Neth. J. Pl. Path. 94 (1988) 149-160

Detection of tobacco rattle virus in different parts of tulip by ELISA and cDNA hybridisation assays

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Accepted 23 December 1987

Abstract

Different parts of tulips cv. Apeldoorn were assayed for the presence of tobacco rattle virus (TRV) by means of ELISA, cDNA hybridisation and immuno-electron microscopy. Assays were periodically performed during the growing season and upon storage of the bulbs. During the growing season in the field the relative TRV concentrations detected by ELISA and cDNA were highest mainly in the basal stem-parts and basal leaf-parts, respectively. When, during storage, infected bulbs were divided into a number of sections, TRV could be detected only in some of the sections, irrespective of the test used. However, nearly all sprouts of infected bulbs, stored at 5 °C for 7 months, appeared to contain detectable amounts of TRV upon testing with ELISA and cDNA. Thus, testing of sprouts may offer a possibility to develop a routine test for TRV in tulip bulbs in due course.

Additional keyword: localisation.

Introduction

Covering a total land area of about 70 km^2, tulips are one of the most important commercially grown bulbous crops in the Netherlands. Of the viruses found in tulip, tulip breaking virus (TBV) and tobacco rattle virus (TRV) occur most frequently (Asjes and Elbertsen, 1982). Both viruses in tulips can be detected in the field by visual inspection as the viruses produce characteristic symptoms (Asjes en Elbertsen, 1982; Van Slogteren, 1958). Screening, however, should be done preferentially with the bulbs to fit into a sample inspection as part of a general quality inspection. TBV is detected reliably by ELISA (Van Schadewijk and Eggink, 1984) in homogenates of tulip and lily bulbs; over 700 000 ELISA assays are carried out each year by the Bulb Inspection Service in the Netherlands to prevent the use of infected propagation material. In contrast, preliminary experiments on the detection of TRV in bulb homogenates yielded unsatisfactory results with ELISA (unpublished results). This may be partly due to technical problems such as a low concentration of the virus or the presence of inhibitory substances in the homogenates. In addition, the rather antigenic variability among tobraviruses (Harrison and Robinson, 1986) may cause a fundamental problem in that probably many antisera are required to detect all possible serotypes.

Moreover, RNA 1, the largest of the two genomic RNAs of TRV, can establish an infection in plants in the absence of RNA 2. Such an infection cannot be detected with an antiserum against the virus, because the RNA-2-encoded coat protein is not produced. RNA 1 infections are known to occur in some potato stocks (Harrison et al., 1983) but have not yet been observed in tulip.

The technique of cDNA hybridisation might offer an alternative way of detecting TRV in tulip bulbs. Available data indicate that different TRV strains show much diversity in the sequence of RNA 2 but share extensive homologies in their RNA-1 sequences (Robinson and Harrison, 1985; Angenent et al., 1986). Probably all known TRV serotypes are detectable by using a single cDNA-1 clone, wether or not RNA 2 is present in the infected plant. Experimental evidence supporting this notion has been presented (Linthorst and Bol, 1986). Previously, cDNA was cloned to RNA 1 of several TRV strains (Cornelissen et al., 1986; Angenent et al., 1986). In this paper we describe the detection of TRV in field-grown tulip plants and in stored bulbs by ELISA, immuno-electron microscopy and cDNA hybridisation. The prospects for a future routine test using either of these techniques are discussed.

Materials and methods

Plant material

A. Field-grown plants. Unless mentioned otherwise, tulips of cv. Apeldoorn were used. The plants were grown in the test field of the Bulb Research Centre at Lisse. This field was known to be free of TRV infection as a result of soil disinfestation by methylbromide treatment. One selected group of plants was over 90% infected with a serotype of TRV that was detected in ELISA by antisera (coded TF and PV) provided by the Bulb Research Centre, Lisse. Another selected group was free of symptoms of virus infection and was used as a healthy control in this study.

