Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 9(2) pp. 027-039, February, 2020 Issue.
Available online http://garj.org/garjas/home

Full Length Research Paper

Perineal Plant Extract as a Culture Medium for Production of Plant Growth Regulators by Molecularly Identified Rhizospheric Microorganisms

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Accepted 13 February, 2020

The feasibility of developing alternative environment friendly media for conventional chemical synthetic media was assessed using perineal plant extracts as locally available cheap materials which contain considerable amount of carbohydrates, proteins and macro elements necessary for microbial growth. In this study, about fifteen bacterial isolates were isolated on *Aloe vera*-based culture medium from *Aloe vera* rhizosphere and ten bacterial and eight actinomycetes isolates were isolated on *Mentha*-based culture medium form *Mentha varidis* rhizosphere. Out of all,MA6 and A13 isolated from *Menthavaridis* and *Aloe vera* rhizosphere respectively produce more gibberellic acid (27.86 and 17.32 µg/ml respectively) on natural medium. Additionally, both isolates were efficient in carbohydrate production MA6 (89µg/ml) and A13 (144µg/ml) on natural medium. Moreover, 9(50 %) isolates and 4(23%) isolates showed maximum Ammonia production qualitatively on peptone water after growing on *Mentha*-based culture medium and synthetic medium respectively. While 1(7%) and 7 (47%) isolates revealed maximum Ammonia production qualitatively on peptone water after growing on *Aloe vera* -based culture medium and synthetic medium. Phylogenetic analysis of 16S rRNA sequence of the two isolates MA6 and A13 showed maximum sequence similarity with *Streptomyces rochei* strain DW3 and *Xanthomonas nasturtii* strain WHRI 8853. *Streptomyces rochei* strain DW3 showed strong antifungal activity against *Fusariumsolani* (18 mm zone of inhibition) and *Xanthomonas nasturtii* strain WHRI showed good antifungal activity against *Fusarium oxysporum* (19 mm zone of inhibition). We hereby recommend switching to use of plant extracts which is environment friendly and also more economic for cultivation as an alternative for synthetic culture media.

Keywords: Plant-based medium, Perineal plants, rhizobacteria, Actinomycetes, PGP activities, antagonism.

INTRODUCTION

A nutrient material prepared for the growth of microorganisms in laboratory is called culture media. Culture media used in the laboratory for the cultivation of microorganism supply the nutrients required for the growth

and maintenance. Nutrient agar (NA) is universally used as a general purpose medium for the cultivation of broad range of bacteria. The feasibility of developing alternative media to different culture media namely NA were assessed using locally available cheap materials because the use of readymade culture media in schools and laboratories has financial limitations. Generally the cheap locally available materials such as cereals and legumes may serve as alternative nutrient media to grow bacteria **Uthayasooriyan *et al*. (2016).** Microbial researches are hindered by high cost of culture media. Therefore we have to try to use various alternative media to reduce the cost involved. In line with the other studies carried out in this area by a number of authors, this group (**Ravathie et al., 2012; Nirmala et al., 2014)** has worked on the possibility of using a number of sources as alternative culture media. Even then, there is a necessity to formulate new media with easily available low cost material as substitutes for NA and PDA **Arulanantham *et al.* (2012) and Osman *et al*.(2012)** mentioned that plant juices and/or extracts are well suited as culture media for microbial growth and fermentations, as they contain all the necessary nutrients as well as growth factors such as amino acids, vitamins and minerals. **Antony *et al*. (2014)** investigated that Protein and carbohydrate rich raw materials like Soya, Potato, dates, Groundnut, Cereals, Cassava, Yam, Pigeon pea, Maize and Beans have been successfully used in formulation of cheap alternative bacteriological media.**(Nour *et al*., 2012)** revealed that colonies of rhizospheric microorganisms (*Azospirillum brasilense*, *Enterobacter agglomerans* and *Klebsiellapneumoniae*) nicely developed on surface-inoculated agar plates prepared from crude and diluted juice of *M. crystallinum* (ice plant) over reference nutrient media.

Rhizobacteria are known as free-living bacteria and actively colonize rhizosphere or around plant roots. Rhizobacteria have the potential to increase plant growth as it is capable of producing plant growth hormones, such as indole acetic acid, gibberellic acid, cytokines and ethylene, side rophores production, antibioticsand able to dissolve phosphate **Shameer and Prasad (2018)** and the application of plant growth-promoting rhizobacteria (PGPR) could allow growers to reduce the use of synthetic fertilizers and increase the sustainability of crop production **Ali *et al*. (2019)**.One Group of PGPR that has an important role in growth promoting of plant is Actinomycetes. **(Jog *et al*., 2012)** reported thatrhizosphere actinomycetes, a group of Gram positive bacteria, have highlighted to be the most potential candidates of biofertilizer agents. Some Actinomycetes genera have been widely developed for increasing agricultural crops productivity including *Actinoplanes*, *Streptomyces* and *Micromonospora*. Streptomyces species isolated from the wheat rhizosphere attributed with high plant growth promoting activities that could significantly promote wheat growth.

