

## Pulsed Field Gel Electrophoresis (PFGE): a DNA finger printing technique to study the genetic diversity of blood disease bacterium of banana

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### ABSTRACT

*Hadhiwiyono, Widada J, Subandiyah S, Fegan F (2011) Pulsed Field Gel Electrophoresis (PFGE): a DNA finger printing technique to study the genetic diversity of blood disease bacterium of banana. Biodiversitas 12: 12-16.* Blood disease bacterium (BDB) is the most important pathogen of bananas in Indonesia. In some field, the disease incidence reaches over 80%. Epidemiologically, the disease is similar to Moko disease in South America and Bugtok disease in the Philippines caused by *Ralstonia solanacearum* race 2. However, BDB is different in phenotype and genotype from the two diseases. Previously BDB was limited in South Sulawesi since 1920s – 1980s and recently was reported in 27 of 30 provinces in Indonesia. Pulsed-Field Gel Electrophoresis (PFGE) is a genomic DNA fingerprinting method, which employs rare cutting restriction endonucleases to digest genome prior to electrophoresis using specialized condition to separate of large DNA fragments. The results showed that PFGE analysis was a discriminative tool to study the genetic diversity of BDB. Based on the PFGE analysis, BDB isolates obtained from different localities in Yogyakarta and Central Java were quite diverse.

**Key words:** blood disease bacterium, banana, PFGE.

### INTRODUCTION

In growing bananas (banana and plantain), blood disease was a major obstacle of banana production in Indonesia (Supriadi 2005). The causal agent of the disease is called blood disease bacterium (BDB) that formerly was called *Pseudomonas celebensis* by Gauman and used to reinstatement by Eden-Green *et al.* (1988), but it is no longer valid and has disappeared in the latest literatures (Supriadi 2005). Epidemiologically, the disease is similar to Moko disease in South America and Bugtok disease in the Philippine caused by *Ralstonia solanacearum* race 2. However, BDB is different in phenotype and genotype from the two diseases (Eden-Green 1994; Eden-Green *et al.* 1998; Prior and Fegan 2005). In the fields, the pathogen is high virulent in cooking banana (ABB genomic group) varieties (Eden-Green 1994) but other varieties were also susceptible to BDB by artificial inoculation (Sudirman and Supeno 2002). The national loss of banana production due to the blood disease was estimated around 36% in 1991 (Muharam and Subijanto 1991). The damage of banana plants in certain districts where ABB genomic group are planted such as Bondowoso and Lombok, were extremely serious. The disease incidence could reach over 80% (Mulyadi and Hernusa 2002; Supeno 2002; Supriadi 2005). Cahyaniati *et al.* (1997) estimated the actual loss from Lampung Province was about 64%. The pathogen has been distributed in 90% of provinces in Indonesia with the

disease incidences varied from 10 thousands to millions of banana cluster (General Directorate of Horticulture of Indonesia 2006; Subandiyah *et al.* 2006).

Up to now, the blood disease is still difficult to control due to a poor fundamental knowledge about ecology and epidemiology of the disease such as how the pathogen survive in soil, whether the pathogen associates with root systems of non-host plants, and whether widespread is the problem in naturally occurring *Heliconia* and *Musa* spp. etc. It is obvious that in-depth studies on the ecology and epidemiology of blood disease bacterium of banana are urgently required (Fegan 2005).

Genetic variability of pathogen is one of the important themes in ecological study on bacterial wilt disease (Persley *et al.* 1985). Ecologically, Cook and Baker (1983) viewed that the occurrence of a plant disease epidemic indicated that some aspect of the biological network is not equilibrium: (i) the pathogen is genetically homogeneous and highly virulent, in high inoculum density, or not in equilibrium with the antagonists; (ii) the abiotic environment is relatively more favorable to the pathogen than to the host and/or antagonists; (iii) the host plant is genetically homogeneous, highly susceptible, and continuously or extensively grown; (iv) the antagonists are absent or in low populations, lack nutrients, or the proper environment to function as antagonist, are inhibited by other microorganisms. Conversely, the absence of a disease epidemic indicates that the pathogen is absent, or its

population genetically diverse, the host is highly resistant or at least minimally diverse, the abiotic environment is unfavorable to the pathogen in a part or all of the time, or antagonists are inhibitory to the pathogen.

