Neth. J. Pl. Path. 88 (1982) 87-98

Purification of tulip breaking virus and production of antisera for use in ELISA

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Accepted 5 February 1982

Abstract

After comparison of various homogenization buffers and clarification methods, tulip breaking virus (TBV) was purified on a large scale by homogenization in 0.1 M tris buffer (pH 9.0), clarification with Triton X-100, and differential centrifugation. The TBV suspensions were absorbed with an anti-host protein antiserum. The purity of the product was verified by mechanical inoculation of test plants, serology, electron microscopy, spectrophotometry, and analytical ultracentrifugation.

The TBV antisera obtained from rabbits after intramuscular injections had titres of 1/1280 to 1/10240 in the microprecipitin test and were mainly used in conjugate dilutions of 1/1000 in ELISA. The absorbance values for healthy tulips and lilies were (very) low. The antisera prepared against TBV from tulip cv. Jack Laan reacted best with TBV in lilies, whereas the antisera prepared against TBV from tulip cv. Texas Flame reacted best with TBV in tulips.

Additional keywords: lily symptomless virus.

Introduction

To improve the general quality of flower bulbs, the decisive and obligatory field inspection for diseases is preceded by a voluntary inspection of a sample grown under glass. In the latter inspection, which will become obligatory in the near future, special attention is paid to virus indexing, varietal trueness, and deviations of various kinds. Because virus indexing is the most important and the most time-consuming part of the sample inspection, it was thought that the planting under glass could be replaced by a laboratory test for the most important virus(es). The sample inspection with respect to varietal trueness, deviations, etc. could then be made in a sample grown in the field, because these factors are less dependent on weather conditions.

In the inspection of tulips, indexing for tulip breaking virus (TBV; potyvirus group; Van Slogteren, 1971) is of primary importance. Large scale testing for TBV is only feasible by the use of a serological test. In comparison with other serological techniques, ELISA is sensitive, time-saving, and suitable for automation (Van Schadewijk, 1981). Moreover, ELISA allows testing of bulbs, as has been demonstrated for lilies with lily symptomless virus (LSV; Beijersbergen and Van der Hulst, 1980 a, b).

A test for TBV is also valuable for the production and certification of virus-free lily stocks. This test can be easily combined with the test for LSV, for which ELISA is al-

ready in use (Van Schadewijk, 1981). Since about 1965, antisera against TBV have been available (Van Slogteren and De Vos, 1966). Because background absorbance is too high, these very early antisera cannot be used in ELISA (J.C.M. Beijersbergen, pers. comm.). Therefore new antisera have to be prepared and thus more advanced purification procedures have to be investigated.

A purification procedure successfully used for other potyviruses (Huttinga, 1973) proved unsuitable for TBV. Therefore, we compared various methods for the purification of other potyviruses (Tomlinson, 1963; Damirdagh and Shepherd, 1970; De Zoeten and Fribourg, 1971; Van Oosten, 1972; Huttinga, 1973) and combined some of them for the purification of TBV and the preparation of antisera for use in ELISA.

Materials and methods

Virus sources. Leaves were collected from TBV-infected stocks of tulips and lilies about two weeks after flowering. Lilies of cv. Enchantment were grown under glass and in the field, and tulips of cvs. Elmus, Halcro, Maureen, and Texas Flame, and the so-called Rembrandt tulips of cvs. Divera and Jack Laan in the field. Stocks of tulips of cvs. Jack Laan and Texas Flame to be used for antiserum production on a large scale, were grown under gauze. Leaves of the virus-free tulip cv. Prominence grown under glass, were collected at flowering. The effect of freezing or freeze-drying of tulip leaves on TBV was detetermined on various tulip cultivars.

