CURRENT PROGRESS ON THE DETECTION OF PLANT VIRUSES, WITH SPECIAL REFERENCE ON ZUCCHINI YELLOW MOSAIC VIRUS

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Abstrak

Kejituan beberapa uji serologi sebagai alat diagnosis penyakit viral pada tanaman dievaluasi dengan menggunakan zucchini yellow mosaic virus (ZYMV). Konsentrasi virus murni terendah yang masih terdeteksi dengan uji *latex flocculation, protein A-coated latex-linked antisera, DAS-ELISA, non-precoated I-ELISA,* dan *precoated I-ELISA* berturut-turut adalah 25ug /ml, 0,1 ug/ml, 5 ug/ml, 1 ug/ml, dan 10 ug/ml. Tingkat pengenceran tertinggi yang masih memungkinkan terdeteksinya ZYMV dalam ekstrak tanaman sakit dengan uji yang sama berturut-turut adalah 500, 4.000, 10^5, 10^6,dan 10^4. Kebanyakan tanaman *Cucurbitaceae* menunjukkan adanya aktifitas ensim peroksidase, sehingga penggunaan *peroxidase conjugate* untuk mendeteksi virus pada kelompok tanaman ini sebaiknya dihindarkan. Meskipun uji serologi merupakan teknologi yang relatif mahal, namun di masa yang datang diharapkan merupakan "mitra" yang baik bagi pakar penyakit tumbuhan dalam membantu memecahkan masalah praktek di tingkat petani.

I. Introduction

In Indonesia, plant viruses cause significant amount of economic losses in agricultural commodities. To guide the management of viral diseases, some informations such as the kind of virus, inoculum potential, serotypes development, geographical distribution of each virus, and other ecobiological data are needed. Consequently, the development of a simple, rapid, sensitive, and specific diagnosis method for viral diseases would be very valuable.

Traditional methods for viral diagnosis that mainly based on the observation of external or internal symptoms are not suitable for the most purposes because (1) a certain virus may induce symptoms that are similar to those induced by other viruses; (2) double or triple infections by viruses frequently occur in one plant; (3) virus multiplication in susceptible hosts does not always lead to visible symptom;

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(4) symptoms also happen to disappear temporaly; and (5) not adaptable for presymptomatic diagnosis.

Another constraint in doing visual recognition for viral diagnosis is that the major visible symptoms of viral diseases such as growth reduction, color deviation, and malformation are not specific to virus infection, so that sympton often confused with those caused by other pathogens and or physiological disaster.

In the limited applications, viral diseases diagnosis based on the specific reaction of viruses on their host plants has been the method of choice for identifiying plant viruses. Moreover, a certain virus can be detected or separated from double or triple infected plants by using differential hosts or indicator plants. However, the use of indicator plants for detecting plant viruses is sometimes troublesome and expensive; insensitive; requiring green house space, time, and energy to grow the indicator plants. Additionally, it takes long time for the inoculated indicator plants to exhibit recoqnizable symptoms.

The problems concerning viral diseases diagnosis can be overcome by using serological reaction-based diagnosis. This article deals with the current achievement in the use of serological assays, mainly enzyme-linked immunosorbent assay (ELISA), for detecting zucchini yellow mosaic virus (ZYMV). Detailed technical information on the assay is already available in the previous reviews (Clark, 1981; Clark and Bar-Joseph, 1984).

II. Zucchini yellow mosaic virus

ZYMV is the provisional name given to a potyvirus that in 1973 caused a widespread epidemic in zucchini squash (*Cucurbita pepo* L.) in northern Italy, which differed from all the other viruses known to infect cucurbits (Lisa *et al.*, 1981). Recently, many isolates of ZYMV have been reported as new destructive pathogen of commercial cucurbits in many places (Purcifull *et al.*, 1984; Provvidenti *et al.*, 1984; Nameth *et al.*, 1985; Ohtsu *et al.*, 1985). The existence of this virus in Indonesia has probably not been reported yet.