Ecologically, to sustain their generation, to survive, to persist and to adapt a life in their habitat or varies unfavorable environmental condition, organism species evolve a complex genetic character and each character is genetically diverse in a species. A complex interaction among many different gene products determines the ability of a bacterium to cause disease (Brown and Caitilyn 2005). Among the genetic characters are pathogenicity fitness, virulence, aggressiveness, penetration capability in host, survival capability in host and/or environment etc.

The capacity to cause plant disease has evolved in relatively small number of bacterial species which are phenotypically and genetically diverse. Below the level of the species the strains that make up these species also vary in genotype and phenotype. Traditionally phenotypic techniques such as substrate utilization profiles and total fatty acid composition have been employed to characterize plant pathogenic bacteria. Recently more reliable DNA-based methods have been applied which provide a more complete understanding of genetic and evolutionary relationships of bacteria (Fegan and Hayward 2004).

Pulsed-field gel electrophoresis (PFGE) is a genomic DNA fingerprinting method, which employs rare cutting restriction endonucleases to digest the genomic DNA of bacteria which is then subjected to electrophoresis using specialized condition for separation of large fragments of DNA (Fegan and Prior 2005). Coenye et al. (2002) suggested that PFGE are the best method to study epidemiology of human bacterial diseases, such as *Neisseria meningitidis* (Popovic et al. 2001), *Campylobacter jejuni* (Ribot et al. 2002), *Stenotrophomonas maltophilia* (Berg et al. 1999, Valdezate 2004) *Staphylococcus aureus* (Schlichting et al. 1993) *Salmonella enterica*, *Shigella sonnei* and *Listeria monocytogenes* (Hunter et al. 2005; Kubota 2005). Clinically, it is an invaluable tool in detecting the occurrence of an outbreak and trying to determine its source (Hentea 2004). This paper reports the evaluation of PFGE analysis as a tool for analyzing diversity of BDB isolates from some district in Yogyakarta and Central Java.

## MATERIALS AND METHODS

The PFGE method used in this study was based upon procedures as described by Popovic et al. (2001) with some modifications. Plugs were prepared with 1 mL of the bacterial suspension with  $OD_{\lambda 600nm}=0.1$  ( $\sim 10^8$  cfu/mL), harvested from 5 days BDB culture on CPG broth. Proteinase-K was used for lysing of the bacterial cells in the plugs with conditioning at 50 °C over night, continued by soaking the plugs in 3 mL TE and shaking for an hour. This washing step was done 4 times, and then the plugs were placed in a final volume of 1 mL TE (Tris-EDTA) and stored at 4°C.

Restriction enzyme *Xba*I was used for digesting the bacterial DNA genome (at 20 units per 20  $\mu$ l prepared in restriction buffer) and incubated at 37 °C for 4 hours. After

digestion, 1 mL of 0.5xTBE was added to the Eppendorf tube to stop the reaction and allowed the plugs to sit in running buffer for at least 15 minutes. The marker was also pre-incubated in the running buffer for 15 minutes.

CHEF type (BioRad™) was used for PFGE running. Running conditions: the electrophoresis chamber was filled with 2.2 L of fresh 0.5xTBE buffer, and chiller was adjusted to get 14°C. The running program was consisted of initial switch time, 2.16s; final switch time, 54.17 s; duration of run, 19 h, angle, 120°; gradient, 6 V/cm with a linear ramping factor. DNA fragments were stained with EtBr. DNA fragments were visualized under UV transilluminator and documented using digital camera. NTSYS soft ware was used to analyze the similarity of DNA banding pattern and dendrogram. PFGE grouping was based on reproducibility of the technique. The reproducibility was determined by repeating PFGE with isolate PTI-1 on three separate occasions. In addition, DNA from this isolate was run three times in each gel as standard for PFGE typing (Valverde et al. 2007).

## RESULTS AND DISCUSSION

Fourteen isolates of BDB were analyzed to investigate the genetic diversity of the isolates. DNA fragments generated by *Xba*I restriction endonuclease digestion of genomic DNAs of the isolates prior to PFGE analysis were presented in Figure 1.

The results showed that PFGE analysis was a discriminative tool to study genetic diversity of BDB. Using 14 isolates from Yogyakarta and Central Java areas, BDB were quit diverse. Digestion of BDB genome with *Xba*I and visualization with PFGE showed that each isolate could generate between 14 to 17 convenient fragments with average  $15.64\pm 0.74$ . Whereas based on position of the fragments within 14 isolates of BDB, the PFGE could generate 21 fragments.

