Neth. J. Pl. Path. 86 (1980) 277–283

Application of enzymes during bulb tissue extraction for detection of lily symptomless virus by ELISA in Lilium spp.

J. C. M. BEIJERSBERGEN and CAROLA TH. C. VAN DER HULST

Bulb Research Centre, Lisse, The Netherlands

Accepted 11 February 1980

Abstract

Lily symptomless virus (LSV) was readily detected with the enzyme-linked immunosorbent assay (ELISA) in leaves of *Lilium* Mid-Century hybrids cvs Enchantment and Destiny and *Lilium speciosum* cv. Brabander. In bulbs of cv. Enchantment LSV could be detected without any additional treatment of the extracts, but for reliable results in cv. Destiny it was necessary to pre-incubate the bulb extracts with cellulase or hemicellulase.

Additional keywords: Lilium Mid-Century hybrid, *Lilium speciosum*, cellulase, hemicellulase.

Introduction

Lily symptomless virus (Allen, 1972) is widespread in many lily cultivars (Asjes et al., 1973). Lilies were freed from LSV by tissue culture (Allen, 1974; Asjes et al., 1974; Van Aartrijk and Blom-Barnhoorn, 1979), because plants infected with this virus usually produce smaller flowers than healthy plants do and are commercially less valuable. Commercial stocks obtained in this way are checked for the presence of LSV by the single immunodiffusion drop test (Van Slogteren et al., 1976), the leaves being tested.

For a certification programme, however, testing bulb tissue is preferable, because for commercial purposes the bulb itself must be certified. The single immunodiffusion drop test is not always sufficiently sensitive to detect the presence of LSV in bulb tissue (Derks and Vink-van den Abeele, 1980) and aspecific reactions have been found to occur occasionally (Van Slogteren et al., 1976).

The enzyme-linked immunosorbent assay (ELISA) is a sensitive tool for the detection of plant viruses (Clark and Adams, 1977) and has been applied in a number of cases (e.g. Adams, 1978; Bossenec and Maury, 1978; Gugerli, 1978; Maat and De Bokx, 1978; Koenig et al., 1979; Stein et al., 1979).

This paper describes the application of ELISA for the detection of LSV in leaves and bulbs of lilies. An attempt was also made to determine whether a strong inhibitory action of bulb extracts in ELISA could be eliminated by the addition of enzymes to the extracts.

Material and methods

Leaves of LSV-infected field-grown plants of *Lilium* Mid-Century hybrid cvs Enchantment and Destiny and *Lilium speciosum* cv. Brabander were harvested in July and

stored in plastic bags at –20 °C. The bulbs were lifted in October and stored at +2 °C in wood-shavings in plastic bags. Virus-free material of cvs Enchantment and Destiny was obtained from tissue-cultured plants raised under insect-proof nylon netting.

Extracts were prepared by homogenizing 2–5 g tissue with an Ultra Turrax in twice the weight of PBS-Tween extraction buffer 0.01 M phosphate, pH 7.4, containing 2% polyvinylpyrrolidine (Clark and Adams, 1977). As tissue was assumed to contain 50% water, this extract is referred to as extract 1/5. The extract was further diluted stepwise to 1/25, 1/125, ... with the same extraction buffer.

The bulb extracts were pre-incubated for about 18 h at room temperature with one of the following enzymes (Sigma) or combinations thereof: α-amylase A 6505, cellulase C 7502, hemicellulase H 2125, pectinase P 4625, and pectin-esterase P 6763. The enzymes were suspended in extraction buffer (25 mg/ml) and added to the extract to a final concentration of 1 mg/ml.

LSV antiserum (titre: 1/2048, determined by a microprecipitation method) was prepared by Derks (Derks and Vink-van den Abeele, 1980) and γ-globulins were purified and conjugated with alkaline phosphatase (Sigma, type VII) after Clark and Adams (1977). ELISA was performed according to Clark and Adams (1977) using 4 μg γ-globulins/ml for coating and an enzyme-conjugate dilution of 1/250. Results were read at 405 nm with a continuous-flow cuvet, lightpath 1 cm, volume 0.08 ml. All samples were tested in duplicate; the results are given as mean absorbance value in the tables and figures.

Results

Leaves. LSV can be detected readily in extracts of lily leaves cv. Enchantment. Even for diluted extracts (1/125) of LSV-infected leaves, absorbance values did not overlap with those of concentrated virus-free extracts (1/5) (Table 1).

Since the specific activity of LSV antisera can vary, depending on the rabbit injected, it should be mentioned that the results given in Table 1 were also obtained, both for LSV-infected and virus-free leaves, with other sera when 1 μg γ-globulin/ml for coating and a conjugate dilution of 1/1000 were used.

