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Use of Serological-Based Assay for the Detection of *Pepper yellow leaf curl Indonesia virus*

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Diseases caused by *Pepper yellow leaf curl virus* infection is considered to be emerging plant diseases in Indonesia in the last five years. One key factor for disease management is the availability of accurate detection of the virus in plants. Polyclonal antibody for *Pepper yellow leaf curl Indonesia virus-Bogor* (PYLCIV-Bgr) was produced for detection of the virus using I-ELISA and DIBA methods. The antibody was able to detect PYLCIV-Bgr from infected plants up to dilution 1/16,384 and cross reaction was not observed with *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), and *Chilli veinal mottle virus* (ChiVMV). Positive reaction was readily detected in membrane containing *Begomovirus* samples from Yogyakarta (Kaliurang and Kulonprogo) and West Java (Bogor and Segunung). Infection of PYLCIV-Bgr in chillipepper, tomato, and *Ageratum conyzoides* was also confirmed using polyclonal antibody for PYLCIV-Bgr in DIBA. Polyclonal antibody for PYLCIV-Bgr is suggested to be included in disease management approach due to its good detection level.

Keywords : DIBA, ELISA, *Geminivirus*

Geminiviridae is a plant virus family whose members produce serious damage to several crops worldwide, especially in tropical and subtropical regions (e.g. Middle East, Africa, Europe, Central America, Japan, United States, Caribbean basin) (Moriones and Naval, 2000; Varma and Malathi, 2003). Viruses of this family are grouped in four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*), which differ in genome organization, host range and vectors (Fauquet et al., 2000). Increases of *Begomovirus* associated epidemics in Indonesia since early 2000 are presumably due to a rise in whitefly infestation in a variety of crops. *Begomovirus* infection has been detected in chillipepper, tomato, tobacco, and weed *Ageratum conyzoides* from various region in Indonesia (Haerani and Hidayat 2003; Hidayat et al., 2008; Sudiono et al., 2005; Sukamto et al., 2005; Sulandari et al., 2006). *Pepper yellow leaf curl virus* (PYLCV) which is the most widely spread virus in Indonesia has been isolated from infected field at Segunung, Bogor, West Java and tentatively called *Pepper yellow leaf curl Indonesia virus-Bogor* (PYLCIV-Bgr) (Hidayat et al., 2006). The virus infection induces foliar chlorosis and curling, reduced leaf size, inhibition of fruit set, and abnormal fruit development (Hidayat et al., 2006; Sulandari et al., 2006). The virus could be transmitted by grafting and whitefly, but it was not mechanically transmitted (Rusli et al., 1999). Insect transmission was occurred in persistent manner transtadially but non-transovarially. The host range of the virus includes among others tomato, eggplant, tobacco (*Solanaceae*); soybean, yard long bean, mung bean (*Leguminosae*); sun flower, ageratum (*Compositae*) (Sulandari et al., 2006). Analysis of sequence identity revealed that PYLCIV-Bgr has 98% nucleotide sequence identity with PYLCIV-LBI and PYLCIV-LBI.I. The last two viruses were collected from the same geographic location, i.e. Lembang, Bandung, West Java but they were isolated from different host plant, i.e. *Lycopersicon esculentum* and *Capsicum annuum*, respectively (Hidayat et al., 2006).

The widespread occurrence of epidemics associated with begomoviruses and their potential threat to crop production, make it essential to develop procedures for their detection in plants for disease management. Several attempts have been made including both serological, polymerase chain reaction (PCR) and DNA hybridization methods. Begomoviruses are serologically related when tested with antibodies prepared to coat proteins of purified begomoviruses (Harrison, 1991). However, antibodies with high quality and specificity were difficult to obtain. This was probably due to the low to moderate antigenicity of the *Begomovirus* coat proteins (CP), together with the low amount of CP recovered from infected plants (Harrison, 1985). Therefore, routine detection and identification using serology has not been as common for begomoviruses as for other plant viruses. In contrast, PCR and nucleic acid hybridization methods have provided sensitive techniques for the detection and identification of begomoviruses in infected plants and viruliferous whiteflies (Aidawati et al., 2002; Briddon and Markham, 1994; Mehta et al., 1994; Rojas et al., 1993).

