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Soil treatments and rootstocks against apple replant problem

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Abstract
Apple orchards planted in late sixties in Himanchal Pradesh and North Western Himalayan region have shown symptoms of declining productivity as these plants have completed their economic life span. Due to limited land, choice of crops for different micro climatic niches and incomparable economic equivalence of other fruits with apple, orchardists are compelled to replant old apple orchard sites with apple only. There has been substantial increase in the proportion of declining orchards which need to be changed. Therefore, standardization of suitable agro-techniques to combat replant problem in apple for better field survival rate and productivity under replant conditions for sustainability of apple industry in the state. In present study there were 20 treatments comprising of four apple rootstocks i.e. Seedling, M.793, MM.111 and M.7 and five different soil management treatments i.e. control, soil fumigation, PGPR, biocontrol and combined (Soil fumigation + PGPR + Biocontrol) with three replications. The pooled data over the years 2015 and 2016 revealed that M.793 rootstock recorded significantly higher values of plant growth and vigour parameters, soil enzyme and biological activities. Among the treatments, highest growth and vigour parameters, soil enzymatic activities and microbial counts were recorded maximum in combined treatment. The interaction between rootstocks and treatments revealed that combinations of M.793 × combined treatment recorded maximum growth and vigour traits, enzymatic activities, bacterial counts, fungal counts and actinomycetes counts compared to other rootstocks and treatment combinations under replant situations.

Keywords: Apple, biological activities, pgpr, replant soil, rootstocks, *Trichoderma viride* (biocontrol)

Introduction
Apple (*Malus* × *domestica* Borkh.) orchards planted in early sixties have Jammu and Kashmir, Himachal Pradesh, Uttrakhand, North Eastern hilly states and south Nilgiri hills in India. It is grown over an area of 277 (in '000 ha) with annual production of 2242 (in '000 MT) and productivity of 8.0 MT (NHB, 2016) [1]. With increasing population, adverse environmental factors and shirking the land resource in hill states, orchardists are compelled to replant old apple orchard sites with apple, which lead to drastic economic loss not only due to uprooting of old trees but also because of poor establishment of new plantations on the same site. As a result, a general decline in the growth and productivity of replanted apple orchards is commonly observed.

Apple replant disease (ARD) is a complex syndrome that occurs in young apple trees in replanted orchard sites (Mai and Abawi, 1981) [2]. Apple replant problem, though reported in the literature for more than century, has yet to have its causes clearly defined. Decline in apple productivity has been attributed to fungi, bacteria, nematodes, toxic agents, insect-pests, nutritional disturbances, enzymatic activates and chemical residues (Benizri *et al.*, 2005) [3]. In general, apple orchards of more than 50 years age have shown much more unfruitfulness. There has been increasing concern about poor growth of apple trees planted at sites where apple tree grew before (Utkhede and Smith, 1994) [4]. After several years, trees may recover from the initial growth depression and eventually reach the size and annual yields of unaffected trees (Haas and Defago, 2005 [5] and Leinfelder and Merwin, 2006) [6]. (Foy *et al.*, 1996) [7]. Despite this partial recovery, cumulative yields and profitability in ARD affected orchards usually remain lower than in unaffected orchards (Peterson and Hinman, 1994) [8].

There has been substantial increase in the proportion of declining orchards which need to be replanted. Therefore standardization of agro-techniques with integration of rootstocks, soil sterilization, biocontrol and PGPR is an attempt to replant problem. The study effect on plant growth and soil biological activities traits of replantation of apple.

Materials and methods

Location and Climate: The experiment was laid out at an elevation of 1250 m above mean sea level at 30° 51'N latitude and 76° 11'E longitude in the Department of Fruit Science, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh. The experimental site lies under the sub-temperate, sub-humid mid-hill agro climatic zone II of Himachal Pradesh where, summer is moderately hot during May-June while, winter is quite severe during December-January. The annual rainfall ranges between 110-120 cm and the major amount of which is received during June to September.

