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Enzyme-linked immunosorbent assay (ELISA) and disperse-dye immuno assay (DIA): comparison of simultaneous and separate incubation of sample and conjugate for the routine detection of lettuce mosaic virus and pea early-browning virus in seeds

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Abstract

Two modifications of ELISA and DIA were compared in relation to sensitivity of detection of two plant viruses and suitability for large-scale routine testing. DIA is a solid phase immuno assay like ELISA, in which the enzyme conjugate is replaced by a dye sol conjugate and substrate incubation is replaced by immediate dissolving of the dye molecules from the conjugate with an organic solvent. Sample and conjugate were incubated separately (ELISA 1, DIA 1) or simultaneously (ELISA 2, DIA 2). The seed-borne viruses viz. lettuce mosaic virus (LMV) and pea early-browning virus (PEBV) were subjected to the assays. DIA detected LMV in a purified extract of *Nicotiana benthamiana.* However, compounds present in crude virus-free and virus-containing plant extracts strongly interfered with DIA, necessitating adaptation of DIA to plant viruses in crude extracts.

With the extracts of lettuce and pea seeds ELISA 2, in comparison with ELISA 1, resulted in equal (LMV) or slightly higher (PEBV) extinction values and in a higher ratio between extinction values of virus-containing and virus-free samples. The higher sensitivity of ELISA 2 in combination with its higher efficiency as a result of simultaneous sample and conjugate incubation, indicates the potential of this method for large-scale indexing.

Additional keywords: seed-borne viruses, lettuce, pea.

Introduction

ELISA, originally a medical immunodiagnostic assay, has been introduced in plant virology by Voller et al. (1976). Because of its suitability it has soon been accepted as the major routine method for indexing of plant material for viruses (Clark and Adams, 1977; Clark, 1981). Its contribution to the production of virus-free propagation material of many economically important crops, viz. potatoes, vegetables, fruit-trees, grapevines, flowerbulbs and many other ornamentals, has been of great economic value and will become increasingly important. The procedure described by Clark and Adams (1977) is widely applied in its original form with slight modifica-

tions, e.g. concerning buffer composition for some host-virus combinations. In 1979 Flegg and Clark described a simplified procedure based on simultaneous incubation of sample and enzyme conjugate to enable the detection of apple chlorotic leafspot virus. This method, used in some routine medical diagnostic programs, may also be of importance for large-scale routine application of ELISA with plant material.

DIA, a diagnostic method developed by Gribnau et al. (1982), who found it at least as sensitive as ELISA, seems promising as an alternative for ELISA in routine application. Advantages of DIA over ELISA are the replacement of relatively expensive enzymes by cheap dye sol particles and the replacement of the substrate incubation step by addition of a simple organic solvent to dissolve the dye molecules from the dye sol conjugate. For DIA this results in an immediate overall staining of the well in case of infected samples.

This paper describes the application of ELISA and DIA with separate (ELISA 1 and DIA 1) and with simultaneous (ELISA 2 and DIA 2) sample and conjugate incubation for lettuce mosaic virus (LMV) and pea early-browning virus (PEBV). Results are analysed with regard to sensitivity and suitability for large-scale indexing of plant material.

Materials and methods

Viruses and antisera. Two seed-borne viruses, LMV (Tomlinson, 1970) and PEBV (Harrison, 1973), were tested. Antiserum to LMV was prepared in 1981 to virus purified from *Nicotiana benthamiana* (see also Van Vuurde and Maat, 1983). Antiserum to PEBV was prepared in 1971 to virus purified from *N. rustica.* Both antisera, prepared at the Research Institute for Plant Protection (IPO), had titres of 1024 in the micro-precipitation test.

Partial purification of LMV for test antigen was from *N. benthamiana.* Infected leaf material was homogenized in citric acid-phosphate buffer (pH 7). After incubation with 1% Triton X-100, the homogenate was filtered through cheese-cloth. Further treatment was by low-speed centrifugation and precipitation with 5% polyethylene glycol 8000. Virus-free *N. benthamiana* was treated in the same way.

