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Research Article

Isolation and Characterization of Plant Growth Promoting Antagonistic Bacteria from Cotton and Sugarcane Plants for Suppression of *Phytopathogenic Fusarium* Species

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Abstract

Background: Plant Growth Promoting Rhizobacteria (PGPR) may be utilized to augment plant growth and suppress the plant pathogens. Objective: The present study was conducted to isolate and characterize the antagonistic bacteria indigenous to cotton and sugarcane rhizosphere in Pakistan, and to evaluate their ability to suppress phytopathogenic *Fusarium* spp. Out of 63 isolates 37 different morphotypes were studied for their antagonistic activity against *Fusarium monoliformae, Fusarium oxysporum* and *Fusarium solani.* Among these 31 strains showed the percentage suppression ranging from 40 to 66% against *Fusarium* spp.

Objectives: The antagonistic bacteria having antifungal activity were studied for different morphological and physiological characteristics using Gram staining and light microscopy. Most of them were Gram negative and tentatively identified as *Pseudomonas* spp. The selected strains were screened *in vitro* for plant growth regulation and antifungal traits.

Material and Methods: Our study included 1000 premature CAD patients that classified into two groups with history of MI (n = 461) and without of MI (n = 539). The polymorphism variants in 10% of samples were determined by PCR-RFLP technique and genotyping of the polymorphism in all subjects was conducted by High Resolution Melting method. Given the two conditions of patients residing in Tehran and also faced with their first episode of MI, 640 out of 1000 study samples that had been previously followed-up were assessed in a retrospective cohort phase regarding long-term major adverse cardiac events (MACE).

Results: Four bacterial strains were able to produce the chitinase enzyme while four other bacterial strains showed protease production. Ten strains were positive for HCN production. Out of 37, eight strains showed phosphate solubilization ranging from 13 to 24 µg/ml. eighteen strains produced indole acetic acid ranging from 5 to 19 µg/ml.

Conclusions: This study identified specific traits in the isolated rhizobacteria which make them good candidates as PGPR and might contribute to enhance growth of crop plants. This information is of general interest and also helpful for devising strategies to manage diseases caused by *Fusarium* in cotton and sugarcane.

Keywords: Fusarium monoliformae; Fusarium solani; Fusarium.oxysporum; *Pseudomonas* spp; biocontrol

1. Background

Cotton and sugarcane are the most important cash crops in Pakistan. These crops are prone to various diseases by fungal pathogens as *Rhizoctonia solani, Colletotrichum, gossypii, Fusarium oxysporum, Fusarium moniliforme, Fusarium solani* and *Verticillium dahlia* (1). The use of fungicides to secure these important crops are most commonly practiced. However, their application is not only costly but also harmful to the environment. So, one of the best ecofriendly practice is to use the Plant Growth Promoting Bacteria (PGPR) in order to combat disease and improve plant growth. Of these biocontrol agents *Pseudomonas spp.* is considered to be the best bacterial agent in controlling fungal disease, in turns improving plant growth (2, 3).

PGPR are generally the free living bacteria which promotes plant growth and health either by direct or

indirect ways (4). There has been a considerable research from 1980's for the potential use of the antagonistic bacteria for plant growth promotion (5, 6). The plant growth promotion is carried out directly by the use of phytohormones and nitrogen fixation. Growth can also be enhanced indirectly by preventing the phytopathogens (7, 8). These Plant Growth Promoting Rhizobacteria (PGPR) influences the plant growth by the production of important plant growth regulators. Of these, Indole Acetic Acid (IAA) is an established plant growth regulating compound (9). They induce the growth regulation through different phytohormones production (9, 10) and the capability of (11, 12) inorganic phosphate and other phosphate (organic) mineralization (9, 13). They also suppress the disease by the synthesis of siderophores, fungicidal compounds and antibiotic enzymes (14). Of all the other bacterial isolates *pseudomonas spp*. are found to be more effective in enhancing the plant growth and grain yield of the treated crops (15-17). These rhizobacteria (PGPR) are very helpful for crops as they have the ability to persistently colonize the roots especially during the growing season (18). Hence, it is necessary to have the knowledge of the native population of bacteria, their identification and characterization is also required for understanding the diversity and allocation of the rhizospheric bacteria of specific crops (19).

Keeping in view the importance of antagonistic bacteria, the current study was focused on the isolation and characterization of potent bacteria against three different *Fusarium* spp. (*F. oxosporum, F. monoliforum and F. solani*). These antagonistic bacteria may be used as biocontrol agents. It is also an attractive alternate to the chemical fertilizers which are the source of environmental pollution and have hazardous compounds that are mostly non degradable and harmful to human health (20).

