

## TEST OF BLOOD DISEASE BACTERIUM (BDB) TRANSMISSION BY POTENTIAL INSECT VECTORS

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### ABSTRACT

**Test of blood disease bacterium (BDB) transmission by potential insect vectors.** Blood disease bacterium (BDB) is one of the important diseases in banana and a major obstacle in developing and increasing banana production in Indonesia. The purpose of this study was to prove the ability of the Drosophilidae insect as a vector in transmitting BDB. The research was conducted at the Insect Biosystematics Laboratory and Plant Bacteriology Laboratory, Department of Plant Protection, Faculty of Agriculture, IPB University. Drosophilidae insects were taken from the field and then reared in laboratory by being fed with ripe bananas to obtain offspring that are free from diseases or pathogens. Imago of the Drosophilidae from rearing was fed by inoculum sources which was infected banana, then inoculated into healthy plants. The plants used were healthy and flowering, heliconia. The results showed that the Drosophilidae insects were able to transmit BDB to heliconia plants that showed symptoms, brownish flower colors and falling flower crowns. Detection of BDB isolated from flower parts and the inside parts of the insects used in transmission test using the PCR method showed positive results.

**Key words:** Drosophilidae, heliconia, transmission test, vector

### INTRODUCTION

Blood disease bacterium (BDB) is one of the important diseases in banana plants and a major obstacle in developing and increasing banana production in Indonesia. (Hadiwiyono, 2011). Transmission of BDB by insects has been reported by several researchers. Pathogens can be transmitted by insects vector from one plant to another after going through: 1) the acquisition feeding period, which is the time needed by the vector to eat on the plant until it gets a pathogen; 2) the inoculation feeding period, i.e. the time needed by the vector to eat in healthy plants until they can transmit pathogens; and 3) retention period, i.e. interval of time vectors can still transmit pathogens (Wijaya, 2007). Furthermore, the accuracy of the vector is added to thrill the stylet on the part of the diseased plant and the proportion of the infective vector influences the rate of disease transmission (Dietzgen *et al.*, 2016). In pathogens that are persistent there is a latent period that is the time needed by the pathogen to be in the vector body until it can be transmitted (Montong & Salaki, 2019). Persistent pathogens are circulative in the vector body, that is, if the pathogen enters through the

stylet into the digestive tract, then together with protein, fat and other elements enter the blood through the digestive tract walls in the mesenteron, then carried by blood flow to the salivary glands and excreted back through the stylet (Dietzgen *et al.*, 2016). Insects reported so far are still potential vectors and there were no specific reports regarding vector insects from BDB. In this study, the insects that positively identified and detected for the presence of BDB inside and outside of the body was suspected to have the potential in transmitting BDB pathogens (Mairawita *et al.*, 2012). To prove this, a BDB transmission test was carried out using the Drosophilidae insect as one of the potential vectors.

### MATERIALS AND METHODS

**Research Site.** This research was conducted at the Insect Biosystematics Laboratory and Bacteriology Laboratory, Department of Plant Protection, Faculty of Agriculture, IPB University.

**Drosophilidae Maintenance.** Drosophilla flies used in this transmission tests was collected from the banana

plantations around Dramaga, Bogor. The flies were caught, maintained, and reared. Rearing of *Drosophilla* flies in the laboratory was carried out to produce second generation, the offspring that were free of BDB.

**Test Plant Preparation.** The test plants used in this research were banana (*heliconia*) which have flowered and were 5 months-old. These *heliconia* plants were maintained in polybags ( $25 \times 25$  cm). The growing media was a mixture of soil and manure with a ratio of 2 : 1. The test plants were kept in large cages of  $150 \times 40 \times 60$  cm and raised to produce flowers (Figure 1). *Heliconia* plants were used as test plants because *heliconia* and bananas belong to family *Musacea*.

