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Trigona Minangkabau Potential as Bacterial Spreader Agent of *Ralstonia Solanacearum* Phylotype IV Cause Blood Disease on Banana Plants

Mairawita [1 +], Suswati [2],Habazar.T [3], Hasyim. A[4] and Nasir.N [5]

[1] Study Program of Agricultural Sciences Concentration Plant Pest and Disease Science, Graduate Studies, University of Andalas, Campus Unand Limau Manih, Padang 25163.

[2] Program Plant Pests and Diseases Andalas Graduate University, Campus Unand Limau Manih.

[3] Vegetable Crops Research Center, Lembang, Boat No. Tangkuban road. Lembang PO Box 8413 517 40 391 Bandung.

[4] Biology Graduate Program Andalas University, Campus Unand Limau Manih.

Abstract. Blood disease bacterium *(blood disease bacteria,* BDB) caused by *Ralstonia solanacearum* Phylotype IV ranks first in the list of banana diseases in Indonesia. To date information on the spread of disease by insects is very limited. Research objectives are: (i) to determine the role as a disseminator of *R.solanacearum* Phylotype IV, (ii) to determine the amount of inoculum BDB borne by each individual insect. The experiment was conducted in May - September 2008. Samples of insects (adult insects, the young insects, larvae, eggs) nectar and pollen were taken from a colony of *Trigona minangkabau* in endemic area Baso BDB plateau (876 m asl) using the method of *sampling purpossive.* active adult insects taken from the banana flower kepok BDB infected and healthy banana flower. BDB isolation of adult insects is done from the caput and abdomen, while for the young insects, larvae and pupae did not do the division of the body. BDB was isolated using rinses and maceration cultured techniqueby using *triphenyl tetrazolium chlorid* (TTC) medium (Baharuddin. 1992). Bacterial population was calculated using the formula Klement *et al.,* (23). Characterization and identification was done by testing BDB morphological, physiological and pathogenicity tests. Parameter studies: population BDB (cfu/g) of each stage of insect development. The results showed that BDB can be isolated from the outside and the inside of the body of an adult, a young insect, pupa, larva also on pollen and nectar but it was not found in eggs. The result of our study indicated that BDB can be isolated from outside and inside part of the body of adult insect, young insect, larvae and also found in pollen and nectar, while BDB was not found in eggs. BDB population was higher in inside part fo the insect body in each phase of the development of the insect. From all phases, the BDB was higher in inner part of the body of adults insect which have visited infected banana flower

Keywords: *Trigona* minangkabau*,* insects spreaders, banana, *R. solanacearum* Phylotype IV, the population of BDB.

1. Introduction

Blood diseases *(Blood disease bacterium,* BDB) caused by *Ralstonia solanacearum* Phylotype IV is a major cause of production decline of banana in Indonesia and it occupy the first position of the list of banana disease in Indonesia (22). This bacteria is deadly and systemically infect the vascular tissue (20) which was first reported in 1907 in Selayar Islands (28). In 1999 the distribution of BDB disease have been reported in West Sumatra (12), six years later it was reported that banana plants in Nagari Tabek Panjang, Bungo Koto Tuo, Simarasok, Padang Tarok and Koto Tinggi, Baso village was also infected, in the highland of District of Agam which is the center production of banana has been infected by BDB with high category (6).

[+] Corresponding author.
E-mail address: mairawitamarlisrahman@gmail.com.

The development and spread of these disease are very fast. Geographical spread of blood diseases in Indonesia ranges from 100 km year $^{-1}$ (18), in Sumatera, it is ranged between 189-203 km year $^{-1}$ (12).Currently all the bananas plant in West Sumatra has been attacked by BDB. Rapid spread and development of BDB was caused by insect as vectors (12).

Species of insects that reported frequently visited banana flowers which are infected by wilt disease and is thought to play a role in the spread of Moko disease (strain SFR) is *Trigona* spp. (Apidae), *Polybia* sp. (Vespidae), and *Drosophila* sp. (Drosophilidae) (29). According to Leiwakabessy *et al.,* (14) *Trigona* spp. is disease-transmitting agents BDB on banana plantations in Lampung. The spread of banana wilt disease in plants caused by *Xanthomonas campestris pv. musacearum* (Xcm) in Uganda is also caused by flower-visiting insects (8; 10). Some families of insects Apinae, Lonchaeidae, Muscidae, Tephritidae and Vespidae found as vectors of disease in planting banana Xanthomonas wilt in Ethiopia (2).

