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Study on Rhizosphericmicroflora of Wild and Transgenic varieties of *Gossypium species* in Monsoon

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Abstract

Many, microorganisms playing an important role in plant growth are used in agriculture system, especially these group of microorganisms called plant growth promoting rhizobacteria (PGPR), which can increase the growth of plant directly and indirectly; acting as biofertilizers, phytostimulators and biocontrol agent. Various number of bacteria including species of Pseudomonas, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Proteus Bacillus, have observed to enhance plant growth. In present study, wild Gossypium species and transgenic Gossypiumhisrsutum sample were collected in monsoon season from four different sampling sites Rhizosphere, Rhizoplane, Endorhizosphere, Bulk soil from Agriculture farm, cotton research centre, Surat, Gujarat. A total Fifty nine bacteria were isolated and in vitro screening was done for different plant growth promoting activities; such as phosphate solubilization, zinc solubilization, Potassium solubilization, Nitrogen Fixation, ACC deaminaseacitivity, phytohormons production, HCN production, ammonia production, Lytic enzymes production, Triphenyltetrazolium tolerance (TTC) activity. In present work, eight bacterial isolates were positive for phosphate solubilization, two zinc solubilization and twenty four potassium solubilization. Nitrogen fixation activity was shown in twenty five isolates. ACC deaminase activity was shown in twenty five isolates. IAA production and Gibberelic acid shown nine and fifty six isolates respectively. Four isolates were positive for HCN production and thirty two for ammonia production. Lipase, protease and amylase enzyme activities were shown twenty three, twenty eight and twenty respectively. Twenty four isolates were tolerance to TTC. From all these traits, eight isolates were showing maximum plant growth promotion activities. As PGPR are environmental friendly and offer sustainable approach to increase production of crop and heath. So PGPR will restrict the use of chemical fertilizer in agriculture area.

Keyword: PGPR, Rhizobacteria, biofertilizer.

Introduction

The growth human populations and fertilizers were used to increase crop production and meet the high demands for food sources. The production cost is increased, and the harmful nature of chemical fertilizers for the environment, which has led to a interest in the use of biofertilizers for enhanced environmental sustainability, lower the cost production and help in good crop yields. I had selected cotton plant because it has advantages like, on the basis of production India is second number due to its favorable weather condition as well as the every part of the cotton plant has its economic important.

The rhizosphere zone has been defined as the volume of soil directly influenced by the presence of living plant roots or soil compartment influenced by the root. Rhizosphere supports very large, live and active microbial population capable of exerting beneficial, neutral and detrimental effects on the plants. Rhizobacteria, presence in rhizosphere zone that gave the beneficial and important effects on the plant growth by direct or indirect traits are called as plant growth promoting rhizobacteria (PGPR)[1]. These PGPR in the rhizosphere are very important for increasing health of host plantand fertility of soil[2]. The activerhizobacteria (fungi and bacteria) improved growthof plant and crop productivity are generally known as Biofertilizer. These term PGPR was first gave by Kloepper and Schrothfor the microorganisms closely interact in the rhizospherezone[3]. The rhizosphere zone is a great important for microbial interactions released by plant roots which are the main food source for rhizobacteria and geochemical cycling of nutrients[4].So Isolation, Identification, Screening and selection of rhizobacteriaand their used in various integrated practices have great application for enhancing the growth of crop and higher agricultural crops yield with maintaining of agro-ecosystems. Rhizobacteria have been reported to direct traits of enhance plant growth by a various mechanisms: Phosphate, potassium, zinc solubilization Nitrogen fixation and plant growth promoting hormones synthesissuch as IAA (Indole-3- acetic acid, GA (gibberellic acid)[5]. Indirect mechanisms involves elimination of harmful microbes, via antibiotics production, lytic enzymes production (Protease, lipase, amylase), hydrogen cyanide, catalase.

