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ORIGINAL ARTICLE

MANAGEMENT OF REPLANT PROBLEM BY THE PRODUCTION OF PLANT GROWTH REGULATORS AND PHOSPHATE SOLUBILISING POTENTIAL OF FLUORESCENT *PSEUDOMONAS* SPP. ISOLATED FROM THE NORMAL AND REPLANT SITES OF APPLE AND PEAR

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ABSTRACT –

Replant problem is a serious problem and common cause of poor growth and delayed cropping of trees planted in old orchard sites. The possible solution to the replant problem is to introduce appropriate novel and indigenous bioagent conferring maximum plant growth promotion activities so as to manage the replant problem of horticultural crops. *Pseudomonas* species especially some members of fluorescent group are the most diverse and versatile group of indigenous microflora that have the potential to produce plant growth regulators such as auxins, cytokinins and gibberellins along with the Phosphate solubilising capacity. In the present study, in total 30 Fluorescent Pseudomomas spp. were isolated from soil samples collected from the rhizosphere of apple and pear in normal and replant sites of Shimla district of Himachal Pradesh and were tested for Phosphate solubilising and Plant growth regulators production. In qualitative method, maximum P solubilisation was found to be 442.85 PSE% and by quantitative method, the phosphate solubilizing activity was recorded in the range of 1.7 µg/ml to 3560 µg/ml. Maximum auxin, cytokinin and gibberellins production were 11.94 µg/ml, 179.48 µg/ml and 347.50 µg/ml respectively.

INTRODUCTION

Replant problem is a serious problem and common cause of poor growth and delayed cropping of trees planted in old orchard sites. It is distributed worldwide and is often encountered in establishing new orchard sites (Mai and Abwai, 1981). This problem of newly planted trees (apple and pea**r) is common in replanted orchards. The decrease in growth is also referred to as specific replant disease (SRD). It is a complex disease syndrome and its etiology is still unclear and may relate to complex of fungi, bacteria, nematodes and soil factors such as pH, moisture, stress and insufficient available phosphorus for vigorous growth of young trees (Catska *et al*., 1982).** The possible solution to the replant problem is to introduce appropriate novel and important indigenous bioagents conferring maximum plant growth promotion activities so as to manage the replant problem of horticultural crops; to have better and effective root colonization and to inoculate planting material or soil. *Pseudomonas* species especially some members of fluorescent group are the most diverse and versatile group of indigenous microflora of almost all the horticulture and forestry crops. They are potential sources of useful metabolites that are important industrially, agriculturally and biotechnologically (Malik, 1990).

A large number of phosphate solubilizing bacteria have been isolated from the rhizosphere of several crops (Nautiyal *et al*, 2000) and these constitute about 20-40% of the culturable population of soil microorganisms. The important genera of phosphate solubilizing bacteria include *Pseudomonas, Achromobacter, Aerobacter, Bacillus, Serratia and Xanthomonas* (Chen, 2006). The plant growth promoting effect of phosphate solubilizing bacteria is considered to be also related to their ability to synthesize plant growth regulating substances (Sattar and Gaur, 1987). The production of Indole acetic acid (IAA), gibberellins and cytokinins by phosphate solubilizing bacteria have been reported earlier by several workers (Sattar and Gaur, 1987; Khalid *et al*., 2004).

MATERIALS AND METHODS

Isolation

Fluorescent Pseudomomas spp. were isolated from soil samples collected from the rhizosphere of Apple and Pear in normal and replant sites of Shimla district of Himachal Pradesh. Rhizospheric soil along with the root pieces from both the plants was collected and stored in plastic bag at 4ºC temperature until further processing. Total bacteria and total fluorescent *P*seudomonas spp. were isolated using dilution plating using appropriate dilutions on nutrient agar and Kings B agar media respectively. Isolates were classified on the basis of colony characteristics such as size, color, shape and texture of each isolate. The probable isolates showing greenish/yellowish fluorescent or pyocyanin pigments were assumed to be fluorescent Pseudomonas. The colonies were restreaked for purification and further observation of colony morphological characterization and pigment production on different media i.e. nutrient agar, Kings, Pikovskayas etc. The most predominant *Pseudomonas* isolates were identified on the basis of morphological, biochemical and physiological tests as prescribed in Bergey's manual of systematic bacteriology and were confirmed from the Department of Basic Sciences, Dr. Y.S Parmar University of Horticulture and Forestry,

