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Comparative Effect of *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas putida* on the Growth of Replanted Apple

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Fluorescent *Pseudomonas* species have a number of traits like antifungal, siderophores, phosphate solubilization, lytic enzymes, HCN, ammonia and plant growth regulators that allow them to act as plant growth promoting and biocontrol agents. They competitively colonize plant roots, play important role in enhancing the growth of plants and in plant disease control. These may be used for soil improvement and to increase soil fertility hence for management of replant problem of apple in Himachal Pradesh. To ensure the sustained availability of PGP and biocontrol agent's in soil formulation development protocol has to be standardized. On the basis of different PGPR activities three indigenous strains viz., *Pseudomonas aeruginosa* C, *Pseudomonas fluorescens* M and *Pseudomonas putida* L were selected for field studies. In all the treatments with individual (L, M and C) and consortia strains (L+M, L+C, M+C, L+M+C) there was an 8.0 to 86.5 per cent increase in plant height as compared to control. The performance of replanted apple was much better in terms of root colonization capacity, plant establishment and increase in plant growth in terms of plant height, number of nodes and branches over their respective control after fifteen months of plantation.

Keywords: *Pseudomonas* sp., bioformulation, apple, replant site.

Apple (*Malus domestica* Borkh.) is one of the most important fruit crop grown and consumed all around the world and has been distributed almost across the whole temperate region in the Northern and Southern hemispheres[1].

Replant problem refers to the poor growth of replanted young trees on the old sites. It is distributed worldwide and is often encountered in establishing new orchards[2]. The disease is a complex syndrome that reduces growth, survival and yield of replanted tree. Replant problem is caused by biotic and abiotic factors[3]. The biotic factors includes the rhizosphere microflora (bacteria, fungi, actinomycetes, nematodes and their interactions) and abiotic factors includes phytotoxins, nutrient imbalance, low or high pH, soil structure and damage, and lack of excess of moisture[4].

Fluorescent *Pseudomonas* species are the most diverse and versatile group of plant growth promoting rhizobacteria. Their potential to synthesize different secondary metabolites with diverse biological activities is the important function of soil fertility and sustainability of crops[5]. The integration of their important traits like production of antifungal antibiotics, iron chelating siderophores, lytic enzymes, plant growth regulators, phosphate solubilization, ammonia and HCN production with ecological fitness of the strains will be prerequisite for designing useful, efficient and effective novel bioagent. The large

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scale application of indigenous plant growth promoting fluorescent *Pseudomonas* sp. may be able to manage replant problem of fruit crops especially apple.

The main objective of this research was to exploit the indigenous potential fluorescent *Pseudomonas* strains viz., *P. aeruginosa*, *P. fluorescens* and *P. putida* in individual and consortial combination to act as biofertilizer which can become one of the most promising biotechnologies to solve apple replant disease and can also improve production with low inputs in fertilizer. The aim of the study was to select more effective combination of fluorescent *Pseudomonas* sp. which can be used for biofertilizer development for management of apple replant problem.

MATERIALS AND METHODS

Isolation of fluorescent *Pseudomonas* sp. from apple rhizosphere

The fluorescent *Pseudomonas* sp. was isolated from the rhizosphere of apple plants in normal and replant sites of Shimla district (Himachal Pradesh). The rhizosphere soil loosely adhering to apple roots were gently teased out with small root pieces in polythene bags and immediately transported to laboratory under cold conditions (4ºC) for further process. The serial dilution agar plate method was used to isolate *Pseudomonas* sp. on King's B medium[6]. The composition of the medium was (g/l^{-1}): Peptone, 20.0; K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 1.5; Glycerol, 15.0 ml. All the twelve isolates from apple rhizosphere were morphologically, physiologically and biochemically characterized so as to select the isolates belonging to *P. aeruginosa, P. putida* and *P. fluorescens* group.

