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Comparative Studies Between *Xanthomonas citri* subsp. *malvacearum* Isolates, Causal Agent of the Bacterial Blight Disease of Cotton

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Abstract Out of 21 bacterial isolates collected during a survey from seven different cotton growing areas representing three districts of Tamil Nadu, India, only eight bacterial isolates of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), now being called (*Xanthomonas citri* subsp. *malvacearum*) were found to be pathogenic to the susceptible cotton variety TCB-209. The eight pathogenic isolates were grouped into clusters A and B based on protein profiles. Cluster A had I_1, I_3, I_5, I_7 and I_8 isolates while cluster B had I_2, I_4 and I_6 isolates. The isolates were morphologically similar, but varied in their cultural and biochemical characteristics. Isolate I_5 collected from Kavalur region showed a very high degree of virulence. Polyclonal antiserum was raised using the most virulent isolate I_5 and the titre was found to be 1:500. The antiserum also reacted positively with the other isolates. Pathogen was detected from different parts of the infected plant through DAC-ELISA and the leaves recorded maximum absorbance value indicating high level of bacterial infection.

Keywords: *Bacterial blight, Cotton, DAC-ELISA, Xanthomonas axonopodis* pv. *malvacearum (X. citri* subsp. *malvacearum)*

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1. Introduction

Cotton is an important fibre crop of India and ranks first in the world in its cultivated area (88 lakh hectares) though it ranks only fourth in production. Its production has increased since 1996-97 from 50 lakh bales to 116 lakh bales in 2001. Diseases are one of the chief constraints in cotton production. Among the different diseases, Bacterial Blight of Cotton (BBC) caused by *Xanthomonas axonopodis* pv. *malvaceulum* (Smith) Vauterin causes considerable yield loss in different cotton growing areas of the country. In India, an average loss of 30 to 35% is reported due to this disease [1,2]. In general, losses due to bacterial blight ranged between 1% and 27% depending on the cultivar and crop age [3]. The disease has become endemic in Tamil Nadu both in winter and summer irrigated cotton crops under favorable weather conditions and in the absence of ruling resistant varieties coupled with an intensive cropping system. The standard chemical recommendation consisting of antibiotics together with copper oxy chloride is not effective unless it is taken up right at the initial stage of this disease and it requires repeated applications. But this can lead to environmental pollution, development of resistance by the pathogen and residual toxicity. Alternatively, the biological control of disease management practice is an eco-friendly approach with long lasting effects, avoiding the problems emanating from the use of chemicals. For accurate breeding strategies and biocontrol measures, few methods and tests have been suggested to avoid field infection of the bacterial blight pathogens. Bhutta [4] and Mehta [5] have suggested using agar plate technique or a Semi-Selective Agar Medium as routine, accurate and sensitive method for the detection of the disease in the seeds. As a bio-control agent, *Pseudomonas fluorescens* CRb-26 was able to suppress the disease due to the pivotal role of its phenolic compound 2, 4-diacetylphloroglucinol against the Bacteria Leave Blight (BLB) pathogen [6]. A mixture of *P. fluorescens* (Pf32, Pf93) and *B. subtilis* (B49) as talc-based powder formulations under greenhouse and field conditions resulted in significant increase in yield, plant height, number of bolls and branches as well as reduced incidence of BLB disease in cotton [7]. Biocontrol agents induced systemic resistance against bacterial blight of cotton (BLC) [8]. Additionally, Pathogen host plant interactions necessitate the evaluation of susceptibility and resistance of cotton based on testing differential series of cultivars, lines and other concepts and techniques like semi-selective medium, pathogenicity tests and rep-PCR fingerprints to races or isolates typing

[9]. Indeed, understanding the genetic variability in pathogen populations is a very important step to control the disease and in developing resistant cotton cultivars [10]. According to gene for gene interaction model between specific pathogen strains and specific plant host varieties [11,12], it is very important to test the effect of unidentified isolates on susceptible as well as in resistant cotton lines characterized by their R(s) genes and the SSR markers. This article describes in details the differences between 8 pathogenic isolates of *Xanthomonas citri* subsp. *malvacearum* isolated from cotton plants grown in different districts of Tamil Nadu in India based on their morphology, pathogenicity, biochemical properties and protein profile. Also, generated polyclonal antiserum for the most virulent bacterial isolate I_5, which is used to detect the BBC from different parts of infected cotton plant.

2. Materials and Methods

2.1. Survey and isolation of different isolates of *X. citri* subsp. *malvacearum*

A survey for the occurrence of cotton bacteria blight disease was conducted in different cotton growing areas of Tamil Nadu *viz*., Srivilliputhur, Aruppukottai, Salem and Coimbatore, India. Leaves showing the typical symptoms of vein blight and angular leaf spot (Figure 1) were cut into 3- 4 mm bits by using a sterile scalpel. The leaf bits were surface sterilized with 0.1% mercuric chloride solution and washed 3 times in repeated changes of sterile distilled water. They were then crushed with a drop of sterile water using a clean glass rod. A loopful suspension was streaked in Petri plates containing nutrient agar medium with the help of a sterile inoculation needle [13]. The plates were incubated at room temperature. Colonies of yellowish slimy growth were noticed after 48 h. The yellowish slimy bacterial colony was sub-cultured into slants of the same medium from a single colony obtained from purified bacterial colony using dilution plate technique and streak plate method [14]. Bacterial isolates were maintained in sterile distilled water to avoid the loss of virulence for further study [15]. Another method of isolation was also tried which involved the washed leaf bits being directly placed on the nutrient agar medium and bacterial colony being further purified [14].

