	+	-	-	-	-	+	+	+	419.2
An-14-Kul	-	+	+	+	+	-	-	-	-	+	+	+	275.4
An-15-Kul	-	+	+	+	+	-	-	-	-	+	+	+	324.1
An-16-Kul	-	+	+	+	+	-	-	-	-	+	+	+	292.9
An-17-Kul	-	+	+	+	+	-	-	-	-	+	+	+	207.7
An-18-Kul	-	+	+	+	+	-	-	-	-	+	+	+	299.6
An-19-Kul	-	+	+	+	+	-	-	-	-	+	+	+	212.5
Ar-1-kul	-	+	+	+	+	-	-	-	-	+	+	+	267.3
Ar-2-kul	-	+	+	+	+	-	-	-	-	+	+	+	232.2
Ar-3-kul	-	+	+	+	+	-	-	-	-	+	+	+	361.1
Ar-4-kul	-	+	+	+	+	-	-	-	-	+	+	+	299.9
Pn-1-Kul	-	+	+	+	+	-	-	-	-	+	+	+	419.2
Pn-2-Kul	-	+	+	+	+	-	-	-	-	+	+	+	116.1
Pn-3-Kul	-	+	+	+	+	-	-	-	-	+	+	+	233.2

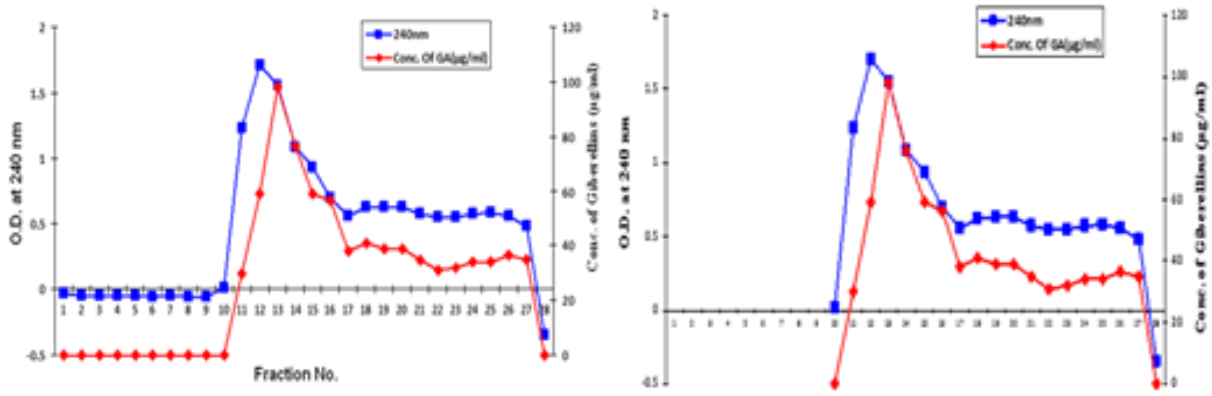
Pn-4-Kul	-	+	+	+	+	-	-	-	-	+	+	+	251.5
Pn-5-Kul	-	+	+	+	+	-	-	-	-	+	+	+	309.6
Pr-1-Kul		+	+	+	+	-	-	-	-	+	+	+	180.6
Pr-2-Kul		+	+	+	+	-	-	-	-	+	+	+	254.7
CD.05													16.41

*MR/VP- Methyl red / Voges Proskauer ;(+) indicates positivity of test; (-) indicates negativity of test
Extracellular Production of plant growth regulators i.e. Gibberellinsexpressed in terms of concentration (µg/ml) in 72h old supernatant as calibrated from the standard curve of gibberellic acid (GA3) 100-1000(µg/ml)

Table 2: Effect of extracted gibberellins like substances from *P. aeruginosa* An-1-kul and An-13-kul on of α-amylase release activity from rice seed without embryo

S. No.	Extracted gibberellins	End of reaction A_{700}	α-amylase activity(% units)
1.	Control	0.325	20
2.	An-1-kul	0.291	34.02
3.	An-13-kul	0.280	38.28
4.	Starch (C)	0.390	-

High Performance Liquid Chromatography Analysis: In the present study we measured the gibberellins amount of two fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul through HPLC and spectrophotometer. Extracted gibberellins from strains An-1-kul and An-13-kul showed optimum peak value at 240 and 280nm and showed presence of gibberellins in fraction number 10 to 17 and 13-22 respectively in the main peak (Figure 1). Maximum excitation wavelength for gibberellins was 290nm (Vessey, 2003) [31].



