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K Pranaya
Department of Plant Pathology, College of Agriculture, Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, Telangana, India

Dr. Bharati N Bhat
Department of Plant Pathology, College of Agriculture, Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, Telangana, India

Dr. G Uma Devi
Department of Plant Pathology, College of Agriculture, Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, Telangana, India

Dr. S Triveni
Department of Plant Pathology, College of Agriculture, Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
K Pranaya
Department of Plant Pathology, College of Agriculture, Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, Telangana, India

Colony, morphological and biochemical characteristics of cotton phyllosphere bacteria and its antagonistic activity against the *Alternaria* leaf spot of cotton

K Pranaya, Dr. Bharati N Bhat, Dr. G Uma Devi and Dr. S Triveni

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Abstract
Cotton crop is known to suffer from number of fungal, bacterial and viral diseases. Among them, *Alternaria* leaf spot caused by *Alternaria* spp. is predominant in causing economic losses to the cotton crop in the country. Phyllosphere inhabitants termed as epiphytes may consists of a variety of bacteria and filamentous fungi. They also play a key role in carbon and nitrogen cycling and help in important environmental processes such as methanol degradation and nitrification. So, bacterial phyllosphere cultures (P1 to P8) were isolated from the Bt and non Bt cotton by leaf imprint method and dilution method. The colony characters of isolates of bacteria pertaining to their shape, size, elevation, margin, texture, appearance and pigmentation were recorded. Gram's staining and endospore staining revealed that P1, P2, P3, P5, P8 were Gram positive, P1, P2, P5 were showing endospore staining and P1, P4, P6, P7 were rod shaped. Biochemical tests revealed that the eight isolates Bt and non Bt cotton were differed in each test and showed positive for the catalase and oxidase test. The isolates P1, P5 and P6 showed positive results to Voges proskauer test. Isolates P2, P4 and P6 revealed positive results of phyllosphere bacteria to Indole test. Whereas, isolates P1, P2 and P8 showed negative reaction to methyl red test. The phyllosphere bacterial isolates P3, P5 and P7 recorded negative reaction to gelatin liquefaction. Screening of phyllosphere microflora against *Alternaria macrospora* was conducted following dual culture technique for all the isolates. Phyllosphere bacterial isolate P5 recorded maximum growth inhibition with 57.41 per cent followed by P4 isolate with 53.52 per cent growth inhibition over the test fungus *Alternaria macrospora*. Whereas least per cent growth inhibition was reported in isolate P6 with 45.63 per cent.

Keywords: Morphological and biochemical, *Alternaria*, cotton

Introduction

Material and Methods

Isolation of phyllosphere microflora Dilution method

Healthy Bt and non Bt cotton plants were collected. The plants were put separately into sterile bags, then transported to laboratory for isolation of phyllosphere microorganisms. From each plant, ten discs of one cm leaf bits were cut with a sterile cork borer. The discs were transferred to sterile distilled water of 100 ml and stirred for one hr. An aliquot of one ml was plated on PDA medium and nutrient agar medium

Leaf imprint method

In order to estimate the bacterial population on adaxial and abaxial leaf surfaces, leaf imprints were made on nutrient agar medium. An intact individual leaf was placed on to nutrient agar plate and was pressed with the smooth end of a sterile glass rod until a clear imprint of the entire leaf was obtained on nutrient agar surface. The plates incubated at 24 ºC for 2-5 days until colony formation. Selection of single bacterial colonies was done based on morphological variation and after purification they were preserved in refrigerator for evaluation by dual culture (Aneja, 2003) [1].

Morphological and cultural characteristics of the bacteria: Pure cultures of bacteria were streaked on nutrient agar plates separately and incubated at room temperature until single colony developed. Individual colony was examined for shape, size, colour, Gram staining, endospore staining, elevation and texture.

