Original Research Article

ISOLATION AND CHARACTERIZATION OF PHYLLOSPHERE MICROFLORA OF MAIZE

ABSTRACT

Maize is renowned as the "Queen of Cereals" and is one of the world's most significant cereal crops. Several foliar and stalk rot diseases affect maize crops. The illness affected the majority of the cultivars that were issued. The influence of overuse of chemical fungicides on the environment and food safety has become a serious problem with the rise of ecological agriculture. Epiphytes are phyllosphere residents who can include a wide range of bacteria and filamentous fungi. Microbial interactions in the phyllosphere repress and promote plant pathogen colonisation and infection of tissues, increasing disease resistance and agricultural crop productivity, implying that phyllosphere microorganisms can play a key role in growth promotion and disease suppression. Bacteria from individual colonies were studied. Individual colony bacteria were examined for shape, size, colour, Gram staining, endospore staining, elevation and texture for morphological studies. Different biochemical tests *viz*., Catalase test, Oxidase test, Voges Prausker's test, Indole test, Methyl red test, Gelatin liquefaction were done for phyllosphere bacteria.

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Key words: Phyllosphere, Bacteria, Maize, Microflora

INTRODUCTION

Several foliar and stalk rot diseases affect maize crops. Turicicum leaf blight, often known as Northern corn leaf blight, is a foliar disease caused by the fungus Exserohilum turcicum (Pass.) Leonard and Suggs. Andhra Pradesh, Telangana, Karnataka, Bihar, Himachal Pradesh, and Maharashtra are among the states in India where this condition is widespread. The influence of overuse of chemical fungicides on the environment and food safety has become a serious problem with the rise of ecological agriculture. Epiphytes (Ruinen, 1961) are phyllosphere residents who can include a wide range of bacteria and filamentous fungi. The internal and external foliar microbiota serves a variety of purposes, including indirect pathogen protection through interactions between non-pathogenic bacteria and foliar plant pathogens (Arnold et al., 2003). Further microbial interactions in the phyllosphere boost disease resistance and agricultural crop productivity, suggesting that phyllosphere bacteria can play a key role in plant growth promotion.

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Materials and methods

Isolation of phyllosphere microflora

Dilution method

Healthy maize plants were collected from several locations in the districts of Karimnagar, Mahaboobnagar, and Ranga Reddy. The plants were separated into sterile bags and sent to the lab for phyllosphere microbe isolation. A sterile cork borer was used to cut ten discs of one cm leaf pieces from each plant. The discs were placed in 100 mL of sterile distilled water and swirled for one hour. A one-milliliter aliquot was plated on PDA and nutritional agar media.

Leaf imprint method

Leaf impressions on nutrient agar medium were used to quantify the bacterial population on adaxial and abaxial leaf surfaces. On a nutrient agar plate, an intact individual leaf was inserted and pushed with the smooth end of a sterile glass rod until a clear impression of the entire leaf was obtained on the nutritional agar surface. Until colony formation, the plates were incubated at 24°C for 2–5 days. The morphological variety of single bacterial colonies was used to select them.

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

In the centre of the glass slide, a drop of sterile distilled water was placed. A loop of inoculum was extracted from the young culture, mixed with water, and deposited in the centre of the slide. To form a thin smear, the suspension was spread out on the slide with the tip of the inoculation loop. The smear was dried in the air and fastened by passing the slide over the flame three to four times. After that, the smear was saturated with crystal violet solution for 1 minute and gently washed with tap water. The slide was then submerged in iodine solution. Iodine solution was drained after 1 minute of incubation at room temperature, followed by washing with 95 percent decolourizer. Following that, it was thoroughly blotted and cleaned with water for 15 to 30 seconds. For 1 minute, the smear was incubated with safranin solution. The slide was gently washed with running tap water and air dried. For each isolate, the slide was inspected under a microscope at 100X magnification with oil immersion and data was collected.

Endospore staining

On a clean slide, a bacterial smear was obtained, air dried, and gently heat fixed. The slides were then soaked with malachite green for 3-5 minutes using a burner flame. To remove the colour, the slides were gently rinsed in running tap water. After the slides had cooled, safranin was poured onto them. The slide was gently washed with running tap water and air dried. The slides were examined at 100 times magnification with oil immersion, and data was collected for several isolates.

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).

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

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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 after full grown of the 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.

Results and Discussion

Isolation of phyllosphere microflora

Healthy maize leaves were collected from Karimnagar, Mahaboobnagar and Ranga Reddy districts for phyllosphere isolation by leaf imprint method and dilution method. Twenty-two bacterial cultures and six fungal cultures were isolated from the phyllosphere and designated as phyllosphere for bacteria P1 to P22 and phyllosphere fungi for F1 to F6 respectively.