Rootstocks establishment

One year old uniform seedling and clonal rootstocks namely seedling, M.793, MM.111 and M.7 were planted in black polythene bags (18″ × 9″ size) containing a mixture of soil, FYM and sand (2:1:1). The optimum level of moisture was maintained in the growing media of polybags by regular irrigation. Planting was done under natural condition, in first week of February, 2014.

Materials and Treatments

The suitable methodology has been used to understand the response of apple seedlings and clonal rootstocks to replant soil. One year polybag raised four rootstocks i.e. M. 793, MM.111, M.7 and Seedling were planted in 50 liters plastic container and filled with soil and FYM (3:1) along with soil ball and application of five soil management treatments viz., control, soil fumigation, PGPR, biocontrol and combined (Soil fumigation + PGPR + Biocontrol) in Factorial Completely Randomized Design with three replications. Planting was done in pots under open field condition, during first week of January, 2015. These rootstocks were grafted with 'Super Chief' in March 2015.

Soil Fumigation and Planting

Soil from replanted orchard site at Habban was brought to the experiment field of Department of Fruit Science. A heap of soil was sterilized with formalin (1:9) formaldehyde solution and covered under polythene sheet for three weeks. Afterword the soil heap was opened and worked in such a way to exclude formaldehyde fumes from soil and after two weeks the manures were mixed and sterilized soil was filled in 50 liters plastic container along with plants of polybag raised seedling and different clonal rootstocks.

Time of application: (PGPR and *Trichoderma viride*)

Plant Growth Promoting Rhizobacteria [(PGPR) 250 ml] and Bio control [(*Trichoderma viride*) 100 gm] were applied at the time of planting in pots and then repeated after every three months up to December 2016.

Soil Enzymes

Urease: The urease enzyme estimation was carried out by method given by Tabatabai and Bremner, (1969) [9]. Five gram of moist soil was taken in a volumetric flask (50 ml), added 0.2 ml of toluene and 9 ml of urea solution and mixed again for a few seconds. Then stopper the flasks and incubated for 2 hours at 37 ^{o}C. After the incubation, added approximately 35 ml of KCL-Ag_2SO_4 solution, swirled the flask for a few seconds and allowed the flask to stand till contents have cooled to room temperature (above 5 minute). Brought up the contents to 50 ml by addition of KCL-Ag_2SO_4 solution and mixed the contents thoroughly. To perform controls, followed the procedure described for assay of urease activity, but made the addition of 1 ml of 0.2 M urea solution after the addition of 35 ml of KCL-Ag_2SO_4 solution. It is recommended that at least three replications are carried out. The reaction shows a linear time course up to 5 hours.

Phosphatase

The phosphtase enzyme estimation was carried out by method given by Tabatabai and Bremner, (1969) [9]. One gram of soil taken in test tube was incubated with 1ml of 5mM buffered sodium p-nitrophenyl phosphate in acetate buffer (pH 5.2) and 0.3ml toluene at 37 °C for 1 hour. Determination of p-nitrophenol involved the colourimetric analysis of the extract obtained by treating the incubated soil sample with 4 ml water, 10 ml of 0.5 M NaOH and by filtering it through Whatman No. 42 filter paper. The suspension obtained by shaking the mixture for 1 minute and absorbance of yellow colour of p-nitrophenol released was determined spectrophotometrically at 420 µ wavelength. The standard curve was prepared by p-nitrophenol (10-100 ppm). The results were expressed as µ mole of p-nitrophenol released per gram soil per hour (µmole p-nitrophenol g^{-1}soil h^{-1}).

Dehydrogenase

The dehydrogenase enzyme estimation was carried out by method given by Casida *et al.*, (1964) [10]. One gram of air - dried soil taken in air-tight screw capped test tube was incubated with 0.2 ml of 3 % 2, 3, 5 – triphenyl tetrazolium chloride (TTC) and 0.5 ml of 1% glucose solution at 28 ± 0.5°C for 24 h. Added 10 ml of methanol and allowed to stand for 6 h. Clear pink coloured supernatant liquid was readings taken with a spectrophotometer at a wavelength of 485 nm (blue filter). Extrapolated triphenyl formazen (TPF) formed from the standard curve drawn in the range of 10 to 90 mg TPF ml^{-1}. The results were expressed as mg TPF formed per h per g soil.