**Inoculum Preparation.** The inoculum source was bananas infected by blood disease bacteria, which was collected from BDB endemic areas in Aceh and the isolates that were derived from banana (Figure 2A). Isolation of BDB from diseased bananas was conducted by cut the banana into pieces, then disinfected with sodium hypochlorite solution for 5 min and rinsed 4 times with sterile water to remove residual sodium

hypochlorite. This tissue then crushed and added with 10 mL of sterile water and gradually diluted 5 times. After that, the bacterial suspension was streaked on the prepared Triphenyl tetrazolium chloride (TZC) media. Observation of the characteristics of BDB colonies on TZC media was carried out after 48–72 h. The BDB colony is round with a size of 1–4 mm, where the middle parts of the colony are red and the edges are clear. Another characteristic observed was that the colonies tended to be sticky to the surface of the medium so it was rather difficult to remove using an aseptic needle. In addition, new colonies form on the third day after they are grown at room temperature. This is consistent with the BDB character reported by Mairawita *et al.* (2012). These pure bacterial colonies were transferred to sucrose peptone agar (SPA) media and incubated for 1–2 d at 28 °C (Figure 2B) and then applied to ripe bananas that would be used as feed for *Drosophilidae* as vector insects in transmission tests (Figure 2C).

**BDB Transmission Test Uses *Drosophilidae* Insects.** The *Drosophilidae* flies that are used as an infective insect agent are obtained from



Figure 1. Confinement containing *heliconia* plants used as test plants.

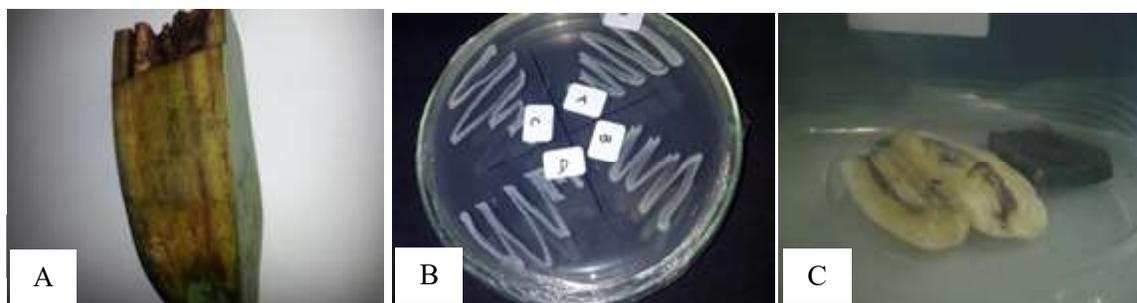


Figure 2. Source of BDB inoculum. (A) Banana infected BDB; (B) BDB isolates on SPA media; (C) Banana smeared with BDB isolates .

the imago stage after the insects were fasted for 6 h (Figure 3A-B). After that, it is allowed to carry out the acquisition feeding period (feeding at the source of the inoculum) for 4 d. After going through the acquisition feeding period, the flies are transferred into the heliconia test plants that have been prepared to carry out the latent period as well as the inoculation feeding period for 4 d (Figure 4). For this transmission test, the number of each test insect being used is 1, 3, 5, 7 and 9 insects/plants. The control plants used the flies that were given the acquisition feeding period on healthy bananas, with the acquisition period, latent period and the inoculation period as the same as the other treatments.

Transmission test was carried out to determine the role of *Drosophilidae* as a vector in transmitting BDB bacteria to banana plants and to obtain information regarding the minimum number of vectors to transmit blood diseases. The variables used in this transmission test were the occurrence of the disease and the incubation period. After going through the inoculation feeding period, the infective insect was removed from the cage and isolated for the presence of BDB both of the outside and the inside of the body. The method of isolation was abased on Wijaya *et al.* (2010).

**Detection of BDB.** The presence of BDB infection in the inoculated plants was detected using PCR. The bacteria was isolated from flower, because it is the location of the transmission treatments. The infective insects for transmission were also isolated and then detected using the PCR method.

