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Occurrence, detection and transmission of *Pseudomonas syringae* pv. *lachrymans* from the seeds of Cucumber (*Cucumis sativus* L.) in Rajasthan

Poonam Arora[1] and Dilip Kumar Sharma*[2]

[1]Department of Botany, Agarwal P.G. College, Jaipur-302 003 Rajasthan, India
[2]Vardhman Mahaveer Open University, Kota, Rajasthan, India

Abstract

The aim of the study is to know the diseases symptoms, incidence, and transmission of seed-borne bacteria (Pseudomonas syringae pv. lachrymans, PSL) from the seeds of cucumber (Cucumis sativus L.) that belongs to plant family Cucurbitaceae. The causal agent PSL causes the Angular leaf spot (ALS) disease. A total of 102 seed and, 50 fruit samples were collected by a major and minor survey from various districts of Rajasthan to study the disease incidence. The collected seed samples were categorized in asymptomatic (healthy-looking) and symptomatic (discolored seeds) in dry seed examination using a stereo-binocular microscope. The collected seed samples revealed 20-100% incidence of bacteria on Kings Medium B. On the basis of morphological, physiological, chemical, molecular and pathogenicity test the bacterium find to be Pseudomonas syringae pv. lachrymans. PSL attack caused tiny, round to irregularly shaped, water-soaked lesions with a yellow halo to appear on cotyledonary leaves and other plant components, depending on disease severity. In the pot experiment and field investigation it was found that the bacterial pathogen produced tiny yellow spots covered by halo on leaves which became necrotic at disease severity. As the disease progresses, the whole leaf collapses due to the reduction of photosynthesis capacity and photosynthetic area of the leaf and finally reduces the yield of the crop.

Keywords: *cucumber (Cucumis sativus L.), Pseudomonas syringae pv. lachrymans (PSL), disease transmission, angular leaf spot, seed discolourations, leaf spots, fruit symptoms.*

I. Introduction

The cucumber (Cucumis sativus L.), a member of the Cucurbitaceae family, is a primitive vegetable that originated in India and is consumed as a cooked or salad produce. [1]. It is a wide-ranging and heterogeneous family that comprises worldwide 118 genera and 825 species[2,3] and 36 genera and 100 species in India[4]. It is widely known as Kheera and Gherkins in the tropics, subtropics and temperate zones of India[5]. In India cucumber are grown as a vegetable that is used for domestic purpose and exported to other countries for foreign income. Bharatpur, Jaipur, Bikaner, Dausa, Hanumangarh, Pali, Sawai Madhopur, Sikar, Sirohi, Karauli and Dholpur are major cucumber-producing districts in Rajasthan.

The disease angular spot (ALS) in cucumber was first discovered in the United States in 1913 and the pathogenic organism was identified in 1915 by Smith and Bryan. In Japan, it was first reported in 1957. In Turkey, it may cause considerable yield losses in both greenhouses and field agriculture [6,7]. The crop is invaded by several fungi, bacteria, viral diseases which reduced the quantitative and quality values of the crop. ALS is caused by *Pseudomonas syringae* pv. *lachrymans* (Smith & Bryan) Young, Dye & Wilkie. The complex species *Pseudomonas syringae* divided into 64 pathovars on the basis of pathogenic characters, and *Pseudomonas syringae* pv. *lachrymans* (PSL) is one of them[8-14]. Following the pathogen's attack, the cucumber leaves developed vein-limited, water-soaked lesions with or without a chlorotic halo. Water-soaked spots on fruits that could be deformed[15-16]. Angular leaf spot (ALS) is limiting its open-field production[17-18]. It could result in large yield reductions in both field and greenhouse crops[7]. Cucumber and other cucurbit producers in Turkey suffered significant harm and financial loss as a result of the disease's proliferation-promoting climate[19, 20]. It caused water-soaked blisters on the leaves, which eventually turned necrotic and decreased the leaf's ability to photosynthesize[21,22]. Depending on the species' susceptibility, ALS can cause yield losses of up to 30%–60% in fruits by reducing the ability to photosynthesis of diseased leaves[16]. Once attacked by PSL on the cucumbers the yields decrease significantly, caused by reduced photosynthetic capacity of the infected foliage, and the disease is difficult to control[22]. In China, during 2014-2016, the disease incidence varied from 15 to 50% in different fields, causing 30–50% of yield losses[14]. The disease is responsible for economic losses in cucumber production worldwide[9, 21]. With 2.1 million hectares and 71.3 million tons produced in China, the USA, and the EU, uninfected cucumber farming is extremely important[23].