The present study is aimed to replace the nutrient source in chemical synthetic media with various locally available cheap materials such as perineal plants that contain considerable amount of protein, carbohydrate, vitamins and amino acids. These good nutrient source media are cheap, commonly available and also using their juice as culture medium for the growth of plant growth promoting rhizobacteria that may be used as biofertilizer to enhance the growth and productivity of commercially important plants.

MATERIALS AND METHODS

Plants of study and preparation of plant juices

The tested plants, *Mentha varidis* and the succulent plants *Aloe vera*, are cultivated in Sirs EL- Layan, El-Menoufia governorate, Egypt. Both plants were perineal and *Aloe vera* was chosen for its availability in arid and semi-arid environments. The profuse biomass of *Mentha* produced enough juice of suitable nutritional composition (Table 1) andjuicy biomass, chemical compositions and nutritional contents of the tested succulent plant (*A. vera*) (Table 2) **Tiwari and Upadhayay (2018)** that facilitate its use as a culture medium. The succulent leaves of *A. vera* and the vegetative parts (leaves and stems) of *M. varidis* were washed, sliced, and then blended with equal aliquots of distilled water (w/v) for 5 min. The resulting slurry homogenate was coarse-filtered through cheesecloth to obtain plant juice; almost 73–82% of the plant fresh weight was recovered as juice. The pH for *M. varidis* juices was in the range of 5.8-6.5 and *A. vera* juices 4-6.2.The plant juices obtained from the tested plants were further diluted with distilled water (v/v); 1:10, 1:20,1:40 , 1:80, and 1:100. Exclusively, such diluted juices were used as such to prepare the plant-based agar culture media (1.5-2% agar, w/v). All media were adjusted to pH 7.0 autoclaved at 1.5 atm., 121° C for 20 min.

The use of perineal plant juice as a culture medium for isolation of rhizospheric microorganisms

For rhizospheric microorganism's isolation, serial dilution techniques were used. Sample suspensions were prepared by adding 1g of the rhizospheric soil (the soil adhered to root surface and in between root) that obtained from Sirs EL- Layan, El-Menoufia governorate, Egypt was added to 10 ml of sterile water (the stock) and shaken vigorously for 10 minute by using vortex, dilute was then settled for a short period. Sterile dilution blanks were marked

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sequentially starting from stock and10^{-1} to 10^{-6}. From each dilution tube 0.1 ml of dilution fluid was transferred into the surface of the plant-based culture media at different diluted juice of both *Mentha* and *A. vera* (in triplicate) and spread with an alcohol sterilized L shape glass rod then incubated at 30^{O} C for 24 - 48 hours. After successful growth of microorganisms, the individual colonies were picked up and purified, then sub-cultured on plant-based agar slants; incubated at 37° C to achieve vigorous growth and then maintained as stock cultures at 4° C for subsequent studies.

Cultivation of plant-based culture medium-microbial isolates on artificial media

Bacterial isolates that have been devolved on plant-based culture media were tested to grow on soil extract agar medium at 30^{O} C for 24 – 48hr and actinomycetes isolates also were grown on starch Casein medium at 30^{O} C for 5-7days. After the incubation all the plates were observed for microbial growth and pigmentation for comparison.

In-vitro screening of isolates for multiples plant growth promoting (PGP) activities

All the isolates were screened for plant growth promoting activities i.e., GA production, carbohydrates production and Ammonia production.

Determination of total gibberellins (GA)

For bacterial isolates, conical flasks (250 ml capacity) containing 100ml of both plant-based and nutrient broth medium were inoculated with 1 ml of 24 h old rhizobacterial cultures, the flasks were incubated on rotary shaker (150 rpm) 36±2 °C for 72 h. Then, flasks were centrifuged at 4000 rpm for 15 min to separate supernatant. On the other hand, the actinomycetes isolates were streaked on plant-based and starch nitrate agar medium and incubated at 28°C. After 5 days, the agar discs containing actinomycetes mycelia were transferred to conical flasks (250 ml capacity) containing 100ml of both plant-based and starch nitrate liquid medium. After 7 days of culture at 30°C and stirring at 150 rpm, they are centrifuged at 3000 rpm for 15 min.

For extraction of total gibberellins, the supernatant (10 to 30 ml) was adjusted to pH 8.6 with 1% NaOH and extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate fraction was evaporated to dryness and held for further purification. The aqueous phase was adjusted to pH 2.8 with 1% HCl and extracted three times with equal volumes of ethyl acetate. The remaining aqueous phase was discarded. This fraction contained acid hormones such as GA (**Shindy and Smith, 1975**). Gibberellic acid was determined using 1ml ethyl acetate extract, 1 ml HCl followed by 1 ml Folin Denis's reagent, 3 ml water and mixed. The test tubes were put in boiling water bath for 5 min, left to cool. The presence of green color indicated the presence of GA and the absorbance was measured at 750 nm according to **Udagwa and Kinoshita (1961**). The amount of GA produced per milliliter culture was estimated using a standard curve.

Determination of total carbohydrates

Total carbohydrate content of either insoluble or soluble sugars was determined as glucose by the phenol-sulphoric acid method (**Dubois *et al.*, 1956**) as follows :-
One ml of the sugar solution (supernatant of bacterial and actinomycetes culture on both natural and synthetic media) was first mixed with 1 ml of 5 % redistilled phenol solution and then with 5 ml of 96 % sulphoric acid. A blank was also prepared using water instead of the sugar solution. After cooling and standing for 10 min, each tube was shaken and placed in water path at 25-30^{O} C for 20 min. The yellow orange color found was then read at 490 nm. A standard curve was constructed in the same way using different concentrations of glucose (10-100 mg ml^{-1}).