Biofertilizers, PGPR enhance the plant growth, their productivity, yield and increase the various nutrient of the host plant which are replace and altered the chemical fertilizers[6]. So increases in yields of crop have been reported by PGPR[7]. So, keeping all these in view, the present study was carried out to

isolate the various plant growth promoting strains from the rhizospheric soils of *Gossypium sp.*

Material and Methods

Collection of sample: Rhizopheric soil sample were collected from Rhizosphere of cotton plant growing at different site of Agriculture farm, Cotton research centre, Surat in monsoon season. Intact root system was dug out and rhizospheric soil sample were carefully taken in sterile plastic bags and store 4°C.Total 5 soil samples were collected for isolation of rhizophericmicroflora.

Isolation of Rhizopheric microflora: The rhizopheric microflorawere isolated from the Bulk soil, Rhizosphere (loosely attach to soil), Rhizoplane (surface bacteria with root adhesion capacity), Endorhizosphere (portions of the cortex and endodermis) samples by serial dilution technique.

Inoculate all the sample on the selective media Pikovskaya agar, Nitrogen free media, Ashby's Mannitol agar, King's media, Bacillus media, Yeast Extract Mannitol Agar. Plates were incubated at room temperature until visible growth observed. All the isolates were colonical characteristics and morphological characterized for Gram reaction and Motility.

Screening of Plant growth promoting traits (PGP) of Rhizobacteria (PGPR)
Direct mechanisms

Phosphate solubilization: The bacteria were screened for solubilization of phosphate as per methodology described by Gupta S. *et.al*[8]. On Pikovskaya agar with insoluble tricalcium phosphate (TCP), a loop full of each culture was put on the agar plates and incubated at 30±0.1 °C for 5 days. The solubilization zone was observed.

Potassium solubilisation: Bacterial isolates were screened for the potassium solubilization ability using spot test method on modified Aleksandrov mediumplates containing either mica powder (A) or KH_2PO_4 (B). A loopful of culture growth of different bacterial isolates was spotted medium plates and observations were taken after 3-4 days of growth[9].

Zinc solubilisation: The bacterialisolates were inoculated into Pikovskaya modified medium which contain 0.1% insoluble zinc compounds (ZnO, $ZnCO_3$ and ZnS). The bacteria were inoculated and incubated at 28ºC for 46- 48 hours. The clearing zones around colonies were measured.

Nitrogen fixation: Bacteria was inoculated in NFb mediumwith or without addition of NH_4Cl as a nitrogen source in plate. The Plates were incubated at 28°C - 30°C for 6-7 days, and growth of bacteria was appeare as identified as Fixation of nitrogen[10].

ACC deaminase activity: The activity of ACC deaminasewas described by Glick *et al*.[11] µl of bacterial culture was inoculated into two agar plates of NFb or NFb-ACC modified (1-aminocyclopropane-1- carboxylate) as nitrogen source. These Plates were incubated at 28°C- 30°C and observed every day forgrowth formation in 4-7 days. Bacteria were re-inoculated. Again these plate were incubated in the same condition. Newly bacterial colonies observed in NFb-ACC modified medium were identified positive for ACC deaminase activity[11].

Phytohormons production: IAA (Indole-3-acetic acid) production 500 µl of 24 h old rhizobacterial cultures were inoculated in 50 ml of Nutrient broth with 0.1% DL-tryptophan. Incubated in the cold incubator Shaker with 180 rpm for 48 h in dark. The cultures of bacteria were centrifuged at 10,000 rpm for 10 min in 4°C - 5°C. IAA was Quantified in the supernatants which done using colorimetric assay[12].

Gibberellic acid production: The gibberellic acid production method by agriculturally beneficial microorganisms was identified by Borrow *et.al*.[13] A quantity of 100 ml of nutrient broth were prepared for isolates and sterilized. 1 ml broth of isolates were added in medium Incubate it at 37ºC for 7-8 days. After 7 days, the culture was centrifuged at 8000 g for 10-15 mins to remove bacterial cells. 15 ml of the culture was pipetted out separately into the test tubes and two ml of zinc acetate solution was added. After 2 mins, 2 ml potassium ferrocyanide solution was added and centrifuged at 8,000 g for 10 mins. 5 mlsupernatant was added to 5 ml of 30% hydrochloric acid These mixture was incubated at 25°C for 75 min. The blank was prepared with 5% hydrochloric acid. Absorbance was measured at 254 nm in UV-VIS spectrophotometer. From the standard graph prepared by using gibberellic acid solution of known quantities, the amount of GA produced by by the culture was calculated.