**DNA Isolation Procedure.** Isolation of bacterial DNA was carried out using the Genomic DNA Mini Kit Geneaid™ method for isolating bananas, *Drosophilidae*, and flowers that had been purified from the transmission test results. Bacterial isolates were taken in amount of 5  $\mu$ L plus RBC Lysis Buffer in amount of 900  $\mu$ L and stirred until homogeneous. Incubated at room temperature for 10 min. Put in a Centrifuge at 12,000 rpm for 5 min. Afterwards, the supernatant was removed. Then, 100  $\mu$ L RBC Lysis Buffer was added to the sample, and re-suspended. After that, 200  $\mu$ L of GB Lysis Buffer was added and gently turned to homogeneous. The suspension then incubated at 60 °C for 10 min. During incubation, once every 3 min the tubes were stirred until homogeneous. The 200  $\mu$ L absolute ethanol were added and vortexed for 10 seconds. The sample (supernatant) was moved to the



Figure 3. Imago insect of *Drosophilidae*. (A) Dorsal; (B) Dorsal lateral.

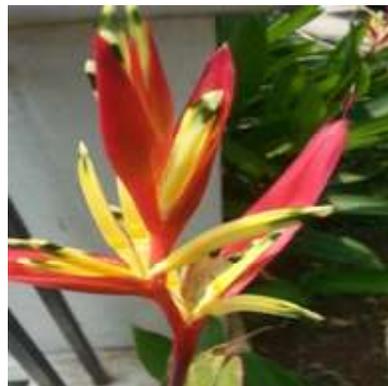


Figure 4. Heliconia plant used in the BDB Transmission test.

GD column. and centrifuge at 12,000 rpm for 5 minutes, then GD column was moved to the new collection tube. Washed by adding 400 µl of W1 buffer. Centrifuged at 12,000 rpm for 30 s. Washed again, by adding 600 µL wash buffer. Centrifuged at 12,000 rpm for 30 s. Centrifuged at 12,000 rpm for 3 min. The GD column was transferred to a 1.5 mL tube. Add an elution buffer in amount of 100 µL. Incubated at room temperature for 3 min. Centrifuged at 10,000 rpm for 30 s. Samples are stored at -20 °C.

**DNA Amplification Using PCR.** Gene fragments were amplified by PCR using primers 759 R (5'-GTGCCCGTCAACTCACTTTCC-3') and 760 F (5'-GTGCCCGTCAGCAATGCGGAAT CG-3') (Sagar *et al.*, 2014). The DNA amplification process was carried out with an initial denaturation process at a temperature of 94 °C for 3 min. Denaturation in the cycle was carried out at a temperature of 94 °C for 15 s, annealed at 57 °C for 30 s, extension at 72 °C for 30 s, final elongation at 72 °C for 10 min to avoid the presence of DNA that has not been fully amplified. The cycle was done 35 times. PCR results can be stored at 4 °C.

**Electrophoresis.** The results of DNA amplification were separated based on the size of the base pair by electrophoresis technique, which used 1.5% agarose gel in a TAE buffer 1X and added 5 µL GelRed per each 50 mL agarose. Agarose gel solution is poured into a mold to make a well of a certain size. A total of 20 µL of PCR results were taken carefully, placed in a gel pit. As a standard molecule, a one kb ladder marker is used. Electrophoresis is run with a potential difference of 100 V for about 30 min. Observation of DNA bands was carried out under UV light and documented.

## RESULTS AND DISCUSSION

**BDB Transmission Test on Heliconia Using Drosophilidae Insects.** Drosophilidae is one of the insects that have been tested (others are Tephritidae and Muscidae) for the presence of BDB bacteria, BDB is also found in the insect's body through isolation (Sahetapy *et al.*, 2019). The sources of inoculum to be infected to the insects are diseased ripe bananas taken from the field and healthy ripe bananas that are smeared with BDB isolate. During the acquisition period, in the confinement fed with diseased banana, the Drosophilidae flies did not survive within 1 day and the flies died so that the flies cannot be used for the treatment. This is because, the flies are normally attracted to the odor produced by the fruit or flowers. Suggests that, whilst

