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Isolation of Antipathogenic Plant Growth Promoting Bacteria and their Characterization for Plant Growth Promoting Activities

H.M. Sherathiya, V.J. Jadeja, G.J. Mehta and B.J. Malviya

Department of Microbiology, Shree M. & N. Virani Science College affiliated to Saurashtra University, AITS Campus, Kalawad Road, Rajkot - 360005, India

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Cotton which known as a 'white gold' as it has predominant position amongst all cash crops in India. But cotton suffers from a number of fungal and other diseases in Gujrat. Wilt caused by *Fusarium oxysporum* and root rot caused by *Rhizoctonia solani* is the most important fungal diseases that provide economic yield loss to farmers. For the control of fungal diseases the use of chemical fertilizers had led to many serious problems, forcing scientists to explore other alternatives. In last few decades many biocontrol agents were found but not been able to survive and not that much effective when applied in to the field condition. To resolve this problem there is a needs to find out such biocontrol agents which is native to the soil ecosystem and shows better survival when applied to the field. Possible way of this constrain is the use of plant growth promoting rhizobacteria (PGPR) as a biocontrol agents. PGPR are universally known to enhance the adaptive potential of their host's plants. The primary aim of this study was to isolate and characterize rhizoplane bacteria associated with native cotton species from soil of Saurashtra region which possess antifungal activity. In this research twelve anti-pathogenic PGPR bacterial isolates which were successfully isolated from cotton rhizosphere were evaluated for their PGP activities.

Key words: Plant Growth Promoting Rhizospheric Bacteria (PGPR), Bio Control, Nitrogen Fixer, Phosphate Solubilisation, Siderophore Producer, IAA Producer, ACC Deaminase Activity, Exopolysaccharide Producer.

Cotton is one of the world's most important commercial crops, and its protection from disease is vital to the many millions dependent on it directly or indirectly. Cotton suffers from a number of fungal and other diseases in India. Of the common fungal diseases, wilt caused by *Fusarium oxysporum* and root rot caused by *Rhizoctonia solani* is the most important as the yield loss due to this disease is considerable[17].

Root rot disease is caused by soil-borne fungi *R.solani*. The loss in yield results due to reduction in plant stand by way of sudden death of plants. The disease appears in patches. Due to this disease perfectly healthy plants may wilt within 24 hours with leaves drooping without showing any discoloration. Roots of affected plants become brown to dark and the bark of the affected roots shred.

Fusarium wilt caused by a soil-borne fungus *Fusarium oxysporum* affects diploid cotton in north India, parts of Gujarat, Maharashtra and Karnataka. In young as well as old plants the initial symptoms are stunting followed by yellowing, wilting and drooping of most of the leaves. In old plants, lower leaves towards the base are affected first followed by younger ones towards the tips. Leaf discolouration appears around the edges and

* To whom all correspondence should be addressed.
E-mail: hiren.sherathiya@gmail.com

progresses towards the midrib and leaves gradually drop. Thus disease causes considerable reduction in yield.

Different fungicides and chemical agents were used to manage this disease. But extensive use of chemicals may create environmental problems and are costly too. Therefore, more recently there has been a resurgence of interest in environmental friendly, sustainable and organic agricultural practices for the biological control of plant pathogens is an attractive preposition as it mimics the nature's own way of balancing the ecosystem[19]. Furthermore, obvious pollution of the environment and the toxic effects of synthetic chemicals on non- target organisms including humans have prompted investigations on fungicides of biological origin[29]. During the last couple of decades, the use of PGPR for sustainable agriculture has increased tremendously in various parts of the world due to their efficacy as biological control and highly specific to certain plant species. There is very little information regarding the use of PGPR metabolites as antagonistic agents against fungal pathogen and biofertilizers in cotton. The aim of the present work was to check for the plant growth promoting activities of potential isolates[17].

The rhizobacteria may be present (i) in the soil surrounding roots, utilizing the metabolites leaked from roots as the growth nutrients, (ii) on the root surface or rhizoplane, (iii) in the root tissue, inhabiting spaces between cortical cells and (iv) inside the cells in specialized root structures or nodules. Host plant-microbial interactions helps in the synthesis of phytohormones which help in plant growth, development and productivity. PGPR, in addition to improvement of plant growth is directly involved in increased uptake of nitrogen, solubilization of minerals, and production of siderophores and make it available to the plant[25].

The enhancement of plant growth by PGPR indicates their potential as bio-fertilizers in the field of agriculture. Though the importance of PGPR is well understood, but efficient PGPR are lacking. Districts of Saurashtra are better cotton producing district in Gujarat but, So far, no study has described or selected anti-pathogenic PGPB associated to cotton plants and no one has characterize rhizoplane bacteria associated with native cotton species and soil of Saurashtra region. Keeping this objective in mind the present investigation was performed and their plant growth promoting abilities were evaluated after screening and isolation of anti-pathogenic plant growth promoting rhizobacteria (PGPR) from the rhizosphere of cotton plants of Saurashtra region[25].

