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Evaluation of plant growth promoting attributes and lytic enzyme production by fluorescent Pseudomonas diversity associated with Apple and Pear

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Abstract- Replant problem is very serious problem which suppress growth and yield of apple and pear in all major fruit growing areas of the world. Fluorescent *Pseudomonas* has potential to synthesize different secondary metabolites with diverse PGPR activities which enhance soil fertility and promote plant growth. Under the present study twenty six *Pseudomonas* strains were isolated from the rhizosphere of apple and pear plants of their normal and replant sites and found that the *Pseudomonas* count was more in normal site as compare to replant site. They were further screened for the production of various PGPR activities and proteolytic enzymes production viz. protease, chitinase and glucanase. AN-1-UHF, AN-5-UHF, PN-7-UHF and PN-13-UHF were selected on the basis of their higher PGPR attributes and proteolytic activities. These strains were further found to be very efficient in antimicrobial activities.

Index Terms- *Pseudomonas*, PGPR, Proteolytic enzymes, Antimicrobial

I. INTRODUCTION

Replant disease is a serious problem which suppress growth and yield of fruits trees in all major fruit growing areas of the world. Catska *et al*., [1] reported that a replant problem in apple and pear orchards appears to be related to soil and rhizosphere microflora. This problem is distributed worldwide and is often encountered in establishing new orchards on old sites [2]. Rhizobacteria that exert beneficial effects on plant growth and development are referred to as plant growth promoting rhizobacteria (PGPR) [3].The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include, the ability to produce plant growth regulators, asymbiotic N_2 fixation, antagonism against phytopathogenic microorganisms by production of siderophores, antibiotics and cyanide solubilization of mineral phosphates and other nutrients [4]. More specifically, the soil-borne fluorescent *Pseudomonas* have received particular attention because of their capacity to produce a wide range of enzymes and metabolites [5]. Antagonistic or biocontrol activity of fluorescent *Pseudomonas* may also be due to the production of different types of cell wall degrading enzymes like chitinase, protease / elastase and β 1,3 glucanase. These enzymes are supposed to degrade the cell wall of various bacterial and fungal plant pathogens. The possible solution to the management of replant problem of horticulture crops is to introduce appropriate novel and important indigenous best plant growth promoting bioagent conferring maximum plant growth promotion and disease controlling activities. So isolation and preliminary characterization of indigenous fluorescent *Pseudomonas* diversity producing these lytic enzymes is important.

II. EXPERIMENTAL SET-UP AND PROCEDURE

2.1 Isolation of Pseudomonas sp. from the Rhizosphere of apple and pear

Collection of Soil Sample

Soil samples were collected from the rhizosphere soil of pear and apple orchards in university campus field under the consideration of "Deptt. Of Fruit Breeding and Genetic Resources", University of Horticulture and Forestry, Nauni, Solan (H.P), India.

Isolation and Identification

Fluorescent *Pseudomonas* sp. were isolated from soil samples and the proposed isolates were characterized on the basis of morphological biochemical and physiological tests as prescribed in Bergey's manual of systematic bacteriology [6].

2.2 Screening of fluorescent Pseudomonas isolates for lytic enzymes

1) ESTIMATION OF PROTEASE ACTIVITY

a) Plate assay (Qualitative)

All *Pseudomonas* sp. strains were screened out for proteolytic activity by well plate assay method on 1% skim milk agar plates. Plates were incubated at 37^{o}C for 24-48 h and observed for proteolysis i.e. clear zone (mm dia) produced around the well (7 mm).

b) Quantitative Assay

Seventy two h old cell free culture supernatant screened as the crude enzyme source. Protease activity was assayed by the method of Morrihara et al., [7] using 2.0% casein in 0.05 M Tris buffer pH 7.0 as a substrate. Casein solution 1ml was incubated with an equal volume of suitable diluted enzyme preparation at

37^0C after 5 min the reaction was terminated by the addition of 3 ml of 5% of Trichloroacetic acid. The mixture was centrifuged at 5000 rpm for 10 min at 4^0C and the supernatant was assayed for protein concentration by use of modified Folin phenol method [8] and the change in the optical density was measured at 660 nm.

2) CHITINASE ACTIVITY

All isolates of *Pseudomonas* sp. were screened out for chitinase activity by well plate assay method on chitin agar plate in which 1% of colloidal chitin was used. Plates were incubated at 37°C for 5-7 days and observed i.e. clear zone (mm dia) produced around the well (7 mm) or culture bit.