Homogenization and clarification. Leaves (fresh, frozen at —20 °C or freeze-dried) were homogenized with a blender in one of the following buffers (w/v:2/5): 0.1 M tris-thioglycollic acid (pH 9.0; Huttinga, 1973), 0.033 M phosphate buffer (pH 7.0 or 8.0; Van Slogteren and De Vos, 1966), or 0.5 M borate buffer (pH 7.2, 8.2, or 9.0; Tomlinson, 1963), all containing 1% sodium sulfite. After being pressed through cheese cloth, the extracts were either shaken with an equal volume of butanol/chloroform (v/v:1/1; Wetter, 1960), stirred with 8% butanol (Damirdagh and Shepherd, 1970), or subjected to low-speed centrifugation (10 min at 1500 *g* or 8000 *g*) and treated with 5% Triton X-100 (Van Oosten, 1972). When carbon tetrachloride/chloroform was used, the organic solvents were mixed together with leaves and buffer (Huttinga, 1973).

Concentration. Virus was concentrated by differential centrifugation (90 min at 26500 *g* or 90000 *g* followed by 10 min at 1500 *g* or 8000 *g*). After high speed centrifugation, the pellets were resuspended in either 0.1 M tris-HCl (pH 9.0), 0.033 M phosphate buffer (pH 7.2), or a mixture of 0.2 M boric acid and 0.05 M disodium tetraborate (pH 7.6). A mixer was used to resuspend the pellets during purification on a large scale. In some experiments the virus was additionally precipitated with 4% polyethylene glycol (PEG; Damirdagh and Shepherd, 1970).

Test plants. Test plants were mechanically inoculated with purified suspensions of TBV-infected and healthy tulips to check for the absence of viruses other than TBV (Asjes, 1972). The test plants included: *Chenopodium quinoa, Nicotiana debneyi, N. tabacum* cv. White Burley, *N. clevelandii, (N. clevelandii* × *N. glutinosa), Gomphrena globosa* and *Tetragonia expansa.* All plants were grown under glass at about 20 °C.

Analytical ultracentrifugation. Sedimentation coefficients were determined in two trials by the graphical method of Markham (1960) using a Phywé analytical ultracentrifuge.

Spectrophotometry. For the determination of virus concentrations, use was made of an extinction coefficient $E^{0.1\%}_{260} = 2.4$, as has been done for other potyviruses (Damirdagh and Shepherd, 1970; Van Oosten, 1972).

Electron microscopy. Leaf extracts and (partially) purified virus preparations were negatively stained with 2.5% PTA (pH 7.2) and examined in a Philips EM 201. For the measurements, tobacco mosaic virus was used as external standard.

Serology. Tulip and lily leaf extracts and (partially) purified virus preparations were tested for TBV and LSV initially by the microprecipitin test (Van Slogteren and De Vos, 1966; Derks and Vink-van den Abeele, 1980), and later by ELISA (Clark and

Fig. 1. Procedure for the purification of TBV

A. Infected tulip leaves
homogenize in 0.1 M tris-thioglycollic acid (pH 9.0) containing 1% Na_2SO_3 (w/v : 2/5)
press through cheese cloth

extract
centrifuge 10 min at 1500 *g*

supernatant
stir with 5% Triton X-100 for 45 min – 1 h
centrifuge 90 min at 90 000 g

pellet
resuspend in c. 1/8 volume 0.1 M tris-HCl (pH 9.0)
centrifuge 10 min at 8000 *g*

supernatant
centrifuge 90 min at 90 000 *g*

pellet
resuspend in 0.1 M tris-HCl (pH 9.0): 2 ml per 100 g initial weight
centrifuge 10 min at 8000 *g*

supernatant

B. add anti-host protein antiserum (c. 0.1 ml per ml supernatant)
allow to stand for 18 h at 4 °C
centrifuge 10 min at 9000 *g*

supernatant
centrifuge 90 min at 90 000 *g*

pellet
resuspend in 0.1 M tris-HCl (pH 9.0)
centrifuge 10 min at 7000 *g*

supernatant (purified virus)

Fig. 1. Procedure voor de zuivering van TBV.

Adams, 1977; Beijersbergen and Van der Hulst, 1980 a, b). Dilution endpoints of TBV containing sap were determined with the microprecipitin test.