Materials and methodology

Soil sample collection and isolation of rhizospheric bacteria

Cotton (*G.hirsutum*) plants of different fields of Saurashtra region were selected for the study. Soil samples from rhizosphere of cotton plants were collected carefully by uprooting the root system and placed in a sterile polythene bag for transport and stored at 4°C. 1.0 gram of rhizospheric soil was suspended in 1.0 ml properly. After 1 h of sedimentation, 1.0 ml of water was taken from the tube and it was mixed in another 9.0 ml of sterile DDW for dilution. 1.0 ml of bacterial suspension was further diluted in another sterile test tube containing 9.0 ml DDW. In this way it was diluted up to 10^{-7} dilution. 100 µl of suspension was kept in solid N- agar plate and spreading was done. It was incubated for 3 days at 30°C in incubator and morphologically different colonies appeared on the plates were isolated, sub cultured in N-agar medium[25].

Screening for plant growth promotion

Cotton (*Gossypium hirustum*) seeds were surface sterilized with 0.1% mercuric chloride for 5 min, rinsed with sterilized distilled water (SDW) and soaked in bacterial suspension (3×10^8 cfu ml^{-1}) using 1% carboxymethyl cellulose (CMC). Air dried seeds were placed on a paper towel (ten seeds per paper) and incubated at 28 ± 2^0C for 4 days in a growth chamber. Percentage germination was recorded along with root and shoot length. Non-bacterized seeds served as control.

Preliminary screening of PGPR isolates for anti-pathogenic activity

Fifty two plant growth promoting isolates out of two hundred eleven bacterial isolates were tested for their ability to produce antifungal substances against *Fusarium oxysporum* and *Rhizoctonia solan*i using a dual-culture in vitro assay on PDA plates. Five µl of each bacterial suspension (10^8 cfu/ml) was placed on the plate. After 48 h incubation at 28°C, a single 6 mm diameter mycelial disc was placed at the centre of plates. Then plates were incubated at 27-29 °C in darkness and after 7 days the zone of inhibition was

measured. This experiment was conducted twice. Bacteria with inhibitory potential were selected for further experiments.

Test for Plant Growth Promoting activities

Test for IAA production

The production of indole-3-acetic acid (IAA) was tested by colorimetric method of Gordon and Weber. Cultures were grown in nutrient broth medium with tryptophan (100 µg/ml) at 30°C with shaking at 80 rpm for three days. 1.5 ml culture was centrifuged at 8000 rpm for 5 min at desired time interval and pellet was discarded and supernatant retained. To 1 ml supernatant, 2 ml Salkowski reagent (1 ml of 0.5 M $FeCl_3$ was mixed in 50ml of 35% $HClO_4$) was added. The sample was incubated at room temperature for 25 min. The optical density of the samples was recorded at 530 nm (blank from respective medium was used). The amount of IAA was quantified with standard of pure IAA prepared separately[25].

Test for Nitrogen Fixation

Cultures were grown in nutrient broth medium.0.1 ml of culture was inoculated in test tube containing Nfb (Nitrogen free bromothymol) semisolid media. All the tubes were incubated at 30°C for 48 h and observed for the growth by the formation of pellicles.

Dinitrogen fixation efficiency analyses of potential isolates were done in semisolid NfB medium. The tubes were incubated for 10 days at 30°C. The amount of N_2 fixation was determined by a Kjeldahl analysis. After incubation, the medium growing isolates was poured into Kjeldahl tubes with the salt mixture (40:2.5:1.5 ratio of K_2SO_4, $CuSO_4$ and metallic selenium) and 3ml of concentrated sulphuric acid were added into the tube. The tubes were digested in digester at 420°C for 20 minutes. After digestion and the tubes were cooled, distilled water was added until the final volume of 50 ml.Twenty mililitres of digested sample were poured into distillation tube and put under distillation apparatus. In a 250 ml erlenmeyer flask, 20 ml of 4% boric acid and 6 drops of Conway reagent (1000mg methyl red, 150mg bromcresol green, 200ml ethanol 96%) were added. The flask was placed under the condenser of the distillation apparatus and the tip of the condenser outlet was beneath of the solution. Distillation was carried out using UDK 132 Semi-Automatic distillation unit and delivery of 30 ml of 40% NaOH and 100 ml of distilled water was automatically poured through the distillation apparatus. The solution containing distilled NH_3, boric acid and mixed indicator was titrated against 0.05N HCL using autotitrator[28].

Test of Phosphate Solubilization

All isolates were plated on standard agar medium (pH 6.8–7.0) containing 5 g of tricalcium phosphate (TCP) as sole phosphorus source for selectively screening the bacteria which have the ability to release inorganic phosphate from tricalcium phosphate[20]. Uninoculated plates and *E. coli* inoculated plates served as controls. After 3-days of incubation at 30°C,clear zones around colonies indicates positive result. Colonies with clear zones were further purified by replating on agar medium supplemented with TCP agar plates for phosphate solubilization.

Quantitative analysis of phosphate solubilization was performed as per the method of Mehta and Nautiyal, 2001. The potential isolates were inoculated in Nautiyal phosphate solubilization broth. The Nautiyal medium comprises 1.0% (w/v) glucose, 0.5% (w/v) $Ca_3(PO_4)_2$, 0.5% (w/v) $MgCl_2$-$6H_2O$, 0.025% (w/v) $MgSO_4$ -$7H_2O$,0.02% (w/v) KCl and 0.01% (w/v) $(NH_4)_2SO_4$. The pH was adjusted 7.0. The culture was incubated for 3 days at 30 ± 2°C. The cultures were harvested by centrifugation at 10,000 rpm for 10 min. The culture supernatant thus obtained was used for quantitative assays. The optical density was measured at 600 nm with spectrophotometer[25].

