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**APPLICATION OF
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A LARGE SCALE INDEXING OF POTATO
VIRUS X, POTATO VIRUS Y, AND
POTATO LEAFROLL VIRUS**

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Introduction

Enzyme-linked immunosorbent assay (ELISA) was first applied to detection of plant viruses by VOLLER *et al.*¹⁷⁾. They showed that several ng of viral antigen per ml could be detected by ELISA. Its sensitivity, that is much higher than conventional serological techniques such as ring-interface precipitin test, microprecipitin test, and Ouchterlony agar gel double diffusion test^{2,18)}, made ELISA wide use in diagnosis of plant viruses. For example, there had been no effective serological method for diagnosis of potato leafroll virus (PLRV) except for immune electron microscopy^{6,11)} and the most common practice had been visual indexing of foliage where a large number of plants were required to be indexed. Since ELISA was introduced, PLRV can be easily detected in extracts from infected foliage, tubers, and roots of potato^{1,3,4,6,7,9,12,13,14)}.

In addition to its high sensitivity, ELISA can also be applied for processing a large number of samples in a short period, and indexing for several viruses at one time is possible. These advantages of ELISA promise its routine use in diagnosis of potato viruses. In this paper we applied ELISA to detections of potato virus x (PVX), potato virus Y (PVY) and PLRV in a large scale indexing and discussed practical problems encountered with it.

Materials and Methods

Production of antiserum and conjugated γ -globulin.

PVX. The viral antigen for antiserum production was purified from infected tomato leaves by the procedure outlined in Fig. 1. The antiserum with a titer of 1/4096 by a slide test was used for the conjugation with the alkaline phosphatase. Typical ELISA reactions are shown in Table 1. When healthy tomato leaves were used as an antigen [1 g leaves/100 ml 0.02 M phosphate buffer, pH 7.4, containing 0.85% NaCl and 0.05% Tween (PBS-Tween)] non-specific absorbance was negligible using concentrations of γ -globulin between 0.1-10 $\mu\text{g/ml}$ and of the enzyme conjugate between 150-600 folds dilutions. Under these conditions, 100 ng/ml of purified antigen could be easily detected. Concentrations of the coating γ -globulin and the enzyme-conjugate used for the large scale indexing were 0.2-1 $\mu\text{g/ml}$ and 400-500 folds dilutions, respectively.

PVY. The viral antigen for antiserum production was purified from frozen infected *Nicotiana sylvestris* leaves by the procedure outlined in Fig. 2. The antiserum with a titer of 1/512 by a microprecipitin test was used for the conjugation with the enzyme. Typical ELISA reactions are shown in Table 2. The ELISA values of healthy *N. sylvestris* leaves were nearly the same as those of PBS-tween buffer, using 0.1-10 $\mu\text{g/ml}$ of γ -globulin and 200-800 folds dilutions of the enzyme-conjugate. For the large scale indexing, 0.2-1.6 $\mu\text{g/ml}$ and 200-500 folds dilutions of the coating γ -globulin and the enzyme-conjugate, respectively, were used.

PLRV. Production of antiserum and enzyme-conjugate have been described previously⁹. The large scale indexing was performed using 2 $\mu\text{g/ml}$ of the coating γ -globulin and 400-500 folds dilutions of the enzyme-conjugate.

ELISA procedure.

Methods described by CLARK and ADAMS²⁰ were followed. γ -globulin was purified by ammonium sulfate precipitation and DEAE cellulose column chromatography, and conjugated with alkaline phosphatase (Boehringer Mannheim GmbH, Grade I) by 0.05% glutaraldehyde. Microtiter plates with 96 wells (Flat bottom microELISA plate, immulon, Dynatech) were used. We used "Jet Pipet" connected with 8 needles (York Instruments, Berkely, Ca.) for coating the plate with γ -globulin, washing with PBS-tween, and charging the wells with the enzyme-conjugate and the substrate. Each sample was duplicated and 30 samples including healthy and infected controls were tested in one plate. ELISA values were read using Corona microplate 2-wavelength photometer MTP-12 equipped with a M-12 printer.