2.3 Screening of lytic enzymes producing Fluorescent *Pseudomonas* isolates for the production of other plant growth promoting activities

3) PRODUCTION AND ESTIMATION OF PLANT GROWTH REGULATORS

a) Auxins

Measurement of auxins was done by quantitative colorimetric method [9] with slight modifications. 2 to 3 drops of orthophosphoric acid was added to 2 ml supernatant and added 4 ml of salper reagent (1 ml of 0.5 M $FeCI_3$ in 50 ml of 30 % $HCIO_4$: prepared fresh). This mixture was incubated for 1h in dark. Absorbance was measured at 535 nm. Concentration of indole acetic acid (IAA) was estimated by preparing calibration curve using indole acetic acid (IAA) as standard (10-100 µg/ml).

b) Gibberellins

The method of Holbrook *et al.* [10] with slight modifications was used for the determination of gibberllins. To 15 ml of supernatant, 2 ml of zinc acetate reagent (21.9 g 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°C for 75 min. For blank 5 ml of 5 per cent HCI was used. Absorbance was read at 254 nm concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA_3) as standard (100-1000 µg/ml).

c) Bioassay of Cytokinins

Radish cotyledons expansion bioassay test was employed [11] for estimation of cytokinins like substances the radish seeds (*Raphanus sativus* L. cv Japanese white) were germinated in total darkness for 48 h. at 28°C. After removing the seed coat, smaller cotyledons were transferred to sterilized Petri dishes containing the test solution/ distilled water (control)/ standard (kinetin) on filter paper strips. 12 cotyledons were placed in each Petri dish and were included at 25°C under fluorescent light for 3 days. Then cotyledon on filter paper strips in Petri dish were blotted, dried and weighed. The bioassay response (final weight-initial weight) was expressed as increase in weight of cotyledons. Concentration of cytokinins present in the extract was calculated of by preparing standard curve by using kinetin as standard (100-1000 µg/ml).

4) PHOSPHATE SOLUBILIZATION

a) Plate Assay (Qualitative)

Pikovskaya's agar plates [12] with known amount of inert phosphorous source were prepared. In well plate assay method, 48hr old supernatant of culture was kept and plates were incubated at 28 ± 2ºC for 48 hrs. Phosphate solubilization expressed in terms of mm diameter of solubilization zone produced around bit and phosphate-solubilizing efficiency (% PSE) was calculated. Plates were incubated at 28 ± 2ºC for 48hrs and observed for solubilization zone produced around the well. Phosphate solubilization expressed in terms of Phosphate solubilization efficiency (% SE) calculated from equation

Phosphate solubilization efficiency (% PSE) = $\frac{Z - C}{C} \times 100$

Where, Z= Diameter of yellow zone (mm)

C= Diameter of colony/bit (mm)

b) Liquid Assay (Quantitative Method)

Quantitative estimation of Phosphate solubilizing activity was done by spectrophotometric method [13, 14, 15]. To 5 ml supernatant 5 ml ammonium molybdate reagent (15 gm of ammonium molybdate in 400 ml distilled water and 342 ml of conc. HCl, total volume was made up to 1 L) was added along with shaking. 1 ml of working solution of cholorostanous acid (40%) (i.e. 0.5 ml of stock solution was added to 65.5 ml H_2O to make final 66 ml) was added. Stock solution was made by dissolving 10 gm $SnCl_2.2H_2O$/25 ml concentrated HCl. Total volume of reaction mixture was made 25 ml. O.D was measured at 660nm using red filter. Corresponding amount of soluble phosphorous was calculated from standard curve of potassium dihydrogen phosphate (KH_2PO_4; 0-10 ppm). Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization, which in turn represents µg/ml of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (0-10 µg/ml).

5) HCN PRODUCTION

Production of hydrogen cyanide (HCN) was checked according to Bakker and Schippers, [16]. All the Pseudomonas were streaked on prepoured plates of King's medium B amended with glycine(1.4 g/l). Whatman No.1 filter paper strips were soaked in 0.5 per cent picric acid in 2 per cent sodium carbonate and were placed in the lid of each petriplates and then Petriplates were sealed with parafilm and were incubated at 30oC for 1-4 days. Uninoculated control was kept for comparison of results. Plates observed for change of color of filter paper from yellow (-) to dark brown (+++) to orange brown (+++++).

6) AMMONIA PRODUCTION

For the production of Ammonia method of Lata [17] was used. *Pseudomonas* sp. were grown in peptone water (5 ml) in tubes. Tubes were incubated at 30°C for 4 days. After 4 days, 1ml of Neissler's reagent was added to each tube. Presence of faint yellow colour (+) indicated small amount of ammonia and deep yellow (++) to brown color (+++++) indicated large amount of ammonia production.