2.2. Pathogenicity of isolates of *Xam*

Purified cultures of *Xam* were multiplied in nutrient broth. After 24-36 h (Lag phase) these cultures were subjected to low speed centrifugation of 12000 *X g* for 15 min at 25°C. The bacterial pellet was re-suspended in sterile distilled water to obtain an optical density (OD) of 0.4-0.45 using G55703AT spectrophotometer at 620 ηm for pathogenicity studies [16]. Susceptible hybrid of cotton (TCB 209, Central Institute for Cotton Research, Coimbatore - 641 003, Tamil Nadu, India) was raised under glass house conditions in pot culture. The plants were irrigated with water and covered with a polythene bag, 24 h before inoculation [16]. The collected 21 isolates were tested on TCB 209 after one month by sand paper technique to prove the pathogenicity [17]. Inoculation was done between 10:00 AM to 6:00 PM on the abaxial surface of leaves. The water congestion was also repeated 24 h after inoculation. The plants inoculated with sterile water served as the control. The bacterium from the artificially infected leaves was re-isolated and compared with respective original isolates.

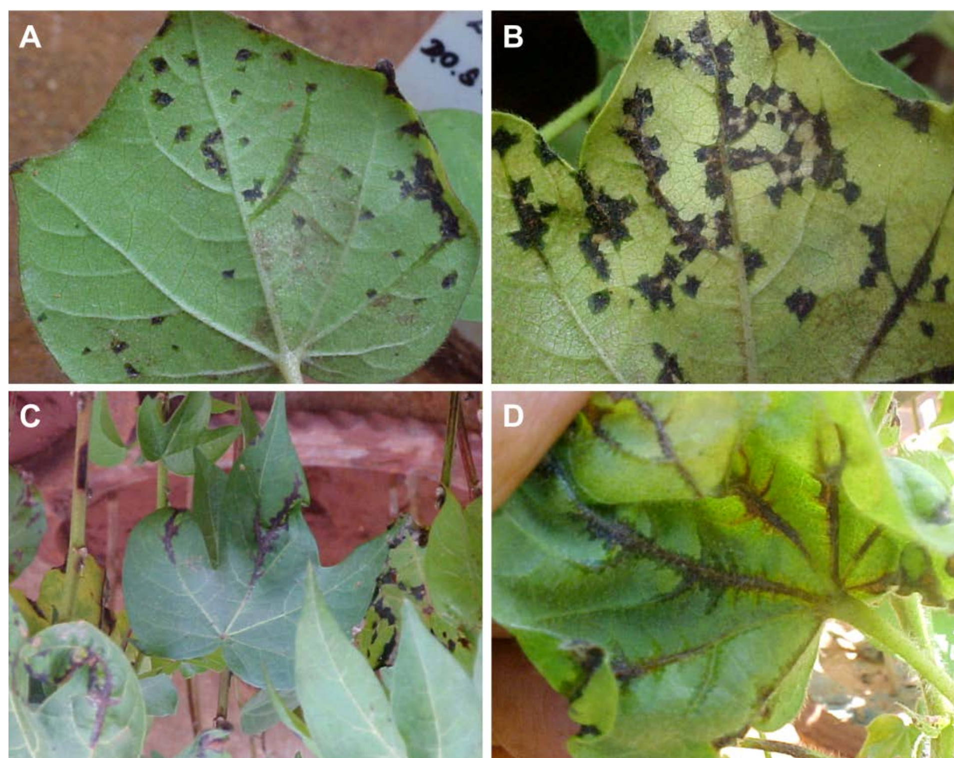

Figure 1. Symptoms of BBC disease by different *Xam* isolates of cotton plant: A and B: Angular leaf spot, C and D: Vein blight