The bacterium is Gram negative and KOH solubility test negative but levan and catalase are positive. It is oxidase, potato rot; nitrate reduction and arginine dihydrolase negative. The bacterium is non-fluorescent, non-hydrolyzing of starch and gelatin[24]. The goal of this research was to examine the transmission and detection of the disease, and this study was carried out because PSL needs appropriate detection

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Dilip Kumar Sharma | drdilipsharma12@gmail.com

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techniques to enhance effective management strategies.

Pathogen isolation and biochemical identification are the primary detection methods, although molecular approaches like rep-PCR and PMA-qPCR have been reported to be employed for PSL detection [25-27]. In addition to offering quick and sensitive detection, loop-mediated isothermal amplification assays (LAMP) [28-31] have been widely used for plant pathogen detection because they can be used outside of traditional laboratory settings and offer a variety of detection strategies [32].

II. Materials and Methods

(I) Incidence of pathogen

All 102 seeds, 50 fruits samples and various infected plant parts of cucumber collected from storage houses, market and farmers field of 16 districts of Rajasthan, were brought to the laboratory to know the disease sign on various parts of plant. In order to isolate the pathogen, the seed samples and other plant components were surface sterilized and incubated under aseptic conditions on moistened blotter papers in the context of Petri plates and Nutrient Agar media. To isolate the pathogen, all of the cucumber seed samples underwent dry seed examination (DSE) and were incubated on a moistened blotter in SBM [33]. The pure form of bacterial colonies were incubated at 30^{o} C for 48 hrs, and were used for various biochemical tests *viz.* Gram's staining, KOH solubility test, and LOPAT[24,34-35], and pathogenicity test[24] to identify of the species of bacteria. The host plant and other plant species were used to investigate the pathogenicity of the pure bacterial isolates that were found using different techniques. The diseased fruit and other plant parts are subjected to incubate on NA media and moistened blotter papers to know the characterization of the bacterial colony i.e. shape, size, elevation, color, pigmentation, margin, and growth pattern.

(ii) Disease transmission

For transmission tests, two naturally infected cucumber seed samples (Lab. ac. no. CU-1412 and CU-1420) with 78 and 82% infection on the standard blotter method (SBM) and 94–100% on Kings medium B were chosen. The 100 seeds per category per sample were sowed on moist blotters (10 seeds/plate) and 1% water agar medium in test tubes (1 seed per test tube, TTSST). The seeds were then incubated at 25±2o C for 12/12 h cycles of light and darkness up to 7 and 14 days, respectively. In the pot experiment, 100 seeds per category per sample were planted in pots (two seeds per pot), and information on symptoms, seed germination percentage, and death was noted. At various phases of plant growth, the pathogen was separated from the affected portion of the plant.

