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Full Length Article

The Occurrence of Blood Disease of Banana in Selangor, Malaysia

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Abstract

Bacterial wilt disease has caused a serious threat to the banana industry in Malaysia. The disease is always associated with Moko disease caused by *Ralstonia solanacearum*. The bacterium forms irregular round, creamy colonies with red centres when cultured on Kelman's tetrazolium chloride (TZC) medium. Moko disease has been interchangeably identified as blood disease as both of them cause similar wilting symptoms on banana. Therefore, the present study was carried out to detect the occurrence of bacterial wilt in Selangor through comprehensive procedures on morphology, molecular, pathogenicity and specificity tests. Isolation of bacteria was conducted on infected plant samples (fruit stalk, fruit pulps, stem and root) collected in the field by plating on TZC medium. Bacteria colonies that are Gram-negative, oxidase-positive, and obligate aerobe were chosen for further identification through polymerase chain reaction (PCR) using 759F and 760R primers. Molecular identification showed the bacteria that colonized the infected plants is confirmed as BDB, which is very closely related to *R. solanacearum*. After Koch's Postulate and pathogenicity test on tomato, wilting symptoms were found on all banana plantlets after two weeks. The bacterium did not cause any wilting symptom on tomato plantlets. The present study revealed the distribution of blood disease in Selangor, after the first report in Perak. It also suggested that both morphology and DNA-based identification are necessary for the detection of the pathogen that causes bacterial wilt in banana. © 2016 Friends Science Publishers

Keywords: Banana; Blood disease bacterium (BDB); Kelman's tetrazolium chloride (TZC); Moko disease; *Ralstonia solanacearum*

Introduction

Bacterial wilt disease is one of the most severe diseases affecting banana plants in countries such as India, Indonesia and the Philippines (Sequeira, 1998). The disease is commonly known as bacterial wilt and is always associated with *R. solanacearum* (Fegan and Prior, 2006). This bacterium is well-known to be one of the world most important phytopathogenic bacteria due to its persistence and wide host-range (Denny, 2006). *R. solanacearum* is a soil and water-borne pathogen that can enter the host plant through wounds that occur on the root (Alvarez *et al*., 2008). It can also be transmitted by insects during pollination (Fegan, 2005). Generally, it is classified into two main groups based on the mode of transmission; the "SFR" (small, fluidal, round) and "A" (Amazon basin) strain and the "B" (banana) strain (Supriadi, 2005). The "SFR" and "A" strain bacteria are readily transmitted by insect and are able to move rapidly from host plants to surrounding plants. Meanwhile, "B" strain bacterium is transmitted through root contact and contaminated planting equipment (Sequeira, 1998). It is recommended that a fallow period of 6 months is needed on field infested by insect-transmitted infection and up to 12 months for soil-transmitted infection (Fegan, 2005).

Blood disease is another type of wilting disease that affects the banana plantations in Indonesia since 1921 (Supriadi, 2005; Remenant *et al*., 2011). It is caused by blood disease bacterium (BDB, previously known as *Psuedomonas celebensis*) that exhibits similar symptoms with those infected by Moko disease (Fegan and Prior, 2006). The symptoms include wilting and yellowing of leaves, vascular discoloration, bacterial ooze and reddish-brown fruit rot (Remenant *et al*., 2011). The disease has caused a depressing scenario to the banana industry due to its aggressiveness and severity of infection.

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In Malaysia, bacterial wilt in banana is always associated with Moko disease (Zulperi and Sijam, 2014). The disease was first reported in the banana plantations in Johor after a severe flood that hit the state in 2007 (Husain and William, 2011). It was then spread to other states in the country that caused severe yield lost. According to Tengku Ab Malik *et al*. (2012), there was about 60% bacterial wilt infection incidences reported in 3200 hectares of banana plantations in Johor. Meanwhile, BDB was first detected in Perak in 2013 (Kogeethavani *et al*., 2013). This suggested that both Moko and blood disease could be invading other provinces of Peninsular Malaysia. Therefore, it is important to identify the causal agent of bacterial wilt in banana in view that both pathogens were being reported in Malaysia.

The detection and identification of the pathogen can be done through morphological observations on selective media and biochemical tests (Denny and Hayward, 2001). DNA-based technique was also used to detect pathogen on latently infected plants or soil, using selective primers (Opina *et al*., 1997; Ito *et al*., 1998). These methods are useful for the detection of viable cells of the pathogen in different hosts and in soil environment. The present study was conducted to identify the causal agent of bacterial wilt in Batang Kali, Selangor and to confirm the pathogen viability through morphology, molecular, pathogenicity and specificity tests. This is very important to generate better understanding on the pathogen and to recommend effective ways to control the spread of the disease.

