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Blood bacterial wilt disease of banana: the distribution of pathogen in infected plant, symptoms, and potentiality of diseased tissues as source of infective inoculums

HADIWIYONO♥
Faculty of Agriculture, Sebelas Maret University. Jl. Ir. Sutami 36a, Surakarta 57126, Central Java, Indonesia. Tel. +62- 271-646994 Fax. +62-271-646655., ♥email: hadi_hpt@yahoo.com

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Abstract. *Hadiwiyono. 2011. Blood bacterial wilt disease of banana: the distribution of pathogen in infected plant, symptoms, and potentiality of diseased tissues as source of infective inoculums. Nusantara Bioscience 3: 112-117.* Bacterial wilt caused by blood disease bacterium (BDB) is the most important disease of banana in Indonesia. The disease was difficult to control due to by poorly understood of ecology and epidemiology of the disease. This paper reports the distribution of pathogen-infected plant, symptoms, and potentiality of diseased tissues as source of inoculums. For studying the distribution of BDB in diseased banana, a number of 14 points of plant organ tissue was sampled and the pathogen was detected by PCR using a couple of specific primer for BDB, 121F, and 121R. In addition to the detection of BDB using PCR, both external and internal symptoms were observed. All the points of detection were also used as source of inoculums to search the potentiality of the tissues as source of infective inoculums. The results showed that BDB was distributed systemically in infected banana. The pathogen infection caused systemic symptom and all part of infected banana were potential as source of infective inoculums.

Keywords: blood disease bacterium, banana, distribution, inoculums, PCR.

Abstrak. *Hadiwiyino. 2011. Penyakit layu bakteri darah pada pisang: distribusi patogen pada tanaman yang terinfeksi, gejala, dan potensi jaringan yang sakit sebagai sumber inokulum infektif. Nusantara Bioscience 3: 112-117*. Layu bakteri yang disebabkan oleh penyakit darah (BDB) adalah penyakit paling penting yang menyerang tanaman pisang di Indonesia. Penyakit ini sulit dikontrol karena ekologi dan epidemiologinya kurang dipahami. Penelitian ini melaporkan distribusi patogen pada tanaman yang terinfeksi, gejala, dan potensi jaringan yang sakit sebagai sumber inokulum. Untuk mempelajari distribusi BDB pada pisang yang sakit, sejumlah 14 titik jaringan dari berbagai organ tanaman dicuplik dan patogen dideteksi dengan PCR menggunakan sepasang primer spesifik untuk BDB, yaitu 121F dan 121R. Selain deteksi BDB menggunakan PCR, baik gejala eksternal maupun internal diamati. Semua titik deteksi juga digunakan sebagai sumber inokulum untuk mencari potensi jaringan sebagai sumber inokulum infektif. Hasil penelitian menunjukkan bahwa BDB terdistribusi sistemik pada pisang yang terinfeksi. Infeksi patogen menyebabkan gejala sistemik dan semua bagian pisang yang terinfeksi berpotensi sebagai sumber inokulum infektif.

Kata kunci: penyakit darah bakteri, pisang, distribusi, inokulum, PCR.

INTRODUCTION

Banana and plantain (*Musa* spp.), hereafter referred to as bananas are important horticultural commodities. In the latest years, export growth of banana fruits from Indonesia is less conducive. The significant growth occurred during 1984-1994 with growth rate in the volume and the value 57.7% and 46.7% (Setiajie 1997). After the years, however, the products tend to stagnant or decline. From 2001 to 2005, Indonesian banana production was 4.30, 4.38, 4.21, 4.20, and 4,28 million tons respectively (General Directorate of Horticulture 2006). It seemed that blood bacterial wilt disease caused by blood disease bacterium (BDB) have involved in the case of low production of bananas (Supriadi 2005).