The results of observation in bananas plantation which is attacked by BDB in highlands (Tabek Panjang, Baso District, Agam regency, West Sumatra Province) indicated that the two most common types of insects visiting banana flowers are: *Trigona minangkabau* (Apidae) and *Drosophila* spp. (1). Research about role of both from insects types in particular *T. minangkabau* (stingless bees) which is also called Galo-Galo by local people, which infected and spread banana disease in highland district in West Sumatra is never been reported before

The purpose of this study were: (i) to determine the role of *T. minangkabau* as disseminators of BDB, (ii) to determine the amount of BDB inoculum borne by each individual insects in the field.

2. Methods

Determination of the study location using purposive sampling method that is based by endemic BDB in the highlands of Baso (S.00 ^{0} 16'02 .2 "-S.00 ^{0} 16'02 .5", E 100 ^{0}27'08 .7 "- E 100 ^{0} 27'12 .2 "), altitude 876 m asl, Baso District, Agam regency, West Sumatera Province. The study was carried out from May to September 2008.

2.1. Sampling

2.1.1. Sampling of T. Minangkabau Imago of Banana Flowers

Imago of *T. minangkabau* (Figure 1) was collected from male flower of healthy and infected bananas which is attacked by BDBusing insect nets (sweep net) at 09.00 to 10.00 am. Insect samples taken from 11 healthy Kepok banana flowers and seven flowers of Kepok sick. The collected insects were put in "killing bottle". Each individual of *T. minangkabau*put in an eppendorf tube which containing 9 ml of sterile water, with three replications, labelled and put into ice box before transported to laboratory for BDB isolation andBDB identification.

2.1.2. Sampling of Egg, Larvae, Pupa, Imago New *T. Minangkabau,* Pollen and Nectar from the Colony *T. Minangkabau*

Egg, larvae, pupa, new phase of *T. minangkabau,* also pollen and nectar was taken from colonies of *Trigona minangkabau* (Fig 2a, b, c, d). Each of development stage of *T. minangkabau* was taken three eggs, put in microtube 9 ml sterile water, labeled and placed in the ice box, and then taken to the bacteriology laboratory for isolation of BDB. Sampling was conducted at 09.00 am. As much as one g of pollen and 100 ml nectar were put in each tube Ependroff containing 9 ml of sterile water.

2.2. Isolation of BDB

2.2.1. From the Outside of the Body Phase of The Imago *T. Minangkabau* Which Visit Male Flowers

Isolation of BDB conducted by using medium Triphenyl Tetrazolium Chlorid (TTC) (17). Isolation of bacteria was conducted from three tail imago *T. minangkabau* as much as 1 g of each insect body parts (head and abdomen) is inserted into a test tube containing 9 ml of sterile water.Then vortex with a speed of 300 rpm. The rinse water was diluted (10^{-2} and 10^{-4}). 10 ml of each dilution was breed with the method of TTC cast on selective media, incubated at room temperature 29°C for 48-72 hours.

BDB isolation is also performed on samples of eggs, larvae, pupa, new phase, pollen and nectar from *T. minangkabau* colony.

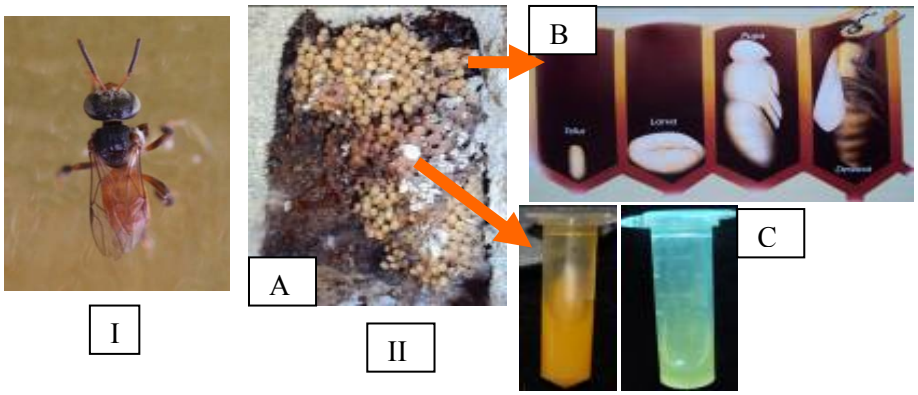

Fig. 1: I: Imago of *T. minangkabau,* II : Development phase and colonies of *T. minangkabau*. A. Colonies of *T. minangkabau*, B. *T. minangkabau* phase of development (egg, flyblow, pupa and adult), C. Pollen and D. Nectar

