Biochemical characterization and antibiotics sensitivity of bacteria associated with black rot of orange (Citrus sinensis) fruit in Bangladesh

Accepted 27th December, 2020

ABSTRACT

Citrus sinensis is a delectable, juicy and prized fruit in Bangladesh, containing great nutritional impact. The present study was conducted for isolation and detection of the phyto-pathogen responsible for bacterial black rot disease of orange as well as evaluation of its antibiotics sensitivity. The pathogen of the disease was isolated from infected fruits of orange and cultured on Luria-Bertani (LB) growth medium. Gram staining and different biochemical tests were exposed for morphological characterization. Antibiotic sensitivity was assessed by disk diffusion method. The causal agents of the disease were gram negative, rod shaped, motile bacteria. In biochemical tests, Kligler iron agar (KIA), Simon's citrate tests were positive for bacterial sample 1 (BS1) and SIM (Sulphide-Indole-Motility) medium, MacConkey agar, catalase (H2O2), potassium hydroxide (KOH) tests were negative. Moreover, SIM medium, KIA, MacConkey agar tests were positive for bacterial sample 2 (BS2) and Simon's citrate, catalase, potassium hydroxide tests were negative. In antibiotic sensitivity's assay, cefixime showed highest 28.0±0.7 mm diameter of inhibition zone against the isolated bacterial strains. The present research work may be helpful for molecular detection of citrus black rot diseases causing pathogens and their biological control techniques in near future.

Keywords: Citrus sinensis, black rot, bacterial pathogen, identification, antibiotic sensitivity.

INTRODUCTION

Orange is the fruit of numerous citrus species under Rutaceae family; it primarily refers to Citrus sinensis, also known as sweet orange. Sweet orange was originated from a backcross hybrid between pummelo and mandarin (Xu et al., 2013). Citrus sinensis one of the most economically important perennial fruit crop in the world. Orange fruit is mainly used for juice extraction (Kimball, 1999); juice contains only about one-fifth the citric acid of lime or lemon (Penniston et al., 2008). Oranges contain rich dietary fiber, vitamin C, antioxidant (Islam et al., 2020), varied phytochemicals, including carotenoids, flavonoid (Aschoff et al., 2015). It also contains various orange aroma, including aldehydes, esters, terpenes, alcohols and ketones (Perez et al., 2008). Diseases are one of the major issues which hamper the fruit quantity and quality. Nowadays, bacterial spot, black pit of fruit, blast, citrus canker, huanglongbing, die back, and variegated chlorosis diseases are very disturbing for citrus growing areas of the world (Ali et al., 2013), including Bangladesh. The disease causes wide injury to orange fruits, and harshness of contamination differs with different varieties (Burhan et al., 2007). Bacterial diseases stance a continuous hazard to citrus cultivation and causes extensive financial impressions in all citrus growing parts almost the world (Mendoça et al., 2017). Some reports have been published on pre-harvest and post-harvest bacterial, viral and fungal diseases of citrus fruits (Mamun and Feroz, 2017; Hasan et al., 2019; Chaity et al., 2019). Therefore, it is essential to identify the newly mentioned disease namely black rot of orange fruit. Unfortunately, there is no report on isolation, identification and efficient control technique of citrus black rot disease causing bacterial pathogens in Bangladesh. The
goal of this study was to isolate and characterize the pathogens responsible for black rot as well as their controlling techniques using some commercial antibiotics. As our knowledge goes, this is the first report on this disease.

MATERIALS AND METHODS

Isolation and purification of bacteria

In the year 2018-2019, eight black rot disease symptoms of orange fruits were collected from RDA market in Rajshahi, Bangladesh. Infected fruits showing typical black rot were brought to the laboratory. The fruits were cleaned by washing with distilled water and were aseptically refrigerated until analyzed within 20 hours. The disinfection and isolation were performed according to Ali et al. (2013) with minor modifications. In brief, the infected fruit pieces were excised with sterile scalpel and then disinfected superficially through the following protocol: 70% alcohol for 1 minute, sodium hypochlorite for 4 minutes and ethanol for 30 seconds. Finally, the samples were rinsed three times in autoclaved distilled water. The samples were then placed in 100 ml of LB liquid medium separately and incubated at 37°C overnight. After that a sterile loop was used to streak the bacteria onto fresh LB agar plates and incubated again for 16 hours at 37°C. Six single colonies were picked and streaked on fresh medium plates for pure culture. Finally, two pure cultures were preserved on LB slant at 4°C for shorter duration for characterization.

Morphological characterization

The gram staining test differentiates bacteria by the properties of cell wall. Generally, the gram-positive bacteria contain large amount of peptidoglycan in its cell wall while the other type of bacteria named Gram negative bacteria contain a little amount of peptidoglycan. So, the cell loses its initial color from the primary stain. Gram-positive bacteria retain the crystal violet dye, and thus is stained violet, while the gram-negative bacteria don’t. When the safranin is added, it stains the gram-negative bacteria as pink color.

