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ISOLATION, SCREENING, CHARACTERIZATION AND ANTAGONISM ASSAY OF PGPR ISOLATES FROM RHIZOSPHERE OF BT AND NON BT COTTON PLANTS

A.B. RAJITHASRI* AND K. SATYAPRASAD

Mycology and Plant Pathology Laboratory, Department Of Botany, Osmania University, Hyderabad-500007, Telangana State, India

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. In our investigation, we have isolated, screened and characterized the PGPR from the rhizosphere soil of Bt and non Bt cotton, were collected from different areas of Khammam and Warangal districts in Telangana, India. Bacterial isolates were screened preliminarily for their antifungal activity against *Colletotrichum gossypi, Fusarium oxysporum, Macrophomina phaseolina* and *Rhizoctonia solani.* 23 antagonistic isolates were tested for their PGP traits, extra cellular enzyme and siderophore production. The present study, suggests that the use of PGPR isolates as inoculants/ biofertilizers might be beneficial for cotton cultivation as they enhanced growth of cotton due to the production of IAA, NH3, phosphate solubilization, siderophore production and also having antifungal activity against phytopathogenic fungi.

KEYWORDS: PGPR, cotton, antifungal activity, extra cellular enzyme activity, siderophore production.

A.B. RAJITHASRI
Mycology and Plant Pathology Laboratory, Department Of Botany, Osmania University, Hyderabad-500007, Telangana State, India

***Corresponding author**

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly. Plant growth promoting rhizobacteria (PGPR) are originally defined as root-colonizing bacteria, that cause either plant growth promotion or biological control of plant diseases[1]. 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[2]. Cotton is an important cash crop in India and in many parts of the sub-tropical world. It is the most important raw material for the Indian textile industry. Cotton is a soft, staple fiber that grows in a form known as a boll around the seeds of the cotton plant. In India nine million hectares (m ha) area was under cotton cultivation and it is one fourth of the global area under cotton cultivation (35 m ha). During 2013-14 India is the 2nd top producer of cotton in the world after China. Therefore, in the present investigation, our attempts were directed towards the selection of indigenous strains exhibiting a number of traits associated with cotton plant growth promoting ability under *in vitro* conditions, as a prelude to use them in field conditions. Further there is little or no information available on this aspect with reference to cotton.

MATERIALS AND METHODS

1. Soil sampling

Soil samples were randomly collected from the rhizosphere of Bt and Non Bt cotton, growing in different locations of Khammam and Warangal districts, Telangana, India and stored at 4^0C. Intact root system was dug out and the rhizospheric soil samples were carefully taken in plastic bags and stored at 4°C. A total of 10 soil samples were collected for the isolation of rhizosphere bacterial isolates.

2. Isolation and preliminary screening of the bacterial isolates

The rhizosphere soil samples were processed within 24h for isolating most predominant plant growth promoting rhizobacteria (PGPR) using serial dilution method[3] on nutrient agar (NA) medium plates. Plates were incubated at 30°C for 24-48 h. Colonies were picked from these plates and maintained as pure cultures at 4°C in equal volumes of nutrient broth and 30% glycerol.

3. Plant Growth Promoting Mechanisms

The isolates that promote cotton growth were analysed for their ability to solubilize phosphate, produce ammonia, hydrogen cyanide (HCN), siderophore and indoleacetic acid (IAA)-like substances.

3.1 Ammonia production

All the bacterial isolates were tested for the production of ammonia in peptone water. 24h old cultures were inoculated into 10 ml peptone water in each tube and incubated for 48 h at 30^0C in the incubator shaker. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production[4].

3.2 Phosphate solubilization

Phosphate solubilization detected by the formation of transparent halos surroundings bacterial colonies on the Pikovskaya agar[5] after 72 hour incubation at 28°C.

