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SCREENING OF RHIZOMICROFLORA ISOLATED FROM THE RHIZOSPHERE OF *AEGLE MARMELOS* FOR MULTIPLE PLANT GROWTH PROMOTING AND ANTIMICROBIAL ACTIVITIES.

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ABSTRACT

The rhizosphere is the area of intense microbiological activity. The population of microorganisms differs qualitatively and quantitatively in the rhizosphere from plant to plant. So the rhizosphere may harbor some interesting organisms. As roots of *Aegle marmelos* are used as drug, the rhizosphere of *Aegle marmelos* may harbor various types of microorganisms. PGPR increase plant growth by various mechanisms like NH_3, HCN, IAA, Siderophore production and PO_4 solubilization. In the present study 21 bacterial isolates from the rhizosphere of medicinal plant *Aegle marmelos* were obtained and characterized on the basis of morphological, cultural and biochemical properties. All isolates were screened for various PGP activities. Interestingly out of 21 isolates 18 (85%) showed NH_3 and IAA production within the range of 9-126µg/ml, 81% produced HCN, 33% produced siderophore and 57% solubilized PO_4. Two isolates AM3 and AM9 were identified as *Bacillus cereus* and *Bacillus pumilis* respectively. AM3 was identified by PIBWin software and AM 9 by 16s rRNA and showed positive results for all 5 PGP activities. While isolate AMH2 showed 4 PGP activities. AM3 and AM9 isolates were selected further for seed germination and pot experiment. AM 3 and AM 9 showed 100% and 96% seed germination respectively, increase in plant height, root length, shoot length, fresh and dry weight as compared to control. *Bacillus cereus*, isolates AMH 2 and AM10 showed antibacterial activity against plant pathogen *Xanthomonas axonopodis* and *Xanthomonas citri*. So these isolates can be used as bio inoculants and bio controlling agents.

KEYWORDS: Rhizosphere, PGPR, medicinal plants, antibacterial activity.

INTRODUCTION

The term rhizosphere was introduced by the German scientist Hiltner to denote the region of the soil which is adjacent to plant roots thought to be of great importance to plant health and soil fertility.[1] The rhizosphere is the zone of soil surrounding a plant root where the biology & chemistry of the soil are influenced by the root. This zone is about 1 mm wide but has no distinct edge. It is an area of intense biological & chemical activity influenced by compounds exuded by roots & by micro organisms feeding on the compounds. Greater rhizosphere effect is seen with bacteria than the actinomycetes and fungi.[1]

Rhizosphere microorganisms differ from one plant to other. The population of microorganisms in rhizosphere and non rhizosphere soil differs qualitatively and quantitatively.[2]

The rhizosphere of medicinal plant may harbor some interesting organisms. *Aegle marmelos* (Bael) tree is found throughout India and other continents in dry forest as well as cultivated. It belongs to family Rutaceae. The roots are sweet, astringent and febrifuge. Useful parts are leaves, fruits and roots. Fruits contain marmalosin, young bark coumarin and umbelliferone, leaves contain an essential oil. Plant pacifies vitiated kapha, body pain, poison, diarrhoea, dysentery, vomiting and intermittent fever. Pulp of unripe fruit is constipating where as that of ripened fruit is laxative. Leaves cure diabetes, cough, inflammation and asthma.[3] They are useful in diarrhoea, dysentery, dyspenia, seminal weakness and stomachalgia. Plant properties affect rhizosphere population through root exudates.[4] Rhizosphere population varies in structure and species composition depending on root zones.

Many researchers used extracts of *Aegle marmelos* on human pathogens. But there were few reports about rhizosphere bacteria of the medicinal plants like *Aegle marmelos.* As roots of *Aegle marmelos* are used as drug, the rhizosphere of *Aegle marmelos* may harbor various types of microorganisms. The present study was carried out to study enzymatic potential, PGPR activities and antimicrobial potential of the rhizobacterial isolates.

In the present study bacteria were isolated from the rhizospheric soil of *Aegle marmelos* and the isolates obtained were studied for their enzymatic potential. All isolates were screened for plant growth promoting activities like NH_3, HCN, IAA and siderophore production and PO4 solubilization and antimicrobial potential. The PGP potential of selected isolates was evaluated by pot experiment.

