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Characterization of Leaf Blight Pathogen, *Pseudomonas syringae* pv. *syringae* of mango in Bangladesh

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Abstract

Bacterial leaf blight of mango caused by Pseudomonas syringae pv. syringae is an emerging disease and great threat for production of healthy mango saplings in different nurseries of Bangladesh. A survey was conducted in nurseries of some selected areas viz. Dinajpur, Rajshahi, Khulna, Mymensingh and Madhupur of Bangladesh to know the status of bacterial leaf blight of mango in terms of its incidence and severity and to characterize the pathogen causing the disease. The results showed that the highest (10.42%) bacterial leaf blight incidence was recorded in Rajshahi followed by Mymensingh, Madhupur and Dinajpur with 9.73, 9.12, 8.64 % disease incidence respectively and the lowest (8.58%) incidence was recorded in Khulna. The maximum (7.34%) severity of bacterial leaf blight was recorded in Rajshahi followed by Dinajpur, Mymensingh and Madhupur with 7.17, 7.13, 7.13% disease severity and the minimum (7.03%) severity was recorded in Khulna. Twenty four bacterial isolates were obtained from the infected leaves by tissue planting method from different locations surveyed. Pathogenicity test on detached mongo leaves confirmed the bacterium was Pseudomonas syringae pv. syringae. The hypersensitivity test in tobacco leaves with P. syringae pv. syringae isolates did not show rapid death leaf of tissues where infiltrated. The causal bacterium, P. syringae pv. syringae was also characterized by a series of biochemical tests viz. KOH solubility test, Gram staining test, Kovac's oxidase test, Temperature sensitivity test, Levan test, Sugar utilization test, Arginine dihydrolase activity, Catalase test and Pectolytic test. The results of the all the tests revealed that the causal organism of leaf blight of mango was Pseudomonas syringae pv. syringae.

Keywords: Characterization, leaf blight, Pathogen, Mango

Introduction

Mango (*Mangifera indica* L.) belonging to the family Anacardiaceae is said to have originated in the region of eastern Indo-Bangladesh, Burma, Malaysia or Thailand[1]. It is also grown all over the tropics and subtropics and one of the most important fruit crops in the world. The leading mango producing countries are India, Pakistan, Mexico, Brazil, Haiti and Philipines. Mango is also grown in Indonesia, Java, Thailand, Bangladesh, Srilanka and Northwest Australia. India is the largest producers with approximately 66% of the total mango production in the world[2]. In Europe, mango orchards are not widespread, but mango plantings in southern Spain have escalated over the last 10 years, presently covering about 800 ha. It is widely grown all over Bangladesh with the quality mangoes solely concentrated in the north-western areas especially greater Rajshahi, Dinajpur and Rangpur[3]. Mango ranks third among the tropical fruits grown in the world. Bangladesh produced 82816 metric ton of mango in 31028 ha of mango orchard during the period of 2008-09[4]. Mango is now recognized as one of the desirable fruit in the world market for its excellent flavor, pleasant aroma, attractive color and taste. Mango has got a unique position in respect of nutritional quality, taste, consumer's preference etc. among the 50 kinds of fruits grown in Bangladesh[5].

Mango is being the most demanding fruit. So the demand for fruit mango is increasing day by day with growing population but declining in production which results in scarcity every year. Disease is one of the predominating causes for lower production of mango in Bangladesh[6]. Mango is reported to be attacked with as many as 18 different diseases in Bangladesh. The major diseases occur in mango are scab, anthracnose, stem end rot, bacterial leaf spot, bacterial leaf blight, powdery mildew, die-back, rust, malformation etc. Bacterial leaf blight has been observed in mango in southern Spain in 1991. The symptoms of the disease are characterized by a rapid enlargement of necrotic lesions in buds and leaves. Mango buds, leaves, and stems are all predisposed to infection, but fruit lesions have not been detected. Disease symptoms comprise necrosis of vegetative and flower buds and bud failure before bud break. Necrotic lesions in buds occasionally outspread to the leaf petiole through the stem. Leaf blight of mango in Bangladesh has been reported by Hossain[7]. They also identify the causal agent to be the bacteria *Pseudomonas syringae* pv. *syringae* but the pathogen was not characterized in details. Hence, a comprehensive survey was made in some nurseries of the selected mango growing areas to know the status of bacterial leaf blight of mango in terms of its incidence and severity in some selected nurseries and to characterize different isolates of its causal organism in Bangladesh.