(iii) Pathogenicity test

The bacterial isolates were artificially inoculated utilizing methods like smothering of seeds, stab inoculation of seedlings at the 3-4 leaf stage of the crop, and other plant parts. The host and other plants, such as round, bitter, bottles, and sponge gourds, were used to test the pathogenicity. Cucumber seeds that were susceptible to PSL were planted in plastic pots with sterilized soil and kept in a standard development chamber at 250°C during the day and 220°C at night. Sodium lamps were used to illuminate the pots for 16 hours[17]. The bacterial inoculums yielded for 24 hrs on Kings medium B (agar medium) at 28^{0}C was used. An optical density (OD 600) of 0050 is estimated by the plate-count technique107 CFU (colony forming unit) ml. for the aqueous suspension in sterile distilled water with an adjusted concentration of 1×107 CFU (colony forming unit) ml. Cucumber plants with three to four leaves were sprayed on the underside of the leaves to inoculate them [36]. For six days, the triplicate-inoculated plants were housed in the chamber above 90% relative humidity and in the dark. The leaves were graded for the severity and symptoms of the condition after seven days.

III. Results and Discussion

(i) Incidence and identification of pathogen: Molecular Characterization

To identify the molecular sequence of pathogens, *Pseudomonas syringae* pv. *lachrymans* in cucumber 16S rRNA tool was used. High molecular weight DNA was examined under UV light, and DNA quality was assessed on a 1.2% Agarose gel. Using a good heat cycler, isolated DNA was cloned using a primer specific to 16S RNA. There is a single distinct 1500 bp DNA band. PCR amplified product undergoes for enzymatic purification through the Sigma Gen Elute Kit as per protocol further subjected to the sequencing process. The 16S rRNA gene sequence of bacterial pathogens associated with cucumber. These were deposited to Gen Bank assigned with NCBI (National Centre for Biotechnology Information) accession no PU877737.1 (*Pseudomonas syringae* pv. *lachrymans*). Genbank flat file of *Pseudomonas syringae* pv. *lachrymans*strain AF2, 16S ribosomal RNA, complete sequence Genbank: PU877737.1 1500 bp linear BACT. 16SrRNA complete sequence, Length-1495 Score-2296 bits (1495), Identities-1410/1495. Seed and fruit samples were collected from the field and market area from 16 districts of Rajasthan. A total of 102 seed samples and 50 fruit samples were collected and subjected to various tests. The collected samples revealed symptomatic [moderately discolored (04.25-48.25%) and heavily discolored (01-36.25%)] and asymptomatic (16.25-93.25%) seeds (Fig. A, Table 1). The symptomatic seeds showed the growth of PSL on incubation on media (Fig.1) and moistened blotter papers (Fig.1B). Sponge gourd and other cucurbit seeds have also been observed to exhibit similar effects [37-38]. According to morphological, biochemical, and pathogenicity tests, the isolated bacterium was identified as *Pseudomonas syringae* pv. *lachrymans* (PSL). The collected seed and fruit samples showed a 20–100% incidence of pathogen on Kings Medium B. in LOPAT. The disease was determined to be an angular spot on cucumbers, and the molecular characterization confirms that the pathogen is PSL.

ORIGIN

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Biochemically the *Pseudomonas syringae*[39] is a gram-negative (motile by more than one polar flagella) fluorescent pytopathogenic pseudomonad. The organism exhibits pathogenicity, and its growth, especially on iron-deficient media like KMB, creates a diffusible yellow-green pigment that fluoresces when exposed to UV light[40]. Arginine dihydrolase is absent. It produces slime in the media with 2-5% sucrose as a result of Levan formation (Fig.2A) and KOH solubility test (Fig 2B). Tests for nitrate generation and oxidase reaction yield negative results, but tests for potato soft rot, tobacco hypersensitivity, and Kovac's oxidase yield were positive results (Fig. 2C). The ideal temperature range for growth is 25-30° C; growth stops at 41°C. Creamy irregular mucoid colony developed around the seeds (Fig.1C) on KMB agar media with high incidence were recorded showed its wide spread occurrence in Rajasthan. Nearly every portion of the plant showed signs of the disease. On leaves, the symptoms first appeared as marginal chlorotic spots that grew larger as they approached the leaf's center. The chlorotic zone gave rise to necrotic patches that became larger, fused together, and filled the entire lamina. *Pseudomonas syringae* generates a hypersensitivity reaction on tobacco leaves and forms smooth, mucoid colonies on nutrient agar with 5% sucrose. To differentiate between pathogenic and saprophytic (such as *P. fluorescens*) fluorescent pseudomonads, a number of biochemical tests [15,41-43]. The foundation of contemporary microbial identification and characterization is DNA-based methods. Significant genetic variation among *P. syringae* pathovars has been shown using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and repetitive sequence PCR (rep-PCR)[44,45]. Recent rep-PCR genetic studies showed that *P. syringae* pv. *lchrymans* and *P. s.* pv. *syringae* were diverse[43].