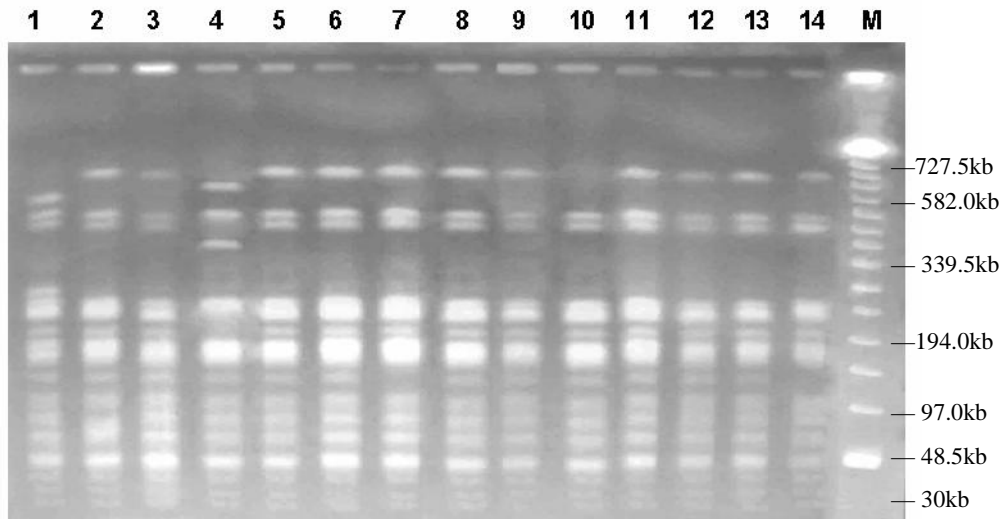
Through analysis with the un-weighted pair group method arithmetic average (UPGMA) to fragment pattern of 14 isolates of BDB generated a tree plots with coefficient of similarity among the isolates (Figure 2). In reproducibility testing, the banding pattern of the reference isolate was obtained a baseline of the level of similarity between three different replicates, at 94%. Therefore, a cut off the value of 94% was used to define as a PFGE type. Based on the criterion, the PFGE analysis generated 5 strains of 14 isolates of BDB. It means that BDB in Yogyakarta and Central Java are diverse. At least 9 isolates from Yogyakarta and Central Java were grouped in one pulsed-field gel types (strain III). The isolates were GRG-1, KRA-1, SRG-1, MGL-1, TMG-1, TMG-2, SMG-1, BTL-1, and KLT-1, implying that those strains were the most common pathogen in the provinces. Whereas the other isolates (strains I, II, IV, V) were the minor strain in the provinces.

The PFGE method is excellent in type ability, reproducibility, and discriminatory (Basim and Basim 2001; Coenye et al. 2002). Whereas Hielm et al. (1998) suggested that PFGE analysis is a reproducible and

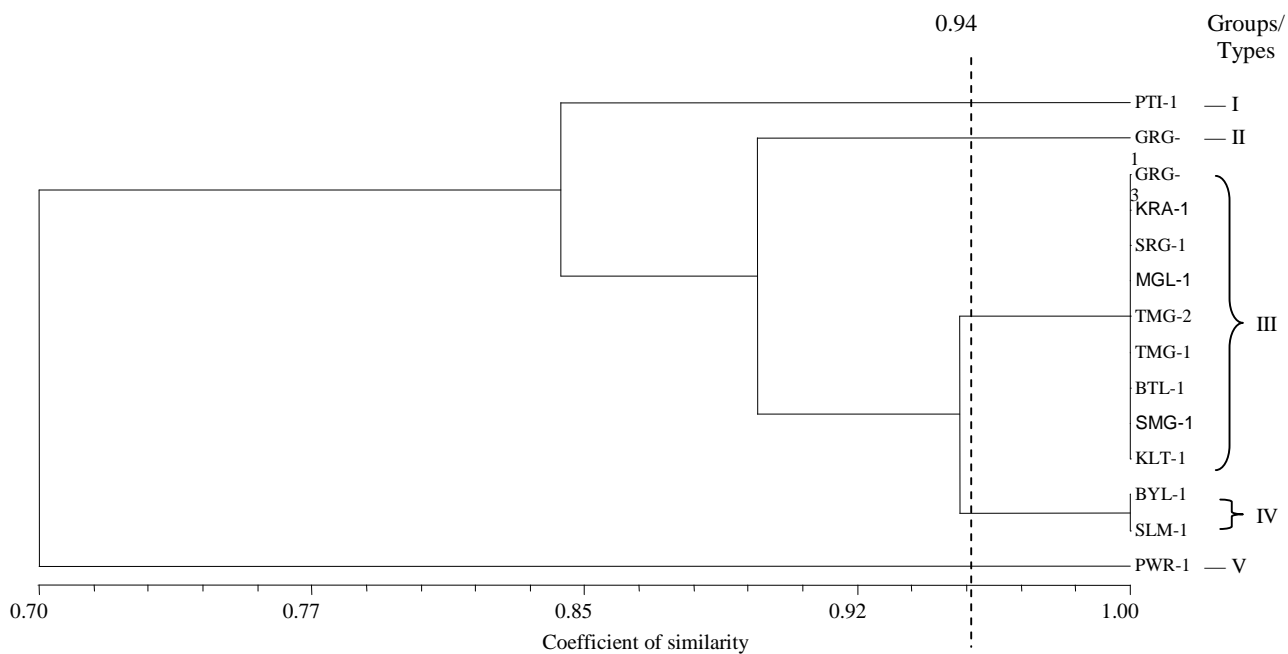
discriminating epidemiological method for studying *Clostridium botulinum* group II at the molecular level. The present results indicated that BDB was a diverse pathogen.