a. Column chromatography of An-1-kul **b.** Column chromatography of An-13-kul

Fig 1: Sephadex G-25 column chromatographic profile of ethyl acetate extracted gibberellins from *P. aeruginosa* An-1-Kul (a) and An-13-kul (b) at wavelength 240 nm

Gibberellins extracted from the culture of fluorescent *Pseudomonas* isolates showed a similar peak to that of the standard gibberellins (Hi-media) in HPLC analysis. The retention time of the extracted gibberellins from both the isolates was 2.352 and 2.322, which matched the retention time of authentic gibberellins of 2.169min (Figure 2).

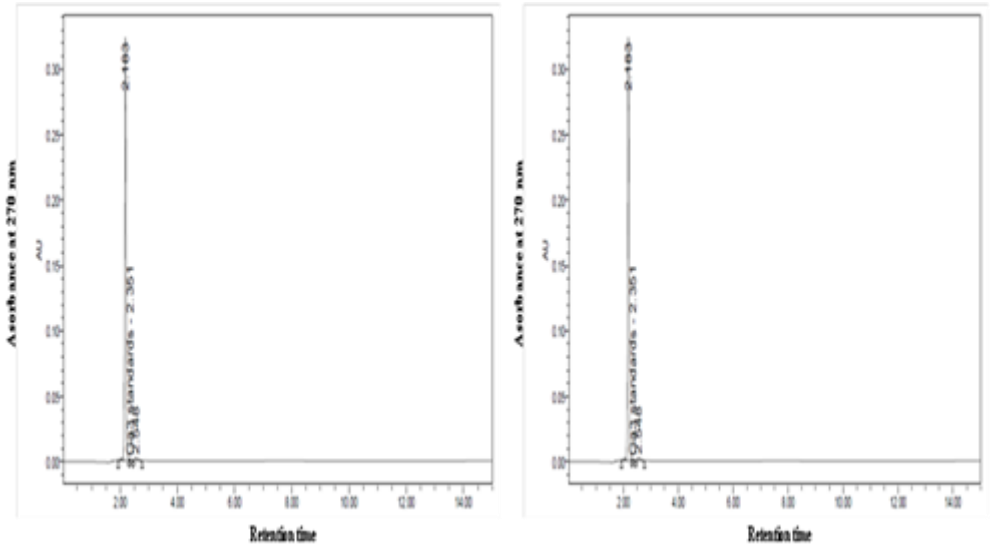


Fig 2: HPLC chromatograph of partially purifed gibberellins from *P. aeruginosa* (a) An-1-kul (b) An-13-kul

The range of gibberellins production from fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul was 0.68µg/ml and 0.64 µg/ml culture respectively (Table 3).

Table 3: HPLC chromatogram characteristics of partially purifed gibberellins from *Pseudomonas* aeruginosa An-1-kul and An-13-kul

S. no.	Name of sample	Retention Time (RT)	% Area	Conc. (µg/ml)	Peak Height
1.	An-1-kul	2.351	97.97	0.68	894
2.	An-13-kul	2.322	100.00	0.64	1010

Discussion

The versatile, predominant and desirable organisms i.e. *Pseudomonas* sp. were isolated. It may attribute to specific choice of media employed for isolation, identification and characterization i.e. nutrient agar and King's B medium. Several others hence supported to use King's B medium for isolation of fluorescent *Pseudomonas* sp. by far the most evidence for the positive effects of PGPR points to bacteria-mediated changes in root growth and morphology (Kloepper *et al*., 2007; Sharma *et al*., 2014) [18, 26]. With the production of different phytohormones like indole-3-acetic acid (IAA), gibberellic acid and cytokinins PGPR can increase root surface and length and promote in this way plant development (Azcon and Barea, 1975; Sharma and Kaur, 2014) [3, 27].