Molecular characterization and *in-vitro* screening of fluorescent *Pseudomonas* sp. for plant growth promoting activities

All the twelve fluorescent *Pseudomonas* sp. were characterized for plant growth promoting activities viz., phosphate solubilization[7], siderophore production[8], HCN production[9], ammonia production[10] and plant growth regulator production[11,12,13] according to their respective methods. Three potential fluorescent *Pseudomonas* isolates L, M and C were selected and molecularly characterized by 16S rRNA technique using *Pseudomonas* specific primers viz., FP-1 (GGTCTGAGAGGATGATCAGT) and RP-1 (TTAGCTCCACCTCGCGGC) in MJ Mini BIORAD personal thermal cycler-100 (PTC-100)[14]. The PCR amplification was as follows: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 2 min with a total of 35 cycles. For DNA sequencing, eluted amplified DNA products of fluorescent *Pseudomonas* isolates L, M and C was purified followed by sequencing in Bioserve Private limited (Hyderabad, India). After sequencing the obtained sequence were analyzed by Basic local alignment search tool (BLAST) for their identification with the NCBI data base[15]. On submission of partial sequence of *Pseudomonas putida* L, *Pseudomonas fluorescens* M and *Pseudomonas aeruginosa* C to GenBank database (NCBI) the accession no. assigned was KF751235, KF751236 and KJ871666

Replant sites for field experiment

The field experiment was conducted with apple rootstocks (MM793 and MM111) in ARD (apple replant disease) infected sites of Shimla distt. (Himachal Pradesh). The two sites at Maggota and Sharontha were selected for conducting field experiment on the bases of age of orchard and symptoms of ARD. A total of eighty apple rootstocks (MM793 and MM111) were purchased from KVK (Rohru) by farmers.

Field application of *Pseudomonas putida* L, *Pseudomonas fluorescens* M and *Pseudomonas aeruginosa* C in replant sites of Maggota and Sharontha (Shimla distt.)

The field experiment was conducted to investigate the effect of individual and consortial fluorescent *Pseudomonas* strains on the growth and establishment of apple rootstock planted in replant sites of Maggota and Sharontha. The fluorescent *Pseudomonas* sp. viz., *Pseudomonas putida* L, *Pseudomonas fluorescens* M and *Pseudomonas aeruginosa* C were grown in nutrient broth at 28±2 °C for 48-72 hr (adjusted to inoculum density 1 x 10^8cfu/ml) and used for the treatment of plant roots and soil of pits individually and in different combinations viz., L, M, C, L+M, L+C, M+C, L+M+C and control. The consortial combination viz., L+M, L+C, M+C, L+M+C was made in 1:1 and 1:1:1 ratio. The control plants were treated with 1/10 diluted nutrient broth.

Five rootstocks of each treatment

were planted in replant site of Maggota and Sharontha after treating roots according to different treatment combination. The plants were treated with fluorescent *Pseudomonas* suspension by dipping their roots in liquid inoculum for 15-30 minutes before planting them in replant site pits pretreated with *Pseudomonas* suspension[16]. The cyclic treatment for successive twelve months were given at monthly interval in rhizosphere of plants by adding 100-150 ml of inoculum ($1x10^8$ cfu/ml) to the basin of plants. The results were compared with uninoculated control. Effect on plant and soil parameters like plant height, number of nodes/leaves, branches and NPK content of soil were studied. The survival percentage of plants, total microbial and *Pseudomonas* count was also determined from the rhizosphere soil after every month of the cyclic treatment. The experiment was carried out in a randomized block design (RBD). The experimental data was analyzed statistically using ANOVA.

