Isolation and characterization of bacterial spot disease of citrus through biochemical approaches and its control measures

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Abstract
Citrus bacterial spot disease is caused by Xanthomonas campestris pv. citrulina, which produced unsightly blemishes on leaves and stems of nursery plants and reduce its market value. The present study was done to identify the pathogen from citrus infected leaves, and find out its biological control. Different biochemical tests including gram staining, motility, Simmons citrate, urease, catalase, potassium hydroxide, Triple sugar iron, Kligler iron agar, MacConkey agar, Kovacs oxidase tests showed positive response against the isolated bacteria. All biochemical test results ensured that, the isolated bacteria was gram negative. Chloramphenicol (30 mcg) revealed the highest antibiotic sensitivity with 32±0.5 mm diameter of zone of inhibition against the isolated bacteria. The raw extract of Allium sativum showed highest antibacterial activity with 16.0±0.5 mm diameter against the isolated bacteria. The study will be helpful to confirm the efficacy of these antibiotics and plant extracts to make biological control of the disease.

Keywords: Bacterial spot, Xanthomonas, Biochemical assays, Sensitivity test, Bio-control

Introduction
Citrus is one of the most edible fruits in worldwide, which belongs to the Rutaceae family. In world, citrus are the second most planting and production fruit plant [1]. It is very effectual secondary metabolites source for nutrition, health, drugs, and other industrial solicitation [2]. Additionally, it reflect various therapeutic qualities. Citrus fruit, like lemons and limes particularly have high concentration of citric acid. Basically, citrus fruit juice used in numerous periodic application for healing blow, scabies and other skin problems [3]. The cooked fruits are used against colds and as perspiration increaser. The citrus juice, which mixed up with water is used against dyspepsia alkaline and fresh juice used against catarrhal angina. The curtail fruit juice is applied to upper stomach vomiting and also applied in chronic disease.

Malus pumila, Citrus variegated chlorosis, Xanthomonas, Biochemical assays, Sensitivity test, Bio-control

Materials and Methods
Plant materials
Disease infected citrus plant leaves were collecting from Rajshahi University Campus, Rajshahi, Bangladesh and were identifying by a research officer of Bangladesh Fruits Research Institute, Binodpur, Rajshahi. Spot disease infected leaves of citrus plant were used as plant material for this present investigation.

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Isolation and purification of causal organism
Spot disease infected leaves were disinfested using a dilute sodium hypochlorite solution (10%) and rinsed thoroughly. Cut the infected area and placed on LB liquid media for allow to growing bacteria and incubated for 16 hours at 37°C. After the bacteria have grown into LB liquid medium, use a sterile loop to streak the bacteria onto a solid nutrient agar media plates and incubated for 16 hours at 37°C. One of creamy white colony was pick up by loop and streaked on another media plate for pure culture.

Morphological Test
Gram staining: Gram staining reagents was prepared by taking crystal violet, gram’s iodine, decolorizes (alcohol), and stain safranin. Bacterial sample was smears on a slide and carefully fixed by heat. One drop crystal violet was place on smears and holds it 1 minute and rinse with distilled water. One drop of gram’s iodine was place on smears for 1 minutes and rinse with distilled water. Added decolorize reagents on the sample. If it is gram negative, removing the crystal violet. Finally one drop of safranin was added for 30 seconds and rinse with distilled water and placed to air dry for several minutes. The slide was observed under 100X microscope along with one drop of immersion oil to examine shape, size, arrangement and staining reaction of bacterial isolates [12].

Motility test: Soft agar medium was prepare in a test tube for motility test. One isolated colony was pick from the culture and inoculates the medium by stabbing the center of the medium to a depth of 1 inch [13].

Biochemical test
Simmons citrate test: Citrate medium [14] was prepare in deionized water and sterilized at 121°C for 20 minutes. After that the media was place on a tube and cooled in a slanted position. The isolated colony was pick with a needle and the slant surface was lightly streaked. The incubation was done at 37°C for 16 hours.

Urease test: Urease medium was prepare to add deionized water. The pH was adjusted to 6.7 and autoclaved at 121°C for 20 minutes except urea. After autoclaved, cooled the media to 50 to 55°C, then urea base was add into the media and mixed thoroughly. The tubes were slanted during cooling until solidified. The isolates were inoculating into the slant and incubate at 37°C for 16 hours [15].