Phytase (Inorganic Phosphate Standard Curve)

Phosphate standard solutions were prepared by initially making 1 ml stock solution of KH_2PO_4 in water. This was then diluted to prepare a range of phosphate standard concentrations ranging from 200 to 1,000 nmol/ml phosphate. To 1.0 ml of each reference standard was added 1.0 ml of 0.5 M tri-chloroacetic acid (TCA) solution and 1 ml of reagent A (prepared fresh by dissolving 5.0 g of $FeSO_4.7H_2O$ in 90 ml of distilled water and adding 10.0 ml of 8.0% (w/v) ammonium molybdate. The 8.0% ammonium molybdate solution was prepared by dissolving 8.0 g of ammonium molybdate in 50 ml of distilled H_2O. Then, 27.0 ml of 10 M H_2SO_4 was added and the volume as made to 100 ml with distilled water. After standing at room temperature for 5 minute, the absorbance of each was determined at 660 nm.

Detection of rhizosphere microbial counts

Microbial counts was performed by standard plate counts technique, Wollum, (1982) [11] by employing different media for different groups of microorganisms.

Suspension of 0.1ml from dilution blank was spread over pre-poured solid media *viz.*, Nutrient Agar, Jensen medium, Jensen, (1987) [12] and Pikovskaya's medium, Pikovskaya, (1948) [13] with the help of glass spreader under aseptic conditions for enumeration of bacteria, free nitrogen fixing bacteria and phosphate solubilizing bacteria, respectively. Plates were incubated in inverted position at 28±2ºC for 48 hours. After the incubation period, the microbial counts was expressed as colony forming unit per gram of soil (cfug^{-1} soil).

Plant Growth traits

Plant height: The plant height was measured from the ground level to the top with the help of a graduated scale and mean was worked out and expressed in centimeters (cm).

Number of feathers

Total number of branches per plant were counted in each plant and treatment.

Leaf area

Ten fully expanded mature leaves were taken from each replication of each treatment. The leaf area was measured with the help of portable Laser (CI- 202), CID Bio-Science leaf area meter and average leaf area of each treatment was calculated and expressed as square centimeter (cm^2).

Plant volume

The total above ground plant volume of each plant was calculated from the data on height and spread measurements according to the formulae suggested by Westwood, (1978) [14] and was expressed in cubic meters (m^3).

1) For a tree that was taller than wide (Prolate Spheroid),
Volume = 4/3 πab^2
2) For a tree that was wider than tall (Oblate Spheroid),
Volume = 4/3 $\pi a^2 b$
Where,
π = 3.14
a = ½ the major axis (height)
b = ½ the minor axis (spread)

Statistical analysis

Data on plant growth and physiological parameters of replanted apple to determine the significance of differences analyzed by using Completely Randomized Design (RBD)-two way analysis of variance (ANOVA) as suggested by Gomez and Gomez, (1984) [15]. In addition to show the interrelationship between rootstocks in combination with soil management treatments and mean values of each studied plant growth and physiological parameters statistical analysis program (SPSS) was used.

Results and discussion

Correlation studies with rootstocks and treatments: The correlation studies of both soil management treatments and rootstocks among the different characters showed that plant volume had positive and significant association with all growth traits and biological activities except fungal and actinomycetes counts with rootstocks and treatments Table 5 to 6, respectively. The path coefficient analysis revealed (Table 7 and 8) that like traits plant height (0.468 cm), urease activity (-0.939 µ mol $L^{-1}g^{-1}h^{-1}$), phosphatase activity (0.102 µ mol $L^{-1}g^{-1}h^{-1}$), phytase activity (0.752 µ mol $L^{-1}g^{-1}h^{-1}$), fungal counts (0.226 10^4cfu/g soil) and actinomycetes counts (0.178 10^2cfu/g soil) had the highest positive direct effect on plant volume. The leaf area (-0.077 cm^2), number of feather (-0.266), dehydrogenase activity (-0.332 µ TPF $g^{-1}h^{-1}$) and bacterial (-0.105 10^5cfu/g soil) had the direct negative effect but correlation with plant volume is positive with treatments.

