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Detection of *Liberibacter asiaticus* causing *Citrus Vein Phloem Degeneration*from Siam Citrus leaves (*Citrus nobilis* var. *microcarpa*) in Singkawang City plantation, Pontianak, West Kalimantan

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The objective of the-present study was to detect the presence of pathogenic fastidious bacterium, *Candidatus* Liberibacter asiaticus using PCR from leaves of Siam citrus showing Citrus Vein Phloem Degeneration (CVPD) symptoms in Singkawang City plantation, Pontianak, West Kalimantan, Indonesia. Citrus leaf samples were collected based on visual observation of symptoms showing CVPD infection. Typical symptoms of CVPD include leaf yellowing (*chlorosis*), vein banding, leaves become stiff, thicker and smaller in size. The pathogenic bacterium, *Candidatus* Liberibacter asiaticus was detected using two specific primers, OI1/ OI2c amplified 16S rRNA gene and A2/J5, amplified ribosomal protein gene of the rpl*KAJL*-rpo*BC*operon (*β*-operon). PCR amplification detected the presence of 1100 bp band using OI1/ OI2c primers, and 703 bp band using A2/J5 primers from symptomatic Siam citrus leaves. PCR products were not detected from healthy plants serve as a control. By using two sets of specific primers to amplify 16S rRNA gene and ribosomal protein gene**,** *Candidatus* Liberibacter asiaticus was detected in symptomatic Siam Citrus leaves in Singkawang City, Pontianak, Indonesia. Detection of the bacterial pathogen causing CVPD is important to prevent the spreading of the disease which could affect the production of citrus fruits.

Key words: *Candidatus* Liberibacter asiaticus, CVPD, PCR detection, Siam Citrus, Singkawang City Pontianak

Tujuan dari penelitian ini adalah untuk mendeteksi keberadaan bakteri patogen, *Candidatus* Liberibacter asiaticus menggunakan PCR dari daun Jeruk Siam yang menunjukkan gejala penyakit *Citrus Vein Phloem Degeneration* (CVPD) di perkebunan Kota Singkawang, Pontianak, Kalimantan Barat, Indonesia. Sampel daun jeruk diambil berdasarkan pengamatan secara visual terhadap daun yang menunjukkan gejala infeksi CVPD. Gejala khas CVPD memperlihatkan daun menguning (klorosis), tulang daun hijau tua (vein banding), daun menjadi kaku, lebih tebal dan lebih kecil ukurannya. Bakteri patogen, *Candidatus* Liberibacter asiaticus dapat terdeteksi menggunakan dua pasang primer spesifik, yaitu, OI1/ OI2c gen 16S rRNAdan A2/J5, gen protein ribosom dari operon rpl*KAJL*-rpo*BC* (*β*-operon). Amplifikasi PCR mampu mendeteksi keberadaan pita DNA sebesar 1100 bp menggunakan primer OI1/OI2c dan pita DNA sebesar 703 bp menggunakan primer A2/J5 dari daun Jeruk Siam yang bergejala CVPD. Pita DNA tidak terdeteksi dari daun tanaman sehat yang berfungsi sebagai kontrol. Penggunaan dua pasang primer spesifik gen 16S rRNA dan gen protein ribosom, *Candidatus* Liberibacter asiaticus terdeteksi pada daun Jeruk Siam yang bergejala di perkebuanan Kota Singkawang, Pontianak, Indonesia. Deteksi bakteri patogen penyebab penyakit CVPD merupakan informasi penting untuk mencegah penyebaran penyakit yang dapat mempengaruhi produksi buah jeruk.

Kata kunci:*Candidatus* Liberibacter asiaticus, CVPD, deteksi PCR, Jeruk Siam, Kota Singkawang Pontianak

Citrus is one of the fruit commodities in Indonesia that is very popular with the community, both as fresh fruit and in processed form. Siam Citrus *Citrus nobilis* var. *microcarpa*) has a high economic value for citrus farmers in Indonesia. In West Kalimantan, the main Siam citrus plantation is located in Singkawang City with a cultivation area of 5776 $m^{2.}$.

