Molecular Detection of Citrus Vein Phloem Degeneration

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ABSTRACT

This research was conducted in order to develop pathogen detection tool using specific monoclonal antibodies and DNA-probes for CVPD or Indonesian citrus greening. Precipitated protein from the sap of CVPD infected citrus leaf midribs was used to immunize 8 weeks Balb/c mice. Each mouse was injected intraperitonially with 200 μl PBS containing 10 μg protein in the present of adjuvant with interval of two weeks and booster injection was given (intra vena) three times everyday after the last injection of the regular immunization. The immune lymphocytes were then fused with NS-1 myeloma cells in the present of PEG 4,000 as fusogen. The hybridomas that producing antibodies were selected against the sap of healthy and CVPD infected citrus leaf midribs by ELISA. The hybridomas producing antibodies reactive to CVPD but not reactive to healthy plants were further cloned and propagated. Total DNA were extracted from CVPD infected citrus leaf midribs using the modification method of SDS-Proteinase K- Lisozyme. The DNA was used as the template for PCR of 16S-rRNA gene using universal primers, the part of the gene using specific oligonucleotides of *Liberobacter asiaticum* and *L. africanum*, Asian and African greening bacterium. There were 6 clones of hybridoma producing specific monoclonal antibodies against CVPD *in vitro*, however only clon DW-0310 that showed consistenly reactive to CVPD. This results indicated that DW 0310 is highly sensitive to CVPD samples originated from many *cultivars* of citrus, *cv.* Siem, Keprok, and Manis from different places (Yogyakarta, Magelang, Purworejo, and Bali) and not reactive to any other plant diseases. The universal primers amplified of 1,500 bp DNA fragment that was cut into about 1,300 and 200 bp by Eco RI, and into 1,250 and 250 bp by Bcl I. Using the specific oligonucleotides of *L. asiaticum* in the fragment of 1,500 bp 16S-rRNA gene, 400 bp fragment was found by amplification, on the other hand, there was no amplification using the specific oligonucleotides of *L. africanum*.

Keywords: CVPD, ELISA, monoclonal antibodies, DNA-probes.

ABSTRAK

Penelitian ini dilakukan dalam rangka pengembangan alat deteksi patogen menggunakan monoklonal antibodi spesifik dan probe DNA untuk CVPD. Protein pada lapisan CVPD yang menginfeksi tulang daun jeruk digunakan untuk mengimunisasi tikus umur 8 minggu. Setiap tikus disuntik/diinjeksi secara intraperitonial dengan 200 μl PBS yang mengandung 10 μg protein adjuvant dengan interval dua minggu. Injeksi lewat vena/pembuluh balik diberikan tiga kali sehari setelah penyuntikan terakhir dari imunisasi reguler. Limposit yang telah terimunisasi lalu difusi dengan NS-1 sel-sel myeloma pada penggunaan PEG 4.000 sebagai fusagen. Hybridoma yang menghasilkan antibodi dipilih untuk menyerang lapisan/bagian daun jeruk yang sehat dan yang terserang CVPD dengan metode ELISA. Hibridoma yang menghasilkan antibodi yang reaktif terhadap CVPD tetapi tidak reaktif terhadap tanaman sehat, selanjutnya diklon dan diperbanyak. Total DNA diekstrak dari bagian daun jeruk yang terinfeksi CVPD menggunakan modifikasi metode dari SDS-Proteinase K-Lisosim. DNA digunakan sebagai templat

untuk PCR dari gen 16S-rRNA menggunakan primer universal, bagian dari gen menggunakan oligonukleotida spesifik dari *Liberobacter asiaticum* dan *L. africanum*, bakteri hijau Asia dan Afrika. Terdapat enam klon hibridoma yang menghasilkan CVPD secara *in vitro*, tetapi hanya klon DW0310 yang memperlihatkan secara konsisten reaktif terhadap CVPD. Hasil ini menunjukkan bahwa klon DW0310 sangat sensitif terhadap sampel CVPD yang berasal dari beberapa kultivar jeruk, seperti Siem, Keprok, dan Manis yang berasal dari tempat berbeda (Yogyakarta, Magelang, Purworejo, dan Bali) dan tidak reaktif terhadap penyakit tanaman yang lain. Primer universal mengamplifikasi 1.500 bp fragment DNA yang dipotong menjadi 1300 dan 200 bp dengan Bcl 1. Penggunaan oligonukleotida spesifik dari *L. asiaticum* di dalam fragmen dari 1.500 bp gen 16S-rRNA, fragmen 400 bp didapatkan melalui amplifikasi, di lain pihak tidak melalui amplifikasi oligonukleotida spesifik dari *F. africanum*.

