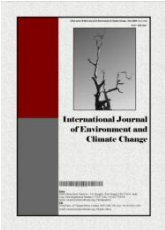

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Isolation and Characterization of Phyllosphere Microflora of Maize

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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 was examined for shape, size, colour, Gram staining, endospore staining, elevation and texture for morphological studies. Different biochemical tests *viz*., Catalase, Oxidase, Voges Prausker's, Indole, Methyl red, Gelatin liquefaction were done for phyllosphere bacteria.

Keywords: Phyllosphere; bacteria; maize; microflora.

1. 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,

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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 [1] 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 [2]. 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.

2. MATERIALS AND METHODS

2.1 Isolation of Phyllosphere Microflora

2.1.1 Dilution method

Healthy maize plants at flowering stage 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 nutrient agar (Hi media).

2.1.2 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 [3].

2.2 Morphological and Cultural Characteristics of the Bacteria

Pure bacteria cultures were streaked separately on nutrient agar plates and cultured at room temperature until a single colony appeared. The shape, size, colour, Gram staining, endospore staining, elevation, and texture of each colony were all investigated.

2.3 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 alcohol as 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.

2.4 Endospore Staining

A bacterial stain was obtained on a clean slide, air dried, and gently heat fixed. Using a burner flame, the slides were then soaked in malachite green for 3-5 minutes. 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 and air dried under running tap water. Data was obtained for numerous isolates while the slides were viewed at 100 times magnification with oil immersion.

2.5 Biochemical Characterization

The Catalase test, Oxidase test, Voges Prausker's test, Indole test, Methyl red test, and Gelatin liquefaction tests were all performed according to conventional protocols [4].

2.5.1 Catalase test

A drop of 3% hydrogen peroxide was applied to a 48-hour-old bacterial colony on a clean glass slide for the catalase test. Catalase activity is shown by effervescence.

2.5.2 Oxidase test

The bacteria were cultured on nutrient agar slants. Hi media oxidase paper discs were left on fully developed cultures for 48 hours. After the bacterial isolates had fully grown, oxidase paper discs were retained in the slants. If the colour changes to purple, it means the outcome was positive.

2.5.3 Voges Prausker's test

Alpha-naphthol and potassium hydroxide were added to the Voges Prausker's broth for the test. A positive result is indicated by a cherry red colour, while a negative result is indicated by a yellow-brown colour.

2.5.4 Indole test

The overnight cultures of the isolates were injected into tryptophan broth tubes, which were then incubated for 48 hours at 28 ±2°C. After incubation, each tube received 10 drops of Kovac's Indole reagent. Isolates that produced a red colour were considered positive for indole synthesis.

2.5.5 Methyl Red test

The test culture was injected into sterilised glucose-phosphate broth tubes and incubated at 28 ±2°C for 48 hours. Five drops of methyl red indicator were added to each tube after incubation and gently shaken. The generation of red colour was regarded as a test failure.

2.6 Gelatin Liquefaction

The test isolates' overnight cultures were injected into sterilised nutritional gelatin deep tubes and incubated at 28 ±2°C for 24 hours. The tubes were then chilled for 30 minutes at 4 degrees Celsius. The isolates that showed liquefied gelatin were considered positive, while those that resulted in gelatin solidification after refrigeration were considered negative.

3. RESULTS AND DISCUSSION

3.1 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.

3.2 Colony Characters of Different Isolates of Phyllosphere Bacteria

Two days after incubation on nutrient agar medium, data on cultural properties of distinct isolates of phyllosphere bacteria were recorded. Table 1 shows the colony features of phyllosphere bacteria isolates, which were round to irregular in shape, medium to large in size, and smooth and shiny (P1, P2, P6, P7, P10, P15, P19, P20, and P22).

The cultural and morphological characteristics of Pseudomonas fluorescens isolates, according to Suman et al. [5], were small to medium size, irregular to round margin, convex elevation, dull white to yellowish green colour with smooth and shining surface.

3.3 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.

3.4 Gram's Staining

Gram's staining was performed on all phyllosphere bacteria isolates (P1 to P22), and the results are shown in Table 2. The majority of the isolates had a positive (purple) response and were rod-shaped.