Preparation of extracts from lettuce seeds. Lettuce seeds from a virus-free lot (cv. Susan) and from a LMV-infected lot (cv. Susan; 4-5% infected seeds) were germinated for four days at 20 °C under artificial light (Van Vuurde and Maat, 1983). Samples of 100 germinated seeds were homogenized with pestle and mortar under addition of small quantities of extraction buffer up to a volume of 2 ml per sample. This extraction buffer was phosphate-buffered saline (PBS; 0.05 M phosphate, pH 7.4, 0.8% sodium chloride, 0.05% sodium azide) containing 2% polyvinyl pyrrolidone (PVP; MW 25 000-30 000) and 0.05% Tween 20.

Preparation of extracts from pea seeds. Batches of 100 dry pea seeds (cv. Finale), either virus free, or including 1 seed with PEBV symptoms, were ground in a large coffee grinder with exchangeable grinder part (Braun MX32/400W). Extracts were prepared by suspending 1 g of pea seed flour in 3 ml of extraction buffer (PBS + 0.05% Tween 20 + 2% PVP).

Serological test methods. Preparation of IgG from the antisera and conjugation with alkaline phosphatase for ELISA was as described by Tóbiás et al. (1982). Conjugation of IgG with Palanil® luminous red G for DIA was as described by Gribnau et al. (1983).

Two variants of ELISA and DIA were applied (Table 1). The procedure for ELISA 1 was performed as described by Clark and Adams (1977) and for ELISA 2 as described by Flegg and Clark (1979) with some modifications (see Table 1). Microtiter plates (M 129A, Dynatech) were coated with IgG (1 μg ml^{-1}) during 18 h at 4 °C. In ELISA 1 incubation with antigen preparations was for 2 h at 37 °C and with enzyme conjugates (0.5 μg of IgG per ml) for 3 h at 37 °C. In ELISA 2, 100 μl of antigen preparation was mixed with 100 μl of enzyme conjugate (1μg of IgG per ml) in the wells of the microtiter plates and the mixture incubated for 3 h at 37 °C.

The 0.05 M phosphate extraction buffer used to prepare the seed extracts was also used to dilute enzyme conjugates. Results were read after 1 and/or 16 h of substrate incubation. For DIA 1 and 2 coating and sample and conjugate incubation were performed as in ELISA 1 and 2, respectively, but the enzyme conjugates were replaced by Palanil® luminous red G-conjugated γ-globulins and the substrate step was replaced by dissolving the dye molecules from the disperse dye sol with dimethylsulphoxide

Table 1. Flow diagrams of two modifications of ELISA and of DIA for the detection of LMV and PEBV.

Coating of microtiter plates with virus-specific antibodies (18 h at 4 °C)			
Washing			
ELISA 1	ELISA 2	DIA 1	DIA 2
Incubation with test antigen (2 h at 37 °C)	Simultaneous incubation with test antigen and enzyme-conjugated virus-specific antibodies (3 h at 37 °C)	Incubation with test antigen (2 h at 37 °C)	Simultaneous incubation with test antigen and disperse dye-conjugated virus-specific antibodies (3 h at 37 °C)
Washing		Washing	
Incubation with enzyme-conjugated virus-specific antibodies (3 h at 37 °C)		Incubation with disperse dye-conjugated virus-specific antibodies (3 h at 37 °C)	
Washing			
Incubation with enzyme substrate (1 h at 20 °C)		Addition of DMSO to dissolve bound dye (shaking 1 min)	
Recording of substrate hydrolysis at 405 nm		Recording of colour intensity at 540 nm	

(DMSO). After addition of DMSO, the plates were shaken for 1 min on a Dynatech microtiter plate shaker before reading at 540 nm.

Results

LMV. Five-fold dilution series of partially purified extracts of *N. benthamiana,* virus free and infected with LMV, made with extraction buffer, and two-fold dilution series of extracts from virus-free and LMV-infected germinated lettuce seeds, made with extraction buffer or with extracts from virus-free germinated lettuce seeds, were used to compare ELISA 1 and 2, and DIA 1 and 2.

The results obtained with the partially purified extracts of *N. benthamiana* are presented in Table 2. They show that LMV could be detected with all four methods, but that ELISA was more sensitive than DIA. The extinction values obtained with the lower antigen dilutions were higher in ELISA 1 than in ELISA 2. The same was true when comparing DIA 1 with DIA 2.

