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Line pattern in Limonium latifolium caused by tobacco rattle virus

JEANNE DIJKSTRA and H. D. VAN DIJKE

Laboratory of Virology, Agricultural University, Wageningen

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Abstract

Tobacco rattle virus (TRV) was isolated from plants of *Limonium latifolium* showing bright yellow or red line patterns and ringspots on the leaves. It was proved that this virus, designated TRV-Lim, was the causal agent of the disease. In its reactions on *Nicotiana clevelandii* it resembled a yellow strain of TRV from Oregon (USA), but the symptoms in *N. glutinosa, N. megalosiphon, N. tabacum* and *Petunia hybrida* were more comparable to those caused by so-called unstable variants of TRV. Dilution end-point was 10^{-6} – 10^{-7}, thermal inactivation at 75 – 80°C, and ageing in vitro 55 – 60 days. The purified virus suspension contained particles of three normal lengths, 70, 102, and 194 nm. The virus sedimented as three components with average sedimentation coefficients of 129, 161 and 206 S, respectively. In purified suspensions TRV-Lim had two different buoyant densities. A serological relationship was found with TRV isolated from Europe and Brazil.

Additional keywords: Limonium dumosum, Limonium vulgare.

Introduction

Some years ago the Plant Protection Service (PD) at Wageningen received diseased plants of *Limonium latifolium* Kuntze (fam. *Plumbaginaceae)* from a nursery. The leaves of the affected plants showed bright yellow or red line patterns and ringspots. In routine transmission tests carried out at the PD a necrotic local lesion was observed once on a tobacco plant inoculated with crude sap from diseased *L. latifolium,* but the test plant was discarded before further transmission experiments could be performed. The diseased *L. latifolium* plants, maintained in the experimental garden of the PD, displayed symptoms throughout the years (Fig. 1).

Initially, transmission experiments performed in the Laboratory of Virology with sap from leaves ground at room temperature gave negative results. However, when partly macerated leaf material was frozen, followed by further maceration and thawing, the inoculum thus obtained induced a few big necrotic local lesions on leaves of *Chenopodium quinoa* and *Nicotiana tabacum* 'Samsun NN' resembling those caused by tobacco rattle virus (TRV).

The aim of the present study was to identify the virus.

Fig. 1. Leaves of *Limonium latifolium* with line patterns and ringspots.

Fig. 1. Bladeren van Limonium latifolium met figuurbont en kringvlekken.

Materials and methods

Isolation, maintenance and propagation of the virus. The virus was isolated by mechanical transmission from diseased *L. latifolium* to *N. tabacum* 'Samsun NN' and *C. quinoa,* maintained in *C. quinoa,* and propagated in *N. clevelandii.* The original isolate was used in all experiments.

Inoculation. Manual inoculations were done with purified virus or water-diluted crude sap from virus-infected plants using carborundum (600 mesh) as abrasive. The original inoculum from diseased *L. latifolium* was prepared by freezing the partly ground leaf material and further grinding of the frozen slurry; after thawing the sap obtained was used for inoculation.

Host range. Seedlings of 20 species and cultivars were inoculated with water-diluted sap from infected *N. clevelandii* leaves. Uninoculated leaves of the test plants were back-tested on *N. tabacum* 'Samsun NN' after about three weeks. Purified virus suspensions were inoculated onto seedlings of *L. latifolium* and *L. dumosum* 'Zilverwit' and onto healthy-looking plants of *L. vulgare.*

Persistence of infectivity in crude sap. Sap from infected *N. clevelandii* leaves was used as inoculum. Infectivity assay was on detached leaves of *N. glutinosa.*

Plant material. All test and assay plants were grown in sterilized soil in the glasshouse at 20 – 30°C.

Virus purification. The virus was purified from leaves of *N. clevelandii* plants. In earlier experiments the purification method according to Lister and Bracker (1969) was used. Later the ether-carbon tetrachloride and the polyethylene glycol (PEG) methods of Huttinga (1972) were adopted. In addition to the last-mentioned method, the virus in the supernatant, obtained after low speed centrifugation (4 000 *g*, 10 min) following the last PEG-NaCl treatment, was given a further purification by density-gradient centrifugation in a zonal rotor (MSE B XV). The rotor was filled with a linear gradient of 15 to 30% sucrose (w/v) in 0.1 M phosphate buffer and loaded with a virus suspension in 0.1 M phosphate buffer containing 2% sucrose. The rotor was accelerated to 121 000 *g*, spun for 5 h and unloaded. The rotor contents were led through a UV absorption meter and collected in fractions. To the virus-containing fractions PEG and NaCl were added to a concentration of 8% and 0.2 M, respectively. The precipitate was collected by centrifuging at 12 000 *g* for 10 min. The pellet was suspended in 0.1 M phosphate buffer pH 7.0. The virus was further purified by two additional cycles of differential centrifugation (100 000 *g*, 90 min and 5000 *g*, 10 min).