2. Objective

The objective of the present study is to check the inhibitory effect of the antagonistic bacteria against the fungal pathogens. The isolation of the most potent antagonistic bacteria that showed various positive characters (PGPR and biocontrol traits) beneficial for the growth of the plants will also be performed. Resultantly, the potent strains can be used for the disease protection. *In vitro* studies will be performed in order to check the disease suppression against the fungal pathogens.

3. Materials and Methods

3.1. Sample Collection

The samples of cotton and sugarcane plants were collected from the experimental farm of Cotton Research Institute (CRI), Multan and Ayub Agriculture Research Institute (AARI), Faisalabad and Jhang areas.

3.2. Isolation and Light Microscopic Studies of Rhizobacteria

These samples were used for isolation of *Pseudomonas* spp. on specific media *i.e.* S1 and King's B media (21, 22). One gram of rhizospheric soil/ sterilized roots was homogenized in 20 mL test tube containing 9 mL saline (0.85% NaCl) separately. The suspension was vortexed and dilutions were prepared up to 10^{-7}. Each dilution (0.1 mL) was spread on plates containing King's B medium incubated at 30±2 °C for 48 h.

The bacterial isolates were studied for colony/cell morphology and yellow pigments production in King's B medium. Pure cultures of pseudomonad's were obtained following successive selection and Gram stain reaction (23). Bacterial strains were stored on LB agar slants at 4°C for short term preservation and in 20% glycerol at -80°C for long term preservation.

3.3. Biochemical Characterization of Rhizobacteria using QTS Kit:

Different biochemical and physiological tests were carried out using QTS – 24 Kit (Desto laboratories, Karachi Pakistan). Single colony of 18 hours old bacterial culture grown on LB plate was suspended in 6 ml sterile saline solution. Liquid paraffin was added to the cups of ADH and H_2S for creating anaerobiosis. The box was covered with the supplied plastic lid and incubated at 37 °C for 18 – 24 hours.

3.4. Fungal Cultures and Growth Conditions

F. oxysporum, F. moniliforme, and *F. solani* obtained from 1st Fungal Culture Bank of Pakistan, Department of Mycology and Plant Pathology, University of Punjab, Pakistan. Potato dextrose agar (PDA) was used for culturing of fungal pathogens.

3.5. In vitro Screening for Antagonism

In vitro inhibition of mycelium growth of *Fusarium* spp. by the bacterial isolates was tested using dual culture assay as described by (24, 25). The percentage growth inhibition was calculated using the following formula (26):

% inhibition = [(R-r/R) ×100]

Where, r is the radius of the fungal colony opposite to bacterial colony and, R is the maximum radius of the fungal colony away from the bacterial colony. All isolates which resulted in more than 30% mycelial growth inhibition against the selected pathogen were stored in nutrient broth supplemented with 15% glycerol at -70°C.

3.6. Detection of Antifungal Metabolites

3.6.1. Protease Production

Protease production was detected as described by Denizci *et al.* (27) on skim milk agar medium added with 0.1% glucose, 0.2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, and 0.5% skim milk. Sterilized Na_2CO_3 (10%) was used to set the pH of the

medium at 10. Inoculated plates were kept at 30±2 °C and observed for zone formation around the bacterial colony.

3.6.2. Chitinase Production

Crab shells were used for the preparation of colloidal chitin according to the modified method described by Mehmood, et al.(28).Production of chitinases by bacterial strains was detected on chitin agar plates (0.5% colloidal chitin, 0.5% Yeast extract, 1% Trypton, 0.5% NaCl) (29). Single colony of bacterial strains was inoculated**.** The Plates were kept in incubator at 30±2 °C. After five days of incubation plates were observed for formation of hollow zone.

3.6.3. Hydrogen Cyanide (HCN)

HCN production by the bacterial strains was detected by growing the antagonistic bacteria on King's B agar medium as described by Shipper et, al. (30). A colour change of filter paper from yellow to brown indicated the cynogenic activity of the bacteria used. The intensity of colour was recorded visually (31).

3.7. Detection of Plant Growth Promoting Traits

3.7.1. Phosphate (P) Solubilization

Bacterial cultures were spot inoculated on the Pikovskaya's agar plate contained tricalcium phosphate as insoluble phosphate source (32). These plates were incubated for seven days at 28±2 °C and observed for the formation of halo zones around the colonies. The phosphate solubilization ability was quantified by phosphomolybdate blue colour method using the spectrophotometer (λ = 882) (33). The experiment was repeated three times and the mean value was calculated.