In facts, previously reported that BDB was limited in Sulawesi Island (Eden-Green 1994) and significant explosive incidence of the disease occurred in the latest

decades (Supriadi 2005). It is possible that blood disease epidemics in out of Sulawesi Islands are related to blood disease epidemic in the first island and it should be confirmed with farther researches using a number of BDB isolates originated from different provinces in Indonesia.



**Figure 1.** DNA fragments generated by Restriction endonuclease digestion of total DNAs from isolates of BDB with *XbaI* after pulsed field gel electrophoresis (PFGE), where 1. PTI-1 (Pati), 2. GRB-1, 3. GRB 3 (Grobogan), 4. PWR-1 (Purworejo), 5. KRA-1 (Kranganyar), 6. SRG-1 (Sragen), 7. BYL-1 (Boyolali), 8. KLT-1 (KLT), 9. SMG-1 (Semarang), 10. BTL-1 (Bantul), 11. TMG-1, 12. TMG-2 (Temanggung), 13. MGL-1 (Magelang), 14. SLM-1 (Sleman), M. Marker (Lambda Ladder).



**Figure 2.** The UPGMA-dendrogram presenting the diversities of BDB isolates based on DNA fragments generated by restriction endonuclease digestion of total DNAs with *XbaI* visualized by PFGE, where 1. PTI-1 (Pati), 2. GRB-1, 3. GRB 3 (Grobogan), 4. PWR-1 (Purworejo), 5. KRA-1 (Karanganyar), 6. SRG-1 (Sragen), 7. BYL-1 (Boyolali), 8. KLT-1 (Klaten), 9. SMG-1 (Semarang), 10. BTL-1 (Bantul), 11. TMG-1, 12. TMG-2 (Temanggung), 13. MGL-1 (Magelang), 14. SLM-1 (Sleman), M. Marker (Lambda Ladder)

Basically, BDB is diverse in population. The spreading BDB to other provinces in Indonesia may lead to an increase the heterogeneousness of BDB due to its internal interaction within strains and with others environmental conditions in where pathogen was introduced. Goto (1990) explained that bacteria have a great capacity to adapt to diverse environments through mutation, gene transfer, of metabolic regulator. Basim et al (1999) opinioned the frequency of horizontal gene transfer among bacterial strains could be in a high level. The authors verified that the frequency of kanamycin resistance transfer to recipient was more than 75 times greater in planta (paper leaves) than in vitro. This gene transfer explains the great frequent among strains of bacterial spot pathogen (*X. axonopodis* pv. *vesicatoria*) in term of polymorphism, which is indicated by the high diversity in population.

Comparing to the diversity of BDB generated by rep-PCR with BOXAIR primer (Subandiyah et al. 2006), this results showed that PFGE is more distinctive than those rep-PCR. rep-PCR could generated 10 fragments with different position and among 20 isolates only isolate 29-LPG and 32-PLU (Central Sulawesi) showed specific fragment pattern. Whereas the PFGE could generate 21 different positioned fragments and generated 5 groups or pulsed field gel types of 14 isolates. Moreover, Coenye et al. (2002) suggested that PFGE was more reproducible than RAPD (random amplified polymorphic DNA) and BOX-PCR finger printing in genomic typing of *Burkholderia cepacia* Genomovar III.