Materials and Methods

Isolation of Pathogen

Field sampling was conducted at a banana plantation in Batang Kali, Selangor in July 2013. The plantation is mainly planted with Nipah (*Musa balbisiana*) and Nangka (*Musa x paradisiaca*) varieties. Most of the leaves turned yellow, wilted and hanged from the junction with the pseudostem. The fruits appeared to be unaffected but the internal pulps showed yellowish-brown discoloration. Infected trees were chopped down and plant parts included fruit, fruit stalk, stem and root were sampled. The infected plant samples produced a stream of bacterial ooze when immersed in water. This is a method that allows fast detection for banana bacterial wilt infections in the field (Agrios, 2005).

Isolation of bacteria was done following the procedure described by Stefani *et al*. (2005) with some modifications. Infected plant parts were cut into small pieces and surface sterilized with 10% sodium hypochlorite (Clorox). They were then left to dry in a laminar flow. Later, the samples were immersed in 10 mL sterilized distilled water (SDW) before macerated. Five, tenfold dilution series were performed on the macerated plant-water samples. Aliquot 10 µL of each dilution was then spread on Kelman's tetrazolium chloride (TZC) medium to obtain single colony of the bacteria. The medium is useful in distinguishing *R. solanacearum* from other bacteria during isolation (French *et al*., 1995). It is also being used to differentiate virulent and avirulent colonies. The cultures were incubated at 28°C and recorded for the growth of the bacteria for 3 days. After 3 days, bacteria colonies that appeared as irregular round, creamy white, red centres were isolated and subcultured back onto similar medium to obtain pure colonies (Fig. 1).

Biochemical Tests

The bacteria colonies were tested with selected biochemical tests for characterisation (Chaudhry and Rashid, 2011; Denny and Hayward, 2001). The tests are useful in eliminating other saprophytic bacteria that are normally occur together with the pathogen. Pure colonies that showed positive results were then transferred and stored in universal bottles containing 10 mL sterilized tap water at 28°C for subsequence use (Denny and Hayward, 2001).

Potassium Hydroxide (KOH) Test

A loopful of bacteria was stirred with a drop of 3% KOH (Sigma-Aldrich) solution on a glass slide using an inoculating wire loop. The formation of slime threads was observed on Gram-negative bacteria whereas negative observation for Gram-positive bacteria.

Catalase Oxidase Test

A loopful of bacteria was mixed with a drop of 3% hydrogen peroxide (H_2O_2) (Sigma-Aldrich) solution on a glass slide using an inoculating wire loop. The formation of gas bubbles was carefully observed with naked eyes. Gram-negative bacteria form gas bubbles whereas negative observation for Gram-positive bacteria.

Kovac's Oxidative Test

Bacterial colony on casamino acid-peptone-glucose (CPG) medium was tested with the tip of an oxidase strip with tetramethyl-p-phenylenediamine dihydrochloride (Kovac's oxidase reagent, Fluka). The bacteria is characterized as oxidase-positive if a purple colour develops after 10 sec; delayed positive if coloration develops within 10–60 sec and negative if no changes of colour.

Triple Sugar Iron (TSI) Medium

The ability of the bacteria to undergo fermentation, produce gas during fermentation or generate hydrogen sulphide (H_2S) was tested with TSI medium. TSI medium (Difco) was prepared in capped test tubes and allowed to form slant upon cooling. Bacteria colony was inoculated onto the slant by streaking over the entire surface of the slant using an inoculating loop. The tubes were incubated at room temperature and observed after 24 h. A colour change from methyl red to yellow indicates as positive for anaerobic

growth. If there is any gas produced during the process, the medium will appear as cracked or accumulation of gas bubbles is observed at the bottom of the tube. The production of H_2S by bacteria is confirmed when black precipitate (ferrous sulphide) is formed at the bottom of the tube, as a result of the reaction between H_2S and ferrous sulphate present in the medium.

Oxidation Fermentation (OF) Basal Medium

OF basal medium was prepared following the procedures described by Chaudhry and Rashid [18]. The medium in the test tubes were allowed to solidify at room temperature. Bacteria colony on CPG agar was embedded into the OF medium in two tubes using a sterilized inoculating loop. One of the tubes was covered with a layer of sterile paraffin to a depth of about 2 cm. The tubes were incubated at room temperature and examined after 24 h. A colour change from olivaceous green to yellow in both tubes indicates as positive for anaerobic growth (fermentation).