2.3. Biochemical properties of the isolates

Several biochemical properties *viz*., utilization of nitrogen sources, carbon compounds, starch and gelatin hydrolysis, H_2S and indole production, methyl red test/ MR test, fluorescent pigments and casein hydrolysis were studied for the eight pathogenic bacteria isolates (*Xam*) in the Lab. Inorganic Nitrogen sources *viz*., ammolonium sulphate, ammolonium nitrate, ammolonium dihydrogen orthophosphate, calcium nitrate, potassium nitrate and sodium nitrate were separately incorporated at 0.1% level into the basal medium consisting of 10.0 g/L glucose, 2.0 g/L KH_2PO_4, 0.2 g/L $MgSO_4$ and 0.2 g/L KCl and the pH was adjusted to 6.8 - 7.0 and sterilized. Different isolates were inoculated and incubated at 28±2°C for seven days and the growth was observed. Three replications were maintained for each nitrogen source. For utilization of carbon compounds. Seven carbon sources *viz*., D-galactose, D-fructose, lactose, maltose, sucrose, D-xylose and dextrose, were separately incorporated at 1% level into the basal medium. Durham's fermentation tube was filled with the medium, dropped with a slant tube, each containing 8 ml of the medium, in the inverted position without air bubble and sterilized. The test tubes were inoculated with the bacterial isolates inoculated at 28±2°C and examined for growth, acid and gas production. Bromothymol blue was used as the indicator for acid production (greenish blue at pH 7.0; greenish yellow at pH 6.4; yellow at 6.2). Un-inoculated tubes served as the control. Three replications were maintained for each carbon source. Different isolates were streaked on peptone sucrose agar medium comprising of 0.2% starch and incubated for 5 days (24 to 48 h). Iodine was used for testing the hydrolysis of starch. A positive result was observed as a clear zone around the growth of the bacteria. Gelatin agar medium containing 3 g/L beef extract, 5 g/L peptone and 120 g/L gelatin [18] was prepared, sterilized and poured in Petri plates under aseptic condition. The isolates were streaked onto a gelatin medium and incubated at room temperature for 24-48 h. When the isolates were ready for examination, the plates were flooded with 0.1% mercuric chloride and incubated for 10 min and decanted later. The zones were observed around the colonies, which indicate the hydrolysis gelatin. To study H_2S production, a sterilized peptone water was taken in test tubes and inoculated with cultures of *Xam* under aseptic conditions. Filter paper strips pre-soaked in saturated lead acetate solution were inserted in the tubes just above the medium and incubated for 72 h. The change in color of the filter paper strips to black color represents a positive reaction [18]. Methyl Red test was performed using MR Voges- Proskauer broth (peptone 7.0 g/L, dextrose 5.0 g/L, potassium phosphate 5.0 g/L)was prepared, dispensed in test tube and autoclaved at a 15 lb pressure for 15 min. The pathogen was inoculated and incubated for 48 h at 35°C. Five drops of methyl red (0.1 g of methyl red was dissolved in 300 ml of 95% ethanol and made up to 500 mL with distilled water) were added. The color development was observed after 5 min. An un-inoculated test tube served as the control [19]. Production of fluorescent pigments on King's 'B' medium was studied by streaking the bacterial isolates on King's 'B' medium plates (20.0 g/L proteose peptone, 1.50 g/L magnesium sulphate, 1.50 g/L dipotassium hydrogen phosphate, 15.0 g/L agar agar, 10.0 ml/L glycerol, pH 7.2) was prepared and sterilized in test tubes [20]. The isolates were incubated at room temperature (28 ± 2°C) for 5 days. Appearance of yellowish green fluorescence was observed under ultraviolet light. Tryptophane broth (10 g/L tryptophan and 3 g/L yeast extract) was prepared and sterilized. The *Xam* isolates were inoculated and incubated for 72-78 h at 28±2°C. Filter paper was inserted and placed just above the medium without contact with the medium. Pink coloration in the paper strip showed the positive result of indole production [21].

For casein hydrolysis. the bacterial isolates were streaked on the sterilized medium containing 10 g/L casein, 1 g/L dipotassium hydrogen phosphate, 1g/L magnesium sulphate, 1 g/L sodium chloride, 5 g/L peptone and 20 g/L agar, pH 7.2 [22]. The pathogen was examined after 48 h of inoculation. The appearance of a transparent zone around the bacterial colony showed a positive result.

2.4. Genetic relatedness among the *Xanthomonas* isolates using similarity index

The isolates were compared for their total cell proteins separated through sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) [23]. Protein fraction of the cell envelope was used to characterize the *Xanthomonas* spp. [24]. One loopful of 36 h-grown fresh culture was inoculated into 50 mL of nutrient broth and incubated in a mechanical shaker at 150 rpm for two days under room temperature (28±2°C). The bacterial cell at its logarithmic phase of growth was harvested by centrifugation at 5000 x *g* for 10 min. The cells were washed, resuspended in Tris-buffer (Tris-Hcl 10 mmol/L, 0.75 M sucrose, pH 7.4) and incubated for 10 min on ice and vortexed. The resulting cell suspension was incubated for 30 min at 4°C before centrifugation at 8000 x *g* for 20 min. The supernatant was used as a total protein source for electrophoresis [25]. The total protein was determined by the method of [26] with bovine serum albumin (BSA) as standard and SDS-PAGE was carried out [27]. The diversity analysis of eight isolates of *Xam* was determined based on the presence or absence of protein bands in SDS-PAGE (Figure 2). Similarity among the isolates was assessed based on Jaccard's coefficient [28]. Based on the dissimilarity coefficient, a dendrogram was constructed using an un-weighted paired grouping method average (UPGMA) and the isolates were grouped into cluster.