Among the isolates of *P. s.* pv. *lchrymans* isolated from cucumber leaves with ALS-infected crops from Poland, three distinct rep-PCR clusters were found. (i) A sizable, internally varied cluster including numerous strains with banding patterns resembling those of *P. savastanoi* pv. *phaseolicola*; (ii) Two strains with banding patterns resembling those of *P. syringae* pv. *syringae*; and (iii) a single strain [43]. AFLP research corroborated this diversity within *P. syringae* pv. *lachrymans* strains by identifying strain groupings that were unrelated to phenotypic traits or geographic origin[46].

(ii) Disease transmission

(a) Petri plate method:

After 48 hours of incubation, a radicle formed. On the seventh day of incubation, the highest seed germination in samples CU-1412 and CU-1420 was 90% and 80% in asymptomatic, 70% and 68% in moderately discolored, and 38% and 30% in significantly discolored seeds, respectively. The ungerminated seeds displayed bacterial ooze, browning, and decaying (Fig. 2D-F). The seedling mortality was 02, 04 and 15% in CU-1412 and 01, 05 and 14% in CU-1420 in the three categories respectively (Fig. 3A, B). It was observed that in the incubation period that the seedlings showed browning at the cotyledonary leaves and redicle.

The cotyledonary leaves show leaf apex browning, rotting after oozing and browning.

(b) Test Tube Seedling Symptoms Test (TSST):
On the fifteenth day of incubation, the germination rates of the three types of seeds on water agar were 91, 65, and 25% in CU-1412 and 85, 59, and 32% in CU-1420. The symptomatic seedling grew very slowly and later developed browning on the plumule and radicle (Fig. 2G, I). In both samples, the mortality rate of seedlings on the fifteenth day was higher for strongly discolored seeds (19 and 22%) than for moderately discolored seeds (08 and 09%) and asympomatic seeds (3 and 3%) (Fig. 3C, D). It was observed that transmission zone of stem and rots showed browning, seedlings collapse and finally died (Fig. 2H).

