ポリクローナル抗体とモノクローナル抗体によるズッキーニ黄斑モザイクウイルス(ZYMV)のdo-immunobinding assay(DIA)

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Dot-immunobinding Assay for Zucchini Yellow Mosaic Virus Using Polyclonal and Monoclonal Antibodies*

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Abstract

The application of dot-immunobinding assay (DIA) for detecting zucchini yellow mosaic virus (ZYMV) was described. The virus showed the same binding activity to nitrocellulose membrane (NCM) in three kinds of coating buffers tested. Antigenicity of ZYMV in DIA could be maintained in leaf extract-dotted NCM which had been stored at −20 C, 6 C, or 20 C at least for six months. The methods were developed for sending samples using infected leaves extract-dotted NCM in replace of fresh leaves and for simplifying the procedure for direct deposition of the samples onto NCM. Seven protocols of DIA tested proved to be applicable to discriminate between healthy and infected pumpkin, with limits of detection ranging from 10^{-3}~10^{-5} dilution of leaf extracts. Rabbit polyclonal antibody against ZYMV cross-reacted with watermelon mosaic virus 2 (WMV-2) in two-step direct-DIA and indirect-DIA but not in DAS-DIA. It did not also cross-react with papaya ringspot virus, type W (PRSV-W) in three DIA protocols. When the three DIA protocols were used with monoclonal antibody against ZYMV, the assays resulted in the same sensitivity as those obtained by using polyclonal antibody, and could also be used to discriminate ZYMV from PRSV-W and WMV-2.

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Key words: dot-immunobinding assay, zucchini yellow mosaic virus, nitrocellulose membrane.

INTRODUCTION

Since the simplification of immunobinding technique[22] to dot-immunobinding assay (DIA) by Hawkes *et al.*[6], this quick convenient immunoenzymatic assay has been successfully exploited in various purposes. DIA has certain practical advantages over the enzyme-linked immunosorbent assay (ELISA), because it is relatively sensitive, simple, rapid, low cost, and requires smaller amount of antigen and other reagents. These advantages have contributed to the increasing use of DIA for detecting many plant viruses such as tobacco mosaic virus[7,15], potato viruses S, X, Y[2], potato leaf roll virus[17], barley yellow striate mosaic virus[10], tobacco ringspot virus and tomato ringspot virus[15].

In the present paper we report the results of comparative studies in applying DIA for detecting zucchini yellow mosaic virus (ZYMV) and its related viruses in cucurbits using different protocols of immunoenzymatic assays. The efficiencies in using different extracting buffers, the reactivity of viruses in DIA after being stored or transported by mail, and the simplication of sample deposition, are also described.

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MATERIALS AND METHODS

Viruses and antibodies. Zucchini yellow mosaic virus (ZYMV, isolate 169), papaya ringspot virus, type W (PRSV-W, isolate M-5), watermelon mosaic virus 2 (WMV-2, isolate M-18) and rabbit antisera to these viruses were the same as in the previous studies[16,19,21]. The monoclonal antibody (MCA) against ZYMV was that described previously[21].

Rabbit anti-chicken IgG was obtained from Miles Laboratories Inc.

Immunoglobulin and conjugate preparations. Immunoglobulins from each rabbit antiserum against the respective viruses or chicken IgG, were prepared using the method described by Clark and Adams[4]. The quail immunoglobulin was the same as in the previous study[19]. Each 1 mg immunoglobulin from rabbit anti-chicken IgG, rabbit antiserum against the respective viruses, and IgG_{2b} from monoclonal antibody (MCA) against ZYMV[21] was conjugated with 1.85 mg of alkaline phosphatase (Grade 1, from calf intestine, Boehringer Mannheim GmbH.) using the procedure described by Clark and Adams[4]. Alkaline phosphatase conjugates of goat anti-rabbit IgG (H+L) immunoglobulin and goat anti-mouse IgG+IgM (H+L) immunoglobulin were purchased from Kirkegaard and Perry Lab. Inc. Alkaline phosphatase labelled protein A was obtained from ICN Biologicals.

Preparation of samples. The crude extract preparations were done by grinding leaf samples from infected plants in 1 : 1 (w/v) of 0.05 M carbonate buffer, pH 9.6, 0.02 M PBS, pH 7.4, or 0.02 M Tris-HCl buffer containing 0.5 M NaCl, pH 7.5 (TBS) using a mortar and pestle.