Sulandari et al. (2004) reported the production of poly-

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clonal antibody to begomoviruses infecting chillipepper. Although the antibody showed its promising application for begomoviruses detection, the quality of the antibody obtained was still unsatisfied. In this study, we described the establishment of polyclonal antibodies prepared to coat proteins of purified begomoviruses. Virus purification method applied in our work was different from those reported by Sulandari et al. (2004). Furthermore, we tested whether the polyclonal antibodies would be useful in developing detection technique for begomoviruses.

Materials and Methods

Virus purification. A modification of method described by Luisoni et al. (1995) and Attathom et al. (1990) was used for purification of the virus. *Datura stramonium* was chosen as host plant for virus (PYLCIV-Bgr) source. Young leaves of infected *D. stramonium* was grounded in 0.5 M phosphate buffer pH 6.0 (2.5 mM Na_2EDTA, 10 mM Na_2SO_3, 1% Triton X-100, 1% 2-Mercaptoethanol, 0.1% Driselase) with 1:3 ratio (b:v). Leaf extract was then incubated over night in 4 °C with a light shaking (100 rpm). Chloroform was added to the leaf extract to reach 15% final concentration, followed by clarification of the leaf extract by centrifugation of 41,000 g (Sorvall rotor SL-250T). The supernatant was separated and centrifuged using high speed of 208,000 g (Hitachi rotor P-70AT) for 2 hr to yield pellet. The pellet was resuspended in 0.5 M phosphate buffer pH 7.0 before subjected to centrifugation (12,000 g). Cessium sulfate (Cs_2SO_4) was added to the supernatant to 30% final concentration, followed by a high speed centrifugation of 247,000 g (Hitachi rotor P-65ST) for 48 hr. Viral zone resulted from the last centrifugation was isolated and then mixed with 0.5 M phosphate buffer pH 7.0 and cold chloroform in 4 °C for 20 min. It was then subjected to a couple of centrifugation of 12,000 g for 5-10 min in 4 °C. The supernatant was separated from the remaining debris and kept for further use as immunogen. Purified virus preparation was analysed using spectrophotometer at A_{260nm} and A_{280nm} to estimate its purity and in 4%/12% discontinuous polyacrylamide gels (SDS-PAGE) according to Laemmli (1970) to determine virus molecular weight.

Production of polyclonal antibody. Polyclonal antibody for PYLCIV- Bgr was produced on rabbits following procedure described by Agindotan et al. (2003). Immunizations were performed by priming 3 female rabbits, approximately 4 months old, four times at interval of 2 weeks. The immunogen (30-50 µg) was emulsified in complete Freund's Adjuvant (FA) and injected intramusculary into the hind legs of the rabbits. After 2 weeks, a booster injection emulsified with incomplete FA (30-50 µg) was given and the rabbits were bled 10 days after the last injection. Blood obtained from immunized animals is allowed to clot and the serum was separated from the clot. In attempt to reduce cross reaction with protein of plant host, the antisera was absorbed with leaf extract of *D. stramonium* as described in Dijkstra and Jager (1998). The antisera presumably contain of antibody for PYLCIV-Bgr was then kept in –20 °C for further study.

Evaluation of polyclonal antibody. Serological assessment using polyclonal antibody for PYLCIV-Bgr was performed using Indirect-ELISA (I-ELISA) according to Stack and Macmillan (2005). Dilution of 1/1, ½, ¼, 1/8, 1/16, 1/32, 1/64, and 1/128 of plant extract and dilution of 1/1, ½, 1/8, 1/32, 1/128, 1/512, 1/2048, 1/8192, 1/16384 of antibody was evaluated to determine the titer of antibody. ELISA result was measured by recording its absorbance value using an ELISA plate reader (BIO-RAD Model 550) at A_{405nm}. Negative-positive thresholds were set at two times the mean of healthy control samples absorbance values.

