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Effect of Indigenous Strains of Fluorescent Pseudomonas Sp. on Growth of Apple Plants in Replant Site of Himachal Pradesh

KEYWORDS: Apple replant disease (ARD), Pseudomonas sp., antifungal activity, 16S rRNA, replant site

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ABSTRACT *Apple replant disease is a complex syndrome causing necrotic lesions on feeder roots, stunted tree growth and reduced cumulative yields. Fluorescent Pseudomonas sp. is most promising group of plant growth promoting rhizobacteria (PGPR) that are involved in plant growth enhancement and plant disease control. The ten fluorescent Pseudomonas sp. were isolated from apple rhizosphere from Shimla Distt. (HP) India. These isolates were screened out for plant growth promoting activities in vitro such as antifungal activity against different plant pathogens, phosphate solubilization, siderophore production, proteolytic activity, HCN and ammonia production. Out of ten isolates, two (An-2-nali and Pn-2-Kho) were selected on the basis of their high production of plant growth promoting activities and were genotypically characterized by 16S rRNA method. The strains were successfully used to study their effect on apple plants planted in replant area of Deola (Distt. Shimla). The results showed a significant increase in various plant, soil parameters and the disease incidence has also been reduced after the cyclic treatment of these strains. The aim of the study was to select more efficient PGPR strain of fluorescent Pseudomonas sp. which can be used for biofertilizer development for management of apple replant problem.*

Introduction

Apple replant disease (ARD) is a complex syndrome that occurs in young apple trees in replanted orchard site (Mai and Abawi, 1981). Symptoms include death of fine feeder roots, stunted growth above- and below-ground, and reduced fruit yields. A diversity of pathogens and parasites have been implicated as causal agents of replant disease and conflicting evidence abounds in the literature as to the importance of these agents in disease development (Jaffee et al,1982; Traquair,1984; Dullahide et al, 1994). Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield (Wu et al, 2005).

Plant growth promoting rhizobacteria (PGPR) especially fluorescent Pseudomonas sp. are highly versatile, diverse and efficient phosphate solubilizer. Fluorescent Pseudomonas sp. are most promising group of PGPR bacteria that are involved in plant growth enhancement and plant disease control (Ahmadzadeh et al., 2006). The large scale application of indigenous plant growth promoting fluorescent Pseudomonas sp. may be able to manage replant problem of fruit crops especially apple.

This study describes the isolation and selection of indigenous Pseudomonas strains with plant growth promoting activities from normal and replant site of apple rhizosphere. The Pseudomonas isolates were characterized based on their production of siderophores, antifungal activity, phosphate solubilizing activity, hydrolytic enzymes, HCN production, ammonia production and were identified by 16S rRNA gene technique. The efficacy of potential strains of fluorescent Pseudomonas sp. on growth of apple plants in replant site of Shimla district was tested. The main objective of this research was to exploit the potential fluorescent Pseudomonas strains for benefit of farmers involved in apples cultivation by solving the major apple replant problem. The main objective of this research was to exploit fluorescent Pseudomonas sp. as biofertilizer which can become one of the most promising biotechnologies to solve apple replant disease and can also improve production and with low inputs in fertilizer.

MATERIAL AND METHODS

Isolation and characterization of fluorescent Pseudomonas sp. by morphological and biochemical methods from apple rhizosphere

The fluorescent Pseudomonas sp. was isolated from the rhizosphere of apple plants in normal and replant site of Shimla (HP). The soil samples were collected in aseptic bags and immediately transported to laboratory under cold conditions (4°C) for further process. The serial dilution agar plate method was used to isolate Pseudomonas sp. on King's B medium. All the ten isolates of fluorescent Pseudomonas sp. were morphologically and biochemically characterized for Gram staining, spore staining, catalase, oxidase, denitrification, gelatin liquification test, lecithinase activity and Tween-80 hydrolyzation.

In vitro characterization of fluorescent Pseudomonas sp. for plant growth promoting activities

All the fluorescent Pseudomonas sp. were characterized for plant growth promoting activities viz., antifungal activity, phosphate solubilizing activity, siderophore activity, HCN production, ammonia production and for production of hydrolytic enzymes.