3.7.2. Production of Indole 3-Acetic Acid (IAA)

The cultures were grown in Okon's malate medium (34) added with tryptophan (100 mg/ L) as the precursor of indole-3-acetic acid. The cultures were incubated in a shaker at 160 x g at 30±2 °C for a week. The production of indole-3-acetic acid by the bacterial isolates was qualitatively determined by using Fe-$HClO_4$ and Fe-H_2SO_4 reagents. For quantitative estimation, bacterial cells were harvested. The supernatant obtained by centrifugation at 8000 rpm for 8 minutes at 10 °C. The supernatant was reduced in volume from 70 to 15 mL using freeze dryer (Martin Christ, Alpha 1-4, Germany). The pH of the sample was adjusted to 2.8. Indole acetic acid was extracted by Tien *et al.* (35) method. Equal volume of ethyl acetate was added to cell free liquid culture medium (supernatant) and mixed in a separating funnel. Ethyl acetate fraction was then evaporated to dryness at room temperature in fume hood. The residues of each fraction were dissolved in 1 mL of ethanol. The samples were analyzed on HPLC using Turbochom software (Perkin Elmer, USA). The elution was performed by using licosorb-C18 column for IAA. Ethanol: acetic acid: water (30:1:70) was used as mobile phase at the rate of 1 mL/ minute for 30 minutes. IAA absorbance was detected on a UV detector at 280 nm wavelength. The concentration of IAA was calculated on the basis of peak height and peak area.

4. Results

4.1. Isolation and Light Microscopic Studies of Rhizobacteri

A total of sixty three bacterial strains were isolated on the basis of colony morphology from rhizosphere, roots, soil and tubers of diseased and healthy cotton and sugarcane plants collected from Multan and Faisalabad District, Pakistan. The bacterial colonies were distinguished on the basis of morphology e.g. round, convex with smooth and wavy margins of different colors. Their size was in range of 1-5 mm in diameter. The bacteria were identified on the basis of colony morphology, cell morphology and Gram staining. Most of them were Gram negative bacteria and were identified as the *Pseudomonas* spp. Out of 37 strains three strains were Gram positive and 34 strains were Gram negative. All bacterial strains except three strains were able to grown on S1 medium. All the tested 37 antagonistic strains were able to grow on King's B medium. Colonies of *Pseudomonas* spp. produced yellow pigments on King's B medium. Most of the strains produced the pigmentation on all three media's i.e. LB, S1 as well as in King's B media.

4.2. Biochemical Characterization of Rhizobacteria

Morphological and physiological characteristics of 37 bacterial strains from rhizosphere are given in **Table 1**. Following morphological characterization, motility and gram staining, the isolates were compared with those of standard species using Bergey's Manual of Determinative Bacteriology (23). All of the bacterial isolates were motile and most of them were Gram negative.

4.3. QTS-24 Kit Test

The three best strains (MRh42, MRp2 and ME1) were tested for QTS testing. The results showed that strains contains most of the biochemical metabolites that are very beneficial in plant growth promotion and enhancing its activity against fungal metabolites. The results are presented in the **Table 2**:

4.4. In vitro Screening for Antagonism

These bacterial strains were subjected to antagonistic test against three different *Fusarium spp*. 16 bacterial strains were found to be positive against *F.oxysporum* and 18 bacterial strains showed antagonistic activity against *F. monoliformae.* While for *F.solani* 7 bacterial strains were able to show best suppression (55.5%).

Table 1. Origin, Strains,Colony morphology (Kings B, S1), Gram staining, and tentative identification of the bacterial isolates. Rh* indicates rhizosphere Rp* Rhizoplane, Es stands for Endosphere.

