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Pelargonium flower-break and pelargonium line pattern viruses in the Netherlands; purification, antiserum preparation, serological identification, and detection in pelargonium by ELISA

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Abstract

Two viruses, detected frequently in the Netherlands in pelargonium, were identified by serology and test plant reactions. Antisera were prepared and an ELISA procedure was developed to detect the viruses in pelargonium.

One of the viruses, PFBV-N, proved to be pelargonium flower-break virus. With the antiserum to PFBV-N, it could be detected reliably throughout the year in *Pelargonium zonale* 'Springtime Irene'.

The other virus, PLPV-N, was serologically closely related to pelargonium line pattern virus (PLPV) and to pelargonium ring pattern virus (PRPV), as were an old virus isolate from 'Saturnus', collected in the Netherlands in 1971 (L128), and PLPV isolates from Yugoslavia (PLPV-Y) and Denmark (PLPV-D). There were only minor differences in host-plant reactions between the virus isolates. Based on these tests, PLPV and PRPV are considered as isolates of the same virus, for which, for practical reasons, the name pelargonium line pattern virus is proposed.

PLPV could be reliably detected by ELISA in *P. zonale* 'Springtime Irene' and 'Amanda' throughout the year with only a few exceptions. In *Pelargonium peltatum* 'Tavira', however, results were erratic due to uneven distribution of virus in the plant. Best results were obtained when petioles of fully expanded leaves were tested.

Additional keywords: alfalfa mosaic virus, arabis mosaic virus, artichoke Italian latent virus, cucumber mosaic virus, indexing, pelargonium leaf curl virus, pelargonium ringspot virus, pelargonium vein clearing virus, pelargonium zonate spot virus, tobacco mosaic virus, tobacco necrosis virus, tobacco rattle virus, tobacco ringspot virus, tomato black ring virus, tomato ringspot virus, tomato spotted wilt virus, virus distribution.

Introduction

Pelargonium mother plants for vegetative propagation must be free of viruses. In the Netherlands, they are therefore inspected by the General Netherlands Inspection Service for Ornamental Plants (NAKS). As symptoms are not always clear, a reliable method of virus detection is needed. For the production of virus-free commercial stock, propagating nurseries are also interested in methods to routinely index their

material for absence of infection, as are plant protection services since some of the viruses are quarantine agents (e.g., tomato ringspot virus).

World-wide, at least 18 viruses have been isolated from naturally infected pelargonium, viz. alfalfa mosaic virus, arabis mosaic virus (ArMV), artichoke Italian latent virus, cucumber mosaic virus (CMV), pelargonium flower-break virus (PFBV), pelargonium leaf curl virus (PLCV), pelargonium line pattern virus (PLPV), pelargonium ring pattern virus (PRPV), pelargonium ringspot virus (PRSV), pelargonium vein clearing virus, pelargonium zonate spot virus, tobacco mosaic virus, tobacco necrosis virus (TNV), tobacco rattle virus, tobacco ringspot virus (TobRSV), tomato black ring virus (TBRV), tomato ringspot virus (TomRSV), and tomato spotted wilt virus (Albouy and Poutier, 1980; Albouy et al., 1980; Allen and Broadbent, 1986; Betti and Canova, 1981; Di Franco et al., 1979; Gallitelli et al., 1983; Quacquarelli and Gallitelli, 1979; Rana and Di Franco, 1980; Stone, 1980; Vovlas, 1974). Of these viruses, six have been reported to occur in the Netherlands. Five of them, ArMV, CMV, TNV, TBRV, and TomRSV, were found only incidentally, but a sixth one much more frequently (Van Hoof et al., 1973). This virus, coded L128, reacted with an antiserum to isolate 1076 of M. Hollings, Littlehampton, UK, which was originally named 'pelargonium vein netting virus' (Stone and Hollings, 1971). Later on the name was changed into 'pelargonium ringspot virus', and finally into 'pelargonium ring pattern virus' (Stone, 1974; Stone, 1980; Stone and Hollings, 1976; correspondence with M. Hollings, 1977), but its identity remained uncertain and nomenclature confusing.

From 1975 till 1980, in the Netherlands attention was paid mainly to the production of virus-free plants by meristem-tip culture and to research concerning the reinfection of virus-free plants (Hakkaart, 1976, 1977, 1978, 1979, 1980, 1981; Hakkaart and Hartel, 1979). Testing was by sap inoculation onto *Chenopodium quinoa*. In one case, besides the virus regularly found, and then believed to be PRPV, CMV was detected in 85 of 120 plants of *Pelargonium zonale* 'Rubin', grown outdoors. In addition to the PRPV-like virus, another virus became prevalent between 1985 and 1987. It differed from PRPV in symptoms on *C. quinoa*. Both viruses proved to be wide-spread.