ZYMV is characterized by cryptogram of R/1:3/*:E/E:S/Ve /Ap, and flexious filamentous particle about 750 nm. Other properties of the virus include the thermal inactivation point of 60-65C (10 min), the dilution end point of 10^4-10^5, and longivity in vitro of 12 days (Ohtsu *et al.*, 1985). It is transmitted naturally in non-persistent or stylet - borne manner by *Aphis citricola* (Purcifull *et al.*, 1984), *Aphis gossypii*, *Myzus persicae* (Lecoq *et al.*, 1981), and *Macrosiphum euphorbiae* (Lisa and Lecoq, 1984).

As a member of potyviruses, the transmission by aphids had been shown to be dependent on the presence of helper component which is present in infected and not in healthy hosts (Lecoq and Pitrat, 1985) as observed in other member of this group (Govier and Kassanis, 1974; Sako and Ogata, 1981). There is no evidence of the transmission by seeds (Nameth *et al*., 1985).

ZYMV causes mosaic, shoestringing, stunting, and fruit and seed deformation in various cucurbits and is non-systemic or symptompless in the most host other than family of Cucurbitaceae including the family of Aizoaceae, Amaranthaceae, Chenopodiaceae, Compositae, Labiatae, Leguminoceae, Ranunculaceae, Solanaceae, and Umbelliferae (Lecoq *et al* ., 1981).

III. Serological detection of plant viruses

The virus consists of nucleic acid coated in a protective shell mainly composed of protein. Though the coat protein gene represents only about 10 % the virus genome, the current advances in the virus detection is mainly based on increased sensitivity of method for detecting protein (Martin, 1984). Recently, we successfully applied two kinds of precipitin-based procedure of immunodiagnosis, namely, latex flocculation, and enzyme-linked immunosorbent assays to detect ZYMV.

1. Latex flocculation (LF) test.-- One of useful application of the precipitin - based reaction is LF test, based on the method reported by Bercks and Querfurth (1969). This relatively simple procedure gives very rapid results and can be applied readily to small or large testing program. However, it is not readily adaptable if the available virus antisera have low titre or contain some inhibitory components which prevent the flocculation reaction.

Improvement of this procedure has been already achieved by coating the latex particles using protein A before sensitization with antibody, and the improved procedure was called protein Acoated latex-linked antisera (PALLAS)test(Querfurth and Paul, 1979).

For detecting ZYMV (Somowiyarjo *et al*., 1986 d; 1987b) LF test was carried out with a suspension of Bacto latex 0.81 um (Difco Lab., Detroit, Michigan) which had been sensitized with 100 ug/ml of gamma-globulin from rabbit antiserum. Latex was sensitized by firstly diluting 1 part of latex suspension with 14 parts of 0.15 M NaCl. The mixture of an equal volume of gamma globulin in 0.05 M Tris-HCl buffer,

pH 7.2, and the diluted latex suspension was incubated for 1 hr at 25°C with occasional stirring.It was then twice washed by subjecting to a centrifugal force at 6,000 rpm for 30 min, sucking the supernatant, and resuspending the pellet with Tris-HCl buffer containing 0.02% polyvinylpyrrolidone (PVP). The final pellet was resuspended in the same buffer containing 0,02 % sodium azide and was stored before used. The tests were done in polystyrene microtitre plates (Wako Pure Chem., Ind. Ltd.). The sensitized latex was applied by adding 25 ul of the suspension to 50 ul of antigen preparation, and shaking the mixture at about 100 ascillations per minute for about 15 min at 30°C. After incubation for 30 min at 25° C, the positive reaction could be observed as visible flocculation. As the results, minimum detectable concentration of purified ZYMV by LF test was 25 ug/ml compared with 0.1 ug/ml for PALLAS test. The comparison of the sensi of LF and PALLAS tests for detecting ZYMV in crude extracts of pumpkin is summarized in table 1.

