Detection of the Asian and African Liberobacter species caused greening disease of citrus with PCR

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Abstract

Greening is a severe and wide spread disease of citrus in two main citrus growing areas of the world, Asia and Africa. Isolation of the greening pathogen has become more difficult since this organism has not been cultured in vitro, and the detection of the pathogen can be done only by electron microscopy. It had recently been characterized from the sequence of its 16S ribosomal DNA. The bacterium is a new species of Liberobacter, that has been recognized as Liberobacter asiaticum and Liberobacter africanum. This paper reports a PCR method to detect these two Liberobacter species in citrus trees by amplification of a 1160 bp fragment of their 16S rRNA. Distinction between the two species was achieved by Xba I restriction analysis on 6% agarose gels. The results showed that Liberobacter asiaticum bands appeared as 3 fragments: 520 bp, 506 bp and 130 bp while the Liberobacter africanum band appeared as 640 bp and 520 bp.

Keywords: Citrus greening - Liberobacter asiaticum - Liberobacter africanum - 16S rDNA - PCR detection.

Introduction

Citrus Vein Phloem Degeneration (CVPD) or greening has severe affected the Indonesian citrus industry for almost 30 years and is need to be anticipated in every effort to guard against on each development and of establishment as well as rehabilitation of citrus production in Indonesia. Recently, CVPD or greening has a new international name Huang Long Bin (HLB) (Garnier and Bove, 1993). The pathogen attacks and moves only in the sieve tube within the phloem tissues, causes phloem degeneration and disturbs plant physiological process. The disease affects all commercial citrus varieties except of Pamsilo (Dwiasutti et al., 1996).

This disease is also severe and wide spread on citrus orchards in China (Huang Lung Bin), South Africa (Greening), Taiwan (Likubin), India (Citrus Dieback), and Philippines (Leaf Motting). The distribution of the pathogen are mainly influenced by infected planting materials and insect vectors both the Asian citrus psylla Diaphorina citri Kuw and the African citrus psylla Triozos citri Delwercia. The caused of the disease is an unculturable phloem restricted bacterium that recently characterized as a Liberobacter asiaticum for Asian strain and Liberobacter africanum for African strain (Murray and Schleifer, 1994; Garnier and Bove, 1993).

The only difference between the African and Asian HLB is that the African strain is heat tolerant while Asian strain is heat sensitive.

Since the HLB organism has not been cultured, it was difficult to characterize the organism and to produce specific reagents for its detection. In 1987, a general procedure to produce monoclonal antibodies has been developed (M As) against phloem-restricted organism (Martins-Gres et al., 1987). The use of M As for the detection of greening organism by DAS-ELISA or immunofluorescence showed that they were highly strain specific and limited to only certain strain but none react with other strain. For instance, M As 10 A6 and 2 D12 (produced against the Poonka strain of the greening organism) reacted with several samples from several origins in Indonesia but none react with samples from other geographical origin (Dwiasutti et al., 1992). Therefore, not all of Indonesian greening strains have been detected by these M As in the field. Gao et al., (1993) reported that HLB from Bali, Indonesia are members of serogroup I and II on result of test of monoclonal antibodies recognizing most Asian strain of HLB organism.

Following the progress of research and technology development, Huang Lung Bin detection can be conducted by more accurate and sensitive method. Until 1993, HLB organism strain from Africa could be distinguished from the Asian strain on basis of temperature sensitivity (Bove et al., 1994); serology (Gao et al., 1993) and genomic properties (Villechaniaux et al., 1993). Jagoueix et al., (1994) stated that to determine the phylogenetic position of HLB organism and evolutionary distance between African and Asian Liberobacter PCR method can be used to amplify, clone and sequence the 16s ribosomal RNA (rDNA) of Asian strain Poonka and African strain Nelspruit Africa of the HLB pathogens. The DNA amplified in this way has the characteristic size of 1160 base pairs. For each cycle, the DNA denaturation step is carried out at 92°C for 40 seconds, and primer annealing step and the primer extension step are carried out simultaneously at 72°C for 90 seconds. This paper discussed the use of PCR for HLB detection and distinction between the Asian and African Liberobacter species.

Materials and Methods

Plant Materials. Several sweet orange (Citrus sinensis Osbeck) trees infected by different HLB Liberobacter strains from Asia and Africa with variation of symptoms were obtained as described previously by Garnier and Bove (1993). Several sweet orange trees showed symptom with different nutrient deficiency and infected by Sodioplora citri and MLO.

<table>
<thead>
<tr>
<th>Samples no.</th>
<th>Code of samples</th>
<th>Origin</th>
<th>Symptoms</th>
<th>Name of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>France</td>
<td>Manganese deficiency</td>
<td>Manganese deficiency</td>
</tr>
<tr>
<td>2</td>
<td>P16</td>
<td>South Africa</td>
<td>Leaf mottle</td>
<td>African greening</td>
</tr>
<tr>
<td>3</td>
<td>USA</td>
<td>South Africa</td>
<td>Leaf mottle, vein cracking</td>
<td>African greening</td>
</tr>
<tr>
<td>4</td>
<td>ML</td>
<td>Screen house</td>
<td>Zinc deficiency</td>
<td>Zinc deficiency</td>
</tr>
<tr>
<td>5</td>
<td>V-65 SS P4</td>
<td>Vietnam</td>
<td>Zinc-deficiency</td>
<td>Asian greening</td>
</tr>
<tr>
<td>6</td>
<td>P070-P30</td>
<td>Indonesia</td>
<td>Leaf curling</td>
<td>Asian greening</td>
</tr>
<tr>
<td>7</td>
<td>P070-P33</td>
<td>Philippines</td>
<td>Vein cracking</td>
<td>Asian greening</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>France</td>
<td>Deficiency</td>
<td>Withers Brown (MLO, phytoplasma)</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>France</td>
<td>Deficiency</td>
<td>Stubbon (Sodioplora citri)</td>
</tr>
<tr>
<td>10</td>
<td>CTH 40-P71</td>
<td>China</td>
<td>Leaf mottle, yellowing, nodrib</td>
<td>Asian greening</td>
</tr>
</tbody>
</table>
The samples were collected from plants maintained in a greenhouse at 30°C during the day and 25°C at night for Asian strain and 25°C during the day and 20°C at night for African strains. Healthy citrus plants were obtained from seeds grown at 25/20°C.