The national loss of banana production due to blood bacterial wilt disease was estimated at around 36% in 1991 (Muharam and Subijanto 1991). The damage of banana mats was extremely serious in certain districts in where ABB genomic group were planted such as Bondowoso and Lombok, the disease incidence could reach over 80 % (Mulyadi and Hernusa 2002; Supeno 2001; Supriadi 2005). Now, the pathogen has been distributed in 90 % of provinces in Indonesia with various disease incidences from 10 thousand to millions of banana clusters (Subandiyah et al. 2006).

Blood bacterial wilt disease remains difficult to control due to poor fundamental knowledge about the ecology and epidemiology of the disease. How long does the pathogen survive in soil? Does the pathogen associate with root systems of non-host plants? How widespread is the problem in the naturally occurring of *Heliconia* and *Musa* spp? It is obvious that in-depth studies on the ecology and epidemiology of blood disease bacterium are urgently

required (Fegan 2005). This paper reports the distribution of pathogen, symptoms, and potentiality of diseased tissues as source of inoculums.

MATERIALS AND METHODS

Sampling method

Plant materials were used in this study determined with purposive sampling method using the criteria: from endemic area of BDB, early symptom of BDB, generative stage, no symptom caused by other diseases or pests. A number of 14 points of plant organ tissue was sampled from infected plants. The main parts of infected plants that were detected for the present of BDB cell were flower, brack, fruit pulp, fruit shelter, fruit stalk, bunch peduncle, middle peduncle, basal peduncle, leaf lamina, midrib, petiole, pseudostem, corm, and root.

BDB-DNA extraction

Bacterial cells of BDB were gained through the following technique. Five thin pieces of the tissue approx. 0.2x0.5x1.5 cm^3 obtained from particular tissue point of diseased bananas were immersed in 5 ml sterile water in test tube and left overnight for oozing. One ml of bacterial ooze was transferred into Eppendorf tube and several samples were centrifuged using microcentrifuge at 13000 rpm for 10 minutes. The supernatants were discarded and the pellets were re-suspended each with 1 ml sterile water for washing potential inhibitors of the PCR. The Supernatants were discarded again and the left pellets were used for DNA extraction. The extraction was done using "MicroLYSIS PLUS" Kit, Microzone ™. The DNA extraction Kit was containing *Taq*-polymerase, Anti-*tag*-polymerase, 2x reaction buffer (6 mM $MgCl_2$), 400µM dNTPs, stabilizer, and blue loading dye.

Each of the clean pellets was added with 20 µl solution of Microlysis Plus. The extraction was run in Automatic Thermocycler Machine (Bio Rad™) with the program as follows. Seven steps of heating were programmed for the extraction, that was step 1: 65 °C for 15 minutes, step 2: 96 °C for 2 minutes, step 3: 65 °C for 4 minutes, step 4: 96 °C for 1 minutes, step 5: 65 °C for 1 minutes, step 6: 96 °C for 30 seconds, and step 7: 20 °C for hold. After cycling, the DNA mixture was stored at -20 °C before using as a template of PCR. Before using, the DNA was centrifuged 10000 rpm for 3 minutes and clean supernatant was used as PCR template.

BDB Detection

The existence of BDB in the tissue points was employed through DNA fingerprinting of PCR (Polymerase Chain Reaction)-based method. PCR was done using "Mega Mix Royal" Kit, Microzone™(Appendix 2) added 0.1% BSA in PCR mix. A couple of BDB specific primers 121F and 121R were used in the DNA amplification (Hadiwiyono 2010).