In vitro antifungal activity

The capacity of fluorescent Pseudomonas sp. isolated from orchard soils to inhibit fungal pathogens that contribute to apple replant disease (ARD) was assessed in vitro. Antifungal activity of each test strain of Pseudomonas sp. against different fungal pathogens viz., Dematophora sp., Fusarium sp., Phytophthora sp., Pythium sp. and Sclerotium sp. was checked by well plate assay method (Vincent, 1947).

Detection of phosphate solubilizing activity

Phosphate solubilizing activity was assessed on Pikovskaya's agar plates (Pikovskaya,1948) with known amount of inert phosphorous source (tricalcium phosphate) by measuring

the pinkish/orange zone produced around the well at 28±2° C for 48 hr. Phosphate solubilization expressed in terms of phosphate solubilizing efficiency (% PSE) calculated from equation.

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

Where,

Z= Diameter of yellow zone (mm)

C= Diameter of colony/bit (mm)

Siderophore activity

Siderophore production was tested by growing Pseudomonas sp. in the universal siderophore detection medium CAS agar (Schwyan and Neilands, 1987)

HCN and Ammonia production

For the production of hydrogen cyanide (HCN), the Pseudomonas sp. was screened out according to Baker and Schippers (1987) and ammonia production was detected by the method given by Lata & Saxena (2003).

Detection of hydrolytic enzymes

Chitinase activity was measured according to Chernin et al. (1995) and protease activity according to Kaur et al. (1988).

Identification of potential isolates of fluorescent Pseudomonas sp. by 16S rRNA technique.

All the Pseudomonas sp. was genotypically characterized by 16S rRNA gene sequencing. The genomic DNA from all the Pseudomonas sp. was isolated according to the protocol provided by manufacturer's (Bangalore GeNei) using DNA isolation kit. The quantification of genomic DNA of each Pseudomonas sp. was done by spectrophotometric method. The quantity of each DNA sample was judged by computing 260/280 ratio and also by performing agarose gel electrophoresis of the DNA samples.

The amplification of genomic DNA was carried out using specific oligonucleotide primer sequences: FP-1 (5′-GGTCTGAGAGGATGATCAGT-3′) and RP-1 (5′-TTAGCTCCACCTCGCGGC-3′) in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100). DNA was amplified in 25µL volumes containing 18.8 µl sterilized distilled water, 0.20 µl Taq DNA polymerase, 2.50 µl Taq buffer A, 1.50 µl dNTPs mixture, 0.50 µl forward specific primer, 0.50 µl reverse specific primer, 1.0 µl genomic DNA (Widmer et al., 1998). Samples were amplified in PTC-100 with a total of 35 cycles consisting of denaturation (1 minute at 94°C), annealing (2 minutes at 55°C) and extension (2 minute at 72 °C). Amplified DNA products were verified by 1.2% agarose gel electrophoresis (stained with ethidium bromide (0.5 mg/ml) in 1×TAE buffer.

The amplified DNA was viewed under the UV trans-illuminator and the image was taken through BIO-RAD gel documentation system using quantity one software.

For DNA sequencing, amplified DNA products of two selected Pseudomonas sp. isolates (An-2-nali and Pn-2-kho) was first purified followed by sequencing in Bioserve Private limited, Hyderabad, India). After sequencing the obtained sequence were analyzed for Basic local alignment search tool (BLAST) for their identification with the NCBI data base (Astschul et al., 1990).

Application of potential fluorescent Pseudomonas sp. viz., An-2-nali and Pn-2-kho in replant field

An apple orchard site (at Shimla) was originally planted with apple plants in year 1925 and then replanted in 1996. The planting failed to establish and exhibited many symptoms of ARD. In December 2011 the pits in the replant site was chiseled to remove as many old roots as possible from the soil. The pits were kept open for the period of one month. These pits were planted again in Feb, 2012 with apple plants procured from regional research station- Mashobra-UHF (Shimla) and were used to study the effect fluorescent Pseudomonas sp. (An-2-nali and Pn-2-kho) on growth and establishment of plants in replant site of Deola (Shimla).