Indirect mechanisms: HCN (hydrogen cyanide) Production Screening of Rhizobacteria for HCN production was done as per methodology Castric. Rhizbacterial culture were inoculated on nutrient agar medium with 4.4 g/l of glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution was placed inside the lid of a plate. Plates were sealed with parafilm Incubated it at 28-30±0.1 °C for 4-6 days. So the development of light brown to dark browncolor indicated hydrogen cyanide production[14].

Production of NH_3: Isolates were tested for the production of ammonia in peptone water. Bacterial cultures were inoculated in 10 ml peptone water. Incubated it for 48-72 h at 28-30 ±2°C. In each tube added Nessler's reagent (0.5 ml).So development of brown colour to yellow was a positive test for production ammonia[15].

Protease production: For protease production rhizobacteria was isolated by Smibert and Kreig methodology. Protease production was determine using Skimed milk agar. Bacterial

cell was spot, inoculated and incubated for 2 days at 28±2 °C. The clearing zones around the colonies were observed.

Lipase production: Screening of rhizobacteriafor protease production was done by Egamberdiyeva and Holfich. Lipase production was determine using tributyreneagar. Bacterial cell was spot, inoculated and incubated for 2 days at 28±2 °C. The clearing zones around the colonies were observed.

Cellulase production: Screening of bacterial isolates for protease production was done as per methodology Cattelan A. J *et al.* Cellulase production was determine using carboxyl methyl cellulose agar. Bacterial cell was inoculated and incubated for 2 days at 28±2 °C. The clearing zones around the colonies were observed.

Amylase production: Screening of bacterial isolates for protease production was done as per methodology Smibert and Kreig. Amylase production was determine using starch agar. Bacterial cell was inoculated and incubated for 2 days at 28±2 °C. The clearing zones around the colonies were observed.

Triphenyl Tetrazolium Tolerance (TTC): Bacterial isolates were tested for the TTC tolerance in triphenytetrazolium chloride agar. Baterial culture was spot and incubated for 3 days. The pink color colonies were observed[16].

Results and Discussion

The rhizopheric soils of cotton plants have more rhizobacetriadue to availibity of more nutrients, macronutrients and micronutrient. Rhizobacteria have beneficial effect on plant growth and yield of crop plant by direct and indirect mechanism.

59bacterial isolates were isolated from the four different site – Bulk soil, rhizosphere, rhizoplane, endorhizosphere of wild and transgenic cotton fromcotton research centre, Surat. List of rhizobacteria isolate from different selective media. From Pikovskaya's medium 11, Nitrogen Free media 10, Ashby's Mannitol Agar 8, King's Medium 12, Bacillus Medium 7, Yeast Extract Mannitol Agar11 were isolated.

Cell morphology gram stain showed that 10.1 % Gram positive and 89.83 % Gram negative and 76.27% motile and 23.72% nonmotile organisms. All isolates has shown significant PGPR activity. All the isolates PGPR activity were designated as shown in table-1.

In present study, beneficial bacteria were isolated from rhizosphere. Isolated bacteria were screened for different plant growth promotion activities. Toenhance crop yields, nitrogenous and phosphatic fertilizers are applied at high rates which cause environmental and economic problems. A total of 8 bacterial isolates were screened for phosphate solublization on modified PVK agar. 24 were screened for the potassium solubilization ability using spot test method on modified Aleksandrov medium plates. Only 1strains could form clearing zone for zinc solubilizing ability. 25 isolates showed nitrogen-fixation activity. ACC deaminase activity was shown in 25 isolates. IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. Bacterial isolates were screened for plant hormone IAA and GA. Mostof the bacterial isolates produced plant growth promoting hormoneIAA. The range of IAA production was 0.001- 0.364 µg/ml. Among all isolates, NFM/BT1/BS/7produced high IAA (0.364µg/ml).56 isolates were positive for of GA_3 range of 0.001- 0.373 µg/ml. KM/W/RH/7 produced high GA_3 (0.373 µg/ml).Another trait of PGPR is the ammonia production. 32 isolates efficient isolateswere able to produced ammonia. HCN production by rhizobacteria has been important for in the biological control of pathogens. Production of HCN was detected in4isolates, which acts as an inducer of plant resistance. Protease, lipase, amylase activity was detected in most of the bacterial isolates that may be potentially very advantageous. 24 isolates were tolerance to TTC.