Nauni, Solan (H.P). The pure culture of selected strains were maintained on the nutrient agar slants at 4°C and were sub-cultured periodically on the same media at 28°C ± 2ºC and also maintained in 20% glycerol at -20ºC.

PHOSPHATE SOLUBILIZATION: ESTIMATION METHOD (QUALITATIVE AND QUANTITATIVE)

For estimation and screening of phosphate solubilizing capacity of bacteria, both qualitative and quantitative methods were used. 100 ml of Pikovskaya's medium with tricalcium phosphate was inoculated with 0.5 ml of 18 hours old culture of each test isolates of *Pseudomonas* sp. and incubated at 28 ± 2ºC for 72 hours under shake conditions (90 rpm). Cell free supernatant was collected by centrifugation at 10,000 rpm for 20 minutes at 4ºC. Supernatant was stored at 4ºC in the form of small aliquots (5ml) and was further used for estimation of phosphate solubilizing activity by plate assay and liquid assay.

Plate assay method

Pikovskaya's agar plates (Pikovskaya's 1948) with known amount of inert phosphorous source were prepared. In bit plate assay method, 48hr old culture bit was placed in center of each plate and plates were incubated at 28 ± 2ºC for 48 hrs. Phosphate solubilization was expressed in terms of mm diameter of yellow colored zone produced around bit and phosphate-solubilizing efficiency (% PSE) was calculated. In well plate assay method, the well (7 or 9mm) was cut with the help of sterile cork borer on the prepoured plates of Pikovaskaya's agar medium. 100 µl of 72 hr old cell free supernatant of each bacterial strain was added to each well. Plates were incubated at 28 ± 2ºC for 48hrs and observed for pinkish/orange zone produced around the well. Phosphate solubilization expressed in terms of Phosphate solubilization efficiency (% SE) calculated from equation:

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

Where, Z= Diameter of yellow zone (mm); C= Diameter of colony/bit (mm)

Liquid assay (Quantitative spectrophotometric method)

Quantitative estimation of Phosphate solubilizing activity was done by spectrophotometric method (Olsen *et al.*, 1954). 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 660 nm 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).

Plant growth regulators

Auxins

For production of plant growth regulators, each isolate of Pseudomonas sp. was grown in nutrient broth for 72 h at 28±2°C under shake conditions. Cell free culture supernatants were harvested by centrifugation (10,000 rpm) for 30 minutes at 4°C and stored for further experimentation at 4°C.

Measurement of auxins was done by quantitative colorimetric method (Gorden and Paleg, 1957) with slight modifications. 2 to 3 drops of orthophosphoric acid added to 2ml supernatant and added 4 ml of salper reagent (1 ml of 0.05M, $FeCI_3$ in 50 ml of 35% $HCIO_4$). This mixture was incubated for 25 minutes at room temperature 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, Hi media) as standard (10-100 mg/ml).

Gibberellins

Method of Holbrook *et* al. (1981) with slight modifications was used for determination of gibberellins. To 15 ml of supernatant 2 ml of zinc-acetate reagent (21.9 g of 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% HCI was added and mixture was incubated at 20°C for 75 minutes. For blank 5 ml of 5% HCI was used. Absorbance was read at 254 nm. Concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA_3, Hi-Media) as standard (100-1000 µg/ml).