the banana is diseased, the fruit was not ripe and the fruit surface is rough, so that the flies cannot absorb the substances contained in the banana. This in fact is in-line to the opinion which states that *Drosophila* has been known as fruit flies in which a characteristic of the ripe fruit or rotted fruit (Opina *et al.* 2007). *Drosophila* takes material from the rotted *Rafflesia patma* flowers and is possible to place their eggs in the soft parts of flowers for the growth of their larvae (Antonacci *et al.*, 2017). On the other hand, in the confinement fed with ripe bananas and smeared with BDB isolates, the insect survived during the acquisition period. These flies were used for the transmission test.

**Disease Incidence and Incubation Period.** BDB transmission test using the insect vector Drosophilidae which was fed for acquisition period, the latent period, and the inoculation feeding period for 4 d showed positive results (BDB is transmitted) except for treatment with 1 insect and in the control treatment.

The incubation period for the bacteria that causes BDB disease ranges from 31.3 to 79.0 d (Table 1). This shows the more insects are infected, the more pathogens are transmitted into the plant tissue, causing shorter incubation period. Concentration of pathogens in the plant system is one of the factors that determine the success of transmission of causes of disease in plants. The rate of spread of disease in natural conditions depends on the density of the vector population, the number of bacterial inoculums in plants, the length of the feeding period of acquisition and the length of the inoculation period (Wijaya *et al.*, 2010).

**Disease Symptoms Observed in the Test Plants.** The first part of the plant showing symptoms is the flower, because inoculation is done on the flower (Mairawita *et al.*, 2012). When the transmission test is carried out, the parts of the flower and the insect are covered so as not to be disturbed by other insects. The obvious symptoms are that the flower changes color to brown and the flower crown begins to fall. Flowers show symptoms first because the nectar is ingested by the insects and its saliva is already containing bacteria and therefore transmits the bacteria to the flowers. This is consistent with the results of a research which shows that inoculation of BDB suspensions in banana flowers can also cause symptoms of the disease. While the symptoms that appear on other parts of the plant are quite long, is because the transmission takes place naturally which is done with the help of insects (Taufik, 2010). Symptoms that were observed in treated plants, shows leaf edges necrosis, leaf yellowing and wilting

(Latumahina *et al.*, 2015). The process of necrosis begins with the transmission of flower tissue by pathogens through the mouth of the insect while absorbing the surface of the flower. The activities carried out by the insect cause injury to facilitate pathogens into plant tissue. The presence of relatively large amounts of pathogens can cause symptoms of chlorosis and even the occurrence of necrosis (Stulberg *et al.*, 2015).

#### BDB Isolation and Detection Using PCR.

Transmission test results show that the insect *Drosophilidae* is able to transmit pathogens to the flower parts of the heliconia plant. The pathogen results of the transmission test is confirmed to be BDB bacteria (Figure 5). The isolation of the BDB was also done

to the *Drosophilidae* insect as a vector and was found as the same isolate. The result shows that BDB bacteria was found in the insect's body part that was crushed, while in the body that is washed with water the bacteria was not found (Figure 6).

Visualization of PCR product on the plant and insect parts tested is shown in Figure 6. Flowers and insects from the transmission test results showed positive PCR results (Table 2). DNA samples came from banana flowers in treatments using 7 and 9 tails *Drosophilidae*. Positive results were also obtained for the *Drosophilidae* insect used in the transmission test. The results of bacterial DNA amplification with *Ralstonia solanacearum* primer 759F and 760R showed a specific band of 280 bp (Figure 7).