Test of Siderophore Production

CAS(Chrome Azurol S) agar plates were prepared containing: 1 mM Chromeazurol-S, 10 ml $FeCl_3$.$6H_2O$ (1 mM) made in 10 m MHCl, and N, N-cetyltrimethyl ammonium bromide (2 mM) (CTAB).This was autoclaved separately and added to 300 ml of nutrient broth containing 2 µM $FeCl_3$ replacing the Fe-EDTA which is the usual ingredient of the medium. 10 ml of iron starved culture was inoculated as spot inoculation on the CAS agar plate and incubated for 72 h at 30°C. Yellow to orange halo zone appearing around the colonies against blue coloured background was recorded as positive test for siderophores production.

The isolates were grown in nutrient broth without any iron source to create iron starvation. Iron starved isolates were inoculated in nutrient broth containing 2µM $FeCl_3$ in place of Fe- EDTA.

Estimation of siderophore was made after 3 days of incubation at 30°C. For this culture supernatant was recovered by centrifugation at 8000 rpm for 5 min.0.5 ml supernatant containing less than 7.5 nmol of iron chelator was mixed with 0.5 ml of CAS assay solution. 2,3-dihydroxybenzoic acid (DHBA) or catechol was used as positive control and uninoculated medium as negative control. Absorbance was measured at 630 nm[25].

Test for ACC (1-aminocyclopropane-1-carboxylate) deaminase activity

Screening for ACC deaminase activity of isolates was done based on their ability to use ACC as a sole nitrogen source. All isolates were grown in 5 ml of TSB medium incubated at 28°C at 120 rpm for 24 h. The cells were harvested by centrifugation at 3000 *g* for 5 min and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and spot inoculated on petri plates containing modified DF (Dworkin and Foster) salts minimal medium, glucose, 2.0 g; gluconic acid, 2.0 g; citric acid, 2.0 g; KH_2PO_4, 4.0 g; Na_2HPO_4, 6.0 g; $MgSO_4.7H_2O$, 0.2 g; micro nutrient solution ($CaCl_2$, 200 mg; $FeSO_4.7H_2O$, 200 mg; H_3BO_3, 15 mg; $ZnSO_4.7H_2O$, 20 mg; Na_2MoO_4, 10 mg; KI, 10 mg; NaBr, 10 mg; $MnCl_2$, 10 mg; $CoCl_2$, 5 mg; $CuCl_2$, 5 mg; $AlCl_3$, 2 mg; $NiSO_4$, 2 mg; distill water, 1000 ml), 10 ml and distill water, 990 ml; supplemented with 3 mM ACC as sole nitrogen source. Plates containing only DF salts minimal medium without ACC as negative control and with $(NH_4)_2SO_4$ (0.2% w/v) as positive control. The plates were incubated at 30°C for 72 h. Growth of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as nitrogen source[3].

To measure ACC deaminase activity, potential isolates were grown in 5 ml of TSB medium at 30°C until they reached stationary phase. To induce ACC deaminase activity under non-stress and drought stress conditions, the cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.5), suspended in 2 ml of modified DF minimal medium supplemented with 3 mM final concentration of ACC without PEG for non-stress condition and with PEG 6000 (-0.30 MPa) for drought stress respectively, and incubated at 30°C with shaking for another 36-72 h[3].

ACC deaminase activity was determined by measuring the production of á-ketobutyrate and ammonia generated by the cleavage of ACC by ACC deaminase[12]. The induced bacterial cells were harvested by centrifugation at 3,000 *g* for 5 min, washed twice with 0.1 M Tris-HCl (pH 7.5), and resuspended in 200 µl of 0.1 M Tris-HCl (pH 8.5). The cells were labilized by adding 5% toluene (v/v) and then vortexed at the highest speed for 30 s. Fifty µl of labilized cell suspension was incubated with 5 µl of 0.3M ACC in an eppendorf tube at 30° C for 30 min. The negative control for this assay included 50 µl of labilized cell suspension without ACC, while the blank included 50 µl of 0.1 M Tris-HCl (pH 8.5) with 5 µl of 0.3 MACC. The samples were then mixed thoroughly with 500 µl of 0.56 N HCl by vortexing and the cell debris was removed by centrifugation at 12, 000 *g* for 5 min. A 500 µl aliquot of the supernatant was transferred to a glass test tube and mixed with 400 µl of 0.56 NHCl and 150 µl of DNF solution (0.1 g 2,4-dinitrophenyl hydrazine in 100 ml of 2N HCl); and the mixture was incubated at 30^0 C for 30 minutes. One ml of 2 N NaOH was added to the sample before the absorbance at 540 nm was measured[3].

The concentration of a -ketobutyrate in each sample was determined by comparison with a standard curve generated as follows: 500 µl á-ketobutyrate solutions of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM were mixed respectively with 400 µl of 0.56 N HCl and 150 µl DNF solution. One ml of 2N NaOH was added and the absorbance at 540 nm was determined as described above. The values for absorbance versus α-ketobutyrate concentration (mM) were used to construct a standard curve[3].