Anti-host protein antisera were prepared with leaf material from healthy tulips of cv. Prominence, treated as indicated in part A of Fig. 1. The resulting suspensions were dialysed at 9 °C for 48 h against 0.1 M tris-HCl (pH 9.0) containing 0.37% formaldehyde. After emulsification of the dialysed suspension with an equal volume of Freund's incomplete adjuvant, 3 rabbits were injected intramuscularly (2 ml in each hind leg) 5 times at weekly intervals. The rabbits were bled weekly, starting 2-3 weeks after the last injection. The 5-6 bleedings yielded about 13 ml serum each. The titres were determined with purified suspensions of healthy tulips by the agar gel double diffusion method.

The TBV suspension from tulip (1ml, 1-2 mg TBV ml^{-1}) were absorbed with the γ-globulin fraction of the anti-host protein antiserum (c. 0.1 ml) and in the first year with LSV antiserum (1/40 ml) as well, both in a concentration of about 1 mg ml^{-1}. After about 18 h at 4 °C, these suspensions were centrifuged at 9000 *g* for 10 min and at 90000 *g* for 90 min. The pellets were resuspended and centrifuged at 7000 *g* for 10 min. The absorbed TBV suspensions (c. 0.5-1.7 mg TBV ml^{-1}) were dialysed and injected as described for the preparation of anti-host protein antisera. Additionally, during the next two years the rabbits (3 per series) received a booster injection intramuscularly and were bled again 5-6 times after each injection.

Titres of the TBV antisera were determined with clarified extracts of TBV-infected tulip cvs. Jack Laan, Halcro, Maureen and Paul Richter or of TBV-infected lily cv. Enchantment, using the microprecipitin test. The extracts were obtained by homogenizing the leaves in 0.05 M tris-HCl (pH 8.0) containing 0.9% NaCl and 10% normal horse serum (w/v:1/2). The extracts were pressed through cheese cloth, and after standing for 1-2 h at room temperature (necessary for the reaction of sap constituents with the horse serum), were centrifuged for 10 min at 1500 *g* or 6000 *g;* The titres were determined after incubation at room temperature for c. 16 h or at 37 °C for 20 min.

For use in ELISA, the γ-globulin fractions of the antisera were coupled with alkaline phosphatase (Clark and Adams, 1977). The absorbance values at 405 nm were measured directly through the plates with a Titertek Multiskan, using *p*-nitrophenylphosphate as substrate.

Results

Virus sources. Storage of TBV-infected lily leaves for about two months at −20 °C before purification resulted in almost complete loss of the virus. The dilution endpoint of the sap from fresh leaves, i.e., 1/32 or more, dropped to 1/8 after storage for six weeks at −20 °C and to 1/2 or 0 after two months. In sap of tulip leaves of cv. Divera stored for six months at −20 °C, many fragments and only a few intact virus particles were observed electron microscopically. The harmful effect of freezing on TBV was also observed with ELISA. Within five days at −20 °C the average absorbance value of 11 TBV-infected tulip leaves dropped from 0.81 to 0.58. There were, however, marked differences between individual leaves, as also found in another experiment (Table 1).

Better results were obtained with freeze-dried tulip leaves stored in bottles under vacuum than with frozen leaves (Table 2). In freeze-dried leaves of tulip cvs. Maureen

Table 1. Effect of freezing of tulip leaves on the detection of TBV.

Tulip cultivar	ELISA absorbance values at 405 nm of individual leaves	
	fresh leaves	leaves after 33 days at −20 °C
Sigrid Undset	>2.0	1.56
	>2.0	0.83
	0.37	0.53
Queen of Night	0.62	0.27
	0.30	0.22

Tabel 1. Het effect van het invriezen van tulpebladeren op de aantoonbaarheid van TBV.

Table 2. ELISA absorbance values at 405 nm of TBV from freeze-dried or frozen leaves of different tulip cultivars.

