

Diversity of Endophytic Bacteria in Symptomatic and Asymptomatic Infected Bananas from Endemic Area of Blood Disease Bacterium Based on RISA

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ABSTRACT

The relationship between the diversity of endophytic bacteria and disease development has been documented on many diseases. In Indonesia, by the millions banana are infected by Blood Disease Bacterium, causing severe damages. In this work, the diversity analysis of endophytic bacteria in infected bananas from endemic area of blood disease bacterium had been conducted using Ribosomal Intergenic Spacer Analysis (RISA). RISA is a Polymerase Chain Reaction (PCR)-based method as a useful assessment tool for the diversity analysis of microbes in the environment. Here, the infected bananas were determined by PCR using specific primer for Blood Disease Bacterium (BDB). Asymptomatic and symptomatic mats samples were analyzed through independent cultureable approach of PCR-RISA using universal primers of operon gene region of rRNA between small subunit (16S) and large subunit (23S) called intergenic spacer region (ISR). The diversity of endophytic bacteria from asymptomatic banana was different from that of symptomatic plants. In addition, the cultivar and habitat type might affect the diversity of endophytic bacteria.

Keywords: PCR, RISA, Blood Disease Bacterium, banana, endophytic bacterium.

1. INTRODUCTION

Blood disease bacterium (BDB) can cause severe losses to banana growers in Indonesia [1]. The diseased plants are not able to produce edible fruits, so the percentage of losses due to the disease has a linear correlation with the disease incidence. In Indonesia, in the fields, it had been reported that disease intensities were very severe in some provinces. In Bondowoso, East Java the disease intensity was reported approximately 97,7% [2], whereas in Lombok it was reached 86,8 percent [3]. The disease is still limited in Indonesia, so it is logic that the disease is listed on the first order of quarantined disease by the Collaboration Web of Asia-Pacific [4].

The relationship of diversity of endophytic bacteria to disease development has been studied on many diseases [5, 6, 7, 8, 9, 10, 11, 12]. In the vascular system, plants associate with endophytic bacteria that ecologically adapt to life and grow within the plant tissues without causing deleterious effect on the plants. Trigalet et al. [13] suggested that endophytic bacteria were promising as biological control agents to vascular disease such as blood disease of bananas. The use of endophytic bacteria as biological control agents has several advantages compared to external bacteria such as antagonists can grow and survive as long as plant growing so they are able to prevent continuously and systematically, so they protect all part of plant. Biological control of endophytic bacteria may be through antibiosis, competition of the infections site, and or induced resistance of the plant [13, 14].

Ribosomal Intergenic Spacer Regions (RISA) is a Polymerase Chain Reaction(PCR)-based analysis method of community structure providing an estimation of the

diversity and composition of microbes [15]. This paper reports the diversity of endophytic bacteria in symptomatic and asymptomatic infected bananas by BDB based on RISA analysis.

2. MATERIALS AND METHODS

Purposive sampling method was applied for determining banana sample in this study with the criteria: endemic area (Karanganyar, Yogyakarta, and Purworejo), generative stage of plant, no symptom caused by other diseases or pests, cultivar Raja Bandung and Kepok Kuning, and two kinds of banana samples, these were infected but asymptomatic (visually showed healthy) and prominent either infected or symptomatic. Each area were sampled for 3 plants of each cultivated variety and each criteria. Microbial diversity analysis was applied on composite samples from 3 plants of sample.

BDB infection status of banana was based on detection using PCR-based method with a pair of specific primer for the pathogen, 121F and 121R. The extraction of BDB and endophytic bacteria was done using "MicroLYSIS PLUS" Kit, Microzone™.

Intergenic Spacer Region (ISR) between gene of small-subunit (SSU) and large-subunit(LSU) rRNA were amplified in 25 µL PCR mix of final concentration, total amount 50 ng DNA of bacteria, 2.5 mL of 10mg mL⁻¹ BSA (Bovine Serum Albumin), 12.5 Mega Mix Royal (Microzone^{RM}) and 1.25 µL of 100 pmol of each primer. The primers were S926f and L189r with running program of PCR-RISA as described by Yu and Mohn (2001). The mixture was heated at 94 °C for 1 minute, followed by running 25 cycles of PCR, Automatic Thermo Cycler

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(BioRad™), was used and the program consist of: 94 °C for 15 seconds, 56 °C for 15 seconds, 72 °C for 30 second, and final extension at 72 °C for 1 minutes.