7) ANTIFUNGAL ACTIVITY

Antifungal activity of each test isolate was checked by standard well/bit plate assay method [18, 19] using malt extract agar (MEA) plates. Plates were incubated at 28 $\pm$ 2°C for 3 - 5 days and observed for inhibition zone produced around the well. Antifungal activity expressed in terms of mm diameter of clear zone around the bit/well and also expressed in terms of per cent inhibition of fungal mycelium as calculating from equation:

$$\frac{C - Z}{Z} \times 100 = \% \text{ inhibition}$$

C : growth of mycelium in control
Z : growth of mycelium in treatment

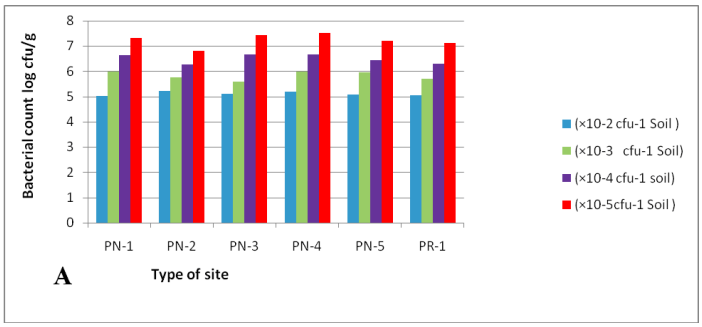
8) ANTIBACTERIAL ACTIVITY

It was checked by well assay method [19]. Plates were incubated at 30^0 C for 24- 48 h and inhibition of growth around the well and expressed as mm diameter of zone produced around the well.

III. RESULTS

Twenty six fluorescent *Pseudomonas* sp. were isolated and their log cfu/g was taken. Bacterial and *Pseudomonas* sp. were found to be more in normal site as compare to replant site. In this study significant difference in total bacterial and total *Pseudomonas* communities in relation normal and replant sites of apple and pear orchards in university field area was observed.

Over all it was found that the *Pseudomonas* population was far less as compared to total population present in normal sites. The variation and less count of *Pseudomonas* may depend upon the genotype and age of plant and also due to the exhausted nutrient level that in turn affect the total bacterial as well as total *Pseudomonas* population in the rhizosphere soil. Yao *et al.*, [2] observed that population shift may be related to change in soil moisture, soil temperature, rhizodeposition and root turn over. According to Weller, [20] the surrounding environment i.e. rhizosphere soil type and nature of plant exudates and organic acids produced by plant roots also influences the nature and type of *Pseudomonas* sp. or other microorganism present in the soil.



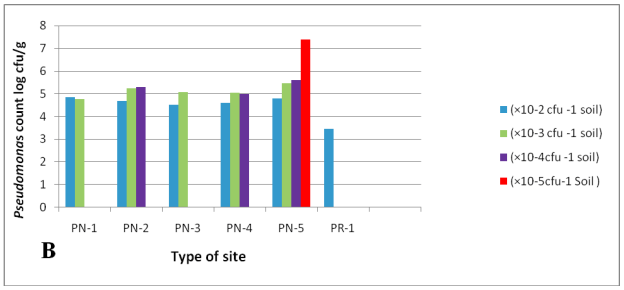


Figure 1: Population of Bacteria (A) and *Pseudomonas* (B) in normal and replant site.

3.1 Lytic Enzyme production

1) PROTEASE ACTIVITY

The production of proteolytic activity was shown by all the 26 isolates of *Pseudomonas* sp. isolated from the rhizosphere of both apple and pear growing in normal and replant sites **(Table 1)**. Here, four isolates of apple showed activity in the range of 27–28mm other four in the range of 20–24 mm and rest four showed activity in the range of 15–19 mm diameter. While five isolates from pear showed activity in the range of 32–38 mm and nine showed activity in the range of 20–28 mm. Selected four isolates from apple and pear showed activity in the range of 24-25 mm and 32-38 mm respectively. Quantitatively six isolates from apple showed maximum activity in the range of 2–3 units/ml while other six isolates showed less activity in the range of 1.6–1.9 units/ml. Eight isolates of pear showed slightly more activity in the range of 3.0–5.5 units/ml. Four isolates showed activity in the range of 2.1–2.7 units/ml and two isolates activity in the range of 1.5–1.8 units/ml. Four strains selected for further study showed maximum activity in the range of 3.0–5.5 units/ml. They all were found to be statistically different from each other.

In agriculture, these proteases producing microorganisms have role as biological control agents for eradication of some fungal plant pathogens. The screening of isolates for protease production on skim milk agar plate according to Cattelan *et al.*, [21] showed that *Pseudomonas* isolates were positive for the proteolytic activities which supported the work done by our study.

2) CHITINASE ACTIVITY

The production of chitinase activity was not shown by all the 26 isolates of *Pseudomonas* sp. Among all of the *Pseudomonas* isolates only been shown by ten isolates ranging between 17-22 mm diameters of clear zone **(Table 1).** It was observed that there was statistical significance different between all the isolates of *Pseudomonas* sp.