3.3 Hydrogen cyanide (HCN) production

Screening of bacterial isolates for hydrogen cyanide (HCN) production was done as per methodology described by Castric[6]. Bacterial cultures were inoculated on HCN induction medium (King's B medium amended with 4.4 g/l glycine). A Whatman filter paper disk dipped in HCN revealing solution (0.5% picric acid and 2% Na2CO3) was placed on lid of petriplate were tightly sealed with parafilm and incubated at 30°C for 4 days. Development of orange-brown color of the paper indicated HCN synthesis ability.

3.4 Siderophore detection

Siderophore was detected by the formation of orange halos surrounding bacterial colonies on CAS agar plates[7] after 48 hour at 28°C.

3.5 Indole acetic acid production

Culture growth conditions

Detection of IAA Culture growth conditions 25ml of Luria Bertini (LB) broth supplemented with 1mg/ml of tryptophan (LBT) was inoculated with 24h old culture and incubated in refrigerated incubator shaker at 30°C for 48hr at 150rpm in the dark. Bacterial cells were harvested by centrifugation at 10000 rpm for 10 min at 4°C. Estimation of indole-3-acetic acid (IAA) in the supernatants was done using calorimetric assay[8].

Calorimetric estimation

The 2ml of supernatant was mixed with two drops of ortho-phosphoric acid and 4 ml of Salkowski reagent (50ml, 35% of perchloric acid, 1 ml 0.5M $FeCl_3$ solution), and incubated in dark at room temperature for 20 min. Development of pink to red color formation was considered positive evidence for IAA production. Absorbance was read at 530 nm. Auxin production was determined by using a standard graph[9]. The IAA production was calculated from the regression equation of a standard curve and the result was expressed as µg/ml over control.

4. Antifungal activity

The antifungal activity of the bacteria was assessed against the 4 test pathogens *Macrophomina phaseolina*, *Colletotrichum gossypi*, *Rhizoctonia solani*, *Fusarium oxysporum* by dual culture plate technique[10] on potato dextrose agar (PDA) medium. The zone of inhibition was measured after 72 h by subtracting the distance (mm) of fungal growth in the direction of an antagonist from the fungal radius. The percent inhibition was calculated using the formula[11].

% inhibition = (R - r) / R ×100

Where 'r' is radial growth of the fungal colony opposite the bacterial colony and, R is the radial growth of the pathogen in control plate measured in mm.

5. Screening of bacterial isolates for hydrolytic enzyme production

The selected antagonistic PGP isolates were screened for their hydrolytic enzyme production like chitinase, cellulase, pectinase, protease, lipase, amylase, gelatinase and catalase activities.

5.1 Production of chitinase

The qualitative chitin production assay was performed on colloidal chitin medium for 4 days at 28±2°C. Clear halos around and beneath the colony were observed around the bacterial colonies indicating the enzymatic degradation of chitin[12].

5.2 Cellulose activity

Cellulose degrading enzymes were screened by using M9 medium supplemented with with yeast extract (0.12% w/v) and carboxyl-methylcellulose (CMC) (1% w/v)[13]. Strains surrounded by a clear halo after 5 days of incubation at 28°C were considered as positive for cellulase production.

5.3 Pectinase activity

Production of pectinase was performed by using M9 medium amended with 4g of pectin per liter[14]. The plates were incubated for 4 days at 28+2°C. The appearance of a clear halo around the colonies indicates pectinase production[13].

5.4 Catalase activity

Catalase test was assayed by adding three to four drops of H_2O_2 on culture which was grown on trypticase soy agar medium (Tripticase 15gm, Phytone 5.0gm, NaCl 5.0gm, Agar 15.0gm, Distilled water 1000ml) for 48hr. The effervescence indicates catalase activity[15].

5.5 Protease activity

The qualitative assay for protease production was determined by a clear zone in sterile skim milk agar plates[16].

5.6 Lipase activity test

The isolates were grown on nutrient agar amended with egg yolk. After 48 h of incubation a turbid zone around the colony indicates

positive lecithinase activity. Agar medium was flooded with a saturated aqueous solution of copper sulphate ($CuSO_4$) and kept for 10-15 min. Formation of greenish blue colour zones around the colony indicated the production of lipase[17].

