

ズッキーニ黄斑モザイクウイルス(ZYMV)検出のためのモノクローナル抗体の利用

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	Somowiyarjo,S. 佐古,宣道 野中,福次
発行元	日本植物病理學會
巻/号	54巻4号
巻号補足	
掲載ページ	p. 436-443
発行年月	1988年10月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



The Use of Monoclonal Antibody for Detecting Zucchini Yellow Mosaic Virus*

Susanto SOMOWIJARJO**, Nobumichi SAKO***,**** and Fukuji NONAKA***

Abstract

Production and diagnostic utility of monoclonal antibody (MCA) to zucchini yellow mosaic virus (ZYMV) in enzyme-linked immunosorbent assay (ELISA) are described. Four selected hybridomas produced MCA of the IgG_{2b} subclass, and the titre of these MCA in ascitic fluid ranged between 10⁷ and 10⁹ which were 1,000 times higher than those in culture fluid. Of the six procedures of ELISA tested, the most sensitive result was obtained when the plates were coated with MCA and rabbit polyclonal antibody (PCA) was used as intermediate antibody (procedure 5). This assay was able to detect ZYMV in purified preparation at detection end point of 0.1~1 ng/ml and in crude extracts of pumpkin leaves at dilution end point of 10⁶~10⁷. The use of MCA ZYMV-45 in non-precoated I-ELISA could discriminate ZYMV and watermelon mosaic virus 2 (WMV-2). Thus, the result represented a major advance in the detection of ZYMV which the use of PCAs in the same assay could not discriminate these two viruses. This assay gave also strong positive reactions with all of ZYMV-infected samples that reacted with PCA in double-antibody sandwich ELISA, thereby providing specific diagnostic tool for ZYMV which is needed for guiding the management of potyviruses on cucurbits.

(Received March 8, 1988)

Key words: zucchini yellow mosaic virus, monoclonal antibody, ELISA.

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV) is one of the world's important members of the potyvirus that recently has been reported as a new destructive pathogen of commercial cucurbits in many areas^{4,11,12,15-17,19-21}. Strong dependency of successful management of this disease on the accurate diagnosis has increased the demand for establishment of rapid and specific diagnostic procedures.

Enzyme-linked immunosorbent assay (ELISA)^{2,3,7,8,13-15,23,24} and other serological assays^{21,25} using polyclonal antibodies (PCA), while providing rapid and sensitive method for diagnosis, are dependent on the specificity and avidity of the antisera used, which are known to vary greatly. In addition, since ZYMV is antigenically related to other viruses^{11,21}, the antisera may cross-react with the related viruses. Current progress in serological assays, specially the adoption of techniques for preparation of monoclonal antibodies (MCA)⁹ has offered the possibility to overcome these problems.

In this paper, the production and diagnostic utility of MCA to ZYMV are described. An abstract of some of these results had been previously reported²⁷.

* This research was supported by a Grant-in-Aid for Developmental Scientific Research I No. 61860005 to the second author from the Ministry of Education, Science and Culture, Japan

** Faculty of Agriculture, Gadjah Mada University, Yogyakarta 55581B, Indonesia ガジャマダ大学農学部

*** Faculty of Agriculture, Saga University, Saga 840, Japan 佐賀大学農学部

**** To whom correspondence should be addressed.

MATERIALS AND METHODS

Viruses and polyclonal antibodies. ZYMV (isolate 169) and watermelon mosaic virus 2 (WMV-2, isolate 18) were obtained from pumpkins cultivated in Okinawa and Miyazaki Prefectures, respectively, and were maintained in pumpkin (*Cucurbita maxima* cv. Hokoakawa) under greenhouse conditions. They were then purified using a method originated by Sako *et al.*²²⁾ The purified viruses were stored at -80°C before use as immunogen or as antigen in the assays.