Field samples of tulips were collected on the following dates in 1986: April 22, May 2-9-15-23-30, and June 13-20-27. Each time 5 tulip plants were harvested, 4 from the stock of TRV-infected plants and one from the non-infected control. In all cases, the plants from the infected stock showed symptoms of TRV infection. Each plant was divided into 15 parts, numbered 1 to 15 (Fig. 1). The first samples were taken after the two leaf stage when symptoms of TRV developed distinctly. On April 22, the tulip plants were about 10-15 cm tall and not all parts of the stem were large enough to be used. After May 9, the flowers were removed and were no longer available for testing. Towards the end of June the tulip plants began to senesce and above ground parts died off at the beginning of July.

B. Stored bulbs. After lifting in the beginning of July, bulbs of tulip cv. Apeldoorn were stored for 2-7 months at the following temperatures: 5, 13, 17, 20 and 25 °C. Sampling took place during this period. In addition, stocks of tulip cvs. Golden Apeldoorn, Candela and Attila were stored at 17 °C. Bulbs were divided into 12 pieces plus the sprout, as shown in Fig. 2. A first cut was made from top to bottom, followed by a second cut perpendicular to the first one, giving four approximately equal parts. Each part was divided into three by taking the outer scale, the second outer scale and the inner scale(s), each part containing the corresponding part of the basal plate. The sprout was collected as a whole from the middle of the bulp.

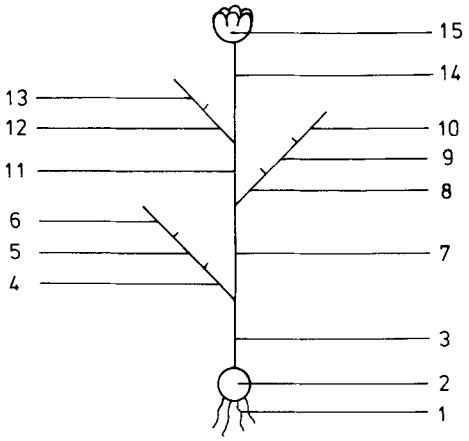

Fig. 1. Division of a tulip plant into 15 parts for assay by ELISA or cDNA hybridisation. (1) roots, (2) bulb, (3) stem part between the bulb and the first leaf, (4-6) basal, middle and top part of the first leaf, (7) stem between first and second leaf, (8-10) basal, middle and top part of second leaf, (11) stem between second and third leaf, (12-13) basal and top part of the third leaf, (14) stem part between third leaf and the flower, (15) flower or flower bud.

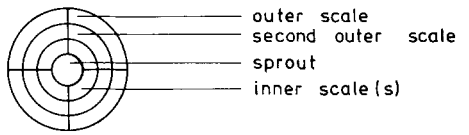

Fig. 2. Division of a tulip bulb into 13 parts for assay by ELISA. A cross-section of the bulb is shown.

Sample preparation for ELISA

A. Field-grown plants. 2 g of each sample was ground in 10 ml of extraction buffer (0.137 M NaCl, 2.7 mM KCl, 95 mM Na_2HPO_4, 13.2 mM KH_2PO_4, 0.1% Tween 20, 6.2 mM NaN_3 pH 7.4) with an ultra-turrax blender for 5-10 seconds at high speed. This homogenate was left to sediment and the supertenant was pipetted into ELISA plates.

B. Stored bulbs. The different bulb parts and the sprout were squeezed through a ribbed rotary press (Pollähne) and immediately diluted with extraction buffer, approximately 5 ml per g. The homogenates were left to sediment at room temperature for at least 1 h. Then the supertenant was pipetted in the test plates.

ELISA procedure

Gamma-globulins and conjugates of antiserum (coded TF) prepared against a serotype of TRV were kindly provided by A.F.L.M. Derks, Bulb Research Centre, Lisse. ELISA was performed according to Clark and Adams (1977), with the following modifications. Microtiter plates (Petra-plastic type F-ELISA) were coated for 20-68 h at 6 °C with gamma-globulins (1 μg/ml) and washed twice with deionized water. Samples were incubated at 6 °C overnight followed by washing. A standard sample of a frozen sap stock of 0.2 g TRV-infected tulip leaf per ml was applied as a reference. Conjugate, labelled with alkaline phosphatase and stored in 50% glycerol, was diluted 1 : 500 in conjugate buffer (0.137 M NaCl, 0.22 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 3.0 mM NaN_3, 2.7 mM KCl, 0.3% Tween 20, pH 7.4) and incubated for 3 h at 37 °C. If