Dot-immunobinding assay (DIA). Nomenclature of the reagents and solutions of DIA was adopted from those described by Hibi and Saito[7]. The assays were carried out on nitrocellulose membrane (NCM) (Bio Rad) which were cut to appropriate size and marked with a grid of 1×1 cm using a soft pencil. They were then dipped in TBS for 15 min and dried on filter paper for 5 min before deposition of the first reagent.

Throughout the following experiments, only the antigen (2 μl) was applied individually to each grid of NCM by using a micropipet. Other reagents or solutions were applied to NCM by two methods. In the first method, NCM was transferred onto a glass plate and covered with about 20 μl of reagent/grid. In the second method, NCM was dipped and incubated in a glass petri dish containing a desired solution or reagent. All reagents were incubated at room temperature (about 25 C). Seven protocols of immunoenzymatic assays compared in this experiment are shown schematically in Table 1, using the acronyms adopted from Al Moudallal *et al.*[1] Following the first incubation of NCM with antigen or antibody in 0.05 M carbonate buffer, pH

Table 1. The protocols of dot-immunobinding assay (DIA) for detecting zucchini yellow mosaic virus

No.	Protocol	Successive steps of assay a)	Detection limitation with Purified virus	Detection limitation with Crude extract
1.	Two-step direct-DIA	AG, AB^{R}-E	0.2 ng	10^{-4}
2.	Double antibody sandwich (DAS)-DIA	AB^{R}, AG, AB^{R}-E	2 ng	10^{-3}
3.	Non-precoated indirect (I)-DIA	AG, AB^{R}, anti-R^{G}-E	20 pg	10^{-5}
4.	Non-precoated I-DIA	AG, AB^{R}, Protein A-E	20 pg	10^{-5}
5.	Non-precoated I-DIA	AG, AB^{Q}, anti-Ch^{R}-E	20 pg	10^{-4}
6.	Precoated I-DIA	AB^{Q}, AG, AB^{R}, anti-R^{G}-E	0.2 ng	10^{-4}
7.	Precoated I-DIA	AB^{R}, AG, AB^{Q}, anti-Ch^{R}-E	20 ng	10^{-3}

a) AG: antigen, AB: antibody, R: rabbit, Q: Japanese quail, Ch: chicken, G: goat, anti-R^{G}: goat anti-rabbit globulin, anti-Ch^{R}: rabbit anti-chicken globulin, E: alkaline phosphatase.

9.6, for 5 min, the unoccupied binding sites on NCM were saturated by incubation with 3% bovine serum albumin (BSA) in TBS (blocking buffer) for 30 min. The NCM was then washed by rinsing briefly in distilled water and shaking gently for 20 min in each of two changes of TBS containing 2% polyvinylpyrrolidone (PVP) and 0.05% Tween 20 (PBS-PVT). The washed NCM was further incubated sequentially with the reagents listed in Table 1. The incubation time for each reagent was 60~90 min, and washing procedure between each step of the assay was the same as that after incubation with blocking buffer. Finally, the NCM was incubated with color development solution which was prepared by mixing fast red TR salt (Sigma) and naphthol AS-MX phosphate (Sigma) as recommended by Banttari and Goodwin[2]. The reaction was stopped within 30~60 min by washing the NCM in distilled water, and the NCM was then air-dried for visual observation and storage.

RESULTS

Cross-absorption of antibodies

Initially, when DIA was applied to detect ZYMV in crude extracts of pumpkin leaves (protocol 3), the high non-specific reaction was observed in healthy samples. The reaction then was overcome when gamma-globulin was cross-absorbed. Prior to depositing the gamma-globulin on NCM, it was diluted with extracts of healthy leaves (1 : 50) in 0.2% BSA in PBS-TVP (antibody buffer) to a concentration of 4 μg/ml and the mixture was incubated for 4 hr at 37 C or overnight at 6 C. This procedure was consequently adopted to prepare the gamma-globulin or the conjugate which was deposited just after the antigen application in all experiments.

Effect of extracting buffers

To determine the optimal conditions of DIA, three kinds of extracting buffers for the antigen which were also used as coating buffers (0.05 M carbonate buffer, pH 9.6, 0.02 M PBS, pH 7.4, and TBS) were evaluated. All three resulted in good detection of ZYMV (Fig. 1). For convenience, carbonate buffer was selected as the extracting and coating buffer in all experiments.

