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The Development of Rapid, Accurate and Simple Identification Techniques for Bacterial Grain Rot (*Burkholderia glumae*) on Rice

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Abstract

Grain Rot Disease (Bacterial Grain Rot) or Bacterial Panicle Blight becomes a serious threat to rice farmers in Indonesia. Agricultural Quarantine Office in Makassar (2016) and Pare-Pare (2017) confirmed that this disease has spread throughout the rice development area in South Sulawesi, but unfortunately Food Crops and Horticulture Protection Service has not made this disease as the main disease that should be monitored regularly. This is probably due to the difficulty of control measures as the symptoms of BG disease which in general occur only when the phase is generative and often the symptoms are confused with other pest symptoms such as blast, bacterial blight, stink bugor others. To overcome this problem, a fast, accurate, and simple method must be developed to diagnose the causal agent. To evaluate this method 8 isolates of *Bulkholderia glumae* were obtained from several varieties and several locations. For comparison, several pathogenic bacterial isolates in rice plants. The KOH3% test results showed that the whole isolates were negative. In the media King's B shows positive in the production of toxoflavin, catalase and negative results in the arginine dehydrolase test, levan production, and gelatin liquefaction tests. The occurrence of maceration symptoms in onion slices provides more convincing evidence for the presence of *B. glumae* bacteria in rice. The hypersensitive reaction test in tobacco cannot be used as a benchmark because only 5 isolates react positively while the other 3 isolates react negatively. Through a series of results the tests showed differences in the characteristics of *B. glumae* bacteria with bacteria that cause rice diseases such as: *Xanthomonas oryzae* pv *oryzae* (leaf blight), *X. oryzae* pv *oryzycola* (leaf streak), so that the method can be used by pest observers and students to determine quickly, accurately, and simple for the presence of *B. glumae* bacteria in rice. The accuracy of the system will then be confirmed through molecular testing with specific primers.

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Keywords

Early detection, Panicle blight, Morphological and physiological characterization.

Introduction

Bacterial Grain Rot on rice caused by the *Burkholderia glumae* bacteria is one of the most severe seed infectious bacteria in many rice-producing regions, including in South Sulawesi, Indonesia. The loss of yield by this disease ranges from 15% which is common until a loss of up to 80% has been recorded as the worst infection (Fang *et al.*, 2009).

B. glumae includes OPTK A2 Group I in rice, which means its existence, has been reported in Indonesia but is limited to certain areas and control measures are being carried out (Baharuddin *et al.*, 2017). This disease is

often misinterpreted by the presence of other pests such as: blast, leaf blight, and walangsangit. The Symptoms of bacterial grain rot diseases are small lesions (1 to 5 mm) with brown margins on the leaves and grains, so that infected grains become empty before they are fully filled so that panicles often remain erect due to loss of grain weight, rachis of panicles remain green, margins of reddish brown may form on individual flag leaf sheaths of infected (Sayler *et al.,* 2006). If no more careful observation made, this disease will be very detrimental and difficult to control because it attacks on generative phase. This disease is a major disease that not only causes economic losses, but also because it is relatively difficult to control due to its diverse pathogens and its genetic traits are susceptible to mutations, especially virulence against rice varieties. Therefore, a fast, accurate, and simple method needs to be developed to identify the causes of grain rot on rice.

Materials and Methods

Isolation of *B. glumae*

Plant samples were obtained from 4 (four) regions, that is Maros Regency in Lau and Bantimurung Districts, and Barru Regencyin Mangkoso and Garessi Districts. Consisting of Ciherang, Mekongga and SL-8 hybrid varieties. The symptomatic grains of the plant are then sterilized by using 2% NaOCl for 1-2 minutes then washed with 3 times of sterile distilled water for 30 seconds. The grains are crushed and 1 ml of distilled water is added, then scratched on NA media. As a comparison, the isolates of *Xanthomonas oryzaepvoryzae* (*X. oryzae*), *B. gladioli*, and *Ralstonia solanacearum* were compared.

Morphological characterization

The bacteria obtained from the purification results were then observed for their morphological characteristics. Morphological observations include the expression of the forms of bacterial colonies, colony colors, forms of elevation colonies, and edges of colonies.

Physiological characterization

KOH 3% solubility

All bacterial isolates were tested using KOH_3% solution to see the gram reaction. Bacterial isolates were tested on glass preparations by dripping 1-2 drops of KOH_3% solution and then mixing the bacteria using an ose needle. Gram negative if the mixture becomes slimy, and gram positive if not slimy (Schaad *et al.,* 2001).

Catalase testing

Catalase test using H_2O_2 solution: Tests were carried out on glass preparations and then given bacteria using ose needles and then drops of H_2O_2 solution on the bacteria. When an air bubble is formed, the catalase reaction is positive, and a negative reaction if no air bubbles are formed.

Bacterial pigmentation in media King's B

The isolates were obtained sub-culture on King's B media and incubated for 2 days at 37^{o}C. This test aims to look at the toxoflavin yellow pigment produced by the bacterium *B. glumae* (Karki *et al.,* 2012).