Table 1. Disease incidence and the incubation period in insect transmission tests

Number of insects in each confinement	Disease incidence* on Heliconia plant (Symptomatic plants/replication)	Incubation period** (d)
Control (healthy plant with no insect)	0/3 (0 symptomatic plant from 3 replications)	-
1 (healthy plant with 1 insect)	0/3 (0 symptomatic plant from 3 replications)	-
3 (healthy plant with 3 insects)	2/3 (2 symptomatic plant from 3 replications)	79.0
5 (healthy plant with 5 insects)	2/3 (2 symptomatic plant from 3 replications)	71.0
7 (healthy plant with 7 insects)	2/3 (0 symptomatic plant from 3 replications)	50.5
9 (healthy plant with 9 insects)	2/3 (0 symptomatic plant from 3 replications)	31.3

\* Disease incidence is number of symptomatic heliconia plants divided by number of replication (3). \*\* Number of days from treatment given until the symptom appear. (-): no transmission.



Figure 5. BDB isolates originating from the results of transmission tests. (A) on TZC media; (B) on SPA media; (C) Heliconia flowers on transmission test results.

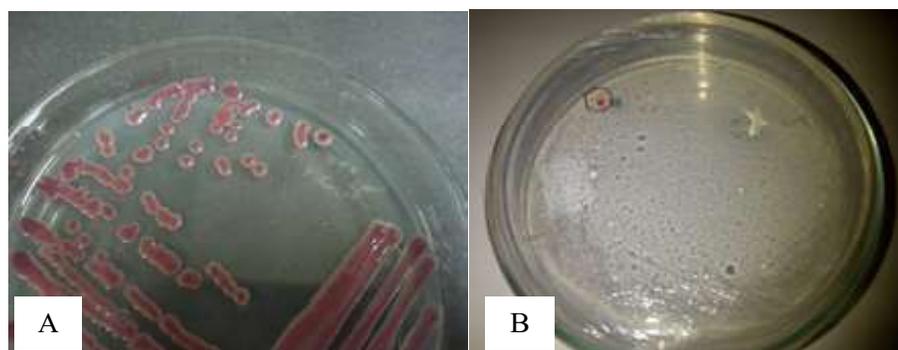


Figure 6. BDB isolates a result of transmission test on TZC media. (A) Inner insect's body; (B) Washed insect.

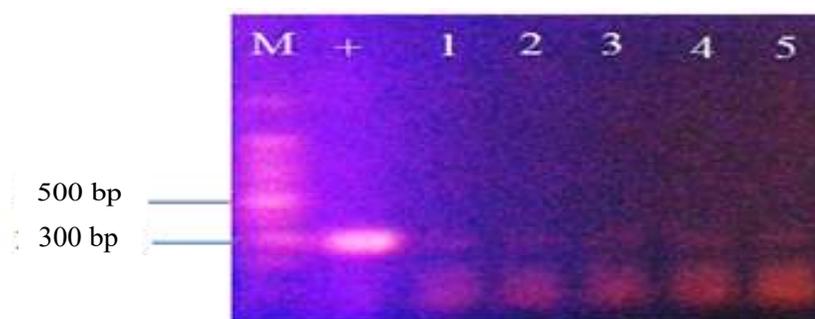


Figure 7. Visualization of BDB DNA from PCR using 759F and 760R primers. (M = marker 100 bp, + = positive control of *Ralstonia solanacearum*, 1 = Banana fruit (BFR), 2 = Banana flower 1 (BFL1), 3 = Banana flower 2 (BFL2), 4 = Green insect 1 (GI1), 5 = Green insect 2 (GI2).

Table 2. PCR Test with primer 759F and 760R on several isolates of insect origin

No.	Isolate code	Origin of isolates		PCR results	Information
		Order	Family		
1	BFR	Banana fruit	Musaceae	+	Not perform
2	BFL1	Banana flower	Musaceae	+	Transmission test
3	BFL2	Banana flower	Musaceae	+	Transmission test
4	GI1	Grind insect	-	+	Transmission test
5	GI2	Grind insect	-	+	Transmission test

## CONCLUSION

The incubation period for the bacteria that causes BDB disease ranges from 31.0 to 79.0 days. Drosophilidae able to transmit the pathogen BDB to healthy heliconia plants. Symptoms that appear in transmission tests are very slow because transmission occurs naturally with the intermediary of the insect. Detection of the presence of BDB by molecular method showed a band at a size of 280 bp in both plant and insect.

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