Isolation, Identification and Chemical Control of Leaf Blight Bacterium on Rice Plants

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Abstract

In this study, a pathogenic bacterial strain was isolated from the infected leaves of rice plants in Maubin area, Ayeyarwaddy Region in Myanmar. In order to identify isolated bacterial strain, biochemical tests, gram staining test, carbon and nitrogen sources were carried out at Department of Botany, Maubin University. In biochemical tests, all five tests (catalase, Voges-Proskauer, methyl-red, urease and citrate) indicated positive reactions. After gram staining, isolated pathogenic bacterial strain was gram-negative bacteria. Among carbon sources, sucrose and glucose were the best whereas glycerol was suitable for the growth of isolated strain. Among nitrogen sources, yeast extract and oat meal were good nitrogen sources while malt extract was suitable for the growth of this strain. This bacterial strain was identified as *Xanthomonas oryzae* that causes leaf blight disease on rice plants. Chemical control on rice pathogens *in vitro* was undertaken by paper disc diffusion assay at Department of Botany, Maubin University. The copper oxychloride showed high antibacterial activity on *Xanthomonas oryzae* while kasugamycin indicated weak activity on *X. oryzae*.

Keywords: Biochemical tests, Chemicals control, Identification, *Xanthomonas oryzae*

Introduction

Bacterial blight is one of the most destructive rice diseases in Asia and historically been associated with major epidemics. It occurs in China, Korea, India, Indonesia, the Philippines, Sri Lanka, Myanmar, Laos, Taiwan, Thailand and Vietnam. The disease also occurs in Northern Australia and Africa. In the late 70s epidemic due to bacterial blight was reported in India. Controls of plant diseases are crucial to the reliable production of food and it provides significant reductions in agricultural uses of land, water, fuel and other inputs. Diseases typically reduce plant yields by 10% every year in more developed countries, but yield loss to diseases often exceeds 20% in less developing countries. Plant disease causes major economic losses for farmers worldwide (Maloy, O.C. 2005).

Bacterial leaf blight has formed one of the major limiting factors in increasing rice production. In Japan, the yield losses varied between 20-50% in severely affected areas. In India, the losses in yield have been estimated in the range of 6-60% and 2-74%. Its occurrence in Africa and America had led to concern about its transmission and dissemination in the 70s (Reddy, 1991).

The objectives of the present research are to collect the infected leaves on rice plants, to isolate pathogenic bacteria from the infected leaves, to identify isolated pathogenic bacteria and to investigate the effects of different chemicals on isolated pathogenic strain *in vitro*.

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Materials and Methods

Collection of Infected Leaves on Rice Plants

The infected leaves on rice plants were collected from the paddy fields in Maubin area, Ayeyarwaddy Region. Then, the infected leaves were kept in the plastic bags to isolate pathogenic microorganisms from the leaves in Figure 1.

Isolation of Pathogenic Bacteria

The infected leaves of rice plants were washed with tap water and cut into many small pieces. The pieces of leaves were placed into the dishes containing two different media. After two days, the disease causing colonies formed around the pieces of leaves in Figure 2. These colonies were collected by a loop and cultured into the test tubes containing different media for pure culture. The two media used to isolate pathogenic bacteria were: medium 1 containing sucrose 1.0 g, yeast extract 0.3 g, NaCl 0.05 g, agar powder 1.8 g, pH 7 and distilled water 100 ml and medium 2 containing nutrient agar power 2.8 g, pH 7 and distilled water 100 ml (Yee Yee Thu *et al.* 2002).