Tulip cultivar	Duration of storage (yr)	Freeze-dried leaves	Frozen leaves
Jack Laan	1	>2.0	0.40
Maureen	2	0.71	0.50
Apeldoorn	8	1.01	
Elmus	8	0.78	
Rauwenhoff	18	1.06	

Tabel 2. ELISA-absorptiewaarden bij 405 nm van TBV uit gevriesdroogde of bevroren bladeren van verschillende tulpecultivars.

and Elisabeth stored for two and 17 years, respectively, intact virus particles were detected electron microscopically.

For purification on a large scale we always used fresh tulip leaves infected with TBV. Leaves collected in the period from flowering to about two weeks after flowering gave better results than older leaves as determined spectrophotometrically. This was caused by the development of *Botrytis* on the older leaves, particularly of cvs. Jack Laan and Divera.

Homogenization and clarification. When leaves were homogenized in borate buffers more virus particles were found to be aggregated than in tris and phosphate buffers. Furthermore, more virus particles aggregated at lower pH values. The highest yields of non-aggregated virus particles were obtained with 0.1 M tris-thioglycollic acid (pH 9.0).

When the first low-speed centrifugation was performed at 8000 *g* for 10 min, the supernatant gave only a faint or no reaction with TBV antiserum, whereas after centrifugation at 1500 *g* the dilution endpoints of the same TBV-containing clarified saps were usually 1/32. Clarification with Triton X-100 gave twice-better results (on average) than clarification with butanol or butanol/chloroform. Especially butanol/

Fig. 2. Length distributions of TBV in crude sap of lily cv. Enchantment (A), after purification with butanol/chloroform and 150 min at 90 000 *g*/10 min at 1500 *g* (B), and using carbon tetrachloride/chloroform and 90 min at 26 500 *g*/10 min at 8000 *g* (C).

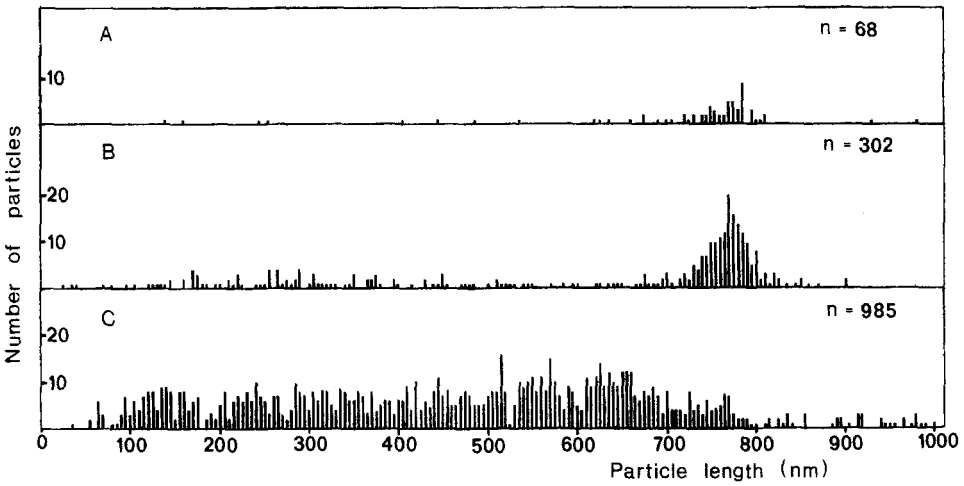

Fig. 2. Lengteverdelingen van TBV in ruw sap van lelie cv. Enchantment (A), na zuivering met butanol/chloroform en 150 min 90 000 g/10 min 1500 g (B) en na gebruik van tetrachloorkoolstof/chloroform en 90 min 26 500 g/10 min 8000 g (C).

Fig. 3 Electron micrograph of TBV purified from tulip cv. Texas Flame with Triton X-100 and anti-host protein antiserum. The preparation was negatively stained with 2.5% PTA (pH 7.2). Magnification bar represents 500 nm.

