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RESEARCH ARTICLE

BIOCHEMICAL CHARACTERIZATION OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* OF OKRA (*ABELMOSCHUS ESCULENTUS* L.) MOENCH IN RAJASTHAN

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ABSTRACT

Bacterial leaf blight disease of okra caused by *Pseudomonas syringae* pv. *syringae* is an emerging disease and great threat for production of okra in Rajasthan. A survey was conducted to know the status of bacterial leaf blight of okra in terms of its incidence and severity and to characterize the pathogen causing the disease. Eighty nine seed samples of okra (*Abelmoschus esculentus* L.) Moench were collected from 15 major okra growing districts of Rajasthan and subjected to dry seed examination. All the seed samples were categorised as asymptomatic, bold discoloured and heavily discoloured seeds collected during 2011; 2012 and 2013 growing seasons of okra. The samples were collected from farmer's field, market and farm storage (houses). Eighty nine seed samples of okra belongings to 15 districts of Rajasthan, India revealed asymptomatic (15.25-83.75%), bold discoloured (07.5-54.25%) and heavily discoloured seeds (02.25-34.75%) in dry seed examination. The discoloured seeds included brown black or water soaked symptoms and such seeds on incubation yielded the bacterium. At seedling stage, seedling shows failure or delayed seeds germination, bacterial oozing and rotting that resulting collapse of hypocotyls and cotyledonary leaves that resulting seedling mortality. So the seed losses seed germination, seedling are symptomatic that increase seedling mortality. High incidence of pathogen was found in Jhalawar, Jaipur, Kota, Sawai Madhopur and Bharatpur. Twenty four bacterial isolates were obtained from the infected seeds and plant material collected from field during the survey. Pathogenicity test on host plant leaves confirmed the bacterium was *Pseudomonas syringae* pv. *syringae* (PSS) but HR test (hypersensitivity test) in tobacco leaves with pathogen isolates showed weak positive reaction of tissues where infiltrated. The causal bacterium was also characterized by biochemical tests and results of the all the tests revealed that the causal organism of leaf blight disease of okra was *Pseudomonas syringae* pv. *syringae.*

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INTRODUCTION

Okra (*Abelmoschus esculentus* (L.) Moench syn. *Hibiscus esculentus* L. of family Malvaceae is an important vegetable crop of Rajasthan. It is a native of the Africa still found growing wild along the river Nile in Egypt as well as in Ethiopia. It is also grown all over the tropics and sub-tropic. India is the world's second largest producer of vegetables next to China. In India, it is grown over an average of 4.52 lakh in mts with a production of 43 Lakh tones (Anonymous, 2011). The important producing states of okra in India are Haryana, Assam, Maharashtra, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal. At present, it is grown in many tropical, subtropical and warmer temperate areas and countries (Kochhar, 2004).

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Leaf blight disease is one of the predominating disease that lower production, nutritive and market value of the crops. The symptoms of bacterial blight disease are characterized by a rapid enlargement of necrotic lesions on leaves. Disease symptoms comprise necrotic lesions on tender leaves, occasionally outspread to the leaf petiole. The causal agent to be the bacterium *Pseudomonas syringae* pv. *syringae.* Crop suffers from a number of phytopathogenic fungal and bacterial species causing severe losses, reduces plating and market value of okra seeds (Schaad and Kendrick, 1975, Neergaard, 1987, 1986, Bradbury, 1986, Schaad, 1989, Richardson, 1986, Agrawal, 2000, Sharma, Jain, Jain and Sharma, 2013).

MATERIALS AND METHODS

A survey was carried out to know the status of bacterial leaf blight during 2011; 2012 and 2013 growing seasons of okra from farmer's field. All the naturally infected seeds and plant parts of okra were collected at different stages of growth from

farmer's fields, houses and market. All the seed samples were categorised as asymptomatic, bold discoloured and heavily discoloured seeds for the study. The seeds and plant material from various fields were collected and brought to laboratory for the isolation of the pathogen (s). The infected seed samples and infected plant tissues of leaves and fruits were surface sterilized and plated on Petriplates containing NA (Nutrient Agar) Agar medium under aseptic conditions. Eighty nine seed samples of okra and infected plant material collected from 15 districts of Rajasthan were subjected to dry seed examination, incubation on moistened blotters and KMB agar medium to find the incidence of PSS in okra.