The results obtained with the dilution ranges of the germinated lettuce seed extracts are presented in Table 3. They show that the extinction values obtained in DIA 1 and DIA 2 with the extracts from LMV-infected germinated seeds did hardly or not exceed those obtained with the extracts from virus-free germinated seeds and were sometimes even slightly lower. In ELISA 1 and 2, there was a clear difference between extinction values of virus-free and virus-containing extracts up to antigen dilutions of 4 or 8. Results in ELISA 1 and 2 and in DIA 2 were not influenced by the nature of the diluent. Reactions with virus-free extracts were weaker in ELISA 2 than in ELISA 1. For ELISA 2, improved contrast was found between virus-free and virus-containing extracts after prolonged substrate incubation (16 hours) at room temperature (Table 4). The extinction values of wells incubated with virus-free extracts were even considerably lower than those of buffer control wells and increased with decreasing concentration of plant material. Extinction values for samples of virus-free plant extracts in ELISA 1, however, were much higher than those of buffer controls.

PEBV. Two-fold dilution ranges of virus-free and PEBV-infected dry pea seeds, prepared with extraction buffer and with virus-free seed extracts, were tested. The results obtained with the four methods are presented in Table 5. They show that in DIA 1 en 2 the extinction values obtained with extracts from PEBV-infected seeds did not exceed those obtained with the corresponding virus-free material.

In ELISA 1 and 2, however, extinction values obtained with all dilutions of extracts from PEBV-infected seeds were higher than those obtained with each of the dilutions of the virus-free seed extracts. Again in ELISA 2 the extinction values obtained with virus-free material were lower than those in ELISA 1. In ELISA 2 substrate incubation of 16 hours at 20 °C strongly increased the difference between extinction values of non-infected and infected samples, especially when these were diluted more than four times with buffer (Table 6). Upon prolonged substrate incubation, ELISA 1, unlike ELISA 2, resulted in values for virus-free extracts >2.00.

Table 2. Extinction values in two modifications of ELISA at 405 nm and in two modifications of DIA at 540 nm, for dilution series of partially purified extracts from *Nicotiana benthamiana* without and with LMV. ELISA read after one hour of substrate incubation at 20 °C.

Dilutions of test antigens[1]	*Nicotiana benthamiana* virus free				*Nicotiana benthamiana* with LMV			
	ELISA 1[2]	ELISA 2	DIA 1	DIA 2	ELISA 1	ELISA 2	DIA 1	DIA 2
undiluted	0.02[3]	0.03	0.03	0.01	2.00	1.00	0.43	0.14
5	0.04	0.04	0.02	0.02	2.00	1.53	0.51	0.13
25	0.04	0.03	0.03	0.02	2.00	1.14	0.22	0.09
125	0.03	0.05	0.05	0.02	0.83	0.58	0.07	0.04
625	0.06	0.05	0.06	0.02	0.25	0.26	0.04	0.02
3125	0.06	0.02	0.02	0.05	0.10	0.09	0.04	0.02

[1] Dilutions made with PBS + Tween + PVP.

[2] ELISA 1 and DIA 1: separate incubation with test antigen and conjugate;
ELISA 2 and DIA 2: simultaneous incubation with test antigen and conjugate.

[3] Results are averages of duplicate wells. Extinction values corrected for absorption by non-hydrolyzed substrate by subtracting from them 2/3 of the average extinction value in 12 wells treated with extraction buffer instead of with plant material.

Table 3. Extinction values in two modifications of ELISA at 405 nm and in two modifications of DIA at 540 nm, for dilution series of extracts from germinated lettuce seeds without and with LMV. ELISA read after one hour of substrate incubation at 20 °C.

Dilutions of test antigens	Extracts from											
	virus-free seeds diluted with buffer[1]				LMV-infected seeds diluted with							
					buffer[1]				virus-free seed extracts			
	ELISA 1[2]	ELISA 2	DIA 1	DIA 2	ELISA 1	ELISA 2	DIA 1	DIA 2	ELISA 1	ELISA 2	DIA 1	DIA 2
undiluted	0.07[3]	0.01	0.23	0.01	0.38	0.38	0.18	0.01	0.43	0.40	0.16	0.01
2	0.07	0.00	0.06	0.02	0.21	0.21	0.08	0.04	0.21	0.22	0.16	0.03
4	0.07	0.00	0.03	0.01	0.12	0.12	0.06	0.03	0.15	0.12	0.24	0.02
8	0.06	0.00	0.03	0.02	0.10	0.07	0.04	0.02	0.09	0.07	0.19	0.01
16	0.06	0.00	0.03	0.00	0.08	0.04	0.04	0.02	0.08	0.04	0.16	0.01
32	0.07	0.01	0.03	0.01	0.07	0.02	0.04	0.02	0.06	0.02	0.11	0.01

[1] PBS + Tween + PVP.