(c) Pot experiment:
In the pot experiment, seed germination began on the seventh or eighth day and persisted for 20 days in seeds that showed no symptoms. In CU-1412 and CU-1420, the percentage of plantlets that fell after 20 days was highest in severely discolored seedlings (37 and 47%) compared to discolored seedlings (16 and 18%) and asymptomatic seedlings (2 and 3%) (Fig. 3E, F). Up until the fruiting stage, the symptoms were noted. After surface sterilization and incubation on NA agar, symptomatic plant portions produced bacterial colonies (PSL) (Fig. 1C). Similar effects were seen on different plant segments during the field survey (Fig. 2J-L).
Initially the seedlings showed browning, wilting and later on plant collapse. The leaves have tiny yellow or off white spot covered by halo or without a halo. These spots become brown to black necrotic at disease progression. No external symptoms were visible in the fruit's surface but internally the browning at the placental and mesocarp region. In this study the bacterium was also found on the various components of leaves. According to reports, bacteria typically enter cucumber tissues through scars, lenticels, hydathodes, and stomata. It grows in the gaps between cells in the tissues of cucumber plants. Cotyledons, leaves, stems, and fruits may exhibit symptoms locally or systemically[6,20].
This study revealed that the disease symptoms initially manifest as lesions, or water-soaked patches, on the underside of leaves. These angular lesions are confined by tiny leaf veins and are accompanied by a slimy white film that seeps out of the lesions in humid conditions. Similarly, lesions eventually show up on the upper leaf surface as irregular reddish-brown spots that may develop into necrosis and systemic infections that affect all kinds of vascular tissue, according to a number of studies[20]. It was observed in this study during the field survey that in high moisture area or high moisture on the leaf surface vigorously help in the growth of symptoms of the disease. According to similar findings, wind and rain are crucial factors in the pathogen's spread [6, 38]. Water-soaked areas under humid conditions are covered in a white exudates that eventually dries and forms a thin white crust on or next to the location beneath the leaf surface.
Small, round to irregularly shaped, water-soaked lesions were discovered on the leaf surfaces during the field investigation. The spots grow until they are constrained by bigger veins, giving them an angular appearance. According to similar observations, Bradbury (1986) noted that symptoms included vein-limited, water-soaked lesions on cucumber leaves, with or without a chlorotic halo, and water-soaked lesions on fruits that might be deformed[15].
In this study the seed in the fruits containing discolouration at hilum region, attached on the placenta and is supposed to be the hilar region as the possible entry of the pathogen. In the present study as per accordance with the findings of various scientists who reported *Pseudomonas syringae* pv. *lachrymans* was found to be seed-borne in cucumber[37,38,47]. The fruit blemishes may burst open and turn chalky white later on it may facilitates the attack of fungi and bacteria, secondarily cause a slimy, foul-smelling fruit rot. Therefore, it is recommended that cucumber seeds need to be subjected to health test for bacteria before sowing. Plants with bacterial spots occasionally displayed considerable defoliation as a result of a severe infection[48,49].

(iii) Pathogenicity tests:
In pathogenicity test, the seedling showed browning in the radicle and plumule followed by rotting and mortality. Six replicate plants of each cultivar per strain were used for the pathogenicity experiments. Mortality was found in two healthy samples 85 and 80%, respectively after inoculation of bacteria. The symptoms of browning and rotting were observed in bottle gourd, round gourd and cucumber but such prominent symptoms were not observed in bitter gourd. Similar findings showed that the bacteria might cause illness in sweet gourds, wax gourds, bottle gourds, snake gourds, sponge gourds, ridge gourds, and cucumbers[38]. Browning, hypocotyle and radicle rotting, and other signs were seen in the host plant during the pathogenicity test. Similar symptoms were seen in sunflower (*Pseudomonas syringae*) [50], tomato (*Ralstonia solanacearum*)[52], brinjal (*Ralstonia solanacearum*) [51], and chilli (*Ralstonia solanacearum*, *Pseudomonas syringae* pv. *syringae*, *Xanthomonas axonopodis* pv. *vesicatoria*) [53]. Additionally, inoculated leaves showed necrotic lesions and discoloration. 83.3% of seeds were infected following artificial infection, according to Parashar and Sharma (1984)[54].
The lower side of each of the three leaves per strain was sprayed to inoculate cucumber plants at the two to three leaf stage[36, 55]. After 24 hours in the dark with 100% relative humidity, the plants were placed in the same chamber with 90% relative humidity for six days. After seven days, the plants began to exhibit symptoms. The majority of PSL cucumber strains produced similar symptoms when inoculated onto cucumber leaves, according to the pathogenicity test results. However, some strains produced numerous tiny chlorotic lesions surrounding dry, light-colored, papery lesions on the cucumber leaves, which were very different from those caused by the *P. syringae* pv. *lachrymans* reference strains, which are thought to be saprophytic.