Catalase test: Transferred a small amount of bacterial colony in the clean glass slide by the use of sterile loop. A drop of hydrogen peroxide was place on a glass slide [16].

Potassium hydroxide test (KOH): In KOH test, one or two isolated colonies from the pure culture were placed on a clean slide. After that added one drop of 0.3M KOH on the top of the colonies and waited for 1 minute and observed [17].

Triple sugar iron agar test (TSI): This test was performed to determine the ability of an organism to attack a specific carbohydrate incorporated in a basal growth medium [18], with or without the production of gas, along with the determination of possible hydrogen sulfide (H₂S) production. The TSI agar slant (long butt and short slant) containing three types of sugars (Dextrose, Lactose and Sucrose) was stab, streak with inoculums and incubate at 37°C for 16 hours.

Kligler iron agar test (KIA): The medium was prepare one liter deionized water and sterilized at 121°C for 20 minutes [19]. The overnight culture of each isolate were stabbing the butt and streaking the surface of the tube as well as incubated aerobically at 37°C for 16 hours.

MacConkey agar test: The medium was prepare by petri plates. The pH was adjusted to 7.1 and sterilized at 121°C for 20 minutes. The medium was pour into the petri dishes and cool to solidify at room temperature in the laminar airflow. The isolated colony were taking with sterile loop and streaking the petri plates. The incubation was done at 37°C for 16 hours [14].

Kovac’s oxidase test: One drop of 1% Kovacs’ reagent (1gm Tetramethy-p-phenylenediamine Dihydrochloride in 100 ml distilled water) was placed on the middle of the Whatman filter paper and platinum loop full of bacterial strain was carefully rubbed on the filter paper [20].

Antibiotic Sensitivity Test
Different types of antibiotics like, Amoxicillin, Azithromycin, Carbencillin, Cefotaxime, Clarithromycin, Chloramphenicol, Gentamycin, Kanamycin, Streptomycin and Tetracycline were used for antibiotic sensitivity test. For this purpose, Kirby-Bauer disk diffusion method has been used [21]. First LB agar medium was prepared for making culture plates. 1-2 ml of isolated bacterial colony were poured in each petri plates and left the airflow cabinet for solidification. Finally each different antibiotics were placing centrally on the respective plates and incubating overnight at 37°C.

Antibacterial Activity Test against Plant Extracts
Four plants extracts like, Allium sativum, Allium cepa, Gingiber officinale, Ocimum basilicum were used for antibacterial activity test. Selected plants were collected, dried, cut in a small piece and pest in mortar pestle. The extracts were stored in a glass bottle. This test was screened by using the disc diffusion method. An inoculum suspension was swabbed uniformly to solidify of LB agar for bacteria and allowed to dry for 5 minutes. Disc of 6 mm in diameter were used. Aliquot of 10, 15, 20μl from each plant raw extract was added into each disc on the seeded medium and allowed to stand on the bench for 30 minutes for proper diffusion and incubated at 37°C for 16 hours [22].

Statistical tools for analysis
Above study were conducted in repeated triplicate for accurate results and statistical analysis. All the data were revealed as mean and standard error using 2013 Microsoft Excel sheet version. The actual values with P<0.5 was calculated statistically significant.

Results
Isolation and Purification
The infected leaves samples placed on LB liquid media showed the bacterial colonies after 16 hours of incubation at 37°C. The turbid condition in the media indicates the bacteria were grown. By streaking method, single colonies were found. The colonies were creamy white in color. The size and shape of colonies were found to be small, medium, convex and mucoid.
Morphological characterization of isolated bacteria

Gram Staining and motility test
In gram staining test, bacterial colonies slide were observed under the light microscope at 100X using oil immersion. The isolates showed to be gram negative, rod shaped and pinkish in color when stained with counter-stained by the safranin which was the indication for gram negative bacterium. After incubated the medium at 37ºC for 16 hours, a positive motility test was observed. The growth area was extending away from the line of inoculation which indicates the isolated bacteria were motile.