The PCR amplification program of DNA was conducted using Automatic Thermocycler Machine (BioRad™). Thermal cycle of PCR program was arranged as described by Fegan (Unpublished) one cycle of initial denaturing at 96 °C for 5 minutes, followed by 30 cycles of 94 °C for 15 seconds, 59 °C for 30 seconds, and 72 °C for 30 seconds, ended with one cycle of 72 °C for 10 minutes, then hold at 11 °C. Amplified DNA fragments were visualized by electrophoresis using agarose gel 2 % (weight/volume) in 0.5XTBE buffer for 30 minutes at 100-volt current. A volume of 1 µg/ml ethidium bromide was added in the melted agarose gel to stain the DNA; subsequently, the gel was poured in a mold to form gel wells by cooling in room temperature for ± 20 minutes. The agarose gel was removed to be soaked in TBE running buffer in the electrophoresis tank. The PCR products at 5 µL volume were loaded into the well on the gel. The DNA fragments were visualized under UV Transilluminator and documented by taking a photograph.

BDB Detection using plantlets-indicator

A volume of one ml of washed bacterial ooze collected as described above was injected in a plantlet, Kepok Kuning having been acclimated for 3 months. If the plantlets were showing wilt symptom of blood disease, the bacterial ooze samples were considered that the tissue positively contain BDB and the tissues were potential as source of infective inoculums.

Redetection of BDB from inoculated plantlets

To make sure that wilting on the plantlets were caused by BDB, re-detection of the pathogen was done using PCR. When the PCR gives a positive result, it means that BDB is distributed in the tissue.

RESULTS AND DISCUSSION

Three cultivated varieties of banana have been achieved from different location in this study, that are Kepok Arab, Kepok Kuning, and Raja Bandung sampled from Sragen, Karanganyar, and Klaten respectively (Figure 1).

For purposing detection of BDB existence in diseased plant by PCR using primers of 121F and 121R, at least 14 tissue points of individual infected plant for each cultivar were sampled. The detection using PCR showed that BDB could be detected from all of the plant tissue points. These results were supported by the established symptoms on indicator susceptible plantlets and re-detection BDB from the seedlings. It occurred on all of cultivars, Kepok Arab, Kepok Kuning, and Raja Bandung (Figure 1, 2, 3). This means that BDB is existent in all of the sample tissue points. Thus, these observations give evidence that BDB is systemically distributed in all parts of infected plants.

For supporting the detection results, the sign (ooze), external and internal symptom on the inoculated seedling was observed. The observations of natural infection got that the bacterial ooze was usually exuded by brack and male flower of inflorescence in the early morning and in wet weather. Yellowing on leaves initiated from leaf margin frequently was observed on early symptom and slow wilting banana. Sometimes, however, the wilting was very fast without through yellowing of leaves previously.

The leaves showed wilting, and some of leaves were felled hanging drop of leaves (Figure 4-A).