100 g of potato virus X infected tomato leaves frozen at -20°C
 ground with 200 ml of 0.1 M sodium citrate, pH 7.0, containing 0.5% 2-
 mercapthoethanol in a motor and a pestle
 stirred with one-half volume of CCl_4 with a magnetic stirrer
 centrifuged at 3,000 rpm for 15 min

Aqueous phase
 polyethylene glycol #6,000 and NaCl were added to 4 g/100 ml and 0.1 M,
 respectively
 stirred with a magnetic stirrer for 1 hr and stood at 6°C for 1 hr
 centrifuged at 4,000 rpm for 20 min

Precipitate
 suspended in 25 ml of 0.05 M sodium citrate, pH 7.0
 stirred with one-half vol. of CCl_4 for 10 min with a magnetic stirrer
 centrifuged at 3,000 rpm for 15 min

Aqueous phase
 centrifuged at 10,000 rpm for 15 min

Supernatant
 centrifuged at 27,000 rpm for 120 min in a Hitachi RP 30 rotor

Pellet
 suspended in 1.5 ml of 0.05 M sodium citrate buffer
 centrifuged at 10,000 rpm for 15 min in a Hitachi RP 30 rotor

Supernatant
 centrifuged in a 10-40% (w/v) sucrose density-gradient buffered with 0.05 M
 sodium citrate, pH 7.0, at 25,000 rpm for 120 min in a Hitachi RPS 27 rotor

Virus zone
 collected by ISCO density-gradient fractionator model 180 equipped with a
 model UA-2 UV monitor
 centrifuged at 35,000 rpm for 90 min in a Hitachi RP 40 rotor

Purified virus
 suspended in 2 ml of 0.05 ml sodium citrate buffer (recovery is about 2.0-2.5
 mg virus/100 g tissue)

Fig. 1. Purification procedure of potato virus X.

TABLE 1. ELISA values of potato virus X at different concentrations of coating γ -globulin and enzyme-conjugate

	Concentration of coating γ -globulin ($\mu\text{g}/\text{ml}$)								
	10			1			0.1		
Purified PVX ¹⁾	0.35	0.28	0.17	0.32	0.33	0.25	0.34	0.31	0.24
Healthy tomato ²⁾	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.00	0.00
PBS-tween buffer	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	$\times 100 \times 300 \times 600$			$\times 150 \times 300 \times 600$			$\times 150 \times 300 \times 600$		
	Dilution of enzyme-conjugate								

1) 100 ng/ml

2) Crude extract (1 g leaf tissue/100 ml PBS-tween)

200 g of systemically potato virus Y-infected *Nicotiana sylvestris* leaves frozen at -20°C
 ground with 600 ml of 0.1 M tris-HCl, pH 7.0, containing 0.05 M disodium ethylenediaminetetraacetate, dihydrate and 1% 2-mercapthoethanol, in a motor and a pestle
 homogenized with one-half vol. of CCl_4 in a blender for 3 min
 centrifuged at 3,000 rpm for 15 min
 Aqueous phase
 polyethylene glycol #6,000 was added to a final concentration of 4 g/100 ml
 stirred with a magnetic stirrer for 1 hr and stood at 6°C for 1 hr
 centrifuged at 4,000 rpm for 20 min
 Precipitate
 suspended in 30 ml of 0.1 M borate, pH 8.2
 homogenized with 15 ml of CCl_4 for 3 min
 centrifuged at 3,000 rpm for 15 min
 Aqueous phase
 centrifuged at 10,000 rpm for 15 min
 Supernatant
 27,000 rpm for 120 min in a Hitachi RP 30 rotor
 Pellet
 suspended in 10 ml of the borate buffer
 centrifuged at 10,000 rpm for 15 min in a Hitachi RP 40 rotor
 Supernatant
 centrifuged at 35,000 rpm for 90 min in a Hitachi RP 40 rotor
 Pellet
 suspended in 2 ml of the borate buffer
 centrifuged at 10,000 rpm for 15 min in a Hitachi RP 40 rotor
 Supernatant
 centrifuged in a 10-40% (w/v) sucrose density-gradient buffered with the borate buffer at 23,000 rpm for 120 min in a Hitachi RPS 27 rotor
 Virus zone
 collected by ISCO density-gradient fractionator model 180 equipped with a model UA-2 UV monitor
 centrifuged at 35,000 rpm for 90 min in a Hitachi RP 40 rotor
 Purified virus
 suspended in 2-4 ml of the borate buffer (recovery is about 200-350 $\mu\text{g}/100\text{ g}$ leaf tissue)