Gamma-globulin against ZYMV from rabbit antiserum and immunoglobulin from egg yolks of immunized Japanese quails (*Coturnix coturnix japonica* Temminck *et* Schlegel) were the same as in the previous studies²³⁻²⁶⁾. Rabbit antiserum to WMV-2 was kindly provided by Dr. V. Lisa, Italy. Isolate 18 reacted clearly with this antiserum (unpublished data).

Immunoglobulin from rabbit anti-chicken IgG (Miles Lab. Inc.) or anti-WMV-2 was prepared by the method described by Clark and Adams³⁾.

Conjugates. Alkaline phosphatase-labeled affinity-purified immunoglobulins from rabbit antiserum against mouse IgG+IgM (H+L) and goat antiserum against rabbit IgG (H+L) were obtained from Kirkegaard and Perry Lab. Inc., and were used at dilution of 5,000 and 1,000 times, respectively. The conjugation of alkaline phosphatase with IgG from rabbit antiserum against WMV-2, ZYMV, and chicken IgG or with IgG_{2b} from MCA was done by method described by Clark and Adams³⁾. These prepared conjugates were used at dilution of 750, 1,000, 2,000, 5,000 times, respectively.

Immunization of mice, cell fusion and cell culture. Two six-week-old BALB/c mice were employed for immunization. Each mouse was given three times of intraperitoneal injections with 4 weeks between injection, using 20 μg , 40 μg and 60 μg of purified virus, successively. The virus was mixed with an equal volume of Freund's complete adjuvant at the first injection, and with incomplete adjuvant at the following injections. Intravenous booster was given at the day of 90, with 100 μg of virus.

Three days after booster, the mice were sacrificed and the spleens were removed aseptically. The B-lymphocyte cells from the spleens were fused with mouse myeloma cells P3-X63-Ag8-U1 using the procedure as described by Köhler and Milstein⁹⁾. Eleven days after fusion, supernatant fluids from growing culture were assayed for the present of specific antibody using non-precoated indirect ELISA (I-ELISA) with the assay condition as used in the previous work²³⁾. This was done in the microtitre plate (Immulon I, Nunc) with antigen of 1 $\mu\text{g}/\text{ml}$ purified ZYMV. The selected hybridomas were cloned under condition of limiting dilution using mouse or rat peritoneal cells as the feeders. Cloning was repeated three times. The hybridomas that were not being propagated were stored in liquid nitrogen in fetal calf serum containing 10% of dimethyl sulfide.

Production and isotype determination of MCA. Ascitic fluids were produced by intraperitoneal injection of hybridomas into mice which had been given injection of pristane (1,6,14-tetramethyl pentadecana) two weeks earlier. Ascitic fluids were harvested 10-15 days after injection. A part of the ascitic fluid was used to prepare IgG using Protein A-Sepharose CL-4B (Pharmacia).

ELISA. Unless otherwise stated, the assay condition was same as that in the earlier experiments^{23,24)}, except that the plates were Immulon II (Nunc).

The diagnostic utility of MCA alone or its combination with PCA was evaluated in the six protocols of immunoenzymatic assays. The steps of the assays were shown at Table 2, using the acronyms as used by earlier workers¹⁾. All reagents were used at volume of 200 μl per well. Depending on the procedure used, the plates were firstly incubated with antigen or antibody in 0.05 M carbonate buffer, pH 9.6, for 4 hr at 37 $^{\circ}\text{C}$. After washing, the unoccupied binding sites on the plates were saturated by incubation with 0.1% bovine serum albumin (BSA) in 0.02 M

PBS, pH 7.4, for 1.5 hr. They were then incubated sequentially with the reagents as listed in Table 2. Following the incubation with conjugate, the plates were incubated with 1 mg/ml of *p*-nitrophenol phosphoric acid disodium salt (Nakarai Chem. Ltd.) for 1.5 hr at 25 C. The ELISA absorbance values were measured with Easy Reader EAR 400 FW (SLT-LABINSTRUMENTS), without terminating the reaction.