Fig. 1. Sheath blight on rice leaves

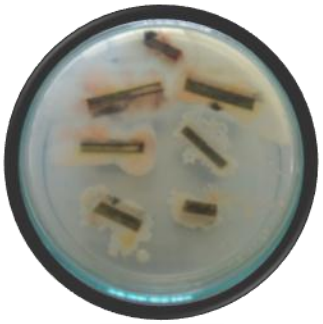


Fig. 2. Isolation of pathogenic bacteria

Biochemical Tests for Pathogenic Bacterial Strain

1. Catalase test

The catalase test is used to differentiate Staphylobacilli (catalase-positive) from Streptobacilli (catalase-negative). The enzyme, catalase, is produced by bacteria that respire using oxygen, and protects from the toxic by products of oxygen metabolism. If the bacteria possess catalase (i.e., are catalase-positive) when a small amount of bacteria isolated is added to hydrogen peroxide, bubbles of oxygen are observed. The test is done by placing a drop of hydrogen peroxide on a microscope slide (Maehly A.C., Chance B. 1954).

2. Voges-Proskauer test

The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which has been inoculated with bacteria. A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result. The test depends on the digestion of glucose to acetylmethyl-carbinol. In the presence of O_2 and strong base, the acetylmethyl-carbinol is oxidized to diacetyl.

Alpha-napthol acts as a color enhances, but the color change to red can occur without it (Baltimore, Williams and Wilkins, 1984).

3. Methyl-Red test

In microbiology, methyl red is used to identify bacteria producing stable acid by mechanisms of mixed acid fermentation of glucose. All enterics initially produce pyruvic acid from glucose metabolism. Some enterics subsequently use the mixed acid pathway to metabolize pyruvic acid to other acids, such as lactic, acetic and formic acids. These bacteria are called methyl-red positive. Other enterics subsequently use the butylene glycol pathway to metabolize pyruvic acid to neutral end products. The bacteria are called methyl-red negative (Clarke H.T.; W.R. Kirner, 1941).

4. Urease test

Urea is common metabolic waste product of protein digestion in most vertebrates that is toxic to most living organisms. Urease catalyses the breakdown of urea into ammonia and carbon dioxide. The test organism is cultured in a medium containing urea and the indicator phenol red. If the bacterial strain is urease, producing the enzyme will hydrolyze the urea to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline shown by change in color of indicator to reddish pink. The change in the color of the medium from orange to pink red due to rise in pH indicate the positive test while the unchanged color of media indicates negative test (Bailey & Scott, 2013).

5. Citrate test

Bacteria are inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. The medium also contains inorganic ammonium salts, which are utilized as sole source of nitrogen. Use of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. If the medium turns blue, the organism is citrate positive. If there is no color change, the organism is citrate negative (MacWilliams, M.P. 2009).

Staining Method of Pathogenic Bacterial Strain

Gram staining reaction was made by method of Anthony Joseph Salle (1948). A bacterial film at room temperature, it was fixed by passing through the flame for several times and primarily stained with ammonium oxalate crystal violet and washed with water followed by the addition of iodine solution. The stained smear was covered with a few drops of alcohol and washed with water. Secondary staining of the smear was made with saffranin solution. After washing the stained slide with water, it was blotted dry and observed under the oil immersion objective. The nature of staining reaction was recorded.

Utilization of Carbon and Nitrogen Sources

Carbon sources were sucrose, glucose and glycerol whereas nitrogen sources were yeast extract, oat meal and malt extract. Basal media for carbon sources were yeast extract 0.3%, K_2HPO_4 0.01 %, $MgSO_4$ 0.01% and $CaCO_3$ 0.01% while basal media for nitrogen sources were glycerol 1.0%, K_2HPO_4 0.01%, $MgSO_4$ 0.01% and $CaCO_3$ 0.01% (Monaghan *et al*. 1999).

Evaluation of Chemicals (*in vitro*) on Pathogenic Bacterial Strain

Isolated pathogenic bacterial strain *Xanthomonas oryzae* was used as test organism. The two medium flasks (each containing sucrose 0.5 g, yeast extract 0.3 g, NaCl 0.05 g, agar 0.45 g, distilled water 25 ml, pH 7) were autoclaved. After autoclaving, at 50°C one loop of bacterial test organism was inoculated into 25 ml of flask. Then, this flask was incubated at room temperature for 2 days.