The two selected Pseudomonas sp. 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 according to the treatments. Two concentrations (full and half concentration; half concentration was made by 1 1 of inoculum and water) of each culture were used along with control (T1; treated with water only). Five plants of each treatment viz., Treatment T1, T2, T3, T4 and T5 were planted in replant site after the treatment of their roots. The plants were treated with Pseudomonas suspension by dipping their roots in liquid inoculum for 15-30 minutes before planting them in replant site pits. The soil of pits was also treated with each inoculum before plantation of treated apple plants. The cyclic treatment for successive ten months were given at monthly interval in rhizosphere of plants by adding 50-100 ml of inoculum (1x10^8 cfu/ml) to the basin of plants. Results were compared with uninoculated control. Effect on plant parameters like plant height, plant girth, leaf size, number of nodes/leaves and soil parameters like NPK content of soil, soil pH and moisture content 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 analysed statistically using ANOVA.

RESULTS AND DISCUSSION

The screening strategy carried out in this paper consisted of the isolation of fluorescent Pseudomonas sp. capable of stimulating plant growth through biocontrol mechanisms (Table 1). Accepting what was earlier stated by Whipps (2001) and Maidack (1996) and other authors (Compant et al., 2005) that effective biocontrol agents often act through the combination of several different mechanisms, a selection procedure that allowed us to find strains that were positive for more than one antagonistic mechanism was designed.

Table1. Morphological and biochemical characterization of fluorescent Pseudomonas sp. isolated from normal and replant site of apple.

Fluorescent Pseudomonas Isolates	Cell shape	Gram staining	Spore staining	Pigment production	Catalase test	Oxidase test	Gelatin liquefication	Tween-80 hydrolyzation
An-3-bagh	Coccobacilli	-	-	Dark Green	+	+	+	-
An-4-bagh	Rods	-	-	Green	+	+	+	-

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An-3-kho	Coccobacilli	-	-	Green	+	+	+	-
An-3-naga	Coccobacilli	-	-	Green	+	+	+	+
An-2-nali	Coccobacilli	-	-	Green	+	+	+	-
An-1-panch	Coccobacilli	-	-	Green	+	+	+	-
An-4-panch	Rods	-	-	Green	+	+	+	-
Pn-2-kho	Rods	-	-	Yellow green	+	+	+	-
Pn-2-panch	Coccobacilli	-	-	Yellow green	+	+	+	-
Pn-3-panch	Coccobacilli	-	-	Greyish	+	+	+	-

+ indicates activity; - indicates no activity.

The screening resulted in a group of bacteria able to produce antifungal activity, phosphate solubilizing activity, siderophores, HCN, ammonia and protease activity thus allowing us to select bacteria showing multifarious plant growth promoting activities (Fig. 1 and 2).