In pelargonium, symptom expression is highly influenced by external conditions. Symptoms are often weak, occur seasonally, or do not show at all (Abo El-Nil et al., 1976a, b; Albouy et al., 1979, 1980; Stone, 1980; Newhart and Romaine, 1980; Welvaert and Samyn, 1985). Several test methods have therefore been used for virus detection and identification, such as sap inoculation to indicator plants, serology, electron microscopy, and combinations of these.

Mechanical transmission to test plants proved to be difficult and the rate of success varied with the season. Special buffer additives were necessary to overcome the effect of transmission-inhibiting substances (Abo El-Nil et al., 1976b; Albouy et al., 1980; Paludan and Begtrup, 1987; Pena Iglesias et al., 1974; Welvaert, 1974; Welvaert et al., 1982).

Stone (1980) and Stone et al. (1981) reported the failure to identify PFBV, PLPV, PRPV, and PRSV directly in pelargonium by serology, including ELISA and immuno electron microscopy. However, such methods have since then been more or less successful for a number of viruses.

Abo El-Nil et al. (1976b) could detect TobRSV and CMV in symptomless commercial pelargonium plants by immunodiffusion, but only after partial purification and concentration.

Albouy and Poutier (1980) and Albouy et al. (1980) successfully detected CMV and TomRSV in pelargonium plants by ELISA. Results could be improved by replacing the standard extraction buffer, phosphate-buffered saline, pH 7.4, containing Tween and polyvinylpyrrolidone (Clark and Adams, 1977), by a tris buffer at pH 8.6 with several additives.

Newhart and Romaine (1980) and Newhart et al. (1982) detected TobRSV by ELISA in crude extracts of pelargonium leaf material, irrespective the presence of symptoms, provided the extract was c. 20 times diluted with extraction buffer. Newhart et al. (1982) could also detect the virus in seed of pelargonium. They found ELISA more reliable than a bioassay during the latent stage of infection. The sensitivity of ELISA could be improved by clarifying the extracts. Romaine et al. (1981) were able to detect the virus by ELISA in crude leaf extracts and in leaf disks.

TomRSV was successfully detected by Powell (1984), both with direct and indirect ELISA in crude sap as well as leaf disks.

Samyn and Welvaert (1980) indicated the prospectives of ELISA for the detection of CMV, while Wiedemann and Oertel (1988) made partially successful trials with ELISA to detect PFBV in pelargonium. Paludan and Begtrup (1987) could detect PFBV and TomRSV all year round by bioassay and immunosorbent electron microscopy.

This paper reports the identification of the two pelargonium viruses most prevalent in the Netherlands. Some of the existing confusion about the identity of PRPV was clarified. Antisera to both viruses were prepared, and ELISA procedures were developed.

Material and methods

Virus isolates and antisera. Netherlands virus isolates were obtained from *P. zonale* 'Springtime Irene' (PLPV-N), collected in the seventies, and from 'Salmon Irene' (PFBV-N), collected in 1986. A purified preparation of isolate L128 from pelargonium 'Saturnus' dated from 1971 and had been stored since in 50% glycerol at −20 °C. Isolates of PLPV were gifts from N. Pleše, Zagreb, Yugoslavia (PLPV-Y) and from N. Paludan, Lyngby, Denmark (PLPV-D). PRPV (isolate 1076) was obtained from A.A. Brunt, Littlehampton, UK.

Antisera to PRPV (isolate 1076), PLCV (isolate 232), PRSV (isolate 1372), PFBV (isolate 991), and PLPV (isolate 1583) were kindly supplied by M. Hollings or A.A. Brunt, Littlehampton, UK. Antiserum to PLPV-Y was obtained from N. Pleše. An ELISA kit for PFBV was from Agdia, Inc., USA. An antiserum to L128 was prepared at IPO-DLO in 1971. An antiserum to tomato bushy stunt virus (TomBSV) was obtained from J. Polák, Prague, Czechoslovakia. Antisera to TomRSV were from A.A. Brunt, and from IPO-DLO (prepared in 1973 to isolate 41 of R. Stace-Smith, Vancouver, Canada).

Names and codes of virus isolates and antisera are reviewed in Table 1.

Virus isolation and mechanical transmission. Virus was isolated from pelargonium by sap inoculation to carborundum-dusted leaves of *C. quinoa*. Inoculum was prepared by macerating pelargonium leaf or flower material with pestle and mortar in approximately 10 volumes of 0.05 M potassium phosphate buffer, pH 7.2, contai-

Table 1. Names and codes of virus isolates and/or antisera.