2.2.2. Isolation of BDB From the Inside of the Body Imago Phase T. Minangkabau which Visit Male Flowers

Part of the head and abdomen *T. minangkabau* separately disinfected with a solution of 5% sodium hypoclorite for 15 minutes. After that it was washed with sterile water 3-4 times and then wind dried, 1 g of each was crushed and added to 9 ml of sterile water. It was diluted 10^{-2} and 10^{-4}. 10 ml of each dilution was breed with the method of TTC cast on selective media, incubated at room temperature 29°C for 48-72 hours. BDB isolation is also performed on samples of egg, larvae, pupa, new phase of *T. minangkabau.*

2.3. Identification of Pathogens in Blood Diseases

Identification carried out to ensure that the bacteria isolated from all phases of the development of insects, pollen and nectar really bring*R. solanacearum* phylotipe IV disease causing blood in banana plants. BDB pure cultured used as a source of inoculums for pathogenicity tests and morphological characterization of the properties (including shape, size, shape edging, color and shape of the surface) and physiological (test gram reaction, pectinase enzyme test, test pathogenicity and hypersensitivity reactions).

2.4. Observation

2.4.1. Population *T. Minangkabau*

Population of *T. minangkabau* calculated based on the number of insects caught in each healthy flower and diseased plants.

2.4.2. Population BDB

Klement formula (23) was used to calculate BDB population on healthy and sick banana flower both in head and abdomen (outside and inside), where :

JB = Ax B; JB = number of bacteria, A = Number of colonies of bacteria, B = the dilution factor

3. Results and Discussion

3.1. Population of *T. Minangkabau* in Banana Flower

In highland of Tabek Panjang, Baso District, Agam regency, West Sumatera Province, the endemic land of BDB it was discovered a population *T. minangkabau* or Galo-Galo with high amounts of the banana flower kepok which affected by BDB than in healthy flowers. In one day, one sick plant of banana kepok, visited by 28.14 ± 13.75 individual in the morning visit is higher (18.00 ± 8.75) than evening (10.14 ± 5.00), while in healthy banana flowers number of the insect found was much lower. The number of which visited healthy flowers in one day 21.81 ± 6.63 individual, on the morning of 13.00 ± 2.64 individual and in the evening 8.81 ± 3.99 individual. Based on these data, there is an increase of visiting of *T. minangkabau* in male sick flower, 29,2% (Table 1). This is because imago attracted by ooze dripping from broken male flower (bacterial). These ooze contains high bacterial propagules.

The high population of which visited the sick banana flower determine the rapid development and spread of blood diseases in the highlands Tabek Panjang. During one flowering period infected banana flower visited by more *T. minangkabau* imago (140.70 ± 68.75) than healthy flower (109.05 ± 33.15). In study conducted on banana flower cultivar Kayinja (banana crew) BXW in Mukono district, Luwero and Mpigi Uganda, flower visiting insect mostly dominated by family of Apidae (*Plebeina denotii* (Vachal) *stingless bee*, Drosophilidae (*fruit flies*) and Chloropidae (*grass flies*).

Table 1: *Trigona minangkabau* population mean (± SD) who visited the flowers healthy and diseased banana kepok highland Tabek Panjang, Baso District, Agam regency, West Sumatera Province

Banana plants	Frequency of arrival (head / day)		Population (tail/day)	Enhancement (%)
	09.00-10.00 wib	15.00-16.00 wib		
healthy plants	13,00±2,64 (11)[a]	8,81±3,99	21,81±6,63	-
diseased plants	18,00±8,75 (7)	10,14±5,00	28,14±13,75	29,02

The high density of *T. minangkabau* kepok visiting banana flowers in the morning due to various factors, including compliance with a source of food and food quality. Sugar content is high in kepok banana flower (26.37 (24.81-27.93)%) compared with other banana (12). Sugar concentration of nectar produced in morning preferred by *T. minangkabau.* According to Yuniana (9) nectar sugar concentration of orange *(Citrus sinensis* (L.) Osbeck) produced higher early morning (38%-39%) and it is preferred by *T. minangkabau, T.itama* and *T.moorei.*

3.2. BDB Insulating Phase Imago *T. Minangkabau* who Visit Banana Flowers

On the surface of the body / body parts of insects trapped in the sick banana flower were found 100.00% positive carrying bacteria BDB bacterial and 90.91% on healthy banana flower (Table 2). This is because of insects activity which visiting sick or healthy banana flower was very high. In the field observations, itwas found that the imago *T. minangkabau* moving on from healthy banana flower to sick banana floweror otherwise to seek pollen and nectar. According to Molina (13), insects have roles for transmission of Moko disease by *R. solanacearum* from one plant to another plant on garden. Besides that, the infected plant looks like normal from outside, these conditions enlarge the chances of disease spreading through insect.