Fig. 2. Purification procedure of potato virus Y.

TABLE 2. ELISA values of potato virus Y at different concentrations of coating γ -globulin and enzyme-conjugate

	Concentration of coating γ -globulin ($\mu\text{g}/\text{ml}$)								
	10			1			0.1		
PVY ¹⁾	2.53	1.10	0.52	2.23	0.91	0.46	1.55	0.84	0.58
Healthy ¹⁾	0.11	0.04	0.03	0.09	0.05	0.02	0.07	0.07	0.01
PBS-tween	0.14	0.06	0.03	0.09	0.04	0.03	0.08	0.02	0.01
	$\times 200$	$\times 400$	$\times 800$	$\times 200$	$\times 400$	$\times 800$	$\times 200$	$\times 400$	$\times 800$
	Dilution of enzyme-conjugate								

1) Crude extract from *Nicotiana sylvestris* leaves (1 g leaf tissue/100 ml PBS-tween)

Large scale-index of potato leaves for PVX, PVY, and PLRV.

Reliability of the indexing by ELISA was examined by comparing it with other conventional indexing methods. The procedures are follows :

- 1) 1st indexing ; About 400 seed potatoes var. Hokkai No. 60 were grown under the green house condition. The plants were indexed for PVX by a slide test, and for PVY and PLRV visually.
- 2) 1st ELISA ; Seventeen days after sprouts had emerged, young leaves were taken. And they (0.5 g) were ground in a mortar and a pestle with 2 ml PBS-tween and stored at -40 C until the first ELISA test. The samples were thawed and centrifuged at 3,000 rpm for a few minutes and the supernatants were used for the first ELISA.
- 3) 2nd indexing ; The potatoes tested by the first ELISA were planted in a field and indexed for PVX by a slide test and for PVY and PLRV visually.
- 4) 2nd ELISA ; The upper leaves of the field grown potatoes were taken in mid August and stored frozen at -30 C until the second ELISA for PVX, PVY, and PLRV.

Results**Storage conditions of PLRV enzyme-conjugate.**

The enzyme-conjugate was divided into two and stored at 4 C and -40 C . Their detection-sensitivity was compared simultaneously at several months intervals using purified PLRV. No significant differences could be detected in ELISA values after 18 months, the maximum duration tested (Fig. 3).

Storage conditions of PLRV coating γ -globulin.

During the experiments, the significant decrease in ELISA values was found when the coating γ -globulin was thawed after storage for 5 months at -40 C , diluted to $1\text{ }\mu\text{g/ml}$, and kept at 4 C for 3 months (Fig. 4). Instead of storing the thawed γ -globulin at 4 C , the plates were coated with the γ -globulin and the storage conditions of the coated plates were examined (Fig. 5). The plates were coated with $1\text{ }\mu\text{g/ml}$ of PLRV γ -globulin, washed four times with PBS-tween and once with distilled water. The plates were then air dried and stored at 4 C and -40 C . One month after the storage, about the same ELISA values were obtained between the plates stored at the two temperatures. Whereas 4 and 11 months after the storage, the plates at -40 C showed the higher values then those obtained from the plates stored at 4 C . After 11 months of the storage, the first coated outer raw of the coated wells showed no ELISA absorbance, while those of the second inner raw had values of 0.22-0.32, when both raws were charged with the same