L128	Virus isolate from pelargonium 'Saturnus' and antiserum, 1971, from the Netherlands.
PFBV	Pelargonium flower-break virus, ELISA-kit, Agdia, USA.
PFBV-N	Virus isolate from *Pelargonium zonale* 'Salmon Irene' from the Netherlands.
PFBV-991	Pelargonium flower-break virus, antiserum to isolate 991 from UK.
PLCV-232	Pelargonium leaf curl virus, antiserum to isolate 232 from UK.
PLPV-D	Pelargonium line pattern virus, isolate from Denmark.
PLPV-N	Virus isolate from *Pelargonium zonale* 'Springtime Irene' from the Netherlands.
PLPV-Y	Pelargonium line pattern virus, isolate and antiserum from Yugoslavia.
PLPV-1583	Pelargonium line pattern virus, antiserum to isolate 1583 from UK.
PRPV-1076	Pelargonium ring pattern virus, isolate 1076 and antiserum from UK.
PRSV-1372	Pelargonium ringspot virus, antiserum to isolate 1372 from UK.
TomBSV	Tomato bushy stunt virus, antiserum from Czechoslovakia.
TomRSV-Brunt	Tomato ringspot virus, antiserum from UK.
TomRSV-IPO	Tomato ringspot virus, antiserum, prepared at IPO-DLO in 1973.

ning 2 or 4% (w/v) polyethylene glycol 8000 (PEG). Further inoculation to *C. quinoa*, *Nicotiana clevelandii*, *Phaseolus vulgaris* 'Bataaf', *Pisum sativum* 'Koroza', and *Vicia faba* 'Kompakta' was with sap prepared in phosphate buffer without PEG, or in water.

Virus purification. Viruses were propagated in *N. clevelandii* grown at 18-22 °C, in a greenhouse, shaded during summer, and with additional light by SON/T-lamps during winter. Inoculated leaves were harvested c. 10 days (PLPV-N) or 2-3 weeks (PFBV-N) after inoculation.

Both isolates were purified similarly. Leaves, buffers and organic solvents were chilled at 4 °C before the procedure was started, and most other treatments were also done at this temperature. Sodium phosphate buffer, 0.02 M, pH 6, was used. For homogenization of leaf material and resuspension of the first virus sediment, 0.02 M sodium thioglycolate, and 0.02 M sodium diethyldithiocarbamate were added. Low-speed centrifugation was for 10 min at 8000 rpm in Sorvall rotors GSA (10 400 *g*) or SS34 (7710 *g*), depending on the volume. High-speed centrifugation was in a Beckman rotor 30 for 2 or 3 h at 27 000 rpm (85 600 *g*). All *g*-values given are at R_{max}.

Hundred g of leaf material was homogenized in a Waring blender in 200 ml of buffer, 25 ml of diethyl ether, and 25 ml of carbon tetrachloride. The homogenate was centrifuged at low speed. This was followed by two cycles of high- (for 2 h) and low-speed centrifugation, with resuspension of the high-speed sediments in 70 and 6-12 ml of buffer, respectively. This was followed by two rate zonal centrifugations, with 1 ml of virus suspension on top of 36 ml of a gradient of 10-40% (w/v) sucrose in buffer, for 2 h at 24 000 rpm in a Beckman rotor SW27 or SW28 (103 800 *g*). Virus fractions were collected, diluted 1:1 with buffer and centrifuged at high speed for 3 h. The sediments were resuspended in 6 ml (after first centrifugation) or 2.5 ml (after second centrifugation) of buffer. The last suspension was mixed with an equal volume of

glycerol, and stored at −20 °C. To estimate virus concentrations, extinction coefficients at 260 nm were assumed to be 6.5 for PLPV-N (assuming a RNA content of 20%, as for carnation ringspot virus) and 4.5 for PFBV-N (as for tomato bushy stunt virus; Martelli et al., 1971).

Antiserum preparation. Rabbits were injected intramuscularly, four times at intervals of three weeks, with 0.25 mg of virus emulsified in Freund's incomplete adjuvant. The first bleeding was three weeks after the last injection. For PLPV-N, this was followed two days later by another intramuscular injection with 1 mg of virus in Freund's incomplete adjuvant. The second bleeding was three weeks after the first. Later on more injections were given and bleedings taken, the intervals between bleedings or between injection and bleeding being three weeks.

Serology. Serological identification of virus isolates and titration of antisera were done with the agar double-diffusion test using 1% (w/v) Difco agar (purified) in saline or 0.8% (w/v) agarose in 0.02 M potassium phosphate buffer, pH 6, both containing 0.05% (w/v) sodium azide. Antiserum dilutions were prepared with 0.1 M tris, adjusted to pH 8 with 0.1 M citric acid, and containing 0.05% (w/v) sodium azide. Virus preparations were diluted with 0.02 M potassium phosphate buffer, pH 6, and containing 0.05% (w/v) sodium azide. Results were read after two days at room temperature.