	Host	Origin	Strains	Colony morphology		Gram staining	Tentative identification
				S1			
				Kings B			
1.	Cotton	Rh*	MRh1	1. Light yellow, shiny.	1. Yellow, not shiny.	1 -	1.*Pseudomonas*
2	Cotton	Rh	MRh4	2.Darkyellow,shiny,scattered	2. Off-white shiny smooth.	2 –	2 *Pseudomonas*
3	Cotton	Rh	MRh6	3. Off white, shiny smooth.	3. Off-white smooth shiny.	3. -	3.*Pseudomonas*
4.	Cotton	Rh	MRh7	4. Off white scattered rough.	4. Off-white not shiny but rose.	4.+	4.*Bacillus*
5.	Cotton	Rh	MRh11	5. Off white smooth margins.	5. Off-white smooth rose.	5 +	5.*Bacillus*
6.	Cotton	Rh	MRh17	6. Gummy off-white margins.	6. Scattered margins shiny appearance.	6.-	6.*Pseudomonas*
7	Cotton	Rh	MRh19	7. Off-white smooth margins.	7. Off-white shiny smooth margins, raised.	7.-	7.*Pseudomonas*
8.	Cotton	Rh	MRh20	8. Off-white smooth margins.	8. Yellow smooth margins, raised, shiny.	8.-	8.*Pseudomonas*
9.	Cotton	Rh	MRh21	9. White not shiny not raised.	9. Off-white in color not smooth not shiny.	9.-	9.*Pseudomonas*
10	Cotton	Rh	MRh22	10. Off white shiny scattered margins not raised.	10. Smooth shiny off-white in color raised smooth ends.	10.-	10.*Pseudomonas*
11.	Cotton	Rh	MRh23	11. Yellow scattered, not raised.	11. Dull, off-white in color not smooth not shiny.	11.-	11. *Pseudomonas*
12.	Cotton	Rh	MRh24	12. Off-white not raised, scattered.	12. Off-white in color raised smooth ends.	12.-	12. *Pseudomonas*
13.	Sugarcane	Rh	MRh25	13. Light yellow scattered not raised.	13. Light green in color rough appearance gives color in media.	13. -	13. *Pseudomonas*
14.	Sugarcane	Rh	MRh26	14. Off-white shiny scattered margins not raised.	14. Off-white not shiny not rose.	14. -	14. *Pseudomonas*
15.	Sugarcane	Rh	MRh27	15. Dark yellow light shiny scattered ends.	15. Bright green in color not shiny rough appearance.	15. -	15. *Pseudomonas*
16.	Sugarcane	Rh	MRh28	16. Gummy off-white smooth, shiny.	16. Off-white in color smooth, shiny smooth edges.	16.+	16. *Bacillus*
17.	Sugarcane	Rh	MRh29	17. Off-white in color not raised not shiny.	17. Light yellow in color, smooth shiny raised.	17. -	17. *Pseudomonas*
18.	Sugarcane	Rh	MRh30	18. Light yellow not shiny.	18. Off-white not smooth but shiny rough appearance.	18. -	18. *Pseudomonas*
19.	Sugarcane	Rh	MRh31	19. Off-white smooth margins.	19. Off-white, raised, rough ends.	19. -	19. *Pseudomonas*
20.	Sugarcane	Rh	MRh32	20. Dark yellow, smooth.	20. Dark yellow not raised rough appearance.	20.-	20. *Pseudomonas*
21	Sugarcane	Rh	MRh33	21. Off-white not shiny.	21. Light yellow, not raised, not shiny.	21. -	21. *Pseudomonas*
22	Cotton	Rh	MRh34	22. Off-white not shiny.	22. Off-white smooth shiny appearance.	22. -	22. *Pseudomonas*
23	Cotton	Rh	MRh36	23. Off-white shiny raised.	23. Off-white smooth shiny, raised.	23. -	23. *Pseudomonas*
24	Cotton	Rh	MRh37	24. Yellow not raised rough.	24. Off-white not raised.	24. -	24. *Pseudomonas*
25	Cotton	Rh	MRh38	25. Off-white not raised.	25. Off-white, not shiny.	25. -	25. *Pseudomonas*
26	Cotton	Rh	MRh42	26.Not grown on S1	26. Light yellow, raised, smooth margins.	26.-	26. *Pseudomonas*
27	Cotton	Rh	MRh44	27. Not grown on S1media.	27. Light yellow, raised smooth margins.	27. -	27. *Pseudomonas*
28	Cotton	Rh	MRh45	28. Off-white not raised	28. Off-white, smooth shiny smooth ends.	28. -	28. *Pseudomonas*
29	Cotton	Rh	MRh46	29. Not grown on S1 media.	29. Off-white, smooth shiny.	29. ND	29. ND
30	Cotton	Rp	MRp1	30.Off white in color	30. Light green, not shiny not smooth.	30. -	30. *Pseudomonas*
31	Cotton	Rp	MRp2	31. Dark yellow smooth shiny.	31. Light green, not raised, not shiny.	31. -	31. *Pseudomonas*
32	Cotton	Rp	MRp4	32. Off-white shiny smooth.	32. Off-white, shiny smooth margins.	32. -	32. *Pseudomonas*
33	Cotton	Rp	MRp7	33. Off-white smooth colonies.	33. Off-white smooth margins.	33. -	33. *Pseudomonas*
34	Cotton	Rp	MRp8	34. Not raised not shiny.	34. Not raised, not shiny, rough ends.	34. -	34. *Pseudomonas*
35	Cotton	Es	ME1	35. Off-white not smooth.	35. Off-white in color, smooth and shiny.	35. -	35. *Pseudomonas*
36	Cotton	Es	ME2	36. Off-white, smooth, shiny.	36. Off-white in color, not smooth, not shiny.	36. –	36. *Pseudomonas*
37	Cotton	Es	ME4	37. Dark yellow smooth shiny.	37. Light yellow in color, smooth shiny, smooth margins.	37.-	37. *Pseudomonas*