Material and Methods

Survey and collection of diseased plant samples: A survey was carried out to know the status of bacterial leaf blight of mango in nurseries of Rajshahi, Dinajpur, Mymensingh, Madhupur and Khulna during July to September 2011. At least ten nurseries from each growing areas were surveyed to record the bacterial leaf blight incidence and severity. The infected leaf samples from different nurseries were brought into the laboratory for the isolation of the pathogen.

Isolation, purification, identification and preservation: The infected leaves were surface disinfected by immersion in 10 % Clorox solution and then rinsed in sterile distilled water. Leaf pieces after drying on blotting paper were transferred to nutrient agar (NA) medium and incubated at 25°C for 2 to 3 days. Bacterial isolates were purified by streaking a single colony of each isolate on NA medium as described by Kelman[8].The isolates of bacteria were preserved in 10% skim milk kept at -20°C refrigerator for subsequent biochemical studies.

Characterization of *Pseudomonas syringae* pv. *Syringae:* Pathogenicity Test: To confirm the isolates of *P. syringae* pv. *syringae,* the pathogenicity test was performed on the detached leaves of mango. A single colony of *P. syringae* pv. *syringae* showing virulent, fluidal, irregular and creamy white color was selected for each group of isolates and multiplied in a NA medium. After 24 hours bacterial isolates were suspended in sterile water. The tip of the leaves was cut about 2.5cm. Then the cut leaves were dipped into bacterial suspension for 24hr. The inoculated leaves were then incubated in a Petridish at room temperature for at least 5-7days for the appearance of symptom.

Host range test: The host range of *P. syringae* pv. *syringae* isolates were tested in some other fruit species such as Guava, Jujube and Litchi by pathogenicity test on detached leaves (described earlier) of those fruit species. This test was performed to observe the reaction or infection ability of *P. syringae* pv. *syringae,* on fruits other than mango.

Hypersensitivity response (HR) test: To determine the pathogenic nature of the isolates, hypersensitive reaction was studied on tobacco (*Nicotiana rustica)* plants by infiltration of bacterial suspension into the intrveinal areas of the tobacco leaves[9].

Biochemical studies for of *P. syringae* pv. *Syringae:* Gram staining reaction: 24 hrs old fresh bacterial cultures for each group of isolates were taken to perform the gram reaction test. Bacterial mass were taken out from culture, spread on clean glass slide followed by drying in air without heat and was flamed it lightly and gently to settle the bacteria. Crystal violet was then poured over the bacterial smear on the slide for 2-3 minutes and washed in tap water for 1 minute and lightly blot dry on a paper towel to remove crystal violet. The smear was flooded with iodine solution for 1 minute and washed in tap water for few seconds and then blot dried. It was decolorized with solvent e.g. alcohol, until the solvent flowed colorlessly from the slide (about 2-4 seconds) and rinse in tap water for 5 seconds. Then the smear was counterstained for about 1 minute with safranin solution and washed briefly in tap water followed by blot dry. One drop emulsion oil was then added on the slide and placed it on compound microscope with 100 x magnification for observation**.**

Potassium hydroxide test: Gram staining results were confirmed by potassium hydroxide test (KOH) 3% as described by Suslow et al.[10]. The bacteria were aseptically removed from Petriplates with tooth pick, placed on glass slide in a drop of 3% KOH solution and stirred for 10 second using a quick circular motion of hand.

Oxidase test: A 24h old bacterial colony on nutrient agar, augmented with 1% glucose was used in this test. A loopful of the inoculum was rubbed on filter paper pervaded with 15%(w/v) freshly prepared aqueous solution of Tetramethyl-p-phenylene diamine dihydrochloride[11].