In the present study total fourteen plant growth promoting traits were checked. The frequency of different positive test were checked. Total 8 isolates were showed phosphate solubilization activity, 2 isolates showed zinc solubilization, 24 potassium solubilization, 25 nitrogen fixation, 25 ACC deaminase activity, 56 gibberellic acid, 9 IAA production, 4 HCN production, 32 ammonia production, 28, 20, 23 respectivily for lipase, protease, amylase production and 24 were showed TTC tolerance activity. From Fifty nine,7 isolates have more than 8 positive PGP traits.

Table–1
Bacterial isolates showing different plant growth promotion activities

Sr No	Colony Code	PGPR traits														
		PSB	KSB	ZSB	NFb	ACC	IAA	GA	HCN	AMP	TBA	SMA	SA	CMC	TTC	Total
1	PM/W/BS/1 PM/W/RH/1 PM/BT1/BS/1 PM/BT1/RH/1	-	-	-	-	-	-	-	-	-	-	+- +	+	-	-	2

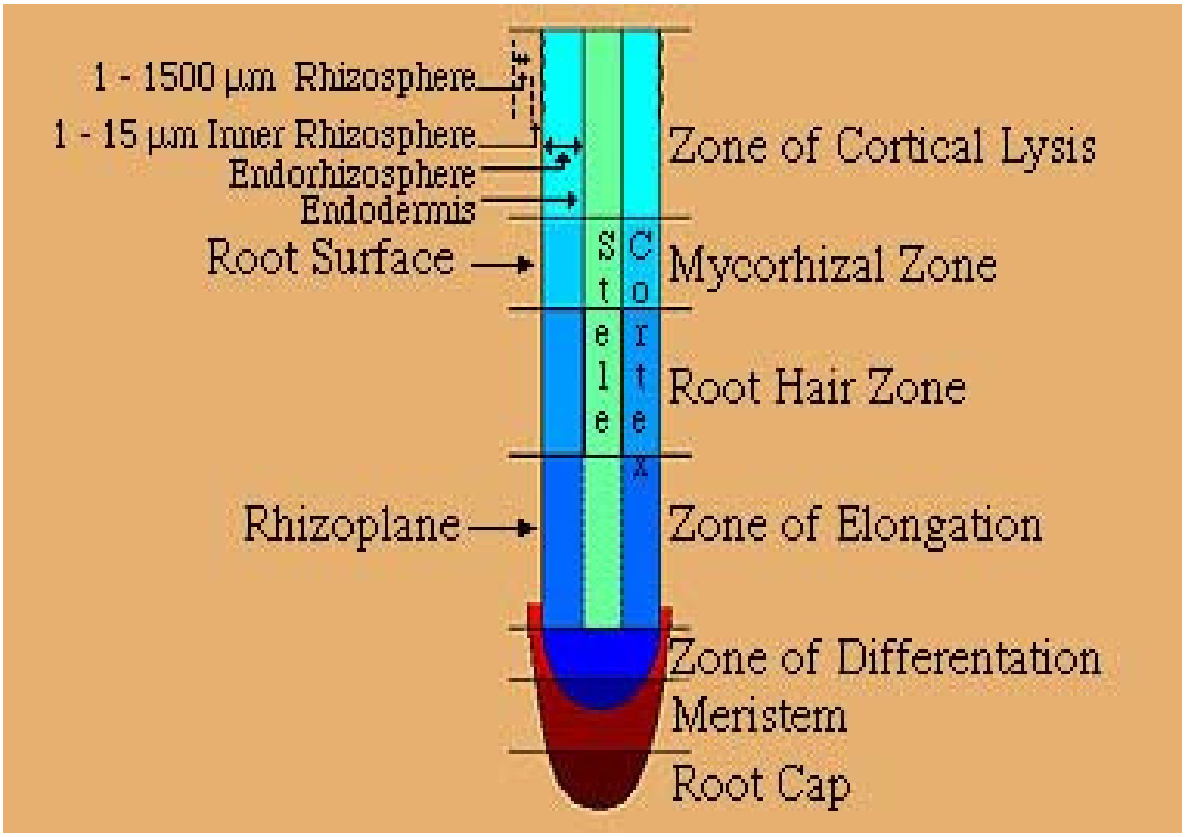
2	PM/W/BS/2 PM/BT2/BS/2 PM/BT2/RH/2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	1
3	PM/W/BS/3 PM/W/RP/3 PM/W/ER/3 PM/BT1/BS/3 PM/BT1/RH/3	-	-	-	-	-	-	-	-	+	-	+	-	-	-	2
4	PM/W/RH/4	+	-	-	+	+	0.132	0.372	-	+	-	-	-	-	+	7
5	PM/W/RP/5 PM/W/ER/5	-	-	-	-	-	-	0.001	-	-	-	+	-	-	-	2
