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Suppression of soil borne fungal pathogens associated with apple replant disease by cyclic application of native strains of *Pseudomonas aeruginosa*

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Abstract: Plant growth promoting fluorescent *Pseudomonas aeruginosa* strains An-E and An- F were used for the suppression of replant disease organisms which were isolated from replant site of apple in Shimla district of Himachal Pradesh. Full and half concentration of individual and consortial strains were used for the experiment. Among all the treatments, full concentration of compatible consortial strains were quite effective in decreasing the deleterious rhizobacterial (197-99 cfu/g) and fungal population (7-0 cfu/g). Total rhizobacterial count starts decreasing after each cyclic application of fluorescent *P. aeruginosa* strains An-E and An-F due to root colonization property of these plant growth promoting strains in the replant site of apple. Establishment of *Pseudomonas aeruginosa* strains at replant site was inversely correlated with decreasing deleterious bacterial and fungal population in the replant site. 70 per cent survival of apple rootstocks was recorded in full concentration of consortial treatment (An-E and An-F) as compared to control after three years of plantation. Four major fungal pathogens viz. *Dematophor anecatrix, Phytophthora cactorum, Pythium ultimum* and *Fusarium oxysporum* were isolated and identified from National centre for fungal taxonomy, New Delhi. These strains can be further exploited and recommended for the management of replant problem of apple.

Keywords: *Pseudomonas aeruginosa,* Replant disease, Microbial count, Soil

INTRODUCTION

Apple is most important horticultural cash crop grown worldwide. The old apple orchard sites (>30 year old) facing a serious problem i.e. replant problem. Apple replant problem is caused by biotic factors (like soil bacteria, nematodes, fungi and actinomycetes) as well as abiotic factors (like insufficient available phosphorus for vigorous growth of young trees, other nutrient imbalances, low or high pH, phytotoxins, poor soil structure, heavy metals and arsenic contamination (Potter, 1999).

Replant disease has significant implications due to the continuous reduction in productivity over the life of the orchard in addition to replacement cost for sick or dead trees. It has been observed that when the apple plants are replanted in the old orchard site, they either die after 2-3 years of age or do not grow well (Mazzola, 2007; Kukreja, 2010). Symptoms of replant disease include severe stunting, shortened internodes, rosette leaves, small root system, decayed and discolored roots and reduced productivity. The replant problem is a complex disease syndrome that affects young trees in replanted orchard sites causing necrotic lesions on feeder roots, stunted tree growth and reduced cumulative yields (Mai and Abawi, 1981).

The etiology of apple replant disease (ARD) is complex and causal agents vary among different sites and regions (Mai and Abawi, 1981). This disease has been attributed to a number of possible abiotic factors (like insufficient available phosphorus for vigorous growth of young trees, other nutrient imbalances, low or high pH, phytotoxins, poor soil structure, heavy metals and arsenic contamination) as well as biotic factors (like soil bacteria, nematodes, fungi and actinomycetes).

Chemical fertilizer application helps in increasing yield at higher cost and may leads to environmental problems. In particular, phosphorus fertilizers present a serious risk of cadmium accumulation in soil (Al-Fayiz *et al*., 2007). Moreover phosphatic fertilizers when added to the soil form tri calcium phosphate in alkaline soil and ferrous phosphate or ferric hydroxyl phosphate and aluminum phosphate in acidic soil (Dave and Patel, 1999).

Microbial inoculants are in use for improving soil fertility during the last century and known to influence plant growth by various direct or indirect mechanisms (Ahmad *et al*., 2008). PGPR promote growth of several annual crops by increasing uptake of nitrogen, iron (through siderophores), phosphorus, synthesis of phytohormones, and by controlling plant diseases. Direct effect of Plant Growth Promoting Rhizobacteria (PGPR) occur when they

produce certain substances such as phytohormones, Phosphate solubilization which directly stimulate plant growth (Upadhyay and Srivastava, 2010).

The large scale application of fluorescent *Pseudomonas* to crops especially apple in replant sites as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers under these harsh and stress conditions and also promote to improve their growth. Hence, they may help to solve the replant problem of apple.

Fluorescent *Pseudomonas* have emerged as the largest and potentially most promising group of plant growth promoting rhizobacteria involved in the biocontrol of plant diseases (Sayed *et al*., 2005). These bacteria are ideally suited as soil inoculants because of their potential for rapid and aggressive colonization. This feature is suggested as a disease control mechanism by preventing the invasion of detrimental soil micro-organisms onto the root surface (O'Sullivan and O'Gara, 1992). Therefore, the present study was carried out with the use of selected plant growth promoting *Pseudomonas aeruginosa* strains which help in suppression of replant disease organisms both *in vitro* and *in vivo*. These fungal pathogens are one of the cause of replant problem of apple.