Production of ammonia

Microbial isolates were tested for the production of ammonia in peptone water **(Dye 1962)**.Freshly grown bacterial and actinomycetes cultures were inoculated in 10ml peptone water in each tube and incubated for 3 days at 36±2 °C and 7 days at 30°C respectively. After incubation 1ml of Nessler's reagent was added to each tube. Development of faint yellow color indicates small amounts of ammonia and deep yellow to brownish color indicates maximum amount of ammonia production **(Cappucino & Sherman, 1992).**

Identification of selected bacterial and actinomycetes isolates

The most efficient bacterial isolate (A13)was subjected to Gram-staining for identification (**Vincent, 1970**) and the cover slip culture method was carried out for morphological characterization of the selected actinomycetes isolate (MA6**)** according to **Kawato and Sinobu (1979)**.

Molecular characterization of bacteria and actinomycetes

1. DNA extraction

Extraction of genomic DNA of bacterial strains was carried out by using the GenElute Bacterial Genomic DNA Kit.

2. PCR amplification and sequencing

The extracted DNA was then identified by 16S rRNA at Sigma Scientific Services Company, 6 of October, El Giza, Egypt DNA was first dissolved in 20 µL of TE buffer and used as the template for the PCR reactions. Total genomic DNA was electrophoresis using 0.8% agarose gel and visualized by UV transilluminator after red gel staining. Amplification of the 16S rRNA gene was performed using a T1-Thermocycler PCR machine.

PCR amplifications were performed in a total volume of 50 µL by mixing 20 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide triphosphate, 1 mM concentrations of each universal primer for 16s rRNA of pA (50 -AGAGTTTGATCCTGGCTCAG-30) and pH (50 -AAGGAGGTGATCCAGCCGCA-30) described by **Edwards *et al*. (1989)** and 3 U of Taq DNA polymerase in 10X Taq buffer A (GeNeI). These reactions were subjected to initial denaturation of 94° C for 6min followed by 35 cycles of 94°C for 45 s, 56° C for 45 s and 72° C for 1 min and a final extension step of 72° C for 5 min using GeneAmp® PCR system 9700 (Applied Biosystems). The PCR products were resolved using 1% agarose gel.

3. Phylogenetic analysis

16S rRNA gene sequence of the isolate was compared with 16S rRNA gene sequences available by the BLASTN search in the NCBI, Gen Bank database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using Clustal X **(Thompson *et al*., 1997)**. The method of **Jukes and Cantor (1969)** was used to calculate evolutionary distances. Phylogenetic dendogram was constructed by the neighbour-joining method and tree topologies were evaluated by performing bootstrap analysis of 1,000 data sets using MEGA 3.1 (Molecular Evolutionary Genetic Analysis).

Antagonistic activities of plant growth promoting rhizobacteria against pathogenic fungi

The possible interaction between the selected bacterial isolates and pathogenic fungi (*Fusarium* sp. and *Rhizoctonia* sp.) was monitored on potato dextrose agar (PDA) medium using a modified agar – plate inhibition zone technique **(Silosuch *et al*., 1994)**. Initially, the pathogenic fungi (*Fusariumsolani*, *Fusarium oxysporum* and *Rhizoctoniasolani*) were grown in 15 cm Petri dishes containing PDA medium and incubated at 28° C for 48 hr. Then, 0.5 cm disks were cut from edge of the actively growing colonies. One disk was transferred to the center of a Petri dish containing PDA medium and a loopful of 48 hrsold *Xanthomonas nasturtii* culture grown on nutrient agar media was inoculated on one side leaving 1 cm from the margin. Then, the dishes were incubated at 28°C for (4-7 days). Zones of inhibition of fungal growth were observed. On the other hand, Potato dextrose agar plates were prepared and inoculated with *Streptomyces rochei* by a single streak of inoculum in the center of the petri dish containing PDA medium. After 7days of incubation at 28°C the plates were seeded with a disk of test fungi at an angle of 90° to the actinomycetes isolate and incubated at 28°C for 5 days in the case of fungi ,Microbial interactions were analyzed by the determination of the size of the inhibition zone **(Madigan *et al*., 1997)**.

RESULTS AND DISCUSSION

This work was undertaken to investigate and assess the potentiality of some plant extracts as sources of nutrient for cultivation of rhizospheric microorganisms. A total of fifteen bacterial isolates were isolated from the rhizospheric soils of *Aloe vera* by using *Aloe vera*-based culture medium with different dilutions 1:10, 1:20, 1:40, 1:80 and 1:100 v/v (juice or sap: distilled water, v/v), while nine bacterial and nine actinomycetes isolates from rhizosphere of *M. varidis* were developed on agar plates of *Mentha*-based culture media prepared from diluted juices (1:10, 1:20, 1:40, 1:80 and 1:100 v/v) of *M. varidis*. Such positive dilution effect was attributed to decreasing the osmotic effect of concentrated nutrients as well as minimizing the inhibitory effect of antimicrobial compounds present in the juices of tested plants Pellizzoni *et al*. (2012). The results revealed that the rhizospheric microorganisms grow successfully on *Aloe vera* and *Mentha*-based culture medium this due to nutritional constituents of *Aloe vera* include amino acids, anthraquinones, enzymes minerals, vitamins, lignin's, monosaccharide's, polysaccharides, salicylic acid, saponins, and steroids Tiwari and Upadhayay (2018) and *Mentha* also contains carbohydrates, protein, lipid, the most predominant mineral found was potassium, sodium, calcium, magnesium and phosphorus Mainasara *et al*. (2018).