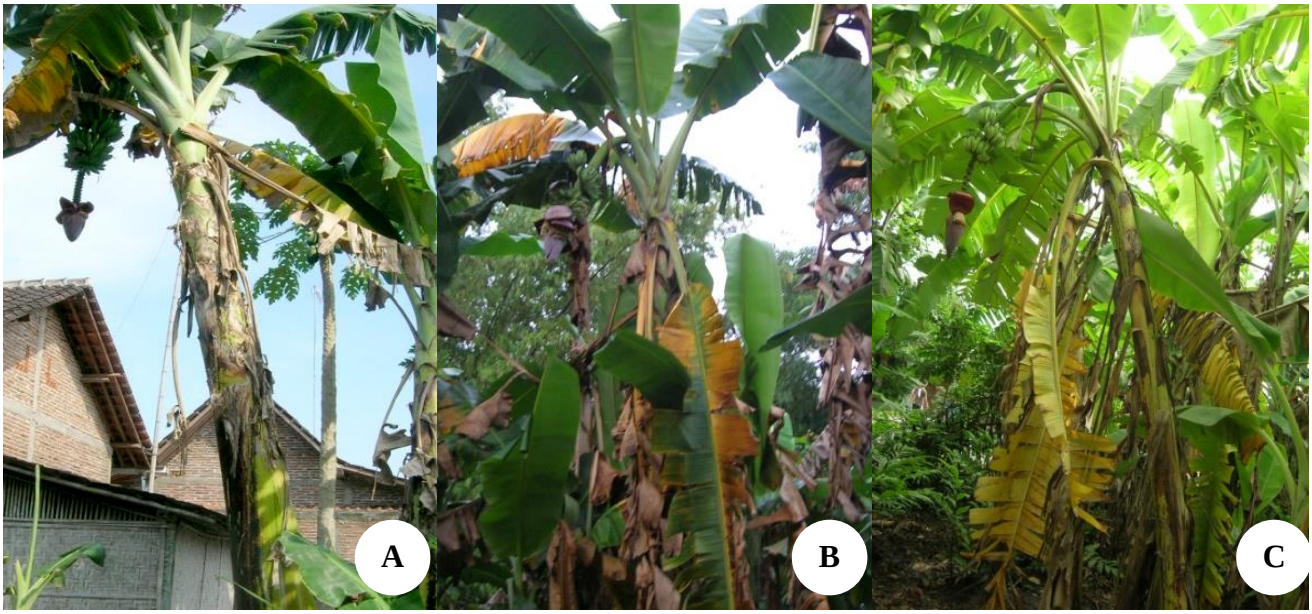

Figure 1.a. Diseased bananas with early symptom used to study on BDB distribution. Cv. Kepok Arab (A) shows bigger canopy than Kepok Kuning, Cv. Kepok Kuning (B) shows smaller canopy than Kepok Arab, Cv. Raja Bandung (C) shows smaller canopy than Kepok Arab and lighter green of leaves than those Kepok Arab and Kepok Kuning (C).