5.7 Amylase activity

The bacterial strains were spot inoculated on starch agar (Beef extract 3.0gm, Peptone 5.0gm, Soluble Starch 2.0gm, Agar 15.0gm, Distilled water 1lit) medium plates. After 48hr of incubation at 30^0C, the plates were flooded with iodine solution for 1min and pour off the excess iodine solution, the appearance of clear zone surrounding the colony indicates positive for starch hydrolysis test[18].

5.8 Gelatinase test

Gelatinase test was performed by inoculating the 24h old culture into gelatin tubes and incubated for 4 to 7 days at 25°C along with uninoculated control. Refrigerate the tubes for 30 minutes, if gelatinase is present, the liquid medium will fail to solidify upon refrigeration[19].

RESULTS AND DISCUSSION

Isolation of PGPR and preliminary screening for their antifungal property

A total number of 76 bacterial strains were isolated from the rhizosphere soils of Bt cotton and non Bt cotton fields from selected regions by serial dilution plate method. From Bt cotton 40 and non Bt cotton 36 bacterial strains were isolated. The Rhizobacteria were screened for antifungal activity against *Macrophomina phaseolina, Colletotrichum gossypi, Rhizoctonia solani, Fusarium oxysporum* and zone of inhibition was taken as an indicator of antifungal property in the dual culture plate method. Among the 76 isolates, only 23 were antagonistic to the four test pathogens. The inhibition percentage was calculated using the formula described by Idris *et al.* (2007) which is (R - r) / R × 100 **(**r: radial growth of the fungal colony opposite to the bacterial colony, R: the radial growth of the pathogen in control (Table 1, Fig:1). Among antagonistic isolates OUR12, OUR13, OUR17, OUR27, OUR36, OUR39 from Bt cotton, OUN3, OUN8, OUN25, OUN26, OUN32 and OUN35 from non Bt cotton exhibited significant antifungal activity (Table.1, Figure.1). Strain OUR27 showed high percentage inhibition (58%) against the charcoal rot pathogen *Macrophomina phaseolina*, isolate OUN26 (66%) against the wilt pathogen *Fusarium oxysporum,* OUN8 exhibited 66% growth inhibition against leaf spot pathogen *Rhizoctonia solani*. On the other hand OUR13 was moderately antagonistic to the wilt pathogen but highly inhibitory against *Colletotrichum gossypi.* The percentage inhibition of 23 strains ranged from 20% to 80%. All the 23 antagonistic isolates were tested for growth promotion traits also.Plant growth promotion was assessed by qualitative and quantitative determination of Indole Acetic Acid (IAA), Ammonia (NH_3), Hydrocyanic acid (HCN) and phosphate solubilization (Table 2, Fig. 1)). Among 23 antagonistic isolates, are known to solubilize phosphorous and all are producing ammonia and IAA. While only 9 (OUR3, OUR12, OUR13, OUR17, OUR32, OUN3, OUN8, OUN29 and OUN30) isolates showed the ability to produce HCN. OUR12, OUR13, OUR36, OUR39, OUN3, OUN8, OUN32 were the best producers of ammonia, High production of IAA was associated with OUN26, OUR12 and OUR13 isolates, while the isolates OUR36 and OUR39 were low producers of IAA. On the other hand, a negative response to HCN production was evident in all the antagonistic isolates except OUR12, OUR13, OUN3, OUN8 and OUN26. Out of 23 PGPR isolates, 6 isolates (OUR12, OUR13, OUR27, OUN3, OUN8 and OUN26) were able to produce siderophores and it is confirmed by the development of orange halos surrounding those colonies Production of extracellular enzymes by microorganisms has an important role in the management of plant pathogens as well as it holds enormous economic potential. In view of the significance of extracellular enzymes all the 23 PGPR isolates selected from the antifungal and growth promotion screening, were tested for their extra cellular enzyme production like chitinase, cellulase, pectinase, catalase, protease, lipase, amylase and gelatinase activity. All the 23 antagonistic