3.5 Endospore Staining

The results of staining all phyllosphere bacteria isolates (P1 to P22) are shown in Table 2. P1, P4, P6, P7, P12, P14, and P16 bacterial isolates generated endospores (green colour) and rod-shaped endospores.

3.6 Biochemical Characterization

As shown in Table 2, twenty-two bacteria isolates were characterised using several biochemical assays, including the Catalase test, Oxidase test, Voges-test, Prausker's indole test, methyl red test, and gelatin liquefaction.

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. Cultural and morphological characteristics of different isolates of fungi on PDA isolated from maize phyllosphere

S. No	Isolates	Colony colour	Colony characters	Fungal genera identified
1.	F_1	White	White appressed centre, appressed margin, white colony with regular margin	*Fusarium* sp. (1)
2.	F_2	Dull white	White fluffy centre, appressed margin, white colony with regular margin	*Fusarium* sp. (2)
3.	F_3	Black	Black appressed centre, appressed margin, black colony with regular margin	*Aspergillus niger*
4.	F_4	Light brown	Light brown fluffy centre, appressed margin, green colony with regular margin	*Aspergillus terreus*
5.	F_5	Green	Green appressed centre, appressed margin, green colony with regular margin	*Aspergillus nidulans*
6.	F_6	Yellow	Yellow fluffy centre, fluffy margin, yellow colony with circular margin	*Aspergillus ochraceus*