Gram staining
A drop of sterile distilled water was placed in the center of glass slide. A loopful of inoculum from young culture was taken, mixed with water and placed in the center of the slide. The suspension was spread out on slide using the tip of inoculation loop to make a thin smear. The smear was dried in air and fixed through mild heating by passing the slide 3 to 4 times over the flame. The smear was then flooded with crystal violet solution for one min and washed gently with flow of tap water. Then the slide was flooded with iodine solution. After incubation at room temperature for one min, iodine solution was drained out followed by washing with 95 per cent decolorizer. After that, it was washed with water within 15 to 30 sec and blotted carefully. The smear was incubated with safranin solution for one min. The slide was washed gently in flow of tap water and air dried. The slide was examined under microscope at 100X power with oil immersion and data was recorded for different isolates.

Endospore staining
A bacterial smear was taken on a clean slide, air dried and gently heat fixed. Then the slides were flooded with malachite green, for 3-5min using the flame of burner. The slides were washed gently in flow of tap water to remove dye. After cooling the slides, safranin was drained on to the slide. The slide was washed gently in flow of tap water and air dried. The slides were observed at 100X with oil immersion and data was recorded for different isolates.

KOH test
One drop of 3 per cent KOH were applied on a microscopic slide. A loop full of bacteria (cultivated for 24-48 h) was transferred to the drop of KOH and stirred carefully. The solution of gram-negative bacteria were viscous and form a mucoid string within 30 sec (Ragavi *et al*., 2019) [6].

Biochemical characterization
Different biochemical tests *viz*., Catalase test, Oxidase test, Voges Prausker's test, Indole test, Methyl red test, Gelatin liquefaction were conducted using standard protocols (Biyyani *et al*., 2016) [3].

Catalase test
Catalase test was performed by taking a drop of 3 per cent hydrogen peroxide and added to 48 hr old bacterial colony on a clean glass slide. The effervescence indicates catalase activity.

Oxidase test
The bacterial isolates were grown in nutrient agar slants. Oxidase paper discs of Hi media were kept on fully grown cultures for 48 hr. Oxidase paper discs were kept in the slants on fully grown cultures of bacterial isolates. If the colour changes to purple it indicates positive result.

Voges Prausker's test
The test was performed by adding alpha-naphthol and potassium hydroxide to the Voges Prausker's broth. A cherry red colour indicates a positive result, while a yellow- brown colour indicates a negative result.

Indole test
Tryptophan broth tubes were inoculated with the overnight cultures of the isolates and incubated for 48 hrs at 28 ± 2ºC. Following incubation, 10 drops of Kovac's Indole reagent was added to each tube. The isolates showing production of red colour was recorded as positive for indole production.

Methyl Red test
Sterilized glucose-phosphate broth tubes were inoculated with the test culture and incubated at 28± 2 ºC for 48 hrs. After incubation five drops of methyl red indicator was added to each tube and gently shaken. Red color production was taken as negative for the test.

Gelatin liquefaction
The overnight cultures of the test isolates were inoculated to sterilized nutrient gelatin deep tubes and incubated for 24 hrs at 28 ± 2ºC. Then the tubes were kept in the refrigerator for 30 min at 4 ºC. The isolates showing liquefied gelatin was taken as positive and those which resulted in solidification of gelatin on refrigeration was recorded as negative.

Screening of phyllosphere bacteria against *Alternaria* sp. *in vitro*
Antagonism test was performed *in vitro* by dual culture method (Landa *et al*., 1997) [4] on PDA. One loop of 48 hr old culture of bacterial isolates were streaked one cm from the outer side of 9 cm PDA plates. Five mm discs of actively growing three-day old fungus was placed at the centre of plates, 2.5 cm apart from the bacteria. Plates inoculated with fungus without bacterial isolates served as control. For each isolate three replicates were maintained. These plates were incubated at 28 ± 2 ºC for 3 days. The growth of *Alternaria* sp. pathogen in the presence or absence of any bacterial isolates was measured. Observations regarding the zone of inhibition radius was recorded after 9 day of incubation and calculated as per the formula given below (Vincent, 1947) [10].

$$I = \frac{C-T}{C} X\ 100$$

Where
I = Per cent inhibition over control
C = Radial growth of pathogen in control (mm)
T = Radial growth of pathogen in treatment (mm)