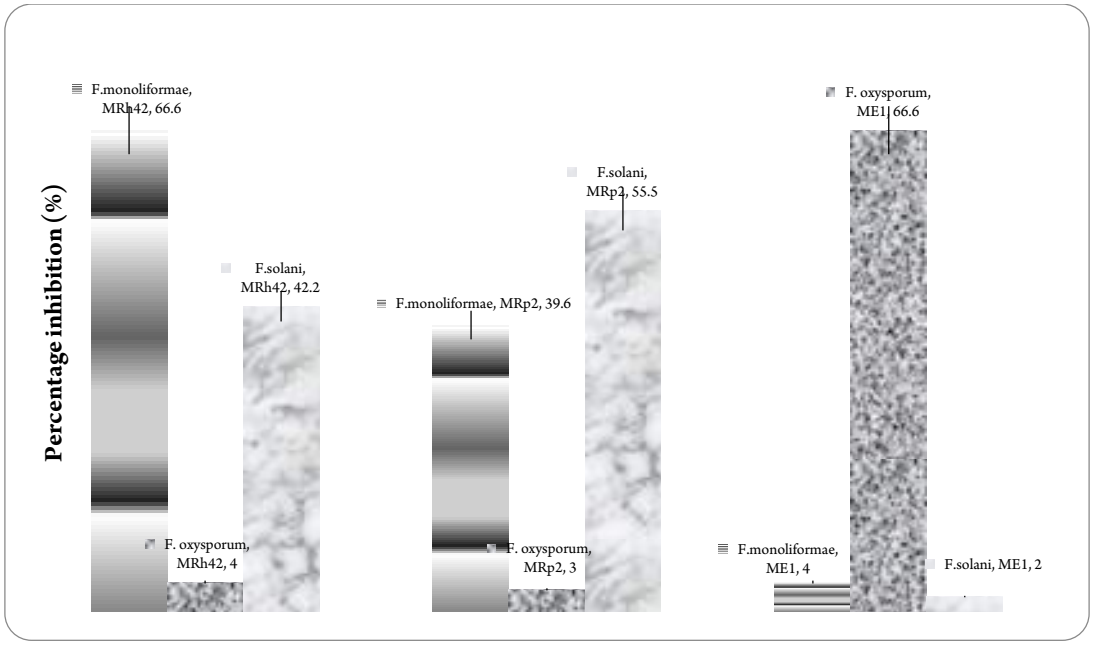

Figure 1. Antagonistic activity of *Pseudomonas* spp. (MRh42, MRp2 and ME1) against *F. monoliformae*, *F. solani* and *F. oxysporum*

MRh 42 is the rhizospheric *Pseudomonas spp*. bacteria that showed suppression against *F.monoliformae* (66.6%). While the activity of MRp2 (39.6%) (Derived from rhizoplane) and ME1 (4%) (Endophytic bacteria) is not as effective and have the activity levels almost half and 4% only. The MRp2 showed the greatest inhibition against *F.solani* (55.5%) while rhizospheric (MRh42) has ten percent less (42.2%) and endophytic strain (ME1) and almost none (2%) activity respectively. ME1 is that endophytic bacteria of *Pseudomonas* spp. which has the highest activity against *F. oxysporum* (66.6) while for that fungus the MRh42 (4%) and MRp2 (3%) almost have no activity. Most of the other strains were antagonistic against two *Fusarium* spp, and none of the single strain is potent against all three strains at a time.

4.5. Detection of Biocontrol Traits

Out of about 37 antagonistic strains 4 bacterial strains MRh11, MRh21, MRh22 and MRh24 were able to produce the chitinase enzyme in the solid or liquid medium that contained chitin as a sole carbon source. It has been reported that chitinase can function in defense against many fungal pathogens and also correlated with induced resistance (36).

Six strains *i.e.* MRh1, MRh6, MRh20, MRh22, MRh42 and MRp1 showed the activity of proteases indicating that these enzymes could be involved in antagonism against the *Fusarium* pathogen. Additionally, some of the strains were HCN producers i.e. MRp1, MRp4, MRp6, MRp19, MRp1, MRh 20, MRh25, MRh30, and MRh33.

4.6. Detection of Plant Growth Promoting Traits

Pink color in calorimetric method (qualitative estimation) indicated IAA production by eighteen antagonistic strains. IAA was quantified by HPLC method, the amount of IAA ranged from 5 to19 ug/ml. Out of isolated strains eight bacterial strains i.e. MRh1, MRh4, MRh6, MRh17, MRh31, MRh37, ME1 and ME4 were able to solubilize the phosphate as indicated by halo zone formation of Pikovskaya's agar medium. The amount of the phosphate was determined by spectrophotometer. The amount of phosphate solubilized by selected bacterial strains ranged from 11 to 30 ug/ml (**Table 2**).