Hydrogen cyanide (HCN) production

HCN production was determined by colour change of filter paper as describe by Alstrom and Burns, 1989. 100 µL of bacterial suspension was inoculated on nutrient agar medium contained 4.4 g L^{-1} glycine. Filter papers were soaked in a reagent solution (sodium carbonate 2% and picric acid 0.5%) and placed in the upper lid of Petri dishes. To prevent volatilization, the plates were sealed with parafilm and incubated at 28°C for 4 days. One plate without inoculation of bacterium was considered as control. If HCN was produced, yellow filter papers changed to cream, light brown, dark brown and eventually turn into reddish-brown.

To compare the bacterial isolates in HCN production semi- quantitatively, isolated bacteria that showed yellow, cream, light brown and dark brown (reddish- brown) colours received 0, 1, 2, 3 and 4 scores, respectively. The scores including 0, 1, 2, 3 and 4 represented no ability and low, medium, high and very high ability of HCN production, respectively[4].

Exopolysaccharides Production

Trypticase soya broth (TSB) with low water potentials (0.5MPa) was prepared by adding appropriate concentrations of polyethylene glycol (PEG 6000) as describe by Sandhya et al. 2009. Which was inoculated with 1% of overnight raised bacterial cultures in TSB[23].

Exopolysaccharide was extracted from 3-day-old cultures raised in TSB (15% PEG 6000 was added to TSB for inducing stress). The culture was centrifuged at 20,000 g for 25 min and the supernatant was collected. Highly viscous cultures were diluted with 0.85% KCl before centrifugation. The pellet was washed twice with 0.85% KCl to completely extract EPS. The possible extraction of intracellular polysaccharides was ruled out by testing the presence of DNA in the supernatant by DPA reagent. Concentration of protein in the supernatant was estimated by Bradford's reagent. Then, the supernatant was filtered through 0.45 µm nitrocellulose membrane and dialysed extensively against water at 4°C. The dialysate was centrifuged (20,000 g) for 25 min to remove any insoluble material and mixed with 3 volumes of ice-cold absolute alcohol and kept overnight at 4°C. The precipitated EPS obtained by centrifugation (10,000 g for 15 min) was suspended in water and further purified by repeating the dialysis and precipitation steps. Total carbohydrate content in the precipitated EPS was determined according to Dubois *et al.*,[9,11].

NH_3 production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 30^0C. 0.5 ml Nessler's reagent was added in each tube. Development of brown to yellow colour was a positive test for ammonia production[2,7].

Catalase Production test

Catalase test was performed by adding 3-4 drops of hydrogen peroxide (H_2O_2) to 48 h old bacterial colony which was grown on trypticase soya agar medium. The effervescence indicated catalase activity[10,24].

Extra cellular enzyme activities

Chitinase Production

Chitinase production was investigated by using chitin medium[7]. The cultures of isolates were prepared by cultivating on N broth medium for 24 h on shaking incubator. About 1 µl suspension of isolates was dropped in to surface of chitin agar medium. Chitinolytic activity was determined by the development of clear zone around the bacterial colony[30].

Cellulase Production

Cellulase production was determined by using the method describes by Miller[17]. M9 agar medium with yeast extract plates were inoculated with individual bacterial isolates and incubated for 3 to 5 d at 30°C. Bacterial growth surrounded by clear halos was considered as positive indication of cellulase production[10].

RESULT AND DISCUSSION

Isolation of rhizospheric bacteria

211 bacterial colonies with different growth characteristics were isolated and purified by further streaking on nutrient agar media and pure cultures were used for further experiments. The criteria taken in to the consideration were pigmentation, elevation, texture and size.

Screening of isolates for plant growth promotion (PGP)

52 isolates out of 211 primary isolates improve plant health and promote growth by increasing of seedling emergence and the vigour when tested in vitro for cotton germination. All 52 PGPR isolates were selected on the basis of their performance in seed germination assay, significantly enhanced the seedling length. Some of the isolates also showed a significant increase in % germination as compared to untreated control. Seeds coated with bacterial isolates showed 100% germination. Seeds coated with bacterial isolates V-3 showed highest seedling length and seedling vigour after 4 days of germination as shown in table 2 below. In this study, seed treatment with the bacterial isolates significantly improved seed emergence together with plant root and shoot length (fig. 1).

The overall improvement in seedling vigour through a significant increase in various physiological parameters suggests that these strains have a plant-growth promoting ability on cotton seedlings and hence could be used for seed inoculation for better establishment of seedlings. The plants with enhanced seedling vigour can help in better establishment of plantations.

Screening for antagonism

Rhizosphere isolates showing plant growth promotion in germination assay was studied for antagonistic activity against the fungal pathogen. Twelve out of 52 bacterial strains showing high antifungal activity against both pathogens that is *Fusarium oxysporum* and *Rhizoctonia solani*., which were selected and used in the subsequent in vitro inhibition tests and field experiments table 3. All these isolates were than identified by biochemical analysis table 1.

Antagonistic activity of the bacterial isolates was evaluated in terms of inhibition zone diameter as an indicator of the reduction in growth of pathogenic fungi. The maximum zone of inhibition was observed in isolate P-3 against *Fusarium oxysporum* and isolate A-4 against *Rhizoctonia solani* as shown in table 3.