All actinomycetes isolates showed fast growth on *Mentha*-based culture medium compared to starch casein medium, these results may be due to the fact that *Mentha* extractis rich in protein, amino acids, fats, fatty acids, carbohydrates, vitamins and the essential elements (Table 1) and additionally some isolates achieved better growth on plant extract medium while others on casein medium and investigated a variable in pigmentation(Table 3). It is possible that the low growth of some actinomycetes isolates on plant-based medium may be due to the presence of antibacterial and phenolic compounds in *Mentha* extract that slightly effect on their growth. All bacterial isolates developed on both *Mentha* and *Aloe vera*-based medium grow on soil extract medium in different rate of growth and pigmentation (Figure 1)

Table 1: The chemical compositions and nutritional contents of *Mentha varidis*

parameters	mg/ 11g fresh weight of mint	parameters	mg/ 11g fresh weight of mint
Protein and Amino Acids		Carbohydrate (c)	850
Protein (a,b)	350	Fiber (d)	790
Tryptophan	5.3	Ash (d)	175
Threonine	15.1	**Fats & Fatty Acids**	
Isoleucine	15.1	Fat	190
Leucine	26.8	Omega-3 fatty acids	38.2
Lysine	15.4	Omega-6 fatty acids	5.8
Methionine	5.1	**Vitamins (e)**	
Cysteine	4.2	Vitamin C	1.5
Phenylalanine	18.6	Niacin	0.1
Tyrosine	11.0	Folate	0.012
Valine	17.5	Retinol	0.022
Arginine	17.2	**Minerals (f)**	
Histidine	7.1	Calcium	21.4
Alanine	18.7	Iron	1.3
Aspartic acid	43.4	Magnesium	7.0
Glutamic acid	40.0	Phosphorus	6.4
Proline	15.2	Potassium	50.5
Serine	14.4	Sodium	3.2
Glycine	17.4	Zinc	0.1
Phytosterols	1.1	Manganese	0.1

(a)**AOAC International, & Latimer, G. W. (2012a)**. Official Methods of Analysis of AOAC International. AOAC International. Chapter 4, P. 25–26

(b)**AOAC International and Latimer, G. W. (2012b**).Official Methods of Analysis of AOAC International. AOAC International. Chapter **4, P. 9–13**.

(c) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A. and Smith, F. (1956): Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, **28(3): 350-356**.

(d) AOAC International. ‘‘Official Methods of Analysis.” (1998).

(e)**Lehotay, S. J. and Hajs˘lova´, J. (2002**).Application of gas chromatography in food analysis. TrAC Trends in Analytical Chemistry, **21(9): 686– 697**.

(f) **Horwitz W. (2012)**: Official methods of analysis of the Association of Official Analytical Chemist. Association of Official Analytical Chemists, Washigton, 758

Table 2: The chemical compositions and nutritional contents of *Aloe vera* Tiwari and Upadhayay (2018)

Chemical Group	Constituents
Amino Acids	Provides 20 of the 22 required amino acids and 7 of the 8 essential ones.
Enzymes	Anthranol,barbaloin, chrysophanic acid, ethereal oil, ester of cinnamonic acid, isobarbaloin, resistannol
Anthraquinones	Provides aloe emodin, aloe tic acid, alovin, Anthracine.
Steroids	Cholesterol, lupeol, camp sterol, sistosterol
Hormones	Auxins and gibberellins
Salicylic Acid	Aspirin like compounds
Saponins	Glycosides
Minerals	Calcium,chromium,copper, iron, manganese, potassium, sodium and zinc
Sugars	Monosaccharide’s:Glucose and Fructose Polysaccharides: Glucomannans/polymnnose
Vitamins	A, B, C, E, choline, B12, folic acid

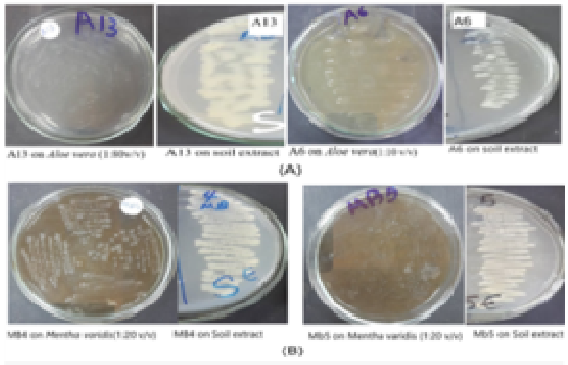

Figure 1: Comparison between growth of bacterial isolates on both natural and synthetic medium, A) Growth of some bacterial isolates on *Aloe vera*-based medium and soil extract medium, B) Growth of some bacterial isolates on *Mentha varidis*-based medium and soil extract medium

Table 3: Growth Characteristics of actinomycetes isolates on both *Mentha varidis*-based medium and Starch casein medium.