RESULTS AND DISCUSSION

Twelve plant growth promoting rhizobacteria were isolated from apple rhizosphere and characterized as belonging to fluorescent *Pseudomonas* sp. on the bases of biochemical and physiological characteristics. Reynolds[17] (2004) also characterized isolates on the bases of biochemical tests including oxidase, catalase, gelatin hydrolysis, nitrate reduction, growth at 4 and 41°C and identified them as *P. fluorescens.*

The screening strategy was carried out to find out an effective PGP fluorescent *Pseudomonas* strain that act through the combination of several different mechanisms. The screening resulted in a group of bacteria able to produce phosphate solubilizing activity, siderophore production, HCN production, ammonia production and growth hormone production thus allowing us to select *Pseudomonas* strains showing multifarious plant growth promoting activities. All the fluorescent *Pseudomonas* sp. showed significant production of phosphate solubilizing activity, siderophores, HCN, ammonia, and growth hormones (Table 1). Overall result showed that fluorescent *Pseudomonas* strains L, M and C produced maximum number of plant growth promoting activities *in-vitro* and also inhibited major fungal pathogens of apple.

Plant growth promoting effect of PGPR strains in different crops were clearly

Table 1. Characterization of fluorescent *Pseudomonas* sp. for plant growth promoting activities

fluorescent *Pseudomonas* isolates	Plant growth promoting traits						
	Phosphate solubilization* (Pi), (µg/ml)	Siderophore production** % SU	HCN production	Ammonia production	Plant growth regulators (µg /ml) Auxins	Gibberellins	Cytokinins
An-1-Jub	17	10.5	++++	+++	17	150	18
An-3-Jub	36	10.5	++	+	19	70	30
An-4-Jub	26	10.5	++++	+++	19	90	22
An-5-Jub	47	2.1	++++	+++	14	120	30
Ar-1-Jub	31	10.5	-	++	13	100	25
An-1-Sh	20	5.2	-	++	21	180	28
An-2-Sh	54	5.2	-	+	07	60	35
Ar-1-Sh	50	10.5	-	+++	22	80	28
Ar-2-Sh	25	15.7	+	++	21	110	28
L	39	23.0	+	+++	16	135	20
M	41	22.4	+	+++	11	140	24
C	30	50.8	+	+++	36	160	16

*Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization which in turn represents µg/ml of soluble inorganic phosphate(Pi) in supernatant as calibrated from the standard curve of KH_2PO_4 (10-100 µg/ml).

**The siderophore unit (% SU) expressed as percent reduction in blue color of chrome azurol-S as compared to reference i.e.

% SU= (Ar-As)/Ar×100

where, Ar = Absorbance of reference solution at 630 nm; As = Absorbance of test solution at 630 nm

demonstrated[18]. Bacterial inoculants are able to increase plant growth, germination rate, improve seedling emergence, responses to external stress factors and protect plants from diseases[19]. This present investigation confirms the earlier work. In this study, inoculation of fluorescent *Pseudomonas* sp. increased all growth and soil parameters as compared to control plants. The performance of replanted apple rootstocks after fifteen months of cyclic treatment with individual and consortium strains of *P. putida, P. fluorescens* and *P. aeruginosa* along with control at two different replant sites is detailed in Table 3. The details of replant site, age of orchard and rootstocks used for field experiment were presented in Table 2. The data was presented as per cent increase over control for plant height, number of nodes and available NPK content of soil.

The growth performance was studied on the basis of plant height, number of nodes/leaves, chlorophyll content and number of branches. On an average in all the treatments with liquid bioformulation of individual (L, M and C) and consortial strains (L+M, L+C, M+C, L+M+C) of *Pseudomonas* sp. there was an increase in plant height as compared to control after 15 months of replantation of apple rootstocks in the range of 8.0 to 86.5 % increase at Maggota and 27.4 to 65.7 % increase at Sharontha as compared to control plants respectively. At Maggota maximum per cent increase in terms of plant height and number of nodes was recorded in formulation with consortia of two strains L+C (86.5 % and 144.5 %) followed by individual strain C (86.1 % and 109.5 %) whereas at Sharontha maximum per cent increase in plant height and number of nodes was observed in formulation C (65.7 % and 69.3 %) Table 3. The maximum number of branches was observed in consortium of three strains viz., L+M+C at both the replant sites. The present experiment revealed that rhizosphere inoculation with *Pseudomonas* sp. resulted in an increased plant height, number of nodes, branches and available NPK content