Biochemical characterization

To conduct the SIM medium test, isolated bacterial strains were inoculated by swab and stab method and incubated for about 30 hours. The SIM medium contains peptonized iron that is used as the indicators of H₂S production. Moreover, the motility of bacteria was observed through the media, if they were motile. In the experiment, Kovac’s reagent was added after 30 hours of bacterial incubation to detect the presence of indole which might be produced through the degradation of tryptophan by the enzyme tryptophanase. Kliger iron agar is recommended for the differentiation of gram-negative enteric bacilli on the basis of the fermentation of dextrose, lactose, and H₂S production. The biochemical test- KIA combines the features of Kliger’s lead acetate medium and Russell’s double sugar agar which contains casein, meat peptones, lactose, dextrose as well as phenol red. The fermentation of lactose and/or dextrose results in the production of acid which changes the colour of the pH indicator (phenol red) and helps in the identification of the bacteria. The ability to utilize citrate as a sole source of carbon (energy) distinguishes certain Gram negative organisms. Along with citrate, the medium contains ammonium ion and other inorganic ions needed for growth. It also contains bromothymol blue, a pH indicator, which becomes green at pH below 6.9, and then turns blue at a pH of 7.6 or greater.

Use of citrate results in the creation of carbonate and bicarbonate as byproducts, thus increasing the pH of the medium responsible for the change of the medium colour from green to blue and this is considered as positive test. MacConkey agar medium has lowered agar content and an adjusted concentration of bile salt and neutral red where the identification of enteric microorganisms is achieved by the combination of the neutral red indicator and lactose. In our test, the medium was autodaved at 121°C for 20 minutes and poured into Petri plates. The isolates were inoculated on the medium and incubated at 37°C for 16 hours. For hydrogen peroxide test, a small amount of bacterial colony was transferred on a clean glass slide by the use of sterile loop. Then a drop of hydrogen peroxide was placed on bacterial colony. The production of oxygen can be seen by the formation of bubbles. The result of the test can be seen with the naked eye and without any aid of instruments. To conduct potassium hydroxide test, one drop of 3% potassium hydroxide solution was placed on a clean microscope slide. Subsequently, a few bacterial colonies were emulsified to the drop of potassium hydroxide to make a dense suspension and stirred continuously for 60 seconds and then gently pulled the loop away from the suspension.

Antibiotic susceptibility assay

Total sixteen types of commercially available and frequently prescribed standard antibiotics (penicillin, neomycin, erythromycin, azithromycin, cefotaxime, oxytetracycline, tetracycline, sulfonamide, gentamicine, vancomycin, rifampicin, amoxicillin, doxycycline, nalidixic acid, kanamycin and ceffixime) were used to evaluate sensitivity of the isolated bacterial strains. Antibiotic susceptibility test was performed to produce a result of susceptible, intermediate (or indeterminate) or resistant for the phyto-pathogenic bacteria. The simple and practical disk diffusion method was applied to test whether the
isolated bacterial strain was susceptible to specific antibiotic or otherwise (Reller et al., 2009). The isolated bacterial strain was grown overnight in LB liquid medium through shaker at 37°C. 0.1 ml (10^{-2} dilution) of bacterial culture was smeared evenly onto the surface of the petriplates containing about 20 ml of LB agar medium. Commercial antibiotic disc were impregnated with standard concentrations of each disc and the culture dishes were incubated at 37°C for 16 hours. The diameter of the zone of inhibition was measured with the help of millimeter scale.

**RESULTS**

**Isolation and purification of bacteria**

Single and pure colonies were isolated from the black rot disease infected fruit lesions tissues grown in LB agar medium. The isolated colonies (BS1 and BS2) were round shaped and pure cultures showed white and cream colors (Figure 1A and C), respectively. In gram staining test, both BS1 and BS2 were small, rode shaped, pink color and gram negative (Figure 1B and D).

**Statistical analysis**

Antibiotic activity index was calculated as: Activity Index (AI) = Da / Db - 1

Here: Da is the diameter (mm) of the growth zone in the experimental dish and Db is the diameter of the growth zone in the control dish. All the above experiments of the present study were conducted triplicate for the consistency of results and statistical purpose. The data were expressed as Mean±SD and analyzed by using Microsoft Excel software of 2013 version.