6	PM/W/RP/6 PM/W/ER/6 PM/BT1/RH/6	-	-	-	-	-	-	0.325	-	-	-	-	+	-	-	2
7	PM/BT1/BS/7 PM/BT1/RP/7 PM/BT1/ER/7	+++	++	-	+	+	-	0.329	-	+	-	+	-	-	+	8
8	PM/BT1/BS/8						0.079	0.330							+	3
9	PM/BT1/BS/9 PM/BT1/RH/9 PM/BT1/RP/9 PM/BT1/ER/9	-	-	-	-	-	-	0.228	-	-	-	-	-	-	+	2
10	PM/BT1/RP/10	-	-	-	+	-	0.072	0.002	-	-	-	+	-	-	-	4
11	PM/BT1/RH/11	_-	-	-	-	-	-	-	-	-	-	-	+	-	-	1
12	NFM/W/BS/1	_-	-	-	-	+	-	0.289	-	-	+	-	-	-	-	4
13	NFM/W/BS/2 NFM/W/RH/2 NFM/BT1/RH/2 NFM/BT1/RP/2	-	-	-	+	+	-	0.323	-	+	-	-	+	-	+	9
14	NFM/W/RH/3 NFM/W/RP/3	-	++	-	+	+	-	0.325	-	-	-	-	-	-	+	5
15	NFM/W/RH/4 NFM/W/RP/4 NFM/BT1/RH/4	-	-	-	+	+	-	0.319	-	+	-	-	-	-	+	5
16	NFM/W/RP/5 NFM/W/ER/5	-	-	-	+	+	0.038	0.230	-	-	+	-	+	-	-	6
17	NFM/BT1/BS/6 NFM/BT1/RP/6 NFM/BT1/ER/6	-	-	-	-	-	-	0.321	-	-	+	+	-	-	-	3
18	NFM/BT1/BS/7	-	-	-	-	-	-	0.364	-	+	+	-	-	-	-	3
19	NFM/BT1/BS/8 NFM/BT1/RP/8 NFM/BT1/ER/8	-	-	-	-	-	-	0.245	-	+	-	+	-	-	-	3
20	NFM/BT1/RH/9 NFM/BT1/RP/9	-	-	-	+	+	0.090	-	-	+	-	-	-	-	-	4
21	NFM/BT1/RP/10 NFM/BT1/ER/10	-	-	-	-	-	-	0.321	-	+	-	-	-	-	-	2
22	AMA/W/BS/1 AMA/W/RH/1	-	-	-	-	-	-	0.326	-	+	-	-	-	-	-	3
23	AMA/W/BS/2 AMA/W/RH/2	-	-	-	-	-	-	0.327	-	+	-	-	+	-	+	8