Table 2. Detail of replant sites and rootstocks used in field experiment

Sr. no	Replant site	Age of orchard	Rootstock replanted	Total rootstock replanted
1	Maggota (Shimla distt.)	>25 years	MM793	40
2	Sharontha(Shimla distt.)	>35 years	MM111	40

Table 3. Effect of cyclic application of individual and consortium strains of *P. putida, P. fluorescens* and *P. aeruginosa* on growth of replanted rootstocks after 15 months at Maggota and Sharontha (Shimla distt.)

Replant site	Treatments (1×10^8 cfu/ml)	Plant height cm, (%I)	Number of nodes(%I)	Number of branches
Maggota	L	40.5	43.7	6.2
	M	8.0	58.0	6.4
	C	86.1	109.5	6.6
	L+M	40.9	68.0	6.6
	L+C	86.5	144.5	6.4
	M+C	42.9	106.5	6.4
	L+M+C	33.2	104.3	6.7
Sharontha	L	48.5	55.6	6
	M	51.1	51.5	6
	C	65.7	69.3	6.6
	L+M	27.4	68.8	6.6
	L+C	28.4	56.2	7.2
	M+C	31.6	60.1	7.4
	L+M+C	38.1	17.4	7.6

% I= percent increase over control

of rhizospheric soil. Similar results have shown previously by Verma *et al*., (2014)[20] who reported that cyclic treatment of fluorescent *Pseudomonas* sp. resulted in significant increase in various plant and soil parameters. Similar increase in plant height was observed in different crops inoculated with *Pseudomonas*, *Azospirillum* and *Azotobacter* strains by other workers[21-24].

The chlorophyll content of leaves of replanted apple rootstocks was also estimated. Results in Table 4 showed that there was not a significant increase in chlorophyll content (a, b and total) in all the treatments as compared to control. The available N, P and K of rhizosphere soil were also assessed before and after the cyclic application of fluorescent *Pseudomonas* sp. (Table 5).The results showed that after application of *Pseudomonas* sp. in the field, there was a considerable increase in available NPK content of rhizosphere soil as compared to control plants. Our results were supported by the findings of Verma *et al*., (2014)[20]

Table 4. Effect of cyclic application of individual and consortium strains of *P. putida, P. fluorescens* and *P. aeruginosa* on chlorophyll content of leaves of replanted rootstocks after 15 months at Maggota and Sharontha (Shimla distt.)

Replant site	Treatments (1×10^8 cfu/ml)	Chlorophyll 'a'	Chlorophyll 'b'	Total Chlorophyll
Maggota	Control	0.16	0.23	0.3
	L	0.16	0.25	0.4
	M	0.18	0.24	0.4
	C	0.17	0.24	0.4
	L+M	0.17	0.24	0.4
	L+C	0.17	0.23	0.4
	M+C	0.17	0.24	0.4
	L+M+C	0.17	0.23	0.4
Sharontha	control	0.15	0.21	0.3
	L	0.17	0.25	0.4
	M	0.18	0.23	0.4
	C	0.17	0.25	0.4
	L+M	0.16	0.22	0.3
	L+C	0.17	0.23	0.4
	M+C	0.16	0.22	0.3
	L+M+C	0.16	0.23	0.3

Table 5. Effect of cyclic application of liquid bioformulation of L, M, C and their consortial formulations on available N, P, K content of rhizospheric soil of Maggota and Sharontha.