Detection of *Begomovirus* using dot blot immunobinding assay (DIBA). Polyclonal antibody for PYLCIV-Bgr was used for detection of *Begomovirus* from different leaf samples. The samples involve tomato plants infected by different isolate of begomoviruses, eggplants, cucumber, chillipepper, and *Ageratum conyzoides* each inoculated with PYLCIV-Bgr using insect vector *Bemisia tabaci* (Aidawati et al. 2002). Young, upper leaves of infected (symptomatic) and non infected plants were harvested and crushed individually in microfuge tubes in tris buffer saline (Tris-HCl 0.02 M, NaCl 0.15 M, pH 7.5) with the ratio of 1:10 (b:v). Tubes were centrifuge at 10,000 g for 2 min, and 10 µl of the supernatant of each sample was spotted on nitrocellulose membrane (Hybond-P, Amersham Biosciences, UK) for DIBA according to Mahmood et al. (1997).

Results

Purified virus preparation. Nucleoprotein concentration obtained following the purification procedure described here varied between 159.65 to 386.10 µg/ml. Spectrophotometric analyses of these purified preparations revealed an ultraviolet light-absorbing spectrum characteristic of a nucleoprotein, with an average $A_{260/280nm}$ value of 1,122. A major protein species of apparent molecular mass of 30 kDa was detected by SDS-PAGE of purified PYLCIV-Bgr preparations (Fig. 1). This band co migrated with total protein obtained from PYLCIV-Bgr infected plant. Comparison of the total proteins from PYLCIV-Bgr infected plant with those from healthy plant indicated that a 30 kDa

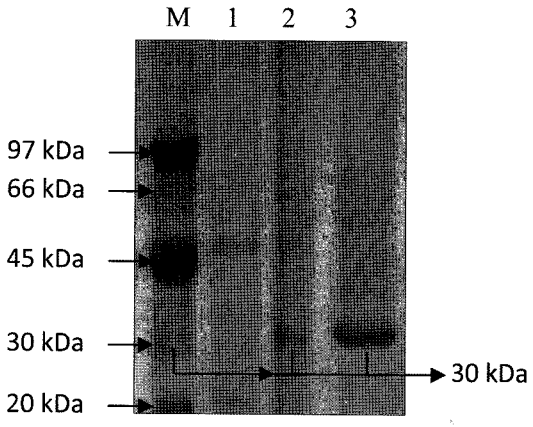

Fig. 1. Protein analysis on SDS-PAGE. M, protein marker; 1, healthy tomato plant; 2, PYLCIV-infected tomato; 3, purified virus preparation.

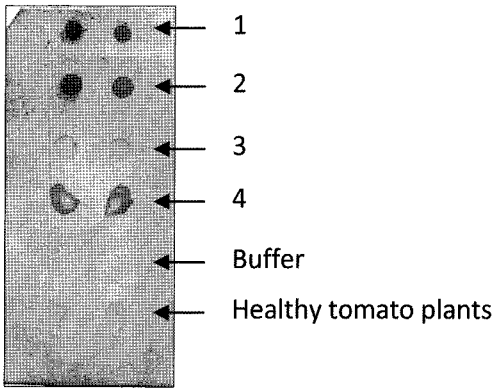

Fig. 2. Reaction of PYLCIV-Bgr polyclonal antibody in DIBA. Samples consisted of tomato plants infected with different field isolates of begomoviruses: (1) Bogor; (2) Kaliurang; (3) Kulonprogo; (4) Segunung.