Temperature sensitivity test: The ability of bacterial isolates to grow at different selected temperature at 27°C, 37°C and 41°C were tested by initially growing isolate on NA for 24h. The bacterial suspension of isolate was prepared from 24 hrs NA culture to 5 ml of nutrient broth. 2-3 drops of bacterial suspension was taken in nutrient broth contained in test tubes which were placed at temperature 27°C, 37°C and 41°C.

Levan test: Sucrose peptone agar or a nutrient agar medium with 5% sucrose is suitable substrate for Levan test. For the test a single colony for each isolate was stabbed with a sterilized tooth pick on NA medium containing 5% sucrose. Then the plates were incubated at 28°C for 2 to 4 days to have distinctive dome shaped colonies.

Sugar Utilization test: Preparation of medium: The medium consists of peptone water to which fermentable sugar was added in the proportion of 1%. Peptone water was prepared by adding 1g of Bacto peptone (Difco, USA) and 0.5 g of sodium chloride in 100 ml distilled water. The medium was boiled for 5 minutes, adjusted to pH at 7.0, cooled and then filtered through filter paper. Phenol red, an indicator at the strength of 0.2% solution was added to peptone water and then dispensed 5 ml in Durham's fermentation tubes, placed inversely. These were then sterilized in autoclave at 121°C maintaining at 15 PSI pressure for 15 minutes.

The sugars used for fermentation test were prepared separately as 10% solution in distilled water (10 g sugar was dissolved in 100 ml of distilled water). A little heat was given to dissolve the sugar completely. The sugar solutions were sterilized using filters. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water and the indicator. Before use, the sugar media was sterilized and

incubated it for 24 hours at 37°C. The carbohydrate fermentation test was performed by inoculating a loop full of nutrient broth culture of the organisms into the tubes containing different sugar media (four basic sugars such as dextrose, sucrose, lactose, and manitol) and incubated for 24 hours at 37°C. Acid production was indicated by the color change from reddish to yellow in the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes.

Arginine dihydrolase activity test: The test was performed to observe the presence of two enzymes that permits certain Pseudomonads to grow under anaerobic conditions. 0.2g peptone, 1g NaCl, 0.06g K_2HPO_4, 0.6g Agar, 0.02mg Phenol red and 2g Arginine HCl was taken in a conical flask and mixed with 200mL water. The conical flask was shaken gently for mixing the chemicals properly. The pH was adjusted at 7.2. These were then sterilized in autoclave at 121°C maintaining at 15PSI pressure for 15 minutes. A fresh culture was stabbed into a soft agar tube of Thornley's medium, sealed with sterilized mineral oil or melted agar and incubated at 28°C. A color change from faint pink to red within four days is positive reaction.

Catalase Test: This test was performed to isolates to check their liveliness. Standard cultures are used for control purpose. One milliliter of 3% hydrogen peroxide (H_2O_2) was placed on the microscope slides. The bacterial isolates collected from the leaf samples were tested for catalase activity. Bacterial isolates were grown in NA medium for 24 hrs and a loopful of bacterial cells was added in the drop of H_2O_2. Bubbles' arising from the solution was recorded as positive reaction. This enzyme is responsible for protecting bacteria from hydrogen peroxide (H_2O_2) buildup, which can arise during aerobic metabolism. Catalase breakdowns H_2O_2 into water and O_2.

Pectolytic activity: Well washed, healthy looking, firm potatoes were surface sterilized and were peeled aseptically. Three standard slices were placed in sterile petridishes and the petridishes were incubated in a moist chamber. Two slices were then inoculated with loopful of bacteria previously grown on Nutrient Agar culture of each isolate and incubated at room temperature for 24-48 hrs for the detection of soft rot symptoms. A control for each isolate was maintained using loopful of sterile water. Pectolytic activity was performed to observe the condition of the sliced potato.