We had similar findings with Chaiharn *et al.*, [22] who found that the ability of antagonistic bacteria to produce proteolytic enzymes, chitinase, β 1,3 glucanase and cellulose at low percentage and the composition of antagonistic mechanisms was specific for each isolate.

3) GLUCANASE ACTIVITY

Out of 26 isolates of *Pseudomonas* sp. isolated from the

rhizosphere soil of apple and pear none of them showed production of activity or clear zones on glucan agar plate **(Table 1)**.

3.2 Screening for different PGPR activities:

1) P SOLUBILIZATION ACTIVITY

Pradhan and Sukla, [23] studied that phosphorous is one of the major nutrients, second only to nitrogen in requirement for plants.

So, in this study 26 isolates from the rhizosphere soil of apple and pear were screened for the production of phosphate solublizing activity by well plate method on Pikovskaya's agar media **(Table 2)**. The phosphate activity was expressed in terms of mm diameter of yellow zone produced around the bit/ well and also expressed as PSE%. By well plate assay method maximum production of phosphate solublizing activity in terms of solubilization zones (mm diameter) was shown by PN-5-UHF

Table 1: Production of three lytic enzymes viz. protease, chitinase and glucanase by fluorescent *Pseudomonas* sp. Isolates

Plant	*Pseudomonas* isolates	Enzyme activity		
		Proteolytic assay		Chitinase assay
		Plate Clear zone (mm dia)* Well	Quantitative Units/ml	Plate clear zone (mm dia) Well
Apple	**AN-1-UHF**	**27**	**2**	**18**
	AN-2-UHF	27	2.2	17
	AN-3-UHF	27	3	20
	AN-4-UHF	24	1.81	0
	AN-5-UHF	**28**	**3.0**	0
	AN-6-UHF	**15**	1.6	0
	AN-7-UHF	17	1.7	19
	AN-8-UHF	20	1.9	20
	AN-9-UHF	22	2	0
	AR-1-UHF	19	1.9	0
	AR-2-UHF	21	2.1	0
	AR-3-UHF	**18**	1.7	0
$CD_{0.05}$		1.68	1.231	1.08
Pear	PN-1-UHF	34	3.5	0
	PN-2-UHF	32	4.09	0
	PN-3-UHF	22	1.8	0
	PN-4-UHF	26	3.6	20
	PN-5-UHF	28	3.8	22
	PN-6-UHF	20	1.5	0
	PN-7-UHF	**38**	**5.5**	**21**
	PN-8-UHF	24	2.7**5**	0
	PN-9-UHF	24	2.2	0
	PN-10-UHF	28	3.1	0
	PN-11-UHF	32	2.7	0
	PN-12-UHF	25	2.1	19
	PN-13-UHF	**32**	**3.1**	**20**
	PR-1-UHF	21	3.0	0
$CD_{0.05}$		1.672	1.885	0.995

**Proteolytic activity / chitinase activity/ glucanase activity expressed in terms of mm diameters of clear zone produced around the well (mm) on skimmed milk / chitin and glucan agar plate respectively at 37^0C for 48 h.*

and PN-11-UHF (38mm) and PSE (442.85%). Seventeen isolates showed phosphate solublizing activity ranged between (25-37 mm) and (257.1-440.14%) where as seven isolates ranged between (23 -17 mm) and (42.8-228.57 %).

All isolates were tested for the phosphate solublizing activity by spectrophotometeric method in terms of inorganic tricalcium phosphate solublization PVK in vitro and expressed as µg/ml of available phosphate. The result showed that maximum solublization of inorganic phosphate and maximum release of phosphate was shown by fourteen isolates in the range of 110-200µg/ml. Eight isolates showed medium range of release of phosphate 52.5–90µg/ml where as four isolates showed minimum range of release of phosphate 30-47.5µg/ml. The four isolates selected for further study, that is two isolates from apple (AN-1-UHF and AN-5-UHF) and two from pear (PN-7-UHF and PN-13-UHF) showed production of phosphate solubilizing activity in the range of 25-28 mm and 32-38 mm by well plate and in the range of 195-200 µg/ml and 167.5-190µg/ml of available phosphorous respectively.

It was observed that there was statistically significant difference between all isolates of *Pseudomonas* sp.

Rodriguez and Fraga, [24] the use of phosphate solubilizing microorganisms as inoculant simultaneously increases the plant crop yield. They found strains from the genus *Pseudomonas*, *Bacillus* and *Rhizobium* species most powerful phosphate solubilizers and supported our findings.

