Biochemical Characterization of Xanthomonas axonopodis pv. malvacearum Isolated from Infected Cotton Plant and It's in vitro Sensitivity against Some Selected Chemicals

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Authors’ contributions

This work was carried out in collaboration between all authors. Author NS designed the study, performed the statistical analysis and wrote the protocol. Author SS carried out the research and wrote the first draft of the manuscript. Authors NS and FMA supervised the study and managed the analyses of the study. Author SS managed the literature searches. Author FMA edited and corrected the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AIR/2017/35626

Editor(s):
(1) Magdalena Valsikova, Horticulture and Landscape Engineering, Slovak University of Agriculture, Nitra, Slovakia.

Reviewers:
(1) Adigun Michael, Crawford University, Nigeria.
(2) Wonni Issa, Agricultural and Environmental Research Institute, Burkina Faso.
(3) V. Vasanthabharathi, Annamalai University, India.
(4) Mohammed Suleiman, Umaru Musa Yar’adua University, Nigeria.

Complete Peer review History: http://www.scientedomain.org/review-history/20763

ABSTRACT

Aim: The aim of our study is to isolate, identify and pathogenicity test of Xanthomonas axonopodis pv. malvacearum (Xam) causing bacterial blight of cotton and to determine the comparative efficacy of some selected chemicals and antibiotic in controlling Xam in vitro.

Place and Duration of the Study: This study was carried out at the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh from January, 2012 to December, 2013.

Methodology: Xanthomonas axonopodis pv. malvacearum was isolated from infected leaves, stems, branches, cotyledons and bolls of cotton. Biochemical characterization and in vitro sensitivity
were evaluated as per the standard methods as described in materials and methods.

**Results:** The bacterium was gram negative, rod shaped and showed positive results in KOH solubility, starch hydrolysis, catalase, citrate utilization, motility indole ureas agar (MIU), gelatin liquefaction test and oxidase test. It produced, slightly raised, blond to bright yellow colour, mucoid colonies on NA medium; circular, flattened or slightly raised, yellow to bright yellow colour, mucoid colonies on YDCA medium and light yellow, mostly circular, slightly flattened on SX medium. *In vitro* evaluation of selected chemicals revealed that Streptomycin sulphate was highly effective against *Xam.* In *in vitro* condition, the highest inhibition zone (35.33, 36.17, 35.17, 33.5 and 32.33 mm after 24, 48, 72, 96 and 120 hrs., respectively) was observed in Streptomycin sulphate @ 0.15% treated plates.

**Conclusions:** Streptomycin sulphate showed higher effectiveness against *X. axonopodis* pv. *malvacearum* compared with selected fungicides.

**Keywords:** *Xanthomonas axonopodis* pv. *malvacearum*; cotton; biochemical characterization; *in vitro* test; chemicals; antibiotics.

### 1. INTRODUCTION

Bangladesh, the second largest apparel producer after China, is also the third raw cotton importer in the world. The Cotton Development Board has set a target of production of 670,000 bales of cotton by 2015 to reduce import of cotton, the basic raw material for the textile sector [1]. Annual requirement of raw cotton for local textile industry would be 4.0 million bales in future. Country’s present cotton production is around 1.0 lakh 29 thousand bales which is a small portion of the total annual requirement of local spinning mills [2]. There are many constraints of production of cotton in Bangladesh. Among them disease is the most serious one [3]. More than 60 diseases were recorded in cotton [4]. Of the major diseases, Bacterial Blight (BB) caused by *Xanthomonas axonopodis* pv. *malvacearum* is an important and destructive disease of cotton affecting yield and fiber quality seriously [5]. In the United States this pathogen caused 73,000 bales loss as a result of foliar and stem phase of bacterial blight [6]. Exact losses caused by this disease are not known in Bangladesh. Bacterial blight is a common disease affecting the growth, development and yield of cotton [7]. The disease affects all the aerial parts of plant and known as angular leaf spot, vein blight, black arm and boll rot depending on the plant part infected [8,9]. Bacterial blight severity is higher at high ambient temperatures (86-97°F) and high relative humidity conditions. The pathogen spread most effectively by splashing water, particularly rain water. Bacteria can enter the plant through natural openings like stomata, nectarines, or through wounds. In Bangladesh for controlling bacterial blight, farmers are using copper fungicides (Cupravit 50 WP), Diathane M-45, and sulfer but not able to manage the disease properly due to the systemic infection nature of the bacteria [10]. That is why we are trying to test the sensitivity of bacteria against the antibiotics as well as against the existing chemicals. Adjustment of sowing date, regular spraying of antibiotics and fungicides, flooding, sanitation etc. may reduce the incidence of the disease [11]. No research work has been critically done in Bangladesh on biochemical characterization, *in vitro* sensitivity of the pathogen, progressive symptoms development, control measures etc. [3]. The present research work was therefore carried out biochemical characterization of *X. axonopodis* pv. *malvacearum* and its sensitivity against some selected chemicals.