In Fig. 1 the ELISA absorbance values obtained with a purified preparation of LSV (Derks and Vink-van den Abeele, 1980) are compared with those for an extract of lily leaves heavily infected with LSV. The purified preparation still showed absorbance at

Table 1. ELISA absorbance values for extracts of lily cv. Enchantment leaves with and without LSV.

	Number of samples extracted	Extract dilution	$E_{405\,nm}$ range
LSV-infected	12	1/5	1.2 –2.0
		1/25	0.94–2.0
		1/125	0.54–0.92
Virus-free	6	1/5	0.04–0.10

Tabel 1. ELISA-absorptiewaarden gevonden met bladextracten van lelie, cv. Enchantment, geïnfecteerd met LSV of virusvrij.

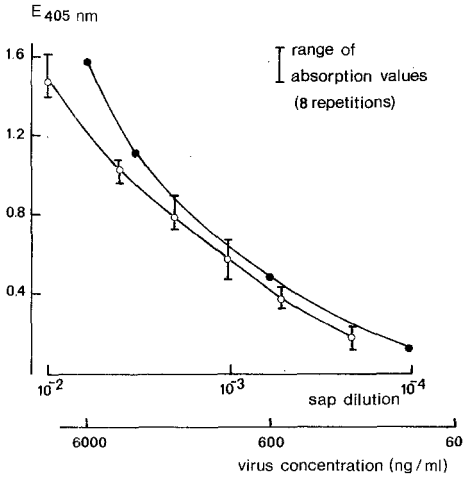

Fig. 1. ELISA absorbance values for purified LSV in extraction buffer (●——●) and leaf extracts of cv. Enchantment with LSV (○——○).

Fig. 1. ELISA-absorptiewaarden voor gezuiverd LSV in extractiebuffer (●——●) en voor bladextracten van cv. Enchantment, geïnfecteerd met LSV (○——○).

about 100 ng LSV/ml. Compairing both curves it can be assumed that the leaf extract 1/1000 contained about 900 ng/ml and hence the original extract 1/5 about 0.18 mg/ml. Because 1 ml extract 1/5 is produced from 0.3–0.4 g fresh tissue, the lily leaves we used contained about 0.45 mg LSV/g fresh weight.

Leaf sap constituents had a slight influence on the test results: compared with the purified preparation, the absorbance value of the 1/100 extract was relatively lower than those of the higher dilutions. The differences between the absorbance values of duplicates were small (about 10%) for the purified LSV within the same experiment and about 25% for the crude extracts.

LSV was also readily detected in leaves of cvs Destiny and Brabander.

Bulbs. The ELISA results for LSV-infected and virus-free bulb tissue are shown in Table 2. The results obtained for bulb extracts of cv. Enchantment were similar to those for leaf extracts and purified LSV preparations: the more diluted the extract (or virus

Table 2. ELISA absorbance values with standard deviation* for extracts of cvs Enchantment and Destiny bulbs with and without LSV (number of bulbs: 8 diseased, 6 virus-free).

Cultivar	Extract dilution	$E_{405\,nm}$	
		LSV-infected bulbs	virus-free bulbs
Enchantment	1/5	0.98 ± 0.20*	0.10 ± 0.04
	1/25	0.58 ± 0.18	0.10 ± 0.04
	1/125	0.30 ± 0.12	0.08 ± 0.04
Destiny	1/5	0.25 ± 0.16	0.10 ± 0.06
	1/25	0.49 ± 0.25	0.08 ± 0.07
	1/125	0.35 ± 0.20	0.09 ± 0.07

Tabel 2. ELISA-absorptiewaarden gevonden met extracten van bollen van de leliecultivars Enchantment en Destiny, geïnfecteerd met LSV of virusvrij.: standaard afwijking.*

Table 3. ELISA absorbance values for a pooled extract of 6 LSV-infected cv. Destiny bulbs after incubation of samples with several enzymes at room temperature for 18 h.

Enzyme preparation added	Extract dilution	
	1/5	1/25
none (control)	0.29	0.41
α-amylase	0.27	0.34
cellulase	0.93	0.69
hemicellulase	1.18	0.71
pectinase	0.88	0.66
pectin-esterase	0.55	0.58

Tabel 3. ELISA-absorptiewaarden voor een bolextract van lelie, cv. Destiny (mengsel van 6 bollen) met LSV, na incubatie van monsters met verschillende enzympreparaten bij kamertemperatuur gedurende 18 h.

suspension) the lower the absorbance value. For bulb extracts of cv. Destiny this pattern differs: diluted extracts (1/25) gave higher extinction values than the extracts 1/5 (Table 2), and the variation between the individual bulbs was greater. This general pattern persisted when the bulb tissue was frozen before extraction, the polyvinylpyrrolidone was omitted from the extraction buffer, or the tissue was homogenized with pestle and mortar. A similar result was obtained when the extract 1/5 had been stored at room temperature for 24 h, heated to 50°C for 15 min, or dialyzed against extraction buffer for 12 h. Nevertheless, from the results obtained with the diluted extracts (1/25 and 1/125) it must be concluded that the extracts 1/5 of cvs Destiny and Enchantment bulbs contained LSV in about the same concentration (Table 2). Because the extract 1/5 of cv. Destiny bulbs is extremely viscous, the possibility that enzymes known to split pectin, cellulose, or starch would enhance the absorbance values obtained with ELISA was investigated.