Biochemical test
In Simmons citrate test, the isolated bacteria showed positive result against Simmons citrate agar, because the medium turns the blue color, which indicates the isolated bacteria were able to utilizing citrate. The isolated bacteria were negative to urease test, because no pink color was formed in the media, which indicates the isolated bacteria were unable to hydrolyzing urea to ammonia. After added hydrogen peroxide on the clean slide, which contains a small amount of bacterial colonies, the oxygen bubbles were clearly indicates the isolated bacteria were catalase positive. In KOH test, the isolated bacteria was gram negative, because it showed viscous strings and formed thread like slime. Color changed of the TSI medium showed the positive result, that’s mean slant yellow and butt yellow color indicates the isolated bacteria were glucose and lactose fermenting. But no H2S was formed in the medium. In KIA test, yellow color in the medium confirmed the positive result, which indicates the isolated bacteria were glucose and lactose fermenting, but no gas was formed in the medium. Characterize the isolated bacteria as MacConkey agar positive, because the medium turns the pink color after overnight incubation at 37ºC, which indicates the isolated bacteria were able to ferment lactose. Finally in Kovac’s oxidase test, the isolated bacteria was gram negative because that gave no purple color after 60 seconds. All characterization results have been presented in a Table 1.

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
<th>Optimization</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>-ve</td>
<td>Small, rod shaped, Pink color colony</td>
<td>Isolated bacteria was gram negative</td>
</tr>
<tr>
<td>Motility</td>
<td>+ve</td>
<td>Growth area extend away from the inoculation line</td>
<td>Isolated bacteria was motile</td>
</tr>
<tr>
<td>Simmons citrate</td>
<td>+ve</td>
<td>Blue color</td>
<td>Isolated bacteria was capable to utilized citrate</td>
</tr>
<tr>
<td>Urease</td>
<td>-ve</td>
<td>No color</td>
<td>Isolated bacteria were not able to hydrolyzing urea to ammonia</td>
</tr>
<tr>
<td>Catalase</td>
<td>+ve</td>
<td>Oxygen bubbles</td>
<td>Isolated bacteria was able to produce catalase enzyme</td>
</tr>
<tr>
<td>KOH</td>
<td>+ve</td>
<td>Viscous and thread like slime</td>
<td>Isolated bacteria was gram negative,</td>
</tr>
<tr>
<td>TSI</td>
<td>+ve</td>
<td>Yellow color</td>
<td>Isolated bacteria were glucose and lactose fermenting, but no gas and H2S was formed</td>
</tr>
<tr>
<td>KIA</td>
<td>+ve</td>
<td>Yellow color</td>
<td>Isolated bacteria were glucose and lactose fermenting, but no gas was formed</td>
</tr>
<tr>
<td>MacConkey agar</td>
<td>+ve</td>
<td>Pink color</td>
<td>Isolated bacteria were lactose fermenting</td>
</tr>
<tr>
<td>Kovac’s oxidase</td>
<td>-ve</td>
<td>Colorless</td>
<td>Isolated bacteria gave no purple color after 60 seconds</td>
</tr>
</tbody>
</table>

Antibiotic Sensitivity Test
In antibiotic test, ten different types of antibiotic discs were used against isolated bacteria. Chloramphenicol revealed the highest significant antibiotic activity with inhibition zone of 13.0±0.5mm against the isolated bacteria. Most of the antibiotic showed a moderate antibiotic spectrum against the isolated bacteria. The results of antibiotic sensitivity test have been presented in a Table 2.

<table>
<thead>
<tr>
<th>Name of Antibiotic</th>
<th>Symbol</th>
<th>Disc potency (µg)</th>
<th>Zone of inhibition (mm) (M±SE)</th>
<th>Sensitivity pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>AML</td>
<td>10</td>
<td>11.0±0.5</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>AZM</td>
<td>15</td>
<td>24.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>CB</td>
<td>100</td>
<td>19.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CTX</td>
<td>30</td>
<td>29.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>CLR</td>
<td>15</td>
<td>25.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C</td>
<td>30</td>
<td>32.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>GEN</td>
<td>10</td>
<td>30.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>K</td>
<td>30</td>
<td>22.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S</td>
<td>10</td>
<td>26.0±0.5</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>TE</td>
<td>30</td>
<td>13.0±0.5</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

Note: Resistant =<10 mm; Intermediate =<10-15 mm; Susceptible =>15 mm [21]