Results and Discussion

Leaf blight of mango a new disease was identified in ten nurseries of each of five areas of Bangladesh viz. Rajshahi, Dinajpur, Mymensingh, Madhupur and Khulna (figure-1 and table-1). The symptoms appear on buds, leaves, and stems of mango but fruit lesions have not been observed. Disease symptoms include necrosis of vegetative and flower buds and bud failure before bud break. Necrotic lesions in buds sometimes spread to the leaf petiole through the stem. Generally, a white creamy gum exudes from necrotic lesions on buds, stems, and less frequently on petioles. Necrotic symptoms also affect flower panicles, causing the most severe economic losses because of the decrease in fruit set. Lesions on leaves start as interveinal, angular, water-soaked spots (1 to 3 mm in diameter) that coalesced, becoming dark brown to black (figure-2). The survey results indicated a regional variation in bacterial leaf blight incidence and severity. The results showed that the highest (10.42%) incidence was recorded in Rajshahi and the lowest (8.58%) incidence was in Khulna (figure-3). At the same time the highest (7.34%) severity was recorded in Rajshahi and the lowest (7.03%) severity was recorded in Khulna (figure-4). Morphological appearance preliminary showed that mango with bacterial leaf blight have shown the presence of bacterial strains resembling *Pseudomonas syringae* pv. *syringae*.

Table-1
Isolates of *Pseudomonas syringae* pv. *syringae* obtained from the infected mango leaf samples collected from some selected areas of Bangladesh

Areas surveyed	Group of Isolates	Number of isolates obtained in each area
Dinajpur	Group 1 (A)	6
Rajshahi	Group 2 (B)	6
Khulna	Group 3 (C)	4
Mymensingh	Group 4 (D)	6
Madhupur (Tangail)	Group 5 (E)	2

Isolation and Identification of the *Pseudomonas syringae* pv. *syringae* isolates: A total of twenty four *P. syringae* pv. *syringae* isolates were obtained from the infected mango leaves collected from different locations surveyed. All of the *P. syringae* pv. *syringae* isolates isolated from infected mango leaves produced cream color or off-white color colonies on NA media after 24 hours of inoculation.

Confirmation test of *P. syringae* pv. *Syringae:* Pathogenicity test: The results of pathogenicity test revealed that all the isolate groups of *P. syringae* pv. *syringae* were able to cause blight symptoms on mango leaves identified by observing dark brown to black blight areas on the leaves (table-2).

Host range test: The host range of *P. syringae* pv. *syringae* was determined by pathogenicity test on detached leaves of the three fruit species viz. guava, litchi and jujube. The result showed *P. syringae* pv. *syringae* causing blight symptoms on mango leaves but did not produce any symptom on the leaves of guava, litchi and jujube (table-2).

Hypersensitivite response (HR) test: The result showed that none of the isolates were able to induce HR (rapid death of local cell of tissues) into the interveinal areas between of tobacco (cv. Banket –A1) and chili leaves.

Biochemical tests: Potassium hydroxide solubility test: The result revealed that an elastic thread or viscious thread was

observed when loop raised from the bacterial solution by toothpick a few centimeters from glass slides in case of all group of *P. syringae* pv. *syringae* isolates of indicating that all groups of *P. syringae* pv. *syringae* isolates are gram negative (table-2).

Gram staining test: The Gram's staining reaction was performed using crystal violate. The microscopic results showed that all of the isolates of *P. syringae* pv. *syringae* did not retain violet color i.e. the isolates retained counter stain (pink color). Therefore, all isolates of *P. syringae* pv. *syringae* representing each group are gram negative and straight or curved rod shaped which is the characteristic feature of any plant pathogenic bacteria (table-2).

Kovac's oxidse test: In this study result showed that all group of *P. syringae* pv. *syringae* isolates were not able to develop deep blue color with oxidase reagent within few seconds which indicated that the result of the test was positive for *P. syringae* pv. *syringae* isolates (table-2).

Temperature sensitivity test: Isolates of *P. syringae* pv. *syringae* grew at 27°C but failed to grow at 37°C and 41°C while conducting temperature sensitivity test (table-2).