Table 1. Detection of ZYMV in crude extracts of infected pumpkin leaves by latex flocculation (LF) and protein A-coated latex-linked antisera (PALLAS) tests[a]

Test	Sample	Visible flocculation at dilution of[b]						
		50	100	500	1,000	2,000	4,000	5,000
LF	Infected	9/9	9/9	9/9	0/9	0/9	0/9	0/9
	Healthy	3/9	0/9	0/9	0/9	0/9	0/9	0/9
PALLAS	Infected	9/9	9/9	9/9	9/9	9/9	9/9	0/9
	Healthy	0/9	0/9	0/9	0/9	0/9	0/9	0/9

a. Data are total numbers of wells from three time experiments

b. Numerator = Number of wells showed flocculation
   Denominator = Number of wells used

2. *DAS-ELISA*.-- One of the outstanding achievement in Plant Virology during the past decade was the adoption of the medical serodiagnosis, double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), for quantitative detection of plant viruses (Voller *et al*., 1976; Clark and Adams, 1977). In this assay, antigen is selectively trapped and immobilized on solid-phase previously coated with antibody.

Enzyme - labelled antibody is then reacted with the immobilized antigen, and after removing unreacted enzyme-labelled antibody, the retained enzyme is assayed by adding a suitable substrate.

According to Clark and Bar-Josheph (1984) the advantages of DAS-ELISA for diagnosis plant viruses include the following:

1. Sensitivity for detecting very small amount of antigen
2. Speed of the assay (6-24 h)
3. Applicable for large scale works
4. Suitable with purified virus and plant extracts
5. Specificity for differentiating serotypes
6. Suitability for both intact and fragmented virions of different size and morphology
7. Possibility of obtaining quantitative data
8. Possibility of automation and standardizing test by production and use kits
9. Low cost and long shelf life of reagents
10. Basic requirement for simple equipment
11. Economical and efficient use of antibodies and antisera.

ZYMV could be successfully detected by using DAS-ELISA with the following procedure of the assays (Somowiyarjo *et al.*, 1985c).
The assays were carried out on polystyrene microtitre plates (Wako Pure Chem. Ind. Ltd.) with three times of 3 - minutes washing between each step of the assays.The washing solution was 0.02 M PBS, pH 7.4, containing 0.05% Tween 20 and 0.05% PVP 40,000(PBS-TPO). The plates were successively incubated with: (1) 200 ul of 2 ug/ml of gamma - globulin form rabbit anti-ZYMV in coating buffer (0.05 M sodium carbonate buffer, pH 9.6) for 4 h at 37 C; (2) 200 ul of antigen in PBS - TPO for 18 h at 6 C; (3) 200 ul of alkaline phosphatase-labelled rabbit anti-ZYMV gamma-globulin diluted 1:1,000 with 0.02 M PBS, pH 7.4, for 4 h at 37 C; and finally with (4) 250 ul of 1 mg/ml of p -nitrophenyl phosphatase (Wako Pure Chem. Ind. Ltd.) in 10% of diethanolamine, pH 9.8, for 1.5 h at 25 C. After terminating the reaction with 50 ul of 3 M NaOH, the DAS-ELISA absorbance values were measured with a spectrophotometer Hitachi Model 200-20 using a micro-cell with a 1 cm light path at wavelength of 405.

In our study, the minimum detectable concentration of purified ZYMV by DAS-ELISA was 5-10 ng/ml. The assay proved to be an accurate tool for diagnosis of ZYMV infected pumpkin, avoiding the non-specific reaction with healthy control. The dilution end point of crude extracts from infected pumpkin leaves assayed with DAS-ELISA was 10^{5} - 10^{6} (Fig. 1) (Somowiyarjo *et al.*, 1985a, 1985b, 1985c).These results were further confirmed with the data obtained on samples collected from the cucurbits cultivating field at farmer level (Sako *et al.*, 1987).