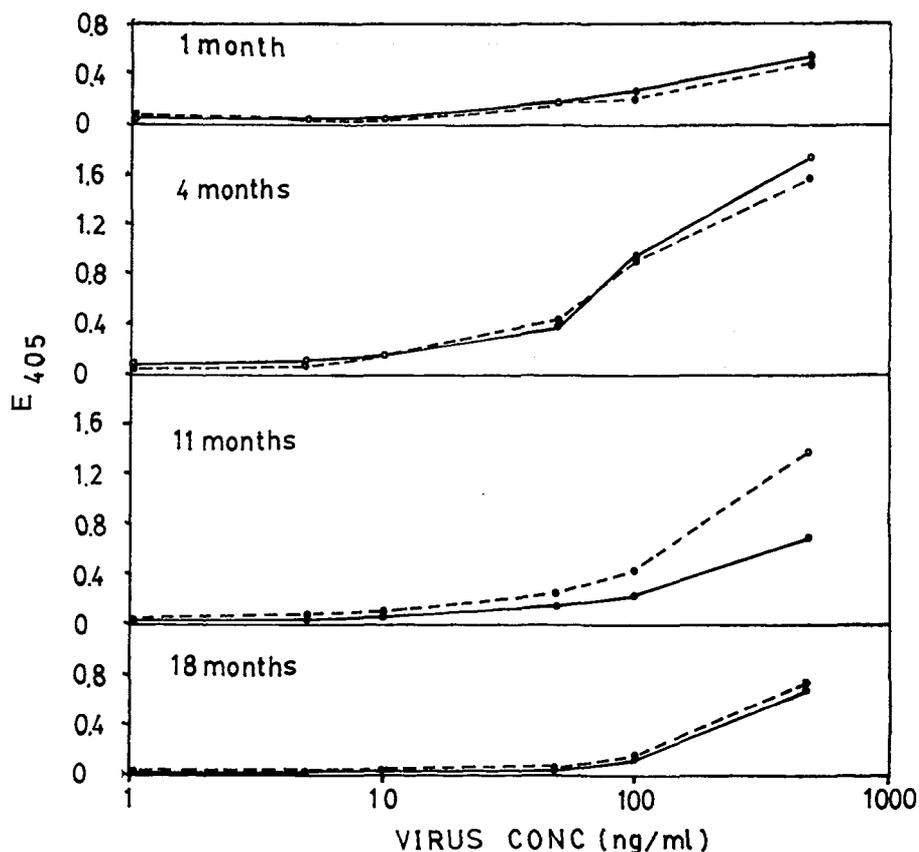


Fig. 3. Effect of storage condition on the detection sensitivity of the enzyme conjugate. The enzyme conjugate of potato leafroll virus was stored at +4°C (○·····○) and -40°C (○—○) and simultaneously compared.

concentration of PLRV antigen (100 ng/ml). The reasons for inability of the first outer row to detect PLRV are unknown at the moment.

Time required for a large scale ELISA for PVX, PVY, and PLRV.

It took 2 hrs and 15 min to prepare antigens from 395 potato leaves. It included 6 persons for maceration of the leaves in a mortar and a pestle, 2 persons for transferring the extracts into glass test tubes. Followings are times required for one person to perform ELISA for one microtiter plate.

Procedure	Time required
Coating the plate with γ -globulin	30-40 sec
Incubation 37 C	4 hr