bulb material was used, 0.4% normal horse serum (Van Schadewijk and Eggink, 1984) was added to the diluted conjugate to inhibit non-specific binding of the conjugate. After washing and incubation for 1 h at 37 °C with p-nitrophenyl phosphate (0.5 mg/ml) as a substrate the absoprtion at 405 nm was determined with a Titertek Multiskan spectrophotometer. ELISA data were converted using the internal standard as a reference, to minimize the effect of variations among plates.

Sample preparation for cDNA-hybridisation assay

A. Field-grown plants. Undiluted extracts from the different plant parts were collected by squeezing small parts through a smooth polished rotary press (Pollähne), and spotted in 2-μl samples onto nitrocellulose filters, which had been pretreated as described by Linthorst and Bol (1986).

B. Stored bulbs. Bulbs of tulip cv. Apeldoorn were divided into four approximately equal parts as described above, frozen in liquid nitrogen and mixed in a Waring blender until powdery. For each gram of bulb material 1 ml of SDS-extraction buffer (0.176 M glycine, 0.024 N NaOH, 0.176 M NaCl, 0.02 M EDTA, 2% SDS, pH 9.0) and 1 ml of water-saturated phenol/chloroform (1/1 : v/v) were added and mixed. The resulting slurry was centrifuged for 20 min at 3000 *g*. The water phase was again extracted with phenol/chloroform and the RNA was precipitated by addition of LiCl to a final concentration of 2 M. The precipitate was dissolved in a small aliquot of water. The RNA was precipitated again by the addition of ethanol to a final concentration of 70% and NaAc to 0.15 M. This precipitate was dissolved in water, in 1% of the volume of the original water phase. 1μl was spotted onto pretreated nitrocellulose. Sprout parts were treated as described for field-grown plant material.

cDNA-hybridisation procedure

A nick-translated ^{32}P-labelled cDNA clone, corresponding to the 3′-terminal 2077 nucleotides of RNA 1 of TRV strain PSG, was used as a probe (Cornelissen et al., 1986). Purified virus suspensions, tulip-plant homogenates or bulb extracts were spotted in 1- or 2-μl samples onto nitrocellulose filters (dot-blot). The test was performed as described by Linthorst and Bol (1986).

Immuno-electron-microscopy procedure

This procedure is based on the method of Milne and Luisoni (1977) with some minor modifications. Grids with a pioloform F-carbon film were coated with 1 : 5 diluted gamma-globulin fraction of anti-TRV serum (coded PV; 1 mg/ml) in 0.07 M phosphate buffer pH 7.2 during 30 min at 37 °C and washed in this phosphate buffer. Bulb-part samples were prepared as described for ELISA (see above), except that Tween 20 was excluded from the extraction buffer and the samples were centrifuged for 5 min at 1400 g. The grids were incubated on a droplet of supernatant sample for 10 min at 37 °C, followed by 50 min at room temperature, and carefully washed with excess demineralized water to remove all traces of phosphate. The grids were laid on droplets of 2% uranyl acetate pH 5.0 for about 1 min. The excess staining solution

was removed with filter paper and the dried grids were examined with a Philips 201 electron microscope.

Results

Distribution of TRV in tulip plants. The distribution of TRV in field-grown tulips was monitored over a 10 week period by both ELISA and cDNA hybridisation. The ELISA data obtained from different parts of the plants in representative tests of three dates during the growing season are shown in Fig. 3. TRV could be detected by ELISA in all parts of the diseased plants (Fig. 3A, B, C) with maximal absorption values occurring in the stem parts (samples 3, 7, 11 and 14), with the highest values prevailing in the basal stem part. During the beginning and the end of the growing season, values within the leaves were highest in the basal part (Fig. 3A and B), while the leaf values were about equal in the middle of the growing season (Fig. 3B). The ELISA results do not point to a gross change in the distribution of TRV in the plants during the 10 weeks of the experiment.