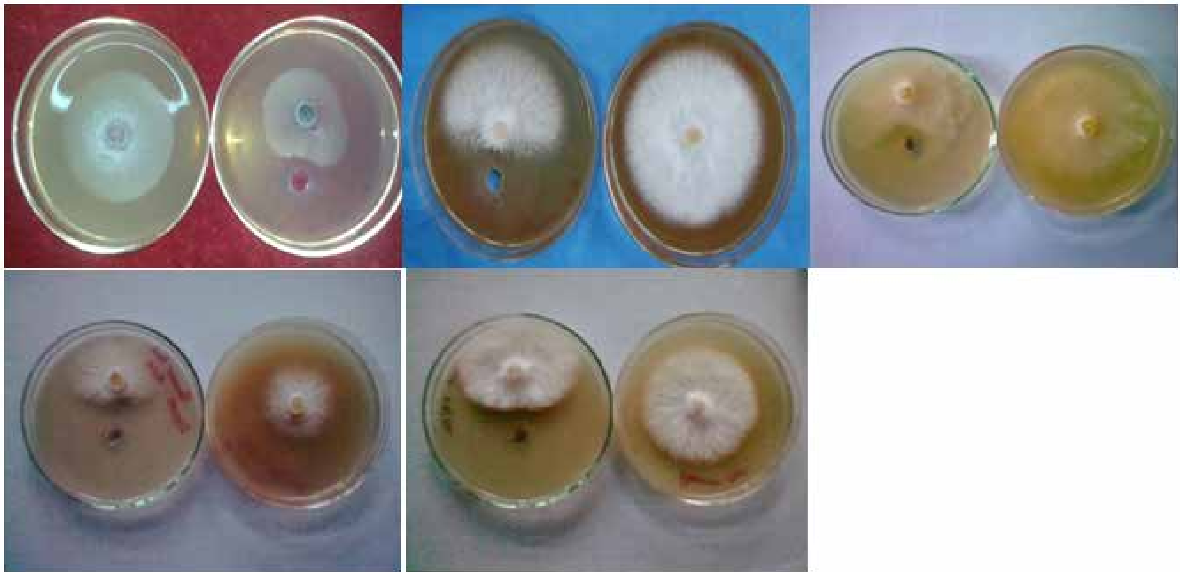

Fig. 1: Antifungal activity showed by potential fluorescent Pseudomonas isolates against different indicator test fungi viz., Fusarium sp. (a) and Pythium sp. (b) Phytophthora sp. (c), Dematophora sp. (d) and Sclerotium sp. (e) with their respective controls.

Fig. 2: Phosphate solubilizing activity, siderophore production and HCN production showed by Pseudomonas isolates isolated from apple rhizosphere

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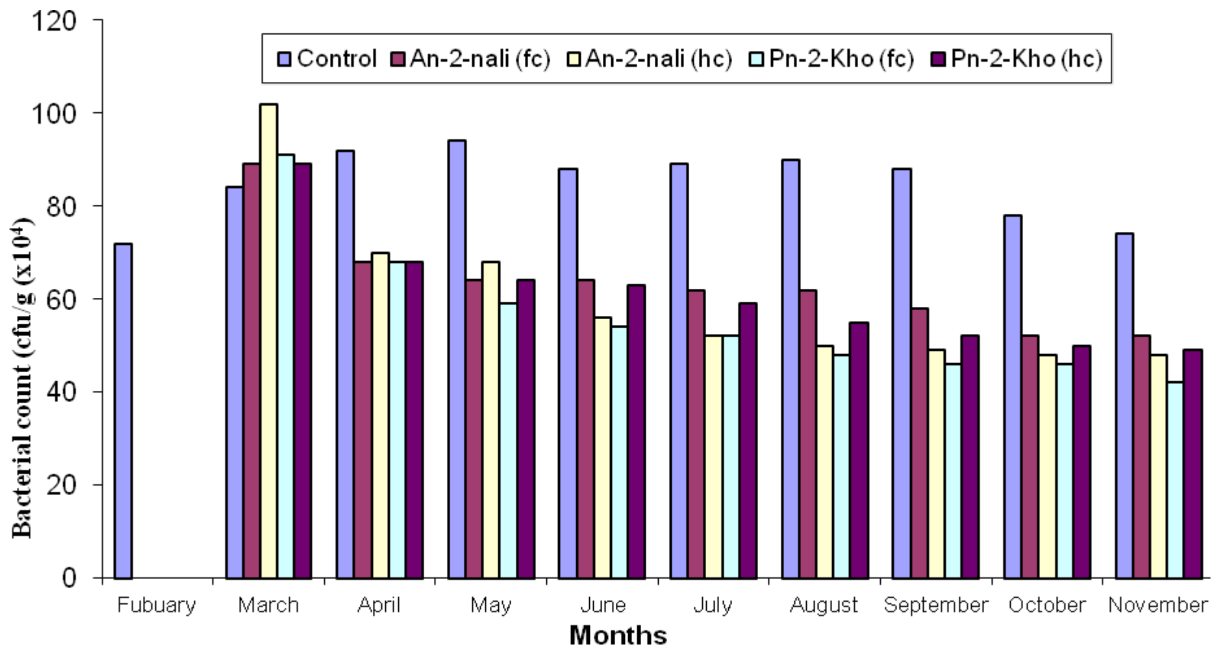

Fig. 3. Effect of cyclic treatment of fluorescent *Pseudomonas* sp. (An-2-nali & Pn-2-Kho) on the rhizobacterial population of apple plants planted in replant site at Deola

All the fluorescent Pseudomonas sp. showed significant production of antifungal activity, phosphate solubilizing activity, siderophores, HCN, ammonia, and protease activity (Table 2 and 3). Overall result showed that two isolates viz., Pn-2-kho and An-2-nali showed the production of maximum number of plant growth promoting activities in vitro and therefore selected for identification by 16S rRNA method.