To isolate and characterize phyllosphere microflora of cotton Isolation of phyllosphere microflora
Healthy leaves were collected from the Bt and non Bt cotton from the College of Agriculture Rajendranagar for phyllosphere isolation by leaf imprint method (Plate 1) and dilution method (Plate 2). Four bacterial cultures were isolated from Bt cotton which are designated as P1 to P4 and other four bacterial cultures are isolated from non Bt cotton and designated as P5 to P8 respectively (Plate 3).
Similar findings were reported by Yadav *et al*. (2010) [11] that the ability of serial dilution plating and leaf imprint methods showed more population abundance on the phyllosphere. Prabakaran *et al*. (2011) [5] reported that a total of ten fungal species belonging to five genera were isolated from surface sterilized leaf segments by dilution plating technique. Akter *et al*. (2014) [2] isolated and identified antagonistic bacteria from phylloplane of rice as biocontrol agents for sheath blight. Ray *et al*. (2014) [8] isolated phylloplane mycoflora of P. bombycina and L. polyantha *viz*., Rhizopus, Mucor, *Alternaria*, Cladosporium, Trichoderma, Pencillium, Cercospora, Curvularia, Aspergillus and Verticilium.

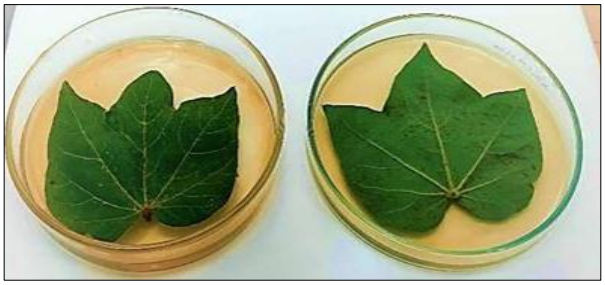


Plate 1: Isolation of phyllosphere microflora by leaf imprint method.

Plate 2: Growth of phyllosphere microflora by dilution plate method

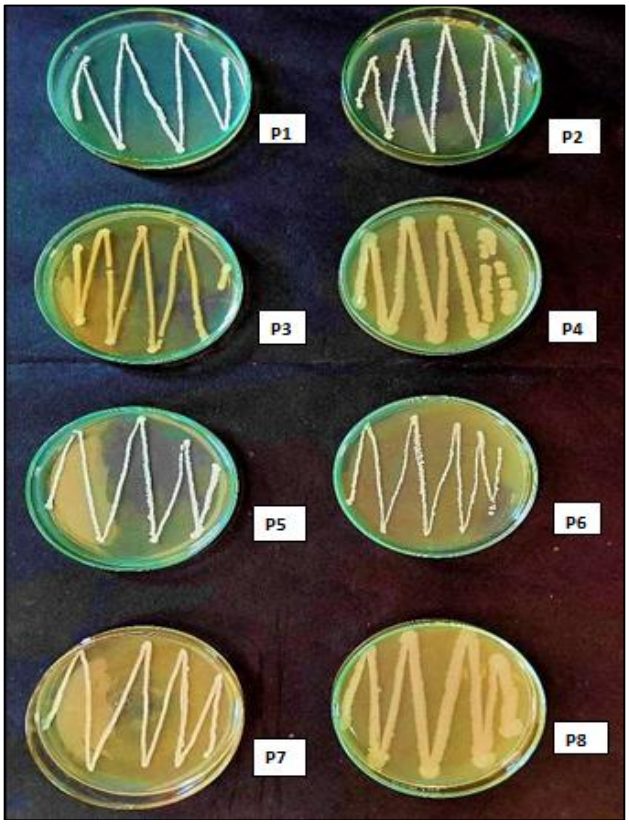


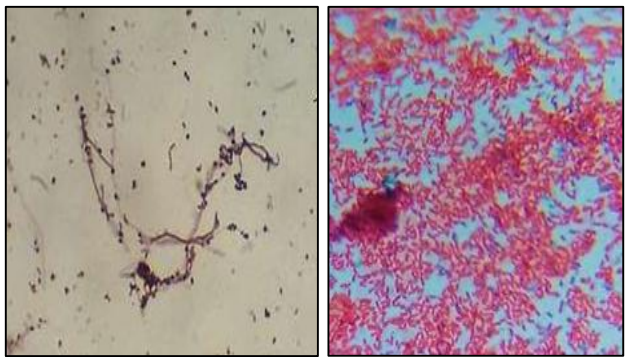
Plate 3: Pure culture of isolates of phyllosphere bacteria

Colony characterstics of different isolates of phyllosphere bacteria

The data pertaining to cultural characteristics of different isolates of phyllosphere bacteria was recorded two days after incubation on nutrient agar medium. The colony characteristics of isolates of phyllosphere bacteria were circular to irregular shape, medium to large size, with smooth and shiny.