MATERIALS AND METHODS

Orchard site: The study was carried out at Magauta area of Shimla District of Himachal Pradesh (India) from 2011 to 2013. Orchard was originally planted with apple crop around 1974 and then replanted in 2005.There planted apple seedlings established poorly and exhibited many symptoms of apple replant disease like poor growth of trees, severe stunting, shortened internodes, decayed and discolored roots and reduced productivity. In 2011, new apple plantation was done in the same orchard.

Collection of soil samples: Soil samples were collected randomly before plantation of rootstocks in the test site to make one composite sample. Total five composite samples were collected from the test site to study the total microbial flora by serial dilution and pour plate method on nutrient agar, King's B medium and potato dextrose agar medium.Total viable counts ofbacteria, fluorescent *Pseudomonas* species and fungi were recorded in terms of colony forming units per gram of soil at dilution10^{-4} in case of bacteria and dilution 10^{-2} in case of *Pseudomonas* and fungal species.

Collection of apple rootstocksand selection of strains: Apple rootstocks variety M-111 (one year old) wereused for plantation in the test site. Total number of treatments were seven including control.Ten rootstocks were taken for each treatment. These rootstocks were selected to study the effect of native best selected plant growth promoting *Pseudomonas aeruginosa* strains An-E (accession no. KJ522923) and An-F (accession no. KJ522924) individually and in combination(1:1). Full and half concentrations were used for each treatment. The liquid formulation of these strains were used to treat the soil of pits with 100 ml inoculums of each treatment for successively two days before plantation. Also the roots of rootstocks were treated with liquid formulations separately before plantation for 30 minutes and planted at appropriate distance in already treated pits. Inoculums of each concentration of liquid formulation was applied at monthly intervals for successive eight month for proper establishment of appropriate concentration of *Pseudomonas* strains in the rhizosphere. Comparison of plant growth studies in terms of plant establishment and % survival was recorded.

Root colonization and determination of total microbial count: To study root colonization of applied *Pseudomonas aeruginosa* strains, rhizospheric soil samples of treated and untreated plants were collected after monthly intervals and were analyzed for total microbial (Total bacterial, *Pseudomonas* and fungal) count using serial dilution and pour plate method on nutrient agar, King' B agar and Potato dextrose agar respectively (Aneja, 2003).

To study the proper establishment of appropriate concentration of fluorescent *Pseudomonas* strains in experimental site, isolations were done from soil samples collected before cyclic application with liquid formulations. The changes in total microbial population after each inoculation was recorded in the form of cfu/g of soil in each treatment after a gap of one month upto one year and after a gap of three months in the second year was done (March 2011 to Nov. 2012).

The four major fungal pathogens isolated from replant site of apple were preserved in potato dextrose agar slants and sent for identification at National Research Centre for Fungal Taxonomy, New Delhi by Dr PN Chaudhary.

Survival percentage: Survival percentage was calculated as the total number of apple root stocks planted and the number of rootstocks survived expressed in terms of percentage survival.

RESULTS AND DISCUSSION

The present study showed that in comparison to treatments with plant growth promoting strains, the total rhizobacterial counts were decreased from 197 to 99cfu/g of soil after cyclic treatment for 24 months in full consortial concentration of An-E and An-F. In treatments with individual strains of An-E half and full, it decreased from 174-73 cfu/g of soil and 140-72cfu/g soil, respectively after 24 month. While in treatment with *P. aeruginosa* strain An-F half and full, it decreased from 178-79 cfu/g and 209-95 cfu /g of soil respectively (Fig. 1).

Table 1. Identification of fungal species isolated from rhizosphere soil of replant sites at Magauta, (Jubbal) in district Shimla.

Fungal isolate	Identification	Identification No.
Ar-F1	*Dematophoranecatrix*	4791.13
Ar-F2	*Fusarium oxysporum*	5790.13
Ar-F3	*Phytophthora cactorum*	5789.13
Ar-F4	*Pythium ultimum*	4790.13

Table 2. Effect of cyclic application of *Pseudomonas aeruginosa* strains An-E, An-F and their consortia on per cent survival of replanted apple rootstocks after three years of plantation.