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Table 1: Incidence of Pseudomonas syringae pv. lachrymans (PSL) in the seeds of cucumber in Rajasthan

S. No.	Districts	Number of seed samples	Number of seed samples infected by PSL	Incidence of PSL on medium KMB
1	Ajmer	5	05(14.75-34.50)	05(20-60)
2	Alwar	4	04(10.50-17.50)	04(25-45)
3	Barmer	4	04(18.00-25.75)	04(55-80)
4	Bharatpur	5	05(5.75-34.00)	05(30-90)
5	Bhilwara	4	04(20.75-35.50)	04(30-70)
6	Bikaner	5	05(7.00-19.25)	05(65-85)
7	Bundi	4	04(23.25-41.50)	04(80-100)
8	Dausa	10	10(19.75-35.50)	10(60-100)
9	Dholpur	4	04(16.75-24.25)	04(40-90)
10	Jaipur	20	20(5.50-47.50)	20(70-100)
11	Jodhpur	5	05(11.50-20.25)	05(30-80)
12	Pali	6	06(5.75-10.75)	06(30-70)
13	Sawai madhopur	8	08(04.25-48.25)	08(55-100)
14	Sikar	5	05(25.75-29.00)	05(64-100)
15	Tonk	10	10(5.00-26.75)	10(50-70)
16	Udaipur	3	03(16.75-37.00)	03(20-50)
	Total	102	102(04.25-48.25)	102(20-100)

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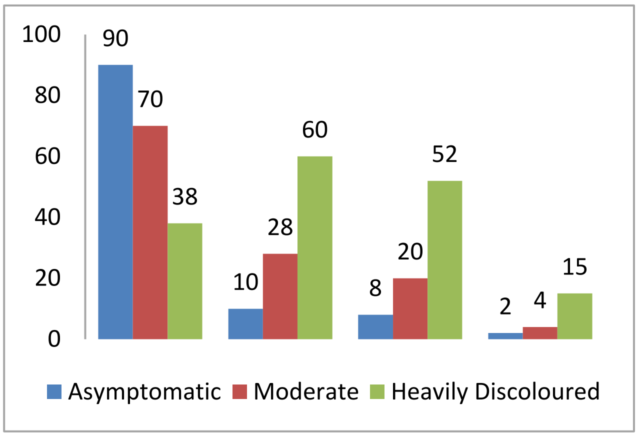
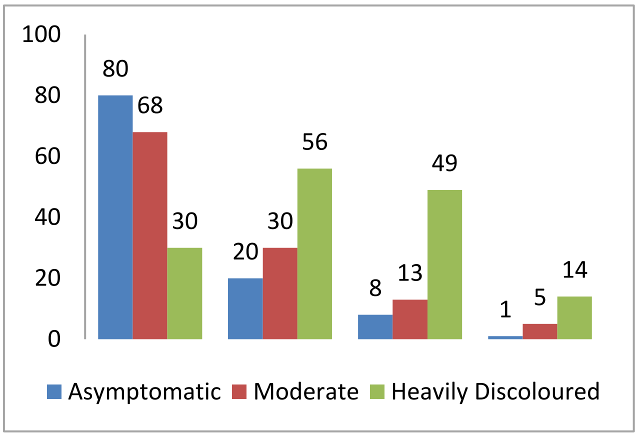
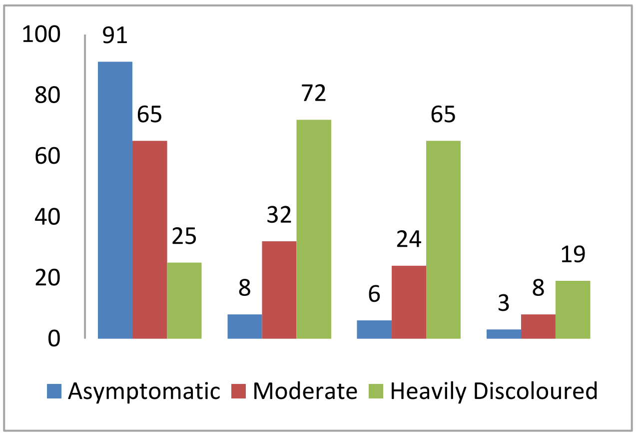
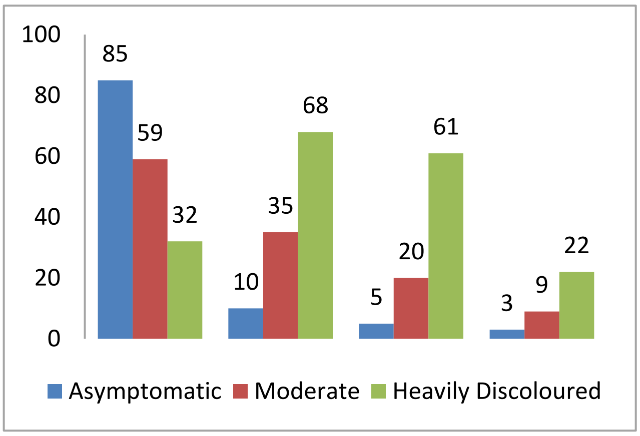




