

Detection of apple chlorotic leafspot virus in apple leaves by Enzyme-Linked Immunosorbent Assay(ELISA)

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Abstract : The standard ELISA(S-ELISA) and the modified ELISA(M-ELISA) were studied to detect apple chlorotic leafspot virus (ACLSV) in the different maturities of apple leaves. The anti-serum against ACLSV was obtained after several times of injection with the purified ACLSV into a rabbit. The optimal concentration for coating IgG and alkaline phosphatase conjugate were 4.15 μ g/ml and 1:100 dilution, respectively. In S-ELISA, 2.5% nicotine solution containing 2.0% polyvinylpyrrolidone and in M-ELISA, phosphate buffered saline containing 0.05% Tween 20(PBS-T), 2.0% polyvinylpyrrolidone and 0.05% thioglycolic acid were suitable for grinding of sample leaves. Eleven samples in 12 young leaf samples showed positive reactions for the young leaves in both S- and M-ELISA. M-ELISA was superior to S-ELISA in apple leaves. Positive reactions were not appeared entirely on the mature leaves sampled in July in both S- and M-ELISA.

Key words : ELISA, apple chlorotic leafspot virus, detection

Introduction

Apple chlorotic leafspot virus(ACLSV)(CADMAN;1963), classified as the genus *Trichovirus* recently(MARTELLI,*et al.* ;1994), is widespread in several rosaceous plants such as apple, pear and *Prunus* spp.(HANSEN and GILMER;1976),and is one of the causal agents of apple topworking disease in Japan, as well as apple stem grooving virus(ASGV) and apple stem pitting virus(ASPV) (YANASE;1974).

Under the post entry quarantine in Japan, these three viruses are indexed by sap inoculation to *Chenopodium quinoa* and graft inoculation to *Malus sieboldii* MO-65(TAKAHASHI,*et al.* ;1988) and *Malus hupehensis* (SAITO,*et al.* ;1988). However, these indexing methods require a long period and are difficult to identify each virus accurately.

Enzyme-linked immunosorbent assay(ELISA) for ACLSV were already reported by FLEGG and CLARK(1979), and YANASE,*et al.* (1986).

In this study, the applications of ELISA were demonstrated for the detection of ACLSV in apple leaves under the post entry quarantine.

Materials and Methods

Virus sources.

Virus sources used in this study are shown in table 1. The virus which was used for the purification was isolated from N-1 apple infected with ACLSV, imported from U.S.A.(The import permit No.3-608). The apple P-1 which was infected with ACLSV, ASGV and ASPV, P-5 with ACLSV and ASGV, and P-203 with ACLSV were provided by Morioka Branch, Fruit Tree Research Station and these three apple trees were maintained in

Table 1. The virus sources used in this study

No.	Plants	Origin	Infected virus
1	N - 1	U.S.A	ACLSV ¹⁾
2	P - 1 (Golden Delicious)	Japan	ACLSV · ASGV · ASPV
3	P - 5 (Fuji)	Japan	ACLSV · ASGV
4	P - 203	Japan	ACLSV ²⁾
5	Wiltshire No.4	Germany	ACLSV ²⁾
6	Wiltshire No.5	Germany	ACLSV ²⁾
7	Gelber edelapfel No.1	Germany	ACLSV ²⁾
8	Gelber edelapfel No.2	Germany	ACLSV ²⁾
9	Marigold No.1	Germany	ACLSV ²⁾
10	Marigold No.2	Germany	ACLSV ²⁾
11	Ida red	England	ACLSV ²⁾
12	Load darby	England	ACLSV ²⁾
Cont.	Healthy seedling	Japan	—

1) The import permit No.3 - 608

2) These plants were under the Post Entry Quarantine in Japan and were suspected to be infected with ACLSV

Yamato Post Entry Quarantine Institute. Six apple trees imported from Germany and two apple trees from England under the Post Entry Quarantine in Japan, were suspected to be infected with ACLSV by graft inoculation to *M. hupehensis*.

Antiserum preparation.

ACLSV was purified from infected *C. quinoa* leaves by using the procedure of JAMES and MUKERJI (1993) to which Triton X treatment was added. The rabbit antiserum against ACLSV was obtained after an intradermal injection with the purified virus(0.125mg) emulsified with Freund's complete adjuvant, subsequent three intramuscular injections with the purified virus(0.25mg) emulsified with Freund's incomplete adjuvant and a final intravenous injection with the purified virus(0.125mg). The antiserum prepared in this study was absorbed by the crude sap of *C. quinoa* (FUJIWARA, *et al.* ;1994) before the purification of IgG and the reduction of the non-specific reaction was checked by dot-immunobinding assay(HIBI and SAITO ; 1984).