Bacteria DNA Extraction

Prior to DNA extraction, bacteria cultures from the stock were streaked on TZC medium and incubated at 28°C for 72 h. The visible bacteria colonies were then streaked onto CPG medium (as a working medium) before transferred to 10 mL of sterilized nutrient-yeast extract-glucose broth (NYGB) to allow propagation of bacteria. The bacteria inoculums were incubated at 28°C for 48 h by rotary shaking with a mechanical shaker (110 rpm).

DNA extraction was performed using the Qiagen Plant-DNA extraction kit, following the protocols provided by the manufacturer. The DNA extracted was checked for visible DNA band under UV illumination. One per cent (1%) of agarose gel was prepared with 1 X Tris-Borate-EDTA (TBE) buffer. Extracted DNA (3 µL DNA and 2 µL loading dye) was loaded in the well of the gel. The electrophoresis was performed at 90 volt for 45 min. The visible DNA was then viewed under UV illumination for documentation.

Polymerase Chain Reaction (PCR)

PCR amplification was performed using primer pair 759F and 760R, following the protocols described by Opina *et al.* (1997). The primers 759F and 760R are common primers used to detect *R. solanacearum* (Opina *et al.*, 1997; Ito *et al.*, 1998). The amplification was conducted in 50 µl volume containing 25 µL DreamTaq PCR Master Mix (DNA polymerase, dNTPs, $MgCl_2$, reaction buffer and nuclease free water), 16 µL nuclease free water, 3 µL of each primer and 3 µL of DNA template. The samples were denatured at 94°C for 30 sec, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 1 min. The final extension was 72°C for 10 minutes. The amplified DNA were then stored at -10°C for future use.

A 5 µL of PCR product (3 µL amplified DNA fragment and 2 µL loading dye) was loaded into the well of 1% agarose gel. Pure BDB colony (accession number: KF208537.1, obtained from Plant Pathology Lab, Horticulture Research Centre, MARDI, Malaysia) and nuclease free water were used as the positive and negative control respectively. The electrophoresis was performed at 90 volt for 90 min. The visible DNA was then viewed under UV illumination for documentation.

Samples with visible DNA bands at approximately 280 bp were purified using Qiagen DNeasy Plant Mini Kit following the protocols provided by the manufacturer. The purified DNA was sent for sequencing to Repfon Glamor Sdn. Bhd., Kuala Lumpur, Malaysia. The sequenced fragments obtained were then aligned and matched with the DNA sequences deposited in the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Koch's Postulate Test

Bacteria inoculums were inoculated onto 2 months' old banana plantlets purchased from a nursery in Banting, Selangor, Malaysia and acclimated in the glass house for 3 weeks. Prior to the inoculation, bacteria from the stocks were streaked onto CPG medium and incubated at 28°C for 72 h. After 72 h, the single colony of the bacterium was transferred into 10 mL SDW to prepare bacteria inoculums. The concentration of inoculums were adjusted to 10^8 cell per millilitre (cell/mL) using Bio-Rad SmartSpec Plus spectrophotometer with SDW (Kusumoto *et al.*, 2004).

Root sections of the plantlets were injured with a sterilized needle (0.5 x 16 mm). Five millilitres (5 mL) of the inoculum was carefully drenched onto the soil. The experiment was conducted in 4 replicates and SDW was used as the control. The plantlets were observed for disease symptoms for 3 weeks.

All the plantlets were harvested at 18 days after inoculation (DAI). The root, stem and leaf pulp of each plantlet were sampled. The samples were subjected to the standard procedures for isolation of pathogen as described earlier for the detection of BDB.

Effect of Pathogen on Tomato Plants

Pathogenicity of the bacterium cultures were then tested on tomato plantlets to determine the ability of the bacterium to affect other solanaceous plants. It is a useful test in distinguishing BDB from *R. solanacearum* that causes Moko disease, with the former is specific to banana (Kusumoto *et al.*, 2004). Bacterial inoculums with a concentration of 10^8 cell/mL were prepared as described. The stem of an 8-weeks' old tomato plantlet was injured with needle and wrapped with a thin layer of sterilized cotton soaked in the bacterial inoculum. The area was then sealed with Parafilm to keep the wounded area moist. The experiment was conducted in triplicates with SDW as the control. The tomato plantlets were observed for wilting or chlorosis on the leaves.

Results

Isolation of Pathogen for Biochemical Test

Biochemical tests were carried out on the pure colonies obtained from TZC medium that categorized as irregular round, creamy white with red centres (Fig. 1). The tests showed that 4 isolates designated as BK1, BK2, BK3 and BK4 were Gram-negative, oxidase positive and obligate aerobe (Table 1). These 4 isolates were selected for further molecular identification based on PCR analysis.