Cultivation of Siam citrus is susceptible to various types of diseases including Citrus Vein Phloem Degeneration (CVPD) or citrus greening which is also known as huanglongbing disease. The disease is caused by a gram-negative bacterium, *Candidatus* Liberibacter of which three species are known as causal pathogens of CVPD, namely *Ca.* Liberacter asiaticus, *Ca.* Liberacter africanus and *Ca. Liberacter* americanus. After they are famous in continent (Bove, 2006). The bacteria are spread by psyllids, *Diaphorina citri* in Asia, Brazil and Florida, and *Triozaerytrea*in Africa. In addition to insect vector, CVPD transmission

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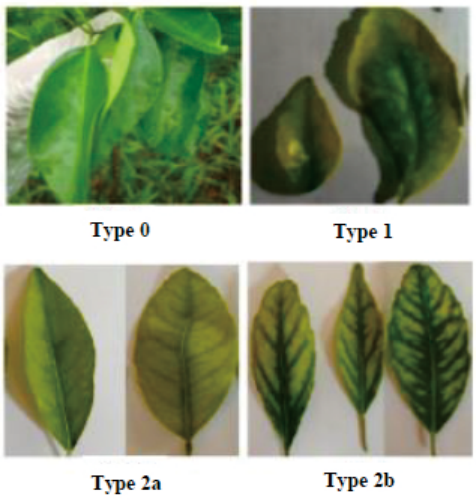

Fig 1 Symptoms of CVPD on Siam citrus leaves in the field. Type 0: healthy leaves. Type 1: partial *chlorosis* at the leaf margins. Type 2a: *chlorosis* between the leaf bones showing vein banding, and the leaves become stiff (mild CVPD symptoms). Type 2b: *chlorosis* between the leaf bones, showing vein banding, leaves are thicker and stiffer (severe CVPD symptoms).

usually occurs through plant seeds that come from propagation by grafting infected buds (Hung *et al.* 2000),

Candidatus Liberibacter asiaticus infected citrus plant by penetrating phloem tissues and vascular systems causing clogging and reducing water transportation (Teixeira *et al*. 2005). Typical symptoms of CVPD disease in citrus leaves those infected with the pathogen *Liberibacter* will show, chlorosis symptoms or yellowish patches (blotching, mottle) irregularities on the leaves and leaf bones (Zubaidah, 2010). However, these visual observation are often misleading as in some cases the symptoms may be related to other biotic and abiotic factors (Lin *et al*. 2010) which showed similar symptoms with CVPD. Among the biotic and abiotic factors that resemble CVPD symptoms including zinc deficiencies (Timmer *et al.,* 2003), stem pitting caused by *Citrus tristeza* virus, Phytophthora root rotand citrus blight (Beattie and Barkley, 2009). According to Pereira *et al.* (2011), diagnostic errors based on visual observation can be higher than 30%. Therefore, visual inspection of the symptoms can lead to misidentification of the correct pathogens.

Due to these the limitations, detection of CVPD pathogen is commonly based on PCR due to its reliability and simplicity. Two specific primers are often used to detect the presence of CVPD pathogens in plant tissues, namely primers OI1/OI2c which was based on 16S rDNA with a DNA target of 1160 bp (Wirawan *et al.*, 2018) and primers A2 / J5 primers, based on ribosomal protein genes (rplA/rplJ genes) of the β-operon sequences (Hocquellet *et al*. 1999). Primers A2 / J5 were used to distinguish between *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter africanus by the size of the PCR products obtained (Hocquellet *et al*. 1999).

In Indonesia, CVPD infection on citrus has increased in citrus producing regions. The disease incidence has increased to 62.34% in East Java, 60% in North Bali, and 70% in Southeast Sulawesi (Nurhadi, 2015). Typical symptoms of CVPD were also observed in Siam citrus plantation in Setapok Village, Singkawang City, Pontianak, West Kalimantan. Thus, the aim of the present study was to determine the pathogen causing symptoms of CVPD in the plantation.

MATERIALS AND METHODS

Sampling of Citrus Leaves. Symptomatic Siam citrus leaves (*Citrus nobilis* var. *microcarpa*) showing CVPD symptoms were collected randomly from a citrus plantation in Setapok Village, Singkawang District, Singkawang City, West Kalimantan, Indonesia. Mild and severe symptoms of CVPD were observed in the plantation, which includes leaf yellowing (chlorosis), vein banding, leaves become stiff, thicker and smaller in size (Fig 1).

The symptomatic leaves were put in plastic bags and stored in a cooler box. The samples were then brought to Genomics and Plant Quality Improvement Laboratory,LIPI Biotechnology Research Center, Cibinong, Bogorand stored in a freezer (-80°C) until

used.