Key words: CVPD, ELISA, monoklonal antibodi, DNA-probe.

INTRODUCTION

Citrus Vein Phloem Degeneration (CVPD) is very destructive disease causes of declining production and killed million citrus trees in Indonesia. The disease was reported as citrus greening caused by unculturable or fastidious gram negative phloem inhabitant bacterium *Liberobacter asiaticum* that was more severe and tolerant to high temperature (Tirtawidjaya, 1980; Garnier *et al.* 1984; Jagaoueix *et al.*, 1994). The disease is spread by vegetative propagation and the insect vector *Diaphorina citri.*

The symptom of CVPD especially on the leaves is chlorosis with dark green around the midribs that is similar to the symptom caused by Zn deficiency, and the lamina is narrower or the leaves are smaller but stiffer than those of healthy plant. The inside symptom is degeneration on the phloem tissue with thicker cell wall and flat sieve (Tirtawidjaja, 1980). The disease develop slowly on individually citrus trees before kill the host plant completely, however, the inoculum is spread quite fast from trees to other trees or orchard by the insect vector. The infected trees with mild symptom that used for source of entres in vegetative propagation will spread the disease to other localities when they were distributed. This problem has been caused continous citrus damage around the islands since early 1970s. The control program needs pathogen detection tools for healthy seedling certification.

Garnier *et al.* (1987) reported the development of some different clones of hybridoma producing monoclonal antibodies that could recognize different isolates of greening bacterium. The monoclonal antibodies were induced by infected phloem of periwinkle that was inoculated with greening pathogen by dodder transmission. Furthermore, the specific sequence in 16S-rRNA gene of *L. asiaticum* and *L. africanum* and also the specific primers of rplKAJL-rpo BC Operon gene of the bacteria were reported (Jagoueix *et al.*, 1994; Planet *et al.*, 1995).

This research resulting on the hybridoma DW 0310 producing monoclonal antibody that was consistenly reactive to CVPD until 30000 fold dilution. The antibody

of clon DW 0310 at 20,000 fold dilution could detect the pathogen on several citrus samples with different citrus cultivars and localities. The candidates of DNA probes for detection of CVPD pathogen were found however further study has to be done in this research.

MATERIALS AND METHODS

Antigen Preparation

The antigen for immunizing mice was prepared by extracting the sap of CVPD infected leaf midribs. The infected leaves of citrus cv. Siem originated from Sleman, Yogyakarta were washed thoroughly using detergent and running water before disinfected by immersing in 70% ethanol for 2 - 3 minutes. The midribs were cut off aseptically and choped finely then grinded completely in a sterile porselin mortar with the addition of PBS 0.01M pH 7.2. The extract was centrifuged at 800 g for 5 minutes at 4°C to separate the rough plant materials. The supernatan with diluted total protein was collected. The total protein was precipitated using saturated ammonium sulphate and dialysed against 10 mM PBS (De-Boer and Schaad, 1990).

Immunization

Balb/c mice was used in the experiments. Eight weeks mouse was immunized three times with the interval of two weeks and booster injection four days before lifting up the lymphocytes organ. The amount of 10 μg precipitated protein in 200 μl solution with the addition of Freund's adjuvant was used for single injection of mice. No adjuvant was added in the protein solution for booster injection.

Cell Fusion, Cloning, Screening, and Hybridoma Propagation

The complete immunized mice were killed to harvest the immune lymphocytes after obtaining the blood from plexus retro-orbitalis about 600 μl. The blood was sedimented by centrifugation to get the polyclonal antiserum. The lymphocyte organ was lifted up aseptically and immune cells were collected and rinsed with RPMI 1640 medium. The confluent culture of myeloma NS-1 was prepared and rinsed with RPMI 1640 medium. The immune lymphocytes and the myeloma cells at the ratio of 10:1 were fussed in the condition of 45% polyethylene glycol 4,000. After a serial washing, the mixture was rinsed with RPMI 1640 medium and resuspended in about 10 ml selective medium to get 10^4 cells per ml suspension. The suspension was dispensed into micro cultural plates with 200 μl per well. The cultures were incubated in the incubator with 5% CO_2 and 37°C for 9-10 days before screening the supernatan antibody (Kohler and Milstein, 1975). The hybridoma cultures in each well was screened for the production of antibody againts the antigent of healthy and CVPD

infected citrus samples using I-ELISA (Koenig, 1981). The cultures that showing the ratio of absorbance (A 405 nm) of CVPD to healthy sample with the result of 2.00 or more were collected for further cloning and screening. The hybridoma clons that produced monoclonal antibodies reactive to CVPD but was not reactive to healthy samples were collected for *in vitro* propagation in 50 ml cultural flash and *in vivo* propagation.

