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Detection of bacterial wilt disease of banana through biochemical approaches and its biological control

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Abstract

The present investigation was done to detect the causal agent of bacterial wilt of banana. Bacterial wilt is caused by gram negative bacteria *Ralstonia solanacearum.* The causal organism was isolated and cultured on LB liquid media at pH 7.4 and growing within 12 to 16 hours. The colonies on LB nutrient media were creamy in color. Morphological test indicated that isolated strains were gram negative because it showed small, rod-shaped, pink color on the media. Extending growth area of culture strain confirmed that isolated bacteria was motile. Biochemical test results showed that the isolated bacteria were Catalase, Potassium hydroxide (KOH), Simmons citrate, Kovacs oxidase, and Urease test positive while only negative to Sulfur indole motility (SIM) test. Among fifteen antibiotics disc, the cefotaxime showed maximum 35±0.5 mm of zone of inhibition against the isolated bacterial strain. In contrast to antibacterial activity test, four plant extracts were used and the test results showed that ethanol extract of *Terminalia arjuna* (Arjun) has a broad spectrum of antibacterial activity with the zone of 12±0.5 mm against the isolated bacterial strain and this effect is increased by increasing the quantity of this compound, which can be used as an alternative for antibiotics.

Keywords: Bacterial wilt, *Ralstonia solanacearum*, Biochemical test, Antibiotic sensitivity, Antibacterial activity test.

INTRODUCTION

Banana (*Musa* sp.) is the fourth most edible global food crop plant after rice, wheat and maize in contrast of its vast production. In Asia, Banana is the most popular cultivated crop and 33 banana germplasm are present in these area (Prahardini *et al*. 2010). Banana has been used for many medicinal objectives. For example, it can be used for different forms of diarrhea treatment, reduce cholesterol from body, decrease the risk of cancer, and gastric ulcers etc. It has excellent source of potassium and banana is an excellent source of potassium, fibers and a number of vitamins (Kumar *et al*. 2012). Jain and his co-workers were observed there are so many different antibacterial and antioxidant activities are present in the seeded banana fruits *in vitro* (Jain *et al*. 2011). The banana cultivation increases annually and there are many disease is affects its production (Shian *et al*. 2012). Among these diseases, bacterial wilt is more concern topics, which caused by *Ralstonia solanacearum* (Elsas *et al*. 2001). Bacterial wilt is an important disease of banana (Ilagan *et al*. 2010). The disease is prevalent throughout the country. And almost all varieties of banana are susceptible to the disease. The primary symptom of the disease is sudden wilting of the plant. The wilted plant later get dried up and even collapse. And the infested bunches are rejected in export market. Therefore, control of the disease is very much important in banana cultivation. The bacterial wilt disease infected banana showed some unique symptoms such as rapid wilting and collapse of the young leaves, yellowish tinge spreads all over the leaves, petiole breaks, appearance of bacterial

ooze and rotting on infected plant parts (Jones *et al*. 1998). Generally, *R. solanacearum* is a soil borne bacterial pathogen, and very diversely in its species and belongs to the group of *Ralstonia*. Due to its diversified species, it can be easily identified by biochemical characterization and also molecular diagnosis such as polymerase chain reaction using specific primer (Nasir *et al*. 2005). Therefore, the present study was conducted to determine the causal pathogen through the different morphological and biochemical and antibiotic sensitivity test. Antibacterial activity of some medicinal plant extracts help to find out the biological control against the isolated bacterial strain.

MATERIALS AND METHODS

Sample collection

Infected leaves were collected from University of Rajshahi and identified by Bangladesh Fruit Research Institute, Binodpur, Rajshahi, Bangladesh.

Isolation and purification

Wilt disease affected plant parts were sterilized by using 10% sodium hypochlorite dilute solution and rinsed thoroughly. Thereafter affected area were cut down by the help of sterilized scalpel and grind in mortar pestle and keep them into Luria-Berteani (LB)broth medium for overnight incubation of bacterial growth(Seal *et al*. 1993). This incubation was done for 12-16 hours at 37°C. The bacterial isolates were purified by streaking method on LB broth media.