Table 3. 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

Fig. 1. Isolation of phyllosphere microflora by leaf imprint method

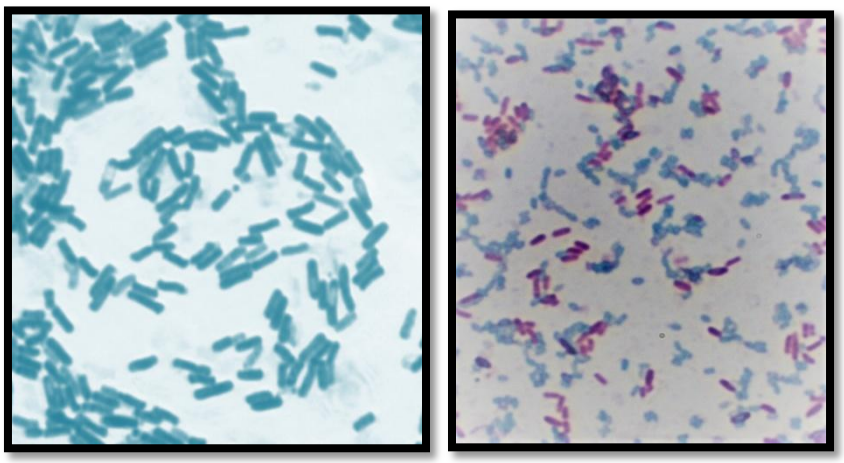

Fig. 2. Gram staining and Endospore staining of phyllosphere bacterial isolate

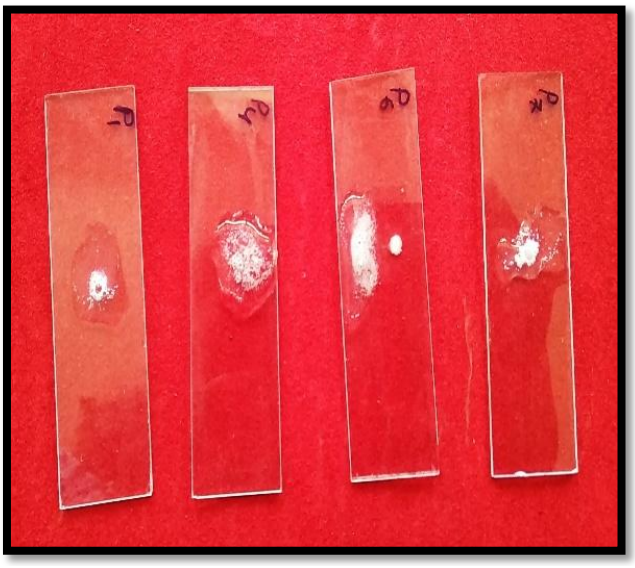

Fig. 3. Catalase test

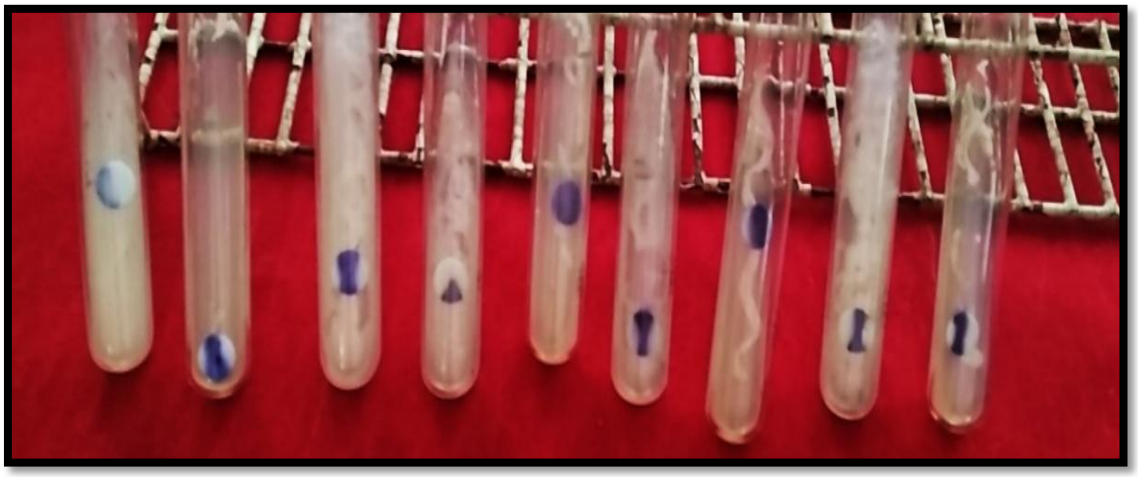

Fig. 4. Oxidase test

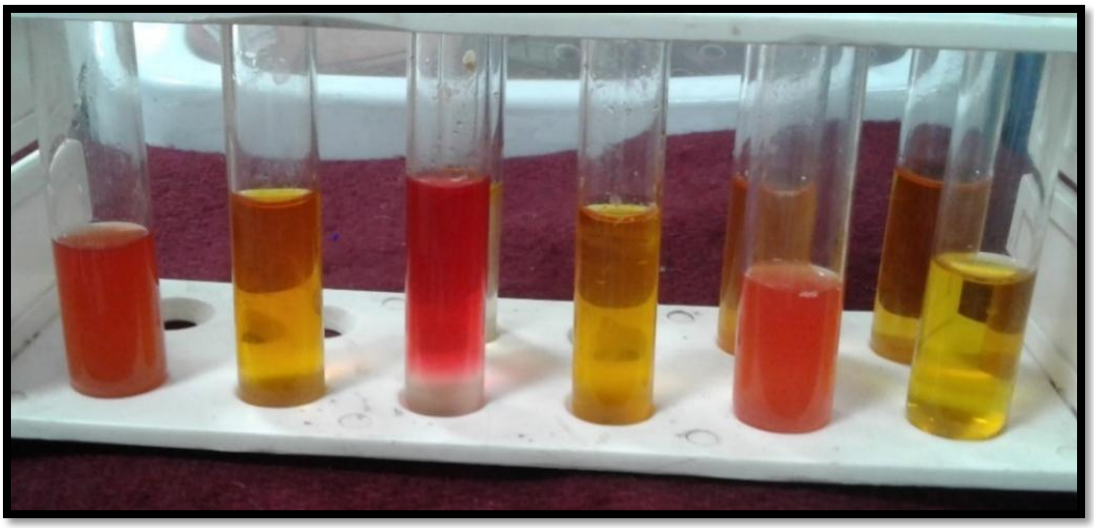

Fig. 5. Methyl red test

Fig. 6. Voges Prausker's test

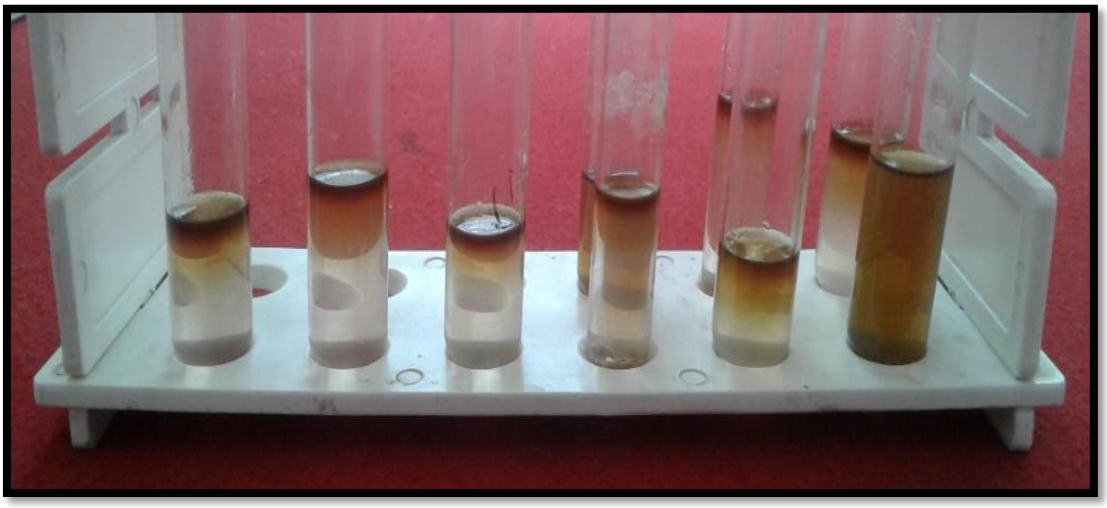

Fig. 7. Indole test

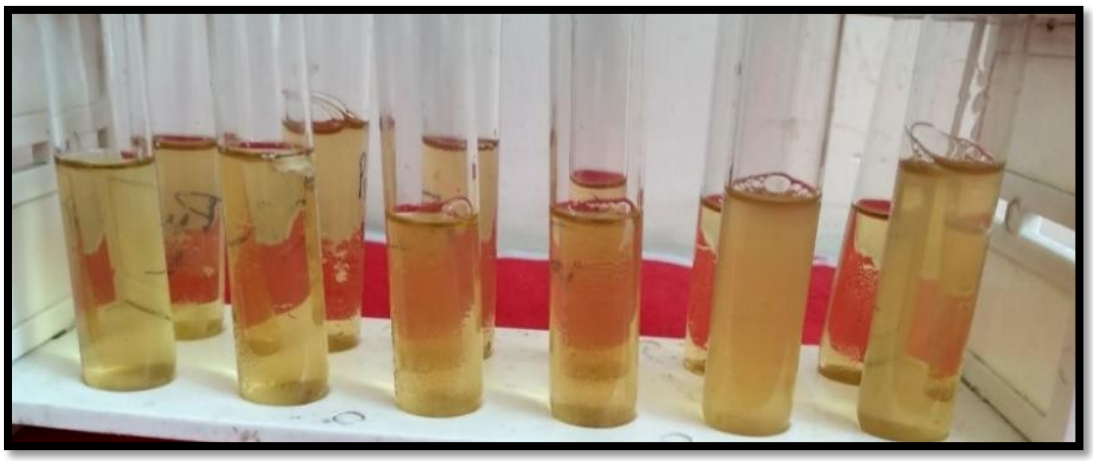

Fig. 8. Gelatin test

3.6.1 Catalase test

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

3.6.2 Oxidase test

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

3.6.3 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.

3.6.4 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.

3.6.5 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.

3.7 Gelatin Liquefaction

The bacterial isolates P8, P15, P17, P18, P19, P21 and P22 recorded negative results Akter et al. [6] 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 [7] 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.

4. CONCLUSION

The leaf imprint method and dilution method were used to isolate twenty-two bacterial cultures (P1 to P22) and six fungus cultures from the phyllosphere. Colony characteristics of bacteria and fungal isolates were documented, including form, size, elevation, margin, texture, appearance, and pigmentation. P1, P4, P6, P7, P12, P14, and P16 were Gram positive, endospores, and rod shaped, according to Gram's staining and endospore staining.

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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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