Indonesian Journal of Biotechnology, June (1999) 271 - 275

Quick detection of citrus greening by PCR method with specific and universal primers

Anang Triwiratno[1], M.E. Dwiastuti[1], A. Supriyanto[1]

1) Tlekung Research and Assessment Installation for Agricultural Technology, PO. Box 22 Batu, Malang, East Java, Indonesia
Phone : (0341) 592683, Fax. : (0341) 593047

Abstract

Huanglungbin or Greening is caused by ploem restricted unculturable bacterium, identified as Liberobacter. In general, Liberobacter found in Indonesia is Liberobacter asiaticum. *The aim of this research was to develop the effective primers for Polymerase Chain Reaction (PCR) method in order to detect citrus greening quickly. Primers used in this research were specific Primers (OI1, OI2c, OA1) and universal Primers (fD1/rP1). The specific Primers (OI1, OI2c) were more effective and specific in detecting citrus greening than those of universal Primers (fD1/rP1). In addition, the advantage of specific primers was that it could be used to amplify DNA greening of 1160 bp only, but could not be used to amplify DNA of other sistemic pathogens.*

Keywords: Huanglungbin (HLB) – liberobacter – polymerase chain reaction – quick detection – specific and universal primers

Introduction

Huanglungbin (HLB) or Greening diseases is caused by phloem-restricted unculturable bacterium namely Librobacter (Planet *et al.*, 1995). The determination of bacterium shows that this pathogen belongs to the family of Sub division Proteobacteria, and it consists of two types those are *Liberobacter asiaticum* for the Asia species and *Liberobacter africanum* for Africa species. However, both of those Liberobacter species genetically have 70 % of similarity (Jagouiex *et al.*, 1994). The disease is transmitted by insect vector such as *Dhiaphorina citri* Kuw. for *Liberobacter asiaticum* and *Trioza erytreae* (Delgwercia) for *Liberobacter africanum* (Garnier & Bove, 1993).

Huanglungbin (HLB), known as Citrus Vein Phloem Degeneration (CVPD) is still considered as the most serious citrus diseases which needs to be anticipated in the establishment and rehabilitation of citrus production in Indonesia. It seems that HLB is caused by *Liberobacter asiaticum* which has heat tolerance. This disease has infected the citrus orchards in several provinces of Indonesia except West Kalimantan province.

The control of HLB diseases through citrus rehabilitation has been done in several provinces in Indonesia. The controlling strategy for HLB in Indonesia has been

formulated and is known as Intregated Management for Healty Citrus Orchard (IMHCO) that facilitates the implementation of diseases control strategy. IMHCO consists of the use of certified viruses-free plants, vector control, well orchard sanitary, optimum tree maintenance and well organized application in one area target (Supriyanto, 1997).

One of the main threat of the IMHCO implementation is the application of vector control and orchard sanitary. Nevertheless, the symptom of HLB disease in the field is still difficult to determine by visual detection, especially by farmer, while indexing method using indicator plants of Madame vinous takes long time. By using indicator plant, the symptom will appear 4-6 months after inoculation (Muharam and Triwiratno, 1990).

Since 1987, monoclonal antibodies method has been developed for detecting this pathogen (Garner & Bove, 1993). In general, this monoclonal antibodies method use either Elisa or Immunofluoresence test. In Indonesia, 10 A 6 clone of monoclonal antibody was very sensitive in detecting CVPD (Dwiastuti *et al.*, 1992). Recently, Polymerase Chain Reaction (PCR) has become common for HLB detection since other methods are time consuming and less sensitive. This method could detect both Liberobacter species, *Liberobacter asiaticum* and *Liberobacter africanum*.

Materials and Methods

The research was conducted in Biology Celuler and Molecular Laboratorium INRA Bordeaux, France and Virologi Laboratory in Tlekung Research and Assessment Installation for Agricultural Technology. Two kinds of Primers, Universal Primer fD1/rP1 and Specific Primer OI1, OI2c and OA1 were used in this research in order to determine the 16S rDNA Liberobacter.