### 2. MATERIALS AND METHODS

#### 2.1 Isolation, Identification and Characterization of Causal Organism

##### 2.1.1 Isolation

Infected leaves were collected from experimental field of Cotton Research, Training and Seed Multiplication Farm, Sreepur, Gazipur (24°12′N 90°28′E). Lesions with green healthy portion of diseased leaves were cut into small pieces. Surface sterilized with 5% sodium hypochlorite solution for 2-3 minutes and washed them three times with sterile water. After surface sterilization, the cut pieces were kept for 30 minutes in 2 ml of sterile water for bacterial streaming (moving out of bacterial populations from the affected leaf tissues after the tissues served in water). One ml of this stock solution was transferred with the help of sterile pipette into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10⁻¹ dilution. Similarly, final
dilution was made up to $10^4$. After preparing different dilution, 0.1 ml of each dilution was spreaded over plate containing Nutrient Agar (NA) medium (bacto agar- 15 g/L, peptone- 5 g/L and beef extract- 3 g/L). For each dilution, three replicated plates were prepared as described by Goszczynska and Serfontein, [12]. Inoculated plates were incubated at 30°C. The plates were observed after 24 hrs and 48 hrs. Then single colony grew over NA plate was restreaked on another plate (NA) with the help of a sterile loop to get pure colony. After purification, bacterium was streaked over NA slant and kept in incubator at 30°C for 48 hrs. Finally the culture tubes were kept at refrigerator at 4°C for further use.

2.1.2 Identification and characterization

Gram's Staining and KOH solubility test were performed to know the Gram reaction nature of the strain of the bacteria.

2.1.3 Biochemical characters

Different biochemical chemical tests such as starch hydrolysis, citrate utilization test, motility indoleureas agar (MIU) test, catalase test, oxidase test, pecteolytic test, gelatin liquefaction test and salt tolerance test were performed as per the standard methods [13,14].

2.1.4 Cultural characters

Most of pathovars of *Xanthomonas* can be differentiated by growth and colony morphology on different media [13]. Growth characteristics of the pathogen were studied by using various differential, selective and semi-selective media. The bacterium was inoculated over NA, YDCA and SX medium and incubated at 30°C for at least 24 hrs and observed the colony characters.

2.2 Tobacco Hypersensitivity Reaction

To determine the pathogenic nature of the isolates, hypersensitivity reaction was performed on tobacco (*Nicotiana rustica*) plants by injection infiltration technique [15]. An aliquot of the inoculums suspension of bacterium (approximately $10^6$ cfu per ml) was prepared in sterile distilled water from a 24 hours culture plate. Then the lower surface of a mature tobacco leaf was infiltrated by pressing a syringe containing the suspension against the leaf and distilled water was used as a negative control. Then it was observed for 72 hours.

2.3 Pathogenicity Test

Cotton plants (CB-9) were grown on pots under nethouse condition to test pathogenicity of *X. axonopodis pv. malvacearum* following standard method [16]. Bacterial cells were grown overnight in NA broth and resuspended in sterile distilled water to a concentration of approximately $10^7$ cfu per ml. An aliquot of the inoculum suspension was injected forcibly into the lower surface of the leaf using a sterile syringe and distilled water was used as negative control. Inoculated plants were maintained at field temperature and were observed for 15 days. Data based on visual symptoms were recorded. *X. axonopodis pv. malvacearum* was reisolated from the infected plants and confirmation test was performed.

2.4 In vitro Evaluation of Some Selected Chemicals against the Bacteria

In *vitro* evaluation of some selected chemicals (Table 1) against the bacterium was done by well diffusion method to measure inhibition zone [17]. Cotton swabs on wooden applicator sticks were prepared. The swabs were kept in a dry tube and were sterilized in the autoclaved [18]. Two test tubes, each containing 10 ml nutrient broth, were taken and inoculated with 48 hours old pure culture of bacteria grown on NA plates. Another test tube containing 10 ml nutrient broth was taken as control. Test tubes were transferred in shaker incubator maintaining 30°C temperature and 150 rpm for 24 hrs. After shaking, the broth culture was spreaded uniformly on NA plate with the help of sterile cotton swabs. One hole of 4 mm in diameter was punched into the same plate. Then it was observed for 72 hours.