The background obtained with the healthy control (Fig. 3) was low, although at the end of the growing season there was an increase in the background obtained with the bulb extract (sample 2, data not shown). By adding 0.4% normal horse serum to the diluted conjugate, this background could be decreased in the bulb experiments later on.

Fig. 4 shows a graphical representation of an assay by cDNA hybridisation of four diseased plants collected at three different sampling dates during the growing season.

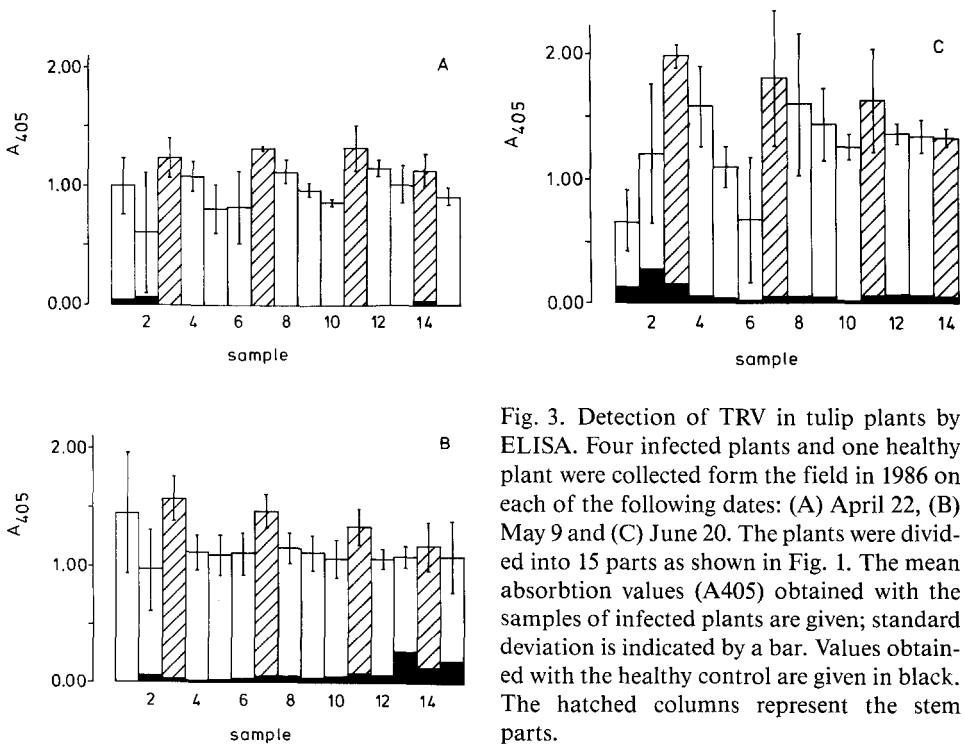

Fig. 3. Detection of TRV in tulip plants by ELISA. Four infected plants and one healthy plant were collected form the field in 1986 on each of the following dates: (A) April 22, (B) May 9 and (C) June 20. The plants were divided into 15 parts as shown in Fig. 1. The mean absorbtion values (A405) obtained with the samples of infected plants are given; standard deviation is indicated by a bar. Values obtained with the healthy control are given in black. The hatched columns represent the stem parts.

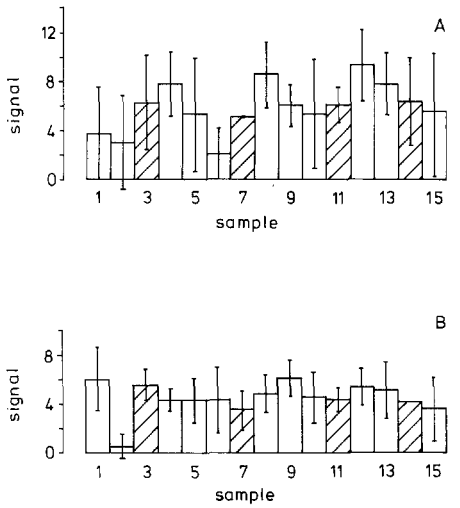

Fig. 4. Detection of TRV in tulip plants by cDNA hybridisation. Samples were collected as described in the legend of Fig. 3. The hybridisation signals are expressed on a zero to ten scale, using the signals obtained with known amounts of purified virus as references (see Fig. 5). The hatched columns represent the stem parts.