Medium	No. of isolates	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Mentha-based medium	MA6	abundant	Gray brown	Dark brown	brown
	MA7	low	white	creamy	non
	MA8	moderate	Creamy brown	Brown	non
	MA9	low	gray	white	non
	MA10	good	Gray brown	Light brown	non
	MA11	good	Gray brown	brown	brown
	MA12	moderate	creamy	brown	non
	MA13	abundant	Gray brown	Light brown	non
	MA18	good	white	white	non
Starch casein medium	MA6	good	gray brown	brown	brown
	MA7	good	White beige	Dark beige	Light beige
	MA8	good	creamy	creamy	non
	MA9	low	white	beige	non
	MA10	moderate	cream beige	light beige	non
	MA11	good	white brown	Pale brown	non
	MA12	low	creamy	beige	non
	MA13	good	creamy	deep beige	Light beige
	MA18	moderate	white	creamy	non

In-vitro screening of isolates for multiples plant growth promoting (PGP) activities

Estimation of total gibberellins

The phytohormone Gibberellin plays a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence **Sharma *et al*. (2018)**. The rhizobacterial isolate from *Aloe vera* rhizosphere produced gibberellin 0.37µg ml^{-1} in isolate A14, the highest of 17.32µg ml-1 in the A13isolate, while rhizobacterial isolate from *Mentha varidis* rhizosphere produced the lowest gibberellin 4.91 µg ml^{-1} in isolate MA13, the highest of 27.86 µg ml-1 in the MA6 isolate Table (4) higher than bacterial isolates from the rhizosphere of some superior local species of Sulawes (2.370 mg L^{-1}) **Gusmiaty *et al*. (2019)**. The production of

Table 4: Production of GA on *Mentha* and *Aloe vera* –based medium and synthetic medium by bacterial and actinomycetes isolates isolated on *Mentha* and *Aloe vera* extract medium.

Mentha extract medium		Synthetic medium		*Aloe vera* extract medium		Synthetic medium	
No of isolates	GA (µg/ml)	No of isolates	GA (µg/ml)	No of isolates	GA (µg/ml)	No of isolates	GA (µg/ml)
MB1	7.96	MB1	8.36	A1	0.57	A1	9.31
MB2	17.52	MB2	9.87	A2	0.81	A2	0.61
MB3	17.12	MB3	7.56	A3	0.75	A3	7.59
MB4	5.15	MB4	5.07	A4	0.45	A4	0.55
MB5	26.25	MB5	13.67	A5	0.79	A5	9.10
MA6	27.86	MA6	18.42	A6	1.53	A6	11.19
MA7	11.27	MA7	10.41	A7	7.98	A7	4.77
MA8	6.19	MA8	0.55	A8	7.94	A8	8.92
MA9	11.27	MA9	10.59	A9	0.65	A9	9.55
MA10	9.87	MA10	0.73	A10	1.07	A10	4.31
MA11	25.45	MA11	1.76	A11	0.75	A11	8.38
MA12	9.02	MA12	11.03	A12	0.47	A12	1.55
MA13	4.91	MA13	0.41	A13	17.32	A13	10.27
MB14	25.05	MB14	4.83	A14	0.37	A14	8.86
MB15	8.46	MB15	8.34	A15	0.75	A15	2.98
MB16	23.64	MB16	5.05				
MB17	15.89	MB17	11.19				
MA18	9.22	MA18	11.23				

gibberellic acid on natural medium was higher compared to synthetic medium this may be due to the absence of some essential growth factors in synthetic medium that enhancement their production.

Total carbohydrates production

Microbial polysaccharides are high molecular weight polymers consisting of carbohydrates. These polysaccharides exist in different forms: covalently bound to the cell surface as capsular polysaccharides or secreted into the surrounding environment as free exopolysaccharides (EPS) **Manjanna *et al.* (2009)**. Both plant growth promoting rhizobacteria and phytopathogenic bacteria are known to produce EPS. EPS forms soil aggregates and maintain soil Water potential during dry season which is one of the vital soil characteristics **Veerapagu *et al.* (2018)**.The results showed that Mentha rhizospheric bacteria produce high amount of carbohydrates on *Mentha*-based medium 89 µg ml^{-1} in MA6 isolate in spite of producing 154µg ml^{-1} on starch nitrate medium, moreover A13 from *Aloe vera* rhizosphere produce 144 µg ml^{-1} of carbohydrates on *Aloe vera* based medium although it produce 82 µg ml^{-1} on nutrient medium Figure (2). **Borgio *et al.* (2009)** reported three bacterial strains, *Bacillus subtilis* NCIM 2063 and *Pseudomonas aeruginosa* NCIM 2862 were examined for their exopolysaccharide (EPS) producing ability at the laboratory level. The highest EPS production was recorded in *P. aeruginosa* (226 µg/ml) grown in nitrogen free medium followed by *B. subtilis* (206 µg/ml) in nitrogen free medium after 7 days of incubation at 37°C.