Test plates: After autoclaving, the conical flask containing 50 ml of nutrient agar medium at 50°C, test organism (1 ml) was added to the flask. Then, the medium and test organism in the flask were thoroughly mixed and poured into the plates.

Chemicals used for paper disc: For evaluation of test bacteria, chemicals were kasugamycin 0.5 g and copper oxychloride 0.5 g, and each was soluble in distilled water 5 mL.

Paper disc diffusion assay: After solidification, paper discs impregnated with chemicals were applied on the test plates. These plates were incubated for 24 hr at room temperature. After 24 hr, inhibitory zones surrounding the test discs were measured. These zones indicated inhibition of the chemicals on the growth of test organism *Xanthomonas oryzae* (Davis and Stout, 1971).

Results

Isolation of Pathogenic Bacterial Strain

For isolation of the disease causing bacteria from the infected leaves on rice plant, these colonies formed around the pieces of infected leaves were collected by a loop and cultured into the test tubes containing different media for pure culture as seen in Figure 3.

Biochemical Tests for Pathogenic Bacterial Strain

In the course of biochemical tests, all five tests (catalase, Voges-Proskauer, methyl-red, urease, citrate) showed positive reactions as shown in Figures 5 and 6. After gram staining test, isolated bacterial strain gave pink color thus it was gram-negative, and its cells were straight rod as shown in Figure 4.

Fig. 3. Pathogenic bacteria

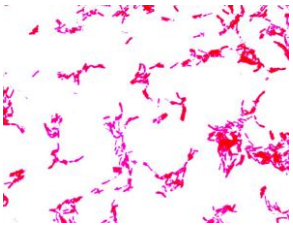


Fig. 4. Staining of pathogenic strain (x100)

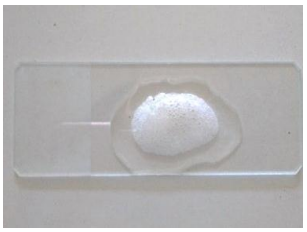
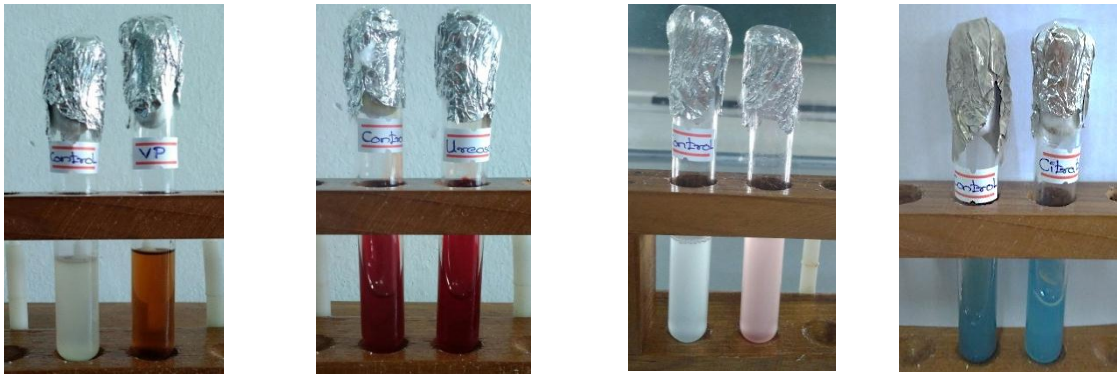


Fig. 5. Catalase test



Voges-Proskauer test Methyl-red test Urease test Citrate test

Fig. 6. Biochmical tests

Utilization of Carbon and Nitrogen Sources of Pathogenic Bacterium

Carbon utilization

Among the carbon sources, the growth of pathogenic bacterium on sucrose and glucose media was good while its growth on glycerol medium was moderate. Thus, sucrose and glucose were the best carbon sources while glycerol was suitable for fermentation. Colony colour of this strain on culture plates of sucrose and glucose was cream while its colony colour on culture plates of glycerol was white as shown in Table 1 and Figure 7.