The pure typical bacterial colonies were incubating at 30^{o}C for 72 hrs. were subjected to various tests namely gram's staining, KOH solubility test, levan formation, oxidase test (Kovacs, 1956), potato soft rot test, nitrate reductase test, arginine dihydrolysis, gelatin hydrolysis test, hypersensitivity test in tobacco and pathogenecity tests (Lelliott and Stead, 1987) for the identification of the bacterial species. For all the tests 24-48 h old cultures (Lelliott and Stead, 1987) and bacterial suspensions were used. The bacterial isolates identified by various methods as described above were subjected to pathogenecity tests (Schaad, 1989) on the host plant and other plant species. The infected plant material like leaves, stem and fruits were surface disinfected and then rinsed in sterile distilled water. The Bacterial isolates were purified by streaking a single colony of each isolate on NA medium (Klement and Goodman, 1967). The isolates of bacteria were preserved for subsequent biochemical studies.

Characterization of *Pseudomonas syringae* pv. *Syringae*

Pathogenicity test

The pathogenicity test was performed on the detached leaves of okra. For the pathogenicity test single colony of PSS showing virulent, fluidal, irregular and creamy white colour was selected for each group of isolates and multiplied in a NA medium. The 24 hours old bacterial cultures were suspended in sterile water was infiltered in leaves. In another experiment, the tip of the leaves was cut, dipped into bacterial suspension for 24hr and incubated in a Petridish at room temperature for at least 3-5days for the appearance of symptom. The host range of PSS isolates were tested in some other crops like chilli, tomato, brinjal for pathogenicity test. This test was performed to observe the reaction or infection ability of Pathogen on other than okra. Hypersensitivity response (HR) test: To determine the pathogenic nature of the isolates, hypersensitive reaction was studied on tobacco (*Nicotiana tabaccum)* plants by infiltration of bacterial suspension into the intrveinal areas of the tobacco leaves (Klement and Goodman, 1967).

Biochemical Studies for of PSS

In Gram staining reaction, 24 hrs old bacterial cultures for each isolates were taken to perform the gram reaction test and add one drop emulsion oil was then added and observed. Gram staining results were confirmed by potassium hydroxide test (3% KOH) [15]. 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. For oxidase test a loopful of bacterial colony (24hrs old) on nutrient agar taken and the inoculum was rubbed on filter paper pervaded with 15% (w/v) freshly prepared aqueous solution of Tetramethyl-p-phenylene diamine dihydrochloride (Lelliott and Stead, 1987). In fluorescence test the ability of bacterial isolates to produced fluorescent pigment was studied on King's medium B. The medium contained proteose peptone (20.0 mg), $K_2HPo_4.3H_2O$ (1.5g), $MgSO_4.7H_2O$ (1.5 g), glycerol (15.0 ml) for 1 litre medium. Plates were incubated at 27^{0}C and the colonies were obtained after 3-4 days. The ability of bacterial isolates to grow at different selected temperature range at 27°C, 37°C and 41°C were tested by initially growing isolate on NA for 24h and transferred to nutrient broth. For levan test Sucrose Peptone Agar or Nutrient Agar medium with 5% sucrose is suitable substrate. 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^{o}C for 2 to 4 days to have distinctive dome shaped colonies.

For study of sugar utilization, 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 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 and autoclave. The sugars used for fermentation test were prepared separately as 10% solution in distilled water. 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^{o}C. The test was performed by inoculating a loop full of 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^{o}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. To study of Arginine dihydrolase activity, 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^{0}C.

A color change from faint pink to red within four days is positive reaction. The catalase test was performed to isolates to check their liveliness. This enzyme is responsible for protecting bacteria from hydrogen peroxide (H_2O_2) buildup, which can arise during aerobic metabolism. This enzyme breakdown H_2O_2 into H_2O and O_2. One milliliter of 3% hydrogen peroxide (H_2O_2) was placed on the microscope slides and added a loopful of 24 hrs old bacterial culture for catalase activity. Bubbles' arising from the solution was recorded as positive reaction. The pectolytic activity was studied by using well washed, healthy, firm potatoes; surface sterilized and was peeled aseptically. Three standard slices were placed in sterile petridishes and incubated in a moist chamber. Two slices were then inoculated with loopful of bacteria of each isolate and