isolates exhibited catalase, protease, amylase and gelatinase enzyme activity. The bacterial isolates OUR1, OUR11, OUR32, OUR40, OUN2, OUN14, OUN23, OUN29, OUN30 and OUN31 could not produce lipase. Lecithinase activity was shown by OUR13, OUR17, OUR27, OUR36, OUR39, OUN3, OUN8, OUN25 and OUN26. OUR12, OUR13, OUR27, OUN3, OUN8 and OUN26 have shown cellulase and pectinase activity (Table3, Fig.1). PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion [20 21]. IAA, a member of the group of phytohormones, is generally considered to be the most important native auxin. Of 23 isolates, all the isolates were positive for IAA production. Among them, three isolates OUR12, OUR13 and OUN26 are found to be good producers of IAA. PGPR isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil[22]. Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rise that represent a possible mechanism of plant growth promotion under field conditions[23]. In our experiments, all antagonistic isolates were able to solubilize phosphate and produce ammonia. Our results have suggested that PGPR are able to enhance the production of IAA, solubilization of phosphorus and ammonia production, thereby improving growth of cotton plant. HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens[24]. In the present work, five PGPR isolates were positive for HCN production, which acts as an inducer of plant resistance. Blumer and Hass[25] [13] reported that IAA production promotes plant growth and HCN production has been proposed as a defense regulator against phytopathogens. Siderophore is one of the biocontrol mechanisms belonging to PGPR groups under iron limiting condition. PGPR produces 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[26]. The bacterial strains that produce different hydrolytic enzymes were effected antagonistically to soil fungi [27] [13]. Production of fungal cell wall degrading enzymes was analysed because this is an important mechanism of fungal inhibition[28]. Production of hydrolytic enzymes involved in pectin degradation, a component of plant cell wall, and in cellulose degradation, a component of not just plant cell wall but also of some fungi from Oomycota, was observed in 6 antifungal isolates. Proteolytic enzyme production was detected as formation of a clear zone around cells on skim milk agar medium for all the 23 selected strains. Catalase activity was detected in all the bacterial strains that may be potentially very advantageous. Amylase and gelatinase activity were observed in all the selected isolates of rhizobacteria. Similar to our findings of multiple PGP activities among PGPR have been reported by some other workers [29 30 31].

Table 1

Inhibition percentage of Macrophomina phaseolina, , Colletotrichum gossypi, Rhizoctonia solani, Fusarium oxysporum by PGPR isolates in Dual culture method

S.No	Isolate	Antifungal activity zone of inhibition (%)			
		Macrophomina phaseolina	*Colletotrichum gossypi*	*Rhizoctonia solani*	*Fusarium oxysporum* f. sp. *vasinfectum*
1.	OUR1	20	40	18	52
2.	OUR3	25	60	22	50
3.	OUR11	28	45	15	54
4.	**OUR12**	32	65	25	57
5.	**OUR13**	40	71	35	57
6.	OUR17	55	63	54	66
7.	**OUR27**	**58**	57	42	57
8.	OUR32	46	46	28	55
9.	OUR36	56	80	24	64
10	OUR39	54	77	40	59
11	OUR40	40	36	36	64
12	OUN2	32	45	23	52
13	**OUN3**	54	**77**	45	54
14	**OUN8**	52	68	**66**	59
15	OUN14	28	45	18	47
16	OUN23	26	35	30	57
17	OUN25	38	65	24	53
18	**OUN26**	52	65	48	**73**
19	OUN29	42	44	23	57
20	OUN30	38	45	40	52
21	OUN31	36	42	28	59
22	OUN32	46	45	36	66
23	OUN35	45	74	42	59

Table 2
Production of NH_3, IAA and HCN by PGPR isolated from cotton (Bt and non Bt) rhizosphere