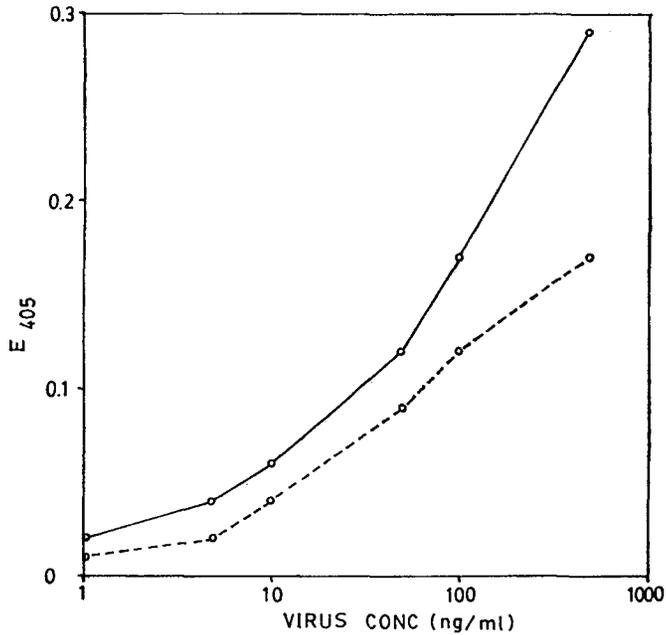


Fig. 4. Effect of storage condition of the γ -globulin on the detection sensitivity. The same lot of purified γ -globulin of potato leafroll virus was stored at -40°C (○—○) and 4°C (○⋯⋯○) for 3 months and compared simultaneously.

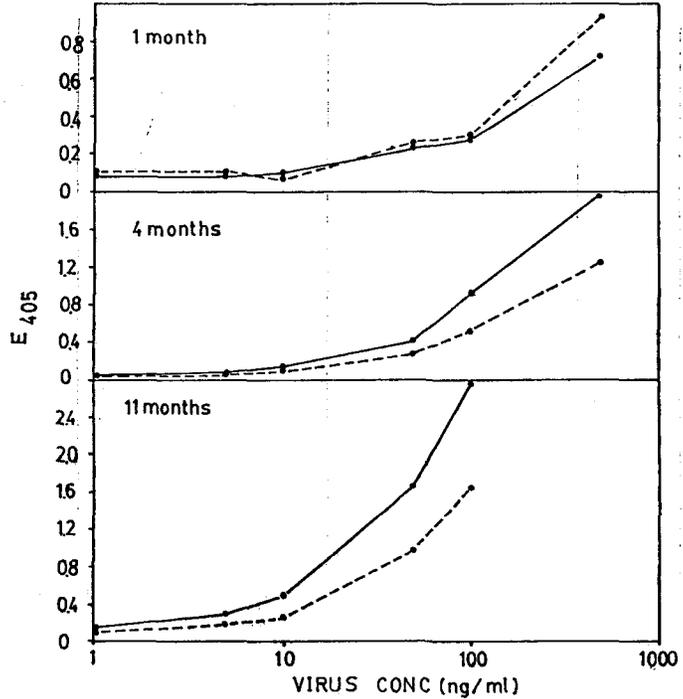


Fig. 5. Effect of storage condition of the plate coated with the γ -globulin of potato leafroll virus on the detection sensitivity. The plates precoated with the γ -globulin were stored at 4°C (○⋯⋯○) and -40°C (○—○) and simultaneously compared.

Washing the plate (4 time)	2 min 30 sec
Charging wells with the antigen	15-25 min
Incubation at 6 C	overnight
Washing the plate (5 times)	3-4 min
Charging wells with the enzyme-conjugate	30-40 sec
Incubation at 37 C	3-5 hrs
Washing the plate (3 times)	2 min
Charging wells with the substrate	30-40 sec
Reading the ELISA values	4 min

one person could process 64 plates in 6 days.

Large scale indexing.

PVX. Samples that considered to be positive in the ELISA reaction invariably showed values higher than 1.2 (70-120% of the positive control) and all the rest remained to have the values less than 0.30 (less than 20% of the positive control) (Fig. 6). Nineteen samples that was shown to be infected by slide tests had high values in the two ELISA tests (Fig. 6). Out of 395 samples, five shown to be infected only in the first slide test had the low values in the two ELISA tests, suggesting the results in the first slide test was questionable. Out of 119 samples, two shown to be infected by the second slide test but not in the first had the high values in the second ELISA test, and were considered to be infected in the field after the first ELISA test. One sample out of 199 samples, that had the high value only in the second ELISA and not detected by the slide tests, is probably due to field infection after the second slide test or misidentification of the sample number.