Test for Plant Growth Promoting activities

Nitrogen fixation

Nitrogen fixation was one of the mechanisms proposed to explain the improvement of plant growth. Isolates A-2 and A-3 able to converts atmospheric nitrogen into ammonium under microaerophilic conditions at low nitrogen levels through the action of nitrogenase and able to grow as a pellicle when grow in Nfb semisolid medium figure 10**.** These two isolates were able to fix nitrogen ranging from 11.0–15.06 mg of nitrogen $kg^{-1}$when measured by micro Kjeldahl method table 5.

Various soil microorganisms live in the plant rhizosphe redue to the rich nutrient availability such as *Rhizobium*, *Azotobacter* and *Azospirillum* enhance plant growth as a result of their ability to fix-N_2.

IAA production

It is evident that all the isolates (100%) showed positive test for IAA production figure 3. IAA production occurred solely in nutrient broth liquid medium supplemented with tryptophan (100 µg/ml). It was found that there was no production in medium lacking tryptophan. Among all the isolate K-1 showed highest IAA production (8.36 ± 0.3 µg/mg dry weight) and S-1 showed the lowest level (0.73 ±0.4 µg/mg dry weight) (Table4).

Bacterial producing IAA survive in the soil environment make use of nutrients exuded by the plant root, proliferate and be able to efficiently colonize the entire root system. So, it would be beneficial for the plant to which it showed interaction. Other complexes interactions between plant and bacterial producing IAA might be due to the fact that IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division, or indirectly by influencing bacterial ACC deaminase activity. [30]

Phosphate solubilization

Out of 12 isolates, four (B-1, V-1, P-3 and K-1) isolates accounting 33.33% produced halo-

Table 1. Plant growth promoting antipathogenic isolates identified by biochemical activities

Isolate	Bactria identified by biochemical activities	Isolation place
K-1	*Pseudomonas putida*	Kutiyana, Porbandar
K-2	*Pseudomonas fluorosence*	Khambha, Amreli
K-4	*Bacillus spp.*	Kalavad, Jamnagar
P-3	*Pseudomonas rhizosphaerae*	Porbandar
J-1	*Bacillus spp.*	Junagadh
U-1	*Bacillus spp.*	Upleta, Rajkot
V-1	*Burkholderia spp.*	Veraval, Junagadh
B-1	*Agromycescerinus*	Bhavnagar
S-1	*Bacillus subtilis*	Surendranagar
A-2	*Azotobacter spp.*	Amreli
A-3	*Azospirillum lipoferum*	Atkot, Rajkot
A-4	*Gluconacetobacterdiazotrophicus*	Amarsar, Rajkot

Table 2. Results of seed germination assay for plant growth promotion

Isolates	Seed Germination Percentage	Total length in cm			Average length in cm	Seedling vigour index
Control	80%	3.8	4.2	4.5	4.1	410
A-1	100%	11	10.8	11.5	11.1	1110
A-2	100%	13	12.5	13	12.8	1280
A-3	100%	9.1	7.2	8.8	8.3	830
A-4	100%	9	7.7	9	8.5	850
A-5	100%	8	11	8	9.0	900
A-6	100%	14.2	11.5	11	12.2	1220
A-7	100%	10.2	10.4	11	10.5	1050
K-1	100%	12	11.5	12.4	11.9	1190
K-2	100%	8.2	8.5	11	9.2	920
K-3	100%	11	10.5	10.9	10.8	1080
K-4	100%	11.8	8.2	12.4	10.8	1080
K-5	100%	12	11.5	12.1	11.8	1180
K-6	100%	8.2	10	8.6	8.9	890
S-1	100%	12	13	12.1	12.3	1230
S-2	100%	9.1	10	9.4	9.5	950
S-3	100%	7.5	8	9.1	8.2	820
S-4	100%	9.9	7.9	8.5	8.7	870
S-5	100%	10	10.4	12	10.8	1080
S-6	100%	11.2	13	11.5	11.9	1190
P-1	100%	9.1	9.7	13	10.6	1060
P-2	100%	8.9	10.6	10.2	9.9	990
P-3	100%	10.5	8.5	8.5	9.1	910
P-4	100%	8.2	9.5	9.4	9.0	900
P-5	100%	13	12.8	16	13.9	1390
P-6	100%	14	12	11.5	12.5	1250
P-7	100%	8.7	9.2	12.3	10.0	1000
J-1	100%	16	10.8	14	13.6	1360
J-2	100%	12.3	12.5	13.3	12.7	1270
J-3	100%	15.1	16.2	14.9	15.4	1540
J-4	100%	14	15.8	15.9	15.2	1520
J-5	100%	10.2	10.7	9.3	10.0	1000
J-6	100%	12	11.8	9.6	11.1	1110
U-1	100%	10	14.4	15	13.1	1310
U-2	100%	15.1	13.4	12.2	13.5	1350
U-3	100%	10.1	12.2	12	11.4	1140
U-4	100%	8.2	9.1	10.1	9.1	910
U-5	100%	14.3	14.9	16	15.0	1500
U-6	100%	15.8	11.8	14.3	13.9	1390
U-7	100%	7.8	9.2	9.8	8.9	890
.V-1	100%	10.5	9	9.5	9.6	960
V-2	100%	10.2	14.9	16.1	13.7	1370
V-3	100%	17.1	15.6	16.1	16.2	1620
V-4	100%	12.6	13	14.2	13.2	1320
V-5	100%	8.2	10.9	9.1	9.4	940
V-6	100%	13.1	10.2	10.4	11.2	1120
B-1	100%	10.2	10.6	13	11.2	1120
B-2	100%	12	13.3	14	13.1	1310
B-3	100%	6.4	7.8	10.2	8.1	810
B-4	100%	9.1	12	8.8	9.9	990
B-5	100%	7.2	6.8	6	6.6	660
B-6	100%	9.1	8.9	9	9	900
B-7	100%	6.2	6	7.2	6.4	640

zones around the growing colony, where as other eight isolates accounting 66.7% either showed growth without any halo-zone formation or failed to grow figure 2.