***P. aeruginosa* strains and their consortia**		**% Survival**		
		Control	**Half**	**Full**
Individual	An-E	40	40	60
Individual	An-F	40	50	60
Consortia	An-E+An-F	40	60	70

Overall, it was recorded that total bacterial count starts decreasing after each cyclic treatment with half, full concentration of individual and consortial application of *Pseudomonas aeruginosa* strains as compared to control. The results are supported by the findings of Kloepper and Schroth (1979) which showed that PGPR increase plant growth indirectly by interacting with root microflora rather than directly producing plant growth producing substances.

It is generally assumed that root colonization by introduced bacteria is essential for biocontrol of root pathogen and that by increasing the population of introduced bacteria on the root should enhance disease control in apple, also after each cyclic application of *Pseudomonas* strain there was gradual decrease in total bacterial count and gradual increase in total *Pseudomonas* count in both the treated seedlings of apple(Suslow, 1982, Shweta *et al.* 2015).

Caesar and Burr(1987)studied the effect of bacterial population size on pathogen population and of disease severity on root cell and growth promotion of apple plants by specific strains of bacteria.

The *Pseudomonas* count in treatment with An-E (half) and An-E (full) concentrations were in the range of 2-17cfu/g soil and 1-19cfu/g soil respectively. In treatment with An-F (half) and An-F (full), the *Pseudomonas* count ranged from 0-10cfu/g soil and 1-18 cfu/g soil respectively. Where as in case of consortial half (An-E + An-F) and full concentration, counts were in the range of 2-18 cfu/g and 3-29 cfu/g respectively. Results showed that there were an increase in total *Pseudomonas* count in consecutive inoculation with *Pseudomonas aeruginosa* strains An-E and An-F. Full concentrations of consortial and individual strains were quite effective in increasing the total *Pseudomonas* count (Fig. 2).

The bacterial community composition especially that of the *Pseudomonas* population, is reportedly a key factor in soil suppressiveness to ARD (Mazzola and Gu, 2002). Soil suppressive to *Rhizoctoniasolani* AG-5 became conducive to this root pathogen when cropped for three years with apple (Mazzola, 1999).

These results indicated towards the possibility of sufficient increase, in number and establishment of

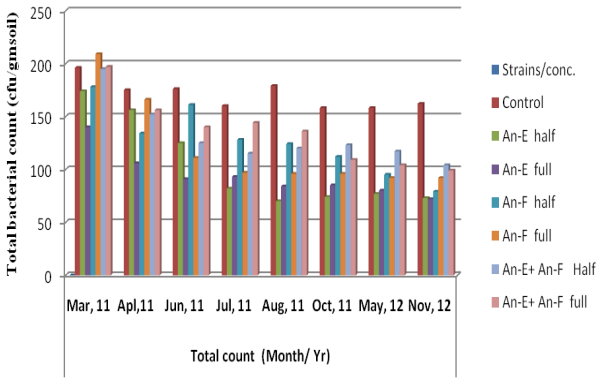

Fig. 1. *Effect of cyclic application of Pseudomonas aeruginosa strains An-E, An-F and their consortia on total bacterial count in rhizosphere of apple orchard.*

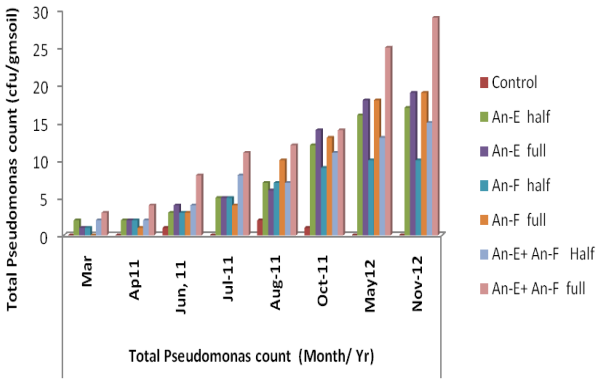

Fig. 2. *Effect of cyclic application of Pseudomonas aeruginosa strains An-E, An-F and their consortia on total Pseudomonas count in rhizosphere of apple orchard.*