The electrophoresis of amplified DNA was done using 6% polyacrilamide gel electrophoresis (PAGE) and stained with silver nitrate solution (0.2% AgNO₃, and 500 µL formaldehyde in 100 mL sterile aquabidest). The similarity of the DNA fragment patterns, were analyzed using software program of NTYSISpc (Version 2.1) to generate tree plot dendrogram.

3. RESULTS AND DISCUSSION

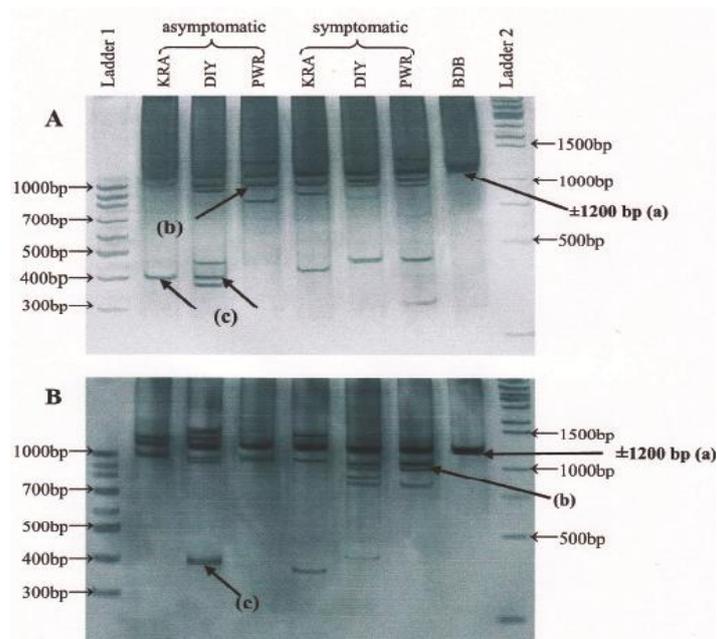
By an assumption that a DNA fragment belong to a species of bacterium, there are two common species of bacterium associated within endosphere of the bananas.. The first bacterium possesses a fragment which is positioned at about 1200 bp and appeared on all of the plant samples. The fragments were positioned in the same fragment belonging to BDB. The second of a common fragment is positioned at about 1000 bp (Figure 1). It means that the bacteria possessing the fragment are not involved in the exhibition of infected banana, because they existed in both healthy and infected bananas.

Generally the results were obvious that RISA generated the diversity structure of endophytic bacteria from the asymptomatic plants led to be different from those of symptomatic diseased plants (Figure 1). The fragment patterns between varieties of banana were different, between origins areas were too. It means that

both cultivar and origin area affects to the diversity structure of endophytic bacteria of the banana.

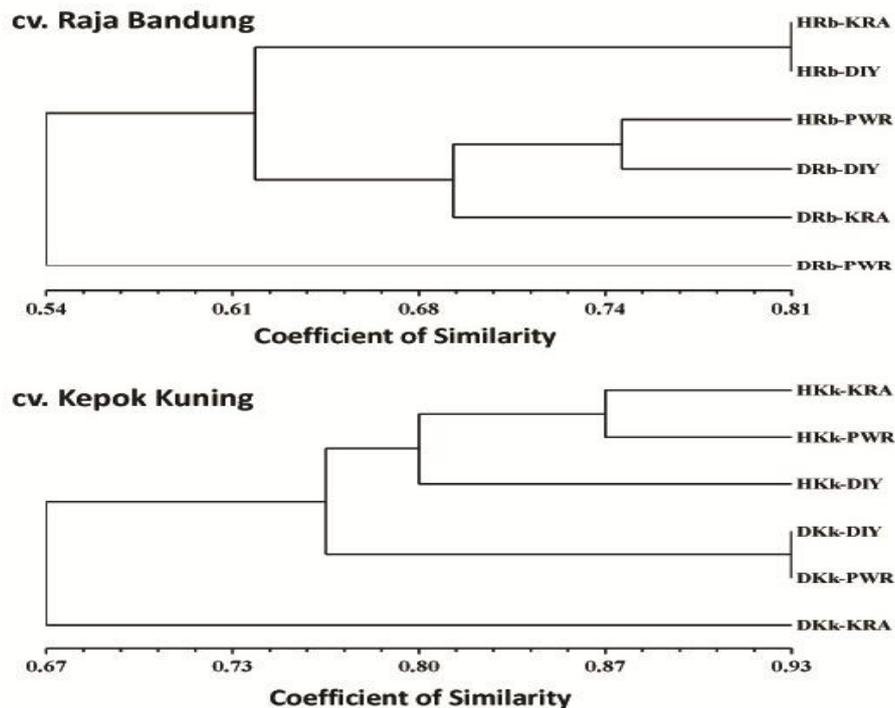
There is an interesting fragment positionate at about 400 bp found in asymptomatic infected plant, in cv Raja Bandung (originate from Karanganyar and Yogyakarta) and in cv Kepok Kuning (originate from Purworejo). The fragments show very thick. It means that population of the endophytic bacteria is abundant in those samples. Possibly, the bacteria having the fragment are involved in the exhibition of banana healthiness on asymptomatic infected banana. Some specific fragments were also found on diseased banana which were not found in asymptomatic banana, but the position of the fragments were variable between cultivars and localities. Possibly, the fragments belong to bacteria which were involved in the establishment of blood disease severity. But it is needed to be studied further.