	AMA/W/RP/2															
24	AMA/W/RH/3 AMA/W/RP/3 AMA/W/ER/3	++	-	-	-	-	-	0.325	-	-	+	+	-	-	+	6
25	AMA/W/RH/4 AMA/W/ER/4 AMA/BT1/RH/4	-	-	-	-	-	-	0.321	-	-	-	-	+	-	-	2
26	AMA/BT1/BS/5 AMA/BT1/RH/5 AMA/BT1/RP/5	-	-	-	-	-	-	-	+	+	-	+	-	-	-	3
27	AMA/BT1/BS/6 AMA/BT1/RP/6	+++	-	-	+	+	-	0.320	-	-	+	+	-	-	+	7
28	AMA/BT1/RP/7 AMA/BT1/ER/7	+++	-	-	+	+	-	0.329	-	+	-	-	-	-	+	6
29	AMA/BT1/RH/8 AMA/BT1/ER/8	-	-	-	-	-	-	0.324	-	+	-	-	-	-	-	2
30	KM/W/BS/1 KM/W/RH/1	-	-	-	-	-	-	0.369	-	+	-	-	-	-	+	4
31	KM/W/BS/2 KM/BT1/BS/2	-	-	-	-	-	-	0.324	-	+	-	-	-	-	-	-
32	KM/W/BS/3 KM/W/RH/3 KM/W/RP/3	-	++	-	-	-	-	0.333	-	+	+	+	-	-	-	5
33	KM/W/BS/4 KM/W/RH/4 KM/W/RP/4 KM/W/ER/4	-	-	-	+	+	-	0.372	+	-	+	+	+	-	-	8
34	KM/W/RH/5	-	+++	-	+	+	-	0.361	-	+	+	+++	+	-	+	9
35	KM/W/RH/6 KM/W/RP/6	-	++	-	-	-	-	0.334	-	-	+	-	-	-	-	3
36	KM/W/RH/7	-	-	-	-		-	0.373	-	+	+	+	+	-	-	8
37	KM/W/RP/8 KM/W/ER/8	-	-	-	+	+	-	0.343	-	+	+	+++	-	-	-	6
38	KM/BT1/BS/9 KM/BT1/RH/9 KM/BT1/RP/9	-	-	-	-	-	0.317	0.373	-	+	+	+	-	-	-	5
39	KM/BT1/BS/10	-	-	-	-	-	-	-	-	-	++	+	+	-	-	4
40	KM/BT1/BS/11 KM/BT1/RH/11 KM/BT1/RP/11	-	-	-	+	-	-	-	-	+	-	-	+	-	-	3
41	KM/BT1/RP/12 KM/BT1/ER/12	-	-	-	-	-	-	0.336	-	+	-	-	-	-	-	3
42	BM/W/BS/1 BM/W/RH/1 BM/W/RP/1 BM/W/ER/1	-	-	-	-	-	-	-	-	-	-	-	+	-	-	1
43	BM/W/RH/2 BM/W/RP/2 BM/W/ER/2 BM/BT2/BS/2 BM/BT2/RH/2	-	+++	-	-	-	-	0.332	-	-	-	-	+	-	+	4