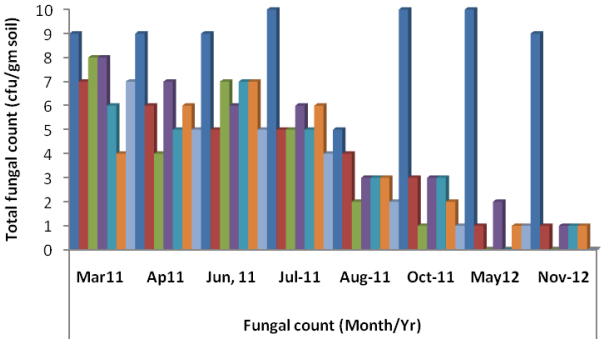

Fig. 3. *Effect of cyclic application of Pseudomonas aeruginosa strains An-E, An-F and their consortia on total fungal count in rhizosphere of apple orchard.*

fluorescent *Pseudomonas aeruginosa* strains in rhizosphere of apple rootstocks planted in replant problem site area. The results also indicated towards the possibility that the bacterial load of *P. aeruginos a*inoculum in the rhizosphere might have decreased the deleterious microflora that may be pathogenic flora of fungal and bacterial origin. The decrease in total bacterial population may be due to production of different types of secondary metabolites i.e. antagonistic, HCN, siderophores, ammonia and/or cell wall degrading lytic enzymes etc. involved exactly in any one of metabolite out of them or combination of one or two metabolite involved have not been ascertained yet. Sarvana kumar *et al.* (2007) studied that some pseudomonads had been recognized as antagonists of plant fungal pathogens and antibiotic producers.

Total fungal count in An-E (half) and (full) concentrations decreased from 7-1cfu/g soil and 8-0 cfu/g soil respectively after cyclic treatment with *P.aeruginosa* strains upto 24 months . In treatments with An-F (half and full concentration), it decreased from 8-1 cfu/g and 7-1 cfu/g, respectively. Fungal counts of consortial strains with half and full concentrations decreased from 7-1 cfu/g soil and 7-0cfu/g soil, respectively. It was observed from the results that total fungal count starts decreasing after monthly application of *P. aeruginosa* strains (Fig. 3).

From the result, it could be revealed that inoculated *Pseudomonas* strains get established after eight or nine months and the effect on root colonization after 24 months in terms of cfu/g of soil was found to be more in treatment with consortial strain (An-E+An-F) in full concentration followed by treatment with bioformulation of individual strains in (An-E and An-F) full concentrations. The present results are in accordance with earlier studies in which they have tried to assess the effect of bacterial population size on pathogen population and of disease severity on root cell and growth promotion of apple plants by specific *Pseudomonas* strains of bacteria (Caesar and Burr, 1987).

The fungal pathogenic organisms associated with apple trees were isolated, purified and preserved in Potato Dextrose Agar slantsand identified as *Pythiumultimum, Demato phoranecatrix, Fusarium oxysporum* and *Phytophthora cactorum* (Table 1). These are the major organisms of apple replant disease.

Survival percentage: Srivastava *et al*. (1999) studied colonization of wheat root by fluorescent Pseudomonads and found that root and shoot length of wheat plant was increased by fluorescent Pseudomonads after 30 days growth in non sterilized soil. After 120- days, the difference between root length of untreated control and treated plant was statistically non-significant.

The percentage survival of apple plants were calculated as the number of plants survived over the total number of plants treated and expressed in terms of percentage-survival (Table 2) after 3 years of plantation. 70 per-cent survival of replanted apple rootstocks after three years of plantation was observed in treatment with full concentration of consortial bioformulation of the strains An-E and An-F (1:1). Individual full concentrations of both the strains (An-E and An-F) and consortial half concentration showed 60 % survival.

Therefore,it was observed from the results that full concentration of two compatible *Pseudomonas aeruginosa* strains showed better results in terms of per cent survival as compared to individual strains.

Conclusion

Fluorescent *Pseudomonas* are effective root colonizer along with plant growth promoting activities. However, the success of our strains may be related to the use of native microflora that got easily established in apple rhizosphere without getting much competence from other microflora. In the present study, full concentration of compatible consortial strains were quite effective in decreasing the deleterious rhizobacterial (197 to 99 cfu/g) and fungal population (7-1 cfu/g and 7-0 cfu/g). Total rhizobacterial count starts decreasing after each cyclic application of fluorescent *P. aeruginosa* strains An-E and An-F due to root colonization property of these plant growth promoting strains in the replant site of apple. 70 per cent survival of apple rootstocks was recorded in full concentration of consortial treatment (An-E and An-F) as compared to control after three years of plantation. They controlled deleterious fungi and bacteria due to production of many indirect PGP activities like broad-spectrum antifungal antibiotics, iron chelating siderophores, HCN, ammonia, supply of nutrients like available phosphorus, iron ions and other small molecules through P-solubilizing enzymes and lytic enzymes etc.

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