Characterization of the isolated bacteria

Different morphological and biochemical tests were done to characterize the isolated bacteria.

Gram staining: Isolated bacteria was picked by the help of loop and spread on a glass slide and then fixed by heat on a very low flame. The crystal violet solution (0.5%) was added onto the top of the glass slide and smear over the slide for 30 seconds and then rinsed with water. It was the inundated with gram's iodine solution for one minute, rinsed with water and then added decolorized with 95% ethanol until colorless runoff. After washing the slide, again added counter stained with safranin for at least 10 seconds, and washed with water. Finally, allow the slide for air dried at room temperature and observed under 100X microscope using immersion oil (Tan *et al*. 2015).

Motility test: Mild agar media was prepared in a test tube for motility test. Picked up one pure colonies on the nutrient media and integrated center of the mild agar medium to a depth of 1 inch by the help of sterilized needle (Schaad *et al*. 2001).

Catalase test: For this test, 3% hydrogen peroxide (H_2O_2) solution was used to observe the production of oxygen bubbles. One of the purified colonies as placed on a glass slide and drop of H_2O_2 solution on the top of the cell (Tan *et al*. 2015).

Potassium hydroxide (KOH): Isolated bacteria were aseptically took from the Petri plates with a tooth pick or an inoculating loop and then placed on a clean glass slide in a drop of 3% KOH solution. After that the mixture was mixed up for 10 seconds and observed the thread like slime (Adler *et al*. 1967).

Simmons citrate test: Citrate media were mixed up in distilled water and sterilized at 121°C for 20 minutes. Thereafter the media were poured into test tube for created slanted position and one of isolated colony was streaked into the surface of the media. This was kept at 37°C for 16 hours (Suslow *et al*. 1982).

Kovacs oxidase test: The Kovacs oxidase reagent (1% tetramethyl-p-phenyl diamine dihydrochloride) was prepared and kept in dark bottle. Then one drop of reagent was placed to a piece of filter paper, which placed on a glass Petri dish. Few numbers of isolated colonies were grazed on the paper saturated with 1% oxidase reagent solution and noted for creating the purple color in 20-60 seconds (Sun *et al*. 2011).

Urease test: In this test, the urease media were prepared and then poured into test tubes. Then small amount of inoculum were placed on a tube and kept into incubator for 16 hours at 37°C (Suslow *et al*. 1982).

Sulfur indole motility (SIM): For SIM test, media were prepared with distilled water and autoclaved at 121°C for 20 minutes. Then media were placed on a test tube. After that, a few amount of inoculum were inoculated into the media test tube and incubated overnight at 37°C for 16 hours. Finally one drop of Kovac's reagent was added and observed (Lelliott *et al*. 1987).

Antibiotic sensitivity test: Antibiotic sensitivity test was done by Kirby-Bauer disc diffusion method (Levin *et al*. 1970). In this test, isolated bacteria were grown in nutrient broth medium, then take 1ml of overnight inoculum and transferred on the nutrient agar plate allowed for drying. Fifteen antibiotics with different disc concentration such as Amoxicillin, Erythromycin,

Gentamycin, Chloramphenicol, Clarithromycin, Ampicillin, Tetracycline, Carbenicillin, Neomycin, Streptomycin, Azithromycin, Kanamycin, Doxycycline, Cefotaxime, and Penicillin were placed in the center of the petri plates and incubated overnight at 37°C for 16 hours (Hudzicki *et al*. 2009).