Table 1. Selected chemicals, their trade name, active ingredient and concentration used in bioassay

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Active ingredient</th>
<th>Chemical name</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cupravit50 WP</td>
<td>Copper Oxychloride</td>
<td>Copper chloride oxide hydrate</td>
<td>0.2</td>
</tr>
<tr>
<td>Indofil M-45</td>
<td>Mancozeb80 WP</td>
<td>N-(2,6 dimethyl phenyl)-N-(methoxyacetyl)-alanine methyl ester (C$<em>{11}$H$</em>{21}$NO$_4$)</td>
<td>0.2</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Streptomycin sulphate</td>
<td>Streptomycin sulphate</td>
<td>0.015</td>
</tr>
<tr>
<td>Sulphate</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
</tbody>
</table>
NA plate at center. Then 100 µl volumes of chemical suspension at different concentrations [Cupravit 50 WP (0.2 and 0.4%), Indofil M-45(0.2 and 0.4%) and Streptomycin Sulphate (0.015 and 0.15%)] were added into the holes. Three replications were maintained for each concentration. Sterile water was used instead of chemical as control. The plates were then incubated at 30°C in incubation chamber. Zone of inhibition around the holes were measured and recorded after every 24 hrs for 5 consecutive days.

3. RESULTS

3.1 Colony of *Xanthomonas axonopodis pv. malvacearum*

Colonies of *Xanthomonas axonopodis pv. malvacearum* on NA plates were yellow, convex, mucoid and circular after 48 hours of incubation (Plate 1A).

3.2 Morphological Characteristics

Under the compound microscope *Xanthomonas axonopodis pv. malvacearum* was rod shaped with round ends, cells appeared singly and also in pairs, gram negative bacteria (red colour) and capsulated. The cells were readily stained with common stains such as crystal violet (Plate 1B). The Bacterium creates thread at KOH solubility test (Plate 1C).

3.3 Pathogenicity Test

The result of pathogenicity test revealed that the bacteria showed typical angular leaf blight symptoms and after isolation gave the same colonial color texture as what these was inoculated. Therefore, these seem to be pathogenic (Plate 4).

3.4 Colony Morphology on Different Growth Media

Colonies of *X. axonopodis pv. malvacearum* on NA medium appered as circular, mucoid, convex, yellow to orange color (Plate 5A). Circular, flattened or slightly raised, yellow to bright yellow color, mucoid colonies were found on YDCA medium (Plate 5B) Bacterium exhibited very poor growth with light yellow, mostly circular, small, flattened, mucoid on SX Medium (Plate 5C).

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th>Results</th>
<th>According to Schaad (1992) [13] and Salle (1961) [14]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch hydrolysis test (Plate 2A)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase test (Plate 2B)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test (Plate 2C)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization test (Plate 2D)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motility indole urease agar (MIU) test (Plate 2E)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquefaction test (Plate 2F)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tobacco hypersensitivity (Plate 3)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Plate 2. Different biochemical tests of *X. axonopodis* pv. *malvacearum*. A. Starch hydrolysis test (positive); B. Catalase test (positive); C. Oxidase test (positive); D. Citrate utilization test (positive); E. Motility indole urease agar (MIU) test; F. Gelatin liquefication test (positive); G. Salt tolerance test (positive)

Table 3. Salt tolerance test for *Xanthomonas axonopodis* pv. *malvacearum* in nutrient broth

<table>
<thead>
<tr>
<th>Time</th>
<th>1%</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>6%</th>
<th>7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48 hr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72 hr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.5 Efficacy of Selected Chemicals against *Xanthomonas axonopodis* pv. *malvacearum* at Different Days After Incubation

A significant variation of inhibition zone was observed against the selected chemicals. The highest inhibition zone (35.33, 36.17, 35.17, 33.5 and 32.33 mm after 24, 48, 72, 96 and 120 hr., respectively) was observed against Streptomycin sulphate @ 0.15%. On the other hand, no inhibition zone was observed in control condition (Fig. 1. and Plate 6). All the selected chemicals give the highest result at 48 hrs. After incubation which was gradually decrease on the successive hrs.