Cytokinins

Radish cotyledons expansion bioassay was employed (Letham, 1971) for estimation of cytokinins like substances. The radish seeds *(Raphahus sativus* cv, Japanese white) were germinated in total darkness for 48h at 28°C. After removing the seed coat, smaller cotyledons were transferred to sterilized petriplates containing the test solution/ distilled water (control)/ standard (kinetin) on filter paper strips. 20 cotyledons were placed in each petridish and were incubated at 25°C under fluorescent light for 3 days. The cotyledons were dried and weighed. The bioassay response (final weight- initial weight) was expressed as increase in weight of cotyledons. Concentration of cytokinin present in the extract was calculated by preparing dosage response curve by using kinetin as standard (100-1000 µg/ml).

Results and discussion

Phosphorus is one of the major essential micronutrients for biological growth and development (Ehrlich, 1990). The concentrations of soluble P in soil is usually very low, normally at levels of 1ppm or less. Pikovskayas

medium was selected for screening the phosphate solubilizing potential, as previous study (Kundu *et al*., 2002 and Srivastav *et al*., 2004) has shown this medium to be most selective, least susceptible to overgrowth by non phosphate solubilizing bacteria and most reproducible, allowing the growth of a broad diversity of phosphate solubilizing bacteria.

The phosphate solubilizing bacteria have the ability to solubilize insoluble mineral phosphate by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO_2 and H_2S (Ivanova *et al*., 2006). Organic acid metabolite production is the major mechanism for the solubilization of insoluble inorganic phosphates. A number of organic acids products including fumaric, lactic, citric, glycolic, malonic, tartaric and succinic acids have been reported in culture filtrates of pure culture of phosphate solubilizing microorganisms (Vazquez *et al*., 2000).

In our study, 19 isolates were isolated from Apple normal site (designated An), 3 from Apple replant site (designated Ar), 5 from Pear normal (designated Pn) and 3 from Pear replant sites (designated Pr). All the indigenous strains of fluorescent *Pseudomonas* isolates from the rhizosphere of apple and pear showed phosphate-solubilizing activity both in Pikovskaya's agar plate and also solubilize tricalcium phosphate in liquid cultures as estimated by spectrophotometric method (Table 1) in accordance to Lata and Saxena (2003). In plate assay method, phosphate solubilizing activity is expressed in terms of mm diameter and phosphate solubilizing efficiency (% PSE) in the range i.e. 24 mm (242.85%) to 41 mm (485.70%). By well plate assay method the range was 17 mm (142.80%) to 32 mm (357.14%). A similar criterion of qualitative performance was also followed by Kundu *et al*., (2002) in categorization of phosphate solubilizers. In liquid assay method, the *Pseudomonas* isolates showed phosphate solubilizing activity which was calculated in terms of µg/ml of orthophosphate solubilization. The phosphate solubilizing activity was recorded in the range of 1.7 µg/ml to 3560 µg/ml.

The formation of halo zone on solid medium is not only the criterion of the ability of the ability of an organism to solubilize P (Ahmad and Jha, 1967) because in some cases there have been contradictory results between plate halo detection and P solubilization in liquid cultures as shown in the study by Dave and Patel (1999), in which out of 38 cultures, 25 did not show zone of phosphate solubilization on agar medium but showed phosphate solubilization in liquid medium. Similar contradictory results were observed by Cakmakci *et al*., (2006) and Fankem *et al*., (2006). In our observation (Table 1), one of our *Pseudomonas* isolates i.e. An-2-panch showed highest P solubilization in quantitative assay but did not show zone of phosphate solubilization on PVK agar medium. This observation is supported by a similar observation by Deubel and Merbach (2005) they realized that their best strain in solubilizing the same Phosphate source in liquid media was one of the strains which could not show clear zone on agar plates.

Production of plant growth promoting substances like IAA, GA and cytokinins by several beneficial microorganisms including phosphate solubilizers have been quantified and related to enhancement of plant growth. The production of hormones has been suggested as one of the mechanisms by which PGPR stimulate plant growth.

Researchers have recently identified cytokinin, gibberellins, auxins and ACC deaminase production by PGPR (Timmusk *et al*., 1999; Vessey., 2003).