Identification of Indole acetic acid was performed qualitatively by spot test and quantified by HPLC. [1] Hydrogen cyanide (HCN) production was detected by plate assay, - represents no production, ++ represents HCN production, +++ represents complete HCN production in plate 2.

Chitinase assay was performed by using chitin as a sole carbon source. 3 Phosphate solubilization: grown on Pikovskaia agar, Bacterial cultures were spot inoculated on the Pikovskaya's agar plate contained tricalcium phosphate as insoluble phosphate source (32). P solubilization was quantified using spectrophotometer [4]. Protease assay was performed and six strains were found positive. All the observations were recorded by repeating experiment thrice with three replicates each time, Mean± standard deviation of each reading was given in **Table 3**.

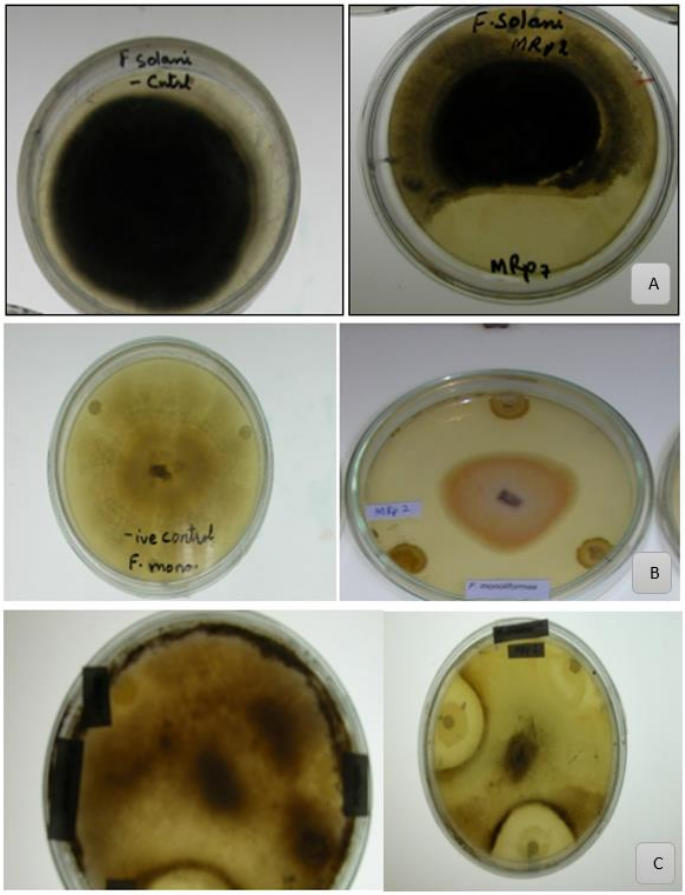

Figure 2. (A) Antagonistic activity of bacterial strains against *Fusarium solani*
Bacterial stain MRp2 showed inhibition zone against the growth of *Fusarium solani*, this strain showed maximum suppression with respect to *F. solani* control.
(B) Antagonistic activity of bacterial strains against *Fusarium monoliformae*
Bacterial stain MRh42 showed inhibition zone against the growth of *Fusarium monoliformae*, this strain had maximum suppression with respect to *F. monoliformae* control.
(C) Antagonistic activity of bacterial strains against *Fusarium oxysporum*
Bacterial stain ME1 showed inhibition zone against the growth of *Fusarium oxysporum*, this strain showed maximum suppression with respect to *F. oxysporum* control.