PVY. Infected and healthy controls had ELISA values of 0.32-0.67 and 0.01-0.12 in the first ELISA and of 0.62-0.81 and 0.02-0.07 in the second, respectively. Although in no case had the infected samples ELISA values below healthy control, the values were continuous from those of healthy to infected controls (Fig. 7). Six samples identified to be infected with PVY by the first visual indexing showed the high ELISA values; 0.21-0.53 (57-100% of the positive control) in the first ELISA and 0.23-0.44 (28-54% of the positive control) in the second. Two samples had high ELISA values in the two experiments but were missed by the first visual indexing. There were samples that had high ELISA values (more than 20% of the positive control) only in one of the two ELISA experiments but was considered to be free by visual indexing. These inconsistent results are probably due to either incomplete washing of the enzyme-conjugate or infection after the second visual indexing.

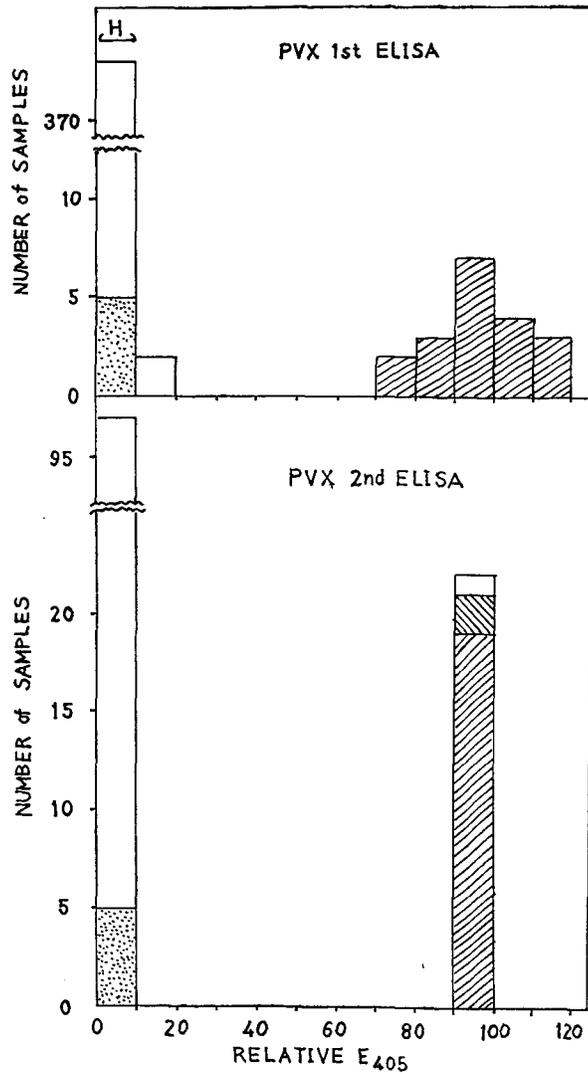


Fig. 6. ELISA values of samples in the large scale-indexing for potato virus X. ELISA values are expressed as an arbitrary unit of absorbance at wavelength of 405 nm relative to a positive control. ELISA value of the positive control was taken as 100.

- : positive in the two slide tests
- : positive only in the first slide test
- : positive in the second slide test
- : negative in the two slide tests