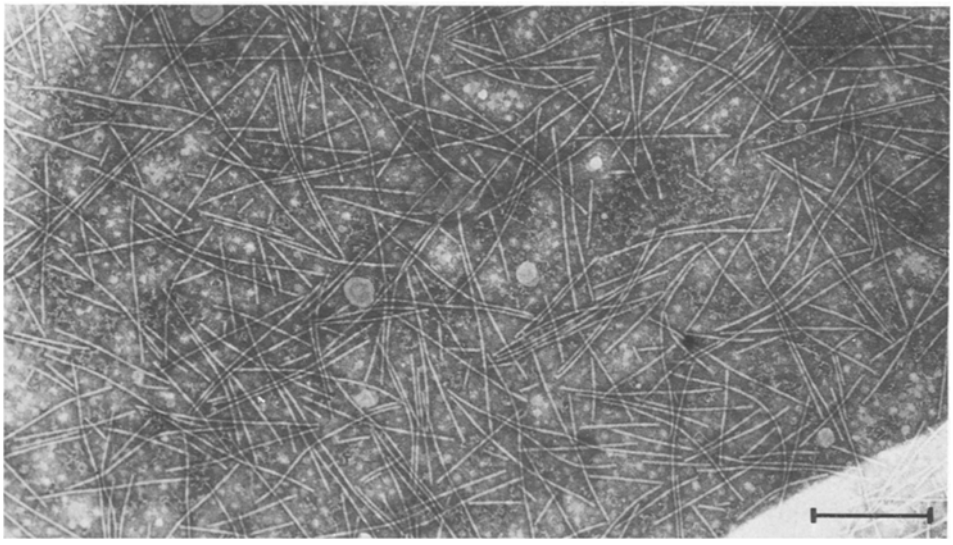

Fig. 3. Elektronenmicroscopische foto van TBV gezuiverd uit de tulpecultivar Texas Flame met Triton X-100 en antiserum tegen plante-eiwitten. Het preparaat werd negatief gekleurd met 2,5% PTA (pH 7,2). De vergrotingsstaaf geeft 500 nm weer.

chloroform gave very variable results: sometimes the virus was very well concentrated, in other cases the dilution endpoints of the purified suspensions were lower than those of the corresponding crude extracts. With carbon tetrachloride/chloroform the virus was fragmented, which was not seen with the other treatments (Fig. 2 and 3). This fragmentation gave rise to higher losses in virus yield during the rest of the purification procedure.

The highest yield of intact virus particles was obtained with Triton X-100, shown by the electron-microscopical, serological, and spectrophotometrical observation. For optimal results, the TBV-containing sap must be stirred with Triton X-100 for 45 min-1 h.

Concentration. At high centrifugal forces (90 000 *g*) the virus particles remained intact except when the extracts had been treated with carbon tetrachloride/chloroform. At lower centrifugal forces (26 500 *g*) the fragmentation effect of carbon tetrachloride/chloroform on TBV persisted (Fig. 2).

In this stage of the purification procedure low speed centrifugation at 8000 *g* was not harmful to TBV.

Precipitation with 4% PEG resulted in a loss of half or more of the total amount of TBV, as determined by the microprecipitin test and electron-microscopical examination.

Absorption with anti-host protein antiserum. With the first antisera prepared against TBV from tulips of cvs. Divera and Jack Laan absorption values were too high when tested with healthy plants in ELISA.

Therefore, anti-host protein antisera were produced to absorb the TBV suspensions. The titres of these antisera ranged from 1/32 to 1/128. The ELISA absorbance values of the TBV suspensions (diluted 1/50) with the anti-host protein antiserum dropped from 0.15 ± 0.02 to 0.03 ± 0.01 as a result of the absorption and subsequent differential centrifugation. At the same time about 46% of the total amount of TBV was lost by this treatment, as determined spectrophotometrically. With ELISA, the loss was about the same (c. 52%).

The complete TBV-purification procedure used in the preparation of antisera, is shown in Fig. 1.

Other viruses infecting tulips. With the use of test plants, none of the other viruses infecting tulips (Asjes, 1972) was detected in the purified TBV suspensions. In studies with the analytical ultracentrifuge, purified suspensions showed two components with sedimentation coefficients of 171 S (LSV) and 153 S (TBV) in 0.1 M tris-HCl (pH 9.0). The presence of LSV was confirmed serologically.