Gibberellic acid (GA_3) and gibberellins like compounds were identified in cultures of various species of *Azotobacter*, *Pseudomonas* and *B*acillus (Katznelson and Cole, 1965) [15]. Microbial synthesis of gibberellins and other metabolites is an important factor in soil fertility (Bashan and Holguin, 1997) [5]. *Azospirillum* sp. is plant growth promoting bacteria whose beneficial effect to be partially due to production of phytohormone including gibberellins (GA_3).Gibberellins are endogenous hormone functioning as plant growth regulators and influencing a range of developmental processes in plants including stem elongation, germination, dormancy, sex expression and fruit senescence. Large scale application of best selected and efficient indigenous strains of fluorescent *Pseudomonas* sp. in specific replant site may be able to manage and control replant disease effectively by increasing the concentration of these hormones in plant rhizosphere that in turn may be effective in increasing root size and volume and also help the young plant to establish properly under these hard and harsh conditions (Verma *et al*., 2001) [30].

Plant growth regulators (PGR_S) like gibberellins, kinetin and indole acetic acid (IAA) were detected by thin layer chromatography (TLC and HPLC) and other conventional methods in culture filtrates of *Azotobacter chroococcum* strains (Jones and Varner, 1967; Sharma *et al*., 2014) [13, 28]. Gibberellins induce the synthesis of enzyme alpha amylase and measurement of alpha-amylase activity is a direct method for assaying gibberellins because primary role of it is to induce the synthesis of enzyme activity (Noggle and Firtz, 1976; Crozier and Reeve, 1977) [23, 8]. HPLC is a powerful method for simplifying the plant hormone identification in comparison with mass spectrophotometric method. In HPLC method the goal is to promote the measurable Gibberellins and reduce the amount of unexpected substances in samples. Plant growth promoting rhizobacteria (PGPR) are group of bacteria that can actively colonize plant roots and can enhance plant growth directly or indirectly. Fluorescent *Pseudomonas* sp. are emerging as largest and potentially most promising group of PGPR bacteria that are involved in plant growth enhancement and plant disease control. Production of Gibberellins is one of the main reasons to promote yield because of inoculation with this bacteria (Sharma *et al*., 2014) [29]. The phytohormone Gibberellins play a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence.

Concluding remarks

The fluorescent *Pseudomonas* sp. are plant beneficial rhizobacteria with all the direct and indirect plant growth promoting activities like plant growth regulators such as auxins, cytokinins and gibberellins. Plant growth regulator production is an important secondary metabolite of fluorescent *Pseudomonas* as it directly involves in the growth and development of plant root, shoots. It is worth to note that strains belonging to *Pseudomonas aeruginosa* are considered safe for use in biotechnological applications. From this experimental study, it can be concluded that extraction and bioassay is important and valuable for evaluation of putative PGPRs and for their products like plant growth regulators and further detailed work is necessary to define the conditions under which PGPR activity and their products can be detected.

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References

1. Aneja KR. Experiments in microbiology, plant pathology and biotechnology. 4th rev. ed. New Age International: New Delhi. 2003; 607.
2. Avinash CS, Shahid A, Agarwal DK. Screening of potential gibberellin producing *Fusarium* strains for the hybrid rice production. Food, Agriculture & Environment. 2003; 1:250.
3. Azcon R, Barea JM. Synthesis of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* and *Azotobacter beijerinckii* related to effects produced on tomato plants. Plants and Soil. 1975; 43:609-619.
4. Bandelier S, Renaud R. Production of gibberellic acid by fed-batch solid state fermentation in an aseptic pilot scale reactor. Process in Biochemistry. 1997; 32:141.
5. Bashan Y, Holguin G. *Azospirillum*-plant relationships: environmental and physiological advances. Canadian Journal of Microbiology. 1997; 43:103-121.
6. Basoacok KS, Aksoz N. Optimization of carbon-nitrogen ratio for production of gibberellic acid by *Pseudomonas* sp. Polish Journal of Microbiology. 2004; 53:117.
7. Bruckner B, Blecschmidt D. The Gibberellin fermentation. Critical Review in Biotechnology. 1991; 11:163.
8. Crozier A, Reeve DR. The application of high performance liquid chromatography to analysis plant hormone. In: Pielt PE, editor. Plant growth regulation: Plant growth substances. University of Lausanne, Switzerland: Springer-Verlag, New York. 1977; 67-76.
9. Elezar M, Escamilla S. Optimization of GA3 production by immobilized *G. fujikuroi* mycelium in fluidized bioreactors. Journal of Biotechnology. 2000; 76:147.
10. Gelmi C, Perez-Correa R. Solid substrate cultivation of *G. fujikuroi* on an inert support. Processes in Biochemistry. 2000; 35:1227.