3.3. BDB Isolation of the Head and Abdomen Imago Phase *T. Minangkabau* who Visit Banana Flowers

The isolation results showed that on the surface and the inside of the head was found colonies of bacteria. The occurrence of bacteria in the digestive system and salivary of *T. minangkabau* suspected brought about at the time of these insects look for nectar from flowers and ooze banana plants which attacked by BDB. the colonies number of BDB was higher in *T. minangkabau* who visit sick banana flower than healthy banana flower. Colonies number of BDB on the surface of the head and abdomen of *T. minangkabau* of sick banana flower, 923 ± 670 cfu/ml and 1423 ± 853 cfu/ml and the inside of the head (15.95 ± 18:58) .10 ^{4} and (22.71 ± 21:15) .10 ^{4} respectively (Table 2). The colonies number of BDB on surface of the head and abdomen *T. minangkabau* of sick banana flower 703 ± 687 cfu/ml and 893 ± 905 cfu/mlrespectively. On the head and abdomen colonies number were isolated: (5.91 ± 9.81) 10 ^{4} cfu/ml and (17.60 ± 14:18) 10 ^{4} cfu/ml. The result of Gold and Bandyopadhyay research (5),colonies number of Xcm wilt cause can be isolated from the body of *Plebeina denotii* which is insect vectors from *Banana Xanthomonas Wilt* (BXW) in Uganda banana plantation. At the head was found (524 ± 185) of bacterial colonies, thoracic (581 ± 193) and abdomen (180 ± 175).

Table 2: The mean number of colonies BDB (± SD) of body parts kepok *T. minangkabau* in healthy and diseased flowers on the highland Tabek Panjang

Insect body parts	The mean number of colonies per insect (UPK / ml)	
	healthy plants	Plants attacked by BDB
surface of the head	703±687	923±670

surface of the abdomen	893±905	1423±853
The inside of the head	(5.91±9.81). 10^4	(15.95±18.58).10^4
The inside of the abdomen	(14.18±17.60). 10^4	(22.71±21.15).10^4

High number of bacterial population inside abdomen because the insect consumed pollen, nectar and ooze of male flower which had been contaminated by BDB.Results of previous research was found that BDB population 3.5 x 10^{11} cfu/ml on male flowers and spathe 2.3 x 10^{11} with symptoms of wilt plants, the leaves are turn yellow, fruit become leak (1). In activities, BDB to take out site pectinase enzyme which cause tissue had attacked and it makes maceration and to export smell which interest flower visitor insect. According to Taneja and Guerin (15), *Rhagoletis pomonella,* and many species of insects attracted to the material plant tissue decay. Protein contained inside fruit has a decomposition into ammonia odor that attracts fruit insects (22).

According to the Eden-Green, (7); Tinzaara *et al.,* (3), the population of bacteria *Xanthomonas wilt* (BXW is found in high amounts in banana nectar, exudates fluid from sheath fracture and ooze of Kayinja banana cultivars (Pisang Awak) who attacked banana *Xanthomonas Wilt* (BXW) in Mukono district, Luwero and Mpigi, Uganda. In male flower nectar has found (1.891 x 10^4 cfu/ml), fresh exudate sheath faults (cfu/ml 3.625 x 10^5 / ml) and ooze (1.896 x 10^{11} cfu/ml) Xcm. When male flowers of sick plants, loss or breakage sheath, the ooze bacteria come out like milk-white drops. Part of insect body contaminated and the bacteria enter tractus digestivus of the insect because the insect suck bacteria ooze and nectar of the infected banana flower.

3.4. BDB Isolation from All Phase Development of *T. minangkabau*

Propagule BDB was found in all phase development of *T. minangkabau,* nectar and pollen except in eggs (Table 3).

Table 3: The results of BDB isolation from the colonies of *T. minangkabau*

Isolation source	Isolated parts	The presence of BDB
Imago	the body surface	++
	in body	+++
Pupa	the body surface	++
	in body	+++
flyblow	the body surface	++
	in body	+++
Egg	surface	-
Polen		+++
Nectar		+

Description: (-) not found colonies of BDB, (+) low number of colonies, (++) moderate number of colonies , (+++) a high number of colonies.