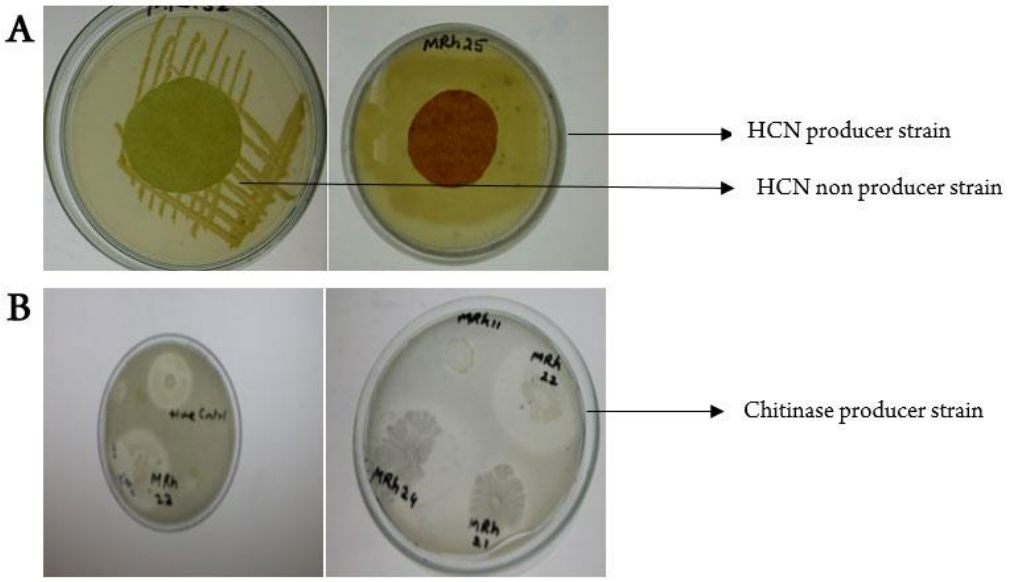

Figure 3. Plate assay for the detection of (A) HCN and (B) chitinase production.
Bacterial stain MRh25 showed HCN production Yellow colour shows no HCN production while orange colour shows HCN production. While MRh22, MRh21 and MRh24 strains were positive chitinase producer as indicated by colour disappearance.

Table 2. Characterization of antagonistic bacteria for growth promotion and biocontrol determinants.

Sr. No.	Isolates of *Pseudomonas* spp.	PGPR traits		Biocontrol traits		
		IAA[1] (ug/ml)	Psolubilisation[4] (µg/ml)	HCN[2]	Chitinase[3]	Protease
1	MRh1	4.6±0.4	11	_	_	+++
2	MRh4	5.0 ± 0.2	18	++	_	_
3	MRh6	-	29	+++	_	+++
4	MRh7	-	14	_	_	_
5	MRh11	15 ±0.3	_	_	++	_
6	MRh17	19±0.2	13	_	_	_
7	MRh19	-	_	++	_	_
8	MRh20	-	_	+++	_	+++
9	MRh21	-	_	_	++	_
10	MRh22	14 ± 0.3	_	_	+++	+++
11	MRh23	15 ± 0.3	_	_	_	_
12	MRh24	_	_	_	+++	_
13	MRh25	16 ± 0.2	_	++	_	_
14	MRh26	_	_	_	_	_
15	MRh27	_	_	_	_	_
16	MRh28	_	_	_	_	_
17	MRh29	_	_	_	_	_
18	MRh30	18 ± 0.2	_	+++	_	_
19	MRh31	-	30	+++	_	_
20	MRh32	_	_	_	_	_
21	MRh33	19± 0.2	_	++	_	_
22	MRh36	17± 0.2	24	++	_	_
23	MRh38	_	_	++	_	_
24	MRh42	5.2±0.8	_	_	_	+++
25	MRh44	_	_	_	_	_
26	MRh45	_	_	_	_	_
27	MRh46	_	_	_	_	_
28	MRp1	4.6±0.4	_	++	_	+++
29	MRp2	18 ± 0.2	_	++	_	_
30	MRp3	_	_	_	_	_
31	MRp4	_	_	_	_	_
32	MRp7	_	_	_	_	_
33	MRp8	_	_	_	_	_
34	ME1	_	16	_	_	_
35	ME2	_	_	_	_	_
36	ME4	_	13	_	_	_

Table 3. Physiological and biochemical tests using QTS-24 kit for characterization of potent bacteria isolated from rhizospheric soil of cotton and sugarcane plants.

Biochemical elements	Bacterial isolates		
	MRh42	MRp2	ME1
ONPG	+	+	+
CIT	+	+	+
MALO	-	+	-
LDC	-	-	-
ADH	-	+	-
H_2S	-	+	+
URE	+	+	+
MAL	+	+	+
MAN	+	+	+
ARA	+	+	+
RHA	+	+	+
SOR	+	+	+
INO	+	+	+
ADON	+	+	+
MEL	+	+	+
RAF	+	+	+

CIT: Sodium Citrate, MALO: Sodium Malonate, LDC: Lysine decarboxylase, ADH: Arginine dihydrolase, ODC: Ornithine decarboxylase, H_2S: H_2S production, URE: Urea hydrolysis, MAL: maltose, MAN: mannitol, ARA: arabinose, RHA: rhamnose, SOR: sorbitol, INO: inositol, ADON: adonitol, MEL: melibiose, RAF: raffinose.