MATERIALS AND METHODS

Materials - Soil sample - Soil samples from rhizosphere region from medicinal plant *Aegle marmelos* Starch agar, Milk agar, Pikovskaya's agar, Peptone water, Nesseler's' reagent, CAS agar plates, Glycine agar, Pikovskaya's agar, Potato dextrose agar, Muller Hinton agar and Glycerol aspargine agar.

Methods

Collection of sample: - For the present study rhizosphere soil samples from Aegle *marmelos* trees from the Dayanand college campus Solapur were collected. These samples were obtained at a depth of 6 to 10 cm. in rhizosphere regions. The soil samples were allowed to air dry at room temperature.

Isolation of bacteria: - Bacteria and actinomycetes were isolated from soil samples by serial dilution technique using nutrient agar for bacteria and glycerol aspargine agar for actinomycetes by incubating at 28 ± 2^0C for 24 hours for bacteria 7 days for actinomycetes. The different colonies were selected and cultural characters were studied and transferred on nutrient agar slants and glycerol aspargine agar slants. Total twenty one different isolates were obtained from two soil samples of two plants from two locations. Isolates were characterized on the basis of morphological, cultural and biochemical characters. Gram staining, motility and endospore staining of isolates were performed. All isolates were studied for different enzymatic activities like catalase, oxidase, hydrolytic enzyme production like amylase and caseinase.

Amylase activity :- Isolates were spot inoculated on sterile starch agar plates separately (peptone - 1gm, NaCl - 0.5gm, Yeast extract – 0.3 gm, starch - 2 gm, agar – 2.5gm, D/W – 100 ml, pH – 7.2) and incubated at 28^0c for 24 hours. After incubation amylase activity was examined by adding dilute iodine solution and observed for zone of clearance around the colony.[5]

Caseinase activity: - Isolates were spot inoculated on sterile milk agar plates separately (nutrient agar 90ml + 10 ml skimmed milk) and incubated at 28^0c for 24 hours. After incubation caseinase activity was examined by observing zone of clearance around the colony.[5]

PGPR potential

All the bacterial isolates were screened for various plant growth promoting activities like NH3, IAA, Siderophore and HCN production and phosphate solubilization.

NH_3 production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube separately and incubated at 28 ± 2^0C for 48-72 hours. Nesseler's reagent (0.5ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production.[5]

HCN production

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lock[6] (and Farah Ahmad *et al.*[7]). Nutrient agar was amended with 4.4 gm glycine per liter and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate and plates were sealed with paraffin wax and incubated at 28± 2^0C for 4 days. Development of orange to red color indicated HCN production.

Siderophore production

All isolates were tested for siderophore production on Chrome azurol S agar medium described by Schwyn and Neilands (1987).[9] Chrome azurol S agar medium plates were prepared and spot inoculated with bacterial isolates separately and incubated at 28±2^0C for 48 -72 hrs. Development of yellow - orange halo around the growth was considered as positive for Siderophore production.

IAA Production: - IAA production was detected by the modified method described by Brick *et al* (1991). Each isolate was inoculated in 5ml of nutrient broth containing 1 mg/ml concentration of tryptophan and incubated at 28 ± 2^0c for 72 hrs. After 3 days the broth was centrifuged at 3000 rpm for 30 minutes. The cell free supernatant was collected and used for detection of IAA production.

One ml supernatant was mixed with 2ml of Salkowski reagent (2ml of 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) and tubes were incubated. Development of pink colour indicated IAA production.

Confirmation of IAA production by using TLC

Selected isolates (AM3, AM4 and AMH2) showing IAA production were inoculated in nutrient broth amended with 1mg/ml tryptophan. 1% inoculum of $O.D_{.600}$ 1.0 was used for inoculation. The inoculated broth was incubated at R.T for 48 hours. After incubation broth was centrifuged at 7000 rpm for 10 minutes. The pH of broth was brought to 3.0 using 1N HCl. 4:1 aliquots of liquid portion of centrifuged sample were extracted three times with ethyl acetate. The organic phase was concentrated to dryness and then diluted with 0.5ml methanol. This solution along with the standard IAA was applied on silica gel plate and TLC was run by using solvent system chloroform: ethyl acetate: Formic acid in 5:3:2 proportion and developed by using Salkowski reagent. Red colour spots were developed. RF value of the standard and IAA produced by the selected isolates was calculated.[10]

Phosphate Solubilization:- The isolates were screened on Pikovskaya's agar plates individually to examine phosphate solubilization as described by Gaur (1990). The isolates were streaked on Pikovskaya's agar plates individually to examine their ability to solubilize Tri calcium phosphate and incubated at 30ºC for 4 days. The isolates showing a clear zone of solubilization around growth indicated PO_4 solubilization.