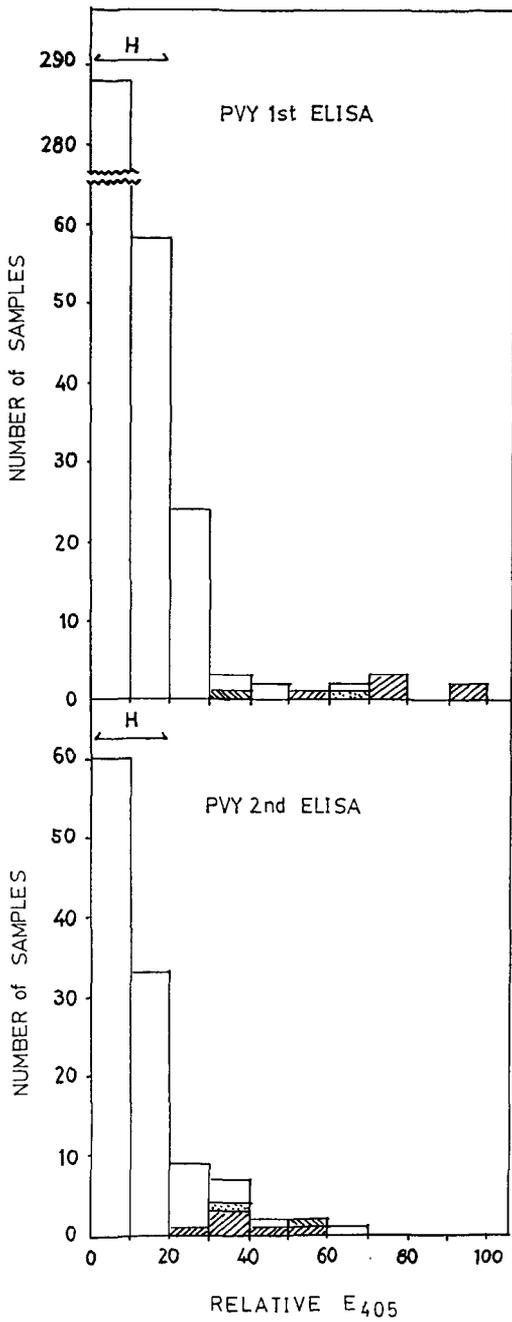


Fig. 7. ELISA values of samples in the large scale-indexing for potato virus Y. ELISA values are expressed as shown in Fig. 6.

- ▨ : positive in the two visual indexing
- : negative in the two visual indexing
- ▩ : individual sample that is negative in the two visual indexings, but had relatively high ELISA values in the two ELISA tests.

PLRV. Out of 382 samples tested, there was only one sample that is considered to be positive. This sample had a high ELISA value in both ELISA tests and detected visually in the second visual indexing (Fig. 8).

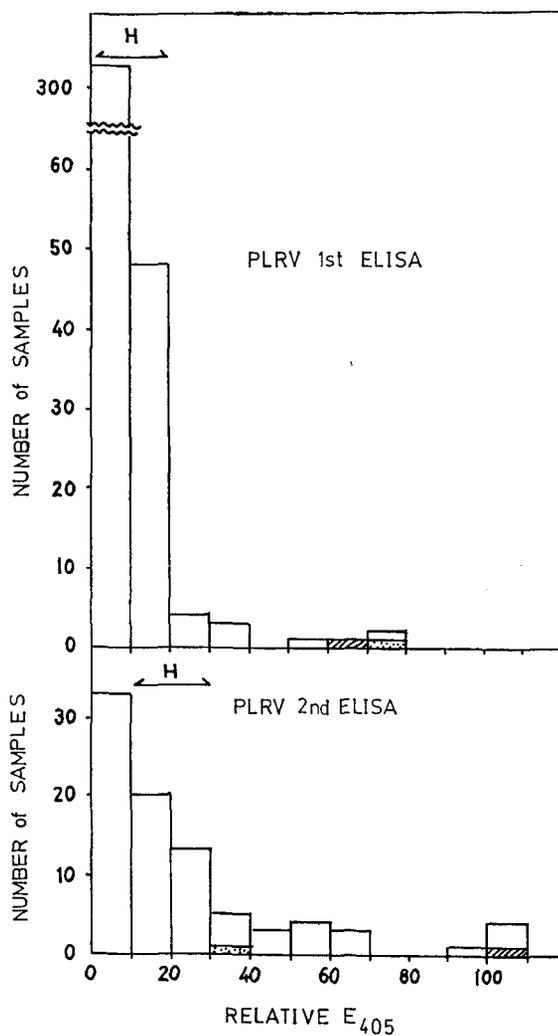


Fig. 8. ELISA values of samples in the large scale-indexing for potato leafroll virus. ELISA values are expressed as shown in Fig. 6.