Gram's staining

All the isolates of phyllosphere bacteria (P1 to P8) were subjected to Gram's staining. Most of the isolates showed positive reaction (purple) and rod shaped and coccus shaped (Plate 4. a, b).



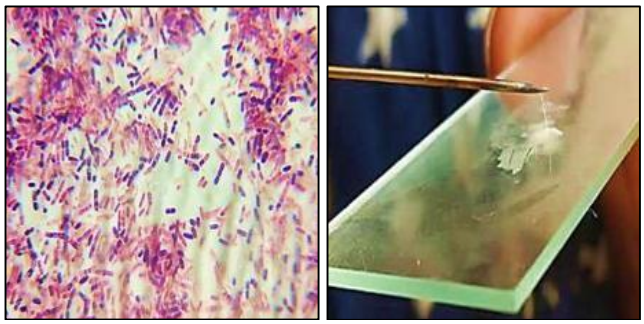
A B

Plate 4: Positive (a) and negative (b) Gram staining of phyllosphere bacterial isolates

Endospore staining

All the isolates of phyllosphere bacteria (P1 to P8) were stained and the results are presented in the Table 5.1 and Plate 4.23. Bacterial isolates P1, P3, P4 from Bt cotton P7 and P8 from non Bt cotton formed endospores (green colour) and rod shaped (Plate 4.1 a, b).

Suman *et al*. (2015) [9] reported that the cultural and morphological characteristics of Pseudomonas fluorescens isolates were small to medium size, irregular to round margin, convex elevation, dull white to yellowish green colour with smooth and shiny which were in conformity with the present results



A B

Plate 4.1: (a) Endospore staining (b) KOH test of phyllosphere bacterial isolates

Biochemical characterization

Eight isolates of bacteria were characterized with different biochemical tests, *viz*., Catalase test, Oxidase test, Voges-Prausker's test, indole test, methyl red test, gelatin liquefaction.

Catalase test

All the eight isolates from Bt non Bt cotton tested positive for catalase and were aerobic (Plate4.2a).

Oxidase test

All the eight isolates from Bt non Bt cotton showed positive for oxidase test. This test revealed that all bacterial isolates have cytochrome oxidase (Plate 4.2b).

Methyl red test

The bacterial isolates P1, P2 from Bt cotton and P8 from non Bt cotton showed negative results. These bacterial isolates

produced acids like lactic acid, acetic acid and ethanol (Plate 4.2c).

Voges prausker's test

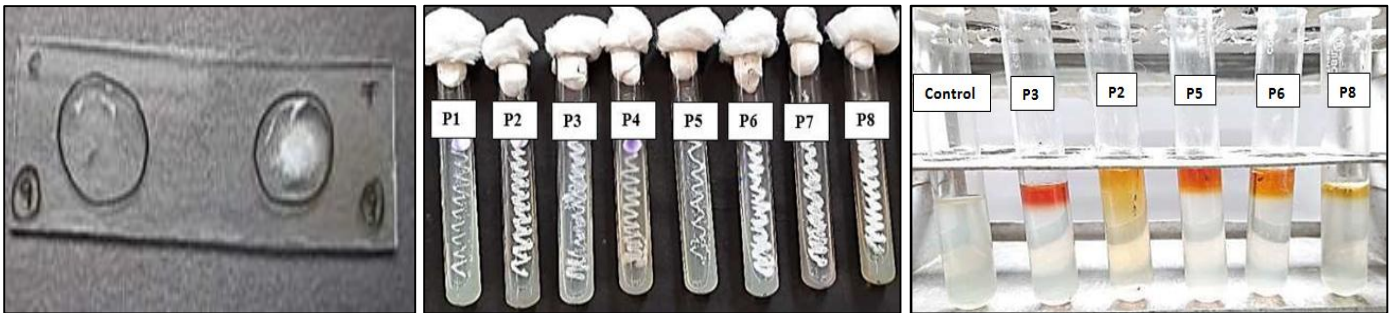
The bacterial isolates P1 from Bt cotton and P5, P6 non Bt cotton showed positive results to acetoin production in bacterial broth culture (Plate 4.3a).