On the basis of these findings, in the first year TBV antisera were prepared with virus suspensions absorbed not only with anti-host protein antiserum but also with LSV antiserum. In the following years the tulip plants grown under gauze were screened for the absence of LSV before TBV purification. In the tulip cultivar Jack Laan about 15% of the plants contained LSV (J.C.M. Beijersbergen, pers. comm.) and in cv. Texas Flame about 1%.

Spectrophotometry. The purified TBV suspensions were almost colourless after absorption with anti-host protein antiserum. The UV spectra before and after absorption showed a maximum at 261 ± 1 nm and a minimum at 245 ± 3 nm. The max:min ratio ranged from 1.03 to 1.13 for TBV purified from tulip cvs. Jack Laan and Divera, and from 1.13 to 1.21 for TBV from cv. Texas Flame. When the leaves were collected too late, i.e., starting two weeks after flowering, the UV spectra did not show a maximum and a minimum. Purified virus suspensions with this type of UV spectrum were not used for antiserum production.

The estimated virus yield ranged between 5 and 21 mg TBV per kg leaves.

Electron microscopy. The purified suspensions were highly homogeneous and contained few impurities. The virus particles were not aggregated or fragmented (Fig. 3).

In 50% glycerol, the virus suspensions could be stored at −20 °C for more than three years without signs of fragmentation or aggregation.

Serology. The determination of titres of the TBV antisera was difficult with leaf extracts obtained according to Van Slogteren and De Vos (1966), because of the unpredictable appearance of spontaneous flocculations. The addition of 10% normal horse serum (Van Slogteren, 1976) to the extraction buffer (tris buffer) resulted, in the microprecipitin test, in clear droplets in which the flocculations with TBV could be clearly observed. With this method spontaneous flocculation was almost completely eliminated, especially when extracts of freeze-dried tulip leaves or leaves frozen at −20 °C overnight were centrifuged for 10 min at 6000 *g*. Only with the tulip cultivar Jack Laan did slight spontaneous flocculation sometimes persist. The titres of the TBV antisera determined in this way ranged from 1/1280 to 1/10240. The conjugate dilution used in ELISA was usually 1/1000. The TBV antisera did not react in ELISA with purified LSV suspensions, thus indicating that the injected TBV suspensions were absorbed completely with the LSV antiserum.

The antisera against TBV from the tulip cv. Texas Flame showed very low absorbance values with healthy tulips and lilies; with the antisera prepared from cv. Jack Laan these absorbance values were a little higher (Table 3). Another difference between these antisera was that the one prepared from cv. Texas Flame reacted best in ELISA with TBV in approximately 50 tulip cultivars tested, and the one prepared from cv. Jack Laan best with TBV in lilies (c. 30 cultivars) and in the tulip cvs. Jack Laan and Divera (Table 3). Serological differences between individual tulip and lily plants were not found to be related to individual differences in symptom expression.

Discussion

Three main problems are encountered in the purification of potyviruses: aggregation between virus particles, aggregation of virus particles with host plant material, and fragmentation of virus particles.

Aggregation between TBV particles could be avoided by using 0.1 M trisbuffer (pH 9.0) except when PEG precipitation was applied, as found for other potyviruses (Huttinga, 1973).

The considerable loss of virus after centrifugation of crude leaf extracts at 8000 *g* suggests the aggregation of virus particles with host-plant material. This hypothesis is

Tabel 3. Serological differences between two antisera prepared against TBV from two different sources as indicated by the ELISA absorbance values at 405 nm of individual tulip and lily plants with and without TBV.

