イネ縞葉枯ウイルス(RSV)圃場検診のための迅速･簡易 ELISA 法

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Rapid and Simplified ELISA for Routine Field Inspection of Rice Stripe Virus

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Key words: rapid ELISA, simplified ELISA, rice stripe virus, dissociation, storage.

Rice stripe virus (RSV), which causes the most serious disease of rice in Japan, is transmitted by the small brown planthopper (*Laodelphax striatellus*) in a persistent manner. The virus is also transovarially transmitted in a high percentage[1]. The incidence of the disease is known to be correlated with the percentage of the viruliferous insects. Therefore, serological assays, such as the passive hemagglutination test (PHA) and latex flocculation test (LF), have been employed every spring for routine field inspection of RSV in individual insects. Although both methods have sufficient sensitivity, each has its characteristic shortcoming, such as the low stability of the red blood cells in PHA and the difficulty of judging the reaction by naked eye observation in LF. To solve these problems, we considered the alternative of using enzyme-linked immunosorbent assay (ELISA), a reliable and highly sensitive method for detecting RSV in plants and insect vectors[2]. The major constraint to the more widespread use of ELISA is the time involved; it normally requires 2-3 days to complete. This problem was solved by the simplified ELISA method which should be excellent for mass-indexing RSV detection.

The antiserum against RSV used had a titer of 1: 2,048 in a preciptin ring interface test. The γ-globulin and its enzyme conjugate were prepared according to Clark and Adams[3]. RSV infected rice plants were maintained and proliferated using viruliferous insects that had fed on infected rice plants.

We first examined rapid ELISA[4], which reduces the incubation time of standard ELISA[3]. Microplates (Immuron 2, Dynatech Laboratories, Inc.) were coated with 1 μg/ml of γ-globulin. The conjugate was diluted to 1: 2,000. Both healthy and infected leaf extracts were diluted in PBS-Tween. Reciprocals of dilution end points of $A_{410} \geqq 0.3$ measured by MR580 microplate autoreader (Dynatech Laboratories, Inc.) were definitely judged as positive reactions by naked eye observation and recorded as

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1) Toriyama, S. (1983). CMI/AAB Description of Plant Viruses No. 269. 2) Horita, M., Tsushima, S., Uyeda, I. and Shikata, E. (1983). Mem. Fac. Agr. Hokkaido Univ. 13 : 551-558. 3) Clark, M. F. and Adams, A. N. (1977). J. Gen. Virol. 34 : 475-483. 4) Korpraditskul, P., Casper, R. and Lesemann, D. E. (1979). Phytopath. Z. 96 : 281-285.

the dilution end point (Table 1, 2).

No coloring was observed by the naked eye in the healthy control samples. Maximum dilution end point (32,000) was observed in 2 hr for coating and 1 hr for both sample and conjugate incubation at 37 C (Table 1). Thus, the complete ELISA process could be carried out with high sensitivity in 5 hr or less compared to the 30 hr normally required. No extensive increase in dilution end point was observed by prolonged incubation times in each treatment. The dilution end point was 4,000 when the plate was incubated 10 min for both coating and sample incubation, and 15 min for conjugate incubation at 37 C in rapid ELISA. This end point did not increase if the incubation time for coating was prolonged from 10 min at 37 C to overnight at 4 C. However, the dilution end point increased up to 8,000 times when the conjugate diluted to 1 : 500 was used in the above procedure (Table 1).

Table 1. Effect of incubation time on ELISA procedure

Incubation time			Reciprocal of dilution at $A_{410} \geqq 0.3$
Coating	Sample	Conjugate[a]	
10 min (37 C)	3 hr (37 C)	3 hr (37 C)	16,000
30 min (37 C)	3 hr (37 C)	3 hr (37 C)	16,000
1 hr (37 C)	3 hr (37 C)	3 hr (37 C)	16,000
2 hr (37 C)	3 hr (37 C)	3 hr (37 C)	32,000
3 hr (37 C)	3 hr (37 C)	3 hr (37 C)	32,000
Overnight (4 C)	10 min (37 C)	3 hr (37 C)	16,000
Overnight (4 C)	30 min (37 C)	3 hr (37 C)	16,000
Overnight (4 C)	1 hr (37 C)	3 hr (37 C)	32,000
Overnight (4 C)	2 hr (37 C)	3 hr (37 C)	32,000
Overnight (4 C)	3 hr (37 C)	3 hr (37 C)	32,000
Overnight (4 C)	overnight (4 C)	10 min (37 C)	8,000
Overnight (4 C)	overnight (4 C)	30 min (37 C)	16,000
Overnight (4 C)	overnight (4 C)	1 hr (37 C)	32,000
Overnight (4 C)	overnight (4 C)	2 hr (37 C)	32,000
Overnight (4 C)	overnight (4 C)	3 hr (37 C)	32,000
10 min (37 C) Overnight (4 C)	10 min (37 C)	15 min (37 C)	4,000→8,000[b]

a) The conjugate diluted to 1:2,000 was used.
b) The concentration of conjugate dilution was increased to 1:500.