Production of ammonia

A faint yellow color and yellow to brownish color developed after the addition of Nessler's reagent which indicates that the test is positive and the PGPR isolates produced ammonia, 50% and 28% of *Mentha* rhizospheric isolates produce high and low amount of ammonia, respectively on peptone water medium after growing on *Mentha*-based culture medium (natural medium), while 22% of isolates produce high amount of ammonia on the same medium after growing on synthetic medium (Table 5). On the other hand, 80% of *Aloe vera* rhizospheric isolates produce moderate amount of ammonia on peptone water medium after growing on Aloe vera-based medium compared to 47% of isolates produce high amount of ammonia on the

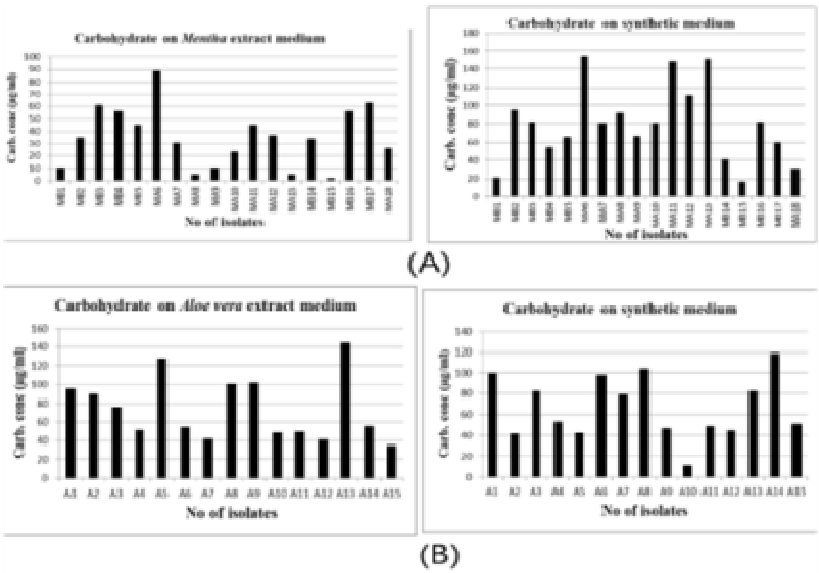

Figure 2: A) Production of carbohydrates on *Mentha*–based medium and synthetic medium by bacterial and actinomycetes isolates isolated on *Mentha*–based medium B) Production of carbohydrates on *Aloe vera* –based medium and synthetic medium by bacterial isolates isolated on *Aloe vera* –based medium

Table 5: Production of ammonia on peptone water by *Mentha* and *Aloe vera* rhizospheric isolates after growing on *Mentha* and *Aloe vera* extract medium (natural medium) and synthetic medium

after growing on *Mentha* extract medium		after growing on synthetic medium		*Aloe vera* extract medium		after growing on synthetic medium	
No of isolates	Ammonia prod.	No of isolates	Ammonia prod.	No of isolates	Ammonia prod.	No of isolates	Ammonia prod.
MB1	+++	MB1	+++	A1	+	A1	+++
MB2	+++	MB2	++	A2	++	A2	+++
MB3	+++	MB3	+	A3	++	A3	+++
MB4	+++	MB4	+++	A4	++	A4	++
MB5	+++	MB5	++	A5	++	A5	++
MA6	+++	MA6	+++	A6	+	A6	+++
MA7	+	MA7	+	A7	++	A7	-ve
MA8	++	MA8	++	A8	++	A8	++
MA9	+	MA9	+	A9	++	A9	+
MA10	+	MA10	++	A10	++	A10	+
MA11	+	MA11	+	A11	++	A11	-ve
MA12	+	MA12	+	A12	++	A12	+
MA13	++	MA13	+	A13	++	A13	+++
MB14	+++	MB14	+	A14	+	A14	+++
MB15	+++	MB15	++	A15	++	A15	+++
MB16	++	MB16	+				
MB17	+++	MB17	+++				
MA18	++	MA18	++				

Note: (+++) high, (++) moderate, (+) low and (-ve) negative

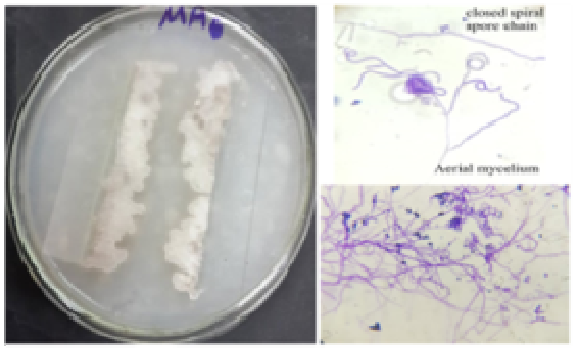

Figure 3: Cover slip culture method for MA6 actinomycetes isolate

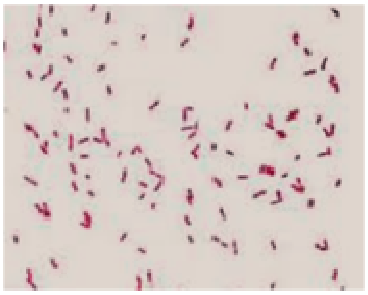

Figure 4: Gram staining for A13 isolate showed Gram negative rod-shaped bacteria

Table 6: The identity of one actinomycetes and one bacterial isolate found in *Mentha* rhizosphere and *Aloe vera* rhizosphere based on 16S rRNA nuclear gene