[2] ELISA 1 and DIA 1: separate incubation with test antigen and conjugate;
ELISA 2 and DIA 2: simultaneous incubation with test antigen and conjugate.

[3] Results are averages of duplicate wells. Extinction values corrected for absorption by non-hydrolyzed substrate by subtracting from them 2/3 of the average extinction value in 12 wells treated with extraction buffer instead of with plant material.

Table 4. Extinction values in ELISA 1 and 2 at 405 nm for dilution series of extracts of non-infected and LMV-infected lettuce seedlings after extended substrate incubation (16 hours, room temperature).

Dilutions of test antigens	Non-infected, diluted with buffer		LMV-infected, diluted with buffer		LMV-infected, diluted with non-infected extract	
	ELISA 1	ELISA 2	ELISA 1	ELISA 2	ELISA 1	ELISA 2
undiluted	>2.00[1]	0.17	>2.00	>2.00	>2.00	>2.00
2	1.73	0.11	>2.00	>2.00	>2.00	>2.00
4	>1.75	0.12	>2.00	>2.00	>2.00	>2.00
8	1.54	0.13	>2.00	>2.00	>2.00	1.35
16	1.46	0.17	>2.00	0.69	>1.95	0.65
32	1.64	0.28	>1.89	0.45	1.53	0.39

[1] Values corrected by adding 0.30 units, being 2/3 of the average value of 12 buffer control wells.

Discussion

ELISA modifications. Both ELISA modifications clearly distinguished between extracts of non-infected and infected material for the virus-host combinations investigated. ELISA 1 gave higher extinction values than ELISA 2 for the dilution series of the purified extract from infected *N. benthamiana* and similar extinction values for the purified extract of non-infected plant material (Table 2). It should be kept in mind, however, that with ELISA 2 the plant extract incubation period was 50% longer, and the plant extract was twice as diluted as in ELISA 1.

As for the crude extracts from lettuce and pea, extinction values of virus-containing extracts were at the same level with ELISA 1 as with ELISA 2 but higher with ELISA 2 when corrected for the extra dilution step. The most interesting aspect of ELISA 2 is the relatively low extinction values of the crude virus-free plant extracts in comparison with ELISA 1, strongly increasing the ratio of the extinction values for infected and non-infected plant material (Tables 3, 4, 5 and 6). Our results with ELISA 2 concerning the reduction of background reaction are in agreement with those obtained for apple chlorotic leafspot in herbaceous and woody host species (Flegg and Clark, 1979) and for apple chlorotic leafspot and apple stem grooving viruses (F.A. van der Meer, personal communication). The reduced sensitivity of ELISA 2 for the detection of relatively high virus concentrations, as observed by Flegg and Clark (1979), is confirmed by our results obtained with purified extract from LMV-infected *N. benthamiana* (Table 2). This may be due to an excess of antigen (see also Clark, 1981).

DIA modifications. Results (Table 2) show that DIA could detect LMV in the purified extract of *N. benthamiana,* demonstrating its prospect as a test method for plant viruses. Differences between virus-free and virus-containing extracts, however, were considerably lower than those obtained with either ELISA modification. About two

Table 5. Extinction values in two modifications of ELISA at 405 nm and in two modifications of DIA at 540 nm, for dilution series of extracts from pea seeds without and with PEBV. ELISA read after one hour of substrate incubation at 20 °C.

Dilutions of test antigens	Extracts from											
	virus-free seeds diluted with buffer[1]				PEBV-infected seeds diluted with							
					buffer[1]				virus-free seed extracts			
	ELISA 1[2]	ELISA 2	DIA 1	DIA 2	ELISA 1	ELISA 2	DIA 1	DIA 2	ELISA 1	ELISA2	DIA 1	DIA 2
undiluted	0.14[3]	0.09	1.42	0.05	0.88	1.06	1.43	0.09	0.88	0.88	1.42	0.04
2	0.14	0.05	1.41	0.07	0.53	0.76	1.43	0.08	0.70	0.73	1.40	0.07
4	0.11	0.04	1.35	0.05	0.40	0.48	1.35	0.07	0.47	0.51	1.38	0.04
8	0.08	0.03	1.21	0.07	0.25	0.26	1.21	0.08	0.37	0.30	1.37	0.03
16	0.07	0.03	0.85	0.06	0.14	0.12	0.82	0.11	0.23	0.26	1.40	0.05
32	0.08	0.03	0.60	0.09	0.09	0.07	0.62	0.10	0.20	0.18	1.42	0.09

[1] PBS + Tween + PVP.