IgG purification and preparation of alkaline phosphatase conjugate

IgG was purified by using Affi-Gel Protein A MAPS-II kit (BIO-RAD). Alkaline phosphatase conjugate was prepared by the method of CLARK and ADAMS (1977).

ELISA

The procedure described by CLARK and ADAMS (1977) was applied as the standard ELISA(S-ELISA) and the procedure by FLEGG and CLARK(1979) was applied as the modified ELISA(M-ELISA). The results were regarded as positive if the absorbance value indicated more than two times that of healthy sample. In the test for the determination of the optimal concentration of coating IgG and conjugate, the S-ELISA was applied and phosphate buffered saline containing 0.05% Tween 20(PBS-T), 2.0% polyvinylpyrrolidone(PVP) and 0.05% thioglycolic acid(TGA) was used as the extraction buffer. Leaf extract of *C. quinoa* infected with ACLSV was used as the test sample. The comparison test of extraction buffers for apple

Table 2. Comparison of the extraction buffers for apple leaves

Buffer	Absorbance value at 405nm			
	S - ELISA		M - ELISA	
	P - 1 ¹⁾	Healthy ²⁾	P - 1	Healthy
A	1.14	0.10	1.39	0.04
B	0.51	0.06	2.22	0.01
C	0.65	0.05	1.87	0.02
D	0.59	0.06	1.45	0.04
E	0.50	0.06	1.18	0.02

A : 2.5% nicotine solution containing 2.0% polyvinyl pyrrolidone (PVP)

B : 0.01M phosphate buffered saline containing 0.05% Tween 20 (PBS - T), 2% PVP and 0.05% thioglycolic acid (TGA)

C : PBS - T, 2% PVP and 0.2% albumin

D : PBS - T, 2% PVP, 0.2% albumin and 0.5% nicotine

E : PBS - T, 2% PVP, 0.2% albumin, 0.5% nicotine and 0.01M sodium diethyldithiocarbamate (DIECA)

1) P - 1 (Golden Delicious) was infected with ACLSV, ASGV and ASPV.

2) Healthy *M. pumila* seedling

leaves was carried out with five buffers shown in table 2. P-1 apple leaf samples were used as positive control and healthy leaves of *Malus pumila* seedling were used as negative control in the test. The extract for ELISA was prepared by grinding 0.3g of leaf sample with 3ml extraction buffer, and that was centrifuged at 5,000 rpm for 4 min before adding to wells of microplate. In the M-ELISA, the conjugate was diluted in PBS-T containing 2% PVP and 0.2% bovine serum albumin, and the diluted conjugate was mixed with the same volume of extract of apple leaf sample grinded with each extraction buffer. For the comparison tests of maturity stages of apple leaves to be suitable for ELISA, young and mature leaves were collected in April and mature leaves were collected in July from each apple tree. Healthy leaves of *M. pumila* seedlings were used as the negative control. The sample of apple leaf was cut with a knife along midrib, and the one side was tested by S-ELISA and the other side was tested by M-ELISA. The solution of 2.5% nicotine containing 2.0% PVP was used as the extraction buffer for S-ELISA, and PBS-T containing 2.0% PVP and 0.05%TGA was used for M-ELISA. The rest of each extract for S-ELISA was tested by sap inoculation with two seedlings of *C. quinoa* for the comparison with ELISA.

In this study, the following buffers and equipments were used; coating buffer : 0.02M sodium carbonate at pH 9.6, substrate buffer : 10% diethanolamine, pH 9.8, washing buffer: PBS-T, flat bottom plates : IMMULON 1, Dynatech Laboratories, Inc. and microplate Reader : Model 450, Bio-Rad.

Results and Discussion

Obtained antiserum

The titer of the antiserum against ACLSV obtained in this study was 320 times by ring test.