Various soil enzymatic activates, microbial counts and growth traits with rootstocks such as plant height (0.295 cm), leaf area (0.012 cm^2), number of feather (0.956), phosphatase activity (1.612 µ mol $L^{-1}g^{-1}h^{-1}$) and phytase activity (0.031 µ mol $L^{-1}g^{-1}h^{-1}$) and fungal counts (0.537 10^4cfu/g soil) had the highest positive direct effect on plant volume. The dehydrogenase activity (-0.381 µ TPF $g^{-1}h^{-1}$), urease activity (-0.017 µ mol $L^{-1}g^{-1}h^{-1}$), bacterial counts (-1.596 10^5 cfu/g soil) and actinomycetes counts (-0.178 10^2cfu/g soil) had the direct negative effect but correlation with plant volume was found to be positive (Table 12 and 14).

Soil biological (Enzymatic activities and microbial counts)

There was a big difference in growth between rootstocks transplanted into treatments and control with replant soil, with those in rootstocks and treatments displaying significantly ($P< 0.05$) higher growth in replant soils over the two years. Plants grafted onto M.793 rootstock had significantly highest dehydrogenase activity (9.32 µ TPF $g^{-1}h^{-1}$), urease activity (42.65 µ mol $L^{-1}g^{-1}h^{-1}$), phosphatase activity (431.43 µ mol $L^{-1}g^{-1}h^{-1}$), phytase activity (5179.23 µ mol $L^{-1}g^{-1}h^{-1}$), bacterial counts (101.53 10^5cfu/g soil) while, fungal counts (14.59 10^4cfu/g soil) and actinomycetes counts (13.67 10^2cfu/g soil) in MM.111. Minimum in activity of different soil enzymes was found seedling rootstock. Different rootstocks did not show consistent influence on rhizobacterial, fungal and actinomycetes counts during both the years of study (Table 4 and 5). Among the treatments, dehydrogenase activity (11.90 µ TPF $g^{-1}h^{-1}$), urease activity (58.81 µ mol $L^{-1}g^{-1}h^{-1}$), phosphatase activity (497.13 µ mol $L^{-1}g^{-1}h^{-1}$), phytase activity (5529.63 µ mol $L^{-1}g^{-1}h^{-1}$), bacterial counts (107.71 10^5cfu/g soil), fungal counts (15.71 10^4cfu/g soil) and actinomycetes counts (14.06 10^2cfu/g soil) were recorded maximum in combined treatment, compared to other treatments however, minimum in control. The highest actinomycetes counts (13.96 10^2cfu/g soil) was observed in control and lowest in biocontrol (Table 1 and 2).

The interaction effect of rootstock and treatment combinations revealed that M.793 rootstock × combined treatment recorded highest values of dehydrogenase activity [12.50 µ TPF $g^{-1}h^{-1}$ (Fig. 5], urease activity [65.16 µ mol $L^{-1}g^{-1}h^{-1}$ (Fig.6)], phosphatase activity [504.67 µ mol $L^{-1}g^{-1}h^{-1}$ (Fig. 7)], phytase activity [5590.83 µ mol $L^{-1}g^{-1}h^{-1}$ (Fig. 8)]. Actinomycetes counts [14.75 10^2cfu/g soil). The bacterial counts [109.14 10^5cfu/g soil (Fig. 9)], fungal counts [17.70 10^4cfu/g soil (Fig. 10)] were recorded in MM.111 × combined however, minimum in seedling × control figures. The interaction between rootstocks and treatments was found to be non-significant in respect of rhizobacterial, fungal and actinomycetes counts during 2015 and 2016. Numerically, all other rootstocks registered higher rhizobacterial, fungal and actinomycetes counts with combined treatment combinations figures 9 to 11, respectively.