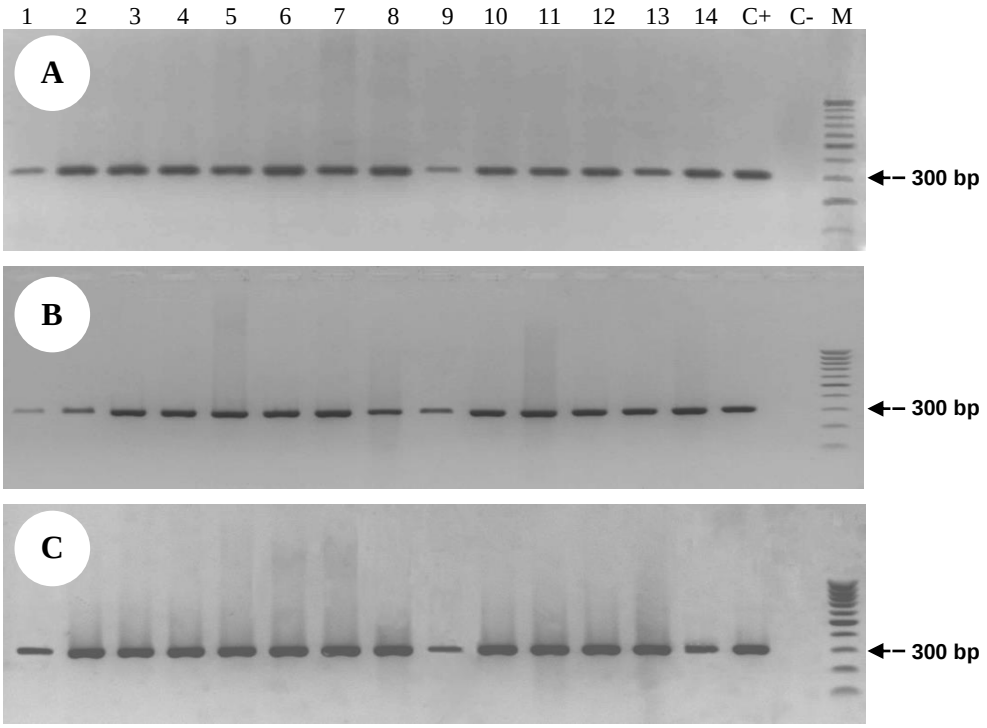

Figure 1.b. Amplified DNA of BDB generated by PCR using primers of 121F and 121R from 14 individual tissue points of cv. Kepok Arab, Kepok Kuning, and Raja Bandung. Male flower (1), Brack (2), Fruit pulp (3), Fruit shelter/coat (4), Fruit stalk (5), Bunch peduncle (6), Middle peduncle(7), Basal peduncle (8), Leaf lamina (9), Midrib (10), Petiole(11), Pseudostem(12), Corm (13), Root (14), positive control-pure culture of BDB (C+), negative control-healthy pseudostem (C), and Ladder (M).

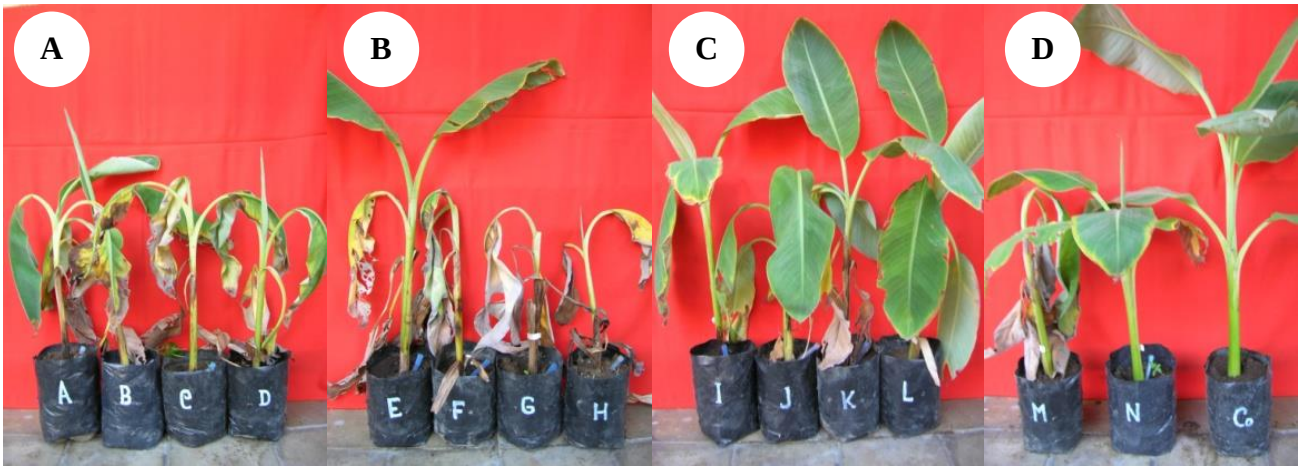

Figure 2. Wilting symptom on seedlings of cv. Kepok Kuning inoculated by washed ooze of tissue points of individual diseased plants of cv. Kepok Arab 5 weeks after inoculation. Male flower (A), Brack (B), Fruit pulp (C), Fruit shelter/coat (D), Fruit stalk (E), Bunch peduncle (F), middle peduncle(G), Basal peduncle(H), Pseudostem(I), Leaf lamina(J), Midrib(K), Petiole(L), Corm (M), Root (N), Control-positive (C+).