Preparation of Citrus Leaf Extraction for PCR. Leaf midribs (0.3 g) were chopped to a fine trace with a disposable razor blade in a plastic petri dish containing 1 ml of TE buffer (10 mM Tris pH 8.0, 0.5 mM EDTA, 1% SDS) to which 0.5 ml of proteinase K was added. The homogenate was transferred to an eppendorf tube and incubated for 2 hours, at 65°C. The suspension was centrifuged for 15 min at 12,000 g and the supernatant mixed with 1 ml of wizard mini prep DNA purification resin (Promega). The resin was transferred to a minicolumn and washed twice with 2 ml of 60% isopropanol. Then, 50 μl of a hot water (80°C) were added, and after 1 min, the column was centrifuged for 10 second minimum at 14000 g in an eppendorf tube. This step was repeated, yielding 100 μl of extract (wizard extract). Eight μl of wizard extract were used for PCR.

Polymerase Chain Reaction (PCR). Specific primers OIC and OIA, defined from the 16S r DNA sequence of Liberibacter asiaticum (strain Poona, India) and primer OIA1 defined from the 16S r DNA sequence of Liberibacter africacum (strain Nelspruit, South Africa) were used in this observation and were evaluated for specific amplification of Liberibacter 16S r DNA. The PCR was performed in 50 μl of reaction mixture containing 0.5 μM of each primer, 200 μM of each of four d NTP (dATP, dTTP, dCTP, dGTP), 78 mM Tris, pH 8.8, 2 mM MgCl₂, 17 mM (NH₄)₂SO₄, 10 μM β-mercaptoethanol, 0.05 % W1 detergent (Gibco BRL), 200 μM BSA, and 2.5 U Taq polymerase (Gibco BRL). A thermocycler of PCR machine (Perkin Elmer Cetus) with the following programs was used for DNA amplification: 35 cycles each at 94°C 40 second (denaturation step) and 72°C for 90 second (primer annealing and elongation). Following amplification, 8 μl aliquots (PCR product) of each reaction mixture and 2 μl of Bromophenol blue (BPP) were analysed by electrophoresis on 0.7% agarose gel and stain in 10 μg/ml ethidium bromide.

Restriction Enzyme Analysis Characterization of Amplified DNA. The aim of this research was to determine the distinction between two species liberibacter: (L. asiaticum and L. africanum). The selected plant materials from previous observation used were: no. (2) greenling from South Africa (P16), no. (3) USA 8 greening from South Africa, no. (5) V85-S5FA Asian greenling from Vietnam, no. (6) PO70-P30 Asian greenling from India, no. (7) PH70-P33 Asian greenling from Philippines and no. (10) CH88-P1 Asian greenling from China. Eight (8) μl of amplified DNA (PCR product) was digested with 20 U of Xba restriction enzyme (Euorgenie) in a final volume of 35 μl 8 μl PCR product, 4 μl TE 10x, 4 μl BSA 10x, 1.5 μl XbaI and 20.5 μl H₂O, centrifuged for short time on 14000 g and incubated over night at 37°C. The next, the DNA was precipitated with sodium acetate (pH 4.8) and 95% alcohol, and then kept over night at -80°C. The digested DNA was analysed by electrophoresis on 4% agarose gels.

Results and Discussion

Figure 1 showed that PCR with the three different mixture of primers were able to distinct all samples (6) infected by Asian and African greening from several countries and no amplification was obtained from all samples with nutrient deficiency symptoms.

Spirillum citri and MLO infected sweet orange plants.

The previous observation showed that primers OIC, OI1 and OIA1 were able to amplify a 1160 bp fragment of greening liberibacter 16S r DNA from purified periwinkle DNA in which African or Asian strain was present in the plant (Jaquiqux et al., 1994; Jaquiqux et al., 1996). Amplification of 16 S rDNA of chloroplast of healthy of nutrient deficiency plants, showed that Spirillum citri and MLO could be detected only by using universal primer which amplify the 1500 bp fragment, but no detection could be obtained using specific primers which amplify the 1160 bp fragment (Jaquiqux et al., 1996).

Conclusion

1. The amplified DNA obtained by using primer OIC/OI1/OIA1 was able to identify all samples infected by liberibacter from different strains: whether Liberibacter asiaticum or Liberibacter africanum.
2. Amplifying the 16S rDNA of chloroplast on healthy and nutrient deficient plant, no Spiroplasma citri or MLO could be detected by specific primer.

3. Distinction between two species of greening has been achieved by X hel restriction enzyme on 4% agarose gel digestion of the amplics. *Librobacter asiaticum* bands appeared as 3 fragments those 520 bp, 506 bp and 130 bp while *Librobacter asiaticum* band appeared as 460 bp and 520 bp fragments.

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

**References**