Present study indicates that the enzyme activates of replant sick apple soil was increased with the application of combined treatment (Soil fumigation + PGPR + *Trichoderma viride*) and M.793 rootstock. Kumar *et al.*, (2014) [16] also reported that the combined application of indigenous PGPR (*B. megaterium*, *A. chlorophenolicus* and *Enterobacter*) significantly increased 17.5%, 79.8%, 78.6% and 26.7% plant height, grain yield, straw yield and test weight under pot

condition and also 29.4%, 27.5%, 29.5% and 17.6% under field conditions which supported our results. These findings are also in conformity with those of Jarak *et al.*, (2012) [17] who also reported the ability of *Trichoderma viride*, *Pseudomonas* sp., *Bacillus* sp. and *Azotobacter chroococcum* strain to enhance maize growth (*Zea mays* L.) under field conditions. These results are also in line with those obtained by Kaur and Reddy, (2015) [18] who found that the highest yield was obtained by bio-inoculation of treatments singly or together with biofertilizer in maize-wheat cropping system. The results are further supported by the findings of Gaind *et al.*, (2006) [19] who also reported that incorporation of compost prepared from paddy straw and fungal inoculants in wheat improved enzymatic activities and phosphorous content of soil. These findings are also in conformity with those of Kaur and Reddy, (2015) [18] who also reported that inoculation of PSB together with rock phosphate fertilizer increased the crop growth parameters (shoot height, shoot and root dry biomass) and grain yield of wheat. Improved enzymatic activities and improved soil phosphorous due to incorporation of compost prepared from paddy straw and fungal inoculants under wheat have been reported by Gaind *et al.*, (2006) [19] which supported present findings. Higher enzyme activities in soil indicate the potential of soil to effect the biochemical transformations necessary for the maintenance of soil fertility, Rao *et al.*, (1990) [20]. The results of present study are in agreement with the observations of Seo *et al.*, (2010) [21], Jarak *et al.*, (2012) [17] and Pesakovic *et al.*, (2013) [22] who reported increased microbial population with bacterial inoculation in strawberry. The findings are in line with the work of Aseri *et al.*, (2008) [23] and Raj and Sharma, (2009) [24] who also reported increased rhizobacterial population with PGPR inoculation. Furthermore, the rhizosphere is known to be a zone of increased microbial activity and consequently enzyme activity.

Positive correlations between soil enzyme activity and soil microbe quantity, microbe diversity and microbe biomass have been observed by many workers (Groffman *et al.*, 2001 [25], Taylor *et al.*, 2002 [26] and Bandick and Dick, 1999) [27]. The soil enzymes are important components of soil, and soil enzymatic activities are correlated significantly with the soil fertility and efficiency of nutrition to plants. They are important indexes for determining the biological activity and productivity of soil (Tuyler, 1974) [28]. Generally, Pb^{2+} can directly interact with the active functional sites of the enzymes, and change their spatial conformation. The activities of urease appears to be more sensitive to pollution than that of other soil enzymes. The soil enzymatic activities in the planted group increased significantly than those of the control group. When a heavy metal replace the active functional sites of an enzyme by combining with their mercapto, amino, or carboxyl, the enzymatic activity inhibition would occur, called enzymatic passivation (Zhou, 1995) [29]. The oligotrophic microbes are very sensitive to metals, which indicated that the growth of microbial community is considerably limited in soils, poor in organic matter and nutrient content (Brookes, 1995) [30]. Further, it is considered that heavy metals mainly inhibit enzymatic reactions through either their complexing with substrate or blocking the functional groups of enzymes or reacting with complex enzyme-substrate (Speir *et al.*, 1995) [31]. Inoculation with AM fungi enrich soil microbe quantities, equilibrate proportion of various microbes, maintain a stabilization of proper proportion of the microbes, enhance soil carbon, nitrogen, and phosphorous cycling power, thus improve the soil enzyme activity (Zhao *et al.*, 2010) [32].