RESULTS

Characterization of MCA

Of the 256 viable cell cultures, 36 secreted antibodies that reacted positively with ZYMV purified preparation (absorbance values in I-ELISA varied from 0.201 to 1.497). Five hybridomas producing high titre antibodies (A_{405} more than 0.70) were cloned, and the remaining hybridomas were stored. Of the five hybridomas generated after three times cloning, four secreted immunoglobulins of the IgG_{2b} subclass which were specific for ZYMV and one secreted immunoglobulin of the IgM subclass which was specific for plant components. The titres of these immunoglobulins in ascitic fluids ranged between 10^7 and 10^9 which were 1,000 times higher than those in culture fluids (Table 1). The MCA-45 having the highest titre of $10^8 \sim 10^9$ was employed in the following experiments.

In the preliminary experiments with non-precoated I-ELISA (procedure 1, see Table 2), 1,000 times dilution of culture fluid and 1,000,000 times dilution of ascitic fluid were found to be the optimum of MCA dilutions and were therefore used for the subsequent assays.

The potential use of MCA from culture media

The usefulness of MCA derived from culture media was evaluated with non-precoated I-ELISA. As can be seen in Fig. 1, the assay was very sensitive, capable of detecting ZYMV in crude extracts from infected pumpkin plants at dilution end point of $10^6 \sim 10^7$. The minimum detectable concentration of purified virus with this assay was 1~10 ng/ml.

Comparative sensitivity of different ELISA procedures

The diagnostic utility of the MCA for ZYMV in six different ELISA procedures was evaluated using MCA from the ascitic fluid with the same virus preparation. The relative sensitivity of these assays with the purified preparation is shown in Table 2. The results indicated that MCA-45 can be used as either intermediate (procedure 1~3) or coating (procedure 4~6) antibodies of ELISA. The MCA reacted well either with ZYMV bound directly to polystyrene plate (procedure 1) or with the virus trapped by polyclonal antibodies from Japanese quail (procedure 2) or from rabbit (procedure 3).

The relative sensitivity of the assays with extracts from infected pumpkin plants is given in Fig. 2. The non-precoated I-ELISA procedure resulted in the same sensitivity as that obtained

Table 1. Partial properties of selected monoclonal antibodies (MCA) against zucchini yellow mosaic virus (ZYMV)

MCA	Subclass	Titre by I-ELISA	
		Culture media	Ascitic fluid
ZYMV-15 ^{a)}	IgM	n.t. ^{b)}	n.t.
ZYMV-44	IgG _{2b}	$10^4 \sim 10^5$	$10^7 \sim 10^8$
ZYMV-45	IgG _{2b}	$10^5 \sim 10^6$	$10^8 \sim 10^9$
ZYMV-75	IgG _{2b}	$10^4 \sim 10^5$	$10^7 \sim 10^8$
ZYMV-147	IgG _{2b}	$10^4 \sim 10^5$	$10^7 \sim 10^8$

a) MCA ZYMV-15 was specific to host component.

b) n.t. = not tested.

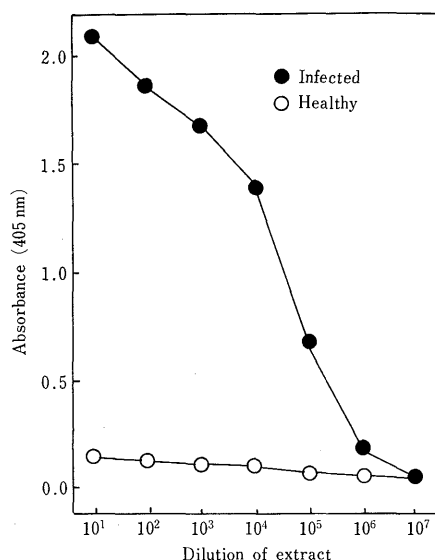


Fig. 1. Detection of zucchini yellow mosaic virus in crude extracts of pumpkin plants by non-precoated indirect enzyme-linked immunosorbent assay (procedure 1, Table 2) using MCA ZYMV-45 from culture media. Each point is the mean of the three experiments using three wells in each experiment.