A B

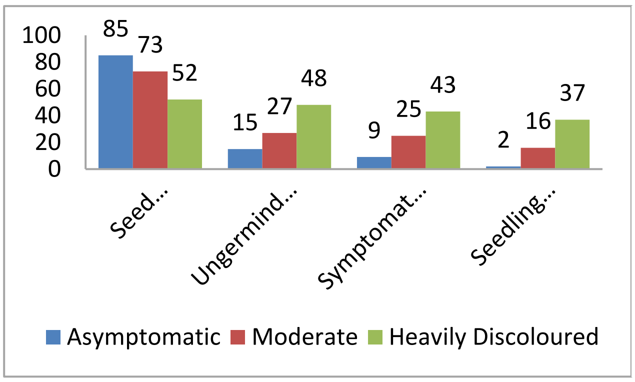
TEST TUBE SEEDLING TEST B

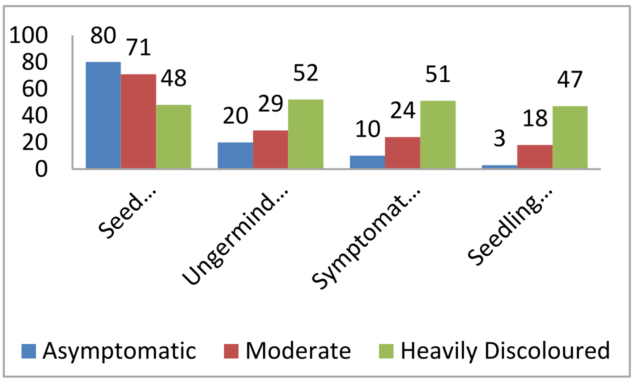




C D

POT EXPERIMENT D





E F

Fig.3 (A-F): Effect on natural seed infection of Pseudomonas syringae pv. lachrymans in Petri plate method, (A) Sample No. CU-1412 and (B) Sample No.CU-1420, on TTSST (test tube seedling symptoms test on water agar), (C) Sample No.CU-1412 and (D) Sample No. CU-1420 and, in pot experiments (E) Sample No. CU-1412 and (F) Sample No.CU-1420

Fig-1: Cucumber seeds infected with Pseudomonas syringae pv. lachrymans (PSL) (A) categorization of seeds of cucumber in asymptomtic, moderately discoloured and heavily dicoloured, (B) Symptoms on Standard Blotter Method (SBM), (C) Characteristic of creamy off white, irregular colonies around the seeds on NA medium left, bacterial colonies around the heavily infected seeds from pot experiment on KMB.