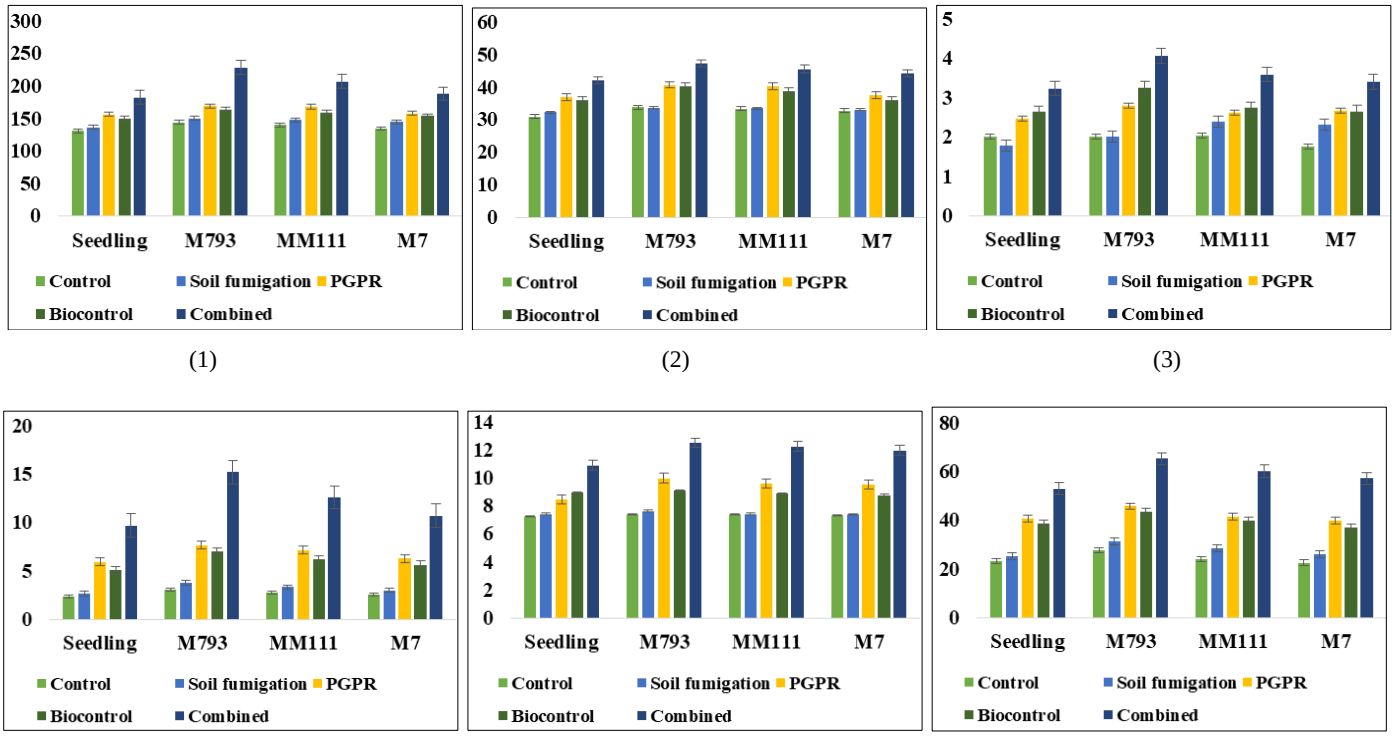

Fig 1: Effect of different rootstocks and treatments on plant height (1), leaf area (2), number of feathers (3), plant volume (4), dehydrogenase activity (5) and urease activity (6) of replanted apple. Vertical bar represent mean of three replication ± SE m and LSD ($p \leq 0.05$).