Levan test: The result showed that all group of *P. syringae* pv. *syringae* isolates were able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (table-2).

Sugar utilization test: The results of Sugar fermentation test clearly showed that all group of *P. syringae* pv. *syringae* isolates obtained from the diseased mango leaves samples were able to oxidize the four (4) basic sugars (Dextrose, sucrose, manitol and lactose) by producing acid and gas(reddish to yellow) (table-2).

Arginine dihydrolase activity: Results of this test showed that the isolates *P. syringae* pv. *syringae* were not able to raise the pH and change the color (table-2).

Catalase test: Results of this test showed that the isolates of *P. syringae* pv. *syringae* were able to raise bubbles on the slide and indicated as positive reaction (table-2)

Pectolytic test: This results showed that *Pseudomonas syringae* pv. *syringae* does not respond against the pectinase enzyme as *Erwinia sp*. does (table-2).

Figure-1
Map showing the areas surveyed for the field based assessment of leaf blight of mango occurring in Bangladesh

A B C D E

Figure-2
Photographs showing the symptoms of bacterial leaf blight of mango from A. =Dinajpur, B. = Rajshahi, C. =Khulna, D. =Mymensingh and E. =Madhupur

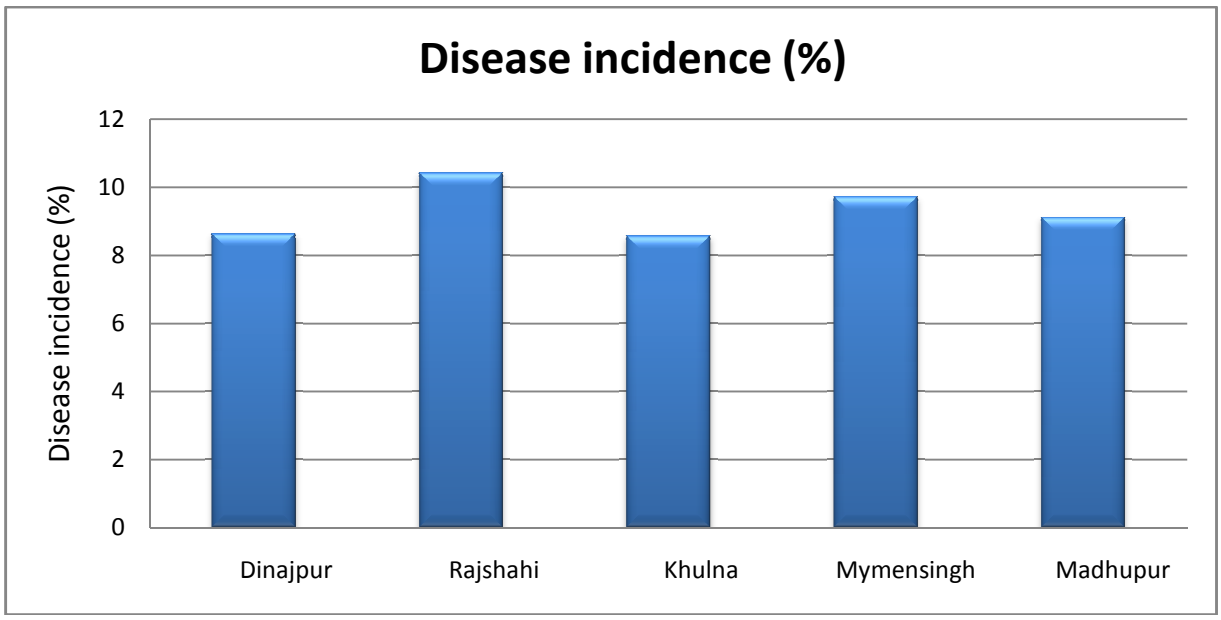

Figure-3
Incidence of bacterial leaf blight of mango in some selected areas of Bangladesh