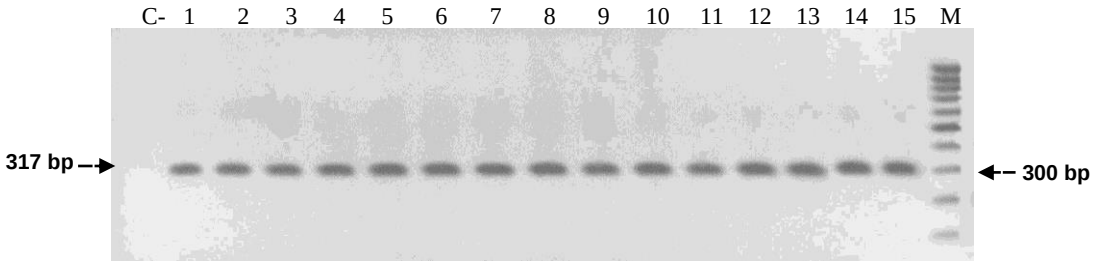

Figure 3. Amplified DNA of BDB generated by PCR using primer 121F and 121R from corm of seedlings of cv. Kepok Kuning inoculated with washed ooze from 14 tissue points of cv. Kepok Arab on 5 weeks after inoculation, Male flower (1), Brack (2), Fruit pulp (3), Fruit shelter/coat (4), Fruit stalk (5), Bunch peduncle (6), Middle peduncle(7), Basal peduncle(8), Leaf lamina(9), Midrib(10), Petiole(11), Pseudostem(12), Corm(13), Root(14), positive control-pure culture of BDB (C+), negative control (C-), and Ladder (M).

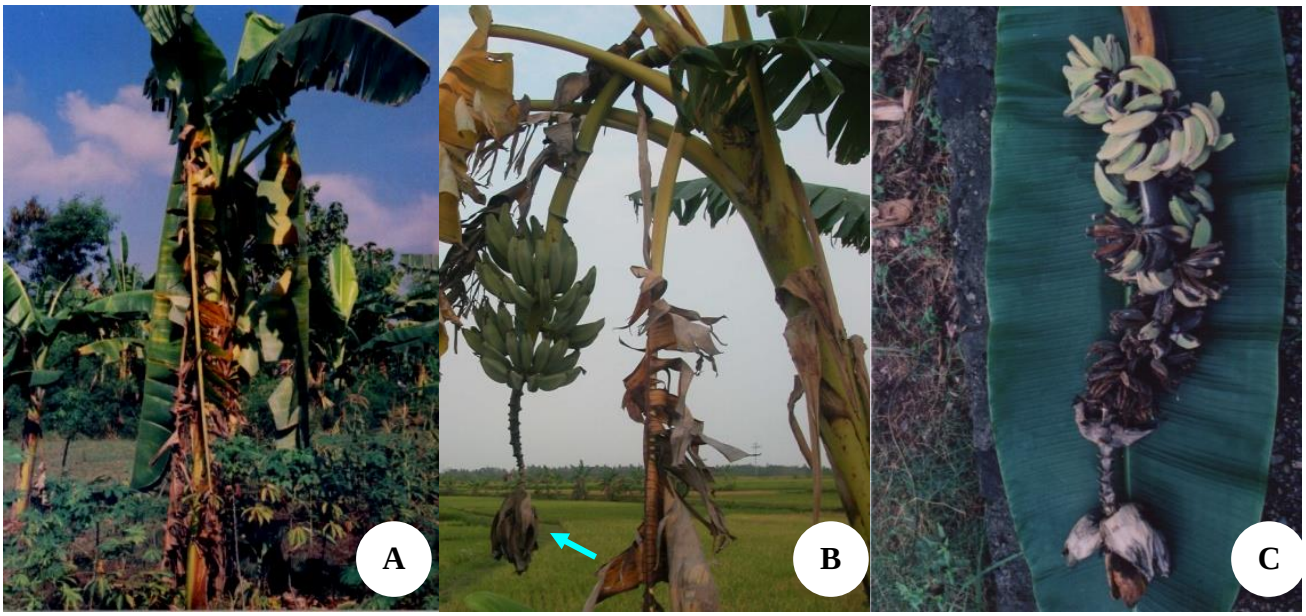

Figure 4. Specific symptoms on bacterial wilt banana caused by BDB. A. Vegetative stage of Kepok Kuning with hanging drop of leaves. B. Generative stage of Kepok Abu with dried inflorescence of flower. C. Bunch of Kepok Kuning with dried inflorescence of flower extending to upper parts or fruits.