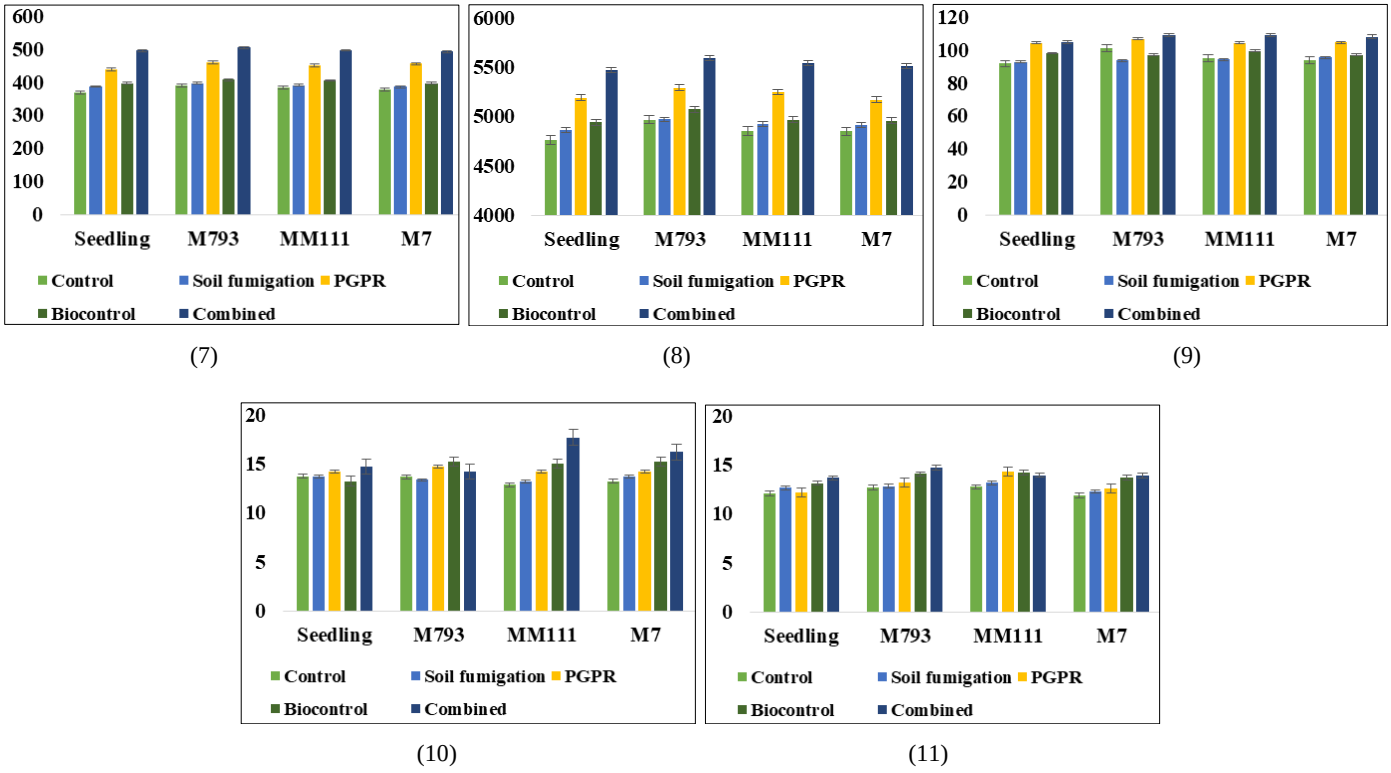

Fig 2: Effect of different rootstocks and treatments phosphatase activity (7), phytase activity (8), bacterial count (9), fungal count (10) and actinomycetes count (11) of replanted apple. Vertical bar represent mean of three replication ± SE m and LSD ($p \leq 0.05$).