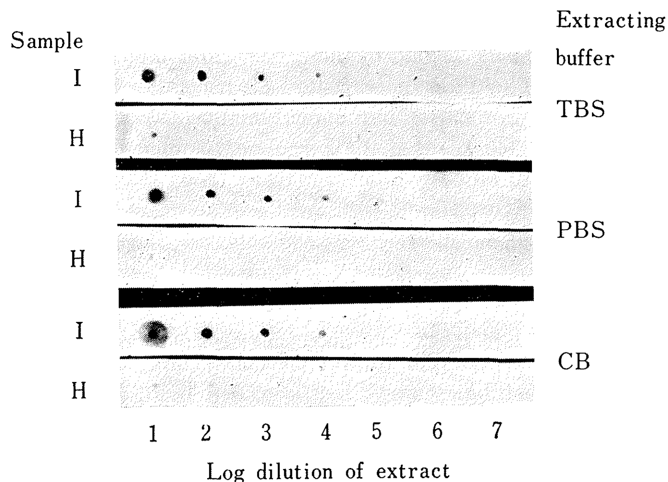

Fig. 1. Sensitivity of dot-immunobinding assay (DIA) for detection of zucchini yellow mosaic virus (ZYMV) using three kinds of extracting buffer. One gram of infected (I) or healthy (H) pumpkin leaf was ground with 1 ml of 0.02 M Tris-HCl buffer containing 0.5 M NaCl, pH 7.5 (TBS), 0.02 M phosphate buffered saline, pH 7.4 (PBS), or 0.05 M carbonate buffer, pH 9.6 (CB), and the extracts were assayed by non-precoated indirect-DIA (protocol 3, Table 1).

Comparative sensitivity of different immunoenzymatic assay

Since considerable differences in the detectability of antigen have been observed with different protocol of immunoassays on microtitre plates of ELISA[1,8], comparative tests of seven different protocols of DIA were made to determine whether the protocol of immunoassays influenced the sensitivity of DIA. The results showed that all protocols tested were applicable in DIA, and were capable of detecting purified ZYMV and discriminating between crude extracts from healthy and infected pumpkin leaves.

The relative sensitivities of the DIA protocols were determined by comparing the ability of 7 protocols to detect ZYMV in purified preparations and in crude extracts of infected pumpkin leaves (Table 1). Protocols 3 and 4 were found to be the most sensititive. They were able to detect ZYMV in purified preparations at a minimum amount of 20 pg and in crude extracts of infected leaves at the dilution end point of 10^{-5}.

Protocols 1 and 6 possessed the moderate sensitivities, whereas protocols 2, 5, and 7 had the lowest sensitivities.

Persistence of DIA reactivity in stored samples

In practice, analysis of a large number of samples is relatively cheaper and more efficient than that of a small number. Thus, if the number of available samples is small, it would be convenient to store the antigen-dotted NCM until a large number of samples is collected. To evaluate the persistence of DIA reactivity of ZYMV in stored samples, the following experiment was performed. NCM was dotted with antigen in crude extracts at dilution of 10^{-1} and air-dried for 5 min. It was then slipped between two pieces of filter papers and put into plastic boxes containing 100 g of $CaCl_2$. After being tightly closed, three boxes were separately stored in temperature of -20 C, 6 C, and 25 C, respectively. The samples were tested every two weeks using protocol 4 (Table 1) of DIA. It was found that the reactivity of ZYMV in DIA could be retained in all conditions tested for 6 months (the longest time tested).

Persistence of DIA reactivity in mailed samples

While the basic equipment for serodiagnosis is relatively simple, at present time it will not be easily obtainable especially in developing countries. For the limited purposes, the problem could be overcome by sending fresh infected plants to the well-equipped laboratories to be tested. A potential drawback in sending fresh samples is the risk of spreading the virus in another areas. Thus, we tried to develop a method of mailing samples for DIA such that we could avoid the direct sending of fresh samples. After air-drying, the virus-dotted NCM was well sealed in plastic bags. The bags were then sealed in paper envelopes, and mailed to the Laboratory of Plant Pathology, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia, with instruction to return the plastic bag as soon as possible.

In the first experiment, we received the samples 15 days after mailing, whereas in the second trial we received the samples 27 days after mailing. Results obtained by procedure 4 of DIA showed that the antigenicity of ZYMV could be retained in mailed samples.

Simple processing of test samples

To test if the time for preparation and deposition of samples to NCM could be reduced, the following experiment was performed. The leaf sample was cut to the size about 0.5×0.5 cm and placed on the NCM using forceps. Using the tip of micropipet, it was then macerated with 5 μl of carbonate buffer. After removing the debris, NCM was processed by DIA (protocol 4) as described above except that Triton X-100 was added into the blocking buffer and TBS-PVT. This procedure permitted us to process 4 samples in about 1 min. Figure 2 shows that the shape of spot obtained by this procedure was irregular, however, the assay was still able to discriminate clearly between healthy and infected leaves.