The results showed that at list one fragment was found in the asymptomatic bananas, which was not found in the diseased plant. The fragment was founded both in cv. Raja Bandung and Kepok Kuning although it was not found in all of asymptomatic plant samples from all localities. The fragment was found in some bananas sampled from Kranganyar and Yogyakarta, but it was not found in the samples from Purworejo.



A: cultivar Raja Bandung, B: cultivar Kepok Kuning, KRA: Karangnyar, DIY: Yogyakarta, PWR: Purworejo, (a): BDB isolate 02-PWR, (b): established on all of samples, (c): just established on the infected asymptomatic bananas.

Fig 1: The DNA fragment pattern of endophytic bacteria of banana generated by PCR-RISA with the primer of S926f and L189r.



HKk: Kepok Kuning asymptomatic, DKk: Kepok Kuning symptomatic HRb: Raja Bandung asymptomatic, DRb: Raja Bandung symptomatic, KRA: Karanganyar, DIY: Yogyakarta, PWR: Purworejo

Fig 2: The UPGMA dendrogram of clustering analysis based on coefficient similarities of the endophytic bacteria community generated by PCR-RISA

As a tool to study on the diversity of endophytic bacteria, PCR-RISA is a discriminative tool. According to the result of clustering analysis based on similarity coefficient of the communities and UPGMA dendrogram in Figure 2, it can be interpreted that from a number of 12 composite samples of banana could delineate 10 different DNA pattern. It means that community structure of the endophytic bacteria in banana is significantly diverse. Each sample could delineate two to seven convenient DNA fragments with the average of 4.42 ± 1.56 .

Plants are thought to differentially influence the survival, growth, and activity of microorganisms in the rhizosphere, phylloplane, and endosphere as well depending on the developmental stage of the plant, part examined, plant species or cultivar, and environmental condition [16]. Halhmann [17] explained that the total population density of endophytic bacteria found in the plants depend on the species, plant genotype, plant tissue, growth stage, and environmental conditions. It can be understood that environmental conditions both edaphic and aerial environment will determine the diversity of endophytic bacteria. Both environments are determined by agricultural practices, in addition macro climate and soil types at the area. Conn and Franco [12] reported their study supporting this statement that soil type affected the diversity and level of endophytic colonization within the plant.

The alteration of environmental conditions caused by the agricultural practices could affect growth of microorganisms. Seghers et al. [18] observed the impact of agricultural practices on the *Zea mays* endophytic community. Their results indicated that the effect of agrochemical is not only limited to microbial community in the bulk soil but also includes the endophytic community.

The present study shows that cultivar and locality affect the diversity structure of endophytic bacteria. Two cultivars were examined in this study, which was designed to monitor the presence and activity of dominant endophytic bacteria at the generative stage of banana. Some researchers revealed that endophytic bacteria population was different among plant species and cultivars at different field areas [12, 19, 20].