Table 1. Morphological characters of pathogenic bacterium on various carbon sources

No.	Carbon Source	Growth	Colony Color
C 1	Sucrose	Good	Cream
C 2	Glucose	Good	Cream
C 3	Glycerol	Moderate	White

Sucrose Glucose Glycerol

Fig. 7. Pathogenic bacterial strain grown on the plates of carbon sources

Nitrogen utilization

The growth of pathogenic bacterium on yeast extract and oatmeal media was good while the growth of pathogenic bacterium on malt extract medium was moderate. Thus, yeast extract and oatmeal were the best nitrogen sources while malt extract was suitable for fermentation. Its colony colour on culture plates of all these nitrogen sources was cream as shown in Table 2 and Figure 8.

Table 2. Morphological characters of pathogenic bacterium on various nitrogen sources

No.	Nitrogen source	Growth	Colony color
N 1	Yeast extract	Good	Cream
N 2	Malt extract	Moderate	Cream
N 3	Oat meal	Good	Cream

Yeast extract Malt extract Oat meal

Fig. 8. Pathogenic bacterial strain grown on the plates of nitrogen sources

Identification of Pathogenic Bacterium

According to the results of biochemical test, gram staining test, carbon and nitrogen sources, isolated pathogenic bacterial strain was identified as *Xanthomonas oryzae* that is one of phytopathogenic bacteria belonging to the family Xanthomonadaceae.

Effect of Chemicals on *Xanthomonas oryzae*

According to paper disc diffusion assay, copper oxychloride showed high antibacterial activity (20 mm) on *Xanthomonas oryzae* while kasugamycin indicated weak activity (12 mm) on *X. oryzae* (paper disc size = 6.0 mm).

Discussion and Conclusion

In this study, pathogenic bacterial strain *Xanthomonas oryzae* was isolated from the infected leaves of rice plants in Maubin area. In order to identify isolated bacterial strain, biochemical tests, gram staining test, carbon and nitrogen sources were carried out. Among carbon sources, sucrose and glucose were the best whereas glycerol was suitable for the growth of *X. oryzae*. Ritchie *et al.* (2009) reported that sucrose was the most suitable carbon source for the growth of some isolates of *X. oryzae*. In this study, yeast extract and oatmeal were good nitrogen source while malt

extract was suitable for the growth of *X. oryzae.* These results are agreement with the statements by Yee Yee Thu *et al*. (2003). They have reported that sucrose, yeast extract and malt extract were the best media for the growth of *X. oryaze*.

Isolated bacterial strain was identified as *Xanthomonas oryzae* according to the results that are in agreements with former researchers (Hopkins *et al*. 1992, Clarke Kirner 1941, Macwilliam 2009). According to paper disc diffusion assay for chemical control, copper oxychloride showed highly antibacterial activity on *Xanthomonas oryzae* while kasugamycin had weakly activity on *X. oryzae.* It was in agreement with Hokkanen and Lynch (1995) who stated that these two chemicals showed antibacterial activity on rice plants.

In conclusion, rice is our staple food and the diseases of rice are estimated to cause annually about 10% loss in rice production. Among *Xanthomonads, Xanthomonas oryzae* pv. *oryzae* causes bacterial blight of rice which is one of the most important diseases of rice in most of the rice growing countries. It is destructive to high-yielding cultivars in both temperate and tropical regions especially in Asia. In this research, chemical control (*in vitro*) on leaf blight bacterial pathogen *X. oryzae* indicated good result. Moreover, various rice varieties should be tested for chemical control with different chemicals. Besides, biological control on various rice varieties should be tested in the further investigations since biological control is better than chemical control due to less side-effect both on living things and on environment.

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