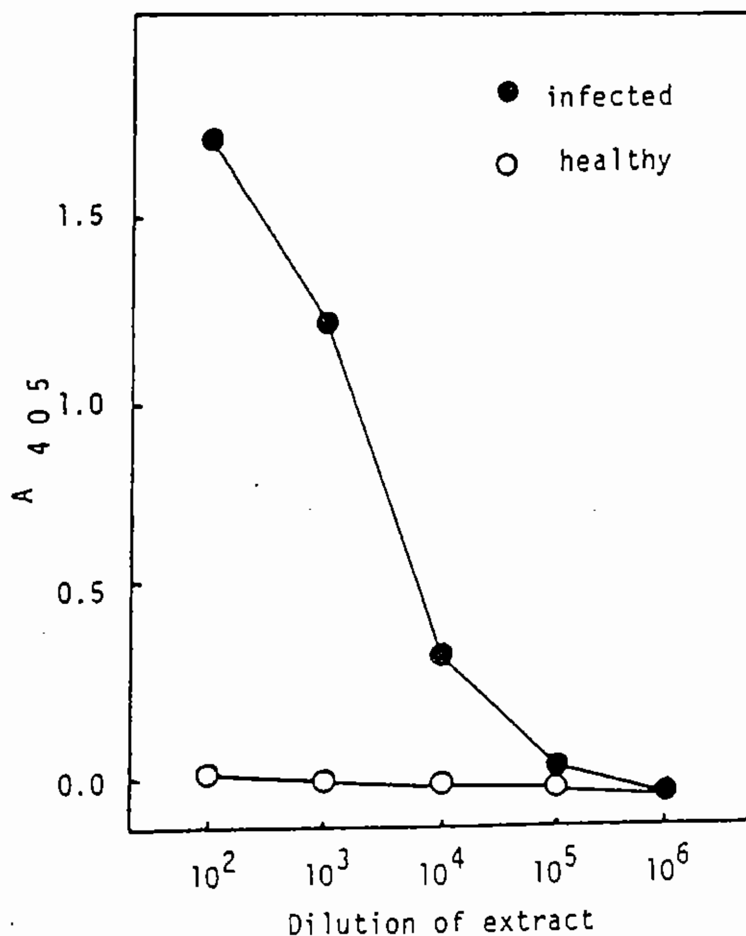

Fig. 1. Detection of ZYMV in crude extracts of infected pumpkin leaves by DAS - ELISA. The microtitre plates were successively incubated with: (1) 200 ul of 2 ug/ml rabbit anti-ZYMV in coating buffer for 4 h at 37C (2) antigen in PBS-T for 18 h at 6 C; (3) conjugate in PBS-T for 4 h at 37 C;and (4) substrate for 1.5 h at 25 C. Each point is the mean of three experiments using three well in each experiment.

3. *Non-precoated I-ELISA*.-- The alternative procedure of DAS-ELISA is non-precoated indirect enzyme-linked immunosorbent assay (I-ELISA), the immobilized antigen is detected by enzyme-labelled anti-immunoglobulin using intermediate of unconjugated specific antibody. The conjugate is not virus(antigen) specific, but is specific for the intermediate antibody. In this assay, antigen is directly immobilized on the solid phase.

For a large-scale testing program where thousands of samples must be tested, the assay has been superior to DAS-ELISA. Not only this assay has broad specificity that enable us to detect viruses that occur in many serotypes, but also offers the utility of a single anti - species IgG conjugate (general conjugate) which is commercially available for all kind of viruses. Moreover, it offers the use of crude antisera instead of purified gamma-globulin that makes the assay can be more readily adopted in developing countries where the equipment is limited.

Non-precoated I-ELISA was also applied for detecting ZYMV with the same conditions of assays as those in DAS - ELISA, except the first coating was with 200 ul of antigen in coating buffer for 4 h at 37 C. After washing, 200 ul of 2 ug/ml of gamma-globulin from rabbit anti - ZYMV was then incubated in plates for 18 h at 6 C. A volume of 200 ul of enzyme-labelled goat anti-rabbit IgG (Kirkegaard and Perry Lab. Inc.) was incubated for 4 h at 37 C prior to washing and addition of appropriate substrate. When alkaline phosphatase conjugate was used, the substrate and it's preparation were same as those in DAS-ELISA. When peroxidase conjugate was used, the substrate preparation was done by the same procedure used by Rowhani *et al.*, (1985).