[2] ELISA 1 and DIA 1: separate incubation with test antigen and conjugate;
ELISA 2 and DIA 2: simultaneous incubation with test antigen and conjugate.

[3] Results are averages of duplicate wells. Extinction values corrected for absorption by non-hydrolyzed substrate by subtracting from them 2/3 of the average extinction value in 12 wells treated with extraction buffer instead of with plant material.

Table 6. Extinction values in ELISA 1 and 2 at 405 nm for dilution series of extracts of non-infected and PEBV-infected pea seed flour after extended substrate incubation (16 hours, room temperature).

Dilutions of test antigens	Non-infected, diluted with buffer		PEBV-infected, diluted with buffer		PEBV-infected, diluted with non-infected extract	
	ELISA 1	ELISA 2	ELISA 1	ELISA 2	ELISA 1	ELISA 2
undiluted	>2.00[1]	>1.71	>2.00	>2.00	>2.00	>2.00
2	>2.00	0.62	>2.00	>2.00	>2.00	>2.00
4	>2.00	0.37	>2.00	>2.00	>2.00	>2.00
8	1.59	0.16	>2.00	>2.00	>2.00	>2.00
16	1.46	0.12	>2.00	>2.00	>2.00	>2.00
32	1.60	0.18	>2.00	>2.00	>2.00	>2.00

[1] Values corrected by adding 0.30 units, being 2/3 of the average value of 12 buffer control wells.

times higher extinction values were obtained with DIA 1 than with DIA 2. Strikingly, the extinction values of DIA for crude extracts of non-infected and infected lettuce and pea differed hardly (Tables 3 and 5).

Comparison of results of DIA 1, obtained with virus-containing plant extracts diluted with buffer, with those obtained with such extracts diluted with virus-free plant extracts (Tables 3 and 5), demonstrates the presence of an interfering compound in the extracts of lettuce and pea. Because microtiter plates were treated similarly in the corresponding ELISA and DIA experiments, this compound must have been present also in the wells filled with plant extracts in the ELISA test series, but without influencing test results. An explanation for this phenomenon might be the difference in charge of the Palanil ® luminous red G-labelled IgG molecules in comparison with the enzyme-labelled IgG molecules. Complexes of the charged dye/IgG particles might have reacted with plant material with a complementary charge, adsorbed at the polystyrene of the microtiter plate.

Prospects of ELISA and DIA modifications for routine indexing. For routine indexing of large series of samples with ELISA, simultaneous sample and conjugate incubation (ELISA 2) will shorten the procedure with one incubation and one washing step. Furthermore, the method will improve the detection of low concentrations of virus, e.g. one slightly LMV-infected lettuce seed in subsamples of one hundred or more non-infected seeds (Van Vuurde and Maat, 1983), because the substrate incubation period can be increased without increasing background reaction. Especially, when results are read without a photometer, the distinction between non-infected and infected samples will be more pronounced, as was shown with ELISA 2 for seed-borne LMV and PEBV after 16 hours of substrate incubation. For this reason results of ELISA 2 will be more reproducible than those of the other tests, which may well stimulate the development of standardized ELISA methods for international attestation. One should be aware, however, of the possible inhibition of the reaction at high virus concentrations. In regard to seed-borne viruses, standardized ELISA procedures are now being developed

under the auspices of the Virus Working Group of the International Seed Testing Association (ISTA).

Several characteristics of DIA, viz. cheap and easy preparation of conjugate, elimination of the substrate incubation step, and simultaneous detection of two different types of antigen (Gribnau et al., 1982) are very promising with regard to large-scale indexing of plant material. In comparison with ELISA, a higher amount of IgG is necessary to prepare the dye sol conjugate, which must be stored freeze-dried or prepared fresh before use (Gribnau et al., 1983). Unlike in antigen detection in human sera, DIA failed to detect LMV and PEBV in crude plant extracts following the procedures described. However, the prospect of DIA as an economically more favourable alternative for ELISA in large-scale routine testing seems to justify further research on adaptation of the method to the detection of viral antigens in plant material.