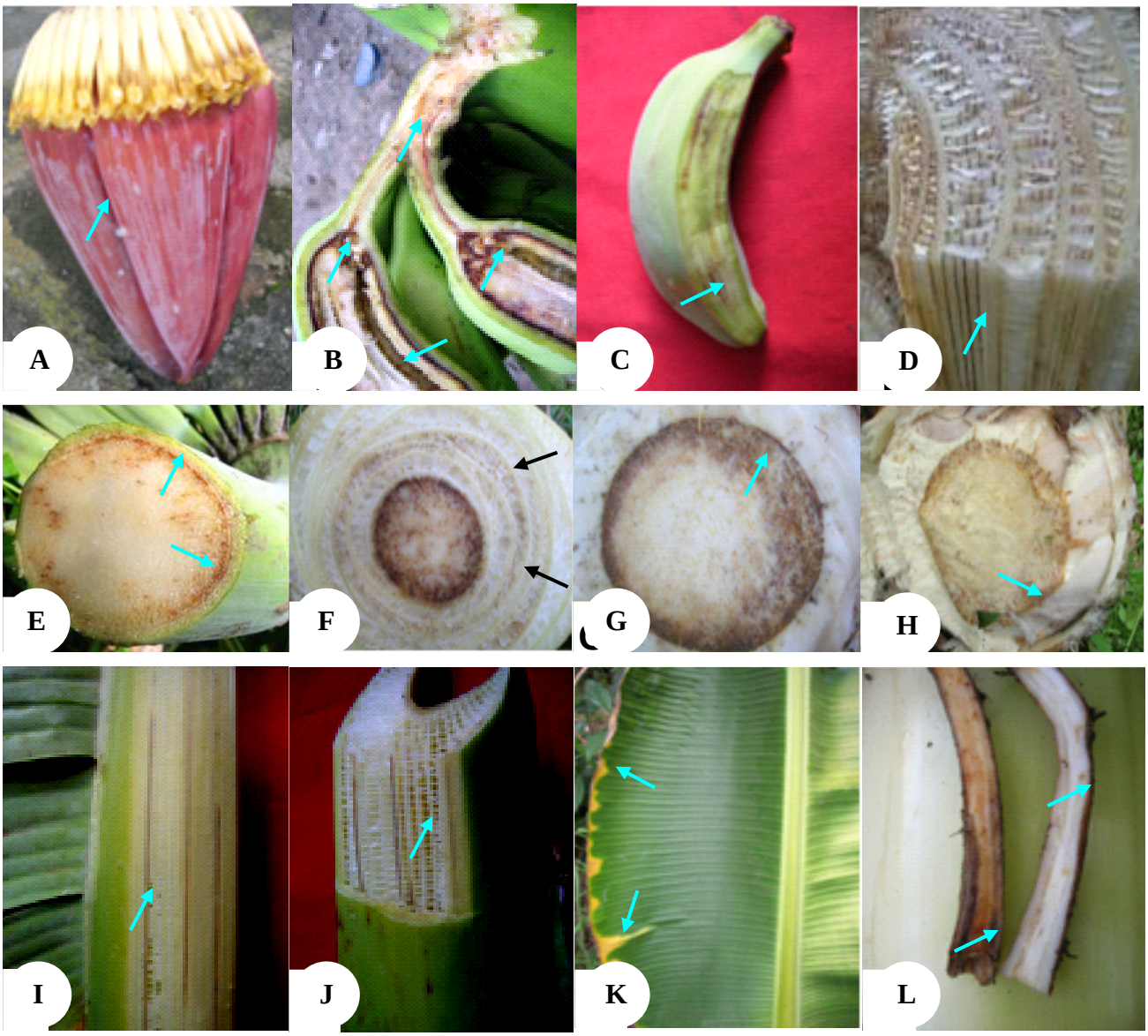

Figure 5. Internal/external symptoms of blood disease on organs of infected plant cv. Kepok Kuning, inflorescence with bacterial ooze (A) browning pulp at along section of the fruit and its stalk (B), browning vessel at the sliced fruit shelter (C), browning vessel at the pseudostem (D), browning vessel concentrated at the peduncle (E), browning vessel concentrated at margin of the middle peduncle (F), browning vessel concentrated at margin of the basal peduncle (G) browning vessel at the midrib (I), browning vessel at the petiole (J), browning vessel at the corm (H), yellowing leaf lamina at the margin (K), and browning diseased root (L).

Wilting of inflorescence flower on generative stage of bananas was observed frequently (Figure 4-B). The wilting inflorescence developed to upper parts of bunch including fruits (Figure 4-C). If peduncle was cut in some points from upper to lower part would be observed a gradual browning in vessel tissues which was observed most severe at the upper part. Discoloration vascular tissues represented by brown dots/points were gradually less frequent on the further lower parts of pseudostem or peduncle. Such symptom can be speculated that the infection is started from the inflorescence. Some diseased plants were in contrary, the symptom was with no or light browning at the upper parts and gradual more severe to the lower part with the most severe in the corm. The latest symptom might be started from the mother plant the growing sucker. Globally, the browning in vessel cells usually can be occurred in the most part of plants, pulp, stalk, fruit shelter, peduncle, middle peduncle and pseudostem, basal peduncle, midrib, petiole, corm, and root (Figure 5).

BDB is difficult to isolate from almost point of diseased plant tissues except the upper peduncle and the bunch, particularly from the fruits. From the fruit, shelter is the most frequent to be able to isolate BDB on CPG medium whereas from lower tissue points it is very difficult due to

the existence of high population of saprophytic that are suppressive the growth of slow-growing BDB. Selective medium for BDB has not been developed yet. Therefore, detection of BDB using culturable-dependent approaches will find technique difficulties. Molecular-based method through culturable-independent should be developed. Detection of pathogenic bacteria using PCR-based method is used for BDB study. In facts, PCR-based method using BDB sequencing primers of 121F and 121R was effective and sensitive.