Table 1. Absorbance value of Enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody for PYLCIV-Bgr

Samples	Absorbance value*	Reaction**
Buffer	0.128±0.029	–
Healthy tomato	0.263±0.001	–
PYLCV-infected tomato	0.996±0.016	+++
CMV-infected tobacco	0.338±0.008	–
TMV-infected tobacco	0.305±0.010	–
ChiVMV-infected chillipepper	0.299±0.006	–

*Absorbance value was measured at 405 nm.
**Negative-positive thresholds were set at two times the mean of healthy control samples absorbance values. Strong reaction was observed for PYLCIV-infected tomato with absorbance value ≥3 times of healthy control samples absorbance values.

Table 2. Absorbance value of ELISA for determining titer of polyclonal antibody for PYLCIV-Bgr

Dilution of antibody	Dilution of plant extract						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Undiluted	+++	+++	+++	+++	+++	+++	+++
1/2	+++	+++	+++	+++	+++	+++	+++
1/8	+++	+++	+++	+++	+++	+++	+++
1/32	+++	+++	+++	+++	++	+++	++
1/128	+++	+++	+++	+	+	+	+
1/512	+++	+++	++	+	–	+	–
1/2,048	++	++	++	+	–	–	–
1/8,192	++	+	+	+	–	–	–
1/16,384	++	+	+	+	–	–	–

Absorbance value was measured at 405 nm. Negative-positive thresholds were set at two times the mean of healthy control samples absorbance values. Reaction was considered strong (+++), mild (++), or weak (+) when absorbance value ≥3 times, (2.5≤⊕<3) , (2≤⊕<2.5) of healthy control samples absorbance values, respectively.

protein was the capsid protein of the virus.

I-ELISA. The result of I-ELISA shows that the antibody produced against PYLCIV- Bgr is of good specificity and good quality. In the cross reaction assay using different types of viruses (*Cucumber mosaic virus*/CMV, *Tobacco mosaic virus*/TMV, and *Chilli vein mottle virus*/ChiVMV) the antibody did not give positive reactions (Table 1). Positive reaction was only observed in PYLCIV-Bgr infected plants with absorbance value more than four times of those of healthy plant. Further dilution assay was conducted to measure the titer of antibody. Infection of PYLCIV-Bgr was detectable in tomato plants in dilution of antibody up to 1/16.384 when the plant extract was diluted up to 1/16 (Table 2). This virus titer is higher than those reported earlier by Sulandari et al. (2004), i.e. 1/10,000.

DIBA. Viral antigens were readily detected on membranes when saps from tomato plants infected by different isolates of *Begomovirus* were spotted (Fig. 2). Positive reaction was indicated by development of purple colors on the membrane at the sample spot. Polyclonal antibody for PYLCIV-Bgr at a ¼ dilution gave different level of positive reaction to different *Begomovirus* samples. The antibody reacted very well with samples from Bogor and Kaliurang but only weakly with samples from Kulonprogo.

Evaluation of antibody reaction was also conducted using plants inoculated with PYLCIV-Bgr by its insect vector, *B. tabaci*. Antibody for PYLCIV-Bgr at a 1/1,000 dilution gave strong reaction to chillippper, tomato, and *A. conyzoides*, but no reaction was noticed with eggplant and cucumber (Fig. 3). Positive reaction was observed on symptomatic as well as non symptomatic leaves indicating that antibody for PYLCIV-Bgr has a good detection level.