Table 3. Results for anti-pathogenic test

Isolates	Diameter of inhibition zones(mm^2)	
	Against *F.oxysporum*	Against *R.solani*
A-2	10.0	11.2
A-3	14.0	18.2
A-4	16.0	15.2
K-1	12.5	11.1
K-2	8.2	8.9
K-4	14.1	12.0
S-1	6.6	9.1
P-3	8.8	20.1
J-1	6.1	9.2
U-1	5.2	4.2
V-1	9.0	16.1
B-1	11.0	16.0

Efficiency of phosphate solubilization was further confirmed by measuring the level of solubilized phosphate in the liquid Nautiyal medium (Table 4). It is evident that P-3, an isolate from Porbandar district of Gujarat showed highest phosphate solubilization (25.17 ± 5.6 µg/mg dry weight) and V-1, an isolate from Veraval district showed lowest phosphate solubilization (11.52 ± 4.5µg/mg dry weight).

Most of the agricultural soils in various parts of Gujarat contain 50 kg/ha of phosphorous, primarily in the unavailable form of precipitated tricalcium phosphate ($Ca_3(PO_4)_2$. Many bacteria are well known to dissolve bound phosphates such as calcium triphosphate,hydroxy apatite and rock phosphate, and enhance the availability of phosphorous for microbial and/or plant growth.

Table 4. Result for IAA production, Phosphate solubilization and Siderophore production

Isolates	IAA production(µg/ml)	Phosphate solubilization(µg /ml)	Siderophore production(µg /ml)
A-2	2.34 ± 0.55	14.52 ± 4.5	14.13 ± 2.15
A-3	5.09 ± 0.65	12.17 ± 3.5	10.84 ± 2.68
A-4	5.23 ± 0.85	12.23 ± 3.7	8.5 ± 1.65
K-1	8.36 ± 0.30	15.66 ± 2.35	8.76 ± 1.38
K-2	4.67 ± 0.60	12.47 ± 2.5	20.22 ± 3.45
K-4	6.26 ± 0.50	14.47 ± 2.5	9.73 ± 2.36
S-1	0.73 ±0.40	15.66 ± 2.35	18.77 ± 2.66
P-3	1.75 ± 0.25	25.17 ± 5.6	15.63 ± 2.67
J-1	3.17 ± 0.65	20.78 ± 4.5	9.73 ± 2.36
U-1	5.72 ± 0.75	16.23 ± 3.7	8.92 ± 2.95
V-1	4.87 ± 0.75	11.52 ± 4.5	12.13 ± 2.15
B-1	3.99 ± 0.6	14.27 ± 3.5	11.73 ± 2.36

Table 5. Results for Nitrogen fixation, ACC deaminase activity and Exopolysaccharide production

Isolates	Nitrogen Fixation mg of nitrogen kg^{-1}	ACC deaminase activity µM/mg protein/h	Exopolysaccharide production µM /mg protein/h
A-2	11.0	-	2.85 ± 0.07
A-3	15.06	-	3.22 ± 0.04
A-4	-	-	-
K-1	-	-	-
K-2	-	-	-
K-4	-	-	-
S-1	-	-	-
P-3	-	-	-
J-1	-	-	-
U-1	-	-	-
V-1	-	3.71 ± 0.025	-
B-1	-	-	-

The highest phosphate solubilizing isolate P-3 (isolated from Porbandardistrict) shows 2-3 times more phosphate solubilizing activity than other isolates and possess all the plant growth potentials tested in this investigation and seems to be a good isolate for use asbio fertilizer. Phosphate solubilization by rhizospheric bacteria is useful character since they can partly meet phosphate demand of the plants. Several workers have reported that seed or soil inoculation with phosphate solubilising bacteria improves solubilization of fixed soil phosphorous and/or applied phosphates, resulting in higher crop yields[13,25,27].

Siderophore production

Siderophore production was detected by means of CAS agar plate assay where blue colour of medium changed to yellow/orange around the growing colonies figure 9. On the basis of yellow/ orange halo zone formation, 9 isolates(S-1, A-4, A-3, K-4, K-2, K-1, P-3, J-1, and U-1) accounting 75% of total isolates showed positive test for siderophore production. Quantitative estimation showed that the highest siderophore (20.22 ± 3.45 µg/mg dry weight) production occurs in the isolate K-2 whereas the lowest (8.5 ± 1.65 µg/ mg dry weight) level was in A-4 (Table 4).