Seeking for Antibody Titer Suitable for Assay Againts the Antigen

The supernatan of hybridoma was used directly without dilution for I-ELISA againts the antigen of healthy and CVPD infected samples. The antibody produced by each hybridoma clon was serial diluted started from 5,000 to 3,000 x then used for I-ELISA againts the both antigens. The antibody that was consistenly reactive to CVPD by the serial dilution was collected. After checkerboard test the antibody with the right titer was used for I-ELISA by several samples of citrus with different cultivars and localities.

DNA Extraction

Total DNA CVPD infected citrus was extracted from the leaf midrids. The DNA extracted using the modification method of Draper and Scott, 1988. Perfectly washed disinfected CVPD infected citrus leaf midribs were choped finely and placed in a sterile porselin mortar then incubated at -70°C for at least 30 minutes. The sample was then added with liquid nitrogen and granded immediatly to become powder. The sample powder was added with extraction buffer (2 M Tris-HCl pH 8.5, of 5 M NaCl, 0.5 M EDTA pH 8.0, 25% SDS), each 0.75-g sample was added with 750 µl extraction buffer. The mixture was centrifuged at 800 rpm for 5 minutes to separate the rough plant materials. The supernatan was transfered into clean tube and added with lisozyme to get the final concentration of 1 mg/ml then incubated at room temperature for one hour. The mixture was added with proteinase-K to get the final concentration of 0.1 mg/ml and further incubated for one hour at 37°C. The mixture was added with 700 µl phenol solution and incubated for 30 minutes at 37°C then centrifuged at 1,200 g for 10 minutes at 4°C. The supernatan was transfered into clean tube and added with 700 µl chloroform isoamyl alcohol and further centrifuged. The supernatan was transfered into clean tube and added with 700 µl isopropanol followed by centrifugation at 1,200 g for 10 minutes at 4°C. The pellet was rinsed with 70% ethanol then resuspended with 200 µl sterile aquabidest. The suspension was added with 1 µl RNA-ses then incubated for one hour at 37°C. Reextraction of phenol and chloroform was conducted to get clean DNA solution. The DNA was precipitated with the addition of 30 µl 3 M sodium acetate and twice volume of cold absolute ethanol. The mixture was incubated at -70°C for at least 30 minutes then centrifuged to get the DNA pellet. The pellet was rinsed with 70% ethanol then resuspended with 200 µl sterile aquabidest and stored for further preparation.

DNA Amplification

The universal primers for amplifying 16S-rRNA gene were used with the sequence of AGA GTT TGA TCA TGG CTC AG as the foward primer (1157) and AAG GAG GTG ATC CAG GCG C as the reverse primer (1158). The specific nucleotides of *L. asiaticum* and *L. africanum* in the gene of 16S-rRNA GCG CGT ATG CAA TAC GAG CGG (4048) and ATG GGT TGC GAA GTC GCG AGG C (4051) (Jagoueix *et al.*, 1994) were used as foward primers for another amplification and the reverse primer was the universal primer (1158)

The reaction volume was 50 µl consist of 2 µl of DNA template, 5 µl each of PCR buffer and DMSO, 2 µl of each of the primers, 2.5 µg Taq polymerase and bring to the total volume of 50 µl using sterile aquabidest. The amplification was procceded with the program of 5 minutes at 92°C of pretreatment, 1 minute at 92°C of denaturation, 30 minutes at 47°C of *annealing*, 1.5 minutes at 72°C of elongation, running for 35 cyclus and 10 minutes of extra extention at 72°C. The PCR was carried out on Coy TempCycler. The PCR products were visualized on 1% agarose. The amplified DNA fragments were purified using Wizard's PCR product purification system (Promega) and the enzyme of Eco RI and Bcl I were used to cut the amplification products.