The effect of plants to endophytic population is well known that it is related to the genotype of plants. Plant exudates alter according to genotypic differences. Therefore the plant genotype can affect the microbial population supported in the endosphere, phylloplane, and rhizosphere. Smith and Goodman [21] had reviewed the host variation in the host specificity of beneficial plant-associated microbes. They discussed host specificity at two levels, quantitative and qualitative. There is either compatible or incompatible interaction in relation to

qualitative host specificity. In nodulation, compatible bacteria infect the plants and form nitrogen-fixing nodules. With incompatible bacteria, early infection may be occurred, but the nodules are neither formed nor are aborted. Araujo et al. [1] reported that community structure of endophytic bacteria from tangerine (*Citrus reticulata*) and sweet orange (*C. sinenses*) were significantly different, endophytic bacteria were recovered less frequently from tangerine than from those of sweet orange.

The endophytic bacteria population is affected by plant genotypes. Siciliano et al. [19] suggested that altering the plant genome could alter exudates composition and hence that microbial population supported in the endophyt. It has been discovered that transgenic canola plants have different rhizosphere and endophytic population compared to the non-engineered plants. Dunfield and Germida [20] investigated the affect of genetically modified plants on the rhizosphere and endophytic bacterial populations supported the fact that plant genotype does affect these population.

This study showed that endophytic bacteria in diseased plant lead to be more diverse than those in asymptomatic plants. Possibly, in the stressed plant caused by the infection affect the endophytic bacterial community. Reiter et al. [11] investigated the response of endophytic bacterial communities in potato infected by *Erwinia carotovora* sub.sp. *atroseptica*. The results of the study indicated that plant stress, due to the infection, increased the diversity in infected plant compared to uninfected control plants. It was also found that pathogen stress had more significant effect on endophytic community than plant genotype.

Araujo et al. [1] supported this phenomena, species of *Methylobacterium* was the genus that were most frequently isolated from symptomatic plants. There was positive association with the occurrence and intensity of symptoms of CVC (Citrus Variegated Chlorosis) caused by *Xylella fastidiosa*. The authors suggested that CVC could play a role in the establishment of endophytic bacteria in the host plant or that endophytic *Methylobacterium* spp. could trigger CVC by a systemic interaction with *X. fastidiosa*.

Based on the present works, a speculation can be made about the role of endophytic bacterial community of plant in blood disease, since it is interesting that a fragment positioned at 400 bp is the most frequently recovered from asymptomatic plants. The relationship of endophytic bacteria to plant healthiness against some pathogens had been studied in many plants [5, 7, 8, 9, 10, 14, 16, 30] Hallmann [17] summarized many endophytic bacterial genus known to control some pathogens or promotion of growth on plants in where the bacteria inhabit. The common genus are *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Enterobacter*, *Curtobacterium*, *Pantoea*, *Bulkholderia*, *Phylobacterium*, *Rhizobacterium* etc. The study of endophytic bacteria inhabiting xylem vessels of

citrus trees revealed that they consist of *Pseudomonas* (40%), *Enterobacter* (18%), Gram-positive bacteria (*Bacillus*, corynforms) (16%), and *Serratia* (6%). Total populations were 1×10^2 to $1 \times 10^4 \text{ g}^{-1}$ tissue. Some of endophytic bacteria were reported to protect plants from diseases. About 4% of endophytic pseudomonads induced hypersensitive reaction on tomato leaves [31]. When a strain of *Bacillus subtilis* that was isolated from xylem vessels of maples was absorbed from root, 55-80% of maple seedlings were protected from the infection of *Verticilium dahliae* [31].

Araujo et al. [1] suggested that *Curtobacterium falccumfaciens* was involved in asymptomatic citrus.

C. falccumfaciens has been also reported as a biological control agent against many pathogens and it has been understood that it acts by triggering induced systemic resistance [32] and by antibiosis [6].

This study was the first attempt to use culture-independent method to evaluate the presence and activity of the dominant populations of bacterial endophytic in relation to exhibition of infected banana by BDB. PCR-RISA was used for this study. The results show that RISA can be used to detect the diversity of endophytic bacteria on the banana. RISA can be an alternative widely applying to microbial diversity analysis including endophytic bacteria [22, 26], in addition to Denaturing Gradient Gel Electrophoresis (DGGE) [1, 11, 22, 23], Terminal Restriction Fragment Length Polymorphism (T-RFLP) [22, 24, 25, 26], and PCR-Single Strand Conformism Polymorphism (PCR-SSCP) [22, 27, 28, 29].

4. CONCLUSIONS

The present study suggests that based on RISA, the endophytic bacteria in the infected bananas are very diverse. The diversity of endophytic bacteria from asymptomatic banana was different from that of symptomatic plants.

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