In the assay using alkaline phosphatase conjugate, the minimum detectable concentration of purified ZYMV by non-precoated I-ELISA was 1 - 5 ug/ml. When antigen of crude extracts were assayed, the assay resulted in the detection limitation of 10^6-10^7 (Somowiyarjo *et al.*, 1985b, 1985c).

In our experiment with peroxidase conjugate, it was found that purified ZYMV could be detected at minimum concentration of 0.5 - 0.1 μg/ml and that detection limitation in crude extracts was 10^7-10^8. However, in the test with crude extracts, we could also obtain high absorbance value from control wells in the plates incubated. In the further studies, it was found that some cucurbit plants have naturally peroxidase activity and that infection by ZYMV is one of the causes for enhancement of the endogenous peroxidase activity in these plants. From the view of the serological detection, the presence of endogenous activity in some cucurbit plants precludes the use of peroxidase conjugate for detecting antigen in crude extracts by non-precoated I-ELISA (Somowiyarjo *et al.*, 1985a, 1985c).

Precoated I-ELISA.-- Despite the widespread acceptance of non-precoated I-ELISA for detecting plant viruses, there is a problem that limit the performance of the assay. The problem with non-precoated I-ELISA is incapability to identify certain binding sites (epitopes) in virus particle (Al Moudalal *et al*., 1984; Koenig and Torrance, 1986). It was hypothesized that virus particle may become partially denaturated when it is directly immobilized on ELISA plates. This problem could be overcome by using precoated I-ELISA, in which the antigen is selectively immobilized on plates previously coated with viral antibody.

A potential drawback to the precoated I - ELISA is that the specific antibody must be produced in two animal species and that the characteristics of the two antisera may be significantly different. In addition of rabbit, chickens are usually used to provide serum or egg-yolk antibody in I-ELISA for detecting plant viruses (Polson *et al*., 1980; Bar-Joseph and Malkinson, 1980). Recently, we recomended the use of small bird, Japanese quail (*Coturnix-coturnix japonica* Temminck *et* Schlegel), as a source of anti-viral immunoglobulin because it offers some advantages over the chicken(Somowiyarjo *et al*., 1986a, 1986b, 1986c, 1987a).

ZYMV could be easily detected by precoated I - ELISA with the same condition of assay that in non-precoated I-ELISA procedure, except the plates were previously coated viral antibody. The ELISA employing quail immunoglobulin on plates coated with gamma-globulin from rabbit antiserum against ZYMV was sensitive as that on non-coated plates, capable of detecting ZYMV in purified preparation at detection limitation of 10 - 50 ug/ml and crude extracts of infected pumpkin leaves at dilution end point of 10^4-10^5 (Somowiyarjo *et al*., 1986c).

IV. Discussion

Serological assay has become a very important tool for viral diseases diagnosis. The versality of the assay,the sensitivity, speed, and precision with which results can be obtained, and the scale of operation make the assay was adopted for many application. Undoubtedly, the assay also offers an alternative tool for detection of viral pathogens that can be very useful to guide crop management decision in Indonesia. However, the assay conditions for various virus-host combination should be examined carefully in order to obtain optimum detection of viruses. It requires trained personnel,well-equipped laboratories, and large sum of money.

Several serological procedures were successfully employed to detect plant viruses in purified preparation of crude extracts of infected plants and viruliferous vectors. To determine the procedure, it is necessary to consider the purpose of the detection and the availability of the equipment because one procedure may have specific advantages over the other in certain condition.

Finally, in developing more accurate and rapid methods for detecting plant viruses, many researches are still needed. One of the study that at the present is being done is the production of monoclonal antibody. In the preliminary tests, it was found that monoclonal antibody gave the precise reagent for detecting AYMV using serological assays(Somowiyarjo *et al*., 1987c).

Undoubtedly, that in the future, serological assay will provide data that will be very useful for extention specialists, consultants, pest control advisors, and plant pathologist to establish the integrated pest management control of viral diseases in Indonesia.

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