**Morphological and biochemical characterization of isolates**

In SIM test, BS1 didn’t produce any sulfide and H_{2}S but BS2 produced, in extended growth both isolates were motile in nature. Also, both isolates didn’t produce any band of red or pink color on the top of the medium. In KIA test, the medium turned into yellow colour, the bacterial strain was lactose and glucose fermenting. In Simmon’s citrate test, the color of bacteria changed from green to prussian blue, which proved the ability of the isolated bacteria to utilize...
Table 1: Morphological and biochemical tests of the isolates

<table>
<thead>
<tr>
<th>Tests</th>
<th>Appearance</th>
<th>BS1</th>
<th>BS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIM</td>
<td>Motile, H₂S and indole production</td>
<td>P, N</td>
<td>P, P</td>
</tr>
<tr>
<td>KIA</td>
<td>Yellow color, non-ferment glucose by bacteria</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Simmon citrate</td>
<td>Production color from green to prussian blue, Isolated bacteria can utilize citrate</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>MacConkey</td>
<td>Pink color for colony, Lactose fermenting</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>No bubbles were produced</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>KOH</td>
<td>No thread like viscous appearance</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

Note: Here, BS1=bacterial sample 1, BS2=bacterial sample 2, P=positive, N=negative

Figure 2. Biochemical characterization of the isolated BS1 and BS2; (a) SIM medium, (b) Kligler iron agar, (c) Simmon’s citrate, (d) MacConkey agar, (e) H₂O₂ and (F) KOH test

citrate compound. In MacConkey test, medium turned into the pink color after overnight incubation at 37°C, the isolated bacteria were capable of lactose fermenting. In catalase test, bubble was not produced due to breakdown of hydrogen peroxide into water. In KOH test, the cells displayed no thread like viscous appearance. This is shown in Table 1 and Figure 2.

Antibiotic susceptibility assay

The highest zone of inhibition with 31.16±0.28 mm followed by 22±0.86 mm was showed by cefotaxime and nalidixic acid against the isolate BS1, respectively. On the other hand, the highest zone of inhibition with 27.5±1.80 mm followed by 19.16±1.75 mm was showed by penicillin and azithromycin against the isolate BS2, respectively. Moreover, the isolate BS1 was susceptible to neomycin, azithromycin, cetotaxime, oxytetracycline, tetracycline, gentamicine, doxycycline, nalidixic acid and kanamycin, while resistance to penicillin, erythromycin, sulfonamide, vancomycin, rifampicin, amoxicillin and cefixime. The isolate BS2 was susceptible to penicillin, erythromycin, azithromycin, cetotaxime, oxytetracycline, rifampicin and amoxicillin, whereas resistance to neomycin, tetracycline, gentamicine, doxycycline, nalidixic acid, and kanamycin resistance to sulfonamide, vancomycin, and cefixime. The details data are given in Figure 3 and 4.

DISCUSSION

Fruits are infected easily with bacterial pathogens by the principle of spread of bacterial infection in fruits supports
that a single infected citrus fruit can be the source of infection to other fruits during storage and on transit (Jay, 2003). Along with antibiotics, the extracts of various plants may be used to treat diseases and are considered as an important source of new antimicrobial agents (Kuda et al., 2004). In current study, we have tried to isolate and identified the bacterial pathogens associated with black rot of orange fruits using morphological and biochemical approaches. Here, we isolate two bacterial strains namely BS1 and BS2. Colonies morphology in LB agar medium and gram staining results confirmed the isolates as gram negative. Advanced biochemical test results were also favoured as *Serratiamarcescens* bacteria regarding to the previous study. [16][17][18] Wang et al. (2015); Schappe et
al. (2020) and Tian et al. (2019) reported that the *S. marcescens* strains were cultured in LB broth medium at 37°C for phage propagation. Casey et al. (2017) reported the *S. marcescens* as gram-negative, rod-shaped bacterium which support our present findings. Tested biochemical results were similar to the previous study (Ali et al., 2017; Zaoti et al., 2018). In antibiotic susceptibility assay, we found some significant inhibitory effect by the tested antibiotics against the isolates. In the previous study, some researchers (Zaoti et al., 2018; Sarkar et al., 2018; Hasan et al., 2018) found similar effect on commercial antibiotics against citrus diseases associated bacterial strains, which support our present findings. Here, we isolated and characterized the bacterial pathogens from rotted orange fruits in vitro and assessed the efficacy of some common antibiotics against. For more confirmation of the isolates, molecular identification and pathogenicity test is needed.

**CONCLUSION**

The morphological and biochemical test performed in the study revealed various characteristics of the phytopathogen responsible for the exaggerated disease. The advanced morphological and biochemical characterization confirmed the identity of the bacteria as *Serratia marcescens*. In control measure, various antibiotics were applied where cetotaximeshowed promising results. So, it can be concluded that the findings of the study may favorin the thorough detection of the pathogenic bacteria as well as finding out proper biological control management of the bacterial disease in near future.

**ACKNOWLEDGMENTS**

The authors would like to thank the Ministry of Education (No.3900.0000.09.06.217/2BS 91/170), Government of the People’s Republic of Bangladesh for financial support to carry out the research.

**REFERENCES**