Antimicrobial activity

Antifungal and antibacterial activity against plant pathogens of bacterial isolates AM3, AM9, AM10 were studied by agar well diffusion method.[12] [8] The bacterial isolates to be tested for their antifungal activity were fully grown in nutrient broth media. The test bacteria *Xanthomonas citri* and *Xanthomonas axenopodis* were grown in nutrient broth and nutrient glucose broth respectively. Fungal pathogens *Aspergillus* and *Fusarium oxysporum* were grown on Sabouraud's agar. The spores were scraped and suspended in 10ml of sterile normal saline solution. Diluted spore suspension (0.1ml) of the fungi was spread on Muller Hinton agar. Wells of 10 mm diameter were punched into the agar medium and filled with 200µl of bacterial culture. The plates were incubated at 28 ± 2^0c for 5-6 days for antifungal and 24 hrs for antibacterial activity. The antifungal and antibacterial activity was evaluated by measuring the growth of inhibition zone against test fungi and bacteria.

Pot experiment

Seed germination:- Healthy green gram seeds were selected for pot experiment. In this experiment first the seeds were surface sterilized by using 0.1% $HgCl_2$ for 3 minutes. After

sterilization seeds were washed with sterile water for 3-4 times. Inoculum of AM3, AM9 was prepared in nutrient broth and diluted for obtaining 10^8 CFU /ml.[14]

The sterilized seeds were immersed in this suspension for coating of PGPR and incubated for 45 minutes on rotary shaker. Seeds were removed, dried in air and sown on Petri plates with wet filter paper for germination. The seeds treated with distilled water were used as control. After 3 days germination was observed and germination percentage and also vigor index was calculated.

In the same manner PGPR treated seeds were sown in plastic pots with sterile soil. Each pot was sown with 10 seeds.

Pot experiment was done in laboratory under the favorable condition for green gram in triplicates. After 15 days plants were removed carefully and washed with tap water. Plant height, root length and shoot length fresh weight and dry weight was measured and recorded for analysis.

RESULTS AND DISCUSSION

In the present study 21 bacterial strains and 2 actinomycetes isolates were isolated from the rhizosphere of two soil samples of *Aegle marmelos* from two locations from Dayanand college campus Solapur.

The organisms isolated were found to be Gram positive cocci, Gram positive spore forming rods, Gram positive non spore forming rods and Gram negative rods.

Table 1: Enzyme activity of rhizobacteria.

SR No	Name of the isolate	Enzymes produced				
		Amylase	Caseinase	Catalase	Oxidase	Urease
1	AM 1	+	+	+	-	-
2	AM 2	+	+	-	+	-
3	AM 3	+	-	+	+	+
4	AM 4	-	-	+	-	-
5	AM 5	+	+	-	-	-
6	AM 6	+	+	-	-	-
7	AM7	-	-	+	+	+
8	AM 8	-	+	+	-	-
9	AM 9	+	-	-	+	-
10	AM 10	-	-	+	-	-
11	AM 11	-	+	-	+	-
12	AMH 1	+++	+	-	-	-

13	AMH 2	+++	+	-	-	-
14	AMH 3	+++	+	-	-	-
15	AMH 4	+++	+	-	-	-
16	AMH 5	-	+	+	-	-
17	AMH 6	-	-	-	-	-
18	AMH 7	+	-	+	+	-
19	AMH 8	+++	+	+	-	-
20	AMH 9	+	+	+	+	+
21	AMH 10	-	-	-	-	-

The enzymatic potential of isolates showed Catalase in 47.61%. Oxidase in 38.09% Amylase and Caseinase in 61.89%, Urease in 19.04% and Phosphate solubilization potential in 52.37% isolates.

Isolate AMH9 showed enzyme activity for all 6 enzymes while AMH6 showed no activity for all enzymes. AMH10 showed only urease activity. AM1, AM3, AM7 and AM8 showed moderate enzyme activity, remaining all isolates showed least enzyme activity.