Antibacterial activity: Four medicinal plants such asleaves of *Ocimum sanctum* (Tulsi), Bulb of *Allium cepa* (Onion), Root of *Asparagus racemosus* (Shatamooli), Burk of *Terminalia arjuna* (Arjun) were used for this investigation. These plant parts were carefully washed and dried in shade for several days, then it was greened into a fine powder (Jorgensen *et al*. 2015). The prepared powder was soaked in ethanol solvents (ratio 1:10, w/v) and shaking at 150 rpm for 24 hour. Filtrated extracts were dried at 40°C. The dried extracts were re-suspended in Phosphate Buffered Saline (PBS) to bring to 500 mg mL^{-1} concentration. The test was screened by disc diffusion method. For preparing aliquot 5 mm in diameter disc were used and 10, 20, 30 µl ethanol extract was soaked into each disc on the bacterial plate and placed on central of the plates for 30 minutes for proper diffusion and incubated at 37°C for 16 hours (Biemer *et al*. 1973).

RESULTS

The infected part showed creamy color in LB liquid medium after overnight incubation. Typical inoculum was picked and confirmed it purification. The colonies were small, convex and mucoid. Structural and chemical properties of bacteria were done. Specific staining techniques help to detect different chemical composition in bacterial cells. Presence or absence of such substance is used for identification of bacteria. These strategies found the fundamental biochemical and biophysical differences in the bacterial cell wall. The isolated strain retained reddish pink colony color, which indicates these were gram negative, while purple color showed gram positive bacteria. In contrast to motility test, isolated bacteria showed positive result, because the inoculation area was extending after overnight incubation. The isolated bacterial strain produced gas bubbles after adding the H_2O_2 solution. In KOH test, the viscous string and thread like slime indicated that isolated bacterium was gram negative. Isolated bacteria turn the citrate media color blue, which confirmed that isolated bacterial strain capable to utilized citrate. In Kovacs oxidase test, isolated bacteria did not produce any purple color, so it confirms isolated bacteria was negative to this test. Isolated bacteria was not able degrade urea in the urease medium. In case of SIM test, the isolated bacteria were unable to form sulfur and indole but isolated bacteria was motile. The summarized results of all biochemical tests were shown in Table 1.

Table 1: Results of biochemical test of bacteria

Test	Results	Remarks
Gram staining test	Negative	Rod shaped, small and pink color
Motility test	Positive	Growth area extending away from the inoculation
Catalase test	Positive	Oxygen bubbles showed isolated bacteria was able to break hydrogen peroxide
KOH	Positive	Thread like slime and sticky staff
Simmons citrate test	Positive	The media color change to blue, which indicates isolated bacteria able to ferment citrate
Kovacs oxidase test	Negative	No color change, addition of Kovac's reagent after 60 seconds
Urease test	Positive	Isolated bacteria turns the media color pink which indicates isolated bacteria able to ferment urease
SIM test	Negative (Motile)	Sulfur production- positive Indole formation- negative

Antibiotic sensitivity test:

For antibiotic sensitivity tests, fifteen different antibiotics have been evaluated *in vitro* against *Ralstonia solanacearum* bacterial species. Most of the tested antibiotic showed high level of antibiotic sensitivity. The obtained results show that, Chloramphenicol, Tetracycline, Neomycin, Kanamycin, and Cefotaxime showed maximum sensitivity against the isolated bacterial strain with inhibition zone more than 35±0.5 mm, while other antibiotic showed moderate antibiotic spectrum against the isolated bacteria. The overall results of antibiotic sensitivity test have been presented in Table 2.

Table 2: Effects of Antibiotic sensitivity test

Name of Antibiotic	Disc potency	Zone of inhibition (mm) + MSE	Response
Amoxycillin	10 µg	12±0.5	Intermediate
Erythromycin	15 µg	10±0.5	Resistant
Gentamycin	10 µg	24±0.5	Susceptible
Penicillin	10 units	10±0.5	Resistant
Chloramphenicol	30 mcg	22±0.5	Susceptible
Clarithromycin	15 µg	9±0.5	Resistant
Ampicillin	10 µg	10±0.5	Resistant
Tetracycline	30 µg	16±0.5	Susceptible
Carbenicillin	100 µg	18±0.5	Susceptible
Neomycin	30 µg	18±0.5	Susceptible
Streptomycin	10 µg	22±0.5	Susceptible
Azithromycin	15 µg	18±0.5	Susceptible
Kanamycin	30 µg	23±0.5	Susceptible
Doxycycline	30 µg	12±0.5	Intermediate
Cefotaxime	30µg	35±0.5	Susceptible