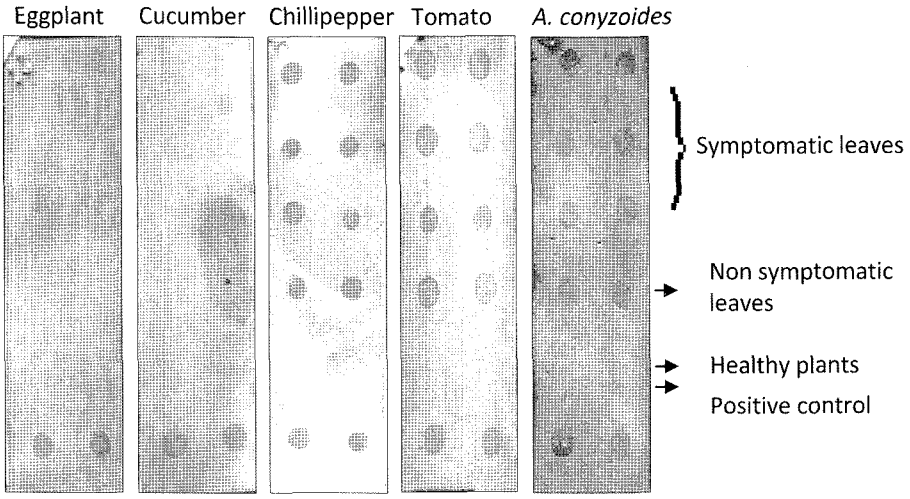

Fig. 3. Deection of PYLCIV-Bgr on inoculated plants (eggplant, cucumber, chillipepper, tomato, *A. conyzoides*) using polyclonal antibody. Tomato plants infected with PYLCIV-Bgr were used as positive control.

Discussion

The increasing importance of pepper yellow leaf curl disease in Indonesia has resulted in the need for accurate detection and identification procedures. Detection is necessary to enable virus reservoir hosts to be identified, virus resistant plant varieties to be evaluated and consequently control measures to be devised or improved. Widespread application of immunological assay using polyclonal antisera has been utilized for decades in the identification of viruses. Although limited success was reported for application of serological assay for begomoviruses, several attempts has been made to improve the quality of polyclonal antibody for begomoviruses (Harrison, 1991).

Polyclonal antibody prepared to PYLCIV-Bgr served as useful serological tools for detection of pepper yellow leaf curl disease using ELISA and DIBA. It was evidenced that the antibody has a good specificity and did not cause cross reaction with non begomoviruses. However, serological cross-reactivity is expected among members of begomoviruses. A comparison of the predicted amino acid sequences of *Begomovirus* coat proteins indicated most of the sequence variability is limited to N-terminal 60 to 70 amino acid residues. The remainder of the coat protein structure is highly conserved among begomoviruses (Padidam et al., 1995). Positive reaction to PYLCIV-Bgr antibody was varied among four isolates of whitefly-transmitted geminiviruses collected from tomato fields in West Java (Bogor and Segunung) and Yogyakarta (Kaliurang and Kulonprogo). In addition to serological relationship, different reaction might be caused by virus concentration in the infected tissue (Dijkstra and Jager, 1998). The higher concentration of the virus the strongest reaction will be produced. It was confirmed that infection occurred in chillipepper, tomato, and *A. conyzoides* following inoculation of PYLCIV-Bgr using *B. tabaci*. Symptoms were not observed on eggplant and cucumber and the virus was not detected. Host range study of begomoviruses reported previously indicated that these two plants were among those that could not be infected (Kato et al., 1998; Sulandari et al., 2004).

Method to produce purified virus preparation applied in our work was different from those reported by Sulandari et al. (2004). *D. stramonium* was chosen as host plant for virus (PYLCIV-Bgr) source as compared to *Physalis floridana* and Driselase was added during tissue extraction to improve virus recovery. The titer of the antibody is higher than those reported earlier, i.e. 1/16.384 as compared to 1/1,000. Based on its sensitivity level the polyclonal antibody produced for PYLCIV-Bgr is very promising for its application for early detection tool. It was shown from the results of DIBA, in which virus infection can be detected with good signals in leaves showing no symptoms. Disease monitoring to detect early infection is important component in disease management strategy, because it will help reducing virus reservoirs in the early stage of disease development. For this purpose, DIBA is probably the method of choice because it is cost-efficient, reliable and practice for mass analyses. Although we have the evidence that serological assay bears great potential for diagnosis of begomoviruses, we still need to improve the methodology to produce antibody. Researches on production of antibody using a recombinant antigen suggested that this is an advantageous procedure compared to the labor-intensive virus increase in herbaceous hosts and cumbersome and expensive virus purification (Nickel et al., 2004).

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