DNA Extraction. For DNA extraction, the citrus leaves were washed with 70% alcohol for 1 min and then washed using three changes of sterile distilled water. The sterile filter paper was used to dry the leaves and cut into small pieces of about 0.5 cm. The small pieces of the leaves were put 1.5 ml micro centrifuge tube, containing liquid LB medium (*Luria Bertani*) and incubated in an oven at 37 ^{O}C for 2-3 days. LB medium is used for the growth of pure bacterial cultures to be isolated.

The next step was isolation of the bacterial plasmid DNA from the leaves in the LB medium. The broth was centrifuged at 12000 rpm for 2 min, the supernatant was removed and 100 µl of solution 1 (1 M Tris-Cl pH 8, 0.5 M EDTA, 0.5 M glucose) was added and incubated in ice for 15 min. After incubation, 200 ml of solution II (10 M NaOH, 20% SDS) was added and incubated in ice for 15 min of which 150 µl CH3COONA solution was added, and incubated again in ice for 15 min. The mixture was centrifuged at 10000 rpm for 5 min and 1 ml of 96% ethanol was added, and centrifugation was repeated in the same condition. After centrifuged, the pellet was washed with 70% ethanol and dried. The pellet was then dissolved in 20 µl Tris EDTA buffer or distilled water and stored at -20^{0}C until used.

The concentration and purity of the plasmid DNA were measured using *Nanophottometer,* at a wavelength of 260 nm, and calculated based on the ratio A280/A260 and A260/A230 ng/µl. DNA purity limits, theorecally the purity of DNA was analysis by the ration 1.8 – 2.0 (Sambrook *et al*. 1989).

Amplification of 16S rDNA Gene and Ribosomal Protein Gene. For PCR amplification, extract from three leaves samples were used. PCR reaction for both genes was performed using *DreamTag* PCR master mix solution (Thermo Fisher Scientific). The PCR reaction was prepared in 12.5 µl reaction containing 1 µl DNA sample, 1 µl forward primer and 1 µl reverse primer, 6.25 µl PCR master mix solution and 3.25 µl Water DNase, RNase-free.

The specific primers used for amplification of 16S rRNA gene were OI1/OI2c primers with atarget PCR product of approximately 1100 bp for *Candidatus* Liberibacter asiaticus (Bove 2006). Another specific primers, A2/J5 primers were based on ribosomal protein gene of the rpl*KAJL*-rpo*BC*operon (*β*-operon) and the expected PCR product was 703 bp for *Candidatus* Liberibacter asiaticus. *Candidatus* Liberibacter africanus produces a band of 669 bp using A2/J5 primers (Hocquellet *et al.* 1999).

PCR was performed in Biometra thermal cycler and PCR conditions used were as follows: initial denaturation at 95^{0}C for 5 min; 40 cycles of denaturation at 94^{0}C for 1 min, annealing at 55^{0}C (primers) and -60^{0}C (primers) for 30 s, extension at 72^{0}C for 1 min; and the final extension was carried out at 72^{0}C for 5 min.

PCR products were analyzed using 0.8% agarose gel electrophoresis, carried out for 60 min, 100V and 400 mA. The marker used was 100 bp DNA ladder (*lamda HindIII*). After electrophoresis, the agarose gel was immersed in ethidium bromide for 15 min, rinses with running water and visualized using UV trans illuminator (UVITEC).

RESULTS

The DNA extraction method used in this study was a modification of the plasmid DNA isolation method based on the principle of alkaline lysis solution. The principle of this method is almost the same as the DNA extraction using Cetyl Trimethyl Ammonium Bromide (CTAB) of which the DNA of the bacteria was extracted from the leave samples. DNA extraction with CTAB is a common method for isolating pathogenic bacteria as members of *Liberibacter.* Research that has been done, successfully detected the presence of the pathogenic bacteria *Candidatus* Liberibacter asiaticus, using two specific primers. The positive reaction of primers OI1/OI2c produced a DNA band of 1100 bp, which was shown in the leaf type 1 sample (Fig 2). Whereas the positive reaction of primers A2/J5 produced DNA bands of703 bp, which was shown in leaf type 2b samples (Fig 3).