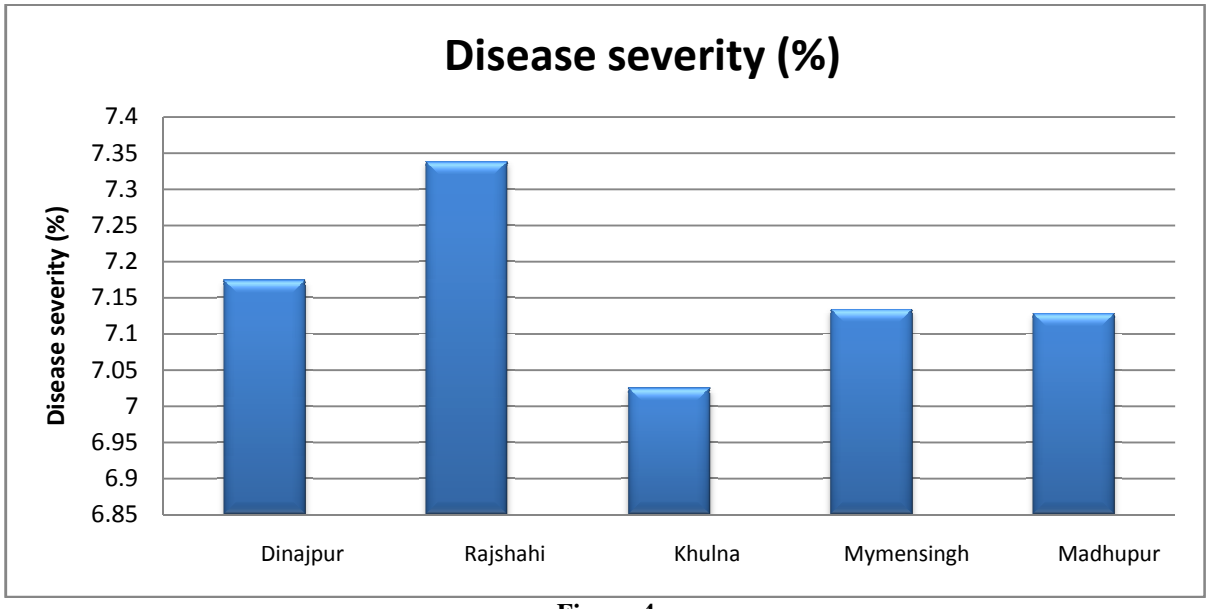

Figure-4
Severity of bacterial leaf blight of mango in some s elected areas of Bangladesh.

Table-2
Biochemical tests of *Pseudomonas syringae* pv. *syringae* isolates causing leaf blight of mango in Bangladesh

Isolate name	Pathogenicity test	Host range Test	Gram staining test	KOH solubility test	Kovac's oxidase test	Levan test	Arginine dihydrolase test	Catalase test	Temperature sensitivity test(°C)			Sugar utilization test				Inference
									27	37	41	Sucrose	Lactose	Maltose	Dextrose	
Group 1	+	-	+	+	-	+	-	+	+	-	-	+	+	+	+	*Pseudomonas syringae* pv. *syringae*
Group 2	+	-	+	+	-	+	-	+	+	-	-	+	+	+	+	*Pseudomonas syringae* pv. *syringae*
Group 3	+	-	+	+	-	+	-	+	+	-	-	+	+	+	+	*Pseudomonas syringae* pv. *syringae*
Group 4	+	-	+	+	-	+	-	+	+	-	-	+	+	+	+	*Pseudomonas syringae* pv. *syringae*
Group 5	+	-	+	+	-	+	-	+	+	-	-	+	+	+	+	*Pseudomonas syringae* pv. *syringae*

Discussion: Bacterial leaf blight was identified as a new disease of mango in many nurseries of Bangladesh. The survey results indicated a regional variation in bacterial leaf blight incidence and severity. These variations of leaf blight incidence and severity may be attributed to the diversity of *P. syringae* pv. *syringae* isolates and also due to the host genotypes and variations of environmental factors prevailing in different locations surveyed.