Majority of the soil contains enough amount of iron(1-6%) but most of them are in ferric form, which is insoluble and thus not accessible to the plants and microorganisms. Many microorganisms including bacteria and fungi have

Fig. 1. Seed germination assay photo after 4 days showing comparison between control and seed treated with bacterial isolates A-1

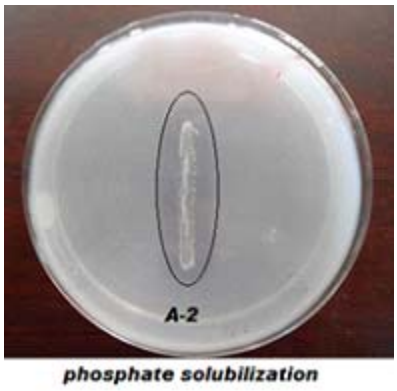

Fig. 2. Zone of clearance on TCP agar plate by phosphate solubilizer

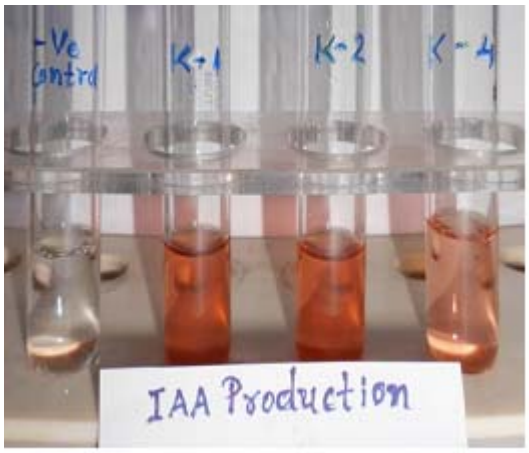

Fig. 3. Brown colour development by IAA producing isolates

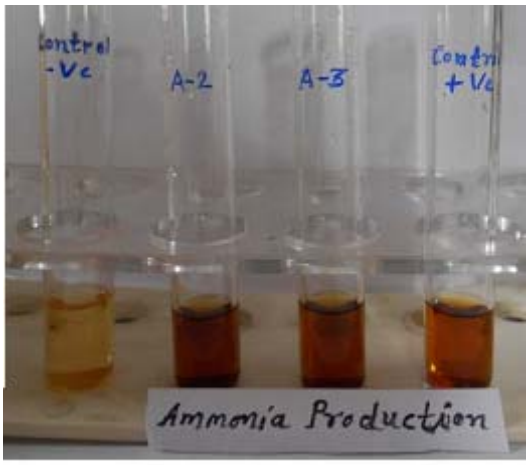

Fig. 4. Brown colour development by Ammonia producing bacterial isolates

developed system for the synthesis of low molecular weight organic compounds, siderophore, which can efficiently solubilise and transport ferric iron. We have analyzed all the isolates for their iron chelating properties and observed that out of 12 isolates, 9 isolates produced siderophore in iron limiting condition[16].

Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, including *Pseudomonas spp*. under iron limiting condition, PGPR produce a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi[30,31].

Screening for ACC (1-aminocyclopropane-1-carboxylate)deaminase activity

All the twelve isolates were screened for ACC deaminase based on the enrichment method, where ACC was used as the sole nitrogen source. Among twelve isolates, one isolates V-1 grew well on DF salt minimal medium with ACC serving as the sole nitrogen source.

The ACC deaminase enzyme activity was assayed by quantifying the amount of á-ketobutyrate produced during the deamination of ACC by the enzyme ACC deaminase. Isolate V-1 utilized ACC as a sole source of nitrogen by the

Fig. 5. Effervesces produce by isolates on addition of H_2O_2

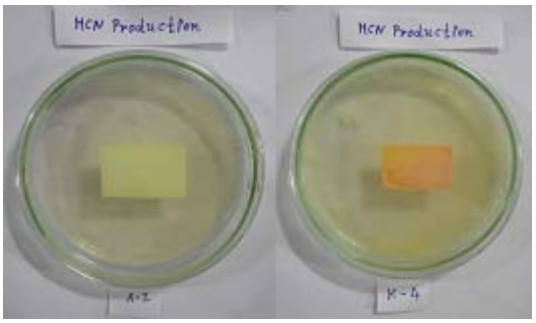

Fig. 6. Brown colour development on paper by HCN producing isolates K-4

Fig. 7. Zone of clearance shown by Chitinase producing isolates

Fig. 8. Zone of clearance shown by cellulase producing isolates

production of ACC deaminase enzyme and it showed the greater amount of ACC deaminase activity (3.71 ± 0.025 µM/mg protein/h of β-ketobutyrate (table 5).

PGPR that have ACC deaminase activity help plants to with stand stress biotic or abiotic by reducing the level of stress ethylene through the activity of enzyme ACC-deaminase that hydrolyzes ACC into á-ketobutyrate and ammonia, instead of ethylene as describe by Glick et al. 1998 and Arshad et al. 2007. In the present study, we screened drought tolerant bacteria having ACC deaminase activity with multiple PGP traits, and found that one out of the screened 12 strains showed ACC deaminase activity[3,6].