Coating immunoglobulin (IgG) and enzyme conjugates for ELISA were prepared according to Tóbiás et al. (1982). IgG was prepared from the third (PFBV-N) or the sixth (PLPV-N) bleeding. Coating IgG was used at 1 μg/ml, and enzyme conjugates were used at concentrations containing c. 0.2 μg IgG/ml.

Testing of pelargonium material. The double-antibody sandwich ELISA (Clark and Adams, 1977) was used, with some modifications. The buffers were as described by Tóbiás et al. (1982), but the extraction buffer was a 0.5 M potassium phosphate buffer, pH 7.05, containing 2% (w/v) polyvinylpyrrolidone (M_r 25 000), 0.5% (v/v) Tween 20, and 0.05% (w/v) sodium azide. Incubation periods for coating, extract, and enzyme conjugate were overnight at c. 4 °C. Recordings were at 405 nm with a Titertek Multiskan ELISA-reader.

Extracts were prepared by squeezing c. 2 cm^2 of leaf material, or c. 0.2 g of petiole in a Pollähne roller press, adding 2 ml of extraction buffer to the rollers of the press and collecting the extract in a glass or plastic tube. Unless otherwise stated, fully expanded leaves were used.

Results

Antiserum preparation. After four injections, the homologous titres of the antisera were 256. Further injections did not (PLPV-N) or hardly (PFBV-N) increase titres. The antisera used for ELISA had titres of 256.

Serological identification of viruses. A crude preparation of PLPV-N in *C. quinoa* reacted with the antiserum prepared to isolate L128 in 1971, and with the antisera to PLPV-1583 and PRPV-1076, as did the purified preparation of L128. The latter did

Table 2. Highest antiserum dilutions giving a positive reaction in double-diffusion tests in agar(ose) gel, performed to identify PLPV-N, PFBV-N, and L128. Antisera were tested at dilutions 4, 16, 64, and 256.

Antisera	Purified virus preparations		
	PLPV-N	PFBV-N	L128
PLPV-N	256	–	256
PFBV-N	–	256	–
L128	64	–	64
PFBV-991	–	256	–
PLPV-Y	16	–	16
PLPV-1583	256	–	256
PRPV-1076	16	–	16
PRSV-1372	–	–	–
TomRSV-IPO	–	–	–
TomRSV-Brunt	–	–	–

– No reaction.

not react with antisera to PFBV-991, PLCV-232, and PRSV-1372.

Crude sap of *C. quinoa* inoculated with PFBV-N reacted with the antiserum to PFBV-991, but not with antisera to PLCV-232, PLPV-1583, PRPV-1076, PRSV-1372, or TomBSV.

The results of one of the tests with purified preparations are presented in Table 2. Antisera were tested at dilutions 4, 16, 64, and 256, and purified preparations undiluted and at dilutions 4 and 16. All antigens reacted with a single precipitation line. Partially purified preparations of non-inoculated *C. quinoa* and *N. clevelandii* were used as virus-free controls. The antisera to PFBV-991, PLPV-1583, and PRSV-1372 reacted with the *C. quinoa* extract, but these reactions did not interfere with the results in Table 2. Antigens did not react with normal rabbit serum. PLPV-N reacted with antisera to L128, PLPV-Y, PLPV-1583, and PRPV-1076, but not with antisera to PFBV, PRSV, and TomRSV. PFBV-N only reacted with the antiserum to PFBV-991.

Placing undiluted purified preparations of PLPV-N and of L128 in adjacent wells in agarose opposite wells with fourfold diluted antisera to PLPV-N, L128, PLPV-Y, or PLPV-1583, single precipitation lines were formed with both viruses. They coalesced without spur formation. Reactions with the antiserum to PRPV-1076 were too weak to detect possible spur formation.

For further identification of PLPV-N and L128, fourfold dilution series of purified virus preparations of these isolates and of PLPV-D and PLPV-Y were tested against four dilutions (fourfold) of antisera to the first three of these isolates and to PLPV-1583 and PRPV-1076. Moreover, in a separate test PRPV-1076 was tested against these antisera except the antiserum to L128. The results are summarized in Table 3. Antigens did not react with normal rabbit serum. The results of spur tests with these antisera and isolates are given in Table 4. The results of both types of tests show that the isolates from the Netherlands, Denmark and Yugoslavia are serologically closely related. Single precipitation lines occurred, and spur formation was only

Table 3. Highest antiserum dilutions giving a positive reaction in double-diffusion tests in agar(ose) gel, performed to further identify PLPV-N and L128.