Molecular Identification

PCR amplification showed the presence of bacterial DNA by 1% agarose gel electrophroresis (Fig. 2). The sequences were then identified using BLAST programme against a database of genomic sequences at NCBI. Sequence homology from the BLAST search at NCBI revealed that the isolates BK1, BK2, BK3 and BK4 were BDB. The sequences were 99 to 100% matched with BDB (accession number: FR854071.1). BDB has very similar morphological characteristics with *R. solanacearum* when cultured on TZC and CPG media. It formed small (approximately 1 to 2 mm in diameter), red-centred creamy colonies on TZC medium after 3 days.

Koch's Postulate Test

Banana plantlets inoculated with BDB inoculums (isolates BK1 – BK4) started to show wilting symptoms at 12 DAI (Fig. 3a). The leaves remained green but appeared wrinkled and slightly curled up. The symptoms were more prominent on the young leaves. Besides that, the midrib and petiole of the leaves wilt, causing them to hang from their junction with the pseudostem. Upon harvested on 18 DAI, 90% of the leaves of all the inoculated plantlets turned yellow and the whole plants wilted (Fig. 3b). The root of the infected plants had little root hair when compared to the control (Fig. 4a). Brown discolorations were observed at the cross sections of the stem and root of infected plants (Fig. 4b).

Re-isolation of the infected plant parts on TZC medium showed that BDB was present in the root, stem and leaf pulps of the plantlets.

Pathogenicity Test on Tomato Plantlets

All the tomato plantlets did not show wilting symptom after inoculated with the bacterial inoculums (Fig. 5). Therefore, the results indicated that the pathogen is BDB.

Discussion

The present study revealed that the banana plants in Batang Kali were infected with BDB, similarly as reported earlier in Perak by Kogeethavani *et al.* (2013). This suggested that the disease is now spreading to other state in Peninsular Malaysia.

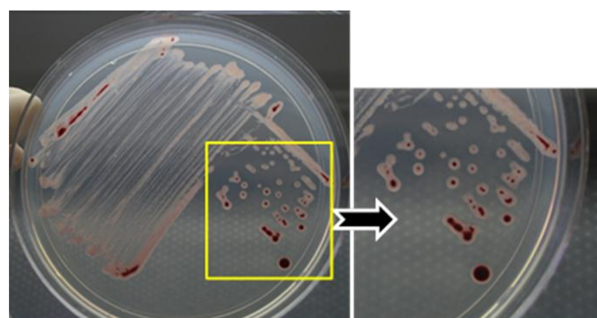

Fig. 1: Pure colonies of bacteria on TZC medium that showed irregular round, creamy white with red centres

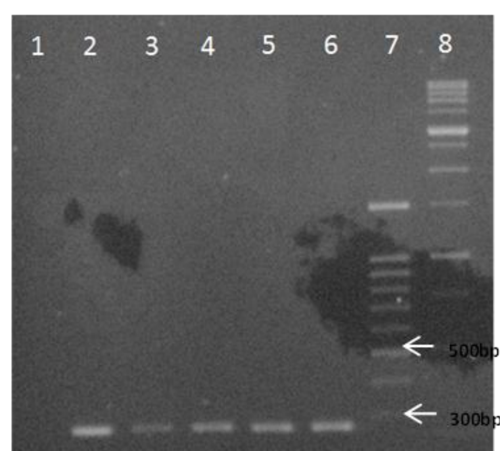

Fig. 2: PCR amplification of genomic DNA for the four bacteria isolated from banana plant parts. Lane 1: negative control (SDW); Lane 2: isolate BK1; Lane 3: isolate BK2; Lane 4: isolate BK3; Lane 5: isolate BK4; Lane 6: positive control (pure culture of BDB, accession number: KF208537.1); Lane 7: 1 kbp ladder and Lane 8: 1.5 kbp ladder

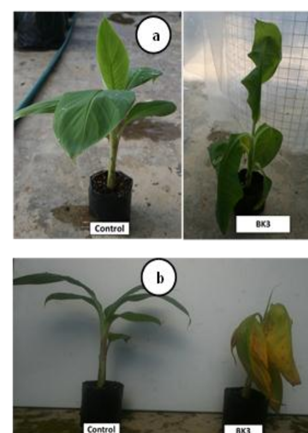

Fig. 3: The symptoms on banana plantlets after Koch's Postulate test compared with control (SDW) (a) Curly and wilt symptom of BDB at 12 DAI (b) Yellowing and wilt symptom of BDB at 18 DAI

Table 1: Observation and indications of biochemical tests on pure bacteria colonies obtained from infected plant parts