2) PRODUCTION OF HCN BY ISOLATES

In addition of antibiosis mechanism that include the disease suppressing effect of siderophores and antibiotics, there are a number of different ways in which PGPR's can inhibit phytopathogen [18] that some *Pseudomonas* are able to synthesize HCN to which these *Pseudomonas* are themselves resistant that may be linked to the ability of these strains to inhibit some pathogenic fungi.

Almost all the isolates of *Pseudomonas* sp. showed HCN production on King's medium **(Table 2)**. Out of 26 isolates of *Pseudomonas* sp. maximum production of HCN (+++++) has been shown by two isolates from apple and four isolates from pear viz. AN-1-UHF, AR-1-UHF, PN-1-UHF, PN-5-UHF, PN-9-UHF and PR-1-UHF. Rest of the ninteen isolates showed production of HCN with range (+++) to (++++).

Although, the role of HCN in disease suppression is not considered to be firmly established. The strains of fluorescent *Pseudomonads* in black pepper were found to produce volatile and non volatile metabolites including HCN [25] against fungal pathogens.

3) PRODUCTION OF AMMONIA

The increasing cost of petroleum products required in nitrogen

fertilizer production has focused attention on the development of biological system for nitrogen fixation in our study **(Table 2)** all the 26 *Pseudomonas* isolates showed ammonia production. Maximum production has been shown by seven isolates including AN-1-UHF, AR-1-UHF and AR-2-UHF from apple and PN-1-UHF, PN-5-UHF, PN-9-UHF and PR-1-UHF from pear (+++++). Rest all of the isolates also showed production of ammonia in the range of (+++) to (++++).

As a part of our investigation, screening of ammonia producing Fluorescent *Pseudomonas* sp. for the development of economical and fermentation process for ammonia production is important. There is indirect evidence of usefulness of *Azotobacter* free living N_2 fixing bacteria in crop improvement under tropical and sub tropical conditions especially with strains excreting high amount of ammonia in addition to a variety of growth promoting factor in presence of carbon rich root exudates [26].

4) PLANT GROWTH PROMOTERS

Pant growth promoting bacteria affected growth of plant by producing metabolites such as plant growth regulators that directly affect the plant growth by facilitating nutrient uptake by plants Kloepper, [27]; Glick, [28]. All 26 isolates were found to be very efficient in producing different plant growth regulators.

Table 2: Different PGPR activities shown by fluorescent *Pseudomonas* isolates

Pseudomonas isolates	Phosphate solubilization activity Assay			Production of HCN	Production of Ammonia	Plant growth regulators		
	Clear zones (mm dia)	S.E. %	Conc. (µg/ml)	Change in color (Yellow To Brown)		Auxins conc. (µg/ml)	Gibberellins conc. (µg/ml)	Cytokinins conc. (µg/ml)
AN-1-UHF		257.1(2.410)	195	+++++	++++	5.2	222	130
AN-2-UHF	25	271.4(2.434)	47.5	+++	+	12.0	216	200
AN-3-UHF		257.14(2.410)	110	++++	++++	4.0	214	80
AN-4-UHF	26	285.1(2.455)	150	++++	++++	7.5	200	130
AN-5-UHF		300(2.477)	200	++++	++++	6.0	298	210
AN-6-UHF	25	257.1(2.410)	67.5	++++	++++	3.0	201	200
AN-7-UHF		228.57(2.359)	115	+++	++++	10.0	150	130
AN-8-UHF	27	228.57(2.359)	185	+++	+++	4.8	140	200
AN-9-UHF		214.0 (2.301)	60	++++	+++	5.8	160	80
AR-1-UHF	28	200(2.301)	45	+++++	++++	4.0	125	80
AR-2-UHF		157.1(2.196)	52.5	++++	++++	3.0	160	250
AR-3-UHF	25	142.82(1.632)	90	++++	+	6.0	146	50
					++++			
	23				++++			
	23							
	22							
	21							
	18							
	17							
$CD_{0.05}$	1.6852	0.0057	0.704			0.743	1.6852	48.67

PN-1-UHF	34		182.5	+++++	++++	3.0	152	130
PN-2-UHF	35	435.5(2.639)	170	++++	+	1.5	250	80
PN-3-UHF	30		147.5	++++	++++	3.5	230	300
PN-4-UHF	32	438.5(2.642)	80	+++	++++	1.0	240	300
PN-5-UHF	38		80	+++++	+++	0.75	245	200
PN-6-UHF	27	415.492.618)	40	++++	++++	0.80	200	130
PN-7-UHF	32		167.5	++++	+	11.0	300	300
PN-8-UHF	28	428.0(2.613)	182.5	+++	++++	0.75	240	270
PN-9-UHF	38		185.0	+++++	++++	4.0	170	270
PN-10-	36	442.8(2.646)	80	++++	+++	11	180	130
UHF	38		67.5	++++	++++	0.50	275	80
PN-11-	30	285.71(2.455)	112.5	++++	+	0.80	230	200
UHF	32		190	++++	++++	15	280	270
PN-12-	22	428.2(2.632)	30	+++++	++++	2.0	130	80
UHF		300(2.477)			++++			
PN-13-					++++			
UHF		442.85(2.639)			++++			
PR-1-UHF					+			
		440.14(2.643)						
		442.85(2.646)						
		415.4(2.618)						
		428.0(2.631)						
		214(1.982)						
$CD_{0.05}$	1.6322	0.0024	0.167			0.901	4.7796	7.142