Table 2. Enzyme-linked immunosorbent assay procedures used for evaluating the reactivity of monoclonal antibody against zucchini yellow mosaic virus (ZYMV)

Procedure	Summary of successive steps of assay ^{a)}	Minimum detectable concentration of purified ZYMV, $\mu\text{g/ml}$
1.	Ag; MCA; anti-M ^R -E	0.01~0.001
2.	ABQ; Ag; MCA; anti-M ^R -E	0.01~0.001
3.	AB ^R ; Ag; MCA; anti-M ^R -E	0.01~0.001
4.	MCA; Ag; ABQ; anti-CH ^R -E	0.1~0.01
5.	MCA; Ag; AB ^R ; anti-R ^G -E	0.001~0.0001
6.	MCA; Ag; AB ^R -E	0.1~0.01

a) Ag: antigen; AB: antibody; MCA: monoclonal antibody; Q: Japanese quail; R: rabbit; anti-M^R: rabbit anti-mouse globulin; -E: enzyme label; G: goat; CH: chicken.

using MCA from culture media, whereas the use of PCA to trap the virus on the plates failed to increase the sensitivity of the assay (Fig. 2A).

The performance of MCA ZYMV-45 as coating antibodies of ELISA to immobilize ZYMV from crude extract of infected leaves was evaluated using three procedures of tests (Fig. 2B). As the results, the MCA could be effectively employed as coating antibodies of the ELISA, but the sensitivity of the assay was influenced by the kind of reagents applied after the virus was immobilized. The same sensitivity was obtained when quail immunoglobulin was used as intermediate antibodies followed by the conjugate of enzyme-labeled anti-chicken IgG immunoglobulin (procedure 4) or when immobilized virus was treated with enzyme-labeled IgG from rabbit anti-serum (procedure 6). The highest sensitivity was found by the assay employing intermediate antibody of IgG from rabbit antiserum (procedure 5).

Serological specificity of MCA in ELISA

ZYMV and WMV-2 are serologically related viruses that often infect the same host^(11,21).

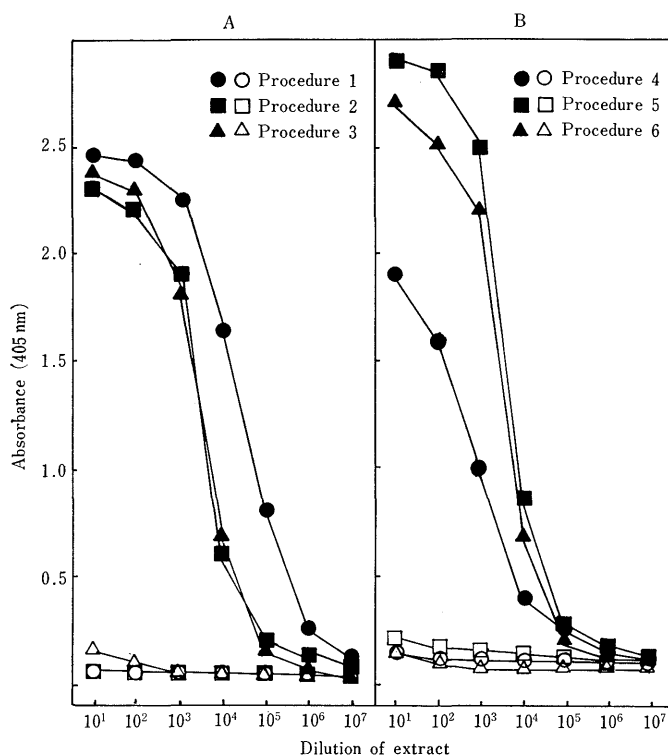


Fig. 2. Comparison of the sensitivity of enzyme-linked immunosorbent assay (ELISA) procedures using intermediate (A) and coating (B) antibodies of MCA ZYMV-45 from ascitic fluid for detecting zucchini yellow mosaic virus in crude extracts of healthy (O, □, △) and infected (●, ■, ▲) pumpkin leaves. See Table 2 for a description of the ELISA procedures. Each point is the mean of three experiments using three wells in each experiment.