Tests	Observations	Interpretation of observation
3 % KOH	Slime threads were observed when tested on bacterial colonies	Gram-negative
Catalse oxidase (3% H_2O_2)	Small gas bubbles were observed on the inoculating loop when tested on bacterial colonies	Gram-negative
Kovac's oxidative	Purple color developed when the strip was tested with bacterial colonies	Oxidase positive
TSI medium	No color changes was observed after 24 hours	Aerobic
OF basal medium	Color change was observed in tubes without paraffin oil. No color change was observed in tubes covered with paraffin oil	Aerobic

The spread of BDB is another serious impact to the banana industry in Malaysia besides the threat of Moko and *Fusarium* wilt. Blood disease was detected in 90% of the provinces in Indonesia and had caused low banana production for five consecutive years (Hadiwiyono, 2011). It was reported that the disease could spread at a speed of 25 km per year (Thwaites *et al*., 2000). This had caused total destruction in certain areas in Indonesia that lead to severe economic loss to the country (Supriadi, 2005). Therefore, the occurrence of BDB in two provinces of Malaysia has generated an urgent need to curb the disease in view of the total destruction of the disease reported in Indonesia.

In Koch's Postulate test, the leaves of infected plants were observed to loss tension and wilted although they appeared green. The root of the infected plants were found to be less developed when compared to the control. This suggested that BDB disrupts the root development of the plants, causing them to wilt eventually. BDB was found at the root, stem and leaf pulp of infected plants when aliquots of serial dilutions were streaked on TZC medium. This suggested that BDB can be systemically distributed to other parts of the plant from the infected site and the whole plant can be the source of infection (Hadiwiyono, 2011).

BDB is very closely related to *R. solanacearum* serologically and they are grouped into the same group as *R. solanacearum* race 2 (Supriadi, 2005). Therefore, they cause similar wilting symptoms on host plants. Previous studies have grouped *R. solanacearum*, BDB and *Ralstonia syzygii* (causal agent for Sumatra disease of cloves) into *R. solanacearum* species complex (Taghavi *et al*., 1996; Remenant *et al*., 2011). The complexity of the species is due to the presence of 16S rRNA sequences of these pathogens that are closely related, but with certain distinctions. The primer pair 759F and 760R is useful in differentiating strains of *R. solanacearum*, BDB and *R. syzygii* (Villa *et al*., 2003). However, BDB is more host specific compared to *R. solanacearum* that causes Moko disease (Supriadi, 2005). Besides banana, *R. solanacearum* will cause wilting on other solanaceous plants. Inoculation of the bacterium into tomato plantlet serves as a useful tool in distinguishing these two pathogens, in addition to molecular identification.

The present study revealed that bacteria form creamy with red centre colonies on the TZC medium do not necessarily *R. solanacearum*. Therefore, identification of the pathogen by morphological characteristics on specific media may not sufficient in determining the identity of a pathogen that causes bacterial wilt in banana. This is particularly

Fig. 4: The developments of banana plantlet root inoculated with BDB and control (SDW) at 18 DAI (a) less development of root hair on plantlet inoculated with BDB; (b) cross sections of infected root and stem that showed brown discoloration of the vascular tissues

Fig. 5: Pathogenicity test of four isolates (BK1, BK2, BK3 and BK4) on tomato plantlets and compared with control (SDW) at 7 DAI

when the occurrence of the pathogen is not common in an area. False identification may lead to inappropriate treatments on the disease. Besides that, no selective medium has been developed for the detection of BDB (Hadiwiyono, 2011). Therefore, molecular identification using specific primers is useful in identifying BDB (Taghavi *et al.,* 1996).

Blood disease was restricted in Sulawesi from its discovery until 1987. The disease is now rapidly spread throughout Indonesia when the quarantine on Sulawesi was broken in 1987 (Fegan, 2005). The battle against the pathogen continues as there is still no effective chemical treatment for the disease. With the invasion of blood disease in Peninsular Malaysia, more efforts should be taken to control the spread of the disease. The cooperation between government agencies, Agriculture Department and farmers

are very important to restraint the dissemination of the disease through quarantine and surveillance. Good agricultural practices (GAP) and knowledge in identifying the early symptoms of the disease are also vital to prevent further spread.

Conclusion

It is confirmed that the banana in Batang Kali, Selangor is infected with BDB. The identification of BDB requires both morphological and molecular techniques for precise detection. It is important to prevent false identification in view that the causal agents of Moko and blood disease are very closely related. The detection of BDB in several provinces in Malaysia generates an urgent need for possible solutions to prevent the spread of the disease.

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