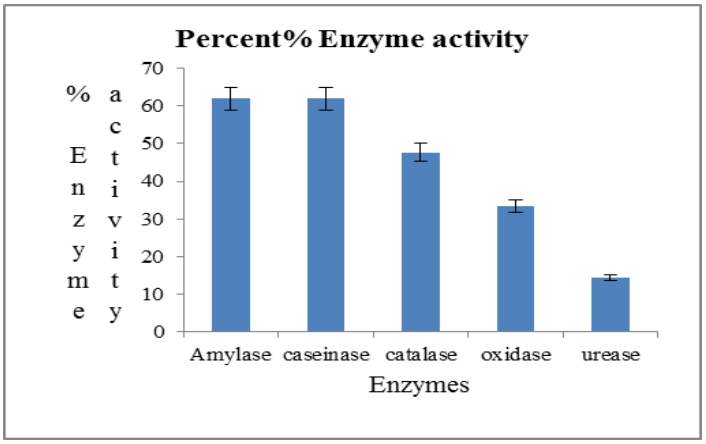


Figure 1: Percent enzyme activity of AM isolates.

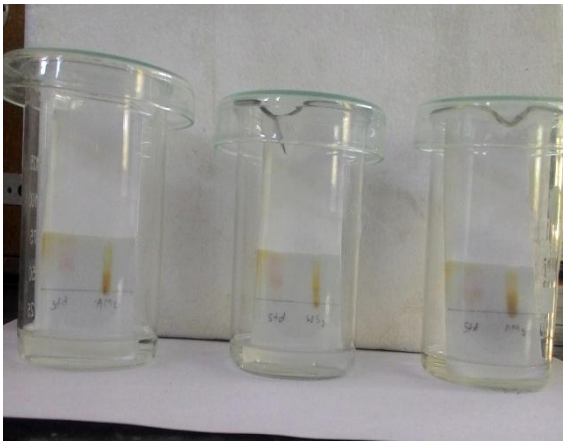
The isolates were also screened for plant growth promoting traits. The results showed that not all isolates possessed all 5 PGP activities. The % of positive isolates varied. The 85.71% bacterial isolates showed NH_3 production and IAA production 57% showed phosphate solubilization, 80.95% isolates HCN production and 33.3% isolates showed siderophore production. Isolates AM3 and AM9 showed positive results for all 5 PGP activities. AMH2 showed 4 PGP activities positive. Two actinomycetes isolates AM7 and AMH2 showed positive results for all 5 PGP activities.

Table 2: PGP activities of rhizobacteria.

Isolate number	NH_3 production	HCN production	Siderophore production	IAA production	Phosphate solubilization
AM 1	+	+	-	+++	+
AM2	+	+	-	+++	-
AM 3	+	+++	+	+++	+
AM 4	+	+	-	+++	++
AM 5	+	+	-	++	+
AM 6	-	-	-	-	++
AM 7	+	+	-	++	+
AM 8	+++	+	-	+++	+
AM 9	+	+	+	++	+
AM 10	+	+	+++	+	-
AM 11	-	++	-	+	+
AMH 1	+++	+	-	+	+
AMH 2	-	-	+	+	+
AMH 3	++	+	-	-	-
AMH 4	+	+	+	+	-
AMH 5	+	+	-	+	-
AMH 6	+	+	-	-	-
AMH 7	+++	+	+	+	-
AMH 8	+++	+	-	+	-
AMH 9	+	+	+	+	+++
AMH 10	+	+	-	+	-

Quantification of IAA produced was also done colorimetrically and amount of IAA produced was calculated from standard graph. The range of IAA production showed different values. The range of IAA produced by the isolates from *Aegle marmelos* is 9-126 µg /ml.

Confirmation of IAA production was done with Thin Layer Chromatography. The purified IAA sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the Rf corresponding to the standard IAA (0.9) as shown in photoplate. It confirmed IAA producing potential of isolates AM3, AMH2 and AM4.



Photoplate 1: Thin layer chromatography.

Photoplate 2: Results of TLC of AM3 and AM 4 isolates.

The isolates showing 5 PGP activities were selected for pot experiment. Two bacterial isolates AM3 and AM9 showed positive results for all 5 PGP activities and selected for pot experiment. Both of them showed 100% and 96% seed germination respectively, increase in plant height, root length; shoot length as compared to control.