Plate 3. Tobacco hypersensitivity test. A. Inoculation of bacterial suspension into the lower surface of tobacco leaf with a sterile syringe; B. Positive reaction and initiation of infection (*Xanthomonas axonopodis* pv. *malvacearum* inoculated)

Fig. 1. Efficacy of selected chemicals against *Xanthomonas campestris* pv. *malvacearum* at different days after incubation

* T1 = Cupravit 50 WP @0.2% ; T2 = Cupravit 50 WP @0.4% ; T3 = Cupravit 50 WP @0.2% + Indofil M-45 @0.2% ; T4 = Cupravit 50 WP @0.4% + Indofil M-45 @0.4% ; T5 = Streptomycin sulphate @0.015% ; T6 = Streptomycin sulphate @0.15%
Plate 4. Pathogenicity test, A. Inoculation of bacterial suspension into the lower surface of cotton leaf with a sterile syringe; B. Symptom of bacterial blight on inoculated leaf of cotton

Plate 5. Cultural characteristics of *Xanthomonas campestris* pv. *malvacearum* on different growth media. A. On nutrient agar (NA) medium; B. On yeast extract dextrose calcium carbonate agar (YDCA) medium; C. On SX agar medium

4. DISCUSSION

The causal organism of BB was isolated from the infected leaves showing typical symptoms of bacterial blight. For confirmation of bacterial blight oozing test was performed. *Xanthomonas axonopodis* pv. *malvacearum* produced yellow, convex, mucoid, colonies of bacterium on nutrient agar medium after 48 hours of incubation at 30°C. The pathogen has also been reported by many researchers throughout the world [19,20,21,22,23]. *Xanthomonas* produce mycoid, circular, convex, yellow, round, glistening and raised colonies on nutrient agar medium [24].

*Xanthomonas axonopodis* pv. *malvacearum* was rod shape, gram negative bacteria. It showed positive reactions in Starch hydrolysis test, Catalase test, Oxidase test, Citrate utilization test, Motility indole urease agar (MIU) test, Gelatin liquefaction test and Salt tolerant test. Similar results has also been reported [19,7,20,24,25,26,27,28]. In the present study, it was observed that *X. axonopodis* pv. *malvacearum* produce circular, flattened or slightly raised, yellow to bright yellow color, mucoid colonies on YDCA medium and light yellow, mostly circular, small, flattened, mucoid on SX Medium. *Xanthomonas* gave yellow, circular, and viscous bacterial colonies on yeast extract nutrient agar (YDCA) after 48-72 h which is supported Hamid et al. [7] and Jabeen et al. [29]. In SX Medium the bacterium gave light yellow to slightly azure, mostly circular, small, flattened, mucoid colonies. A similar result has also been reported by many researchers [19,20,21,30,31]. With the present study, it was observed that *X. axonopodis* pv. *malvacearum* can tolerate up to 3% salt concentration after 72 hours of incubation which is supported by researchers [20,32]. The present study revealed that after infiltration the lower surface of a mature tobacoo leaf with *X. axonopodis* pv. *malvacearum*, the infiltrated area became dry and necrotized within 72 hours which confirmed the pathogenic nature of the bacteria. A same result has also been reported [19,20,33,34].
Plate 6. Bioassay of some selected chemicals against the bacteria, A. Cupravit 50WP (0.2%); B. Cupravit 50WP (0.4%); C. Cupravit 50WP + Indofil M-45 (0.2%); D. Cupravit 50WP + Indofil M-45 (0.4%); E. Streptomycin sulphate (0.015%); F. Streptomycin sulphate (0.15%); G. Control

Among the selected chemicals viz. Cupravit 50 WP, Indofil M-45 and Streptomycin sulphate evaluated in the present in vitro investigation, Streptomycin sulphate @0.15% (36.17 mm) and Streptomycin sulphate @0.015% (31.00 mm) exhibited significantly superior efficacy in inhibiting the growth of Xanthomonas axonopodis pv. malvacearum while Cupravit 50 WP + Indofil M-45 @0.4% (28.17 mm) and Cupravit 50 WP + Indofil M-45 @0.2% (24.33 mm) were found moderately effective and Cupravit 50 WP @0.4% (18.67 mm) and Cupravit 50 WP @0.2% (17.5 mm) were less effective. The results obtained in the present study were in agreement with the reports of earlier workers [20,35,36]. Menegium et al. [37] reported that copper as well as mixture of copper with mancozeb were effective in controlling Xanthomonas spp.

5. CONCLUSIONS

Xanthomonas axonopodis pv. malvacearum was isolated and identified from cotton leaf on the basis of pathogenicity test, tobacco
hypersensitivity test, morphological, biochemical test and growth characters on differential media viz. YDCA medium and SX Medium. In vitro evaluation of selected chemicals revealed that Streptomycin sulphate was highly effective against X. axonopodis pv. malvacearum. Streptomycin sulphate @ 0.15% amended culture resulted the highest inhibition zone of 36.17 mm after 48 hrs. Streptomycin sulphate @ 0.15% can be tested against different races of reference isolates of Xam before field application.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

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