S.No	Isolate	PGP Traits			
		P-solubilization	NH_3 production	IAA production µg/ml	HCN production
1.	OUR1	+	+	42	-
2.	OUR3	++	++	70	+
3.	OUR11	+	+	40	-
4.	OUR12	++	+++	83	++
5.	OUR13	++	+++	83	++
6.	OUR17	++	++	46	++
7.	OUR27	++	++	46	-
8.	OUR32	++	++	49	+
9.	OUR36	+++	+++	8.5	-
10.	OUR39	++	+++	8.5	-
11.	OUR40	+	++	46	-
12.	OUN2	+	+	46	-
13.	OUN3	++	+++	59.5	-
14.	OUN8	++	+++	36.5	+
15.	OUN14	++	+	10	-
16.	OUN23	++	++	27	+
17.	OUN25	+	+	33	-
18.	OUN26	++	++	86	-
19.	OUN29	+	++	42	+
20.	OUN30	+	+	73	+
21.	OUN31	+	++	10	-
22.	OUN32	+	+++	53	-
23.	OUN35	+	++	66	-

- = No production; + = Weak production;++ = medium production; +++ = high production

Table 3

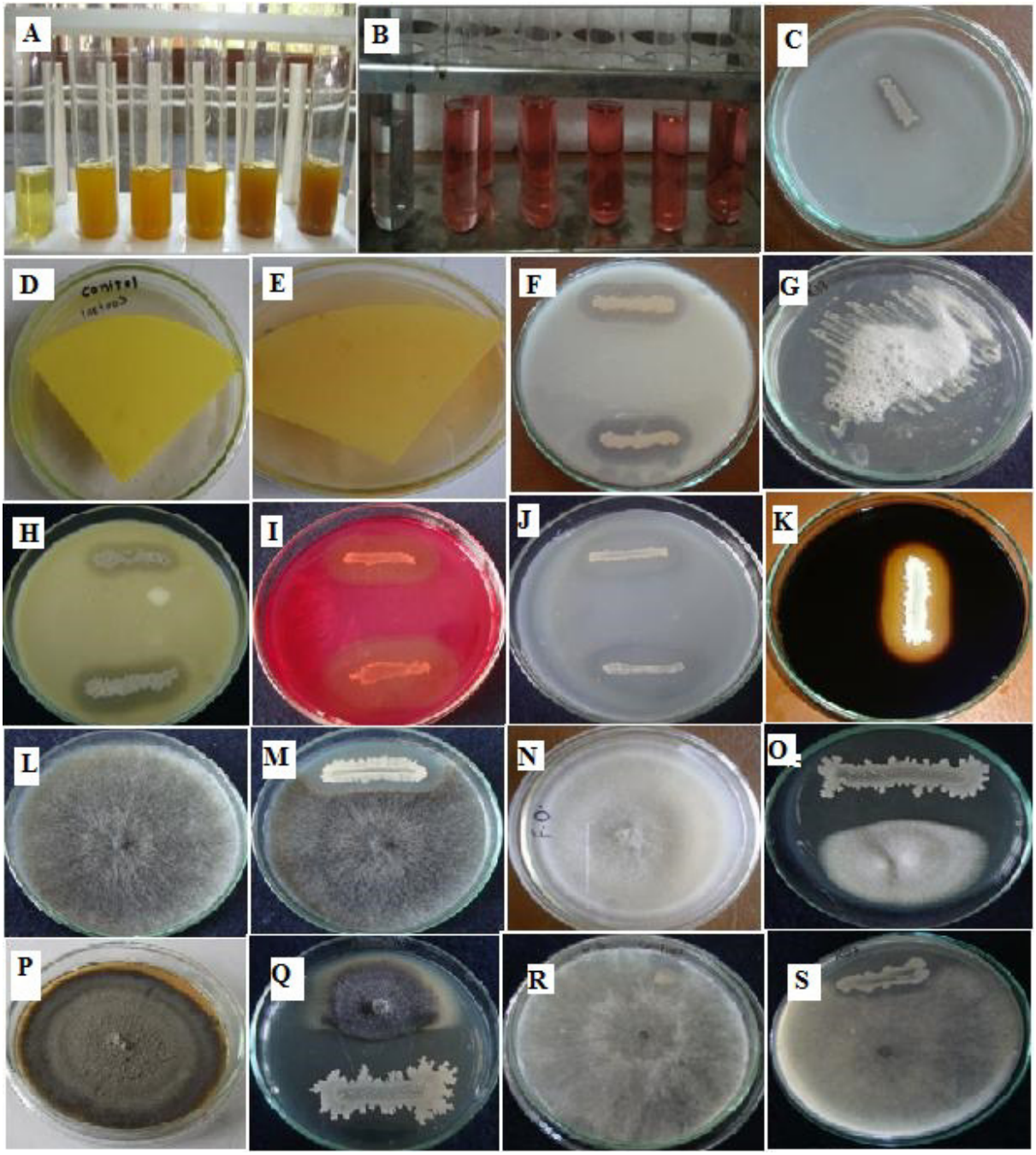
Extracellular enzyme activity of selected rhizobacterial isolates from cotton rhizosphere