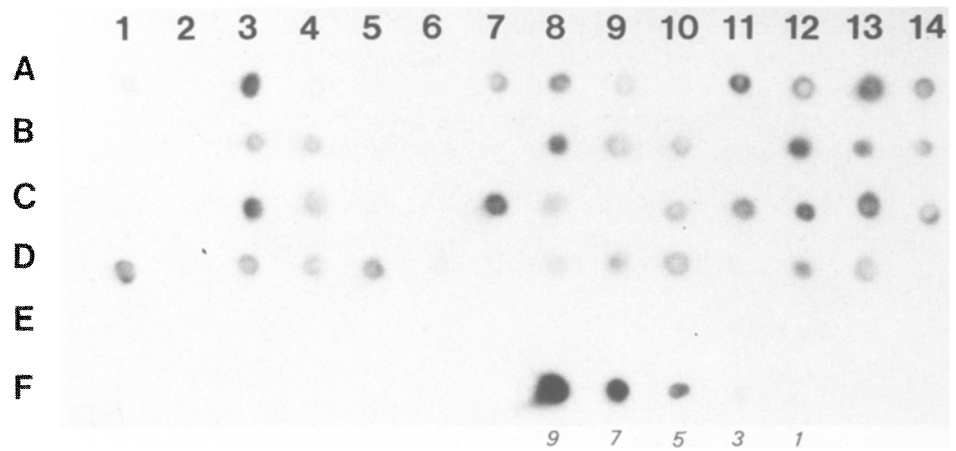

Fig. 5. Dot-blot showing the presence of TRV in different parts of tulip plants. On May 23, 1986, four diseased plants (A to D) and one healthy plant (E) were collected from the field; each was divided into 14 parts as indicated in Fig. 1 (the flowers were no longer available for testing). Sap form the 14 samples of plants A-E was spotted onto nitrocellulose and the filter was hybridised to the TRV-specific probe. In row F known amounts of purified TRV nucleoprotein were spotted to provide a reference scale that was used to quantitate the signals obtained with the plant extracts: (1) 0.56 ng, (3) 2.8 ng, (5) 14.0 ng, (7) 70.0 ng, (9) 350 ng.

Fig. 5 shows an autoradiogram of the dot-blot made in the fifth week of the experiment with four diseased plants and one healthy control. Bakground signals with healthy controls were negligible in all experiments.

The results obtained with the cDNA-hybridisation technique were similar to those of the ELISA assay with two exceptions. Firstly, the cDNA signal obtained with the bulb extract was relatively low or not detectable at all (sample 2 in Figs. 4 and 5).

Secondly, the signals obtained with the basal parts of the leaf were usually somewhat higher than the signals produced by the stem parts, whereas the reverse was found in ELISA.

Detection of TRV in bulbs by ELISA. We investigated the possibility to detect TRV by ELISA and cDNA hybridisation in stored bulbs from batches that were known to contain a high incidence of TRV infection. The bulbs were stored at temperatures between 2 and 25 °C for a period of seven months. In initial experiments multiple assays of a single bulb gave variable results, suggesting that the virus was not evenly distributed in the infected bulb. Therefore, bulbs to be tested were divided into 12 pieces as shown in Fig. 2, and homogenates of all pieces and of the sprout were assayed by ELISA. Fig. 6 shows the ELISA results, obtained with four individual bulbs per