The optimum concentration for coating IgG and conjugate

The optimum concentrations of coating IgG and conjugate were supposed to be 4.15 μ g /ml(1:200) and 1:100 dilution, respectively. (Fig.1, Fig.2)

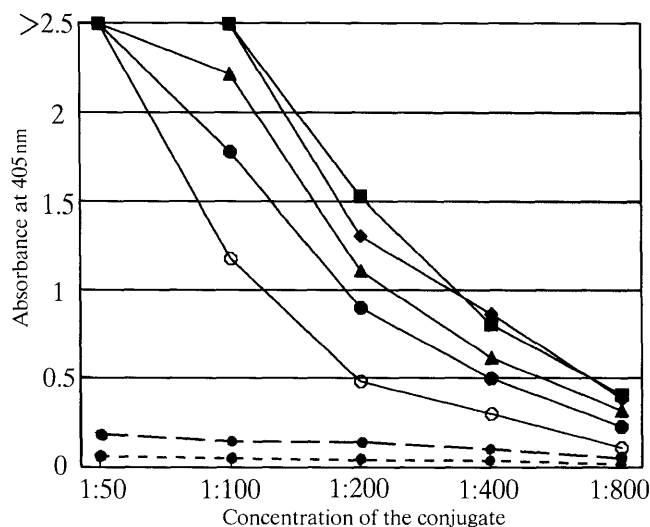


Fig. 1. Effect of different concentrations of coating IgG on the detection of ACLSV in extracts of infected *C. quinoa* leaves. —■—; 1:50, —◆—; 1:100, —▲—; 1:200 (4.15 μ g/ml), —●—; 1:400, —○—; 1:800, —●—; Healthy control, 1:50, —●—; Healthy control, 1:200.

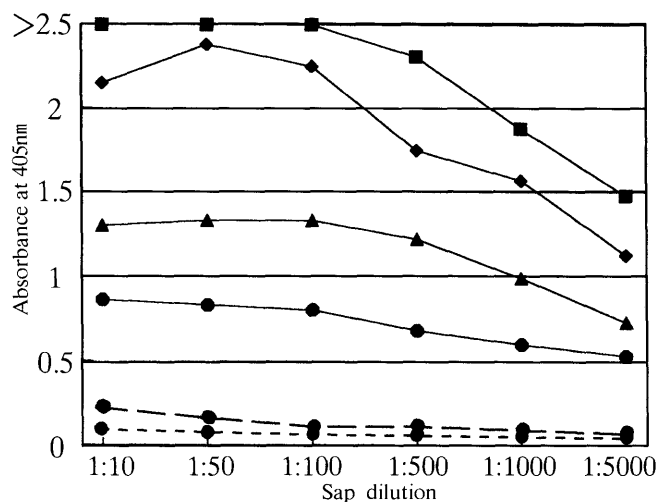


Fig. 2. Effect of different concentrations of conjugate with 4.15 μ g/ml of coating IgG on the detection of ACLSV in diluted extracts of infected *C. quinoa* leaves. —■—; 1:50, —◆—; 1:100, —▲—; 1:200, —●—; 1:400, —●—; Healthy control, 1:50, —●—; Healthy control, 1:100.

Comparison of the extraction buffers for apple leaves

The results of this test were shown in table 2. As the highest absorbance values were obtained by 2.5% nicotine solution containing 2.0% PVP in S-ELISA and PBS-T containing 2.0% PVP and 0.05% TGA in M-ELISA, these buffers were supposed to be suitable.

Comparison of maturity stages of apple leaves

The results of this test are shown in fig.3. On the samples collected in April, 11 samples in 12 young leaf samples showed the positive reactions in both S- and M-ELISA, and some samples of mature leaves did not show positive (4/12 in S-ELISA, 2/12 in M-ELISA). There was no positive reaction in the mature leaves collected in July. In the inoculation test with two plants of *C. quinoa* which was carried out simultaneously, the numbers of samples showing positive reaction were less than that in both S- and M-ELISA. The apple P-5 which did not show any positive reaction in all cases, was supposed to be extremely low in the virus concentration considering that the apple P-5 had been maintained under the high temperature condition in the greenhouse.

Although FLEGG and CLARK(1979) described ACLSV could not be detected by S-ELISA, YANASE *et al.*(1986) reported ACLSV could be detected by S-ELISA. The results of our studies showed both S- and M-ELISA could be used practically for young leaves. The sensitivity of M-ELISA was seemed to be superior to that of S-ELISA, because two mature leaf samples collected in April showing negative reactions in S-ELISA showed positive in M-ELISA.