Isolate code	**Related species**	**Query cover** (QC)	**E-value**	**Similarity (%)**	**ACC number**
MA6	*Streptomyces rochei* strain DW3	100	0.0	98.9	MN135856.1
A13	*Xanthomonas nasturtii* strain WHRI 8853	98	0.0	99	KX518637.1

same medium after growing on synthetic medium Table (5). The ammonia is useful for plant as directly or indirectly. Ammonia released by diazotrophs is one of the most important traits of PGPR's which benefits the crop. This accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.5. It suppresses the growth of certain fungi and nitrobacteria due to it potent inhibition effect. It also upset the microbial community and inhibits germination of spores of many fungi **Patel and Saraf (2017)**.

The two most potential isolates of PGP production on natural medium MA6 and A13, MA6 on *Mentha*-based culture medium was undergo to cover slip culture method it was exhibited gray brown aerial mycelium with brown substrate mycelium. The isolate produced brown diffusible pigment. It had a flat elevation and a rough surface Figure (3). Additionally, isolate No. A13 on *Aloe vera* was undergo to gram staining and showed that it was gram negative rod-shaped bacteria Figure (4).

Identification of plant growth promoting rhizobacteria (PGPR) based on 16S rRNA gene sequencing

The Query Cover (QC) for two species of bacteria(A13) and actinomycetes (MA6) had a value of 100% and 98% respectively (Table 6). The E- value of 0.0 indicated the number of alignments with a score equal to or higher than expected to occur in the database by chance. Therefore,

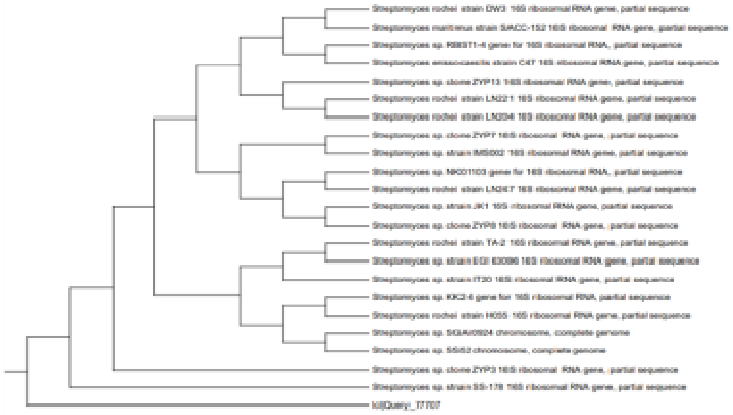

Figure 5: The phylogenetic tree based on sequencing of 16S rRNA gene using MEGA4 software (Tamura *et al*., 2007) illustrating the genetic relationship of *Streptomyces rochei* and closely related *Streptomyces* species.

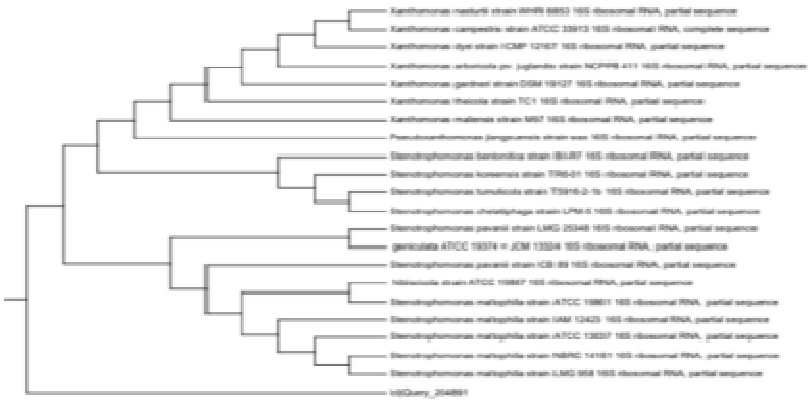

Figure 6: Phylogenetic tree based on sequencing of 16S rRNA gene using MEGA4 software (Tamura *et al*., 2007) illustrating the genetic relationship of *Xanthomonas nasturtii* and closely related bacterial species.

the lower the E-value was, the more significant the score and the better quality of BLAST alignment search were. E-value was low in this study since the sequence of loci as the marker was very short. In this study, the 16S rRNA nuclear gene had a length of about less than 1300 base pairs (bp). Therefore, the search for similarities with the limited query sequence was performed. According to (**Claverie and Notredame, 2003;Santosa*et al*., 2018)**, DNA sequences have a high similarity if the Query Cover value approaches 100% and the E-value approaches 0.0. Based on the Query Cover (QC), the E-Value of 0.0, and similarity, showed that the two isolate strains were *Streptomyces rochei* ***s***train DW13 with QC (100) and similarity (98.9%) and *Xanthomonas nasturtii* strain WHRI with QC (98) and similarity (99%).

The 16S rRNA gene has a characteristic size of about 500 bases until 1550 bpfor the 16S rRNA used for sequencing measuring 1300 bp. Where in that area is a converse area. The use of 16S rRNA is often used in prokaryotic organisms rather than 23S rRNA because of its higher variation.