Purified antigens	Antisera to isolates				
	PLPV-N	L128	PLPV-Y	PLPV-1583	PRPV-1076
PLPV-N	256*	256*	64*	256*	16
L128	256	256	64	256	16
PLPV-Y	256	256	64	256	16
PLPV-D	256	256	64	256	16
PRPV-1076	256	nt	64	256	16
Virus-free *Nicotiana clevelandii*	–	–	–	4	4

* Highest antiserum dilution tested; – no reaction; nt not tested.

Table 4. Results of serological spur tests to compare PLPV-N, L128, PLPV-Y, PLPV-D, PLPV-1583, and PRPV-1076.

Pairs of antigens in adjacent wells	Antisera				
	PLPV-N (4)*	L128 (4)	PLPV-Y (undil.)	PLPV-1583 (4)	PRPV-1076 (undil.)
PLPV-N	–	–	+	–	–
L128	–	–	–	–	–
PLPV-N	–		–	–	–
PLPV-Y	–		–	–	–
PLPV-N	–			–	–
PLPV-D	–			–	–
PLPV-N	–		+	+	–
PRPV-1076	–		–	–	–
PLPV-Y	–	–	–	–	–
PLPV-D	–	–	–	–	–
PLPV-Y	–		+	–	–
PRPV-1076	–		–	–	–
L128		–	–		
PLPV-Y		–	+		
L128		–			
PLPV-D		–			
L128	–	–	–	+	–
PRPV-1076	–	–	–	–	–

* ( ) Antiserum dilution used.
– No spur; when both isolates of the pair are indicated – , complete coalescence occurred.
\+ Spur.

observed using the antisera to PLPV-Y and/or to PLPV-1583 with PLPV-N and PLPV-Y when they were placed in adjacent wells with L128 or with PRPV-1076, and with L128 when it was placed in adjacent wells with PRPV-1076. The isolates reacted with the antiserum to PLPV-1583 as well as with the antiserum to PRPV-1076. Testing the antiserum to PRPV-1076 (undiluted) in the spur test, lines obtained with virus-free *N. clevelandii* crossed with those obtained with the virus preparations (not presented in Table 4).

Original plants of pelargonium 'Salmon Irene' were tested in ELISA, using a test kit from Agdia for PFBV. They were found positive for this virus.

Reactions of test plants. For biological comparison, the virus isolates PFBV-N, PLPV-N, PLPV-D, PLPV-Y, and PRPV-1076 were inoculated onto a small host range. The results are summarized in Table 5.

The reactions to PFBV-N were as follows:

- *C. quinoa*: numerous chlorotic and some necrotic local spots after 5-8 days; occasionally small systemic chlorotic spots.
- *N. clevelandii*: in inoculated leaves yellow diffuse spots after 2-3 weeks or occasionally symptomless infection; no systemic infection.
- No infection was found in *P. vulgaris* 'Bataaf', *P. sativum* 'Koroza' and *V. faba* 'Kompakta'.

The reactions of the test plants to isolates PLPV-N, PLPV-D, PLPV-Y, and PRPV-1076 were mutually similar:

- *C. quinoa*: numerous chlorotic local spots after 4-5 days, most (a few for PLPV-Y) of them becoming necrotic 1-2 days later; occasionally systemic chlorotic spots (chlorotic spots and rings for PRPV-1076).
- *N. clevelandii*: in inoculated leaves a few faint diffuse spots (∅ 3-5 mm) after 10-14 days (2-3 weeks for PRPV-1076) or symptomless infection and for PLPV-Y no clear

Table 5. Summary of test plant reactions after inoculation with PFBV-N, PLPV-N, PLPV-D, PLPV-Y, and PRPV-1076.

Virus isolates	Test plants				
	Chenopodium quinoa	*Nicotiana clevelandii*	*Phaseolus vulgaris*	*Pisum sativum*	*Vicia faba*
PFBV-N	L S/–	L/l –	– –	– –	– –
PLPV-N	L S/–	L/l S/s	l –	L –	L s/–
PLPV-D	L S/–	L/l S/s	l –	L –	L s/–
PLPV-Y	L S/–	l S/s	l S/–	L –	L s/–
PRPV-1076	L S/–	L/l S/s	l S/–	L –	L s/–

L Local symptoms.
l Latent local infection, antigens detected by ELISA.
S Systemic symptoms.
s Latent systemic infection, antigens detected by ELISA.
– No symptoms and no antigens detected by ELISA.
/ Or.

symptoms; systemic mild chlorotic mottle, vein clearing, and mild rugosity or symptomless infection.
- *P. vulgaris* 'Bataaf': in inoculated leaves symptomless infection; occasionally systemic mild mosaic (not yet found for PLPV-N and PLPV-D).
- *P. sativum* 'Koroza': several local grey-brown (brown for PLPV-Y) necrotic, more or less angular spots (∅ c. 1 mm) after 9-14 days; no systemic infection.
- *V. faba* 'Kompakta': several local dark-brown necrotic spots (∅ 1-5 mm) after 6-9 days, with a dark-green ring in yellowing leaf parts (for PRPV-1076, several local dark-brown diffuse spots after 10 days with a transparent upper surface). No or symptomless systemic infection.