Colony characters 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 (P1, P2, P6, P7, P10, P15, P19, P20 and P22) and presented in the Table 1.

Suman *et al*. (2015) 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

Colony characteristics of fungal isolates

The data pertaining to cultural characteristics of isolates of phyllosphere fungi isolates was recorded five days after incubation on PDA medium. The colony characters of fungal

isolates were round, appressed to fluffy margin and colour of the colony at center and margins were black, green, light brown and dull white were recorded and depicted in the Table 1.

Gram's staining

All the isolates of phyllosphere bacteria (P1 to P22) were subjected to Gram's staining and the results are presented in the Table 2. Most of the isolates showed positive reaction (purple) and rod shaped.

Endospore staining

All the isolates of phyllosphere bacteria (P1 to P22) were stained and the results are presented in the Table 2. and Plate. Bacterial isolates P1, P4, P6, P7, P12, P14 and P16 formed endospores (green colour) and rod shaped.

Biochemical characterization

Twenty-two 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 as described in the Table 2.

Catalase test

All the twenty-two isolates tested positive for catalase and were aerobic.

Oxidase test

All the twenty-two isolates were positive for oxidase test. This test revealed that all bacterial isolates have cytochrome oxidase.

Voges prausker's test

The bacterial isolates P1, P6, P7, P14, P16, P17 and P22 showed positive results to acetoin production in six bacterial broth culture.

Indole test

Bacterial isolates P2, P3, P5, P8, P10, P11, P13, P15 and P21 revealed positive results. These bacterial isolates have the ability to split the amino acid tryptophan into indole.

Methyl red test:

The bacterial isolates P1, P4, P6, P7 and P22 showed negative results. These seven bacterial isolates produced acids like lactic acid, acetic acid and ethanol.

Gelatin liquefaction:

The bacterial isolates P8, P15, P17, P18, P19, P21 and P22 recorded negative results

Akter *et al.* (2014) isolated 325 bacteria and 14 of them were identified as fluorescent *Pseudomonas* by morphological and biochemical characterization. Fifty *P. fluorescens* and 28 *Bacillus* strains were isolated from rhizospheric soil and root nodules of pigeon pea, biochemically characterized and identified as *P. fluorescens* and *Bacillus.* Malleswari (2014) studied antagonistic activity of diverse bacterial isolates *in vitro* against *Macrophomina phaseolina*. On the basis of colony morphology and biochemical characteristics the isolate was identified as *Bacillus* sp.

Conclusion

Twenty-two bacterial cultures (P1 to P22) and six fungal cultures were isolated from the phyllosphere by leaf imprint method and dilution method. The colony characters of isolates of bacteria and fungi pertaining to their shape, size, elevation, margin, texture, appearance and pigmentation were recorded. Gram's staining and endospore staining revealed that P1, P4, P6, P7, P12, P14 and P16 were Gram positive, endospores and rod shaped.

Biochemical tests revealed that all the twenty-two isolates were positive for the catalase and oxidase test. Isolates P1, P6, P7, P14, P16, P17 and P22 showed positive results to Voges proskauer test. Isolates of phyllosphere bacteria P2, P3, P5, P8, P10, P11, P13, P15 and P21 revealed positive results to Indole test. Whereas, isolates P1, P4, P6, P7 and P22 showed negative reaction to methyl red test. The phyllosphere bacterial isolates P8, P15, P17, P18, P19, P21 and P22 recorded negative reaction to gelatin liquefaction.

References

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Table 1. Cultural and morphological characters of different isolates of bacteria on NA medium isolated from phyllosphere of maize

Isolates	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation
P1	Circle	Entire	Raised	Moderate	Smooth	Shiny	Cream
P2	Circle	Entire	Convex	Moderate	Smooth	Shiny	Light pink
P3	Irregular	Irregular	Flat	Small	Smooth	Dull	Nil
P4	Circle	Entire	Raised	Small	Rough	Dull	Light cream
P5	Circle	Irregular	Flat	Moderate	Rough	Dull	Yellow
P6	Circle	Entire	Raised	Small	Smooth	Shiny	Light cream
P7	Circle	Irregular	Convex	Small	Smooth	Shiny	Light cream
P8	Irregular	Irregular	Flat	Moderate	Rough	Dull	Orange
P9	Irregular	Irregular	Flat	Moderate	Smooth	Dull	Light brown
P10	Circle	Entire	Slightly raised	Small	Smooth	Shiny	Yellow
P11	Irregular	Entire	Flat	Moderate	Smooth	Dull	Light brown
P12	Circle	Entire	Raised	Moderate	Rough	Dull	Transparent
P13	Circle	Entire	Convex	Small	Rough	Dull	White
P14	Irregular	Irregular	Flat	Moderate	Rough	Dull	Cream
P15	Circle	Entire	Raised	Moderate	Smooth	Shiny	Light green
P16	Irregular	Entire	Slightly raised	Moderate	Rough	Dull	White
P17	Circle	Entire	Flat	Small	Rough	Shiny	Nil
P18	Irregular	Entire	Raised	Large	Rough	Shiny	Light cream
P19	Circle	Entire	Raised	Small	Smooth	Shiny	Yellow
P20	Circle	Irregular	Raised	Small	Smooth	Shiny	Dark pink
P21	Irregular	Irregular	Flat	Small	Smooth	Dull	White
P22	Circle	Entire	Convex	Large	Smooth	Shiny	Yellowish green