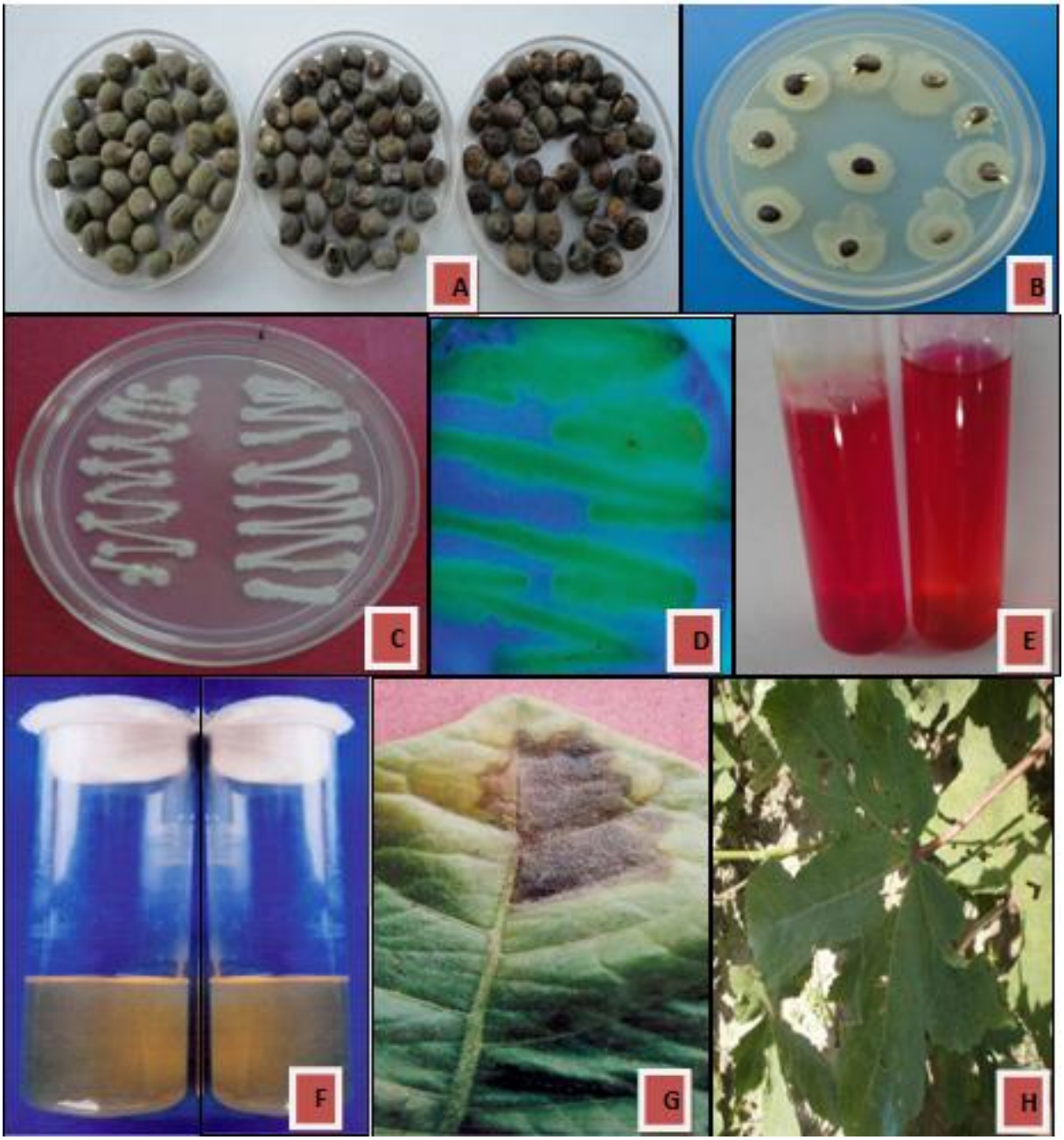

Fig.1. Identification and Infection of *Pseudomonas syringae* pv. *syringae* in okra seeds, (A= seed categorization into asymptomatic (left), moderate discoloured (middle) and heavily discoloured (right), B= characteristic of off white colonies on and around seed on incubation on NA medium, C= pure bacterial colony NA medium, D=bacterial colonies showing fluorescence on KMB medium, E=Arginine dihydrolase test, F= gelatin liquefaction test, G= HR test on tobacco leaf after 10 days, H= symptoms of leaf spots in field.