Each test plant was assayed by ELISA for the presence of antigen in inoculated and non-inoculated leaves. The antiserum to PFBV-N did not react with any test plant inoculated with PLPV-N, PLPV-D, PLPV-Y or PRPV-1076, nor with *P. vulgaris*, *P. sativum* or *V. faba*, inoculated with PFBV-N. The antiserum to PLPV-N did not react with any test plant inoculated with PFBV-N. Virus was only detected by ELISA in non-inoculated leaves of *C. quinoa* and *P. vulgaris* when symptoms were present.

Testing of pelargonium material. The reliability of ELISA for pelargonium, using the antisera to PFBV-N and PLPV-N, was examined by regularly testing pelargonium plants during at least one year.

Of *P. zonale* 'Springtime Irene' naturally infected with PFBV, 5-28 plants were tested with the antiserum to PFBV-N. The results are given in Table 6. Virus-free

Table 6. Absorbance values ($A_{405\,nm} \times 100$) in ELISA after 30 min of substrate incubation, obtained by testing 5-28 PFBV-infected plants of *Pelargonium zonale* 'Springtime Irene', using antiserum to PFBV-N.

Year - week	Value range	Mean value*	Number of plants tested
1988 - 33	114 - > 200	171	5
36	64 - 142	109	5
43	48 - 120	89	6
49	77 - 159	116	6
1989 - 4	8 - 38	19	7
10	10 - 76	43	7
16	22 - 115	65	25
24	12 - > 200	141	21
30	54 - 200	144	28
36	14 - 128	60	28
43	5 - > 200	143	28
50	40 - > 200	164	28
1990 - 8	71 - 91	80	10
Virus-free controls	0 - 1	0	1-5

* For calculating the mean value, > 200 was regarded as 200.

Table 7. Absorbance values ($A_{405\,nm} \times 100$) in ELISA after 60 min of substrate incubation, obtained by testing 2-8 PLPV-infected plants of *Pelargonium zonale* 'Amanda' and 2-12 PLPV-infected plants of 'Springtime Irene', using antiserum to PLPV-N.

Year - week	Amanda			Springtime Irene		
	value range	mean value	number of plants tested	value range	mean value	number of plants tested
1988 - 36	110 - 117	114	2	75 - 110	93	2
43	27 - 35	31	2	3 - 18	11	2
49	108 - 122	115	2	74 - 75	75	2
1989 - 4	57 - 73	65	2	23 - 39	31	2
10	39 - 57	48	2	12 - 19	16	2
16	3 - 110	79	8	7 - 111	49	6
24	21 - 30	25	6	21 - 37	27	8
30	20 - 66	43	8	1 - 62	32	10
36	59 - 77	66	8	30 - 118	78	11
43	38 - 97	80	8	25 - 123	54	12
50	44 - 74	61	8	21 - 145	67	12
1990 - 8	61 - 86	75	8	36 - 89	60	10
Virus-free controls				0 - 3	1	1-5

plants of 'Springtime Irene' gave absorbance values of 0.00 or 0.01, and even after 5 h of substrate incubation, these values did not exceed 0.02. From January till March 1989 absorbance values tended to be relatively low, but compared to those of the virus-free controls they were still clearly positive after 1-5 h of substrate incubation.

The reliability of ELISA with the antiserum to PLPV-N was tested on 2-8 plants of *P. zonale* 'Amanda' and 2-12 plants of 'Springtime Irene', both naturally infected with PLPV. Table 7 shows that for both cultivars the absorbance values were rather stable throughout the year. The virus-free controls of 'Springtime Irene' gave absorbance values from 0.00 to 0.03. For 'Amanda' there was one false-negative in April 1989 and for 'Springtime Irene', one in October 1988 and one in July 1989.

In ELISA, the antiserum to PLPV-N also reacted with extracts of *Pelargonium peltatum*, but results were erratic. Plants of *P. peltatum* 'Tavira' that had been positive in ELISA, were therefore further investigated by inoculating one half of a leaf onto *C. quinoa* and testing the other half by ELISA. All four combinations of positive and negative in bioassay and ELISA were found. To investigate the origin of these whimsical results, each leaf of two plants was cut into six parts, and these as well as the petioles were tested in ELISA. The results obtained with one plant are shown in Fig. 1. Most of the petioles and most parts of the older leaves were found positive. When the middle leaf-parts were positive, the basal leaf-parts were also found positive. Similar results were obtained with the other plant of 'Tavira', and with a plant of *P. peltatum* 'Decora Lila'. The latter, however, had one leaf in which the virus was found in the