Table 2. Biochemical characterization of phyllosphere bacteria isolated from maize

S.No	Isolate	Catalase test	Oxidase test	Voges prausker's test	Indole test	Methyl red test	Gelatin test	Gram's staining	Shape	Endospore staining
1.	P_1	+	+	+	-	-	+	+	Rod	+
2.	P_2	+	+	-	+	-	+	-	Coccus	-
3	P_3	+	+	-	+	+	+	+	Coccus	-
4	P_4	+	+	-	-	-	+	+	Coccus	-
5	P_5	+	+	-	+	-	+	-	Rod	-
6	P_6	+	+	+	-	-	+	+	Rod	+
7	P_7	+	+	+	-	-	+	+	Rod	+
8	P_8	+	+	-	+	-	-	+	Coccus	-
9	P_9	+	+	-	-	+	+	-	Rod	-
10	P_{10}	+	+	-	+	+	+	+	Rod	-
11	P_{11}	+	+	-	+	-	+	+	Coccus	-
12	P_{12}	+	+	-	-	+	+	+	Rod	+
13	P_{13}	+	+	-	+	-	+	-	Rod	-
14	P_{14}	+	+	+	-	+	+	+	Rod	+
15	P_{15}	+	+	-	+	-	-	-	Rod	-
16	P_{16}	+	+	+	-	+	+	+	Rod	+
17	P_{17}	+	+	+	-	+	-	+	Coccus	-
18	P_{18}	+	+	-	-	-	-	+	Rod	+

19	P_{19}	+	+	-	-	+	-	-	Rod	-
20	P_{20}	+	+	-	-	-	+	-	Coccus	-
21	P_{21}	+	+	-	+	-	-	+	Coccus	-
22	P_{22}	+	+	+	-	-	-	-	Coccus	-

+ Positive

- Negative

Figure 1 : Isolation of phyllosphere microflora by leaf imprint method

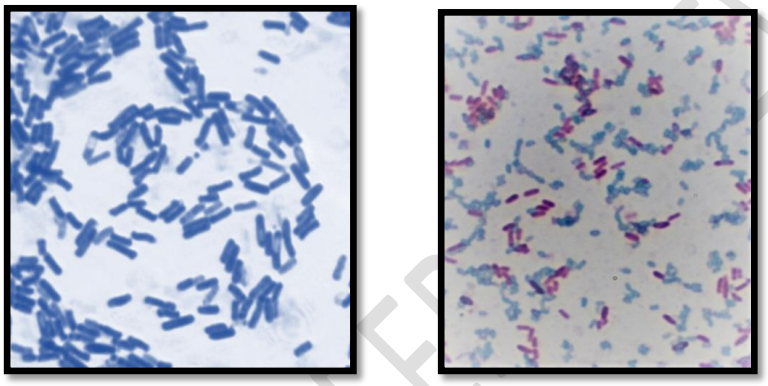


Figure 2 : Gram staining and Endospore staining of phyllosphere bacterial isolate