2.5. Production of polyclonal antibody against the isolate I_5 (*Xam*)

Antigen was prepared using a log phase culture (36 h old) of *Xam* (isolate 5) and centrifuged at 13000 x *g* for 30 min at 4°C. The resulting pellets were washed twice with 0.01 M phosphate buffered saline (PBS) by repeated centrifugation, at 8000 x *g* for 10 min at 4°C. The re-suspended pellet was sonicated with a Fisher Sonic Dismembrator Model 300 for 2 min. The protein content was assessed [26] and 200 μg of protein were used for each injection. Antiserum was raised against *Xam* in a

New Zealand white rabbit by four intramuscular injections. Two hundred μg of protein in PBS (pH 7.4) was emulsified with an equal volume of complete and incomplete (alternatively) adjuvant. Totally four injections were given weekly and followed by one booster injection. The rabbit was bled at weekly intervals for three weeks after the last injection [29]. The blood was allowed to clot for an hour at room temperature and kept overnight in refrigerator at 4°C. After refrigeration, serum was decanted from the clot and centrifuged at 2000 rpm for 10 min to remove cell debris. Sodium azide was added at 0.025% and serum was mixed with equal volume of glycerol and stored at -20°C [30].

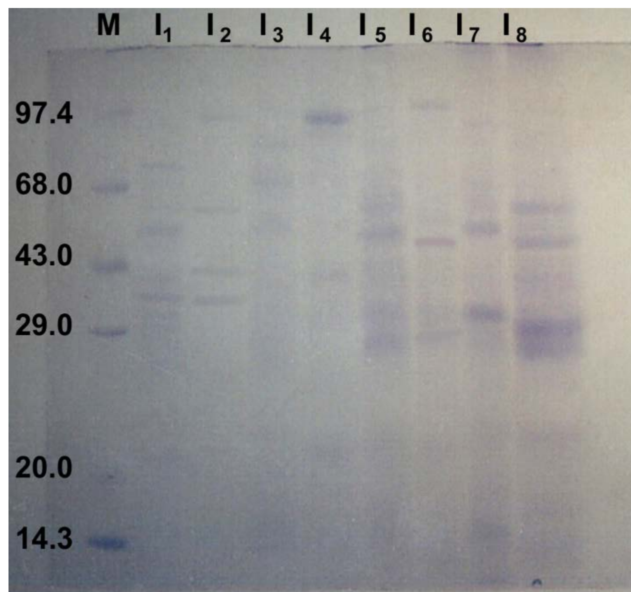

Figure 2. Protein profile of eight different isolates *of X. citri* subsp. *malvacearum.* M: Protein marker (kDa), I_1- I_8: *Xam* isolates

2.6. Serological detection of *Xanthomonas axonopodis* pv. *malvacearum*

The titre of the polyclonal antibody raised against *Xam* (I_5) was measured by indirect ELISA [31]. The sonicated antigen was diluted in phosphate buffered saline PBS (pH 7.4) and added in microtitre plate (100 µl/ml) with dilutions of 1:50, 1:100, 1:200, 1:500, 1:1000 and 1:10000. The plates were incubated for 2 h at 37°C, emptied and washed three times with PBS-Tween 20 (PBS-T). Each washing was done for 3 min. Different dilutions of the antibodies (1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:10000) raised in the rabbit were prepared in PBS-TPO (containing 2% polyvinylpyrolidone (PVP) and 0.02% ovalbumin) and added at the rate of 100 µl/well. The plates were incubated for 2 h at 37°C. After incubation period, the plate was washed three times with PBS-T. One hundred µl of alkaline phosphatase (ALP) conjugated anti-rabbit immunoglobulin was added (diluted in PBS-TPO, 1:7000). After the incubation period of 2 h at 37°C, the plate was washed three times with PBS-T. Then p-nitrophenyl phosphate was dissolved in substrate buffer (Diethanolamine pH 9.8) and 100 µl well^{1-}was added. The plate was kept for 15-30 min at room temperature for color development and the reaction was stopped by adding 50 µl of 3 M NaOH. The color intensity was read at 405 ηm in microplate reader. Sterilized distilled water and a buffer served as control. The immunometric assay of antibody was tested using Indirect ELISA; different dilutions of antigen 1:50, 1:100, 1:200, 1:500, 1:1000, 1:10000 were tested against different dilutions of antiserum 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:10000. Buffer and sterile distilled water served as the control. Absorbance was recorded at 405 ηm.

2.7. Detection of *Xam* from different parts of cotton plant

Different isolates of *Xam* were prepared from different parts of a cotton plant at 1:50 dilution and indirect ELISA technique was done with 1:500 dilution of antiserum. The color development was read at 405 ηm. The buffer alone served as the control.

2.8. Statistical analysis

All experiments were repeated at least twice. The repeated experiments were analyzed separately and showed similar trends. Data from individual experiments was subjected to analysis of variance using the IRRI STAT version 92-1 package developed by the International Rice Research Institute, Biometrics Unit, Philippines. The treatment means were compared by Duncan's Multiple Range Test (DMRT) [32] at 5% level of significance.