Indeed due to the high level of sensitivity, PCR-based detection protocol is an interesting detection tool. This method, however, detects dead cells, viable but not culturable, and culturable (Louws et al. 1999). For monitoring the risk of disease caused by the abundance of pathogen inoculums, BIO-PCR was devised to circumvent for this problem (Schaad et al. 1995). Samples are first plated on selective media to propagate culturable cells and subject to PCR analysis. Unfortunately, the selective media for BDB has not been available yet. For handling this problem, detection using susceptible plant indicator could be used to circumvent to the problem. In this study, susceptible seedlings cv. Kepok Kuning was used for indicator in the detecting BDB. The results showed that all of sample tissue points were existed by viable or infective cells of BDB, indicated by the establishment of symptom generated by inoculation on plantlets with washed ooze of BDB from diseased plant. These results also indicate that all of plant parts are potential as source of inoculums for disseminating or transmitting of BDB.

These works reveal evidence that BDB invades systemically in diseased banana. It suggested that the bacterium is vascular competence. The bacteria life and do their reproduction in along vascular system of the host plant. Eden-Green (1994) mentioned that BDB infection is systemic and usually spreads throughout the rhizome, affecting the young sucker, which may show wilting and act as source of inoculums. The systemic infections were not just indicated by the existence of BDB in all point of samples of diseased plants but also by visible sign, external and internal symptoms. In facts, sometimes browning vascular system is appeared, especially in advance disease symptom (Figure 4, 5). It can be speculated that systemic symptom of blood disease is caused by the existence of BDB colonizing all of points of infected plant host.

CONCLUSION

The results showed that BDB was distributed systemically in infected banana. The pathogen infection caused systemic symptom and all part of infected banana were potential as source of infective inoculums.

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