11. Gomi K, Matsuoka M. Gibberellin signaling pathway. Current Opinion Plant Biology. 2003; 6:489.
12. Holbrook AA, Edge WLW, Bailey F. Spectrophotometric method for determination of gibberellic acid in gibberellins. ACS Washington, D.C. 1961; 159-167.
13. Jones RL, Varner JE. The bioassays of auxins. Planta. 1967; 72:155-167.
14. Kahlon SS, Malhotra S. Production of gibberellic acid by fungal mycelium immobilized in sodium alginate. Enzyme Microbiology Technology. 1986; 8:613.
15. Katznelson H, Cole SE. Production of gibberellins like substances by bacteria and actinomycetes. Canadian Journal of Microbiology. 1965; 11:733-741.
16. Khalifah RA, Lowell N, Lewis Coogins CW. Differentiation between indole acetic acid the Citrus auxin by column chromatography. Plant Physiology. 1966; 41:208-210.
17. King EO, Wood MK, Raney DE. Two simple media for the demonstration of pyocyanin and fluorescein. Journal of Laboratory Clinical Medicine. 1954; 44(2):301-307.
18. Kloepper JW, Reddy SM, Rodreguez-Kabana R, Kenney DS, Kokalis- Burelle N, Ochoa NM. Application for Rhizobacteria in Transplant Production and Yield Enhancement. Acta Horticulture. 2007; 631:217-229.
19. Kumar PKR, Lonsane BK. Immobilized growing cells of *Gibberella fujikuroi* P-3 for production of gibberellic acid and pigment in batch and semi-continuous cultures. Applied Microbiology and Biotechnology. 1986; 28:537.
20. Lindow SE, Desurmont C, Elikins R, McGourtyg, Clark E, Maria TB. Occurrence of indole-3-acetic-acid producing bacteria on pear trees and their association with fruit russet. Journal of American Phytopathology. 1988; 88(11):1150-1157.
21. Mahadvan A, Sridhar RK. Method in Physiology, 3rd eds. Plant Physiology. 1986; 282.
22. Martin RF, Domenech C, Olmedo EC. Ent-Kaurene and squalene synthesis in *F. fujikuroi* cell-free extracts. Phytochemistry. 2000; 54:723.
23. Noggle R, Firtz J. Introductory Plant Physiology, Niall India Pvt. Ltd. Jay Print Press. New Delhi, 1976.
24. Rademacher W. Gibberellin formation in microorganisms, Plant Growth Regulator. 1994; 15:613-616.
25. Santos MGE, Couto MCM, Rebelo HL. Ion-selective electrodes based on metalloporphyrins for gibberellic acid determination in agricultural products. Anal of Bioanalytic Chemistry. 2003; 375:511-516.
26. Sharma S, Kaur M. Effect of different nutrient sources on the production of plant growth regulators (PGRs) by fluorescent *Pseudomonas* species. Indian Journal of Applied Ressearch. 2014; 4(10):468-471.
27. Sharma S, Kaur M, Srivastava DK, Gambhir G. *In vitro* evaluation of plant growth regulators on tissue culture bioassay produced by *Pseudomonas* species. International Journal of Agriculture, Environment & Biotechnology. 2014; 4(7):137-147.
28. Sharma S, Prashad D, Kaur M. Isolation of fluorescent *Pseudomonas* strain from temperate zone of Himachal Pradesh and their evaluation as plant growth promoting rhizobacteria (PGPR). The Bioscan. 2014; 9(2):323-328.
29. Sharma S, Verma PP, Kaur M. Isolation, purification and estimation of IAA from *Pseudomonas* sp. using High performance liquid chromatography. Journal of Pure and Applied Microbiology. 2014; 8:3203-3208.
30. Verma A, Kukreja K, Pathak DV, Suneja S, Narula N. *In vitro* production of plant growth regulators (PGRs) by *Azotobacter chroococcum.* Indian Journal of Microbiology. 2001; 41:305-307.
31. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil. 2003; 255:571-586.
32. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 1991; 173:697-703.
33. Widmer F, Seidler RJ, Gillevet PM, Watrud LS, DiGiovanni GD. Highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (*Sensu Stricto*) in environmental samples. Applied Environment Microbiology. 1998; 64(7):2545-2553.