- ▨ : positive in the two visual indexings
- : negative in the two visual indexings
- ▣ : individual sample that is negative in the two visual indexings, but had relatively high ELISA values in the two ELISA tests.

Another sample had a marginal ELISA value in the first LEISA and the high in the second, but was missed in the visual indexings (Fig. 8). Several samples had high ELISA values only in one of the two ELISA tests, and were indexed as healthy visually. We could not determine whether this is due to the incomplete washing of the plate or the infection occurred shortly before the harvest in August.

Discussion

Although there are some samples with inconsistent results between ELISA and other conventional methods, it is reasonable to conclude that ELISA test is reliable for indexing PVX, PVY, and PLRV using potato foliage. All samples that were found to be infected with PVX by two slide tests consistently showed positive ELISA values. In the case of PLRV and PVY, all samples that were found to be infected by visual indexing showed positive ELISA values.

Differences in the ELISA values of PLRV and PVY between infected and healthy samples were not as much as those of PVX in the present experiments. This made difficult to evaluate the samples with the value intermediate between those of healthy and infected controls. Increasing the concentration of the enzyme-conjugate could improve the evaluation. The other possible factor is the preservation of antigenicity of the sample during the storage. In this experiment, the samples were stored frozen at -40 C for several months before the ELISA. It is possible that a small fluctuation of the temperature during storage decreased the antigenicity of the virus. The antigenicity was well preserved by desiccating samples in silica gel, because we obtained a clear cut ELISA values using desiccated leaves in the case of PLRV^{7,10}. It should be mentioned that successful indexing can be achieved only after the background value of the healthy controls are low enough. One of the common technical problems encountered would be the use of an antiserum contaminated with antibody to healthy plant materials. In preliminary experiments, we tried ELISA indexing using PVS purified from the infected potatoes only by organic solvent clarification and differential centrifugations omitting rate-zonal density gradient centrifugation. The antiserum was apparently contained antibodies to healthy plant materials and the ELISA values of the healthy plant controls were as high as the infected materials. The second problem would be an incomplete washing of the wells after charging them with the enzyme-conjugate. This often results inconsistent background values of the duplicated wells of the same sample and high background values of the healthy controls. We recommend to

wash the wells with 0.3 ml of PBS-tween more than four times; more thoroughly washing than that of CLARK and ADAMS' original description.

For the large scale indexing, it is essential to simplify the protocols and avoid manual handlings as much as possible to minimize variations due to individual technical skill. As it was pointed out before, washing process is the most critical step and should be standardized before the large scale indexing. Another important point is to reduce the time required for each step to process large number of samples efficiently. Preparation of the sample antigen still requires substantial length of time and amount of labor, and needs to be improved. In the case of fresh leaves, extraction with a plier is worth trying instead of macerating tissue with a motor and a pestle. Incubation times of γ -globulin, test sample, and enzyme-conjugate could be reduced^{8, 10}, and should be determined experimentally. Reading the ELISA values with an automated spectrophotometer equipped with a recorder is indispensable.

It was found that the plate could be coated with the γ -globulin before the test and the plate could be stored at -40°C as long as 4 months without significant loss in the detection sensitivity. This helped for one person to index about 400 samples for three viruses in less than a week by preparing the coated plates beforehand.

Summary

Enzyme-linked immunosorbent assay was applied to a large scale indexing of potato virus X, potato virus Y, and potato leafroll virus in potato leaves. Enzyme-conjugate could be stored at either 4°C or -40°C with no significant differences in detection sensitivity. Diluted coating γ -globulin could not be stored at 4°C when it was once thawed. However, a plate coated with the γ -globulin could be stored at -40°C for up to 4 months. In a large scale indexing, 395 potato leaves were indexed. Potato virus X was clearly indexed whereas some samples showed ELISA values intermediate between healthy and positive controls in the case of PVY and PLRV. Problems encountered with the large scale indexing were discussed.

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