Table2. Characterization of fluorescent Pseudomonas sp. for antifungal activity against indicator test fungi viz., Dematophora sp., Fusarium sp., Phytophthora sp., Pythium sp. and Sclerotium sp.

Fluorescent Pseudomonas Isolates	Percent inhibition of fungal pathogens				
	Dematophora sp.	Fusarium sp.	Phytophthora sp.	Pythium sp.	Sclerotium sp.
	% I*	% I*	% I*	% I*	% I*
An-3-bagh	27.27 (31.48)	21.73 (4.76)	17.18 (4.26)	22.91 (4.89)	0.00 (1.00)
An-4-bagh	38.02 (38.07)	7.31 (2.88)	23.07 (4.90)	0.00 (1.00)	0.00 (1.00)
An-3-kho	39.39 (38.88)	0.00 (1.00)	18.75 (4.40)	0.00 (1.00)	0.00 (1.00)
An-3-naga	0.00 (1.00)	7.31 (2.88)	0.00 (1.00)	0.00 (1.00)	17.30 (24.58)
An-2-nali	34.84 (36.18)	0.00 (1.00)	18.75 (4.44)	0.00 (1.00)	25 (29.99)
An-1-panch	35.21 (36.40)	0.00 (1.00)	0.00 (1.00)	28 (5.38)	25 (29.99)
An-4-panch	40.90 (39.76)	0.00 (1.00)	25 (5.09)	0.00 (1.00)	0.00 (1.00)
Pn-2-kho	37.87 (37.98)	28.26 (5.40)	25 (5.09)	0.00 (1.00)	31.57 (34.19)
Pn-2-panch	0.00 (1.00)	0.00 (1.00)	11.53 (5.34)	0.00 (1.00)	23.07 (28.71)
Pn-3-panch	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
$CD_{0.05}$	0.028	0.021	1.5	1.07	1.52

***Antifungal activity expressed in terms of percent inhibition (% I) of mycelial growth of indicator test fungi by well plate assay method.**
Percent inhibition (%I) = C-Z/ C× 100
Where, C: Growth of mycelia in control. Z: Growth of mycelia in test.

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Table3. Characterization of fluorescent Pseudomonas sp. for multifarious plant growth promoting activities.

Fluorescent Pseudomonas Isolates	Phosphate solubilizing activity	Siderophore Activity	HCN Production	Ammonia Production	Protease activity
	Available phosphate (µg/ml)*	% Siderophore Unit (%SU)**	Change of color (yellow to brown)		(mm diameter)
An-3-bagh	285	52.25	++	++	44
An-4-bagh	155	51.04	++	+	43
An-3-kho	260	40.70	++++	+++	45
An-3-naga	310	50.24	+++	++	31
An-2-nali	370	60.70	++	++++	46
An-1-panch	255	51.44	++	++	29
An-4-panch	325	42.10	+	+	46
Pn-2-kho	410	58.70	++++	+++	40
Pn-2-panch	195	56.41	+++	+++	18
Pn-3-panch	315	55.82	++	++	30

+ - Indicatesvery light brown
++ - Indicates light brown
+++ - Indicatesdark brown
++++ -Indicatesorange brown

* Phosphate solubilizing activity expressed in terms of tri-calcium phosphate solubilization which represents µg/ml of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (10-100 µg/ml).

**The siderophore units (% SU) expressed as % 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

Sequences of two selected isolates of Pseudomonas sp. viz., An-2-nali and Pn-2-kho were determined, followed by extensive sequence analyses. Similarity searches revealed that the sequences were of predominantly Pseudomonas origin. On blast, An-2-nali showed 99% homology with Pseudomonas aeruginosa strain NBAII CK-19 E with accession number HQ162480.1. and Pn-2-kho showed 96% homology with Pseudomonas aeruginosa strain RM3 with accession number FJ805450.1.

Plant growth promoting effect of PGPR strains in different crops were clearly demonstrated (Wu et al., 2005). Bacterial inoculants are able to increase plant growth and germination rate, improve seedling emergence, responses to external stress factors and protect plants from diseases (Lugtenberg, 2002). This present investigation confirms the earlier work. In this study, inoculation of fluorescent Pseudomonas sp. increased all growth parameters to some extent as compared to control plants. The performance of replanted apple plants after two year of cyclic treatment with An-2-nali and Pn-2-kho (full concentration and half concentration) along with control is detailed in Table 4.