Table 3. Cross-reactivity of zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2 (WMV-2) in two enzyme-linked immunosorbent assay (ELISA) procedures using polyclonal antibody (PCA) and monoclonal antibody (MCA)^{a)}

Procedure of assay	Antibodies ^{b)}	Absorbance at 405 nm with virus	
		ZYMV	WMV-2
Double-antibody sandwich ELISA	Rabbit anti-ZYMV	0.901	0.102
	Rabbit anti-WMV-2	0.093	0.540
	MCA ZYMV-45	0.837	0.076
Indirect ELISA	Rabbit anti-ZYMV	2.576	1.478
	Rabbit anti-WMV-2	0.901	1.411
	MCA ZYMV-45	2.221	0.080

a) The viruses were used as antigen of the assays at concentration of 1 µg/ml.

b) The PCA and MCA globulins were used at concentration of 2 µg/ml and 0.1 µg/ml, respectively.

Thus, an experiment was done to evaluate the ability of the ELISA procedures to discriminate these viruses using PCA and MCA. The result is given in Table 3.

It was found that double-antibody sandwich (DAS)-ELISA could easily discriminate between

Table 4. Detection of zucchini yellow mosaic virus in field samples using polyclonal antibody (PCA) in double-antibody sandwich (DAS)-ELISA and monoclonal antibody (MCA) in indirect (I)-ELISA ^{a)}

Crop	No. of samples		
	Total	Reacted with PCA in DAS-ELISA	Reacted with MCA in I-ELISA
<i>Cucumis sativus</i> L.	29	24	24
<i>Cucurbita maxima</i> Duch.	40	39	39
<i>Luffa cylindrica</i> Roem.	1	1	1
<i>Momordica charantia</i> L.	4	1	1

a) The crude extracts of samples were used as antigen at dilution of 100 times. The average of absorbance values at 405 nm obtained with PCA and MCA were 0.721 and 1.741, respectively.

ZYMV and WMV-2, regardless of the kind of antibodies used. By contrast, I-ELISA could discriminate these two viruses only when MCA was used.

Diagnostic utility of MCA

To examine the diagnostic utility of the MCA for ZYMV that naturally infected commercially grown cucurbit crops, 74 of samples were collected from Miyazaki Prefecture and assayed with MCA ZYMV-45 in non-precoated I-ELISA. These samples were also assayed with PCA from rabbit antiserum in DAS-ELISA using the assay conditions as previously reported²³⁾. Table 4 shows that ZYMV could be detected on *Cucumis sativus* L., *Cucurbita maxima* Duch., *Momordica charantia* L., and *Luffa cylindrica* Roem. by using the two ELISA procedures.

DISCUSSION

This study demonstrated that the MCA to ZYMV, which is obtainable in very high titre, can be employed for detection of the virus using the methods described. While the application of these methods with PCA for virus diagnosis is limited by the availability and variability of the antisera^{10,14,18)}, the availability of large quantities of high specific MCA to ZYMV would aid in providing diagnostic information of this virus.

As previously reported with other viruses such as tobacco mosaic virus^{1,5)} and potato virus X⁸⁾, the structural form of the virus immobilized on antibody-coated plates used in ELISA trends to be present as intact particle and that directly on the plates is as denatured one. Since MCA may recognize antigen only in one of these structural forms, the suitability of the protocols of ELISA used to detect antigen employing MCA should be examined carefully. The good reactivity of the MCA to ZYMV particles directly bound to polystyrene plates (procedure 1, see Table 2) and also with antibody-trapped virus particles (procedure 3 and 4) indicated that the MCA could recognize the binding site that is accessible in intact or partially denatured virus particles. This would be advantageous, offering the use either with precoated or non-precoated I-ELISA procedures.