3. Results and Discussion

Cotton leaves showing typical vein blight symptoms were collected from different areas of Tamil Nadu and were treated as different isolates with an aim to assess their variability. Out of the 21 bacterial isolates collected from different cotton growing areas of three districts (Salem, Virdhunagar and Coimbatore) of Tamil Nadu, India, only eight different bacteria isolates of *Xam* named as I_1, I_2, I_3, I_4, I_5, I_6, I_7 and I_8 were found to be pathogenic to the susceptible cotton variety TCB-209 (Table 1). The isolates I_1, I_2, I_3, I_4, I_7 and I_8 were collected from Kavalur, Srivilliputtur, Kumaralingapuram, Aruppukottai, Kattukottai and Coimbatore respectively and the isolates I_5 and I_6 from Kalpakanoor village. All the pathogenic isolates collected slightly differed in their morphological, cultural and biochemical characters but differed significantly in their virulence**.** Isolates I_2 and I_4 were less smooth in texture compared to other isolates. The growth of these isolates was moderate. The colonies of other isolates were smooth, slimy, glistening profuse with pale to deep yellow color (Table 1). All the isolates of *Xam* were positive to starch hydrolysis, gelatin liquefaction, H_2S production and casein hydrolysis. This is contradictory with the results obtained by [33] about the difficulty to differentiate few strains of *Xanthomonas axonopodis* pv *manihotis* and *Xanthomonas axonopodis* pv *cassava* based on physiological and cultural reactions. The ability to liquefy the gelatin and hydrolyze starch was reported as a characteristic of the genus *Xanthomonas* [34]. Studies on the utilization of various inorganic nitrogen indicated that the isolate I_5 was significantly different from all the other seven isolates by

recording the highest mean growth (0.78 OD) in all the six nitrogen sources. All the isolates extent utilized ammolonium dihydrogen orthophosphate to the maximum except I_2 and I_7. The isolates I_2 and I_7 grew better when sodium nitrate was used as the nitrogen source which recorded an OD value of 0.75 and 0.76 respectively. Among the 8 pathogenic isolates, I_5 had a better growth in all the nitrogen sources followed by I_6. The isolate I_2 showed the least utilization of all the nitrogen sources tested (Table 2).

The results on utilization of different carbon compounds by *Xam* showed that the growth of *Xam* isolate I_5 was maximum in dextrose (0.91 OD), Lactose (0.89 OD), D-fructose (0.89 OD), D-galactose (0.85 OD), maltose (0.81 OD) and compared to other isolates. The results clearly explained the differential utilization pattern of carbon sources by the different isolates of *Xam* (Table 3). All the eight isolates gave a positive result to starch hydrolysis, gelatin liquefaction, H_2S production and caesin hydrolysis and showed negative result to indole production, fluorescence pigment production on King's "B" medium and methyl red test. Many workers have reported the existence of isolate variations in the pathogen *Xam* [35,36,37]. The most virulent isolate I_5 grew profusely in all the N sources tested. Likewise, D-xylose, lactose, D-fructose, maltose and D-galactose as carbon sources supported better growth. Singh [38] and Singh and Verma [39] reported that glucose and galactose were the best carbon sources for the growth of *Xam*. Extracellular polysaccharides have been reported as the virulence factor and the composition of EPS did not vary qualitatively [40,41]. However, utilization of carbon source might result in a quantitative variation of EPS resulting in an alteration in virulence. Interestingly, Suresh [42] suggested the use of modified D-5 medium with 30°C to be as ideal in maximizing the growth of the bacterial blight of rice (*Xanthomonas oryzae* pv. *Oryzae*) and get the best recovery of the bacterial colonies ($154.33 x 10^5$ cfu/ml).

The genetic relatedness among the *Xam* isolates was studied using similarity index and the data were subjected to cluster analysis using NTSYS program for all the isolates to estimate similarity indices and genetic relatedness among the isolates. The similarity index (SI) values were computed as a ratio of the number of similar bands to the total number in pair-wise comparison of the isolates. The SI values obtained for each pair of bands among the eight *Xam* isolates are shown in Table 4. Based on the data, the genetic distances were used to construct a dendrogram for the eight isolates following the UPMGA method using NTSYS program [43]. Option SAHN was performed which resulted in the dendrogram (Figure 3). From the dendrogram generated, the eight isolates were grouped into two major clusters namely A and B. The cluster A had the isolates I_1, I_3, I_5, I_7 and I_8 while cluster B had I_2, I_4 and I_6. Within the cluster A, the isolates I_1 and I_3 showed the least similarity (54.8%) at the molecular level. In the cluster B the isolate I_2 and I_4 showed more similarity to each other (77.41%) than to the isolate I_6. Though the isolates I_5 and I_6 were from the same geographical location they showed a similarity of only 54.8% which suggested that within the same area, variation existed among the isolates (Table 4).