Indole test

Bacterial isolates P2, P4 from Bt cotton and P6 non Bt cotton revealed positive results. These bacterial isolates have the ability to split the amino acid tryptophan into indole (Plate 4.3b).

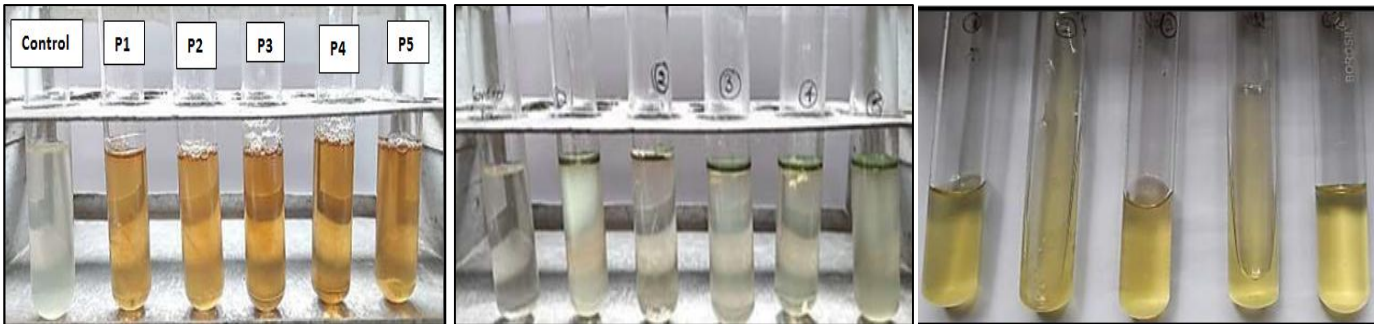
Gelatin liquefaction

The bacterial isolates P3 from Bt cotton and P5, P7 non Bt cotton recorded negative results (Plate 4.3c). From the above it is observed that the isolates of phyllosphere bacteria from Bt and non Bt cotton showed varied morphological, cultural and in biochemical characterization



A B C

Plate 4.2: Biochemical characterization of different isolates of phyllosphere bacteria (a. Catalase test b. Oxidase test c. Methyl red test)



A B C

Plate 4.3: Biochemical characterization of different isolates of phyllosphere bacteria (a. Voges Prausker's test b. Indole test c. Gelatin test)

Screening of phyllosphere bacteria against *Alternaria macrospora*

The phyllosphere microflora isolated from Bt and non Bt cotton were tested for their antagonistic activity against *Alternaria macrospora* by dual culture technique. A total of eight cultures were tested (Table 1, Plate 4.4). Among the eight isolates tested P5 showed maximum growth inhibition of 57.41 per cent with radial growth of 38.33 mm and followed by P4 isolate (53.52%) with radial growth of 41.88 mm respectively over the test fungus *Alternaria* spp. The bacterial isolates P3 (53.33%), P7 (51.04%), P2 (50.44%), P1 (46.56%), P8 (46.55%) and P6 (45.63%) were distinct from each other. Similar findings were reported by Akter *et al*. (2014) [2], who isolated and identified antagonistic bacteria from phylloplane of rice as biocontrol agents for sheath blight pathogen. Selected bacterial isolates were found promising in reducing fungal growth of about 48.22 to 68.4 per cent under *in vitro* conditions.