Formation of fluorescens pigment

All isolates were seen under the fluorescens light. If it produces a glowing green color the result is positive, if not then the result is negative which means that the bacteria do not produce pigment fluorescens.

Arginine dehydrolase

Tests were carried out in a test tube filled with 5 ml Arginine media and then inoculated with bacteria then closed using vaseline / agar and incubated at 28^{o}C for 3 days. If the purple media color changes to red, the result is positive

Gelatin liquefaction

Inoculate bacteria on agar gelatin media and incubate for 3 days. Wet the surface with 5-10 ml of acid mercuric chloride solution. A clear area shows bacteria able to hydrolyze gelatin (positive reaction).

Onion assay

A total of 5 ml of bacterial suspension with concentration of 5x10^{5} CFU in 10mM $MgSO_4$ was applied to 2 mm wound on the inner surface of the onion made with the tip of the micropipette.

Round onion scale was inoculated with bacteria and then incubated at 30^{o}C for 48 hours (Karki *et al.,* 2012). Positive results indicate the presence of maceration on the onion layer.

Results and Discussions

Sampling

Sampling was carried out by looking at the symptoms of grain rot disease at the location (Fig. 1), the symptoms in the form of grains experiencing browning starting from the base of the grains and severe symptoms causing the grains to appear green to fade and wither and empty but panicle rachis remained green.

As many as 8 bacterial isolates were found originating from Lau district, Maros as many as 3 isolates tat is MKBt04, CHBL03, and CHDa isolates, from Bantimurung district, Maros, 2 isolates that is MrBtm 2.2 isolates and MrBtm 2.3 isolates, from Mangkoso District, Barru as many as 1 isolate namely BrMks1 isolates, as well as from Garessi district, Barru as many as 1 isolate, BrGKa isolates 1.2. All of them are *B. glumae* bacteria.

Morphological characteristics of bacterial isolates

Morphological form of *B. glumae* bacteria is yellowish white to grayish white with a smooth round shape and has a convex elevation. Based on the results of the observation, morphological characters were obtained for each bacterial isolate (Table 1 and Fig. 2).

In this study the morphological characters seen are those that appear directly from the colonies in the agar media. The morphological characters of *B. glumae* bacterial isolates obtained have different characters. The difference in morphology in each isolate lies in the color of bacterial colonies, this difference can indicate phenotypic variation of each isolate and can also show differences in the level of virulence of each isolate (Tsushima *et al.,* 1986). Unlike the comparable bacteria that also cause disease in rice plants, namely *Xanthomonas oryzae* bacteria which have the color of yellow colonies and *R. solanacearum* which has a brownish yellow color if grown on NA media, and B. gladioli which is white. Other morphological characteristics also refer to the study of Tsushima *et al.,* (1996) which states that the form of *B. glumae* colonies is divided into two, namely type A round, smooth, and convex, and type B with various (irregular) colonies.

Physiological characteristics of bacterial isolates

Physiological tests were carried out on 8 isolates that had been obtained, the tests included KOH3% solubility, Catalase, bacterial pigmentation on King's B Media, formation of pigment fluorescens arginine dehydrolase, levan production, gelatin liquefaction, and onion assay (Table 2).

All bacterial isolates showed the same physiological characteristics as *B. glumae*. The KOH3% test results showed that all isolates react positively to KOH3% because it produces mucus, which means the bacteria have a negative gram. Catalase test results showed that all isolates reacted positively because they produced bubbles which meant that the bacteria could convert H_2O_2 to H_2O and O_2 (Table 1). Deepali Singh and Karuna Vishunavat (2015) stated that biochemical tests for *B. glumae* bacteria showed a positive test for the solubility of KOH and catalase. According to Schaad *et al.,* (2001) that all *Burkholderia* strains produce positive catalase

B. glumae is a gram-negative, non-fluorescent, rod-shaped bacterium, with 1-3 polar flagella, and measures 0.5-0.7 µm and produces yellow-green pigments which are soluble in water in some media (Cho *et al.,* 2007).

The results of testing toxoflavin yellow pigment produced by *B. glumae* in King's B media showed that all isolated bacterial isolates produced yellow pigment toxoflavin after incubation at 37^{o}C. Cui Zhou-qi *et al.,* (2016) stated that the most important phytotoxins produced by *B. glumae* are bright yellow pigments. The production of toxins depends on the growth temperature and reaches a maximum level of 37°C, and no toxoflavin is detected at 25°C - 28°C. According to Schaad *et al.,* (2001), *B. glumae* colonies have distinctive characteristics when grown on agar media, which are diffuse nonfluorescent. The color of the colonies with diffusible greenish yellow pigments is similar to the *Pseudomonas fluorescens* colonies. Whereas it is different from the other 2 comparable bacteria, *X. oryzae* and *R. solanacearum* which do not produce yellow pigments. The yellow pigment toxoflavin in *B. glumae* bacteria is a virulence factor from these bacteria which causes grain rot in infected rice (Karki *et al.,* 2012).