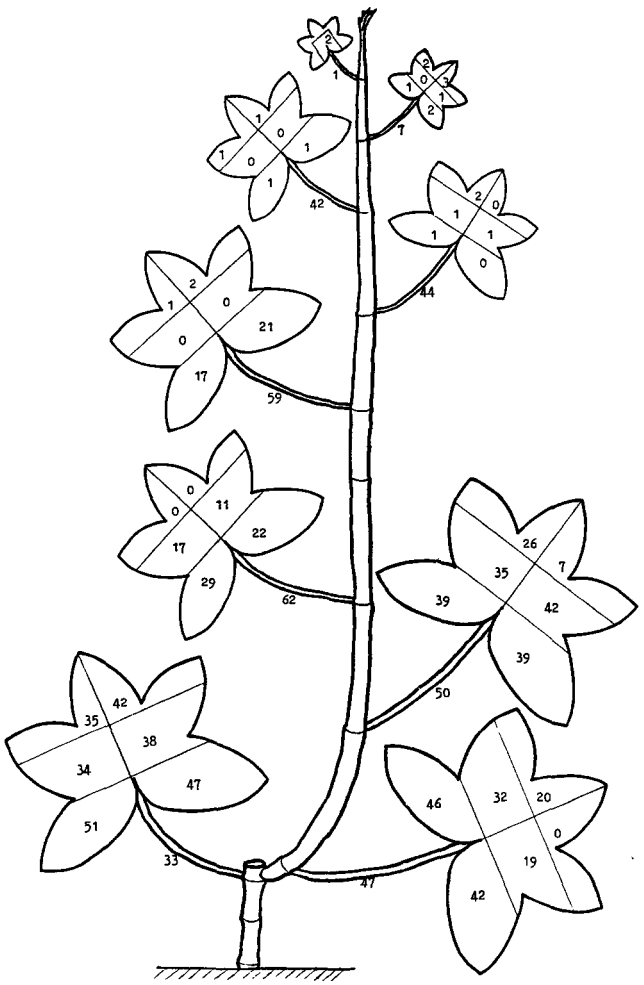

Fig. 1. Virus distribution in a plant of *Pelargonium peltatum* 'Tavira'. Absorbance values ($A_{405\,nm} \times 100$) in ELISA after 60 min of substrate incubation are given for petioles and leaf-parts, using the antiserum to PLPV-N.

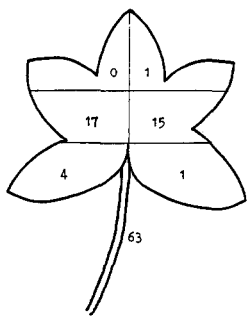

Fig. 2. Virus distribution in a leaf of *Pelargonium peltatum* 'Decora Lila', located at the sixth node from the top. Absorbance values ($A_{405\,nm} \times 100$) in ELISA after 60 min of substrate incubation are given for petiole and leaf-parts, using the antiserum to PLPV-N.

Table 8. Absorbance values ($A_{405\,nm} \times 100$) in ELISA after 60 min of substrate incubation, obtained by testing the lowest leaves and petioles of 22 PLPV-infected plants of *Pelargonium peltatum* 'Tavira', using the antiserum to PLPV-N. Tests performed by NAKS.

Year -week	Leaves			Petioles		
	value range	mean value	% > 10	value range	mean value	% > 10
1990 - 8	6 - 42	16	55	6 - 82	43	86
12	16 - 46	29	100	21 - 48	29	100
17	4 - 84	28	73	8 - 119	37	95
22	6 - 55	25	73	6 - 44	17	86
26	2 - 41	18	73	2 - 69	15	55
30	5 - 41	14	41	5 - 74	23	59
34	4 - 48	27	73	4 - 46	32	86
39	7 - 88	25	73	7 - 100	36	89
43	9 - 50	30	91	9 - 92	46	95
48	13 - 63	35	100	11 - 74	48	100
51	10 - 113	42	95	10 - 77	37	91
1991 - 4	7 - 90	34	68	6 - 77	39	86
Virus-free 'Rouletta'	0 - 10	1				

petiole and the middle leaf-parts, but not in the basal leaf-parts (Fig. 2).

The NAKS, thereafter, tested the lowest leaf and its petiole of 22 naturally infected plants of *P. peltatum* 'Tavira' once a month during a whole year. The results are summarized in Table 8. The first time, all 22 plants gave a positive result for leaf and/or petiole. The mean absorbance values were higher for petioles than for leaves except in May, June, and December 1990. The percentage of positive results for petioles was higher than for leaves, except in June and December 1990.