In some applications, the usefulness of non-precoated I-ELISA was limited by the fact that binding of virus particles on the plates could be strongly inhibited by host components during the first coating step of ELISA^{7,13)}. The inhibition phenomenon was not observed in the present experiment as coating the plates with antibody from Japanese quail (procedure 2) or rabbit (procedure 3) failed to increase the sensitivity of the assays. This fact further supports the diagnostic utility of non-precoated I-ELISA for ZYMV. Additionally, while slight enhancement of the ELISA sensitivity could be obtained by the use of MCA as coating antibody (procedure 5), the use of MCA in non-precoated I-ELISA can be done in shorter time and does not require any effort for producing another PCA.

The applicability of serodiagnosis procedures depends to a large extent on their serological specificity with the related viruses. While broad range of serological specificity can be ideal for another purposes^{2,14}), it happens to decrease their usefulness in providing precise identification of the viruses. In agreement with previous workers^{2,7,14}), the broad specificity of I-ELISA employing PCA was observed (Table 3). Thus, it can give misdiagnosis of ZYMV with other serologically related virus, such as WMV-2. Conversely, the use of MCA in the same assay could successfully be used to discriminate the two viruses. The narrow specificity of I-ELISA using MCA as that of DAS-ELISA is attractive because the former assay can be done simply using a single antimouse IgG conjugate which is available commercially.

In addressing the limitation of MCA as diagnostic reagents, it has been reported that some MCA may be too specific for virus diagnosis^{6,18}), recognizing only rare isolates or small group of isolates in the fields^{14,18}). In the present study, the MCA could strongly react with field samples that reacted with PCA (Table 4). This indicated that the applicability of the MCA for diagnosis of ZYMV is not limited by the problem of too narrow specificity of the assays.

Finally, it is anticipated that in the future MCA-based assays might provide precise diagnosis and other tools for further epidemiological study on ZYMV.

We gratefully acknowledge the advices of Prof. Takeshi Watanabe, Kyushu University for setting up the hybridoma techniques. We are also indebted to the Hitachi Scholarship Foundation for providing scholarship to the first author.