Hetal *et al*[17] studied IAA production potential of bacterial isolates from the rhizosphere of crop plants like cowpea, cotton and groundnut and identified maximum IAA producing species as *Serratia ficaria* and *shigella* species. Malleswari D. *et al*[16] isolated bacterial cultures from the rhizosphere of various medicinal plants including *Withania somnifera* and reported 84.9% isolates as IAA producers and also studied other PGP activities of the isolates. Mahalaxmi *et al*[12] reported IAA production by *Azospirillum, Azotobacter, Pseudomonas* and *Bacillus* strains isolated from the rhizosphere of tomato. Ajay kumar *et al*[18] isolated bacteria from the rhizosphere of French bean plants and reported that almost all isolates are IAA producers. Basharat Ali[19] isolated different *Bacillus* species from the

rhizosphere of different plant species and reported IAA production potential in them. Sutthinan Khamna[20] and Thangapandian[21] isolated actinomycetes from the rhizosphere of medicinal plants and reported positive IAA production and identified most of the actinomycetes as *Streptomyces*.

Potential isolates were also tested for antimicrobial activities against plant pathogens viz. *Xanthomonas citri* and *Xanthomonas axenopodis* and fungal pathogens *Aspergillus* and *Fusarium oxysporum.* Isolates AM3 shown antibacterial activity against *Xanthomonas citri* and AM3 and AM10 shown antibacterial activity against *Xanthomonas axenopodis.* The results of antifungal activity showed that all three isolatesAM3, AMH2 and AM 10 have activity against *Fusarium oxysporum* and all tested bacterial isolates except AM3 and AMH2 shown antifungal activity against *Aspergillus* sp. (Table 1).

Table 3: Antimicrobial activity of bacterial isolates from *Aegle marmelos.*

Mean zone diameter of inhibition against				
Isolate number	***X. citri***	***X. axenopodis***	***Fusarium oxysporum***	***Aspergillus sp.***
AM 3	17.86±0.808	15.6±5.45	33.6±10.44	-
AM 10	-	21.5±1.28	16.1±1.15	11.96±0.152
AM 11	-	-	-	10.53±0.503
AMH 1	-	-	-	11.23±0.493
AMH 2	21.3±0.964	-	23.73±1.44	-
AMH 7	-	-	-	11.03±0.55

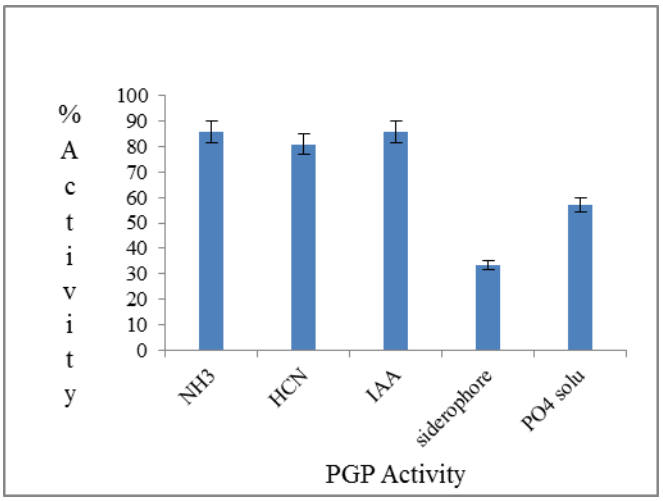


Figure 2: Percent PGPR activity of bacterial isolates.

Photo plate 2: PO_4 Solubilization by bacterial isolates.

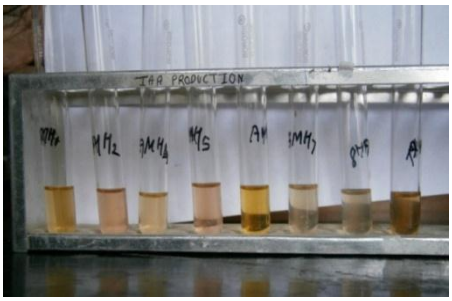


Photo plate 3: IAA production by bacteria.