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm (Islam, *et al*. 2017)

Antibacterial activity test using some plant extracts

The ethanol extract of *Terminalia arjuna* (Arjun) revealed the highest significant antibacterial activity with inhibition zone more than 12±.5 mm. In contrast, ethanol extract of *Allium cepa* (Onion), *Ocimum sanctum* (Tulsi), *and Asparagus racemosus* (Shatamooli) showed antibacterial activity with mean inhibition zone of 8±0.5 mm, 9±0.5mm and10±0.5mm respectively. The results of this current investigation clearly indicate that, the antibacterial activity varied in respect to the plants, type of solvent and type of tested microorganism. The total results of antibacterial activity test have been presented in Table 3.

Table 3: Effects of antibacterial activity of some plant extracts

Name of plant extract	Dose of plant extract (zone in mm) (M±SE)			Sensitivity pattern against isolated bacteria
	10µl	20 µl	30µl	
Ocimum sanctum	7±0.5	8±0.5	9±0.5	Resistant
Allium cepa	6±0.5	7.5±0.5	8±0.5	Resistant
Asparagus racemosus	7±0.5	9±0.5	10±0.5	Resistant
Terminalia arjuna	10±0.5	10±0.5	12±0.5	Intermediate

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm (Islam *et al*. 2017)

DISCUSSION

Fast identification help to detect bacterial wilt disease which is essential because it induced by other pathogens, insects, or mechanical damage at the stem, fruit, and leaves (Agiros *et al*. 1997, Champoiseau *et al*. 2009). Therefore, the wilt disease infected parts were collected and the strain of *Ralstonia solanacearum* was isolated in LB liquid medium and purified. Several morphological and biochemical tests were done to characterize the *Ralstonia solanacearum.* The isolation of causal organism was done on the basis of colony color and growth on the particular media (Cheung *et al*. 2009). In gram staining test, the isolated bacterial strain showed pink color, rod shaped size, which indicates isolated bacteria were gram negative. These results are similar with the findings of Suslow *et al*. 1982. The motility test was confirmed isolated bacterium was motile and these similar findings were founding in Adler *et al*.1967. The isolated bacteria formed oxygen bubbles after addition of H_2O_2 on the slide, that's indicates bacteria were catalase positive [10]. In contrast, Suslow *et al*. 1982 performed KOH test to finally confirm that, gram negative bacteria of wheat, so our isolated bacterium was clearly indicates as gram negative, because the viscous strings and thread like slime was obtained during this test. Sun *et al*. 2011, found similar results in different pathogenic bacteria for citrate test, because isolated bacteria turns the citrate media plate color in blue. After 60 seconds of adding Kovacs reagent the bacteria gives no purple color, which indicates the isolated bacterium were gram negative. The urease test result was confirmed by Cheney and Collins, 1995 that isolated bacteria showed positive response. Among the fifteen antibiotics discs, most of the tested antibiotic showed highest antibiotic sensitivity spectrum. By using Kirby-Bauer disc diffusion method, the obtained results showed that, Chloramphenicol, Tetracycline, Neomycin, Kanamycin, and Cefotaxime showed maximum sensitivity against the isolated bacterial strain with inhibition zone more than 35±0.5 mm, while other antibiotic showed moderate antibiotic spectrum (Hudzicki *et al*. 2009). Finally, four medicinal plants were used for antibacterial activity test. Among these, the ethanol extracts of *Terminalia arjuna* (Arjun) gives the highest sensitivity pattern with the inhibition zone more than 12±0.5 mm against the isolated bacteria and rest of them showed resistance pattern. So, this effect is increased by increasing the quantity of this compound, which can be used as an alternative for antibiotics. However, the overall investigation was help to isolate the bacteria which were responsible for bacteria wilt disease of banana and evaluate its biological control or management system.

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Conflict of interest

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

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