	Antiserum prepared against TBV from tulip	
	cv. Texas Flame	cv. Jack Laan
Tulip cultivar		
Jack Laan diseased	0.40	0.80
	0.41	>2
	0.11	1.80
	0.89	>2
Texas Flame diseased	0.38	0.05
	0.54	0.52
Makassar diseased	1.03	0.59
	1.41	0.53
	0.94	1.64
healthy	0.04	0.05
Queen of Night diseased	0.76	0.29
	1.27	0.50
	1.13	0.41
Yellow Present diseased	1.08	0.98
	1.35	0.52
	1.57	0.42
	0.84	0.83
healthy	0.03	0.05
Lily cultivar		
Enchantment diseased	0.14	0.94
	0.18	0.36
	0.24	0.32
healthy	0.02	0.03
Concorde diseased	0.52	1.80
	0.24	0.41
	0.37	0.34
	1.30	>2
	0.89	1.24
healthy	0.03	0.04
Utaka diseased	0.17	0.76
	0.15	0.49
	0.17	0.96
healthy	0.02	0.00
Stirling Star diseased	0.74	0.40
	0.74	1.40
	1.25	1.80

Tabel 3. Serologische verschillen tussen twee antisera, die bereid zijn tegen twee verschillende bronnen, zoals blijkt uit de ELISA-absorptiewaarden bij 405 nm van afzonderlijke tulpe- en lelieplanten met en zonder TBV.

supported by the finding that centrifugation at 8000 *g* after treatment with Triton X-100, which dissolves cell membranes and chloroplasts (Van Oosten, 1972), did not have a negative effect.

The fragmentation of TBV we observed was caused by freezing of the leaf tissue and treatment of the sap with carbon tetrachloride/chloroform and not by the high centrifugal forces as reported for other potyviruses (Huttinga, 1973). In the purification of plum pox virus high centrifugal forces were not harmful either (Kerlan et al., 1975).

The method of an anti-host protein antiserum, routinely applied by De Zoeten and Fribourg (1971) in the purification of flexuous rod-shaped viruses, was very useful for the production of TBV antiserum, because of the low absorbance values with healthy tulips and lilies in ELISA.

To prevent strain specificity of the TBV antisera and/or conjugates in ELISA (Koenig, 1978), the Rembrandt tulip cv. Jack Laan was chosen as source for virus purification. These Rembrandt tulips showed full, self, and average flower-break symptoms (Van Slogteren, 1971). Nevertheless, the antiserum against TBV from cv. Jack Laan reacted better with TBV in lilies than with TBV in tulips. The differences between the antisera prepared against TBV from cvs. Jack Laan and Texas Flame do not seem to be associated with differences in symptom expression and cannot be explained by differences in titres of antisera and/or dilutions of conjugates either. Other possible causes are under investigation, for instance the existence of serotypes (Beijersbergen and Van der Hulst, 1982), the presence of an unknown virus in tulips (Van Schadewijk and Eggink, 1982), and an influence of the host plant.

The maintenance of TBV suspensions in glycerol at −20 °C without visible fragmentation makes it possible to test the quality of a conjugate in the course of time and to compare conjugates tested at different times. These are important aspects in the distribution of conjugates.

The detection of LSV in Rembrandt tulips was unexpected, because of the incidental occurrence of this infection in tulips (Asjes et al., 1973; Derks and Asjes, 1975), but it is not surprising in view of the cultivation method (without virus inspection) and the indistinctness of the symptoms (Derks and Asjes, 1975; Derks and Vink-van den Abeele, 1980), particularly in tulips simultaneously infected with TBV (Asjes et al., 1973).

Samenvatting

Zuivering van tulpemozaïekvirus en produktie van antisera voor gebruik in ELISA

Na vergelijking van verschillende homogenisatie-buffers en klaringsmethoden werd het tulpemozaïekvirus (TBV) op grote schaal gezuiverd door gebruik te maken van 0,1 M tris-buffers (pH 9,0), klaring met Triton X-100 en differentieel centrifugeren. De virussuspensies werden geabsorbeerd met antiserum gemaakt tegen plante-eiwitten. De zuiverheid van het produkt werd vastgesteld door mechanische inoculatie van toetsplanten, serologie, elektronenmicroscopie, spectrofotometrie en analytische ultracentrifugering. De TBV-antisera, verkregen na intramusculaire injecties van konijnen, hadden titers van 1/1280 tot 1/10240 in de microprecipitatietoets en werden in ELISA meestal in conjugaatverdunningen van 1/1000 gebruikt. De absorptiewaarden

met gezonde tulpen en lelies waren (zeer) laag. De antisera bereid tegen TBV uit de tulpecultivar Jack Laan reageerden het best met TBV in lelies en de antisera tegen TBV uit de tulpecultivar Texas Flame het best met TBV in tulpen.