Table 4.Effect of cyclic treatment of fluorescent Pseudomonas sp. (An-2-nali and Pn-2-kho) on the growth of apple plants planted in replant site at Deola (Shimla) of Himachal Pradesh

Treatments (1x 10^8cfu/ ml)	Plant height (cm)	Plant girth (cm)	Leaf area (cm^2)	Number of nodes
T1 (Control)	49.40	1.28	19.68	11.40
T2 (An-2-nali (fc))	55.20	1.80	31.94	9.60
T3 (An-2-nali (hc))	79.00	1.64	23.45	18.20
T4 (pn-2-kho (fc))	79.20	1.44	25.72	25.60
T5 (Pn-2-kho(hc))	37.60	1.28	29.01	5.00
$CD_{0.05}$	46.73	0.074	9.76	15.79

Table 5. Effect of cyclic treatment of fluorescent Pseudomonas sp. (An-2-nali and Pn-2-kho) on NPK content of soil.

Treatments	N (Kg/ha)	P (Kg/ha)	K (Kg/ha)
T1 (Control) (at the start of experiment)	313.60	28.00	100.57
T2 (An-2-nali (fc))	360.64	71.68	140.00
T3(An-2-nali (hc))	344.96	62.72	137.31
T4 (pn-2-kho (fc))	376.32	78.4	164.41
T5 (Pn-2-kho(hc))	351.32	64.96	138.65

The growth performance was studied on the basis of plant height, plant girth, leaf area and number of nodes. The present experiment revealed that rhizosphere inoculation with Pseudomonas sp. resulted in an increased plant height, plant girth, leaf area and number of nodes. Similar increases in plant height and leaf area were observed in different crops inoculated with Pseudomonas, Azospirillum and Azotobacter strains by other workers (Martinez-Toledo, 1988; Shaukat et al., 2006a; Shaukat et al., 2006b; Siddiqui and Shaukat, 2002). The above results revealed that the treatment T2 (An-2-nali (fc)) and treatment T4 (pn-2-kho (fc)) were better than treatment T3 (An-2-nali (hc)), treatment T4 (pn-2-kho (fc)) and treatment T1 (control). From the results it can be concluded that the full concentration of both the fluorescent Pseudomonas sp. i.e., An-2-nali and Pn-2-kho was best as compared to half concentration. It had also been found that all the treatments were better as compared to control.

The available N, P and K of rhizosphere soil were also assessed before and after the cyclic application of An-2-nali and Pn-2-kho (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.

The decrease in total bacterial population was also observed in treatment T2, treatment T3, treatment T4 and treatment T5 (Fig 3) with a gradual increase in total Pseudomonas count (Fig 4).

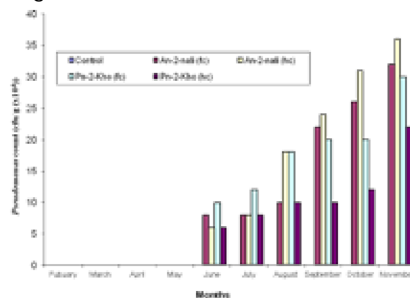

Fig. 4. Effect of cyclic treatment of fluorescent *Pseudomonas* sp. (An-2-nali & Pn-2-Kho) on the rhizobacterial population of apple planted in replant site at Deola

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Burd et al. (2000) 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 girth, plant height and leaf area as compared to control plants clearly showed the beneficial role of Pseudomonas as a rhizobacteria. Such an improvement might be attributed to phosphate solubilizing capacity of bacteria as well as the ability of these microorganisms to produce growth promoting substances (Salantur et al., 2006) .

CONCLUSION

The results of this study suggest that plant growth promoting fluorescent Pseudomonas sp. isolated from rhizosphere of apple has potential to be used successfully for replant problem of apple. The Pseudomonas sp. has shown increase in various plant and soil parameters after two year of cyclic treatment. So it can be concluded from the present study that fluorescent Pseudomonas sp. can be further used for biofertilizer development to overcome the replant problem in apple orchards.

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