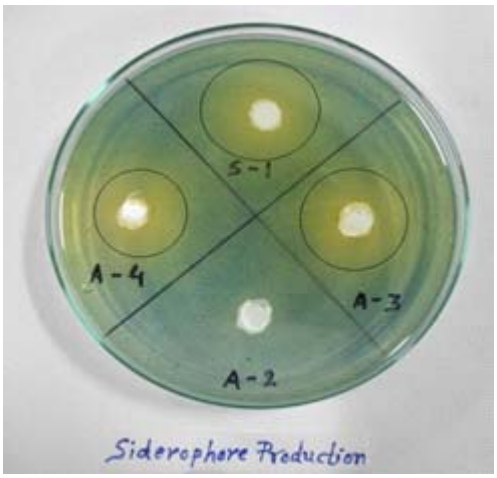

Fig. 9. Zone of development by siderophore producing isolates

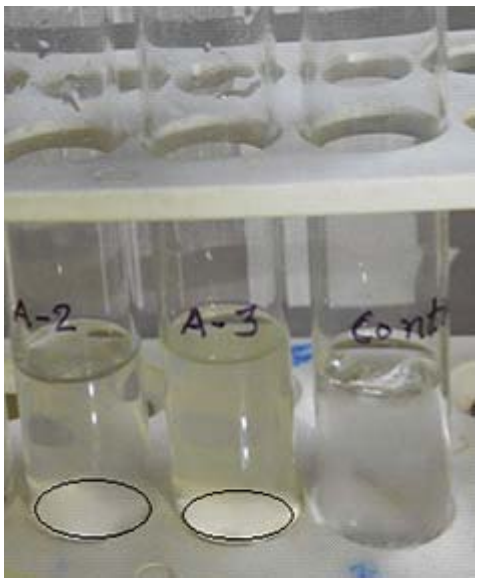

Fig. 10. Pellicle growth in Nfb semisolid medium by nitrogen fixer

HCN production

All the isolates were then subjected to HCN production test, development of reddish brown colour on paper shows positive result figure 6. Out of 12 isolates four (K-1, K-2, K-4,P-3) were recorded positive for HCN production. HCN producing bacteria were categorized in 4 groups with very high, high, medium and low ability. About 33.33% of the isolates showed ability in producing HCN. From which P-3 showed strong results while other gave moderate and low results. HCN, produced by many soil microorganisms and it is postulated to play a role in biological control of pathogens. Production of HCN by certain strains of *Fluorescent pseudomonads* has been reported which involved in the suppression of soil borne pathogens[1,26].

Exopolysaccharides production

The isolate A-3produced3.22 ± 0.04 mg/mg protein EPS and A-2 produced 2.85 ± 0.07 mg/mg protein (table 5). Exopolysaccharides possess unique water holding and cementing properties, thus found to be playing a vital role in the formation and stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation as observed by Roberson and Fireston[21]. Role of exopolysaccharide material has been suggested in the protection of A-2 and A-3 cells against desiccation. Hartel and Alexandre observed a significant correlation between the amount of EPS produced by cowpea *Bradyrhizobium* strains and their desiccation tolerance[13]. Probably EPS can provide a microenvironment that holds water and dries more slowly than the surrounding microenvironment, thus protecting bacteria from drying and fluctuations in water potential[3,15].

NH_3 production

Seven selected isolates (A-2,A-3,A-4, J-1, U-1, V-1, and B-1) out of twelve were positive for ammonia production figure 4. Ammonia productions were found to be associated with plant growth promoting attributes of isolates such as ACC deaminase activity and nitrogen fixing activity.

Catalase Production test

All the isolates were found to be positive for catalase production figure 5. It has been reported that bacterial strains showing catalase activity shown highly resistant to environmental, mechanical and chemical stress[10].

Extra cellular enzyme activities

Chitinase production and cellulase production

Out of twelve bacterial isolates two shows zone of degradation when grown on chitin agar plate. Isolates U-1 and B-1 shows chitinase production, which might be responsible for their anti-pathogenic attributes figure 7.

M9 agar medium with yeast extract plates were inoculated with individual bacterial isolates and incubated for 3-5 d at 30°C. Bacterial growth surrounded by clear halos was considered as positive indication of cellulase production figure 8. S-1 and B-1 showed cellulase activity.

Several studies have demonstrated that production of lytic enzymes by *Pseudomonas* strains was most effective in controlling the plant root pathogens including *F. oxysporum and R. solani*[21].

CONCLUSION

Some of the above-tested isolates exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. Similar to our findings of multiple PGP activities among PGPR have been also reported by some other workers while such findings on indigenous isolates especially in the region of Sautashtra in Gujarat were not explored.

The rhizosphere is a hot spot of microbial interactions as exudates released by plant roots are the main food source for microorganisms and a driving force for their population density and geochemical cycling of nutrients. Thus screening and selection of effective PGPR and their utilization in integrated practices is of great importance for enhancing the growth and yield of agricultural crops with maintaining sustainability of agro-ecosystems.

There are sound reasons for evaluating indigenous rhizosphere bacteria as possible fungal control agents since naturally occurring strains can be found that are competitive and persistent under harsh environmental conditions, including moisture or temperature extremes, soil acidity or alkality, and salt and draught tolerance. Moreover, a better knowledge of these bacteria and their implications on plant physiology could change traditional crop management practices regarding plant nutrition and defence mechanisms. For a maximum exploitation of the plant-bacteria association, effective bacteria must be selected in plant studies that take specific ecological conditions into consideration, e.g., crop management, soil, temperature, etc. Thus the bacteria isolated in this study with mechanisms such as phytohormone production in the rhizosphere and other PGP activities can contribute to improve the ability of the host plant to survive in extreme environments.

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