Experiment 1. The purpose of this experiment was to determine the suitability of primers in detecting samples from screen house. Twenty five samples of citrus leaves were collected from Luwus Citrus Foundation Block (FB) and 5 samples from Sukasade Budwood Multiplication Block (BMB). Poona (India) isolate was used as a positive control and healthy sweet oranges was used as a negative control. All plants were kept in a controlled screen house at a temperature of 25° - 30° C.

Experiment 2. The purpose of this experiment was to determine the specificity of the primers in detecting 16S rDNA from HLB and procaryotes. The samples were collected from plants infected by systemic pathogen such as Citrus Tristeza Virus (CTV), Citrus Vein Enation Virus (CVEV), HLB from India, HLB from Batu and healthy plants used as a negative control.

Wizard Extract Preparation for PCR. Leaves midrib (0.3 gr) were chopped by using disposible razor blade in the sterilized room at 20° - 25° C. This slice midrib was mixed with 1 ml TE Buffer (10 mM Tris pH 8.0, 400 mM EDTA, 1% SDS) and 0.25 mg Proteinase K followed by incubation in a water bath at 65° C for 2 hours or over night. The suspension was subsequently centrifuged at 14,000 rpm for 15 minutes. The suspension was mixed with 1 ml Resin, and incubated for 5 seconds. The suspension was then transferred to a minicolumn and vacuumed by using multi vacuum promega until all liquid was drawn. Then, it was washed twice with 2 ml 80 % Isoproponal and each of washing was centrifuged for 10 seconds. Finally, this material was added with 50 µl hot water (80° C) twice and centrifuged respectively for 10 seconds at 12,000 RPM.

PCR Process. Buffer taq 10 X (780 mM Tris Hcl pH 8.8, 20 mM $MgCl_2$, ($6H_2O$), 170 mM $(NH_4)_2SO_4$, 100 mM B Mercaptoethanol) was made early to prepare PCR MIX. The PCR MIX was made from 200 µl of Buffer Taq 10 X, 100 µl detergent w1 (Gibco BRL), 80 µl dNTP, 20 BSA, 20 µl Primer and sterilized H_2O.

Two kinds of Primers used in this experiment were prepared based upon Liberobacter 16S rDNA (OI1/OI2c/OA1) and Procaryotic 16S rDNA (fD1/rP1). The PCR product was mixed with 25 µl MIX, 2 µl Wizard Extract, 22,5 µl sterilized water and 0.5 µl Taq Polimerase, and finally covered with 10 µl liquid paraffin.

The programs used for DNA amplification were: 92° C for 45s, 55° C for 45s and 72° C for 90s for thirty five cycles for the universal primer (fD1/rP1) and 92° C for 40s, 72° C for 90s for thirty five cycles for the specific primer (OI1/OI2c/OA1).

Electrophoresis and Staining. 0.7% agarose gel and electrophoresis buffer TAE 1X (0.004 M Tris Hcl, 0.02 M Sodium Acetate, 0.001 M EDTA pH 7.8) were used to analyze amplification process by using horizontal electrophoresis. The well was filled with 8 µl PCR and 2 µl BPB (Brom phenol blue). As a control, 1 kb ladder was used in this electrophoresis to display a various fragments of molecular weight. Electrophoresis was adjusted at 80 - 100 Volt, 175 mA.

The result of electrophoresis was stained with 10 mg/ml Ethidium Bromide for 10 minutes.

Results and Discussions

Experiment 1. The result of this experiment indicated that the specific Primer OI1/OI2c/OA1 only detected samples containing *Liberobacter asiaticum* and *Liberobacter africanum*. Samples collected from Luwus Foundation Block and Sukasade Budwood Multiplication Block did not give any amplyfication product of Liberobacter DNA (lanes 4-33 Fig.1), suggesting that those samples did not contain both *Liberobacter asiaticum* and *Liberobacter africanum*. Similar result was also found in healthy control (lane 2). A single band emerged in the positive control (lane 3), which was used to determine the *Liberobacter asiaticum* amplification. The results also showed that no other DNA contamination was observed as no band was detected in the PCR control (lane 1).

