

## Isolation and Characterization of Novel Apple Rhizosphere Bacterium as Fungal Antagonist

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Strain An-F was isolated from the rhizosphere soil of replant site of apple orchard at Shimla district H.P. and identified as *Pseudomonas aeruginosa* on the basis of biochemical tests and by 16S rRNA sequences. This bacterium exhibits a broad-spectrum antifungal activity towards phytopathogenic fungi. The antifungal metabolite by An-F was extracted and characterized using TLC and HPLC (High pressure liquid chromatography). Production of siderophores, phosphatase, HCN, ammonia, indole-3-acetic acid (IAA), gibberellins and cytokinins in An-F were determined. The antifungal metabolite produced by An-F has been identified as 2, 4-diacetylphloroglucinol on the basis of TLC and HPLC data. Present study reports the production of 2,4-diacetylphloroglucinol as well as IAA, gibberellins and cytokinins for the first time by a saprophytic *P. aeruginosa* strain An-F isolated from the replant site of apple. Because of the production of siderophore, growth hormone, protease and phosphatase and its innate fungicidal potential, this strain can be used as biofertilizer and antagonist against a range of phytopathogenic fungi.

**Key words:** Replant site, *Pseudomonas aeruginosa*, 2, 4-Diacetylphloroglucinol, Biocontrol.

Apple (*Malus domestica*) replant disease (ARD) is distributed worldwide and is often encountered in establishing new orchards on old sites (Yao *et al.*, 2006). Symptoms include death of fine feeder roots, stunted growth above- and below-ground, and reduced fruit yields (Rumberger *et al.*, 2007). Causes of replant disease are fungi, bacteria, actinomycetes, nematodes, and their interactions. Other causes cited by researchers include fungi from the following genera: *Pythium*, *Thielaviopsis*, *Rosellinia*, *Phytophthora*, *Cylindrocarpon*, *Fusarium*, *Rhizoctonia*, *Penicillium* and *Alternaria* (Rutkowski *et al.*,

2000b; Benizri *et al.*, 2005). Certain fluorescent pseudomonads from soil have been shown to promote plant growth by inhibiting bacteria and fungi that are deleterious to plants. The production of antibiotic substances by some strains has been recognized as a major factor in the suppression of many root pathogens. In this study, we report on the isolation of a fluorescent *Pseudomonas* strain that can inhibit a number of plant root pathogens by producing DAPG. DAPG is a phenolic compound displaying a remarkably broad spectrum of toxic activity against bacteria, fungi and nematodes. It is one of the three phloroglucinols that have been isolated from fluorescent pseudomonads. We describe the isolation of this compound and also the development of an HPLC assay for its quantitative detection in culture media.

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## MATERIALS AND METHODS

### Bacterial strain

*Pseudomonas aeruginosa* strain An-F (NCBI GeneBank accession no. KJ522924) isolated from the rhizosphere of apple of Shimla district (H.P.). The *Pseudomonas* culture was maintained in 20% glycerol at -20°C and was revived on nutrient agar and employed for the present study. Antifungal activity was checked by well plate assay method (Vincent, 1947) using dual culture technique against *Dematophora necatrix*, *Fusarium oxysporum*, *Phytophthora cactorum* and *Pythium ultimum* phytopathogens isolated from the replant site of apple rhizosphere. On one side of pre-weighed sterilized malt extract agar (MEA) plates, 72 h old culture bit of indicator fungi was placed. On the other side of plate, 100 µl of 72 h old cell free supernatant of bacterial strain was added to well. 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.

### Detection of siderophore, phosphatase, hydrogen cyanide and ammonia production.

Quantitative detection of siderophore production in liquid medium of Chrome-azurole-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). Estimation of phosphorous (Bray and Kurtz, 1945 and Olsen *et al.*, 1954) was done quantitatively in PVK broth supplemented with 5.0 g/l tri-calcium phosphate (TCP) and solubilization of phosphorous was calculated using standard curve of  $\text{KH}_2\text{PO}_4$  (100-1000 µg/ml). For estimation of hydrocyanic acid (HCN) production by *Pseudomonas* sp., a color change in the sodium picrate containing filter paper strip was observed from yellow to orange brown to dark brown (Bakker and Schippers 1987). For the detection of ammonia production, the method of Lata & Saxena (2003) was used. *Pseudomonas* isolate was grown in 5ml of peptone water in tubes at 28±2°C for 4 days. After 4 days, 1ml of Nessler's reagent was added to each tube. Presence of very light brown color (+) indicates small amount of ammonia production and light brown (++) to orange brown color (++++) indicates large amount of ammonia production.