DISCUSSION

Plasmid DNA isolation method based on the principle of alkaline lysis was applied in this study because experiments with the CTAB method have been carried out, however it was not successful in extracting bacterial DNA from symptomatic citrus leaf extract. In contrast, CTAB method was used by Ruangwon and Akarapisan (2006) and Taufik *et al.* (2010) to extract DNA of *Candidatus* Liberibacter asiaticus from symptomtic citrus leaves of which PCR amplification was successfully amplified the 16S rRNA gene.

PCR amplification using OI1/OI2c primers produced 1100 bp band from leaves extract showing type 1 symptoms (partial yellowing on the leaf vein).

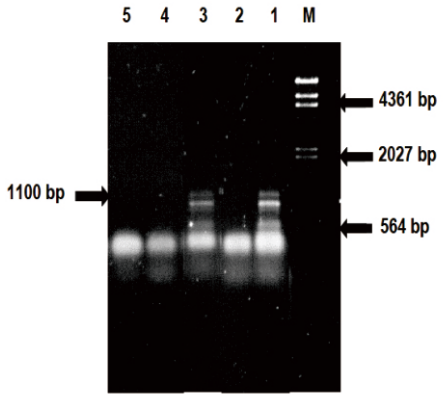

Fig 2 Amplification products using OI1/ OI2c primers. Lane 1; type 1 symptoms leaves : partial chlorosis (positive sample *Ca.* Liberacter asiaticus); Lane 2: water control (negative control *Ca,* Liberibacter asiaticus); Lane 3: Type 1 symptoms : partial chlorosis (positive sample *Ca.* Liberacter asiaticus); Lane 4: type 2a symptoms : mild CVPD symptom (negative sample *Ca,* Liberibacter asiaticus); Lane 5: type 2b symptoms : severe CVPD symptom (negative sample *Ca,* Liberibacter asiaticus). M: 100 bp marker.

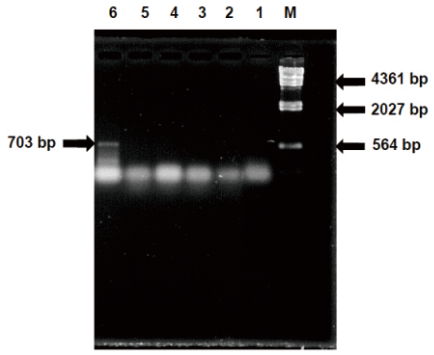

Fig 3 PCR amplification using primers A2/ J5. Lane 1 : water control (negative control *Ca,* Liberibacter asiaticus); Lane 2 : healthy leaf : type 0 (negative control *Ca,* Liberibacter asiaticus); Lanes 3 and 4: leaves extract from type 1 symptoms : partial chlorosis (negative sample *Ca,* Liberibacter asiaticus); Lane 5 : leaves extract from type 2a symptoms : mild CVPD symptoms (negative sample *Ca,* Liberibacter asiaticus); Lane 6 : leaves extract from type 2b symptoms : severe CVPD symptoms (positive sample *Ca.* Liberacter asiaticus). M: 100 bp marker.

PCR amplification using A2/J5 primers produced 703 bp band (Figure 3) which was only detected from leaves extract from Type 2b leaves (severe CVPD symptoms). The results of the PCR amplification from the type 1 and type 2b leaves extract indicated the presence of *Ca.* Liberibacter asiaticus in the symptomatic Siam citrus leaves showing symptoms of CVPD in Setapok village, Singkawang city, Pontianak, West Kalimantan.

The size of the PCR products obtained was in accordance to the size of the PCR products stated by Bove (2006) for OI1/OI2c primers and Hocquellet *et al*. (1999) for A2/J5 primers for detection of *Ca.* Liberibacter asiaticus. Amplification using specific primers OI1/OI2c only showed positive DNA band reactions in leaf type 1, and result in negative DNA bands from samples of type 2b (mild CVPD symptoms) and leaf type 2b leaves (severe CVPD symptoms). According to Wirawan *et al*. (2004), although leaf samples showing chlorosis indicating CVPD symptoms, PCR amplification did not produce any band which may be due to low concentration or uneven distribution of the bacteria in the leaves tissues, which may affect the DNA concentration from the leaves extract. Huang (1979), also indicated that due to low concentration of the bacteria in citrus host, the detection of the pathogen is difficult. Based on the results of measurements of DNA purity in this study 1,800 ng/µl (leaf type 1), 1,724 ng/µl (leaf type 2a) and 1,865 ng/µl (leaf type 2b). Other possible explanations could *Ca.* Liberibacte rasiaticus but caused by abiotic and biotic factors such as nutrient deficiencies and infection be the symptoms shown on the leaves were not related to by other microbes of which the symptoms are similar with CVPD causal pathogen. Wirawan *et al.* (2004) never

found two typical protein molecules in plants attacked by CVPD, namely virulent protein (toxin) from the pathogenic bacteria *Ca.* Liberibacter asiaticus and citrus plant receptor proteins, these two protein molecules interact with each other and influence ion transport into the plant cell so that plants lack mineral elements of Zn, Mn, and Ca. The reaction of the two proteins is what is suspected, be the cause of plant leaves experiencing chlorosis.