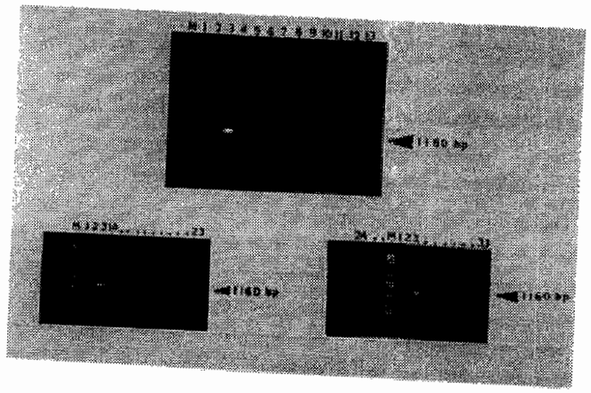

Figure 1. Electrophoresis on 0.7% Agarose gel of amplification with Primers OI1/OI2c/OA1 from water (lane 1), DNA Extracted from healthy (lane 2), *Liberobacter asiaticum* infected sweet Orange Seedling from Poona India (lane 3), sample from Luwus Foundation Block (lane 4 to 28) and sample from Sukasade Budwood Multiplication Block Block (lane 29 to 33) Marker 1 kb ladder Gibco, BRL (M)

It was observed that the universal primer fD1/rP1 gave amplification product of 1500 bp of the 16s rDNA. This amplification product could not differentiate between *Liberobacter asiaticum* (lane 3) and healthy control (lane 2) or the samples from other plants (lanes 4-33). Universal primers did not differentiate between Liberobacter asiaticum

and healthy plants with 16s rDNA amplification by using fD1/rP1 (Figure 2).

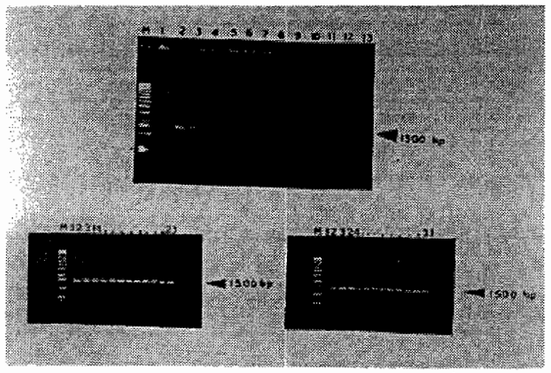

Figure 2. Electrophoresis on 0.7% Agarose gel of amplification with Primers fD1/rP from water (lane 1), DNA Extracted from healthy (lane 2), *Liberobacter asiaticum* infected sweet Orange Seedling from Poona India (lane 3), sample from Luwus Foundation Block (lanes 4 to 28) and sample from Sukasade Multiplication Block Block (lane 29 to 33). Marker 1 kb ladder Gibco, BRL (M).

Experiment 1 showed that the 16s rDNA amplification product (1160 bp) using the specific primer OI1/OI2c/OA1 could differentiate the *Liberobacter asiaticum* and healthy plants (Fig. 1), while 16S rDNA amplification with universal primer fD1/rD1 could not differentiate them.

Experiment 2. Fig.3 showed that there were amplification products with the size of 1500 bp by using universal primer fD1/rP1 from healthy plants (lane 3), Indian *Liberobacter asiaticum* (lane 5), Batu *Liberobacter asiaticum* (lane 7), Citrus Triteza Virus (lane 9), and Citrus Vein Enation Virus (lane 11). Amplification product of 1160 bp was observed by using specific primer O11O11c/OA1 from Indian *Liberobacter asiaticum* (lane 6) and Batu *Liberobacter asiaticum* (lane 8).