Antibacterial Activity Test against Plant Extracts
In antibacterial susceptibility test, raw extracts of four different plants were used for evaluate the control measures of this disease. Among these, the raw extracts of Allium sativum revealed the highest antibacterial activity with16.0±0.5mm diameter of zone of inhibition in20µl/disc concentration followed by 14.0±0.5mm diameter zone of inhibition for same extract in 15µl/disc concentration against the isolated bacteria. The raw extract of Gingiber officinale showed the lowest antibacterial activity with 5.0±0.5mm diameter of zone of inhibition against the isolated bacteria. Likewise, the other raw extract of plant showed moderate antibacterial activity. The test results have been presented in a Table 3.
Discussion
In this study, bacterial spot disease affected leaves were collected from the citrus plant and the Xanthomonas campestris pv citruleno was isolated and purified. Several biochemical tests such as Gram staining, Motility, Simmons citrate, Urease, Catalase, KOH, TSI, KIA, MacConkey agar, Kovac’s oxidase test was done to characterize the isolated bacteria as gram negative bacteria. The isolated bacteria was gram negative, which shows pink color and rod shaped size in staining procedure [23]. The motility test which was accurately confirmed the isolated bacteria was motile [13]. The isolated bacteria was able to utilized citrate and the media color change it to blue [14]. The isolated bacteria showed negative result against the urease test which confirmed the bacteria was not able to hydrolyze urea [15]. The isolated formed bubbles after added hydrogen peroxide on the top of the bacterial colonies, which was confirmed our bacteria was catalase positive [16]. In addition, Suslow et al. (1982) [17] performed KOH test to finally confirmed characterized gram negative bacteria of wheat, so our isolated bacteria was clearly indicates as gram negative. In TSI test, the gram negative bacteria observed positive results against these two test [14]. The slant and butt yellow color indicates the isolated bacteria was glucose and lactose fermenting. No H₂S was formed, because no black precipitation was found in the medium. The KIA test confirmed the isolated bacteria was glucose and lactose fermenting but no gas was formed in the medium.
Taylor and Silliker, (1958) [19] performed the KIA test in Salmonellae food samples which was clearly characterized between gram positive and gram negative bacteria. The isolated bacteria turns the MacConkey agar media pink color after overnight incubation, which indicates the isolated bacteria was able to ferment lactose [14]. Kovac’s reagent gives no purple color after 60 seconds, which indicates the isolated bacteria was gram negative bacteria [20]. In antibiotic test, Chloramphenicol showed highest 32.0±0.5mm diameter zone of inhibition, while tetracycline showed the lowest 5.0±0.5mm diameter zone of inhibition against isolated bacteria. Inhibitory assay, Allium sativum extract revealed the highest 16.0±0.5mm diameter of zone of inhibition and Gingiber officinale extract showed the lowest 5.0±0.5mm diameter of zone of inhibition against isolated bacteria. Kovac’s reagent gives no purple color after 60 seconds, which indicates the isolated bacteria was gram negative bacteria [20]. In antibiotic test, Chloramphenicol showed highest 32.0±0.5mm diameter zone of inhibition, while tetracycline showed the lowest 5.0±0.5mm diameter zone of inhibition against isolated bacteria. Kovac’s reagent gives no purple color after 60 seconds, which indicates the isolated bacteria was gram negative bacteria [20]. In antibiotic test, Chloramphenicol showed highest 32.0±0.5mm diameter zone of inhibition, while tetracycline showed the lowest 5.0±0.5mm diameter zone of inhibition against isolated bacteria. Kovac’s reagent gives no purple color after 60 seconds, which indicates the isolated bacteria was gram negative bacteria [20].

Table 3: Effects of some plant extracts against isolated bacteria

<table>
<thead>
<tr>
<th>Name of plant extract</th>
<th>Zone of inhibition in different doses (mm) (M±SE)</th>
<th>Sensitivity pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10µl</td>
<td>15 µl</td>
</tr>
<tr>
<td>Allium sativum</td>
<td>11.0±0.5</td>
<td>14.0±0.5</td>
</tr>
<tr>
<td>Allium cepa</td>
<td>7.0±0.5</td>
<td>8.0±0.5</td>
</tr>
<tr>
<td>Gingiber officinale</td>
<td>5.0±0.5</td>
<td>7.5.0±0.5</td>
</tr>
<tr>
<td>Ocimum basilicum</td>
<td>6.0±0.5</td>
<td>7.5.0±0.5</td>
</tr>
</tbody>
</table>

Note: Resistant = <10 mm; Intermediate = <10-15 mm; Susceptible = >15 mm

Conflict of interest
The authors declare that there is no conflict of interests regarding the publication of this paper.

References
13. Adler J, Dahl MM. A method for measuring the motility...
of bacteria and for comparing random and non-random motility, Microbiology. 1967; 46(2):161-173.