The symptoms of CVPD observed in Singkawang City plantation were similar with CVPD symptoms reported by Wijaya (2003) in East Java citrus plantation, Karangasem Regency. Similar CVPD symptoms were also reported by Ardiartayasa *et al.* (2006) in eight villages, Katung, Belancan, Bayung Gede, Cloud, Chess, Pengotan, Evening and Pelaga villages in Denpasar, Bali, Indonesia, with citrus leaves showing mild to severe chlorosis. by Taufik *et al.* (2010) also reported the same chlorosis symptoms and vein banding in the citrus plantation in Konawe Selatan Regency, Southeast Sulawesi, Indonesia.

Symptoms of chlorosis and vein banding observed in the field were in accordance with the symptoms described by Wijaya (2003) of which the chlorosis in CVPD citrus leaf become irregular due to reduced formation of chlorophyll in the leave and according to Nurhayati *et al.* (2016), to diagnose CVPD in citrus plants chlorophyll content which can be the basis of reference, namely around 47.06 SPAD. Thus, the leaves become stiff but the leaf vein remains dark green. According to Susanti *et al.* (2014), infection by *Ca.* Liberibacter asiaticus causing chlorosis might also indicate physiological disorders as masses of bacterial cells can inhibit the transportation of nutrients to and from the phloem leading to degeneration of phloem cells. These conditions affected the transportation of nutrients to other parts of the citrus plant. Hence the reduced chlorophyll formation in leaves which experience chlorosis results in a decrease in photosynthetic activity in plants. Wirawan *et al.* (2018), also stated that lorosis occurs through transmission of vector insect stillets in plant tissue when pathogens suck in plant fluids and are in the phloem tissue then scattered to the parts of the plant along with the translocation of organic matter, too many pathogens result in chlorosis and phloem leaf necrosis.

Both specific primers, OI1/ OI2c and A2/J5 have been applied in several studies related to CVPD in Indonesia. For instance, studies by Taufik *et al.* (2010) and Meitayani *et al*. (2014) using OI1/ OI2c detected *Candidatus* Liberobacter asiaticum from infected citrus in Sulawesi Tenggara and Karangasem Regency. The PCR products obtained were 1100 bp band indicated the presence of *Candidatus* Liberobacter asiaticum.

By using A2/J5 primers, 703 bp band of *Candidatus* Liberobacter asiaticum was also reported by Hocquellet *et al*. (1999) from diseased citrus from various citrus orchards in Bali, Indonesia; South Africa and Mauritius. In a study by Ruangwong and Akarapisan (2006), using A2/J5 primers produced 703 bp band of *Candidatus* Liberobacter asiaticum amplified from diseased citrus plants in Chiang Mai, Chiang Rai and Phrae provinces. In the present study, by using A2/J5 primers, PCR products wereonly detected from plant extract with severe symptoms of CVPD (type 2b leaves sample). Whereas in leaf type 1 and leaf type 2a samples, did not show any DNA bands. This is thought to be due to the smaller primary target, in accordance with the statement of Hocquellet *et al,* 1999 that the target DNA using primers A2 and J5 are smaller than the primary targets designed by other 16S rRNA primers, so that DNA degradation during amplification and electrophoresis can be reduced.

Early detection of CVPD especially in a young citrus plants is important to prevent spreading of the disease. Thus, a suitable DNA extraction method is important to obtain good quality DNA for PCR amplications.

As conclusion, PCR amplification using two specific primers OI1/ OI2c and A2/J5 were able to amplify bacterial DNAfrom symptomatic leaves extract of Siam Citrus collected from Setapok Village, Singkawang City, west Kalimantan, Pontianak, Indonesia. Therefore, the bacteria isolated from Siam citrus leaves showing CVPD symptoms were *Candidatus* Liberibacter asiaticus.

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