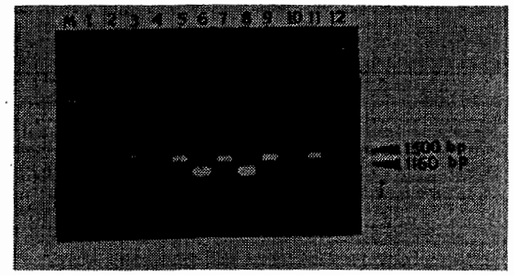

Figure 3. Eletrophoresis on 0,7% agarose gel of DNA amplification with rDNA universal primers fD1/rP1 (lane 1, 3, 5, 7, 9 and 11), Liberobactr 16 S rDNA Specific Primers O11/OI2c/OA1 (lanes 2, 4, 6, 8, 10 and 12) with water (lanes 1,2) healty plant (lanes 3, 4), *Liberobacter asiaticum* from Poona (lanes 5, 6), *Liberobacter asiaticum* from Batu (lanes 7, 8), Citrus Triteza Virus (lanes 9, 10), Citrus Vein Enation Virus (lanes 11, 12) and, M1 kb Ladder.

Amplification of CTV and CVEV were observed using universal primer for Procaryotic 16 rDNA (lane 9 and 11), but no amplification emerged using spesific primer (lane 10 and 12). Universal primer could be applied for amplification of Procaryotic 16s rDNA of healthy plants, *Liberobacter asiaticum*, CTV or CVEV. On the contrary, specific primer was only limited for amplification of Liberobacter 16s rDNA. Amplification did not occur in health plants, CTV and CVEV. Finally, the specific primer OI1/OI2c/OA1 is needed for quick detection of HLB pathogen, in order to support the Indonesian Citrus rehabilitation program.

Conclusion

1. There were no DNA amplification using specific primer OI1/OI2c/OA1 from all samples collected from citrus mothertrees in Luwus Foundation Block and Sukasade Budwood Multiplication Block, suggesting that all of mother trees were not infected with *Liberobacter* sp.
2. Universal primer was not specific for detecting Liberobacter 16s rDNA. Amplification product of 1500 bp was observed using all samples, including Citrus Tristeza Virus (CTV) and Citrus Vein Enation Virus (CVEV).
3. Specific primer is needed to run quick detection of HLB by PCR in order to support citrus rehabilitation program in Indonesia.

References

Dwiastuti, M.E., A. Triwiratno, A. Muharam (1992) Deteksi cepat CVPD pada jeruk dengan teknik immonofluorescence. *Journal Hort.* vol. 2(4): 46 - 51.

Garnier, M. and Joseph-Marie Bove (1993) Citrus greening disease and the greening bacterium. *In*: Weathers L.G. and Cohen M. (eds) *Proc. 12th Conf. Int. Org. Citrus. Virol. Riverside.* pp : 212 - 219.

Jagoueix, S., Bove, J.M. and Garnier, M. (1994) The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of proteobacteria. *Int. J. Sys. Bacteriol.* 44: 397 - 86.

Jagoueix, S., Bove, J.M. and Garnier, M. (1994) PCR detection of the two "candidatus" liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes* (1996) 10 : 43-50.

Muharam, A. and A. Triwiratno (1990) Teknik indeksing virus dan patogen sistemik lainnya. *Makalah Pelatihan Pembibitan Jeruk Bebas Penyakit.* FAO/UNDP di Tlekung, 20p.

Planet, P., Sandrine Jagouix, Joseph M. Bove, Monique Garnier (1995) Detection and characterization of the African Citrus Greening liberobacter by amplification, cloning and sequencing of the rpl KAJL-rpo BC operon current Microbiology vol. 30 (1995) pp : 137 - 141.

Supriyanto, A. (1997) Pengelolaan Terpadu Kebun Jeruk Sehat: Menuju terwujudnya sentra produksi jeruk berorientasi agribisnis yang terlanjutkan. *Makalah Intern IPPTP Tlekung.*