RESULTS AND DISCUSSION

The cell fusion resulted on the number of about 250 clones of hybridoma, however after the last cloning and screening there were only eight clones produced mAbs that reactive to CVPD but negative to healthy samples. The clones were *in vitro* propagated in 50 ml cultural flash and the supernatan was rechek for I-ELISA against CVPD and healthy samples, the results are shown in Table 1. The eight clones of hybridoma were stored in liquid nitrogen before they were further propagated.

There were only 6 clones of hybridoma effective to produce monoclonal antibodies, whereas the other two clones were fail. Furthermore, from the 6 clones of harvested antibodies, only clon DW 0310 was consistenly reactive to CVPD until the dilution of 30,000 fold shown in Table 2.

The results suggested that hybridoma clon DW 0310 was the most stable in producing mAb reactive to CVPD. The other clones probably lost their chromosom responsible for producing mAbs against CVPD during the development, however these clones may propagated in a bioreactor with controled environment.

The results on the I-ELISA using the antibody of DW 0310 at the dilution of 20,000 fold against several citrus originated from different localities suggested that some of the samples gave positive reaction and some others were negative (Table 3).

Table 1. The absorbance (A 405 nm) of supernatan mAbs obtained from flash culture against citrus samples in I-ELISA.

No	Hybridoma	CVPD against mAb	Healthy against mAb	Ratio CVPD: Healthy	CVPD against Buffer	Healthy against Buffer	Note*
1.	DW 0301	0.858	0.350	2.422	0.268	0.225	+
2.	DW 0302	1.065	0.435	2.448	0.268	0.225	+
3.	DW 0303	1.133	0.425	2.666	0.268	0.225	+
4.	DW 0304	1.071	0.413	2.593	0.268	0.225	+
5.	DW 0304	2.160	0.594	3.636	0.192	0.196	+
6.	DW 0305	1.161	0.422	2.751	0.268	0.225	+
7.	DW 0305	1.914	0.421	4.546	0.192	0.196	+
8.	DW 0305	2.057	0.423	4.843	0.192	0.196	+
9.	DW 0306	1.100	0.427	2.576	0.268	0.225	+
10.	DW 0306	2.136	0.659	3.241	0.192	0.196	+
11.	DW 0306	2.173	0.513	4.235	0.192	0.196	+
12.	DW 0307	1.399	0.647	2.162	0.451	0.387	+
13.	DW 0307	1.331	0.608	2.189	0.451	0.387	+
14.	DW 0310	1.140	0.401	2.843	0.210	0.228	+

*Positive when the ratio of A 405 nm of CVPD healthy = or > 2.000

Table 2. The absorbance (A 405 nm) from I-ELISA of antibody DW 0310 with serial dilution.

No	Dilution (fold)	CVPD against mAb #	Healthy against mAb #	CVPD against Buffer	Healthy against Buffer	Ratio of CVPD: Healthy	Note*
1.	10 000	1.528	0.673	0.240	0.242	2.270	+
2.	11 000	1.592	0.642	0.270	0.263	2.480	+
3.	12 000	1.244	0.591	0.270	0.263	2.105	+
4.	13 000	1.279	0.509	0.270	0.263	2.513	+
5.	14 000	1.340	0.481	0.270	0.263	2.786	+
6.	15 000	1.458	0.541	0.270	0.263	2.695	+
7.	16 000	0.269	0.050	0.250	0.248	5.380	+
8.	17 000	0.378	0.032	0.250	0.248	11.812	+
9.	18 000	0.337	0.037	0.250	0.248	9.108	+
10.	19 000	0.204	0.085	0.250	0.248	2.400	+
11.	20 000	0.217	0.020	0.250	0.248	10.850	+
12.	21 000	0.206	0.005	0.250	0.248	41.200	+
13.	22 000	0.193	0.046	0.250	0.248	4.196	+
14.	23 000	0.183	0.015	0.250	0.248	12.200	+
15.	24 000	0.234	0.057	0.250	0.248	4.105	+
16.	25 000	0.263	0.040	0.250	0.248	6.575	+
17.	26 000	0.244	0.043	0.250	0.249	5.674	+
18.	30 000	0.127	0.021	0.250	0.248	6.048	+

: After minus the A 405 nm of buffer *Positive when the ratio of A 405 nm CVPD : healthy = or > 2

Table 3. The absorbance (A 405 nm) of I-ELISA using DW 0310 antibody at the dilution of 20 000 fold.