5. Discussion

The isolation and characterization of the antagonistic bacterial strains were carried out against three lethal fungal species of *Fusarium* (*F.monoliformae, F.oxysporum,and F.solani*). The focus of the present study was to inhibit the effect of the disease causing fungus by identifying the most potent bioantagonistic bacteria so that they can be used in future as biocontrol agents. Biological control by antagonistic bacteria is one of the indirect mechanisms of growth promotion that are responsible for the suppression of disease by reducing the time in which a plant is in the susceptible state. Therefore, by this way the incidence of diseases in cotton and sugar cane plants can be reduced. Rhizosphere and endorhizosphere are considered to be the main areas of the antagonistic bacteria (37). So, the isolation of *Pseudomonas spp*. bacteria was carried out from all the three main areas of plant roots (rhizosphere (MRh), rhizoplane (MRp) and endophytes (ME).

After isolation these all strains out of 67 strains were isolated from cotton and sugarcane plants, 3 strains were found to efficiently suppress the growth of *Fusarium* spp (F.*monoliformae* (66%), *F.oxysporum* (66.6%), *and F.solani* (55.5%). They were also tested for their colony / cell morphology and Gram staining. Most of them were Gram negative and tentatively identified as *Pseudomonas* spp. MRh 42 is the rhizospheric bacterium that has the highest activity against *F.oxysporum* (66.6%). The MRp2 showed the greatest inhibition

against *F.solani* (55.5%). ME1 is that endophytic bacteria of *Pseudomonas* spp. which has the highest activity against *F.monoliformae* (66.6%). Most of the other strains were antagonistic against two *Fusarium spp*, and none of the single strain is potent against all three strains at a time. MRh42 and ME1 are thus the two strains which are most effective against *F.oxysporum* and *F.solani* with greatest percentage of inhibition (66.6%). Therefore, both the rhizospheric and endophytic bacteria of *Pseudomonas spp.* in this study is found to be useful in inhibiting the growth of two species of *Fusarium*. Thus they can be helpful in reducing the disease risk if further applied in pot and field study. These results are inagrement with previous studies in which the *Pseudomonas spp*. are combating and defending the fungus infection in cash crops (1-3).

These three bioactive strains along with others were also checked against various PGPR (IAA and P-solubilisation) and biocontrol traits (HCN, Chitinase, and Protease). Moreover, these hormones and enzymes are also used in wide range of biotechnological applications, especially in agriculture for biocontrol of phytopathogenic fungi and harmful insects (38). MRh 42 is an active IAA and protease producer. The MRp2 strain is an active IAA and HCN producer. ME1 strain in addition to its activity against *F.solani* is an active P-solubilizer (**Table 2**). Thus these three strains are not only active in suppression of fungal pathogens but they are also the active PGPR and biocontrol trait producer. Thus the combination of these properties along with bioantagonistic activity make them more potent in combating the fungal growth and is thus a good alternate to pesticides (39-41)

Of the remaining strains, four bacterial strains MRh11, MRh21, MRh22 and MRh24 were able to produce the chitinase enzyme and four other bacterial strains MRh1 and MRh6, MRh20, MRh22 were positive for protease. Ten strains were found to be an active HCN producer. As it has already been reported that under specific environmental conditions and in certain plant species, some strains of rhizospheric *Pseudomonas* spp. and some of their metabolites such as HCN may help to enhance plant defense against pathogen and hence they can inhibit development of plant diseases (42).

The phosphate solubilization and indole acetic acid production tests showed 18 strains were positive for IAA (5 to 19 µg/ml) and eight strains had the ability to solubilize the inorganic phosphate ranging from 13 to 24 µg/ml. The phosphate solubilization and IAA production alleviate plant growth and indirectly limiting the pathogenic effects on plants (43). Therefore, the present study concludes that isolated antagonistic strains can be used as efficient candidates for biofertilizer production as well as for suppression of fungal pathogens. Antagonistic bacteria may be considered as biological control agents for several obvious reasons, like rapid growth, easy handling and its potential against various fungal pathogens. However, *in vivo* plant assays need to be undertaken to ascertain their full potential.

6. Conclusions

The study highlights potential biocontrol and PGPR bacteria with antifungal activity. They may be a protective tool to reduce deleterious effect of phytopathogenic *Fusarium* spp. Of all the Pseudomonads spp. that were isolated from rhizosphere, rhizoplane and endphytes which were tested against three forms of *Fusarium* spp. The rhizospheric and endophytic strains (MRh42 and ME1) were most effective against *F.oxysporum* and *F.solani* with greatest percentage of inhibition (66.6%). While most of the other strains are active against three different forms of *Fusarium* spp. This information is of general interest and also helpful for devising strategies to manage diseases caused by *Fusarium* spp. in cotton and sugarcane.

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