Literature cited

1. Al Moudallal, Z., Altschuh, D., Briand, J.P. and van Regenmortel, M.H.V. (1984). *J. Immunol. Methods* 68: 35-43.
2. Clark, M.F. (1981). *Ann. Rev. Phytopathol.* 19: 83-106.
3. Clark, M.F. and Adams, A.N. (1977). *J. gen. Virol.* 34: 475-483.
4. Davis, R.F. and Mizuki, M.K. (1987). *Plant Disease* 71: 40-44.
5. Jaegle, M. and van Regenmortel, M.H.V. (1985). *J. Vir. Methods* 11: 189-198.
6. Kascak, R.J., Rubenstein, R., Merz, P.A., Tonna-DeMasi, P., Fersko, R., Carp, R.I., Wisniewski, H.M. and Diringer, H. (1987). *J. Virol.* 61: 3688-3693.
7. Koenig, R. (1981). *J. gen. Virol.* 55: 53-62.
8. Koenig, R. and Torrance, L. (1986). *Ibid.* 67: 2145-2151.
9. Köhler, G. and Milstein, C. (1975). *Nature* 256: 495-497.
10. Lankow, S.H., Woodhead, S.H., Patterson, R.J., Massey, R. and Schochetman, G. (1984). *Plant Disease* 68: 1100-1101.
11. Lisa, V. and Lecoq, H. (1984). *CMI/AAB Descriptions of Plant Viruses*. No. 282.
12. Lisa, V., Boccardo, G., D'Agostino, G., Dellavalle, G. and D'Aquilo, M. (1981). *Phytopathology* 71: 667-672.
13. Lommel, S.A., McCain, A.H. and Morris, T.J. (1982). *Ibid.* 72: 1018-1022.
14. Martin, R.R. (1985). *HortScience* 20: 837-845.
15. Menassa, R., Makkouk, K.M. and Abbasher, A.A. (1984). *J. Phytopathology* 115: 152-159.
16. Nameth, S.T., Dodds, J.A., Paulus, A.O. and Kishaba, A. (1985). *Plant Disease* 69: 785-788.
17. Nameth, S.T., Dodds, J.A., Paulus, A.O. and Laemmien, F.F. (1986). *Ibid.* 70: 9-11.
18. Nowinski, R.C., Tam, M.R., Goldstein, L.C., Stong, L., Kuo, C., Corey, L., Stamm, W.E., Handsfield, H.H., Knapp, J.S. and Holmes, K.K. (1983). *Science* 219: 637-644.
19. Ohtsu, Y., Sako, N. and Somowiyarjo, S. (1985). *Ann. Phytopath. Soc. Japan* 51: 234-237.
20. Provvidenti, R., Gonsalves, D. and Humaydan, H.S. (1984). *Plant Disease* 68: 443-446.
21. Purcifull, D.E., Adlerz, W.C., Simon, G.W., Hiebert, E. and Christie, S.R. (1984). *Ibid.* 68: 230-233.
22. Sako, N., Matsuo, K. and Nonaka, F. (1980). *Ann. Phytopath. Soc. Japan* 46: 639-646.
23. Somowiyarjo, S., Sako, N. and Nonaka, F. (1985). *Ibid.* 51: 569-575.
24. Somowiyarjo, S., Sako, N. and Nonaka, F. (1986). *Ibid.* 52: 653-659.
25. Somowiyarjo, S., Sako, N. and Nonaka, F. (1987). *Ibid.* 53: 266-268.
26. Somowiyarjo, S., Sako, N. and Nonaka, F. (1987). *Proc. Assoc. Pl. Prot. Kyushu* 33: 237 (Abstr.).
27. Somowiyarjo, S., Sako, N. and Nonaka, F. (1988). *Ann. Phytopath. Soc. Japan* 54: 85 (Abstr.).

和 文 摘 要

Susanto SOMOWIJARJO・佐古宣道・野中福次：ズッキーニ黄斑モザイクウイルス (ZYMV) 検出のためのモノクローナル抗体の利用

ZYMV に対するモノクローナル抗体 (MCA) を作製し、その有用性を ELISA 法により調べた。4 種類の抗体産生ハイブリドーマが産生する腹水中の MCA のサブクラスはすべて IgG_{2b} で、その力価は $10^7 \sim 10^9$ 倍で、培養上清液中でのそれは $1/1,000$ であった。試験した 6 種類の組合せの ELISA 法のうち、最高の感度を示したのは、プレートに MCA でコートし、ポリクローナル抗体 (PCA) を二次抗体として用いたときであった。この方法は、純化 ZYMV で $0.1 \sim 1 \text{ ng/ml}$ 、カボチャ罹病葉粗汁液中で $10^6 \sim 10^7$ 倍希釈までの検出限界を示した。MCA (ZYMV-45) を間接 ELISA 法 (一次抗体を処理しない方法) で用いると、ZYMV と watermelon mosaic virus 2 を識別できた。したがって、同法に PCA を用いた場合、この 2 種のウイルスを区別できなかったが、この MCA を用いる方法では、両ウイルスの識別が可能となった。本法により圃場から採集された試料について検出試験を行ったところ、PCA を用いた二重抗体法による判定結果とすべて同一であった。以上の結果は、ウリ科作物の病原 potyvirus を区別する場合に、有用な方法を作出したことを明らかにしている。