### Detection and estimation of plant growth promoting phytohormone production.

Quantitative measurement of auxins was done by colorimetric method (Gorden and Paleg, 1957) with slight modification. The gibberellin was estimated colorimetrically by the method of Holbrook *et al.*, (1961). For bioassay of cytokinin, the radish cotyledon expansion test was employed (Latham, 1971). The bioassay response (final weight - initial weight) was expressed as increase in weight of cotyledon.

### Effect of media on the production of antifungal activity

Six media i.e. King's broth, PPM broth, YM broth, Syncase broth, Succinate broth and nutrient broth were used for growth and production of antifungal metabolites for antifungal activity. Antifungal activity was checked by well plate assay method (Vincent, 1947) using dual culture technique against *Dematophora necatrix*, *Fusarium oxysporum*, *Phytophthora cactorum* and *Pythium ultimum*.

### Detection of antibiotic

Extraction of antibiotic was carried out by following the method described earlier (Rosales *et al.*, 1995) with slight modifications. Briefly, pre-inoculum of bacterium was prepared in 5 ml of KB broth and allowed to grow at 30°C for 19h. A 100ml of Syncase medium ( $\text{Na}_2\text{HPO}_4$  5gm,  $\text{K}_2\text{HPO}_4$  5gm, Glucose 5gm,  $\text{NH}_4\text{Cl}$  1.18gm,  $\text{Na}_2\text{SO}_4$  0.089,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  0.042gm,  $\text{MnCl}_2 \cdot \text{H}_2\text{O}$  0.004 gm, Casein hydrolysate 10gm dissolved in 1000ml of distilled water) was inoculated with 1% of pre-inoculum incubated at 30°C for 4 days on rotator shaker. The fermentation culture was centrifuged at 3500 rpm for 20 minutes. The collected supernatant of *Pseudomonas* sp. was acidified to pH 2.0 with 1N HCL. Extraction was done with equal volume of ethyl acetate. Ethyl acetate fraction was reduced to dryness in vacuum. Residue was redissolved in 2 ml methanol separately. 10 µl samples were chromatographed on Silica-G TLC plates and Chloroform: methanol (50:50) 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.

**HPLC apparatus**

HPLC system consists of a Waters pump 515, rheodyne injection valve with a 20 µl loop and an analytical column (250mm × 4.6mm, 5µm). The spectrophotometric dual > absorbance detector 2487. The reagents for mobile phase preparation i.e. acetonitrile : water acidified with 0.5% glacial acetic acid ( 50: 50) were of HPLC grade and all mobile phases used were filtered and degassed on a Millipore HPLC filtration system with 0.22 micron filter paper. All samples were run at a flow rate of 1ml/ min and detected at a wavelength of 270 nm.

**RESULTS AND DISCUSSION**

In this study, we demonstrated that 2, 4-diacetylphloroglucinol producing bacteria can be isolated from the apple rhizosphere and can be effective in the biocontrol of apple phytopathogenic fungus. The fluorescent bacterial antagonist was gram negative, rod shaped and produces greenish pigment on King’s B medium.

It was found to inhibit the mycelial growth of all fungal pathogens tested. *Pseudomonas aeruginosa* An-F showed maximum inhibition against *Fusarium oxysporum* 45.23%I followed by *Phytophthora cactorum* 40.38%I. It also inhibit *Dematophora necatrix* 20%I and *Pythium ultimum* 13.15%I (Table1).

Investigation on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development on new pesticide products. Production of siderophores, phosphatase, HCN, ammonia and plant growth regulators i.e. IAA, gibberellins and cytokinins evidently suggests a potential plant growth promoting ability of the strain An-F (Table 2).