Fig. 1. Identification of *Pseudomonas syringae* pv. *syringae* using morphological and biochemical characterstics

incubated at room temperature for 24-48 hrs for the detection of soft rot symptoms. Pectolytic activity was performed to observe the condition of the sliced potato. To study the gelatin liquefaction test, gelatin medium containing beef extract (13 g), peptone (5 g), gelatin (120 g) added in 1 litre distilled water was used. The test tube containing 4-10 ml medium sterilized incubated at 27^0C.

RESULTS

A total of 89 seed samples of 15 districts revealed asymptomatic (15.25-83.75%), bold discoloured (07.50-54.25%) and shrivelled-discoloured (02.25-34.75%) seeds (Fig. 1 A, B). The discolourations varied from brown to black spots and water-soaked translucent shining areas (Fig. 2 A). The incidence of pathogen was observed (10-100%) on KMB agar medium (fig.1 b, c). The symptomatic seeds on incubation yielded the growth of PSS. Heavily discoloured seeds on bisecting found distorted embryo and cotyledons. The bacterial colonies isolated from various seed samples were produced off white, circular, entire, shiny and raised colonies on Nutrient Agar. Bacterial leaf blight was identified as a new disease of okra in Rajasthan (Fig. 1 H). During field visit the symptoms were observed on buds, leaves and stems but lesions on fruit have not been observed (Fig. 2 b-f). Necrotic lesions on leaves

Fig. 2. Infection of *Pseudomonas syringae* pv. *syringae* in okra seeds, (A= seed categorization into asymptomatic (extreme left), moderate discoloured (middle two rows) and heavily discoloured (right two rows), B=symptoms showing in standard blotter method, C-F= symptoms on seedling in the field at cotylodenary leaves, leaf and fruits, G= bacterial culture in conical flasks, H=effect of culture filtrates on seeds germination.

Figure 2. Effects and symptoms of *Pseudomonas syringae* pv. *syringae on seed, seedling and plant*

Table 1. Biochemical tests of *Pseudomonas syringae* pv. *syringae* isolates in okra causing leaf blight in Rajasthan

Isolates name	Gram staining test	KOH Solubility Test	KMB Fluores cence test	Levan test	Kovac's oxidase test	Arginine dihydrolase test	Catalase test	Host range Test	Patho Genicity test	Temperature sensitivity test (oC)			Sugar utilization test				inference
										27	37	41	D	L	S	M	
A	+	+	-	+	-	-	+	-	+	+	-	-	+	+	+	+	PSS
B	+	+	-	+	-	-	+	-	+	+	-	-	+	+	+	+	PSS
C	+	+	-	+	-	-	+	-	+	+	-	-	+	+	+	+	PSS
D	+	+	-	+	-	-	+	-	+	+	-	-	+	+	+	+	PSS
E	+	+	-	+	-	-	+	-	+	+	-	-	+	+	+	+	PSS

D=dextrose, L=lactose, S=sucrose, M=maltose, PSS= *Pseudomonas syringae* pv. *syringae,* KOH Solubility test

sometimes spread to the leaf petiole or up to stem. Lesions on leaves start as interveinal, angular, water-soaked spots that coalesced, becoming blight symptoms (Figure 2). Morphological appearance preliminary showed that okra with bacterial leaf blight have shown the presence of bacterial strains resembling *Pseudomonas syringae* pv. *syringae*.

Isolation and identification of the *Pseudomonas syringae* PV. *syringae* isolates

A total of fourteen isolates of PSS were obtained from the infected seed samples of okra collected from 15 districts surveyed. All the 24 isolates classified in 5 groups, isolated from infected okra seeds and leaves produced cream color or off-white color colonies on NA media after 24 hours of inoculation.

Confirmative test of *Pseudomonas syringae* PV. *syringae*

Pathogenicity test: The results of pathogenicity test revealed that all the isolate groups of PSS were able to cause blight symptoms on okra leaves identified by observing dark brown to black blight areas on the leaves (Table-2, Fig. 2 A). Host

range test: The host range of PSS was determined by pathogenicity test on detached leaves of another host like chilli, tomato, brinjal etc. The result showed PSS causing blight symptoms on okra leaves but not produce any symptom on the leaves of these said hosts (Table 2). The results of Hypersensitivity response (HR) test showed that none of the isolates were able to induce HR of tissues into the interveinal areas between of tobacco (Fig. 1 g).