Table 3: Antibacterial and antifungal activities best selected isolates of fluorescent *Pseudomonas* sp.

Pseudomonas isolates	Antibacterial activity (mm dia clear zone)			Antifungal activity (% Inhibition)			
	Xanthomonas sp.	*Bacillus Subtilis*	*Bacillus Cerus*	*Fusarium oxysporum*	*Dematophora sp.*	*Alternaria sp.*	*Pythium sp.*
Control	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
AN-1-UHF	16		16	38(38.06)	41.53(38.33)		
AN-5-UHF	19	14	14		46(42.71)	27.0(31.30)	31.42(33.91)
				36.2(36.99)			
		15				31.0(33.83)	34.3(35.85)
PN-7-UHF	17		14	38.0(38.0	0	36.5(37.1	
PN-13-UHF	18	15	16	6)	44.6(41.86)	7)	30.0(33.21)
				36.2(36.9		25.0(30.0)	
		16		9)			34.2(35.79)
$CD_{0.05}$	1.88	1.88	1.88	(1.1170)	(6.4639)	(1.88)	(5.96)

Patten and Glick, [29] observed that production of auxins i.e. indole acetic acid (IAA) is wide spread among plant associated bacteria and promot plant growth. According to Elzar and Phinney, [30] 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. The above literature supported the work and the results obtained are

given below:

(a) Auxin Production

The maximum production of auxins among *Pseudomonas* isolates isolated from the rhizosphere of apple has been shown by seven isolates from apple viz, AN-1-UHF, AN-2-UHF, AN-4-UHF, AN-5-UHF, AN-7-UHF, AN-9-UHF and AR-3-UHF and three isolates from pear PN-7-UHF, PN-10-UHF and PN-13-UHF in the range of 5.2-15µg/ml **(Table 2)**. Rest all the isolates show auxins production in the range of 0.50 – 4.8µg/ml.

It was observed that there was statistically significant difference between all isolates of *Pseudomonas* sp.

(b) Gibberellins Production

The production of gibberllins was shown by all the 26 isolates of *Pseudomonas* sp. varied in the range between 125-300µg/ml range **(Table 2)**. The maximum gibberllin production was observed by sixteen isolates of *Pseudomonas* sp. ranged between 200-300µg/ml and were followed by six isolates that showed production in the range of 150-180µg/ml where as four isolates showed comparatively minimum production of gibberellins in the range of 125-146µg/ml. It was observed that there was statistically significant difference between all isolates of *Pseudomonas* sp.

(c) Cytokinin Production

Cytokinin production µg/ml was measured by raddish cotyledon expansion bioassay among the isolates of *Pseudomonas* sp. isolated from the rhizosphere of apple and pear, maximum production was shown by thirteen isolates **(Table 2)** out of 13, five isolates were from the rhizosphere of apple viz. AN-2-UHF, AN-5-UHF, AN-6-UHF, AN-8-UHF and AR-2-UHF and eight isolates were from rhizosphere of pear viz. PN-3-UHF, PN-4-UHF, PN-5-UHF, PN-7-UHF, PN-8-UHF, PN-9-UHF, PN-12-UHF and PN-13-UHF. The P-solubilization range between 200 - 300µg/ml followed by six isolates that all showed the 130µg/ml of production. Rest of the seven isolates showed less production of cytokinins that ranged between 50-80µg/ml.

It was observed that there was statistically significant difference between all isolates of *Pseudomonas* sp. Four isolates that is AN-1-UHF, AN-5-UHF, PN-7-UHF and PN-13-UHF were selected isolates for further characterization and they showed production of auxins between 5.2–11µg/ml, gibberellins between 222-330µg/ml and cytokinins between 210-300µg/ml.

4) Antimicrobial activity of partial purified protease by well plate bioassay

Best isolates were selected on the basis of PGPR activities and maximum production of proteolytic activities. They were further used for anifungal and antibacterial activities.

Inhibitory effect against various types of microorganisms may also be due to the lytic or cell wall degrading enzymes produced by the microorganisms [31]. Microbial proteases are increasingly recognized as important virulence factors for a variety of pathogens.