Table 1. Morphological and cultural characteristics of different isolates *of Xanthomonas axonopodis* pv. *malvacearum* collected from different cotton-growing areas of Tamil Nadu

Isolate No.	Place of collection	District	Variety	Symptom	Texture	Appearance	Colour	Growth
I_1	Kavalur	Salem	LRA-5166	ALS	Smooth	More slimy, glistening	Deep yellow	Profuse
I_2	Srivilliputhur	Virudhunagar	SVPR-2	ALS & VB	Less smooth	Less slimy, glistening	Pale yellow	Moderate
I_3	Kumaralingapuram	Virudhunagar	MCU-5	VB	Smooth	More slimy, glistening	Pale yellow	Profuse
I_4	Aruppukottai	Virudhunagar	LRA-5166	VB	Less smooth	Less slimy, glistening	Yellow	Moderate
I_5	Kalpakanoor	Salem	Suvin	ALS & VB	Smooth	More slimy, glistening	Pale yellow	Profuse
I_6	Kalpakanoor	Salem	SVPR-2	ALS & VB	Smooth	More slimy, glistening	Deep yellow	Profuse
I_7	Kattukottai	Salem	Suvin	ALS & VB	Smooth	More slimy, glistening	Deep yellow	Profuse
I_8	CICR (Coimbatore)	Coimbatore	LRA-5166	ALS & VB	Smooth	More slimy, glistening	Yellow	Profuse

ALS – Angular leaf spot; VB – Vein blight.

Table 2. Utilization of nitrogen sources by different isolates *of Xanthomonas axonopodis* pv. *malvacearum*

Isolate	OD value at 620 ηm*						
	Ammonium sulphate	Ammonium nitrate	Ammonium dihydrogen ortho phosphate	Calcium nitrate	Potassium nitrate	Sodium nitrate	Mean
I_1	0.57f	0.55f	0.79c	0.61d	0.69d	0.72cb	0.65d
I_2	0.47g	0.41g	0.62g	0.65c	0.69d	0.75a	0.59f
I_3	0.60e	0.63d	0.73e	0.61d	0.60e	0.66f	0.64ed
I_4	0.62d	0.61e	0.71f	0.59e	0.71cb	0.69e	0.65d
I_5	0.76a	0.82a	0.98a	0.71b	0.72b	0.73b	0.78a
I_6	0.73b	0.79b	0.83b	0.74a	0.69d	0.71dc	0.74b
I_7	0.73b	0.75c	0.71f	0.73a	0.75a	0.76a	0.74b
I_8	0.64c	0.81a	0.77d	0.71b	0.76a	0.61g	0.73cb

CD (p = 0.05); I = 0.01; N = 0.01; I x N = 0.02.
*Mean for three replicates. Means followed by the same letters in a column are not significantly different by DMRT at 5% level of significance.

Table 3. Utilization of carbon sources by different isolates of *Xanthomonas axonopodis* pv. *malvacearum*

Isolate	OD value at 620 ηm*							
	Sucrose	D-Xylose	Lactose	D-Fructose	Maltose	D-Galctose	Dextrose	Mean
I_1	0.78dbc	0.73c	0.81b	0.80dcb	0.72b	0.66ed	0.91a	0.77c
I_2	0.77e	0.71dc	0.87a	0.88a	0.81a	0.78cb	0.89a	0.81b
I_3	0.81b	0.86a	0.87a	0.88a	0.82a	0.81a	0.81cb	0.83a
I_4	0.88a	0.81b	0.79cb	0.87a	0.81a	0.85a	0.85b	0.83a
I_5	0.78dbc	0.84a	0.89a	0.89a	0.81a	0.85a	0.91a	0.85a
I_6	0.81b	0.84a	0.88a	0.82b	0.81a	0.79b	0.91a	0.82a
I_7	0.85a	0.81b	0.89a	0.81cb	0.82a	0.79b	0.93a	0.84a
I_8	0.79cb	0.84a	0.87a	0.81cb	0.83a	0.69d	0.85b	0.82a

CD (p = 0.05); I = 0.037; C = 0.035; I x C = 0.06

*Mean for three replicates. Means followed by the same letters in a column are not significantly different by DMRT at 5% level of significance.

Table 4. Similarity coefficient for different isolates of *Xanthomonas axonopodis* pv. *malvacearum*

Isolates	I_1	I_2	I_3	I_4	I_5	I_6	I_7	I_8
I_1	1.0000000							
I_2	0.2903226	1.0000000						
I_3	0.5483871	0.2580645	1.0000000					
I_4	0.4838710	0.7741935	0.3225806	1.0000000				
I_5	0.6774194	0.2580645	0.7096774	0.3870968	1.0000000			
I_6	0.5806452	0.6774194	0.4838710	0.7419355	0.5483871	1.0000000		
I_7	1.6129032	0.3870968	0.7096774	0.3225806	0.6451613	0.4193548	1.0000000	
I_8	0.7096774	0.1612903	0.7419355	0.2903226	0.7419355	0.3870968	0.6129032	1.0000000

Table 5. Immuometric assay of antibody

Antigen dilution	Antiserum dilution (absorbance 405 ηm)									
	1:50	1:100	1:200	1:500	1:1000	1:2000	1:5000	1:10000	Buffer	DW
1:50	0.151	0.167	0.297	0.371	0.357	0.300	0.160	0.132	0.092	0.092
1:100	0.142	0.161	0.218	0.358	0.350	0.291	0.152	0.132	0.090	0.092
1:200	0.138	0.152	0.211	0.361	0.353	0.291	0.156	0.137	0.092	0.092
1:500	0.131	0.141	0.201	0.317	0.301	0.270	0.250	0.118	0.092	0.092
1:1000	0.122	0.320	0.200	0.311	0.291	0.255	0.111	0.101	0.091	0.092
1:100000	0.090	0.091	0.098	0.099	0.099	0.094	0.093	0.090	0.092	0.092

Population mean + 2 SD = 0.317.