No.	Sample code	CVPD against mAb #		CVPD against buffer	Healthy against mAb #	Healthy against buffer	Ratio CVPD: healthy	Note*
	**	repl. 1	repl. 2					
1.	MS-1	0.363	0.356	0.305	0.067	0.287	5.366	+
2.	PS-1	0.374	0.476	0.305	0.067	0.287	6.343	+
3.	PS-2	0.398	0.475	0.305	0.067	0.287	6.515	+
4.	YS-1	1.595	1.990	0.305	0.067	0.287	26.754	+
5.	.YS-2	1.095	1.137	0.305	0.067	0.287	16.657	+
6.	YS-3	0.782	0.810	0.374	0.032	0.303	24.875	+
7.	BS-1	0.289	0.290	0.374	0.032	0.303	9.047	+
8.	MS-2	0.693	0.661	0.374	0.032	0.303	20.313	+
9.	BK-1	0.974	1.063	0.205	0.211	0.198	4.827	+
10.	MK-1	0.511	0.557	0.374	0.032	0.303	16.688	+
11.	MM-1	0.111	0.064	0.374	0.032	0.303	2.734	+
12.	MM-2	0.097	0.105	0.205	0.211	0.198	0.479	-
13.	Psg-1	0.129	0.137	0.205	0.211	0.198	0.628	-
14.	YB-1	0.028	0.007	0.305	0.067	0.287	0.261	-
15.	YG-1	0.006	0.090	0.305	0.067	0.287	0.716	-
16.	BN-1	0.043	0.033	0.374	0.032	0.303	1.188	-
17.	BL-1	0.036	0.011	0.374	0.032	0.303	0.391	-

#: After minus A 405 nm of buffer

*Positive when the ratio A 405 nm of CVPD : healthy = or > 2.00

**Locality/*cultivar*, MS = Magelang/Siem, PS = Purworejo/Siem, YS = Yogyakarta/Siem, BS = Bali/Siem, MK = Magelang/Keprok, BK = Bali/Keprok, MM = Magelang/Manis, PSg = Purworejo/Sangres, YB = Yogyakarta/Bangkok, YG = Yogyakarta/Gulung, BN = Bali/Nipis, BL = Bali/Limau.

Using the antibody of DW 0310 the samples of citrus cv. Siem seemed to have higher A 405 nm compared to other cultivar, however the antibody also recognized the pathogen in the samples of citrus cv. Keprok and Manis. Different localities of the sample origin tested were not resulted in quite different absorbance. It could be because of the environment of the sample localities were not quite different, and it was also possible that the samples were originated from the same of mother plants that were distributed to some other areas.

Citrus cv. lime and lemon or the hybrids were reported to be less susceptible or more tolerant than mandarin and sweet oranges (Lang *et al.*, 1985). The local cultivars of Nipis, Limau, and Gulung are related to lime and lemon, therefore, the strain of the pathogen could be different and having different antigenic epitopes than that attacking more susceptible cultivars. Citrus cv. Siem and Keprok are related to mandarin, while cv. Manis is Sweet orange which are quite susceptible to CVPD.

The results suggested that the antigen of precipitated protein obtained from the sap of infected leaf midribs where the CVPD pathogen was concentrated was able to

induced monoclonal antibodies on Balb/c mice. The screening of hybridoma was conducted against the samples of both infected and healthy plants. This method may be useful for any other similar plant diseases with unculturable pathogen.

The results on the DNA amplification of 16S-rRNA and its part were discussed below. Using the universal primers the DNA fragment of about 1,500 bp was amplified from the CVPD samples originated from different localities. The fragment was cut into about 1,300 and 200 bp by Eco RI. This result was different from that reported by Jagoueix *et al.* (1994) that the fragment cut into 900 and 600 bp by Eco RI. On the other hand, using the enzyme of Bcl I the result was the same as that reported before which were cut into about 1,250 and 250 bp.

Using the specific oligonucleotide of *L. asiaticum* in the gene of 16S-rRNA as the foward primer and the same reverse primer for the gene, CVPD infected samples resulted in the DNA fragment of about 400 bp, where as using the oligonucleotide spesific of *L. africanum* as the foward primers, no amplification was occured. This result may further investigated that it is possible to be any difference in the DNA sequence of the strain of CVPD in Indonesia and that studied before (Jagoueix *et al.*, 1994).

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