The other method examined was the simplified ELISA in which the number of manipulations is reduced by simultaneous incubation of virus antigen and conjugate with solid-phase antibody[5]. We studied the effect of incubation time on the sensitivity of simplified ELISA. A 25 μl conjugate diluted to 1 : 250 was combined with a 175 μl sample in each well making the final concentration of conjugate dilution 1 : 2,000. The maximum dilution end point (16,000) was obtained when the sample-conjugate mixture

5) Stobbs, L. W. and Barker, D. (1985). Phytopathology 75 : 492-495.

was incubated for 1 hr at 37 C (Table 2). The dilution end point slightly decreased to 8,000 when the incubation time was decreased to 15 min. Virus antigen in infected plant was detected up to 16,000 when the conjugate concentration was 1: 62.5 (final concentration 1: 500). Although the incubation times could be reduced to 10, 10 and 15 min for the coating, sample and conjugate incubation periods, respectively in rapid ELISA, the process is much more simple in the simplified ELISA because the virus incubation and the following washing could be eliminated without serious loss of sensitivity.

Table 2. Effect of incubation time on simplified ELISA

Incubation time		Reciprocal of dilution at $A_{410} \geqq 0.3$
Coating (4 C)	Sample-Conjugate[a)] (37 C)	
Overnight	15 min	8,000→16,000[b)]
	30 min	8,000
	1 hr	16,000
	2 hr	16,000

a) The concentration of final conjugate dilution was 1:2,000.
b) The concentration of conjugate dilution was increased to 1:62.5 (final concentration 1:500).

The simplified ELISA was used for large-scale field testing of viruliferous individual insects. One insect was put into each well of a coated plate which had been washed. The insect was smashed with a vinyl cloride rod (φ6 mm), which was then wiped with a paper towel and used repeatedly. Next, 200 μl of conjugate was added to each well, and the plate was incubated at least 15 min at 37 C and subjected to the assay described above. The time involved included a 15-min incubation, washing before and after the introduction of sample and conjugate, addition of substrate and 15 min to 1 hr incubation after the last procedure. Thus, one cycle of testing could be completed within 2 hr with this simplified ELISA.

The effect of preserving the coated plates on the sensitivity was examined. Plates with γ-globulin were stored for 1, 3, 6, and 8 months at 4 C after incubation for 1 hr at 37 C and then used for simplified ELISA to detect the virus antigen in infected plants. No significant decrease in sensitivity was observed with up to 6 months of storage. Plates kept for 8 months showed decreased sensitivity and slightly increased non-specific reaction to healthy leaf extracts. Thus, plates coated with γ-globulin can be successfully stored for 6 months in a refrigerator. The plates stored in a freezer also could be used (−10 C, data not shown).

Reuse of the microplates was considered by examining the elution of RSV antigens and their respective conjugates from the plate-bound antibody using a method shown before[6)]. Plates previously used for the ELISA were washed twice with PBS-Tween, rinsed with distilled water, and finally shaken dry. They were then incubated in 0.2 M glycine-HCl (pH 2.2) buffer, 0.1 M HCl or PBS-Tween for 1 hr at room temperature.

6) Bar-Joseph, M., Moscviz, M. and Sharafi, Y. (1979). Phytopathology 69 : 424-426.

After removal of the solution, the plates were washed three times with PBS-Tween and then virus-containing or healthy leaf extracts were added to each well and processed by the simplified ELISA. The dissociation efficiency (DE) of various treatments was calculated using the formula of Bar-Joseph[6],

$$DE=\left(1-\frac{Aa-Ab}{Ab-Ac}\right)\times 100$$

in which

Aa=ELISA value (A_{410} nm) in wells that contained infected sap before and healthy sap after elution

Ab=infected sap both before and after elution

Ac=healthy sap both before and after elution.

The 0.2 M glycine-HCl (pH 2.2) buffer gave DE=89% and 0.1 M HCl gave DE=92%. Treatment of PBS-Tween as a control gave DE=60%. Thus, the plates could be reused to great economic advantage. New plates for coating were not needed and could be processed from the antigen incubation. Although a slight non-specific reaction was observed, the recycled plates proved to be useful because of the pronounced reactions of positive ones.

In additional experiments, γ-globulin bound to the microplate was thoroughly removed principally according to the method shown by Banttari and Petersen[7]. The plate soaked in a 1.5 M NaOH-ethanol mixture for 1 hr was washed with distilled water and dried. The cleaned plate could be used repeatedly and interchangenably for ELISA tests of different viruses (data not shown).

We conclude by emphasizing the convenience of our simplified ELISA for routine field inspection of RSV. It can be done using stored plates or recycled plates and is more efficient than the first-mentioned rapid ELISA. Simplified ELISA also offers adequate sensitivity when compared with standard ELISA and should be very useful for mass-indexing programs, as much as PHA or LF, in routine RSV detection.

和 文 摘 要

高橋義行・大村敏博・匠原監一郎・土崎常男：イネ縞葉枯ウイルス（RSV）圃場検診のための迅速・簡易 ELISA 法

多数の保毒虫および罹病イネからイネ縞葉枯ウイルス（RSV）を迅速に検出する目的で，酵素結合抗体法（ELISA 法）の簡易化を検討した。抗体を含むコーティング液を加えたプレートは，4 C，半年間の保存の後にも有効に使用することができた。また使用済プレートは 0.1 M HCl による1時間の処理で RSV を解離させることによって再使用が可能となった。これらの保存または再使用プレートを用いることで，コーティング処理を省くことができ，さらに試料とコンジュゲートを同時に添加することで，従来の試料処理（一晩，4 C）も省略した簡易 ELISA 法を確立した。この簡易 ELISA 法を用いて，信頼度の高い診断を2時間以内に行なえるようになった。

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7) Banttari, E. E. and Petersen, A. C., Jr. (1983). Plant Disease 67 : 18-20.