The arginine test aims to determine whether a bacterium is able to dehydrolase amino acids. According to Deepali Singh and Karuna Visnhunavat (2015) that the arginine dehydrolase test in *B. glumae* bacteria showed negative results. Likewise with the Levan production test in which *B. glumae* bacteria did not form convex and slimy colonies on Levan media while the *X. oryzae* bacteria formed colonies that were convex and slimy.

Table.1 Morphological characters Isolates of bacteria that have been isolated

No.	Isolates	Varieties	Place of origin	Color	Form	Edge	Elevation
1	MKBt04	Mk	Lau, Maros	Grayish white	Circular	Entire	Convex
2	CHBL03	Chr	Lau, Maros	Yellowish white	Circular	Entire	Convex
3	MrBtm 2,2	SL-8	Bantimurung, Maros	Yellowish white	Circular	Entire	Convex
4	MrBtm 2,3	SL-8	Bantimurung, Maros	Yellowish white	Circular	Entire	Convex
5	BrMks1	Mk	Mangkoso, Barru	Creamy white	Circular	Entire	Convex
6	BrGKa1,2	Chr	Lau, Maros	Creamy white	Circular	Entire	Convex
7	CHDa	Chr	Garessi, Barru	Yellowish white	Circular	Entire	Convex
8	BrGKi2	Mk	Garessi, Maros	Yellowish white	Circular	Entire	Convex
9	*X. oryzae*		comparison	Yellow	Circular	Entire	Convex
10	*B. gladioli*		comparison	White	Circular	Entire	Convex
11	*R. solanacearum*		comparison	Brownish yellow	Circular	Entire	Convex

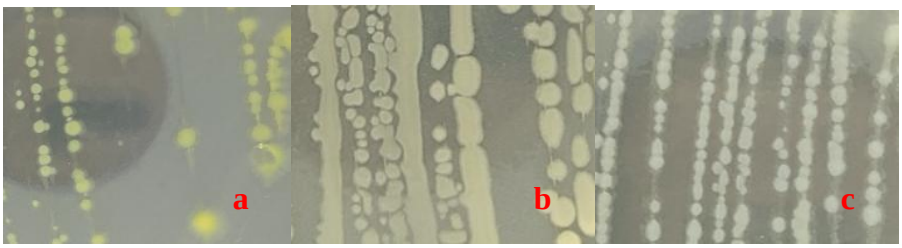
Table.2 Physiological characteristics of the tested isolates

No.	Isolates	KOH 3% solubility	Catalase	Toxo flavin pigment	Fluorescens Pigment	Arginin Dehidrolase	Levan production	Onion Assay	Gelatin liquefaction
1	MKBt04	+	+	+	-	-	-	+	-
2	CHBL03	+	+	+	-	-	-	+	-
3	MrBtm 2,2	+	+	+	-	-	-	+	-
4	MrBtm 2,3	+	+	+	-	-	-	+	-
5	BrMks1	+	+	+	-	-	-	+	-
6	BrGKa1,2	+	+	+	-	-	-	+	-
7	CHDa	+	+	+	-	-	-	+	-
8	BrGKi2	+	+	+	-	-	-	+	-
9	*X. oryzae*	+	+	-	-	-	+[a]	N	+[a]
10	*B.gladioli*	+	+	+	-	-[b]	-[b]	N	+[b]
11	*R.solanacearum*	+	+	-	-[c]	-[c]	-[c]	N	-[c]

[a]Shankara *et al.*, (2017), [b]James *et al.*, (2005), [c]El-Habba *et al.*, (2016), N = not tested

Figure.1 Symptoms of bacterial grain rot in the field caused by *B. glumae* bacteria

Figure.2 Characteristic morphology of *B. glumae* based on color. a) yellowish white, b) creamy white, and c) grayish white



None of the test isolates showed positive arginine dehydrolase test and levan production, so that the isolates showed the character of *B. glumae* bacteria.

The formation of maceration on the onion inoculated with bacterial suspense indicates the ability of bacteria to produce pectinase enzymes which can damage the cell wall of onions. Based on the results of the study, all bacterial isolates can cause maceration on the onion even though with different maceration levels. According to Karki *et al.*, (2012) which states that the formation of maceration in onions shows the ability of bacteria to cause disease, indicating the level of virulence.

The last physiological test is the gelatin liquefaction test. This test aims to determine whether a bacterium has an enzyme gelatinase that is able to hydrolyze gelatin or not. According to Deepali Singh and Karuna Visnhunavat (2015) said that *B. glumae* bacteria were able to hydrolyze gelatin, which was characterized by the appearance of a clear zone around the test bacteria on gelatin media given mercuric chloride acid. While *X.oryzae* and *B.gladioli* bacteria showed positive results for gelatin liquefaction test.

It is concluded, based on a series of morphophysiology test results of bacterial isolates obtained from symptoms of rice grains rot, several methods can be used as a standard for diagnosing *B. glumae* quickly, accurately and simply that is white to yellowish white, KOH_3% solubility, catalase, and gelatin liquefaction react positively. In the media King B fluorescence pigment is not formed, but toxoflavin is formed, especially in virulent strains. Not able to hydrolyze arginine, unable to produce levan, but capable of causing maceration (rot) in onions.

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