Specificity of DIA

To choose the appropriate protocols of DIA it is necessary to know the ability of the protocols to discriminate between closely related viruses, especially those which can infect the same crops. Because of their simplicity in application, procedure 1, 2, and 3 were selected for this

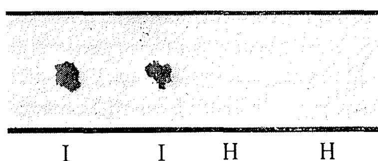

Fig. 2. Dot-immunobinding assay (DIA) for detecting zucchini yellow mosaic virus (ZYMV) in extracts of infected (I) and healthy (H) pumpkin leaves employing simple method for sample preparation. The sample was directly macerated on the nitrocellulose membrane (NCM) using a tip of micropipet with about 5 μl of carbonate buffer. The NCM was then assayed with non-precoated indirect-DIA (protocol 4, Table 1).

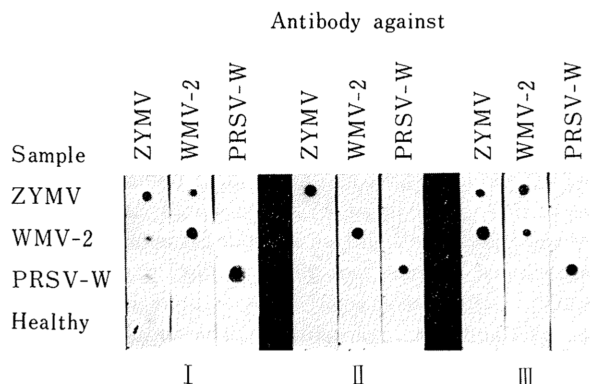

Fig. 3. Reciprocal dot-immunobinding assay (DIA) for zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus-2 (WMV-2), and papaya ringspot virus, type W (PRSV-W) using polyclonal antibodies. The crude extracts of pumpkin leaves (10^{-1} dilution) were dotted to nitrocellulose membrane, and were assayed with two-step direct-DIA (I), double antibody sandwich (DAS)-DIA (II), and non-precoated indirect-DIA (III).

experiment. The experiment was done by reciprocal assay employing three potyviruses of cucurbitaceous plants and their rabbit antisera, namely PRSV-W, WMV-2, and ZYMV. It was found that two-step direct-DIA (protocol 1) and non-precoated I-DIA (protocol 3) were able to discriminate between ZYMV and PRSV-W, but failed to discriminate between ZYMV and WMV-2 (Fig. 3-I and 3-III). Furthermore, discrimination of these three viruses could only be achieved easily by employing DAS-DIA (protocol 2) (Fig. 3-II).

The use with monoclonal antibody (MCA)

Since MCA against ZYMV has been produced and employed successfully to improve ELISA[21], the experiment was done to see if the same MCA (MCA ZYMV-45) could be used to improve the diagnostic utility of DIA. It was found that the use of MCA in the protocols 1, 2, and 3 of DIA resulted in the same sensitivities as those obtained by employing polyclonal antibody (data are not shown), but it could increase the ability of indirect- and two-step direct-DIA in discriminating between ZYMV and WMV-2 (Fig. 4).

DISCUSSION

The results reported here indicate that the applicability of DIA for detecting ZYMV competes with the well established serological method of ELISA[12,18,19] and others[9,20]. In the preliminary experiment, DIA could not achieve high accuracy for virus detection, mainly because of its non-specific reaction with samples of healthy pumpkin leaves. Success in eliminating this

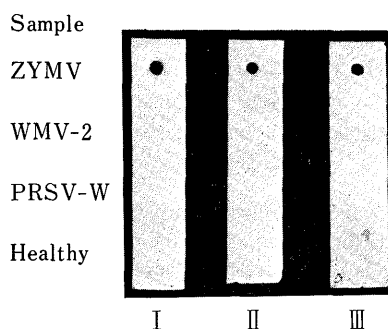

Fig. 4. Demonstration of the ability of monoclonal antibody against zucchini yellow mosaic virus (ZYMV) to discriminate ZYMV from watermelon mosaic virus-2 (WMV-2) and papaya ringspot virus, type W (PRSV-W) in crude extracts of pumpkin leaves (10^{-1} dilution) using double antibody sandwich dot-immunobinding assay (DAS-DIA) (I), two-step direct-DIA (II), and indirect-DIA (III).

non-specific reaction by using cross-absorbed antibodies or a highly specific monoclonal antibody indicates that the problem was caused by the characteristics of the antibodies used and not by the poor performance of the assay system. The excellent protein-binding properties of the NCM used in DIA have probably allowed the assay to easily show the "noise" of the antibody which was not measurable by using other assays.