References

Asjes, C.J., 1972. Tulip veinal streak, a disorder probably caused by tobacco ringspot virus. Neth. J. Pl. Path. 78: 19-28.

Asjes, C.J., Vos, N.P. de & 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.

Beijersbergen, J.C.M. & Hulst, C.Th.C. van der, 1980a. Application of enzymes during bulb tissue extraction for detection of lily symptomless virus by ELISA in *Lilium* spp. Neth. J. Pl. Path. 86: 277-283.

Beijersbergen, J.C.M. & Hulst, C.Th.C. van der, 1980b. Detection of lily symptomless virus (LSV) in bulb tissue of lily by means of ELISA. Acta Hortic. 109: 487-493.

Beijersbergen, J.C.M. & Hulst, C.Th.C. van der, 1982. Can two serotypes of tulip breaking virus be present in tulip stocks? Acta bot. neerl. 31: 242.

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.

Damirdagh, I.S. & Shepherd, R.J., 1970. Purification of the tobacco etch and other viruses of the potato Y group. Phytopathology 60: 132-142.

Derks, A.F.L.M. & Asjes, C.J., 1975. Lily symptomless virus in tulip. Neth. J. Pl. Path. 81: 14-21.

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.

Huttinga, H., 1973. Properties of viruses of the potyvirus group. 1. A simple method to purify bean yellow mosaic virus, pea mosaic virus, lettuce mosaic virus and potato virus Y^N. Neth. J. Pl. Path. 79: 125-129.

Kerlan, C., Dunez, J. & Bellet, F., 1975. Méthodes simples de purification du virus de la sharka (plum pox virus). Ann. Phytopathol. 7: 287-297.

Koenig, R., 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. J. gen. Virol. 40: 309-318.

Markham, R., 1960. A graphical method for the rapid determination of sedimentation coefficients. Biochem. J. 77: 516-519.

Oosten, H.J. van, 1972. Purification of plum pox (sharka) virus with the use of Triton X-100. Neth. J. Pl. Path. 78: 33-44.

Schadewijk, A.R. van, 1981. Toepassing van de ELISA-methode voor het aantonen van virussen in bloembolgewassen. Bloembollencultuur 92: 417-420.

Schadewijk, A.R. van & Eggink, J., 1982. A new virus in tulips as one of the possible explanations for serological distinction between antisera against tulip breaking virus (TBV). Acta bot. neerl. 31: 242-243.

Slogteren, D.H.M. van, 1971. Tulip breaking virus. Commonw. Mycol. Inst./Assoc. Appl. Biol., Descr. Pl. Viruses, No. 71.

Slogteren, D.H.M. van, 1976. A single immunodiffusion drop test for the detection of lily symptomless virus. Ann. appl. Biol. 82: 91-95.

Slogteren, D.H.M. van & Vos, N.P. de, 1966. Tulip breaking virus, its serological behaviour and serological relationship to a virus isolated from lily. In: A.B.R. Beemster & J. Dijkstra (Eds), Viruses of plants. Proc. int. Conf. Pl. Viruses. July 1965, Wageningen: 320-323.

Tomlinson, J.A., 1963. Effect of phosphate and borate on the infectivity of some viruses during purification. Nature 200: 93-94.
Wetter, C., 1960. Partielle Reinigung einiger gestreckter Pflanzenviren und ihre Verwendung als Antigene bei der Immunisierung mittels Freundschen Adjuvans. Arch. Mikrobiol. 37: 278-292.
Zoeten, G.A. de & Fribourg, C.E., 1971. Instability of some flexuous rod-shaped virus particles during purification. Acta Hortic. 23: 278-281.

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