Treatments (1×10^8 cfu/ml)	Available macronutrients NPK(kg/ha) % increase over control (%I) after 15 month											
	Maggota						Sharontha					
	N	% I	P	% I	K	% I	N	% I	P	% I	K	% I
L	313	11.7	54	35.0	146	10.6	290	2.1	35	16.6	145	13.2
M	295	5.3	48	20.0	140	6.0	296	4.2	46	53.3	142	10.9
C	325	16.0	58	45.0	157	18.9	324	14.0	38	26.6	138	7.8
L+M	316	12.8	67	67.5	164	24.2	298	3.5	34	13.3	137	7.0
L+C	290	3.5	69	72.5	148	12.1	328	15.4	42	40.0	146	14.0
M+C	336	20.0	58	45.0	154	16.6	312	9.8	54	80.0	154	20.3
L+M+C	340	21.4	66	65.0	142	7.5	295	3.8	47	56.6	148	15.6
Control	280		40		132		284		30		128	

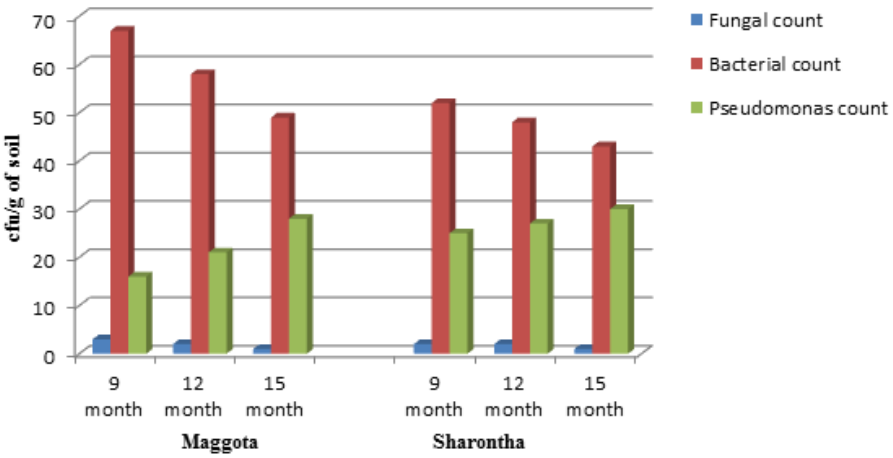

Fig. 1. Effect of cyclic application of fluorescent *Pseudomonas* sp. on rhizobacterial and fungal population in apple rhizosphere

After cyclic application of liquid formulations of both individual and consortial strains (L, M, C, L+M, L+C, M+C, L+M+C) in field the decrease in total bacterial and fungal population was observed with a gradual increase in fluorescent *Pseudomonas* population (Figure 1). After fifteen months of plantation, there was an increase of 20 to 46 cfu/g of soil in total fluorescent *Pseudomonas* sp. count in all the bioformulation treated rootstocks at both the sites. The results indicated towards the possibility of sufficient increase in number and establishment of *Pseudomonas* species in rhizosphere of apple plants planted in replant sites of apple orchards. They also might have decreased the deleterious microflora up to some extent.

Burd *et al*., (2000)[25] reported that plant growth promoting rhizobacteria might enhance plant height and productivity by synthesizing phytohormones, increasing the local availability of nutrients, facilitating the uptake of nutrients by the plants decreasing the heavy metal toxicity in the plants antagonizing plant pathogens. The increased plant height and number of nodes/leaves as compared to control plants clearly showed the beneficial role of *Pseudomonas* as a rhizobacteria. Such an improvement might be attributed to phosphate solubilising capacity of bacteria as well as the ability of these microorganisms to produce growth promoting substances[26].

The results of this study suggest that plant growth promoting fluorescent *Pseudomonas* sp. isolated from apple rhizosphere has potential to be used successfully for replant problem of apple. There was a considerable increase in various plant and soil parameters after fifteen months of cyclic treatment of fluorescent *Pseudomonas* formulation. So it can be concluded from the present study that the individual and consortium of fluorescent *Pseudomonas* strains can be further exploited for bio fertilizer development to overcome the replant problem in apple orchards.

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