Frey et al. (1996) characterized a number isolate of *P. solanacearum*. Comparison of among data obtained clearly identified that PFGE is the most discriminatory method delineating 17 pulse-field gel types, rep-PCR and bacteriocin typing identified nine rep-PCR profile types and nine bacteriocin groups. In a research employed by Smith et al. (1995), showed that genomic fingerprinting of *P. solanacearum* by PFGE revealed a level of genetic diversity previously unrealized using both ERIC-PCR and BOX-PCR. Basim et al. (1999) used PFGE for investigating the frequency of chromosomal gene transfer by conjugation and PFGE could verified the transfer gene occurrence among strain of *Xanthomonas axonopodis* pv. *vesicatoria* in a natural niche of the bacterium. Zhang and Geider (2005) observed isolates of *Erwinia amylovora* using PFGE analysis, and through this way the bacteria were grouped and differentiated into several strains correlated to their origin. So it is useful to hints about course and distribution of the plant disease.

## CONCLUSION

Pulsed-field gel electrophoresis (PFGE) analysis was a discriminative tool to study the genetic diversity of blood disease bacterium (BDB). Based on the PFGE analysis, BDB isolates obtained from different localities in Yogyakarta and Central Java were quit diverse.

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## REFERENCES

- General Directorate of Horticulture of Indonesia (2006) Distribution pattern of plant pests and diseases. [www.deptan.go.id/ditlinhorti](http://www.deptan.go.id/ditlinhorti). [Indonesia].
- Basim H, Stall RE, Minsavage GV, Jones JB (1999) Chromosomal gene transfer by conjugation in the plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*. *Phytopathology* 89: 1044-1049.
- Basim E, Basim H (2001) Pulsed-field gel electrophoresis (PFGE) techniques and its use in molecular biology. *Turk J Biol* 25: 408-418.
- Berg G, Roskot N, Smalla K, (1999) Genotypic and phenotypic relationships between clinical, and environmental isolates of *Stenotrophomonas maltophilia*. *J Clin Microbiol* 37: 3594-3600.
- Brown D, Caitilyn (2005) Understanding the molecular basis of bacterial wilt disease: a view from the inside out. In: Allen C, Prior P, Hayward AC (eds). *Bacterial wilt disease and the Ralstonia solanacearum* species complex. APS Press, St. Paul, Minnesota USA.
- Cahyaniati C, Mortensen N, Mathur SB (1997) Bacterial wilt of banana in Indonesia. Technical Bulletin. Directorate Plant Protection of Indonesia, Jakarta.
- Coenye T, Spilker T, Martin A, LiPuma JJ (2002) Comparative assessment of genotyping method of *Burkholderia cepacia* genovar III. *J Clin Microbiol* 40: 3300-3307.
- Eden-Green SJ (1994) Diversity of *Pseudomonas solanacearum* and related bacteria in South East Asia: new direction for moko disease. In: Hayward AC, Hartman GL (eds) *Bacterial wilt: the disease and its causative agent, Pseudomonas solanacearum*. CAB International, California.
- Eden-Green S.J, Supriadi, Hastati SY (1998) Characteristics of *Pseudomonas celebensis*, the cause of blood disease of banana in Indonesia. In: Proceedings of the 5<sup>th</sup> international congress of plant pathology (abstract). Kyoto, August 20-27, 1998.
- Fegan M (2005) Bacterial wilt diseases of banana: evolution and ecology. In: Allen C, Prior P, Hayward AC (eds) *Bacterial wilt disease and the Ralstonia solanacearum* species complex. APS Press, St. Paul, Minnesota USA.
- Fegan M, Prior P (2005) How complex is the “*Ralstonia solanacearum* species complex”? In: Allen C, Prior P, Hayward AC (eds) *Bacterial wilt disease and the Ralstonia solanacearum* species complex. APS Press, St. Paul, Minnesota USA.
- Fegan M, Hayward C (2004) Genetic diversity of bacterial pathogen. In: Gillings M, Holmes A (eds) *Plant microbiology*. Bios Scientific, London.
- Frey P, Smith JJ, Albar L, Prior P, Saddler GS, Trigalet-Demery D, Trigalet A (1996) Bacteriocin typing of *Burkholderia (Pseudomonas) solanacearum* race 1 of the Frence West Indies and correlation with genomic variation of pathogen. *Appl Environ Microbiol* 62: 473-479.
- Goto M (1990) *Fundamental of bacterial plant pathology*. Academic Press, San Diego, CA.
- Hentea C (2004) Pulsed-field gel electrophoresis: a microbial experience. [http://www.cdc.gov/pulse/net/publication/2004/Hnetea\\_e.htm](http://www.cdc.gov/pulse/net/publication/2004/Hnetea_e.htm).
- Hielm B, Bjorkroth J, Hyytia E, Koreala H (1998) Genomic analysis of *Clostridium botulinum* group ii by pulsed-field gel electrophoresis. *Appl Environ Microbiol* 64: 703-708.
- Hunter SB, Vauterin P, Lambert-Fair MA, van Duyn MS, Kubota K, Graves L, Wrigley D, Barrett T, Ribot E (2005) Establishment of universal size standard strain for use with the pulsed-field gel electrophoresis protocols: converting the national databases to new size standard. *J Clin Microbiol* 43 : 1045-1050.