Biosynthesis of bioactive substances is a specific property of some species or even some strains of microorganisms. This property depends greatly upon the conditions of cultivation of microorganisms. Although good growth may occur in many media, secondary metabolites may only be produced in a specific medium (Bentley and

**Table 1.** Potential of An-F for antifungal activity.

Strain	% inhibition			
	<i>Dematophora</i>	<i>Fusarium</i>	<i>Phytophthora</i>	<i>Pythium</i>
An-F	20	45.23	40.38	13.15

**Table 2.** Potential of An-F strains for plant growth promoting activities.

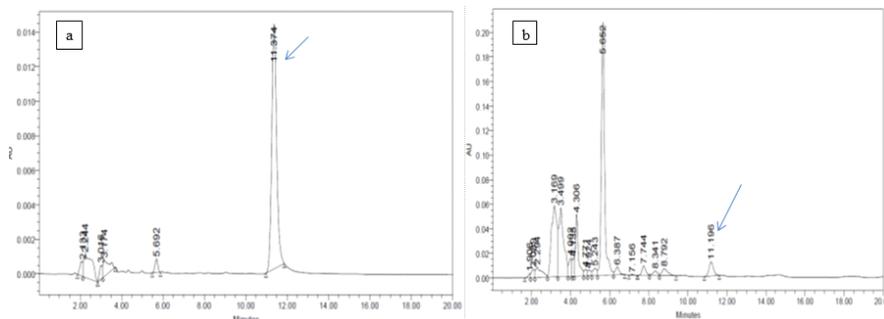
Siderophore %SU	Phosphate solubilizing µg/ml	HCN	Ammonia	Plant growth regulators		
				Auxins µg/ml	Gibberellins µg/ml	Cytokinins µg/ml
57.86	49	+	++++	20	630	23

**Table 3.** Effect of media on production of antifungal activity by An-F strain.

Fungus Pathogens	Media					
	Antifungal activity (mm dia. and % inhibition)					
	KMB	PPM	YM	Syncase Media	Succinate Media	NB
	%I	%I	%I	%I	%I	%I
<i>Dematophora necatrix</i>	0	23.91	27.14	31.11	28.84	20
<i>Fusarium oxysporum</i>	22.22	23.91	28.07	45.23	20.45	14
<i>Phytophthora cactorum</i>	0	25.71	28.84	47.87	25	40.38
<i>Pythium ultimum</i>	25	18.36	23.07	23.91	9.75	13.15

**Table 4.** Quantity of DAPG produced by *Pseudomonas aeruginosa* An-F.

S. no.	Name of sample	Retention Time (RT)	Conc. (µg/100ml)
1	DAPG standard	11.374	
2	An-F	11.196	3.02

**Fig. 1.** HPLC chromatograms of authentic 2, 4-diacetylphloroglucinol (a) and extract of *Pseudomonas aeruginosa* strain An-F (b) after four days of incubation at 30°C in Syncase broth.

apple phytopathogens. Maximum percent inhibition was showed by syncase media against all tested pathogens (Table 3). DAPG is an antibiotic known to be active against a broad spectrum of microorganisms and is involved in the suppression of many plant diseases (Defago 1993; Keel *et al.*, 1990; Keel *et al.*, 1992; Weller and Thomashow 1993). The metabolite DAPG extracted in ethyl acetate fraction of the An-F strain produced bright yellow colour spot at Rf value of 0.8 in the TLC plate. HPLC analysis of extract showed the presence of 2, 4-diacetylphloroglucinol when compared with reference compound (Table 4, Fig.1). Earlier, a number of strains of *Pseudomonas* have been shown to produce phloroglucinol (Broadbent *et al.*, 1976; Garagulya *et al.*, 1974). The severity of tobacco black root rot was reduced when soil was amended with phloroglucinol (Keel *et al.*, 1992). Phloroglucinol antibiotics are phenolic metabolites produced by bacteria with broad antibacterial and antifungal spectrum and phytotoxic properties (Thomashow and Weller 1996). Keel *et al.*, (1990) and Raaijmakers *et al.*, (1997) reported that phloroglucinol – producing strains of *P. fluorescens* have been shown to be effective against root pathogens viz., *Fusarium oxysporum* in tomato, *Thielaviopsis basicola* in tobacco and *Gaeumannomyces graminis* var. *tritici* in wheat.

Kiel, 1962). Development of medium that produce high yields of a desired secondary metabolite is still empirical to a considerable degree (Demain, 1973). In our study experiment on media was focused on the identification of a suitable growth medium for *Pseudomonas* stain An-F that can induce higher level of antifungal activity against

Beside this, phloroglucinol induced defense mechanism against fungal infection (Tomas Lorente *et al.*, 1989). Because of the innate potential of producing siderophore, DAPG, IAA, phosphatase and broadspectrum fungal antibiosis, the strain An-F can be used as biofertilizer as well as a biocontrol agent.

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