Pectinase, cellulase, and especially hemicellulase greatly enhanced the ELISA absorbance value of the LSV-containing extract 1/5 of the cv. Destiny bulbs (Table 3).

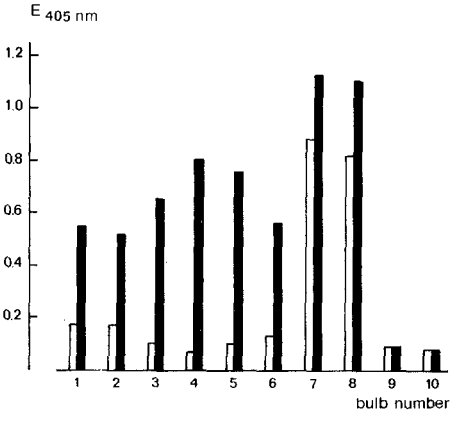

Fig. 2. ELISA absorbance values for extract 1/5 of individual lily bulbs of cv. Destiny after incubation (■) or without (□) hemicellulase at room temperature for about 18 h. (Bulbs 1–8: with LSV, bulbs 9 and 10: without virus).

Fig. 2. ELISA-absorptiewaarden voor extracten 1/5 van individuele bollen van cv. Destiny, na incubatie met (■) of zonder (□) hemicellulase bij kamertemperatuur gedurende ca. 18 h; bollen met LSV: 1–8, virusvrije bollen: 9 en 10.

Several combinations of the enzymes were tested, but the best results were always obtained when hemicellulase was present. None of the enzymes added to virus-free extracts 1/5 cv. Destiny bulbs or to the extraction buffer showed absorbancy in ELISA.

In exceptional cases the absorbance values of extracts 1/5 of individual cv. Destiny bulbs were high enough (0.8) to justify the conclusion that LSV was present in the extract (Fig. 2). Yet hemicellulase then also improved the results.

Discussion

The detection of any virus in a plant by ELISA is based on the fact that an extract of a virus-diseased plant induces a relatively high absorbance in the assay and an extract of a virus-free plant a relatively low one (Clark and Adams, 1977), These conditions were readily fulfilled for leaf extracts of the three lily cultivars tested for LSV, viz. Enchantment, Destiny, and Brabander. Extracts of cv. Destiny bulbs seem to have one or more substances that inhibit at least one of the LSV-induced reactions in ELISA. Such inhibitory substances have also been found in gladiolus corms (Stein et al., 1979), and in some woody plant parts (Clark and Adams, 1977).

The concentrations of these inhibitory substances in the lily bulb extracts possibly differ from cultivar to cultivar; for cv. Enchantment they play a minor role. But even for cv. Destiny, where the effect is pronounced, individual bulbs of the same stock differ in this respect.

Since the bulb extracts 1/5 of cv. Destiny are extremely viscous it may be assumed that the extract contains gummy substances that influence the mobility of the virus particles and hamper fixation of the virus to the attached γ-globulins during incubation of the extract in the well. This problem can be eliminated by the use of various enzymes or simply by dilution with buffer. The latter method is only appropriate if the concentration in the tissue is sufficiently high to permit detection of the virus in dilute extracts.

In the present study the pH of the extracts, the type of buffer, and the presence of plant constituents may have influenced the activity of the enzymes in different ways. Moreover, the specific activity of the commercially available enzyme preparations differ greatly and batches sometimes contain other enzyme impurities. Therefore, it can only be concluded in general that crude preparations of such enzymes as hemicellulase, cellulase, and pectinase have a favourable effect on the detection of LSV in bulb extracts of lilies. More detailed experiments to elucidate the nature of the inhibitory substances are in progress.

The question as to whether ELISA can be used for all commercially available cultivars of lilies in a mass screening program for detection of LSV in the bulbs is also under investigation.

Acknowledgments

The authors are greatly indebted to Dr P. K. Schenk, former director of the Bulb Research Centre, for encouraging this study.