Biochemical tests

Potassium hydroxide solubility test revealed that an elastic thread or viscous thread was observed by all PSS isolates and gram negative (Table 2). Gram staining test showed that all of the isolates of PSS are gram negative and straight or curved rod shaped which is the characteristic feature of any plant pathogenic bacteria (Table 2). The result of oxidse test showed that all 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 pv. *syringae* isolates (Table 2). In fluorescence test, a fluorescent green pigment, water soluble under long wave length (366 nm) under UV lights was observed and result found positive. Gelatin liquefaction test containing bacterial pathogen was found liquefaction negative. Temperature sensitivity test showed that the isolates of PSS grew at 27°C but failed to grow at 37°C and 41°C while conducting temperature sensitivity test. The results of levan test showed that all isolates were able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (Table 2). The sugar utilization test clearly showed that all group of isolates obtained from the diseased okra seeds, twig and leaves were able to oxidize the four (4) basic sugars (dextrose, sucrose, manitol and lactose) by producing acid and gas (red yellow) (Table 2). Arginine dihydrolase activity by the bacterium showed the results that isolates of PSS change pH and color (Table 2, Fig. 1e). The test was performed to observe the presence of two enzymes that permits certain Pseudomonads to grow under anaerobic conditions. The catalase test showed that the isolates of PSS were able to raise bubbles on the slide and indicated as positive reaction. This results of pectolytic test showed that pv. *syringae* does not respond against the pectinase enzyme as *Erwinia sp*. does (Table 1).

DISCUSSION

Bacterial leaf blight disease was reported from Australia (Goss (Olga, 1962); Brazil (Kimura, Ribeiro and Robbs, 1982), Romania (Stancescu and Zurini, 1987) in *Hibiscus esculentus* caused by *Pseudomonas syringae* pv. *syringae.* Hibiscus leaves var. Apple Blossom commonly affected by purplish-black recorded dots 7.25 inch in diameter. The symptoms appeared on fruits and leaves under low RH and high temperature conditions. *Pseudomonas syringae* was found associated with *Xanthomonas campestris* pv. *esculenti* on leaves increasing the severity of the leaf blight. Seeds were smaller, irregularly shaped, chestnut coloured and strongly fluorescent under UV light (Kimura, Ribeiro and Robbs, 1982, Stancescu and Zurini, 1987). Bacterial leaf blight was identified as a new disease of okra in Rajasthan. The survey results indicated a regional variation in bacterial leaf blight incidence and severity, these variations of disease incidence and severity may be attributed to the diversity of (*Pseudomonas syringae* pv. *syringae*) PSS 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 okra by pathogenicity test and different biochemical tests. It was showed the hypersensitive response on the tobacco leaf and the hypersensitive reaction is most distinctly manifested when 1 to 3 days old bacterial cultures were used (Gvozdyak *et al.,* 2001). None of the isolates in this study induce HR in either tobacco or other host leaves. 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.

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 (Suslow Schroth and Isaka, 1982) and this test indicated that the isolates of *P. syringae* pv. *syringae* are gram negative. All the species and strains of *Pseudomonas* are Gram negative rod shaped, and have historically been classified as strict aerobes (Hassett *et al.,* 2002). Most non-pathogenic Pseudomonads are positive, whereas pathovars of *Pseudomonas syringae* and *P. savastanoi and P. viridiflava* are negative (Kovacs, 1956). In this study different isolates of *Pseudomonas syringae* pv. *syringae* did not grow at the temperature 37^{o} C and 41^{0} C (Ayres *et al.,* 1919). The isolates of *P. syringae* pv. *syringae* were able to produce round or circular domed shaped colonies in sucrose medium (Young *et al.,* 1994). It had been 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 (Ayres *et al.,* 1919). The acid production in sugar fermentation test by PSS 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 PSS isolates indicated by the production of red to yellow color.

The pathogen PSS showed negative reaction in arginine dihydrolase test (Lelliott and Stead, 1987). Catalase test for PSS isolates obtained from okra leaves gave positive reaction by the appearance of arising bubbles. The pectolytic test evidently showed that all groups of isolates causing leaf blight of okra was *P. syringae* pv. *syringae*. Bradbury (1986) 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* (Bradbury, 1986). 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 okra leaves not contain too high pectic substance as like as in potato. Such type of study was also reported in mango leaves from Bangladesh (Islam *et al*., 2003).

Conclusion

The findings of the study clearly indicated that leaf blight of okra is caused by *Pseudomonas syringae* pv. *syringae* seems a great threat for raising quality and healthy okra plants 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 and to adopt a proper management strategy suitable for the integrated disease management programs.

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