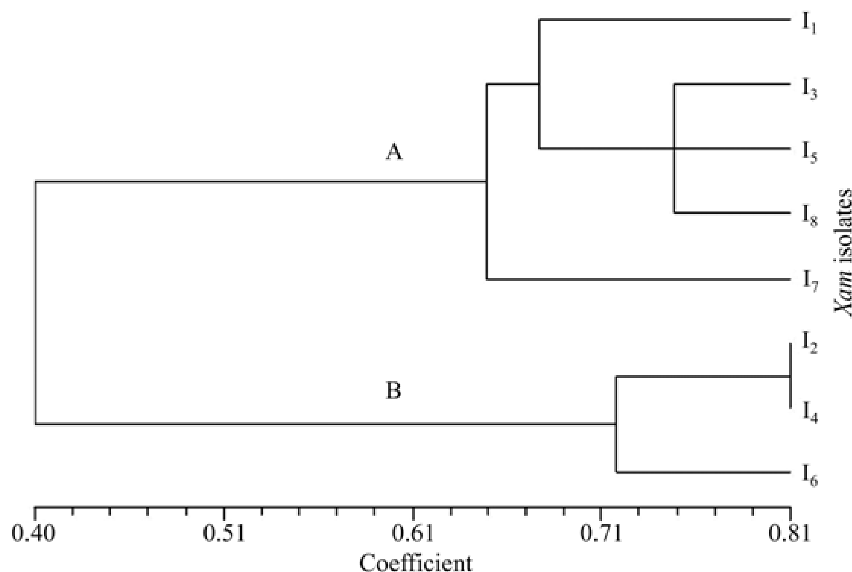

Figure 3. Cluster analysis of protein patterns of eight *X. citri* subsp. *malvacearum* isolates collected from different places of Tamil Nadu

Xam isolates from different geographical locations exhibit wider differences in their electrophoretic pattern of total cell proteins. The isolates were classified into 2 major groups, Clusters A and B. The cluster A had the isolates I_1, I_3, I_5, I_7 and I_8 while the cluster B had the isolates I_2, I_4 and I_6. This indicated that variation existed within the same area. Relatedness of the isolates or strains based on total cell protein had no bearing on the virulence of the isolates. However, Borkar and Verma [40] have reported that the protein content of EPS had a positive correlation with virulence. Similar variation was observed by Dristig and Dianese [25], among 25 isolates of *Pseudomonas solanacearum* from five different regions of Brazil. Nandakumar [44], also studied the variation between 11 isolates of *Pseudomonas fluorescens* collected from all over Tamil Nadu and the isolates were classified into four groups.

The antiserum raised against the virulent isolate I_5 reacted at a dilution of 1: 500 with the antigen, diluted to 1: 50. The antiserum and the antigen diluted to 1: 1000 and above did not react with each other as with their dilution at 1: 500 (Table 5). It was observed that a positive correlation existed between the *Xam* isolates and the antiserum raised against the isolate (I_5) reacted with the other isolates, indicating that the isolates were related to each other. The I_5 isolate was found to be the most virulent one than the others and recorded the maximum absorbance value (0.93 OD), followed by I_6, I_8, I_4, I_3, I_7 and I_1 (Figure 4). Among the different plant parts tested for the presence of the pathogen, *Xam* was detected from leaves, dried leaves and seeds using the rabbit *Xam* antibody (dilution 1: 500). The leaves recorded maximum titre value among the various parts (Figure 5). Polyclonal antibody was raised against purified *Xam* in New Zealand white rabbit and was used to detect *Xam* presence in leaves, dried leaves and seed. Morton [45], Lin [46] and Thaneechai and Schaad [47] have reported the production of polyclonal antibodies against *Xanthomonas vesicatoria* in pepper, *X. campestris* pv. *campestris* in crucifers and *Erwinia amylovora* in apple.