Discussion

Reactions in double-diffusion tests were generally more clear with agarose in phosphate buffer, pH 6, than with agar in saline. Virus preparations reacted with a single precipitation line, indicating that we worked with homogeneous antigens.

The virus from 'Springtime Irene' (PLPV-N) reacted not only with the homologous antiserum and with antisera to PLPV (PLPV-Y and -1583), but also with antisera to L128 and PRPV-1076. L128 reacted similarly. It originated from pelargonium 'Saturnus'. As the British isolate 1076, it was originally believed to be pelargonium vein netting virus (Stone and Hollings, 1971; Van Hoof et al., 1973). Later on, isolate 1076 was considered to be PRPV (see Introduction). At the time of our original experiments with L128, PLPV had not yet been described. Our serological tests indicate that L128 and PLPV-N are closely related, but not identical. PLPV-N could not be differentiated from PLPV-D or PLPV-Y in any of the tests. PLPV-N and PLPV-Y differed from PRPV-1076 by spur formation with antisera to PLPV-Y and/or

PLPV-1583. L128 differed from PLPV-N, PRPV-1076, and from PLPV-Y (Table 4). However, differences were minor in all cases. Reactions of PRPV and PLPV isolates on test plants were also similar, with some minor differences. PLPV-Y on *C. quinoa* is less necrotic, the colour of the spots produced by PLPV-Y on *P. sativum* is slightly darker and the spots produced by PRPV-1076 on *V. faba* are more diffuse than with the other isolates. The reactions of host plants to PLPV-Y are the same as described by Pleše and Štefanac (1980). The host-plant reactions to isolate 1076 differ from those described by Stone (1974): we did find occasional systemic infection in *C. quinoa* and *P. vulgaris*, but not in *P. sativum*; local symptoms in *P. vulgaris* were absent; local symptoms in *P. sativum* showed at least one week earlier, and in *V. faba* local symptoms did occur. These differences and the variation in severity of the reactions, reaction-time and occurrence of systemic infection may depend on experimental conditions and on the virus concentration in the inocula. Therefore, as the PLPV and PRPV isolates differ only slightly, they may be considered as isolates of one virus. After consultation of A.A. Brunt, Littlehampton, UK (correspondence, 1990), for practical reasons we strongly propose to use the name pelargonium line pattern virus rather than pelargonium ring pattern virus, although the latter would have historical precedence. On the basis of our results, we will now name L128 and PLPV-N pelargonium line pattern virus.

Reactions of test plants to PFBV-N correspond with those described for PFBV (Hollings and Stone, 1974; Stone and Hollings, 1973). These results, together with those obtained by agar(ose) double-diffusion tests and ELISA proved the virus from 'Salmon Irene' (PFBV-N) to be pelargonium flower-break virus.

To estimate concentrations of PFBV and PLPV in purified preparations, extinction coefficients at 260 nm were assumed to be 4.5 for PFBV and 6.5 for PLPV. Pleše and Štefanac (1980) used 8.0 for PLPV. Since the real values have not been determined, they will likely need correction. At the time of purification, we considered PFBV to be a tombusvirus, and assumed PLPV to have a RNA-content of 20%. However, PFBV and PLPV are now considered to be a member and a tentative member, respectively, of the carmovirus group (Morris and Carrington, 1988).

For routinely testing pelargonium samples, the low pH (2-3) of crude extracts from this plant species has to be taken into account. A stronger buffer was therefore used than the phosphate-buffered saline (PBS), described by Clark and Adams (1977). The potassium phosphate buffer (0.5 M, pH 7.05) was chosen on the basis of experiences in the ELISA detection of viruses in begonia, extracts of which also have a very low pH (unpublished results). PBS does give good results as found with the Agdia test kit. However, extracts should be diluted sufficiently, and with our extraction buffer sodium sulphite and egg white did not have to be added. In fact, the buffer is a 10-times concentrate of the potassium phosphate buffer, pH 7.2, used in IgG and enzyme conjugate preparation (Tóbiás et al., 1982). Albouy and Poutier (1980) preferred a tris buffer of pH 8.6 with several additives. We did not try this buffer.

Results of indexing *P. peltatum* by ELISA for the presence of PLPV were rather erratic, because of the uneven distribution of the virus. The reliability could be improved when using petioles instead of leaf parts. The low percentage of positive results in June and July 1990, however, indicates that test results obtained in summer

should be interpreted with care.

PFBV has also been found in *P. peltatum* (NAKS, personal information). We have no indication so far, that the problems met in the detection of PLPV also hold for PFBV, but the possibility cannot be excluded.

When testing *P. zonale* for PFBV and PLPV, the ELISA procedure used is reliable throughout the year, though fluctuations in absorbance values and incidental false-negatives for PLPV do occur. The latter may be obviated by testing at least twice.

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