- Kubota K, Barrett TJ, Ackers ML, Brachman PS, Mintz ED (2005) Analysis of *Salmonella enterica* serotypic pulsed-field gel electrophoresis patterns associated with international travel. *J Clin Microbiol* 43: 1205-1209.
- Mulyadi, Hernusa T (2002) Blood disease intensity on banana caused by bacterium of *Pseudomonas solanacearum* in Bondowoso. In: Proceeding of the 16th congress and national seminar of the Indonesian Phytopathological Society. Bogor, 22-24 August 2001 [Indonesia].
- Persley GJ (ed). 1985. Bacterial Wilt Disease in Asia and the South Pacific. ACIAR Proceedings No. 13, 15-24, Canberra, Australia.
- Popovic T, Schmink S, Rosentein NA, Ahello GW, Reeves MW, Plikaytis B, Hunter SB, Ribot EM, Boxrud D, Tondella MI, Kim C, Noble C, Mothershed E, Besser J, Perkins BA (2001) Evaluation pulsed-field gel electrophoresis in epidemiological investigations of meningococcal disease outbreaks caused by *Neisseria meningitidis* serogroup. *J Clin Microbiol* 39: 75-85.
- Prior P, Fegan M (2005) Diversity and molecular detection of *Ralstonia* race 2 strains by multiplex PCR. In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. APS Press, St. Paul, Minnesota USA.
- Ribot EM, Fitzgerald C, Kubota K, Swaminatan B, Barrett TJ (2002) Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J Clin Microbiol* 39: 1889-1894.
- Schlichting C, Branger C, Fournier JM, Witte W, Boutonnier A, Woltz C, Goulet P, Doring G (1993) Typing of *Staphylococcus aureus* by pulsed-field gel electrophoresis, zymotyping, capsular typing, and phage typing resolution of clonal relationships. *J Clin Microbiol* 20: 2599-2605.
- Smith JJ, Offord LI, Holderness M, Saddler GS (1995) Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonas solanacearum*) race 3 in Kenya. *Appl Environ Microbiol* 61: 4263-4268.
- Subandiyah S, Hadiwiyono, Nur E, Wibowo A, Fegan M, Taylor P (2006) Survival of blood disease bacterium of banana in soil. In: Proceeding of the 11th international conference on plant pathogenic bacteria. Edinburgh, 10-14 July 2006.
- Sudirman, Supeno B (2002) Screening some varieties of banana to infection of blood disease of banana. In: Proceeding of the 16th congress and national seminar of the Indonesian Phytopathological Society. Bogor, 22-24 August 2001 [Indonesia].
- Supeno B. 2002. Isolation and characterization of banana blood disease in Lombok. In: Proceeding of the 16th congress and national seminar of the Indonesian Phytopathological Society. Bogor, 22-24 August 2001 [Indonesia].
- Supriadi (2005) Present status of blood disease in Indonesia. In: Allen C, Prior P, Hayward AC (eds) bacterial wilt disease and the *Ralstonia solanacearum* species complex. APS Press, St. Paul, Minnesota USA.
- Valdezate S, Vindel A, Martin-Davila P, Del Saz BS, Baguero F, Canton R (2004) High genetic diversity among *Stenotrophomonas maltophilia* strains despite their originating at a single hospital. *J Clin Microbiol* 42: 693-699.
- Valverde A, Hubert T, Stolov A, Dagar A, Kopelowitz J, Burdman S (2007) Assessment of genetic diversity of *Xanthomonas campestris* pv. *campestris* isolates from Israel by various DNA fingerprinting techniques. *Plant Pathol* 56: 17-25.
- Zhang Y, Geider K (2005) Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. *Appl Environ Microbiol* 63: 4421-4426.