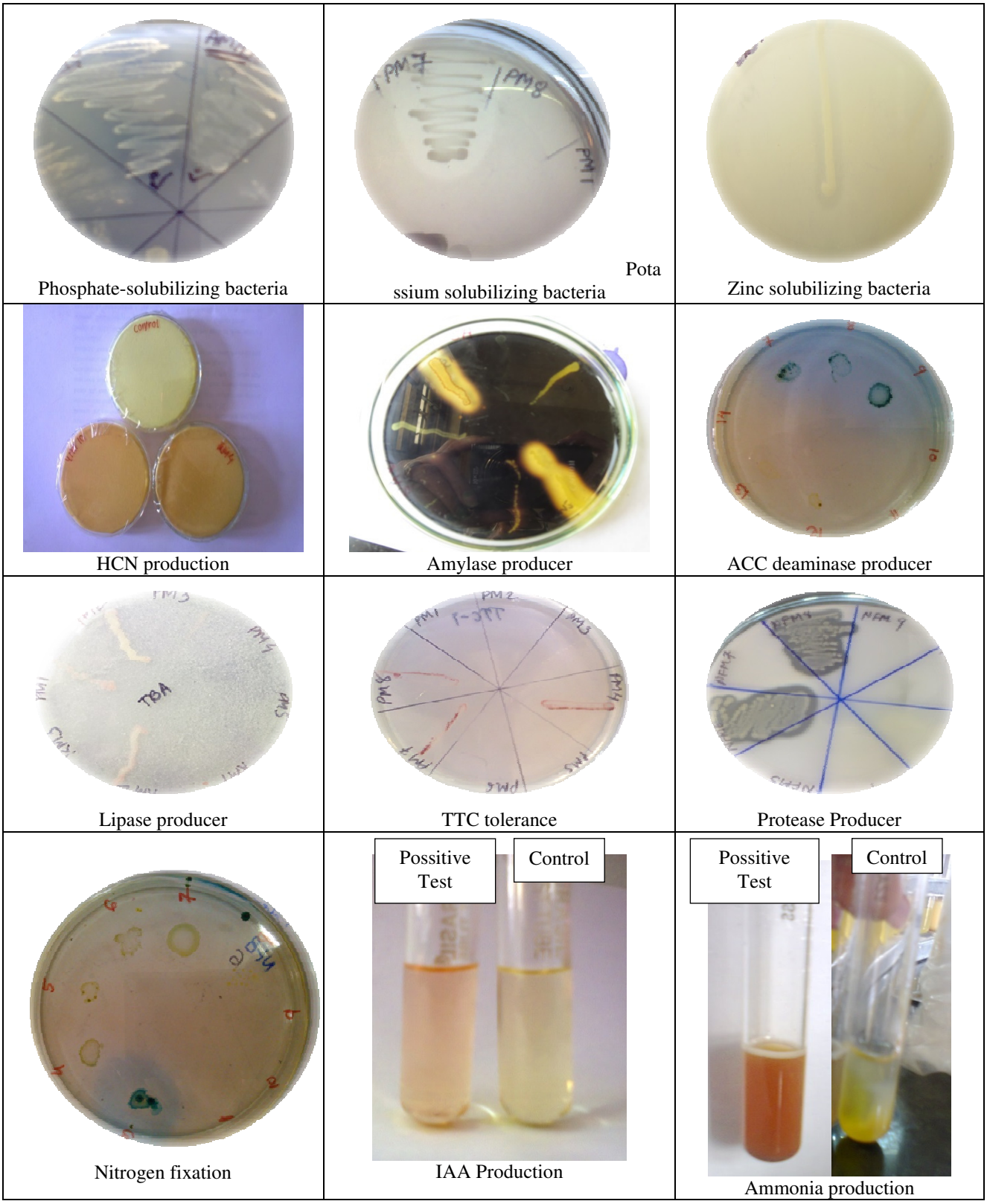
44	BM/W/BS/3 BM/W/RH/3 BM/W/RP/3 BM/BT1/RP/3 BM/BT1/ER/3	-	++	-	+	+	-	0.364	+	-	+	+	+	-	+	9
45	BM/BT1/BS/4 BM/BT1/RH/4 BM/BT1/ER/4	-	-	-	-	-	-	0.342	-	+	+	-	-	-	+	4
46	BM/BT1/RH/5 BM/BT2/RP/5 BM/BT2/ER/5	-	-	-	-	-	0.083	0.336	-	+	+	-	-	-	-	4
47	BM/BT2/BS/6 BM/BT2/RH/6 BM/BT2/RP/6	-	-	-	-	-	-	0.362	-	-	+	+	+	-	-	4
48	BM/BT2/RH/7 BM/BT2/RP/7	-	++	-	+	+	-	-	-	+	+	+	+	-	-	7
49	YMA/W/BS/1 YMA/W/RH/1 YAM/W/ER/1 YAM/BT1/RH/1	-	+++	-	+	+	-	0.334	-	-	-	+	+	-	+	7
50	YMA/W/BS/2 YMA/BT1/BS/2 YAM/BT1/RH/2 YAM/BT1/RP/2	-	+++	-	+	+	-	0.321	-	-	-	+	-	-	+	6
51	YMA/W/BS/3	-	++	-	+	+	-	0.370	-	+	-	+	-	-	+	7
52	YMA/W/RH/4	-	+++	-	+	+	-	-	-	+	+	+	-	-	+	7
53	YMA/W/RH/5 YMA/W/RP/5	+	+++	-	-	-	-	0.320	-	-	+	-	-	-	+	5
54	YMA/W/RH/6 YMA/W/RP/6 YMA/W/ER/6 YMA/BT1/BS/6	-	++	-	+	+	-	0.321	-	+	-	+	+	-	+	8
55	YMA/W/RP/7 YMA/W/ER/7 YMA/BT1/BS/7 YMA/BT1/RP/7	-	++	-	-	-	-	0.331	-	+	-	-	-	-	-	3
56	YMA/BT1/BS/8	-	++	-	-	-	-	0.324	-	+	-	+	-	-	-	4
57	YMA/BT1/RH/9	-	++	-	+	+	-	0.324	-	-	-	-	-	-	-	4
58	YMA/BT1/RH/10 YMA/BT1/ER/10	-	++	-	-	-	-	0.325	+	+	+	-	-	-	+	6
59	YMA/BT1/RP/11 YMA/BT1/ER/11	-	+++	-	+	+	-	0.319	-	-	+	+	+	-	+	8