In our study, the Pseudomonas isolates were found to produce cytokinins like substances in nutrient broth at 28 ± 2ºC under shake conditions in the range from 51.20 to 179.48 µg/ml. Production of Cytokinins by plant growth promoting rhizobacteria including *Pseudomonas*, *Azotobacter*, *Rhizobium* and *Bacillus* has been reported to synthesize cytokinins in pure culture (Arshad and Frankenberger, 1993; **Anith** *et al*., **1999).**

Butak (2006) reported that the optimal cultural parameters for GA_3 production by *Pseudomonas* sp. isolated from wastes of processed olive were affected by physiological conditions (incubation temperature, pH of the growth media, incubation period and incubation condition). In our study, the highest level of gibberellic acid (347.50 µg/ml) produced was obtained in nutrient broth when the bacteria culture was incubated at 28° C for 48 h at pH 7 on rotary shaker and in dark.

In our studies, all of the *Pseudomonas* isolates from the rhizosphere of apple and pear were grown in nutrient broth for 48h. In cell free supernatant, maximum production of gibberellins like substances shown in the range 15.20 to 179.48 µg/ml. The gibberellins production by phosphate solubilizing microorganisms was also demonstrated by Anith *et al.,* (1999). Vikram, (2007) also showed the production of Gibberellic acid by the phosphate solubilizing bacteria isolated from the crops grown in vertisols. The amount ranged from 0.6 to 9.8 µg/25 ml of broth respectively.

Statistics

The results under laboratory conditions on various parameters were subjected to statistical analysis as per method outlined by Gomez and Gomez (1976). The CD at 5 % level was used for testing the significant differences among the treated means. Values in parenthesis in the tables are log-transformed values.

CONCLUSIONS

Replant problem is a common cause of poor growth and delayed cropping of fruit trees especially apple and pear planted in old orchard sites. Overcoming replant problem is critical for the successful establishment of high-density orchards. From the study it is concluded that the isolates belonging to *Pseudomonas* genera are among the most predominant phosphate solubilizing bacteria and plant growth regulator producers. Thus the use of *Pseudomonas* should be increased in agriculture because it offers an attractive way to replace chemical fertilizers, pesticides, and supplements.

Table 1. Phosphate solubilizing potential of fluorescent Pseudomonas isolated from rhizosphere of apple and pear in normal and replant sites

Plant	*Pseudomonas* Isolates	Phosphate solubilizing activity				
		Assay				
		Plate				Quantitative
		Bit method		Well method		
		Yellow zone (mm dia)	SE%	Yellow zone (mm dia)	SE%	Available phosphate (µg /ml)
Apple	**An-1-Naga**	**36**	**414.28 (2.61)**	**30**	**328.57 (2.51)**	**495**
	An-2-Naga	35	400.00 (2.60)	28	300.00 (2.47)	555
	An-3-Naga	40	471.40 (2.67)	25	257.14 (2.41)	274
	An-4-Naga	33	371.42 (2.57)	24	242.85 (2.38)	414
	An-1-kho	35	400.00 (2.60)	28	300.00 (2.47)	121
	An-2-kho	33	371.42 (2.57)	24	242.85 (2.38)	335
	An-3-kho	**36**	**414.28 (2.61)**	**32**	**357.14 (2.55)**	**590**
	An-4-kho	30	328.57 (2.51)	22	214.20 (2.33)	3390
	An-1-bagh	30	328.57 (2.51)	26	271.40 (2.43)	307
	An-2-bagh	33	371.42 (2.57)	28	300.00 (2.47)	210
	An-3-bagh	32	357.10 (2.55)	26	271.40 (2.43)	3300
	An-4-bagh	32	357.10 (2.55)	28	300.00 (2.47)	182
	An-1-panch	34	385.70 (2.55)	29	314.28 (2.50)	207
	An-2-panch	0	0.00 (0.00)	0	0.00 (0.00)	3560
	An-3-panch	41	485.70 (2.68)	22	214.28 (2.33)	310
	An-4-panch	40	471.40 (2.67)	0	0.00 (0.00)	435
	An-1-nali	35	400.00 (2.60)	28	300.00 (2.47)	585
	An-2-nali	34	385.70 (2.58)	0	0.00 (0.00)	185
	An-3-nali	35	400.00 (2.60)	28	300.00 (2.47)	249
	Ar-1-Kho	**36**	**414.28 (2.61)**	**28**	**300.00 (2.47)**	**285**
	Ar-2-Bagh	28	300.00 (2.47)	22	214.20 (2.33)	185
	Ar-3-Nali	24	242.85 (2.38)	17	142.80 (2.15)	1.7
$CD_{0.05}$		**1.12**	**1.08 (.002)**	**0.888**	**.958 (.002)**	**0.986**
Pear	Pn-1-panch	36	414.28 (2.61)	24	242.85 (2.38)	390
	Pn-2-panch	**36**	**414.28 (2.61)**	**26**	**271.40 (2.43)**	**785**
	Pn-3-panch	35	400.00 (2.60)	26	271.40 **(2.43)**	467
	Pn-1-kho	37	428.57 (2.63)	24	242.85 **(2.38)**	267
	Pn-2-kho	**38**	**442.85 (2.64)**	**27**	**285.71 (2.45)**	**425**
	Pr-1-panch	35	400.00 (2.60)	18	157.10 (2.19)	210
	Pr-2-panch	30	328.57 (2.51)	17	157.10 (2.19)	300