Among the DIA protocols compared, the two-step direct-DIA in which NCM-bound virus was directly assayed using an anti-virus conjugate, has not been widely used in the field of plant pathology. While the sensitivity of two-step direct-DIA was comparable to that of others, the use of this protocol could significantly simplify the DIA procedure by reducing the time and number of steps of the assay. This protocol has also been successfully employed to detect purified goat anti-human IgG[3].

The persistence of the DIA reactivity of ZYMV in the stored and mailed samples should aid in the development of diagnosis and virus identification by post which can allow cooperation between well-equipped laboratories and districts where facilities for diagnosis are unavailable. Sending of sample-dotted NCM is obviously easier and cheaper than sending fresh materials for immunoelectron microscope[13] or microtitre plates for the use with conventional ELISA[11]. If neccessary, the sample-dotted NCM may also be treated by glutaraldehyde to further destroy the virus infectivity, thereby completely eliminating the risk of spreading the virus by transportation[5]. The treatment with glutaraldehyde has been also reported to enhance the adherence of antigen to NCM without destroying its antigenicity[14].

The simple method of sample deposition to NCM mentioned above seems to be promising for further use in routine field surveys not only for ZYMV but also for other viruses which infect cucurbit plants. The method is rapid and requires only a simple device, thus allowing it to be used anywhere during the survey. The use of NCM to collect the samples during a field survey has the other advantages of being easy to carry and avoiding deterioration of the samples during long distance transportation. In practice, multiple deposition of a sample is required to enable us to assay a sample using several antibodies against different viruses.

Another crucial point in the DIA procedure, when it is employed to detect virus in field samples, is its ability to discriminate between closely related viruses. In agreement with the results obtained using microtitre plates[21], it was found that the protocol of DIA greatly influenced the specificity of the assay. Thus the DIA, employed a double antibody sandwich form of assay resulting in the narrow specificity, and that employed indirect form gave the broader specificity.

The utility of MCA ZYMV-45 to discriminate between ZYMV and WMV-2 in double antibody sandwich and indirect forms of immunoenzymatic assay has been previously demon-

strated using conventional ELISA[21]. Results obtained in the present study show that the same MCA could also be used to discriminate between the viruses in DIA even in the simplest protocol tested, namely two-step direct-DIA (protocol 1, Table 1).

Finally, it is anticipated that increasing the sensitivity and simplicity of DIA will lead to DIA's rapid adoption for diagnosis of many viruses, especially in poorly- or moderately-equipped laboratories.

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和 文 摘 要

Susamto SOMOWIYARJO・佐古宣道・野中福次：ポリクローナル抗体とモノクローナル抗体によるズッキーニ黄斑モザイクウイルス (ZYMV) の dot-immunobinding assay (DIA)

DIA により ZYMV の検出実験を試みた結果，このウイルスのニトロセルロース膜 (NCM) への結合能は，試験した3種類の緩衝液間で，差異を認めなかった。ZYMV の抗原性は罹病葉の汁液を塗付した NCM を −20C，6C あるいは 20C下で6カ月間保存したのちでも，失われず，DIA によりウイルスの検出ができた。罹病葉の汁液を塗付した NCMを生葉試料の代りに郵送する方法について試験し，罹病葉から NCM 上に試料を塗付する簡易な手法を工夫した。7種類のプロトコールを用いた DIA すべてにより，健全と罹

病カボチャ葉が識別でき，その検出限界は罹病葉の汁液で 10^{-3}～10^{-5} 倍希釈であった。ZYMV に対するポリクローナル抗体を用いる直接法および間接法では，watermelon mosaic virus 2 (WMV-2) との交差反応が認められたが，二重抗体法では認められなかった。これらの DIA では，papaya ringspot virus, type W (PRSV-W) との反応は認められなかった。ZYMV に対するモノクローナル抗体を用いて，これら 3 種類の DIA を試みたところ，いずれの方法でもポリクローナル抗体を用いたときと同じ感度を示し，ZYMV と WMV-2 および PRSV-W を識別できた。