Table 1: Effect of isolates of phyllosphere microflora on the mycelial growth of *Alternaria macrospora* under *in vitro* conditions

Phyllosphere bacterial antagonist isolates	Linear mycelial growth (mm)	Per cent inhibition of *Alternaria macrospora* over control
P1	48.13	46.56 (42.60)
P2	44.60	50.44 (45.25)
P3	42.00	53.33 (46.91)
P4	41.83	53.52 (46.99)
P5	38.33	57.41 (49.27)
P6	48.93	45.63 (42.42)
P7	44.06	51.04 (45.84)
P8	48.10	46.55 (42.98)
Control	90.00	00.00 (00.00)
C.D.	1.096	0.597
SE(m) ±	0.362	0.198
C.V.	1.411	0.673

*Average of three replications.

Figures in parentheses indicate angular transformed value.

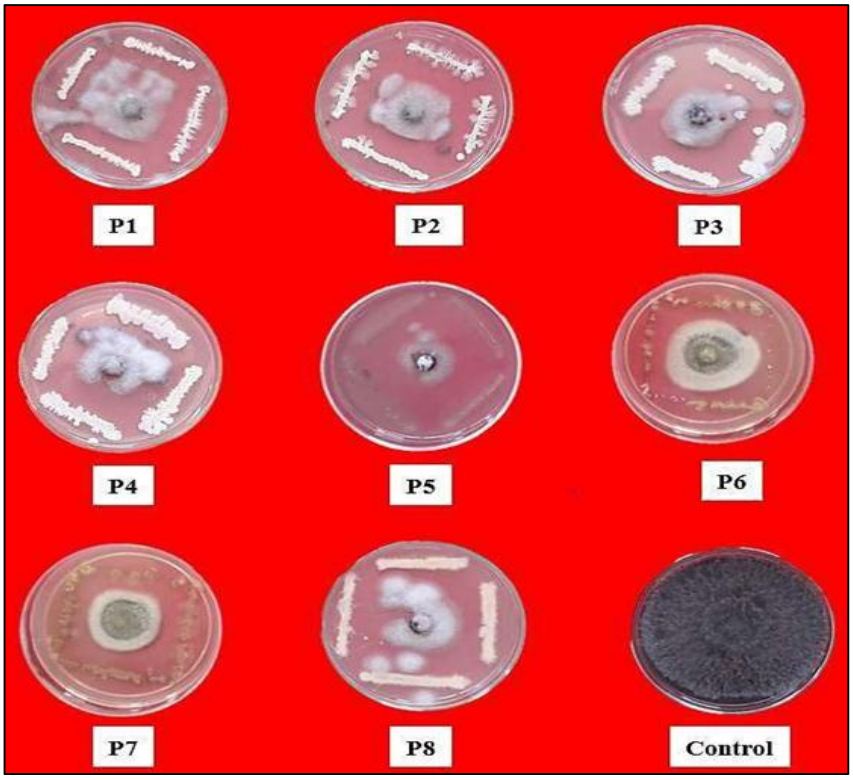


Plate 4.4: Evaluation of phyllosphere bacteria on radial mycelial growth and inhibition of *Alternaria macrospora* under *in vitro* conditions

Discussions

Phyllosphere microorganisms play important role in suppression of the pathogen and induce growth promoting activities in plants. In the present study, healthy Bt and non Bt cotton leaves were collected from the College of Agriculture, Rajendranagar for isolation of phyllosphere microflora by leaf imprint method and dilution method. Four bacterial cultures were isolated from Bt cotton which are designated as P1 to P4 and other four bacterial cultures were isolated from non Bt cotton and designated as P5 to P8 respectively. Biochemical tests revealed that all the eight isolates were positive for the catalase and oxidase test. The isolates P1, P5 and P6 showed positive results to Voges proskauer test. Isolates P2, P4 and P6 revealed positive results of phyllosphere bacteria to Indole test. Whereas, isolates P1, P2 and P8 showed negative reaction to methyl red test. The phyllosphere bacterial isolates P3, P5 and P7 recorded negative reaction to gelatin liquefaction. Screening of all eight bacterial phyllosphere microflora against *Alternaria macrospora* (N2A isolate) by dual culture technique showed that the isolate P5 recorded maximum growth inhibition with 57.41 per cent which was significantly superior and was followed by P4 isolate with 53.52 per cent growth inhibition against the test fungus *Alternaria* sp. and least per cent growth inhibition was reported in isolate P6 with 45.63 per cent.

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