Contamination in all development phase of *T. minangkabau* except the egg stage by BDB it is possibly because workers imago had been contaminated by BDB when searching nectar, pollen and food source (ooze, pollen and nectar) which collected from banana flower infected by BDB. By worker bees, ooze, pollen and nectar contaminated with that bacteria was fed to larvae, pupa and imago. In the beginning of this research it was found the propagule of BDB inside pollen and flower nectar of Pisang Kepok were infected by BDB in endemic land on Tabek Panjang highland and Pasar Usang (1).

According to Atkins (24), bacterial cells attached to body surface of insects as contaminants and enter into digestive tube of insects. These cells will be blown away at the time of insect feeding, sucking nectar or lay of eggs (oviposition). Sources of bacterial inoculum that is spread by insects originated from male flowers and ooze of bacteria which can survive for three years.

3.5. Isolation and Identification of Bacteria

Results of bacterial isolation from *T. minangkabau* imago in field and inside nest / colony found that bacterial colony with characteristic are pink, with measuring 0.5 - 4.5 mm, irregular, convex and *non-fluidal,* with or without a pink center formation (Fig. 2). These characteristics in accordance with the characteristics of BDB colony description by Eden-Green (19), Schaad *et al,.* (11) and Baharuddin (16).

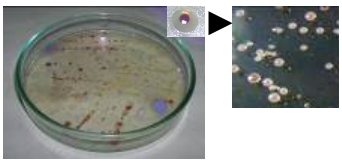

Fig. 2: Bacterial colony from *T. minangkabau* in TTC medium, 48 hours after incubation.

Gram test results showed that bacterial colony found was a group of gram negative (Figure 4a), hydrolyze pectin (Figure 3b). Hypersensitivity reaction test showed a positive result indicated the presence of necrotic regions in injected a suspension of bacteria (Fig. 3c). The results of pathogenicity tests showed that these bacteria are pathogenic to banana plants with an incubation period of 3-4 days with a scale of 4 (all the leaves wilt) and a high level of virulence (scale 5). Typical symptoms of blood disease of the whole leaf wilting occurred at 3-4 days after inoculation (hsi) (Figure 4). These results assure that the bacterial colony is a colony of BDB.

Table 4: Gram test results, hypersensitive reaction test and pathogenicity test colonies of bacteria isolated from the body of *T. minangkabau*

Phase of development / body / food	Gram test	Pectinase test	Hypersensitivity reactions	Pathogenicity test
surface of the head	Negative	Positive	Positive	Positive
in head	Negative	Positive	Positive	Positive
surface of the abdomen	Negative	Positive	Positive	Positive
in the abdomen	Negative	Positive	Positive	Positive
Egg	Negative	Positive	Positive	Positive
Flyblow	Negative	Positive	Positive	Positive
Pupa	Negative	Positive	Positive	Positive
Polen	Negative	Positive	Positive	Positive
Nectar	Negative	Positive	Positive	Positive

According to Buddenhagen and Elasser, (26) colonies of bacteria are transmitted by insects is a highly virulent strain and strain termed SFR (Small, Fluidal and Round). According to Leiwakabessy (14), *Ralstonia solanacearum* strains isolated from family of Apidae (Hymenoptera) in the affected banana plantations in Lampung BDB has a very high level of virulence on banana plants Barangan with an incubation period of 7 dai.

Fig. 3: Gram test results, hypersensitive reaction, and pectinase test of *Trigona minangkabau*. Description: a. Gram test results, isolated from colonies of *T. minangkabau*; b. Hypersensitive reaction in leaves of *M. jalapa* plant at 48 hours after inoculation. c. Test pieces of pectinase on potato tuber

Fig. 4: Testing the pathogenicity on banana kepok plants age of 4 months after acclimatization. Description: a. BDB isolates from the surface of the head. b. BDB isolates from head. c. BDB isolates from abdominal surface. d. BDB isolates from abdomen. e. BDB isolates from eggs. f. the control. g. BDB isolates from larvae. h origin. BDB isolates from pupa. i. BDB isolates from pollen. j. BDB isolates from nectar.

4. Conclusion

From the results of this study it can be concluded that *T. minangkabau* has tremendous potential to spread the BDB. Bacterial cells are found in high amounts on the surface of the head / abdomen and the inside of the head/abdomen and the imago in the field, new imago, pupa, larva, eggs, pollen and nectar in the hive/colony *T.minangkabau* BDB positive.Virulence of bacteria high in banana seedlings kepok.

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