S.No	Isolate	Cellulase	Pectinase	Catalase activity	Protease activity	Lecithinase	Lipase activity	Amylase activity	Gelatinase activity
1.	OUR1	-	-	+	++	-	-	+	++
2.	OUR3	-	-	+	++	-	+	+	++
3.	OUR11	-	-	+	++	-	-	+	++
4.	**OUR12**	+	++	+++	++	-	++	+++	++
5.	**OUR13**	+++	+++	++	++	+++	++	+++	++
6.	OUR17	++	+	++	++	++	+	+++	++
7.	**OUR27**	+++	+++	++	++	+++	+	+++	++
8.	OUR32	-	-	+	++	-	-	++	++
9.	OUR36	-	-	++	++	++	++	++	++
10.	OUR39	-	-	+	++	++	++	++	++
11.	OUR40	-	-	+	++	-	-	+	++
12.	OUN2	-	-	+	++	-	-	+	++
13.	**OUN3**	++	++	+	++	++	+	++	++
14.	**OUN8**	+++	+++	+	++	+++	+	++	++
15.	OUN14	-	-	+	++	-	-	+	++
16.	OUN23	-	-	+	++	-	-	++	++
17.	OUN25	-	-	+	++	++	++	++	++
18.	**OUN26**	+++	+++	+	++	+++	+	++	++
19.	OUN29	-	-	+	++	-	-	++	++
20.	OUN30	-	-	+	++	-	-	+	++
21.	OUN31	-	-	+	++	-	-	+	++
22.	OUN32	-	-	+	++	-	+	+	++
23.	OUN35	-	-	+	++	-	+	+	++

- = No production; + = Weak production; ++ = medium production; +++ = high production

Figure 1

A. Ammonia Production; B. IAA Production; C. Phosphate solubilization; D, E. HCN production; F. Protease activity; G. Catalase activity; H. Lipase activity; I Cellulase activity; J. Pectinase activity; K. Amylase activity;. Antifungal activity L. Macrophomina Phaseolena control, M. M. p./OUR27; N. Fusarium oxysporum control, O. F.o./OUN26; P. Colletotrichum gossipi control, Q. C.g./OUN3; R. Rhizoctonia solani control , S. R.s./OUN8.



CONCLUSION

In our investigation, 23 PGPR isolates were screened on the basis of their antagonistic activity against four test pathogens. The selected set of isolates was further tested for specific PGP traits and extracellular enzyme production *in vitro*. Six potential bacterial isolates were selected for further study which performed multiple PGP characteristics by producing hydrolytic enzymes. Based on such type of study is necessary as it advocates that use of PGPR as inoculants or bio fertilizers is an efficient approach to replace chemical fertilizers. This study illustrates the significance of screening of rhizobacteria under *in vitro* conditions for multiple PGPR traits. This can lead to the selection of effective PGPR isolates which as a result of their multiple PGPR traits can prove to be effective in improving the productivity of cotton crop and maintenance of soil fertility.

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