Some proteolytic enzymes especially elastase, subtilisin, pronase also possess bacteriolytic properties against different gram positive and gram negative bacteria [31]. Several studies have demonstrated the production of Lytic enzyme production by rhizospheric bacteria were involved in the control mechanisms against plant root pathogens including *Fusarium oxysporium* and *Rhizoctonia Solani* [32].

In our study antimicrobial activity of proteolytic enzyme producing fluorescent *Pseudomonas* sp. were checked by well plate assay method against three indicator test bacteria viz. *Bacillus subtilis, Bacillus cerus, Xanthomonas sp.* on nutrient agar plate at 30^0C for 48h **(Table 3)** and against four indicator test fungus viz. *Dematophora sp., Fusarium oxysporum, Alternaria sp. and Pythium sp.* **(Table 3).**

Antibacterial Activity against:

(a) *Xanthomonas* sp.

The maximum zone of inhibition against *Xanthomonas* sp. on pre-lawned nutrient agar plate was shown by AN-1-UHF (19mm) diameter followed by PN-13-UHF (18mm) and PN-7-UHF (17mm) and minimum inhibition zone was shown by AN-5-UHF (16mm).

(b) *Bacillus subtilis*.

The maximum inhibitory zone against *Bacillus subtilis* was shown by PN-13-UHF (16mm) diameter followed by PN-7UHF and AN-5-UHF (15mm) and minimum inhibition zone was shown by AN-1-UHF (14mm).

(c) *Bacillus cerus*.

The maximum inhibition zone was shown by partial purified fraction from AN-1-UHF and PN-13-UHF (16mm) and minimum inhibition zone was shown by AN-5-UHF and PN-7-UHF (14mm).

Antifungal activity against:

(a) *Fusarium oxysporum*

The maximum percent inhibition was shown by AN-1-UHF and PN-7-UHF (38.0%) followed by AN-5-UHF and PN-13-UHF (36.2%).

(b) *Dematophora* sp.

The maximum percent inhibition was shown by AN-5-UHF (46%) followed by PN-13-UHF (44.2%) and AN-1-UHF (41.53%) where as PN-7-UHF showed no inhibition against *Dematophora* sp.

(c) *Alternaria* sp.

The maximum percent inhibition was shown by PN-7-UHF (36%) followed by AN-5-UHF (31.0%) and AN-1-UHF (27.0%) where as PN-13-UHF showed minimum percent inhibition against *Alternaria* sp. (25%).

(d) *Pythium* sp.

The maximum percent inhibition was shown by AN-5-UHF (34.3%) and PN-13-UHF (34.2%) followed by AN-1-UHF (31.4%) whereas, PN-7-UHF showed minimum percent

inhibition against *Pythium* sp. (30%).

IV. CONCLUSION

Present study aims at the isolation and characterization of indigenous plant beneficial *Pseudomonas* species and its influence on the growth of pear and apple isolated from the normal and replant sites of rhizosphere of apple and pear so as to select and develop more efficient indigenous plant growth promoting and disease suppressing bioagents and also be used for production of lytic enzymes viz. protease, chitinase and β 1,3 glucanase. Microbial proteases are increasingly enzymes especially elastase, subtilisin, pronase also possess bacteriolytic properties against different gram positive and gram negative bacteria. Several studies have demonstrated the production of Lytic enzyme by rhizospheric bacteria which involved in the control mechanisms against plant root pathogens including *Fusarium oxysporium* and *Rhizoctonia Solani*. In this study 26 *Pseudomonas* strains were isolated from normal and replant sites of apple and pear plants and screened out for lytic enzyme activities and for different plant growth promoting activities. Maximum production of proteolytic activity was given by best selected isolates viz. AN-1-UHF, AN-5-UHF, PN-7-UHF and PN-13-UHF. Best selected isolates showed antimicrobial activity against bacterial and fungal pathogens.

REFERENCES

[1] Catska V, Vancura V, Hudska G and Prikryl Z. 1982. Rhizosphere micro-organisms in relation to the apple replant problem. *Plant and Soil* 69:187-197.

[2] Yao S, Merwin I A, Abawi GS and Thies JE. 2006. Soil fumigation and compost amendment alter soil microbial community composition but do not improve tree growth or yield in an apple replant site. *Soil Biology and Biochemistry* 38:587-599.

[3] Ashrafuzzaman M, Hossen FA, Ismail MR, Hoque MA, Islam MZ, Shahidullah SM and Meon S. 2009. Efficiency of plant growth promoting rhizobacteria for the enhancement of rice growth. *African Journal of Biotechnology* 8:1247-1252.