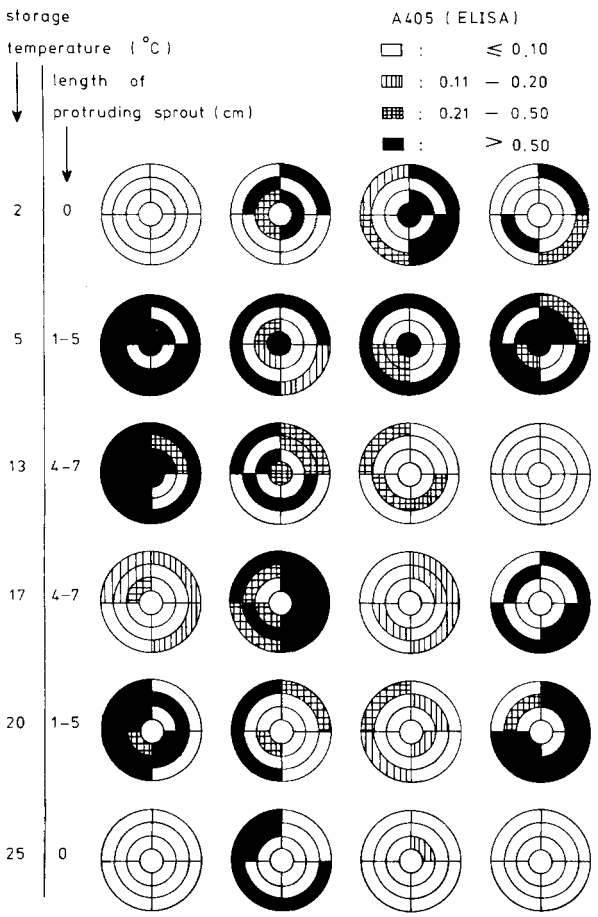

Fig. 6. Distribution of TRV in tulip bulbs as detected by ELISA. Bulbs were stored at six different temperatures in the range of 2-25 °C. After seven months of storage the bulbs were divided into 12 scale parts and the sprout as indicated in Fig. 2. Sprout development depended upon the temperature as indicated. Homogenates of the sclale parts and the sprouts were analysed by ELISA; the absorption values (A405) are indicated in the diagrams representing the bulbs. The results obtained with 4 bulbs at each temperature are given.

temperature treatment, from the tulip cv. Apeldoorn stored at six different temperatures for seven months. In four out of the 24 bulbs no TRV was detectable in any of the 12 parts and in the sprout, while two bulbs gave doubtful results. With the other 18 bulbs TRV was detectable in a variable number of pieces. When a known amount of purified TRV was added to homogenates of the parts of an uninfected bulb, all samples yielded a comparable absorption in ELISA. This demonstrates that the variability in the detection of TRV in the parts of infected bulbs was not due to an uneven distribution of an inhibitory substance. Similar results were obtained with the cultivars Golden Apeldoorn, Attila and Candela in ELISA.

Data obtained by immuno-electron-microscopy were in close agreement with the ELISA results. In samples yielding high ELISA values relatively large numbers of virus particles were seen, whereas few or no virus particles were detectable in samples with low ELISA values. This confirms that in infected bulbs TRV is unevenly distributed.

As there is no regular pattern in the distribution of TRV in the infected bulbs shown in Fig. 6, an infection can easily be overlooked if just one piece of a bulb is assayed. When all samples from an infected bulb were mixed, the ELISA signal was often severely reduced or lost, probably because the virus was diluted below the level of detection. Fig. 6, however, reveals that the sprouts of all infected bulbs stored at 5 °C gave positive signals in ELISA. In some sprouts from bulbs stores at 2, 5 and 13 °C TRV could be detected by ELISA, but not in those from bulbs stored at 17, 20 and 25 °C (Fig. 6). No correlation was found between te presence of TRV in sprouts and sprout length (Fig. 6).

To investigate the influence of temperature in more detail, additional numbers of bulbs that had been stored at 2, 5 and 13 °C were assayed by ELISA. Bulbs of which one or more of the scale parts yielded a positive absorption value were considered to be infected. Table 1 shows that by this criterium 13 out of the 15 bulbs stored at 2 °C were infected. Of these 13 bulbs 5 yielded a positive signal when the sprout was tested by ELISA (i.e. 38%). A similar screening of 44 bulbs stored at 5 °C yielded 36 infected

Table 1. Detection of TRV by ELISA in sprouts of bulbs stored at different temperatures for 7 months.