The different groups of isolates of *P. syringae* pv. *syringae* were confirmed as the causal organism of bacterial leaf blight of mango by pathogenicity test and different biochemical tests. Lelliott and Stead[12] confirmed *P. syringae* pv. *syringae* as the causal organism of leaf blight of mango by pathogenicity test. *P. syringae* pv. *syringae* was also confirmed as leaf blight pathogen of mango by pathogenicity test on mango leaves as reported by Cazorla et al.[13]. Gvozdyak et al.[14] showed the hypersensitive response on the tobacco leaf and the hypersensitive reaction is most distinctly manifested when 1 to 3 days old bacterial cultures of *P. syringae* pv. *syringae* were used. None of the isolates in this study induce HR in either tobacco or chilli leaves. The other cultivars of tobacco need to be tested to find out the clues. However, it may be explained that the cultivars of tobacco and chilli used in this study for HR may not possess the corresponding resistance gene against the effectors. Suslow et al.[10] reported that the KOH technique is far easier and faster to distinguish gram negative and gram positive bacteria than the traditional Gram-strain in which dyes are employed. KOH solubility test indicated that the isolates of *P. syringae* pv. *syringae* are gram negative. Hassett, et al.[15] also reported that all species and strains of *Pseudomonas* are Gram-negative rod shaped, and have historically been classified as strict aerobes. Kovacs[11] reported that most non-pathogenic Pseudomonads are positive, whereas pathovars of *Pseudomonas syringae* and *P. savastanoi and P. viridiflava* are negative. In this study different isolates of *Pseudomonas syringae* pv. *syringae* did not grow at the temperature 37° C and 41^0 C. This result is in accordance with the opinion given by Ayres et al.[16] (1919). The result of sugar utilization test is supported by many previous scientists like Young et al.[17] who reported that the isolates of *P. syringae* pv. *syringae* were able to produce round or circular domed shaped colonies in sucrose medium. Ayres et al.[16] reported that *P. syringae* pv. *syringae* was able to ferment four basic sugars (Dextrose, sucrose, manitol and lactose) by oxidizing and to produce acid and gas in carbon source utilization test. The acid production in sugar fermentation test by *P. syringae* pv. *syringae* isolates was indicated by the color change from reddish to yellow; gas production was noted by the appearance of gas bubbles in the inverted Dhuram's tubes and the oxidation of sugar manitol by the *P. syringae* pv. *syringae* isolates indicated by the production of red to yellow color. The pathogen *P. syringae* pv. *syringae* showed negative reaction in arginine dihydrolase test which clearly goes with the work of Thornley[18]. Catalase test for *P. syringae* pv. *syringae* isolates obtained from mango leaves gave positive reaction by the appearance of arising bubbles. The pectolytic test evidently showed that all groups of isolates causing leaf blight of mango was *P. syringae* pv. *syringae*. Bradbury[19] also reported that pectolysis *P. syringae* pv. *syringae* has no ability to degrade the pectolytic substance of potato at the point of inoculation after 24 hrs at 22^0 C while the test was observed positive in case of *Pseudomonas viridiflava*, *P. marginalis* and *E. carotovora* subsp. *carotovora.* The reason for not producing soft rotting symptoms on sliced potato tuber by *Pseudomonas syringae* pv. *syringae* is the absence of pectinase enzyme. It is caused because, *P. syringae* pv. *syringae* infecting mango leaves not contain too high pectic substance as like as in potato.

Conclusion

The findings of the study clearly indicated that leaf blight of mango is caused by *Pseudomonas syringae* pv. *syringae* which was observed in all nurseries of different locations surveyed. The disease seems a great threat for raising quality and healthy mango saplings in order to get higher fruit production. However, the management strategies still not developed due to lack of the identification of the causal organism. The findings of the present study would definitely be useful to design a

comprehensive molecular based analysis of the pathogen *Pseudomonas syringae* pv. *syringae* and to adopt a proper management strategy suitable for the integrated disease management programs.

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