Plant Growth traits

In the present study, different rootstocks and soil management treatments recorded significant increase in plant growth and soil biological activities. Among the rootstocks, M.793 rootstock had significant increase in plant height (179.42 cm), number of feathers (5.24), leaf area (40.07 cm^2) and plant volume (1.52 m^3) however, minimum in seedling rootstock (Table 4). Among the treatments, plant height (203.64 cm), number of feathers (5.29), leaf area (46.43 cm^2) and plant volume (2.07 m^3) were recorded maximum in combined treatment, compared to other treatments however, minimum in control (Table 2). Among the rootstocks and treatment combinations interaction combined treatment × M.793 rootstock recorded maximum plant height (226.50 cm), number of feathers (6.41), leaf area (48.51 cm^2) and plant volume (2.50 m^3) compared to other rootstock and soil treatment combinations given in figures 1 to 4, respectively. Similar results were obtained by (Thakur, 2017 [33], Misratia *et al.*, 2013 [34], Tukey *et al.*, 1962 [35], Fallahi *et al.*, 2002 [36], Chandel and Chauhan, 1992, [37]) which supported the results obtained in the present study. Seedling rootstock was found to be more sensitive to replant problem because of their susceptibility to soil borne disease in particular. In general, replant sites have more pathogens, thereby, directly affecting the plant growth and development of new saplings. Comparatively, the clonal rootstocks (M.793, MM.111 and M.7) have been reported to be more tolerant to soil borne diseases (Andreev, 1984 [38] and Kviklys *et al.*, 2007 [39]) and have more biomass of adventitious roots. Production of plant growth regulators such as auxin, gibberellins and cytokinins by the plant growth promoting rhizobacteria has been suggested as possible mechanisms of action affecting plant growth. The findings are in line with reports of (Thakur, 2017 [33], Ferree and Warringon, 2003 [40], Rana and Chandel, 2003 [41], Karlidag *et al.*, 2007 [42], Kirad *et al.*, 2009 [43], Tripathi *et al.*, 2014 [44], Kipkoriony and Fusao, 2006 [45]) who also recorded increased plant height and spread with the application of plant growth promoting rhizobacteria and *Trichoderma viride*.

Rumberger *et al.*, (2004) [46] reported that apple rootstock genotype had a stronger effect on the rhizosphere soil microbial community composition than did the pre-plant soil treatments in soils. Present findings reveal 2 years later, rhizosphere communities of bacteria, fungi, and actinomycetes still clustered roughly together by rootstock genotype (Fig. 8 to 10, respectively). Plant species specific rhizosphere microbial communities have been reported widely (Marschner *et al.*, 2001 [47], Miethling *et al.*, 2000 [48], Westover *et al.*, 1997 [49]) as have changes in rhizosphere microbial communities due to intra-specific variation (Carelli *et al.*, 2000 [50], Cattelan *et al.*, 1998 [51], Di Giovanni *et al.*, 1999 [52]). In our experiment, the same scion variety ('Super chief') was grafted onto four different apple rootstocks. The rhizosphere of M.793 had the highest cultureable soil bacterial counts compared with the other rootstocks, and this rootstock also produced the highest plant growth during 2015 and 2016.

In present study, rootstocks strongly affected rhizosphere microbial community composition (Fig. 8 to 10, respectively). This suggests that rhizosphere fungi and bacteria communities may be more influential in the promulgation or suppression of ARD than bacteria and oomycetes at this site. These findings are similar to those of Mazzola that also implicated the involvement of fungi and pseudomonads in ARD (Gu and Mazzola, 2003 [53], Mazzola, 1997 1998 [54]). Rootstocks were not only a main factor contributing to observed changes microbial composition in the rhizosphere, but were also a dominant factor for tree growth and yield. Rootstock genotype selection is thus a promising alternative for managing ARD (Shengrui *et al.*, 2006) [55].

Conclusion

From the present investigation it can be concluded that M.793 and combined treatment (Soil fumigation +PGPR

+Biocontrol) were most effect an individual basis of influence of plant growth traits and soil biological activities. However, the consortium of M.793 rootstock and combined treatment recorded significant increase in plant growth and soil biological activates in the apple under replant sick soil under pot culture studies. Most of growth and soil biological traits had positive correlation with plant volume.

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