It seems that young leaves collected in spring were suitable (YANASE, *et al.*;1986), (KARESOVA, *et al.*;1995) and the concentration of ACLSV in the leaves fell as the season progressed (FLEGG and CLARK ;1979). The results of our tests were similar to their descriptions. Therefore, it suggests that the detection of

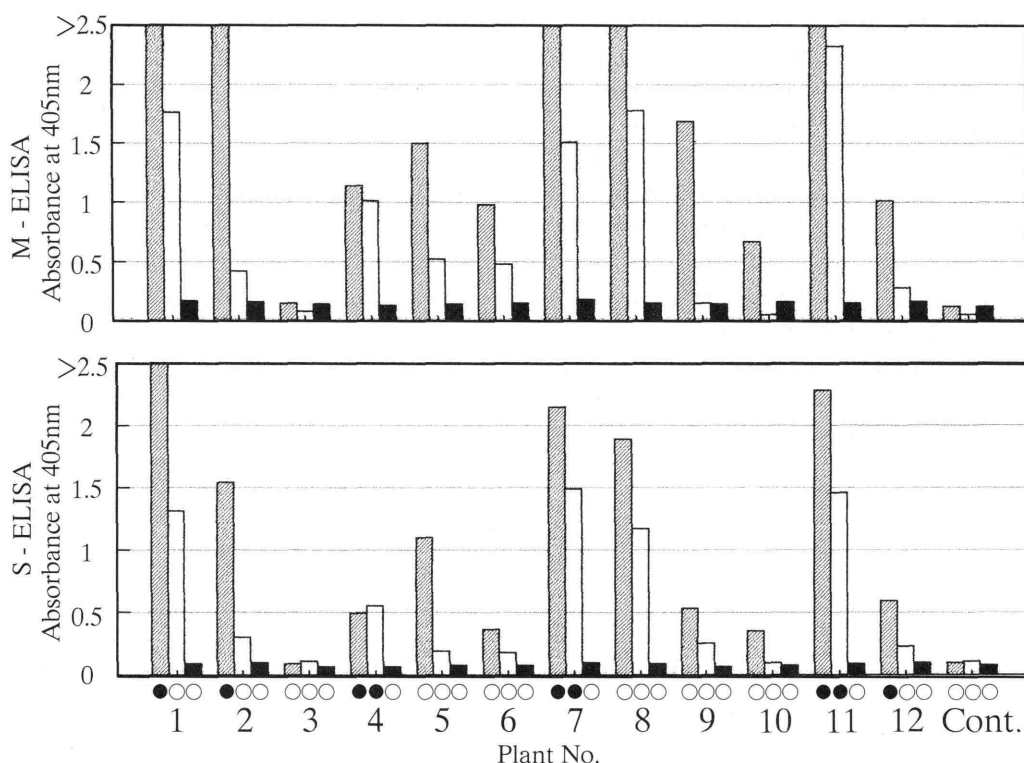


Fig.3. Comparison of young and mature apple leaves collected in different seasons. And sap inoculation test with two seedlings of *C. quinoa*. Open circles indicate negative results and solid circles indicate positive results of the inoculation test, respectively. ▨ ; Young leaves collected in April, □ ; Mature leaves collected in April, ■ ; Mature leaves collected in July.

ACLSV in apple tree should be carried out on young leaves.

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和 文 摘 要

リンゴ葉からのエライザ法による
apple chlorotic leafspot virus の検出

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リンゴ葉を用いた apple chlorotic leafspot virus (ACLSV) の ELISA 法の導入についての検討を行った。ウイルスを純化し、抗血清を得、IgG を純化し、コンジュゲートを作製した。IgG とコンジュゲートの最適濃度は、それぞれ $4.15 \mu\text{g/ml}$ と 100 倍であった。リンゴ葉に対する最適な磨碎 buffer は、standard-ELISA (S-ELISA; M.F. CLARK and A.N. ADAMS, 1977) に対しては 2.5% ニコチン + 2% PVP, modified-ELISA (M-ELISA; C.L. FLEGG and M.F. CLARK, 1979) に対しては

PBS-T + 2% PVP + 0.05% TGA であった。次に、ACLSV に感染したリンゴ 12 株を用い、それぞれの株の 5 月の若葉と成熟葉及び 7 月の成熟葉に対して S-ELISA 及び M-ELISA を行ったところ、4 月の若葉に対しては S-ELISA 及び M-ELISA ともに ACLSV の検出が可能で、M-ELISA は S-ELISA より検出感度は高いこと、及び検査にあつたては、春の若葉を使うのが重要であり、夏の成熟葉からは検出が困難であることが判明した。