2. Amplifying the 16S rDNA of chloroplast on healthy and nutrient deficient plant, no Streptococcus citri or MLO could be detected by specific primers.

3. Distinction between two species of greening has been achieved by X bal restriction enzyme on 4% agarose gel digestion of the amplicons. Liberibacter asiaticum bands appeared as 9 fragments those 520 bp, 306 bp and 130 bp while Liberibacter asiaticum band appeared as 640 bp and 520 bp fragments.

4. For epidemiological study, it is now possible, using X bal digestion of the amplified DNA, to identify the liberibacter species present in plant tissues.

References


Jagnoueix, S., Joseph M. Bove and M. Garnier (1994) The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the proteobacteria. International Journal of Systematic Bacteriology 44: 397-86.


Abstract

Huanglongbing or Greening is caused by phloem restricted unculturale bacterium, identified as Liberibacter. In general, Liberibacter found in Indonesia is Liberibacter 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 (OI, O2; O3), and universal Primers (FD1/F1P1). The specific Primers (OI, O2; O3) were more effective and specific in detecting citrus greening than those of universal Primers (FD1/F1P1). In addition, the advantage of specific primers was that it could be used to amplify DNA greening of 750 bp only, but could not be used to amplify DNA of other systemic pathogens.

Keywords: Huanglongbing (HLB) – liberibacter – polymerase chain reaction – quick detection – specific and universal primers

Introduction

Huanglongbing (HLB) or Greening disease is caused by phloem-restricted unculturable bacterium namely Liberibacter (Planet et al., 1995). The determination of bacterium shows that this pathogen belongs to the family of Xylem division Proteobacteria, and it consists of two types those are Liberibacter asiaticum for the Asia species and Liberibacter africanum for Africa species. However, both of those Liberibacter species genetically have 70 % of similarity (Jagnoueix et al., 1994). The disease is transmitted by insect vectors such as Dstaphorina citri Kuw. for Liberibacter asiaticum and Triozia erytrea (Delgvercia) for Liberibacter africanum (Garnier & Bove, 1993). Huanglongbing (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 Liberibacter 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 Integrated Management for Healthy Citrus Orchard (IMHC). This technology facilitates the implementation of diseases control strategies. IMHC consists of the use of certified virus-free plants, vector control, well orchard sanitation, optimum tree maintenance and well organized application in one area target (Supriyanto, 1997).

One of the main threats of the IMHC implementation is the application of vector control and orchard sanitation. Nevertheless, the symptom of HLB disease in the field is still difficult to determine by visual detection, especially by farmers. While indexing methods using indicator plants of Madame vinaceae takes long time. By using indicator plants, the symptom will appear in 4-6 months after inoculation (Muhsram and Triwiratmo, 1990).

Since 1987, monoclonal antibodies method has been developed for detecting this pathogen (Carey & Boev, 1993). In general, this monoclonal antibodies method use either Elisa or Immunofluorescence test. In Indonesia, 10 A 6 clone of monoclonal antibody was very sensitive in detecting CVIFD (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 Liberibacter species, Liberibacter asiaticum and Liberibacter africanum.

**Materials and Methods**

The research was conducted in Biology Celuler and Molecular Laboratorium INRA Bordeaux, France and Virologi Laboratory in Deokung Research and Assessment Installation for Agricultural Technology. Two kinds of Primers, Universal Primer F1D1-rP1 and Specific Primer OII, OIIc and OAI were used in this research to determine the 16s rDNA Liberibacter.

**PCR Process.** Buffer tag 10 X (780 mM Tris Hcl pH 8.8, 20 mM MgCl₂, 6H₂O, 170 mM (NH₄)₂SO₄, 100 mM β Mercaptoethanol) was made early to prepare PCR mix. The PCR mix was made from 200 µl of Buffer Tag 10 X, 100 µl of Cerebro PS 1 (Gibco BRL), 80 µl of dNTP (Biosynthesis), 20 µl Primer and sterilized H₂O.