[4] Sarvanakumar D, Vijaykumar C, Kumar N and Samiyappan R. 2007. ACC deaminase from *Pseudomonas fluorescens* mediate saline resistance in groundnut plants. *Journal of Applied Microbiology* 102:1283-1292.

[5] Sarvankumar D and Samiyappan R. 2008. Fluorescent *Pseudomonas* mixture mediate disease resistance in rice plants against rot *(Sarocladium oryzae*) disease. *Biocontrol* 54:273-286.

[6] Kreig NR and Holf JG. 1984. Bergeys Manual of Systematic Bacteriology. William and Wilkins, Baltimore, USA.

[7] Morihara K. 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. *Journal of Bacteriology*. 88:745-757.

[8] Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248-254.

[9] Gordon SA and Paleg LG. 1957. Observation on the quantitative determination of indoe acetic acid. *Physiologia Plantarum* 10:37-38.

[10] Holbrook AA, Edge WJW and Fremor TR. 1961. Spectrophotometric method for determination of gibberellic acid. In: Gibberellins 159-167. ACS Washington DC.

[11] Letham DS. 1971. Regulator of cell division in plant tissue. XII A cytokinin bioassay using excised radish cytoledons. *Physiologia Plantarum* 25:391-396.

[12] Pikovsakaya RE.1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species .*Microbiologia* 17:362-370.

[13] Dickman SR and Bray RH. 1940. Colorimetric determination of phosphate. Indus and Engineering chemical Analytical Edition 12:660-665.

[14] Bray RH and Kurtz LT.1945. Determination of total organic available forms of phosphorus in soil. *Soil Science* 23:343-353.

[15] Olsen SR, Cole CV, Whatanable FS and Dean LA. 1954. Estimation of available phosphorus by extraction with sodium bicarbonate. *US Department of Agriculture* 939.

[16] Baker AW and Schippers P. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas spp*. Mediated plant growth stimulation. *Soil Biology and Biochemistry* 19:451-457.

[17] Lata and Saxena AK. 2003. Characterization of plant growth promoting rhizobacteria. In: Training manual on Biofertilizer Technology (eds.) A K Saxena. IARI Delhi pp. 24-25.

[18] Voisard C, Keel C, Haas D and Defago G. 1989. Cyanide production of *Pseudomonas fluorescens* helps suppress black root of tobacco under genotobiotic conditions. *EMBO Journal* 8: 351-358.

[19] Fleming HP, Etchells JL and Costilow RH. 1975. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Applied Microbiology* 30:1040-1042.

[20] Weller DM.1988. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* 26:379-407.

[21] Cattelan, ME, Hartel PG, Uhrmann JJ. 1999. Screening of plant growth promoting rhizobacteria to promote early soybean growth. *Soil Scence Society of American Journal*. 63:1670-1680.

[22] Chaiharn M, Chunhaleuchanon S, Kozo A and Lumyong S. 2008. Screening of rhizobacteria for their plant growth promoting activities. *Kmitl Sci. Tech*. 8.

[23] Pradhan N and Sukla LB. 2006. Solubilization of inorganic phosphate by fungi isolated from agriculture soil. *African Journal of Biotechnol*ogy 5:850-854.

[24] Rodriguez H and Fraga R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* 17:319-339.

[25] Diby P, Kumar A, Anandaraj M and Sarma YR. 2001. Studies on the suppressive action of fluorescent *Pseudomonads* on *Phytopthora capsici*, the foot rot pathogen of black pepper. *Indian Phytopathology* 54:515.

[26] Narula N and Gupta KG. 1987. Detection of ammonia excretors on agar plates. *Zentralbl. Mikrobiology* 142:183-185.

[27] Kloepper JWE. 1993. Plant growth promoting rhizobacteria as biological agents. In: Soil microbial ecology. Application in agricultural and environmental management, FB Metting (eds.), Marcel Dekker, New York. Pp 255-274.

[28] Glick BR. 1995. The enhancement of plant growth by free living bacteria. *Canadian Journal of Microbiology* 4:109-117.

[29] Patten C and Glick BR. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian Journal of Microbiology* 42:207-220.

[30] Phinney BO. 1983 The history of gibberellins. In: The Biochemistry and Physiology of Gibberellins (Ed. Crozier A.) vol 1. Praeger Publishers USA pp 19-52.

[31] Kaur M, Gupta M, Tripathi KK and Gupta KG. 1989. Lytic effect of *Pseudomonas aeruginisa* elastase on gram positive and gram negative bacteria. *Zentrablatt Bakt. Inernational Journal of Microbiology* 127:153-157.

[32] Nagrajkumar M, Bhaskaran R and Velazhaban R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath of blight pathogen. *Micrbiological Research* 159:73-8.

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