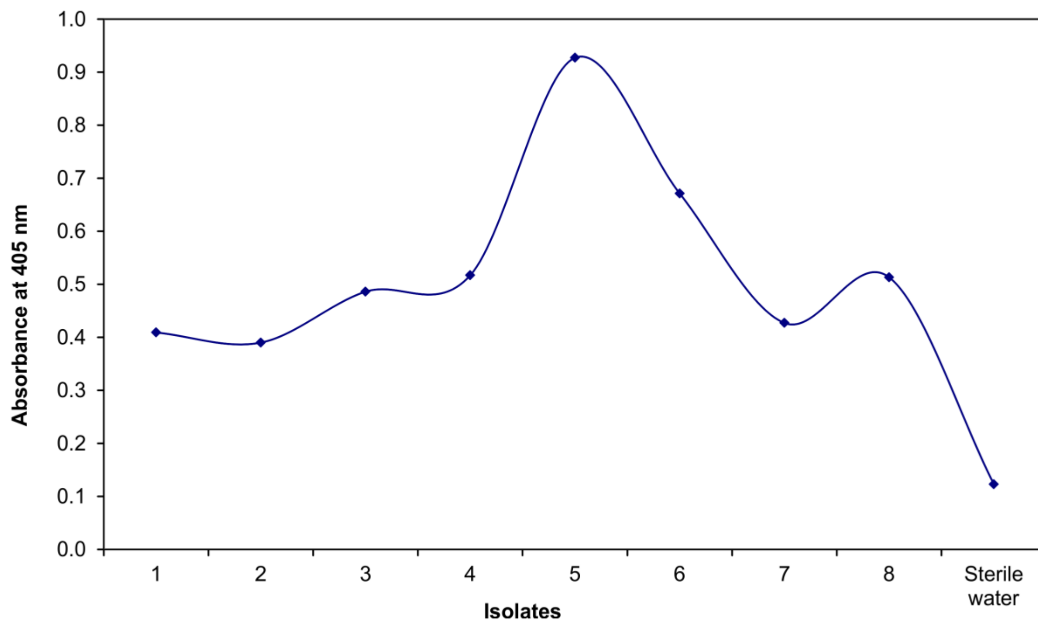

Figure 4. Correlation of eight *X. citri* subsp. *malvacearum* isolates with antiserum

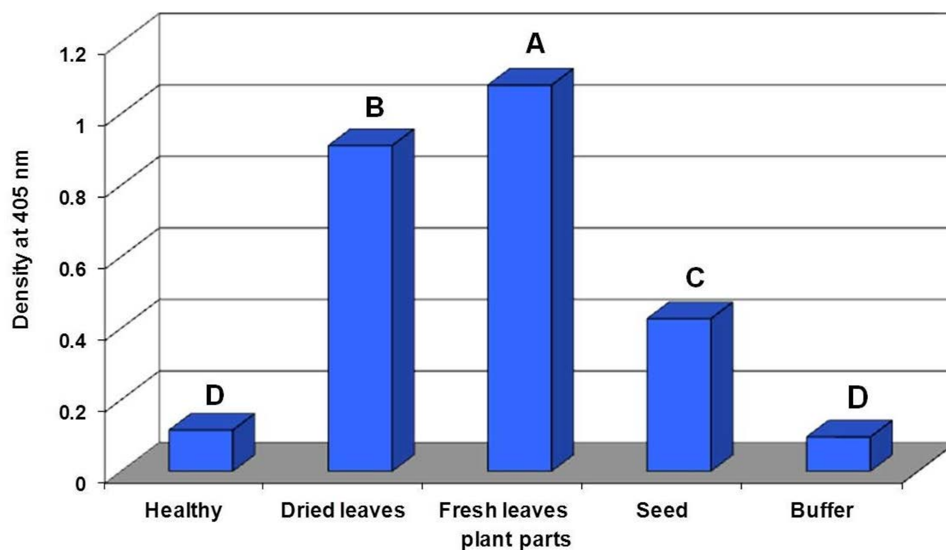

Figure 5. Serodiagnosis of *X. citri subsp. malvacearum* from different parts of cotton plant through ELISA test

Indeed, using ELISA gave an accurate indication of the presence of bacteria on different cotton plant parts, while using a PCR can only help to detect un quantitatively the presence of a pathogen in various plant parts after infection naturally or artificially as was exhibited in banana explants infected by *Xanthomonas campestris* pv. *musacearum* causing Banana Xanthomonas wilt [48]. Some additional physiological responses and microscopical observation assays at plant tissue and cellular levels can deepen the distinguished characterizations of these isolates. Genome sequencing is increasingly becoming cheaper, faster and available as was carried out on two strains (race 18 and 20) of *Xanthomonas citri* pv. *malvacearum* [49], and two highly virulent strains of *Xam* strain (GSPB2388) from Sudan, and a strain of race 18 (GSPB1386) from Nicaragua [50,51]. Sequencing these isolates or at least the most virulent I_5 will lead to indicate the pathogenicity genes and factors to better envisage new pyramided resistance and epidemiological control and surveillance tools.

4. Conclusion

Out of 21 bacterial isolates collected from different cotton growing areas of Tamil Nadu, only eight pathogenic bacterial isolates of *Xanthomonas axonopodis* pv. *malvacearum* (Xam) exhibited typical symptoms of bacterial blight disease on susceptible cotton variety TCB-209. Isolates differed in their ability to utilize carbon, nitrogen sources and total cell proteins. Isolates were grouped into two clusters based on the % similarity at molecular level. Polyclonal antiserum was raised and DAC-ELISA was performed to detect *Xam* from different parts of infected plants. Variability of *Xam* isolates in cultural, morphological, pathogenicity and genetic levels might be the reason for virulent or avirulent nature of the isolates. The results of this study would be quite useful in the identification of various *Xam* isolates based on biochemical tests and genetic relatedness. Further research to the most pathogenic *Xam* isolates, identify the genes of virulence that involved in diseases occurrence, and also management of BBC disease using effective biocontrol agents, are recommended.

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