Bulbs with positive ELISA in[1]		Storage temperature (°C)					
scale part(s)	sprout	2		5		13	
		A405[2]	n	A405	n	A405	n
+	+	1.09 ± 0.15	5	1.30 ± 0.33	34	1.40 ± 0.50	7
−	+			0.86	1		
+	−	0.03 ± 0.03	8	0.07	1	0.04 ± 0.04	3
−	−	0.03 ± 0.04	2	0.06 ± 0.01	8	0.06 ± 0.02	4
Total number of bulbs tested			15		44		14

[1] Plus sign: positive ELISA (A405 >0.20); minus sign: negative ELISA (A405 $\leqslant 0.20$).
[2] Mean absorbtion and standard deviation obtained with homogenates of sprouts from the indicated number of bulbs (n) is given.

Table 2. ELISA values obtained with sacle parts from healthy bulbs stored at different temperatures.

Storage temperature	A405[1]	Number of scale parts tested
2 °C	0.04 ± 0.03	36
5 °C	0.07 ± 0.02	120
13 °C	0.04 ± 0.02	48

[1] The ELISA procedure included addition of 0.4% normal horse serum to the diluted conjugate.

bulbs of which 97% were scored as positive when the sprout was tested by ELISA. The single bulb from this experiment that has been listed as 'infected' without giving a signal in the sprout (Table 1) showed a positive ELISA value in one of its twelve scale parts. Among the 14 bulbs stored at 13 °C, 10 appeared to be infected and 70% of these yielded a positive signal upon testing the sprout. From these infected stocks stored at 2, 5 and 13 °C 82, 86, and 71%, respectively were determined bij ELISA as being infected.

In summary, the results of Table 1 demonstrate that the best correlation between the ELISA's on scale parts and sprouts was achieved with bulbs stored at 5 °C. Although the ELISA data obtained with the scale parts from infected bulbs were quite variable, the sprouts of these bulbs consistently yielded relatively high and constant absorption values in ELISA. Finally, Table 2 illustrates the low background with scale parts from healthy bulbs when normal horse serum had been used in ELISA.

Detection of TRV in bulbs by cDNA hybridisation. Purified virus added to a homogenate of healthy bulbs gave a much lower signal upon cDNA hybridisation than virus diluted in buffer, indicating that inhibitory substances in the homogenate interfered with the assay. This inhibition could be eliminated by treating the homogenate with SDS and phenol/chlorofrom. Fig. 7 shows an experiment in which one healthy bulb (A) and three TRV-infected bulbs (B, C, D) were divided into four approximately equal parts. After phenol/chloroform extraction of the homogenates of these parts, the RNA

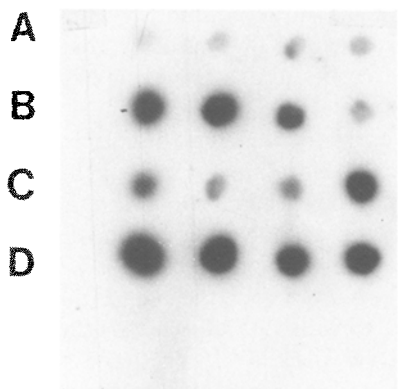

Fig. 7. Detection of TRV in tulip bulbs by cDNA hybridisation. One healthy (A) and three infected bulbs (B,C,D) were stored at 20 °C. After three months each bulb was divided into four equal parts and homogenates of these parts were extracted with phenol and SDS. Purified nucleic acid was spotted onto nitrocellulose and the filter was hybridised to the TRV specific-probe.

was precipitated with LiCl and ethanol to remove DNA and polysaccharides. A low background was obtained in the cDNA hybridisation test with preparations from healthy bulbs (Fig. 7A). However, significant signals were obtained with extracts from infected bulbs; the results of Figs. 7B and C demonstrate that TRV was unevenly distributed in these bulbs, which is in agreement with the ELISA data. In the sprouts of infected bulbs that had been stored at 5 °C for seven months, TRV could easily be detected by spotting sap obtained by squeezing a small part through a ribbed rotary press (Pollähne), and hybridising the filter to the cDNA probe. The data obtained by testing sprouts from bulbs that had been stored at different temperatures completely paralleled the results with ELISA described in the previous paragraph. No signal was obtained with sap of sprouts from healthy bulbs (result not shown).