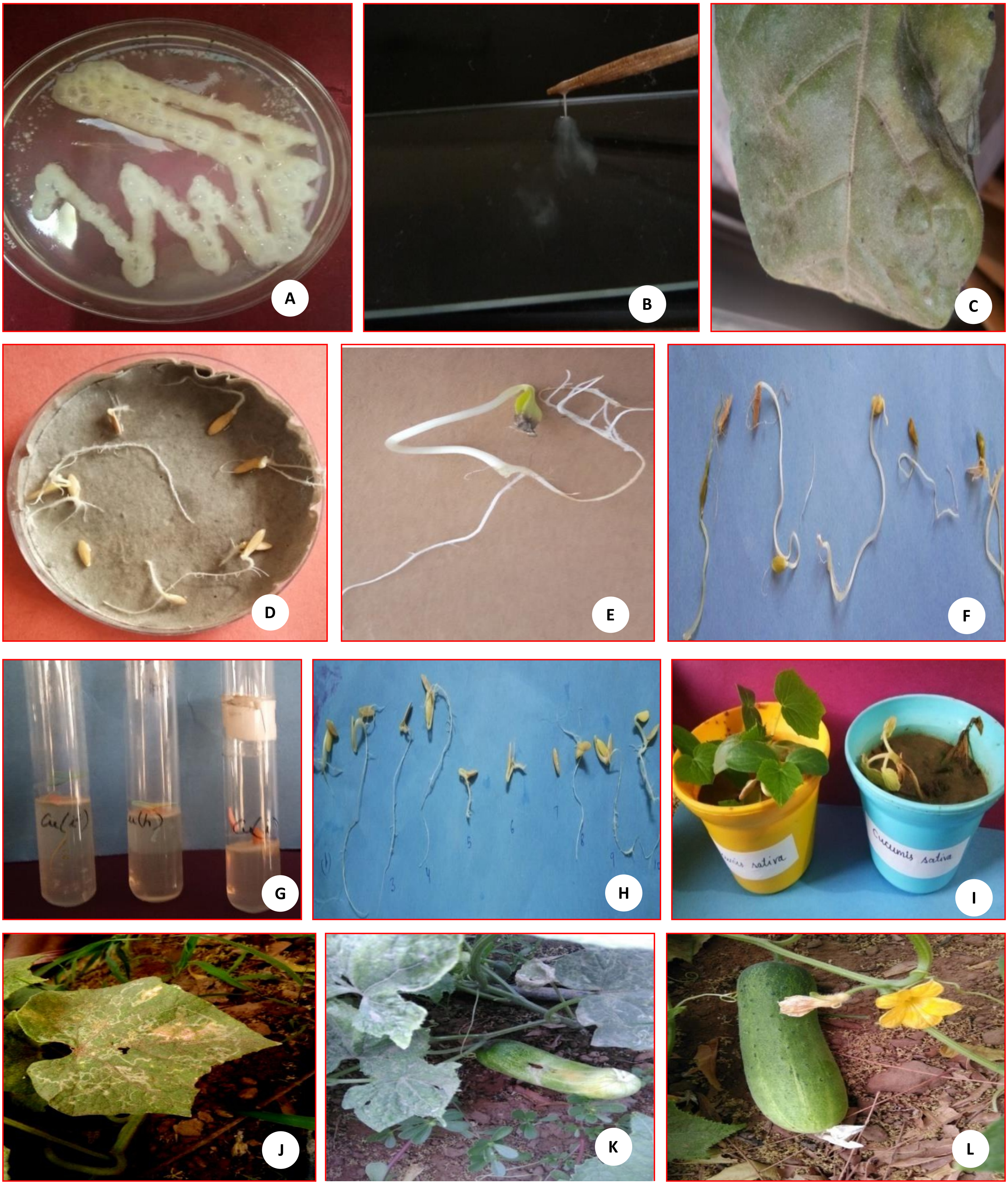


Fig-2A : Identification of Pseudomonas syringae pv lachrymans (A) pure colonies of PSL showing Levan formation, (B) positive response to KOH test (C) Hypersenstivity Test on Tobacco, (D-F) pathogenicity test in Petri plate method, showing browing and oozing on plumule and followed to mortality, (G) Test Tube Seedling Symptom Test (TTSST) on water agar, (H-I) Pot experiment showing symptoms on leaves and seedlings, (J-L) symptoms on leaves flower and fruit in the field infected with PSL.

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