Two kinds of Primers used in this experiment were prepared based upon Liberibacter 16s rDNA (OII/OIIc/OAI) and Polycoryne 16s rDNA (F1D1/F1P1). The PCR product mixture was mixed with 25 µl of PCR buffer, 2 µl of Wizard extract, 22.5 µl of purified water and 0.5 µl of Taq Polymerease, and then covered with 10 µl of liquid paraffin.

The programs used for DNA amplification were: 92°C for 45s, 55°C for 30s, and 72°C for 90s for thirty cycles for the universal primer (F1D1/F1P1) and 92°C for 90s, 72°C for 90s for thirty cycles for the specific primer (OII/OIIc/OAI).

**Electrophoresis and Staining.** 0.7% agarose gel and electrophoresis buffer TAE 1X (0.004 M Tris Hcl, 0.002 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 6 µl of PCR and 2 µl of Brown phenol blue. As a control, 1 kB ladder was used in this electrophoresis to display a variety of molecular weight. Electrophoresis was adjusted at 300 - 100 Volts, 175 mA.

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

**Results and Discussions**

**Experiment 1.** The result of this experiment indicated that the specific Primer OII/OIIc/OAI only detected samples containing Liberibacter asiaticum and Liberibacter africanum. Samples collected from Laven Foundation Block and Sukasada Buddow Multiplication Block did not give any amplification product of Liberibacter DNA (lanes 4-35 Fig. 1), indicating that those samples did not contain both Liberibacter asiaticum and Liberibacter 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 Liberibacter asiaticum amplification. The result also showed that no other DNA contamination was observed as no band was detected in the PCR control (lane 1).
and healthy plants with 16s rDNA amplification by using ID1/IDP1 (Figure 2).

![Figure 2: Electrophoresis on 0.7% agarose gel of DNA amplification with Primers ID1/IDP1 from water (lane 1), DNA extracted from healthy (lane 2), Liberobacter asiaticum-infected sweet Orange Seedling from Poona India (lane 3) sample from Lusus Foundation Block (lane 4 to 28) and sample from Saka-sade Budwood Multiplication Block Block (lane 29 to 35). Marker 1 kb ladder Gibco, NEL (D)].

Experiment 1 showed that the 16s rDNA amplification product (1160 bp) using the specific primer OII/OIIc/OAI could differentiate the Liberobacter asiaticum and healthy plants (Fig. 1), while 16s rDNA amplification with universal primer ID1/IDP1 could not differentiate them.

Experiment 2 Fig.3 showed that there were amplification products with the size of 1500 bp by using universal primer ID1/IDP1 from healthy plants (lane 5), Indian Liberobacter asiaticum (lane 5), Batu Liberobacter asiaticum (lane 7), Citrus Tristeza Virus (lane 9), and Citrus Vein Enation Virus (lane 11). Amplification product of 1160 bp was observed by using specific primer OII/OIIc/OAI from Indian Liberobacter asiaticum (lane 6) and Batu Liberobacter asiaticum (lane 8).

![Figure 3: Electrophoresis on 0.7% agarose gel of DNA amplification with rDNA universal primers ID1/IDP1 (lane 1, 3, 5, 7, 9 and 11), Liberobacter 16s rDNA Specific Primers OII/OIIc/OAI (lanes 2, 4, 6, 8, 10 and 12) with water (lanes 1, 2) healthy plants (lanes 3, 4), Liberobacter asiaticum from Poona (lanes 5, 6), Liberobacter asiaticum from Batu (lanes 7, 8), Citrus Tristeza Virus (lanes 9, 10), Citrus Vein Enation Virus (lanes 11, 12) and 1 kb ladder.]

Amplification of CTV and CVEV were observed using universal primer for Procorvotica 16r DNA (lane 9 and 11), but no amplification emerged using specific primer (lane 10 and 12). Universal primer could be applied for amplification of Procorvotica 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 healthy plants, CTV and CVEV. Finally, the specific primer OII/OIIc/OAI 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 OII/OIIc/OAI from all samples collected from citrus mother trees in Lusus Foundation Block and Saka-sade 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