Key: PM- Pikovskaya's Media, NFM- Nitrogen Free Medium, AMA- Ashby's Mannitol Agar, KM- King's Medium, BM- Bacillus Medium, YMA-Yeast Extract Mannitol, W-Wild Cotton, BT1-Transgenic (Bt-1) Cotton (*Gossypiumhisrsutum)*, BS – Bulk Soil, RH – Rhizosphere, RP – Rhizoplane, ER – Endorhizoshere, PSB – Phosphate solubilizing Bacteria, KSB – Potassium Solubilizing Bacteria, ZSB – Zink Solubilizing Bacteria, NFb – Nitrogen fixation, ACC – ACC deaminase activity, IAA – Indole-3-acetic acid((µg/ml), GA - Gibberellic Acid(µg/ml), HCN - Hydrogen cyanide production, AMM – Ammonia Production, TBA – Lipase Acitiviy, SMA – Protease Activity, SA – Amylase Activity, CMC – Cellulase Activity, TTC – TriphenylTetrazolium tolerance Activity.

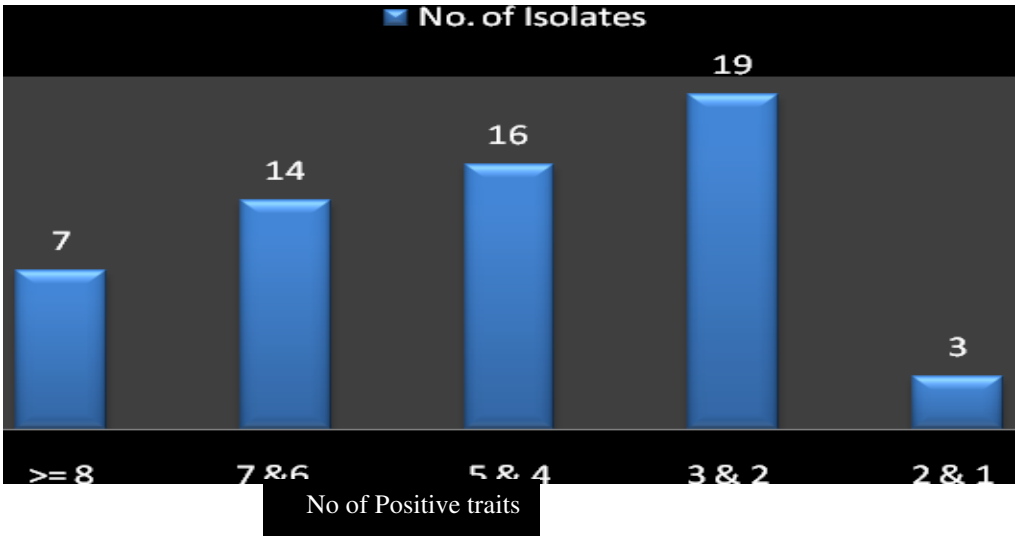
Figure–1
Wild cotton and transgenic cotton



Figure–2
Sampling site for Rhizobacteria



Figure–3
Isolates and their PGP traits



Figure–4
Comparative study of total positive traits in PGPR

Conclusion

The search for PGPR and their investigation of their mode of action are increasing at a rapid used as commercial "Biofertilizer". Thus, the future of this technology looks extremely bright.

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