A phylogenetic relationship based on 16S rRNA nuclear gene

The construction of phylogenic tree (Figure 5&6) described the phylogenetic relationship of the two species, namely *Streptomyces rochei* ***s***train DW13and *Xanthomonas nasturtii* strain WHRI.

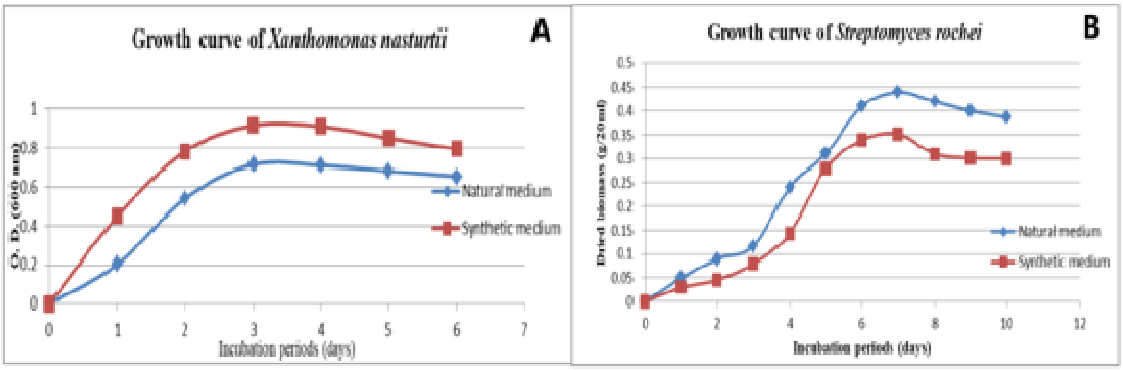

Figure 7: Growth curve of A) *Xanthomonas nasturtii* B) *Streptomyces rochei*

Table 7: Antimicrobial activity of *Xanthomonas nasturtii* and *Streptomyces rochei* isolates

Pathogenic fungi Isolates	Diameter of zone of Inhibition (mm)		
	Fusariumsolani	***Fusarium oxysporum***	***Rhizoctoniasolani***
Xanthomonas nasturtii	**11**	**19**	**14**
Streptomyces rochei	**18**	**13**	**12**

In our findings, the amount of phytohormone (GA3) produced by *Streptomyces rochei* were lower than those reported with that of **Zamoum *et al.* (2017)** who found that*Streptomyces rochei*was positive for gibberellin (GA3) production (86.6 µg ml^{-1}).**(Coplin and Cook, 1990)**also pointed out the EPS of *Xanthomonas campestris* were found to contain sugars such as glucose, mannose and glucuronic acid.

The growth curve of *Xanthomonas nasturtii* and *Streptomyces rochei* on both natural and synthetic medium showed that *Xanthomonas nasturtii* produced high biomass on synthetic medium after three days while *Streptomyces rochei* yielded high biomass after seven days on natural medium Figure (7).

Several rhizobacteria and actinobacteria have been reported as potential agents for the biocontrol of various soil-borne pathogenic fungi and as stimulators of plant growth (**Goudjal *et al.*, 2014; Zamoum *et al.*, 2015 and Ibrahim *et al.*, 2016**).

Xanthomonas nasturtii and *Streptomyces rochei*showed positive antifungal activity against all targeted fungi (*Fusariumsolani*, *Fusarium oxysporum* and *Rhizoctoniasolani*) in vitro. It was found that *Xanthomonas nasturtii* strain WHRI showed good antifungal activity against *Fusarium oxysporum* (19 mm zone of inhibition) Table (7), **Kumer and Shyam (2014)** found that *Xanthomonas luminescens* was effectively suppressed the growth of *Pyriculariaoryzae* Cav. the cause of rice blast disease. Additionally, the strongest activity of *Streptomyces rochei* strain DW3 was observed against *Fusariumsolani* (18 mm zone of inhibition) Table (7), this result was contrast with **Zamoum *et al.* (2017)**who found that the strongest activity was observed against the fungus *Rhizoctoniasolani* (32±0.9mm) and the lowest activity showed against *Fusarium oxysporum* (16±1.6mm) and showed 18±1.9mm zone of inhibition against *Fusarium solani* that agree with our results.

CONCLUSION

Based on this study it is concluded that the using of plant materials as a nutrient media supporting growth of bacteria and actinomycetes. With the rising costs of manufactured dehydrated media in the local markets, this plant – based culture medium (sources of nutrients) would provide a cheaper alternate for use. The isolated rhizospheric microorganisms grow well in natural medium and some of these organisms grow well in synthetic medium. *Streptomyces rochei* strain DW3 and *Xanthomonas nasturtii* strain WHRI 8853 were efficient in plant growth promoting substances production. Consequently, this notable feature opened promising possibilities for their investigation in crop improvement and growth of rhizospheric microorganisms on cheap and available plant material. As the use of synthetic pesticide and fertilizers

pose serious threat to human health and environment, the use of plant growth promoting endophytic actinobacteria can emerge as alternative tools for sustainable, organic and environmental friendly agricultural crop production.

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