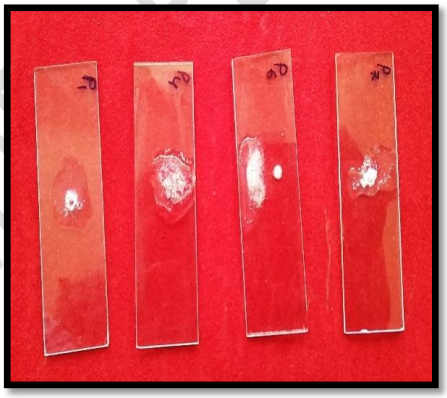


Figure 3: Catalase test

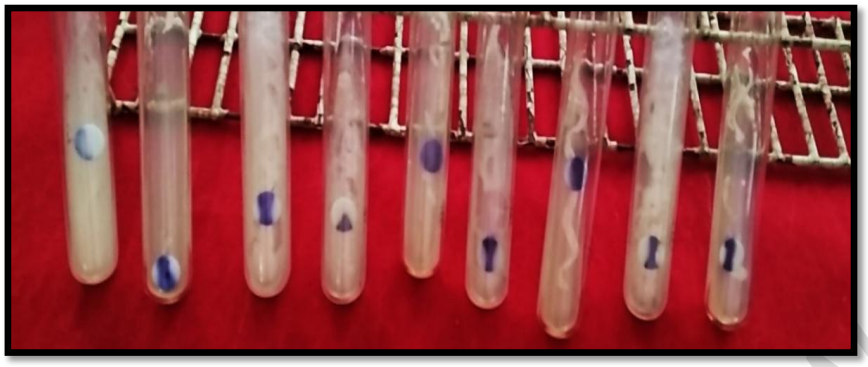
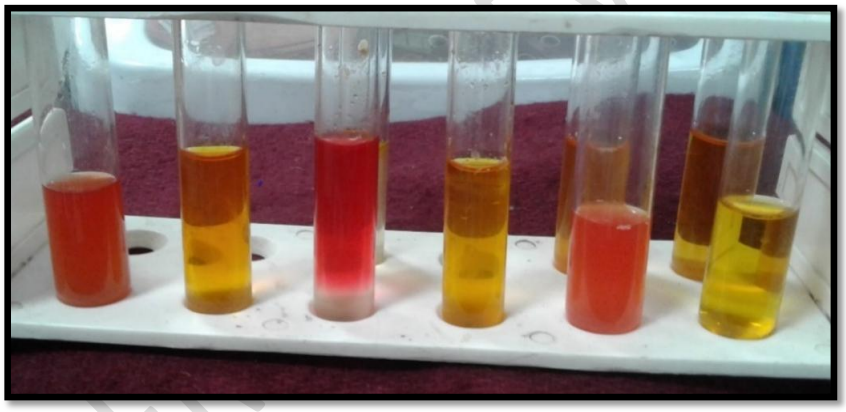


Figure 4: Oxidase test

Figure 5: Methyl red test



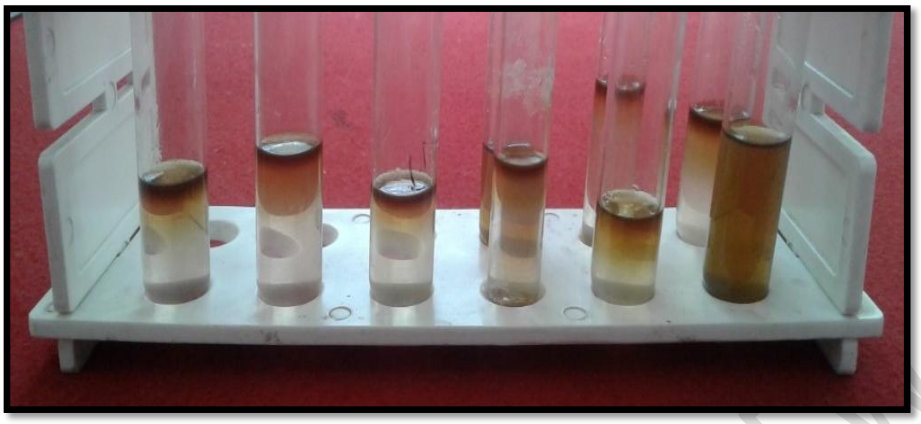


Figure 6: Voges Prausker's test

Figure 7: Indole test

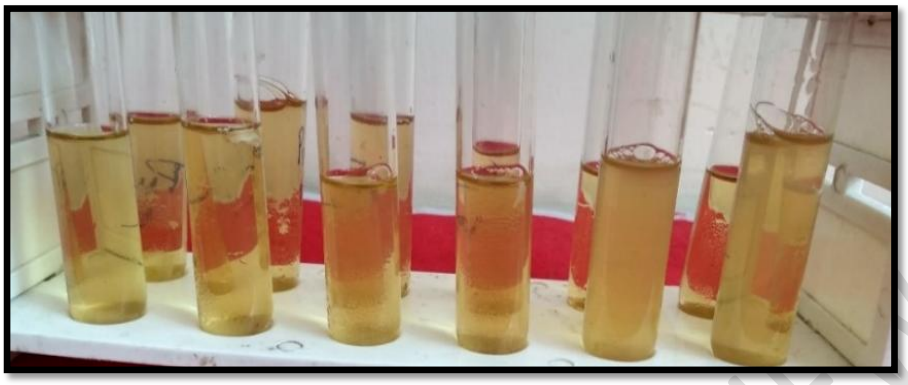


Figure 8: Gelatin test