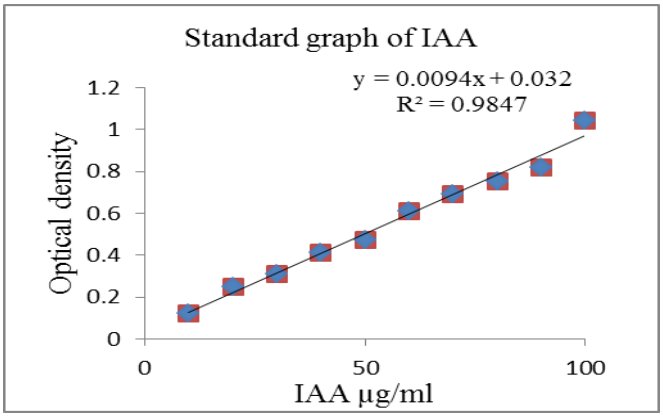


Figure 3: Standard graph of IAA.

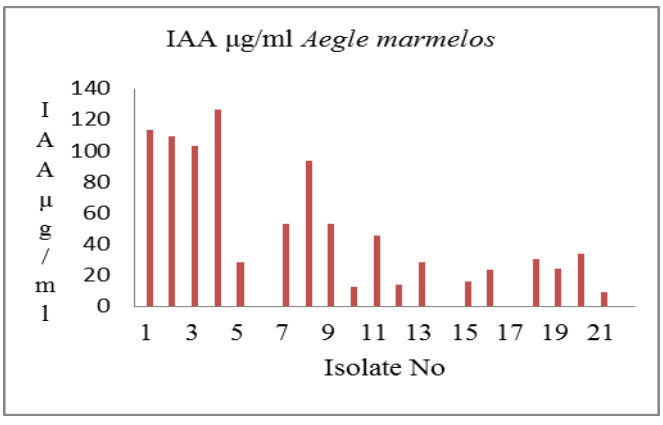


Figure 4: Quantification of IAA by bacteria.

Photo plate 5: % Seed germination on treatment of bacterial isolates.

Photo plate 6: Pot experiment with AM9and AM3 isolates.

Photo plate 7: Root length, shoot length seed germination results with actinomycete isolates.

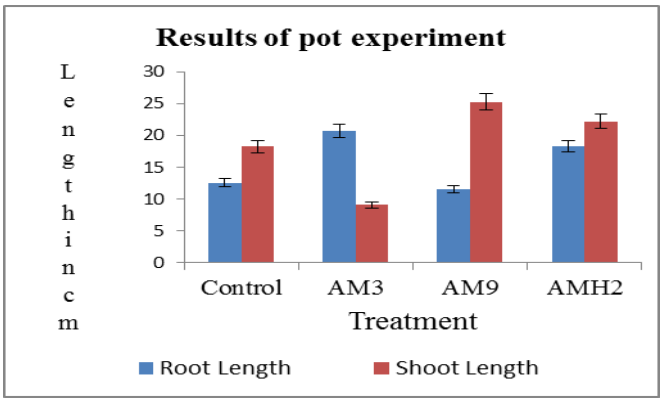


Figure 5: Effect of seed inoculation by PGPR on root and shoot length.

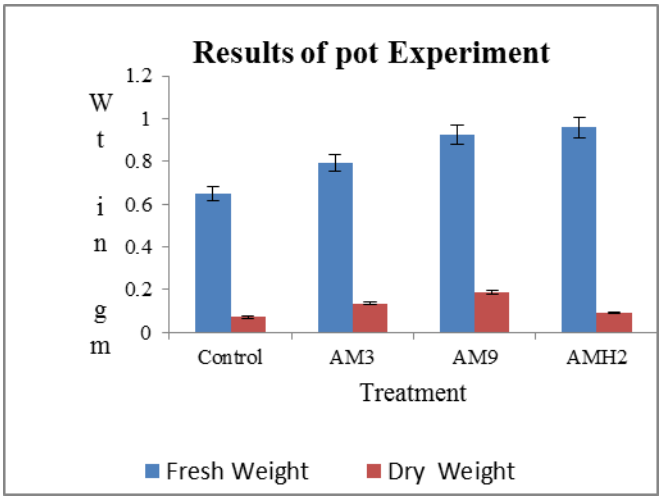


Figure 6: Effect of seed inoculation by PGPR on fresh & dry Weight of roots.

CONCLUSION

Rhizosphere microflora of medicinal plants like *Aegle marmelos* has greater plant growth promoting potential and can be used as bioinoculants for plant growth and development or as supplement for fertilizer.

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