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Samenvatting

ELISA en DIA, vergelijking van gelijktijdige en gescheiden incubatie van monster en conjugaat voor het aantonen van slamozaïekvirus en vroege-verbruiningsvirus van erwt in zaden

Twee modificaties van ELISA en DIA werden vergeleken met betrekking tot hun gevoeligheid voor het aantonen van twee plantevirussen en hun geschiktheid voor routinematige toepassing. DIA is een serologische toetsmethode die veel overeenkomst vertoont met ELISA, maar waarin het enzymconjugaat is vervangen door een conjugaat met gedispergeerde kleurstofdeeltjes en de incubatie met enzymsubstraat door het direct oplossen van de kleurstofmoleculen van het conjugaat met een organisch oplosmiddel. Incubatie van monster en conjugaat vond zowel gescheiden (ELISA 1, DIA 1) als gelijktijdig (ELISA 2, DIA 2) plaats. Twee met zaad overgaande virussen, te weten slamozaïekvirus (LMV) en vroege-verbruiningsvirus van erwt (PEBV) werden bij het onderzoek betrokken. Met DIA kon LMV worden aangetoond in een gezuiverd extract van *Nicotiana benthamiana*. In ruwe planteëxtracten bleken echter stoffen voor te komen die in DIA sterke niet-specifieke reacties tot gevolg hadden. Verder onderzoek is dan ook noodzakelijk om DIA geschikt te maken voor het aantonen van plantevirussen in ruwe extracten van planten. Betere resultaten werden verkregen met de beide ELISA-modificaties. Met de extracten uit slazaad en erwtezaad gaf ELISA 2 vergelijkbare (LMV) of iets hogere (PEBV) extinctiewaarden dan ELISA 1. Bovendien was de verhouding tussen de extinctiewaarden van virusziek materiaal en die van virusvrij materiaal, bij ELISA 2 hoger dan bij ELISA 1. De grotere gevoeligheid van ELISA 2 en de grotere doelmatigheid ten gevolge van de gelijktijdige incubatie van monster en conjugaat duiden op de bijzondere geschiktheid van deze methode voor routinematige toepassing op grote schaal.

References

Clark, M.F., 1981. Immunosorbent assays in plant pathology. A. Rev. Phytopath. 19: 83-106.

Clark, M.F. & Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. 34: 475-483.

Flegg, C.L. & Clark, M.F., 1979. The detection of apple chlorotic leafspot virus by a modified procedure of enzyme-linked immunosorbent assay (ELISA). Ann. appl. Biol. 91: 61-65.

Gribnau, T., Roeles, F., Biezen, J. van de, Leuvering, J. & Schuurs, A., 1982. The application of colloidal dye particles as label in immunoassays: Disperse(d) dye immunoassay ('DIA'). In: Gribnau, T.C.J., Visser, J. & Nivard, R.J.F. (Eds), Affinity chromatography and related techniques. Elsevier Scientific Publishing Company, Amsterdam, p. 411-424.

Gribnau, T., Sommeren, A. van & Dinther, F. van, 1983. DIA – Disperse dye immunoassay. In: Chaiken, I., Wilchek, M. and Parikh, I. (Eds). Affinity Chromatography and Biological Recognition. Academic Press, Inc., New York, p. 375-380.

Harrison, B.D., 1973. Pea early-browning virus. C.M.I./A.A.B. Descriptions of Plant Viruses No. 120, 4 pp.

Tóbiás, I., Maat, D.Z. & Huttinga, H., 1982. Two Hungarian isolates of cucumber mosaic virus from sweet pepper *Capsicum annuum)* and melon *(Cucumis melo):* identification and antiserum preparation. Neth. J. Pl. Path. 88: 171-183.

Tomlinson, J.A., 1970. Lettuce mosaic virus. C.M.I./A.A.B. Descriptions of Plant Viruses No. 9, 4 pp.

Voller, A., Bartlett, A., Bidwell, D.E., Clark, M.F. & Adams, A.N., 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. gen. Virol. 33: 165-167.

Vuurde, J.W.L. van & Maat, D.Z., 1983. Routine application of ELISA for the detection of lettuce mosaic virus in lettuce seeds. Seed Sci. & Technol. 11: 505-513.