Samenvatting

Het gebruik van enzymen bij het vervaardigen van extracten voor het aantonen van symptoomloos lelievirus met ELISA

In de bladeren van secundair geïnfecteerde planten van de leliecultivars Enchantment, Destiny en Brabander kon LSV met ELISA gemakkelijk worden aangetoond. In bladextracten van 'Enchantment' kon nog ca. 100 ng LSV/ml extract worden teruggevonden. Bij toepassing van de standaard-methode van extraheren (2 g weefsel malen in 4 ml extractiebuffer) kon ook in de bolschubben van 'Enchantment' LSV worden aangetoond; bij 'Destiny' gelukte dat aanzienlijk minder goed. Wijzigingen in de extractie-procedure of een speciale behandeling van het standaard-extract (dialyseren, verhitten tot 50 °C etc.) leidde niet tot een betere aantoonbaarheid van het virus met ELISA. Enzymen als hemicellulase, cellulase en pectinase, toegevoegd aan het standaard-extract van bolschubben van 'Destiny' vóór de toetsing (incubatie gedurende 18 h bij kamertemperatuur), verbeteren de aantoonbaarheid van het LSV aanzienlijk.

References

Aartrijk, J. van & Blom-Barnhoorn, G. J., 1979. Some influence of α-naphtylacetic acid on the differentiation of meristems of *Lilium* speciosum 'Rubrum' nr. 10 in vitro. Acta Hortic. 91: 269–280.

Adams, N. A., 1978. The detection of plum pox virus in *Prunus* species by enzyme-linked immunosorbent assay (ELISA). Ann. appl. Biol. 90: 215–221.

Allen, T. C., 1972. Lily symptomless virus. Commonw. Mycol. Inst./Assoc. Appl. Biol., Descr. Pl. Viruses, no. 96.

Allen, T. C., 1974. Production of virus-free lilies. Acta Hortic. 36: 235–240.

Asjes, C. J., Vos, N. P. & Slogteren, D. H. M. van, 1973. Brown ring formation and streak mottle, two distinct syndromes in lilies associated with complex infections of lily symptomless virus and tulip breaking virus. Neth. J. Pl. Path. 79: 23–35.

Asjer, C. J., Bunt, M. H. & Slogteren, D. H. M. van, 1974. Production of hyacinth mosaic virus-free hyacinths and lily sumptomless virus-free lilies by meristem tip culture. Acta Hortic. 36: 223–228.

Bossenec, J. M. & Maury, Y., 1978. Use of the ELISA technique for the detection of soybean mosaic virus in soybean seeds. Annls. Phytopath. 10: 263–268.

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,

Derks, A. F. L. M. & Vink-van den Abeele, J. L., 1980. Purification of lily symptomless virus. Use and value of antisera against intact and pyrrolidine-degraded virus for testing lilies and tulips. Neth. J. Pl. Path. 86: 239–250.

Gugerli, P., 1978. The detection of two potato viruses by enzyme-linked immunosorbent assay (ELISA). Phytopath. Z. 92: 51–56.

Koenig, R., Dalchow, J., Leseman, D. E. & Preil, W., 1979. ELISA as a reliable tool for the detection of Chrysanthemum virus B in chrysanthemums throughout the year, Pl. Dis. Reptr. 63: 301–303.

Maat, D. Z. & De Bokx, J. A., 1978. Enzyme-linked immunosorbent assay (ELISA) for the detection of potato viruses A and Y in potato leaves and sprouts. Neth. J. Pl. Path. 84: 167–173.

Slogteren, D. H. M. van, Derks, A. F. L. M., Blom, G. J., Bunt, M. H. & Vink, J. L., 1976. A further simplified single immunodiffusion drop test for the detection of lily symptomless virus in *Lilium* species. Acta Hortic. 59: 29–36.
Stein, A., Loebenstein, G. & Koenig, R., 1979. The detection of cucumber mosaic virus and bean yellow virus in gladiolus by enzyme-linked immunosorbent assay (ELISA). Pl. Dis. Reptr. 63: 185–188.

Address

Laboratorium voor Bloembollenonderzoek, Postbus 85, 2160 AB Lisse, the Netherlands.

Book review

D. Godan, 1979. Schadschnecken und ihre Bekämpfung. 467 pp., 128 figs, 51 tables and 12 pages with colour photographs. Verlag Eugen Ulmer GmbH & Co., Stuttgart.ISBN 3-8001-30440-0. Price DM 118.

This is a monograph on slugs and snails that are in some way harmful to plants, animals or humans. It deals with terrestrial, amphibian and aquatic gastropods which are either phytophagous or important as intermediate hosts for parasites in higher animals and as vectors of viruses, bacteria and fungi. The book is divided into three main chapters. The first one covers morphology, physiology, taxonomy and ecology; the second one the damage gastropods can cause, including image of injury, methods to estimate population density and tresholds of control, and the third chapter deals with control in its broadest sence. The book concludes with 65 pages of references, a list of scientific names of the Molluscs and of organisms for which they can act as host, and a subject index.