	Pr-3-kho	28	300.00 (2.47)	22	214.20 (2.33)	275
$CD_{0.05}$		**1.73**	**2.75 (0.00)**	**1.49**	**0.20 (0.000)**	**1.619**

Values in paranthesis are log-transformed values

Table 2. Production of plant growth regulators i.e. auxins, gibberellins, cytokinins by fluorescent *Pseudomonas* sp. isolates from the rhizosphere of apple and pear

Plant	***Pseudomonas* Isolates**	*Plant Growth Regulators*		
		Conc. (µg/ml)		
		Auxins*	GIBBERELLINS**	*Cytokinins****
I. APPLE	An-1-Naga	10.11	336.60	51.20
	An-2-Naga	4.40	211.20	153.84
	An-3-Naga	6.00	175.14	51.20
	An-4-Naga	5.10	244.64	76.92
	An-1-kho	2.60	255.70	102.56
	An-2-kho	5.10	127.88	102.56
	An-3-kho	9.55	283.56	76.92
	An-4-kho	9.01	111.20	51.20
	An-1-bagh	8.47	172.36	76.92
	An-2-bagh	3.63	33.36	51.20
	An-3-bagh	4.12	111.20	128.20
	An-4-bagh	9.77	88.96	102.56
	An-1-panch	4.99	33.92	102.56
	An-2-panch	4.45	30.02	128.20
	An-3-panch	9.23	216.84	179.48
	An-4-panch	11.51	88.96	153.84
	An-1-nali	9.55	33.30	76.92
	An-2-nali	3.36	133.44	128.20
	An-3-nali	5.48	189.04	51.20
	Ar-1-Kho	7.92	136.22	102.56
	Ar-2-Bagh	3.90	175.14	51.20
	Ar-3-Nali	3.04	139.000	76.92

II. C $D_{0.05}$		0.516	0.316	0.294
Pear	Pn-1-panch	3.90	200.16	51.20
	Pn-2-panch	9.12	44.75	153.84
	Pn-3-panch	4.45	88.96	51.20
	Pn-1-kho	4.12	200.16	76.92
	Pn-2-kho	11.94	347.50	179.42
	Pr-1-panch	3.36	88.96	51.20
	Pr-2-panch	5.64	77.84	102.56
	Pr-3-kho	4.56	102.86	76.92
III. C $D_{0.05}$		0.38	0.031	0.441

Production of plant growth regulators viz auxins* gibberellins** cytokinins*** expressed in terms of concentration (µg/ml) of each regulators produced extracellularly in supernatant as caliberated from the standard curve of IAA (10-100 µg/ml); gibberellic acid GA_3 (100-1000 µg/ml); and Kinetin (100-1000 µg/ml)

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