Discussion

By ELISA and cDNA hybrididation TRV could be detected in tulip plants showing symptoms of TRV infection. The results indicate that the virus was present in all parts of the plants throughout the growing season with maximal concentrations occurring in the basal parts of the stem and leaf. However, it is possible that the relative detection levels were influenced by inhibitory substances present in the plant homogenates. In the bulb, such substances were probably responsible for the low detection of TRV by cDNA hybridisation.

Large-scale routine testing for TRV in tulips should be done preferentially with the bulbs after harvest. Serological screening of bulbs by the Dutch Bulb Inspection Service is done from August to March. During this period the bulbs are stored at temperatures usually below room temperature. The finding that in infected bulbs TRV was localized at an unpredictable position makes it impossible to take a representative sample from the bulb. In a homogenate of a complete bulb the virus concentration was usually too low to be detected. However, the observation that in developing sprouts from infected bulbs stored at 5 °C for 7 months, the virus was detectable by ELISA and cDNA hybridisation with a high accuracy, offers a perspective for a future routine assay. As the visual incidence in the field of this stock was not exactly the same as found by ELISA and cDNA-hybridisation assay, this should be investigated further. The temperature of storage seems to be rather critical; in the sprouts of bulbs stored at 2 °C or 13 °C detection of TRV was less accurate. Apparently, it is not the growing rate of the sprout that determines whether or not the virus is detectable. Sprouts from bulbs stored at 5 °C and 20 °C had about the same size but TRV was detectable only in the former. A storage temperature of 5 °C is used to obtain early flowering of tulips in greenhouses in December or January. Also, Stein et al. (1986) used 6 °C for storage of gladioli to increase the level of bean yellow mosaic virus detection in the corms. This indicates that specific processes can take place at this temperature which may influence the multiplication and/or spread of virus in the bulb. It should be analysed whether the seven months of storage used in this study can be reduced to a shorter period without the loss of TRV detection in sprouts. Although ELISA and cDNA hybridisation were equally usable to detect TRV in sprout material, the two techniques have their specific advantages and disadvantages. ELISA for TRV does not require the introduction of new facilities and a new technology at the Bulb Inspection Service. However, in this study the assays were done on a selected group of tulips infected with

a single serotype of TRV. At present antisera have been prepared to several TRV serotypes occurring in tulips in the Netherlands. In a study that will be described elsewhere we found indications that a routine assay based on ELISA would require additional antisera.

The cDNA hybridisation technique has the advantage that a single probe detects all TRV serotypes that have been encountered in tulips thus far (manuscript in preperation). The disadvantage of a large-scale routine assay based on cDNA hybridisation is the requirement for the introduction of a new technology, the use of radioactive probes being the major obstacle. Frequently, a switch to non-radioactive probes such as biotin (Forster et al., 1985) is accompanied by a loss of sensitivity, but is may be worthwhile to explore this possibility in more detail.

Samenvatting

Aantoonbaarheid van het tabaksratelvirus in verschillende delen van de tulp met behulp van ELISA en cDNA-hybridisatietoetsingen

Verschillende delen van tulp cv. Apeldoorn werden getoetst op de aanwezigheid van tabaksratelvirus (TRV) met behulp van ELISA, cDNA-hybridisatie en immuno-elektronenmicroscopie. Tijdens het groeiseizoen en de bewaring van de bollen werden regelmatig toetsingen uitgevoerd. Gedurende het groeiseizoen op het veld werden de relatief hoogste TRV concentraties voornamelijk gevonden in het basale deel van de stengel en het okselgedeelte van het blad met respectievelijk ELISA en cDNA-hybridisatie. TRV bleek gelokaliseerd aanwezig te zijn in een of meer stukjes van een gedeelde bol, onafhankelijk van de gebruikte toetsmethode. Bijna alle spruiten van geïnfecteerde bollen die gedurende 7 maanden bij 5 °C bewaard waren, bleken bij het toetsen met behulp van ELISA en cDNA aantoonbare hoeveelheden van TRV te bevatten. Het toetsen van spruiten biedt de mogelijkheid te zijner tijd een routinetoets voor TRV in tulpebollen te ontwikkelen.

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