Extinction coefficient. The optical density of purified virus preparations was measured in a Zeiss spectrophotometer. Absorbance values were not corrected for light scattering. Virus concentrations were estimated using an extinction coefficient $E_{260}^{0.1\%}{}_{1\,cm}$ of 3.00, the value given for TRV by Harrison (1970).

Sedimentation coefficients. The sedimentation coefficients were determined on a Spinco Model-E analytical centrifuge. Centrifuge runs were done with a purified virus suspension in phosphate buffer of pH 7.0 at 20°C. The values calculated according to the graphical method developed by Markham (1960) were corrected for the ion strength of the buffer.

Buoyant density. Equilibrium centrifugation was performed with a suspension of 0.25 mg virus/ml in 30.3% CsCl at 78 100 *g*. Via refractive indices the buoyant density at 25°C (ϱ^{25}) of the virus was determined.

Electron microscopy. Both leaf dips from diseased *L. latifolium* plants and purified virus suspensions were stained with 2% potassium phosphotungstate (pH 6.5). The mixtures were mounted on carbon-reinforced formvar-coated copper grids and examined with a Siemens Elmiskop I electron microscope. For length measurements of the virus particles tobacco mosaic virus from *N. tabacum* 'White Burley' was used as an internal size standard (Bos, 1975).

Serology. Antiserum was produced in a rabbit injected intravenously with 1.2 mg of purified virus on the 1st, 10th and 20th day. Thereafter, the rabbit was injected intramuscularly on the 30th and 45th day with 11.4 mg and 15 mg of virus, respectively, emulsified with Freund's complete adjuvant. On the 60th day blood was collected. The microprecipitin test according to Van Slogteren (1954) and the Ouchterlony

double diffusion were used for serological tests. In the former purified virus suspensions were used, in the latter both purified virus suspensions and sap from plants of *N. tabacum* 'White Burley' infected with different TRV isolates, viz. a TRV maintained in the laboratory for many years (TRV-Lab) and TRV from the peony *(Paeonia hybrida)* cultivars Mai Fleuri and Sarah Bernhard (TRV-P(MF) and TRV-P (SB), respectively).

Antisera to different TRV isolates viz. Italy 5 and Italy 6 originally isolated from maize at Chioggia and from wheat at Mesola, respectively (Van Hoof et al., 1966), L 20, an isolate from gladiolus with notched leaf symptoms (Cremer, 1966), and Lisse, an isolate from tobacco (Bos and Van der Want, 1962) as well as antiserum to the Dutch pea early-browning virus (PEBV) were obtained from Ing. D.Z. Maat, Research Institute for Plant Protection at Wageningen. Antisera to the TRV isolates PRN (the type strain of TRV) and CAM (an isolate from Brazil) were provided by Dr. B. D. Harrison, Scottish Horticultural Institute, Invergowrie, Scotland.

Results

Host range. Mechanical transmission from infected *L. latifolium* plants was difficult, but from the few local lesions appearing after inoculation of sap from the latter plants to *C. quinoa* the virus could easily be transmitted to other test plants. The plants used in the host range studies reacted as follows:

Beta vulgaris 'Groeningia'. Irregularly shaped necrotic local lesions and chlorotic mottling; no systemic infection.

Chenopodium amaranticolor. Many pin-point white-necrotic local lesions; no systemic infection.

Chenopodium quinoa. Irregularly shaped necrotic local lesions and withering leaves; no systemic infection.

Cucumis sativus 'Lange Gele Tros'. Pin-point necrotic dots in chlorotic spots on the inoculated cotyledons, followed by larger necrotic spots; no systemic infection.

Datura stramonium. Necrotic and chlorotic concentric ringspots and chlorotic line pattern on the inoculated leaves; no systemic infection.

Gomphrena globosa. Reddish vein discoloration, reddish brown local lesions and white papery spots; no systemic infection.

Lycopersicon esculentum 'Moneymaker'. Line pattern, yellow concentric local ringspots and necrotic lesions (Fig. 2); usually symptomless systemic infection.

Nicotiana clevelandii. Irregularly shaped necrotic local ringspots and large necrotic lesions; systemic line pattern, bright yellow veinbanding and bulging of the leaves (Fig. 3).

Nicotiana glutinosa. Big brown necrotic local (ring)spots; no systemic infection.

Nicotiana megalosiphon. Many necrotic local lesions, veinal necrosis with oak-leaf pattern; systemic leaf malformation, veinal necrosis and oak-leaf pattern.

Nicotiana rustica. Irregularly shaped necrotic local (ring)spots and yellow line pattern, sometimes in the form of a maze, beginning at the base of the leaf; systemic necrosis of veins and stems.

Nicotiana tabacum 'Samsun NN'. Big necrotic ringspots, sometimes accompanied with arched necrotic spots on the inoculated leaves; systemic necrosis of veins and stems.

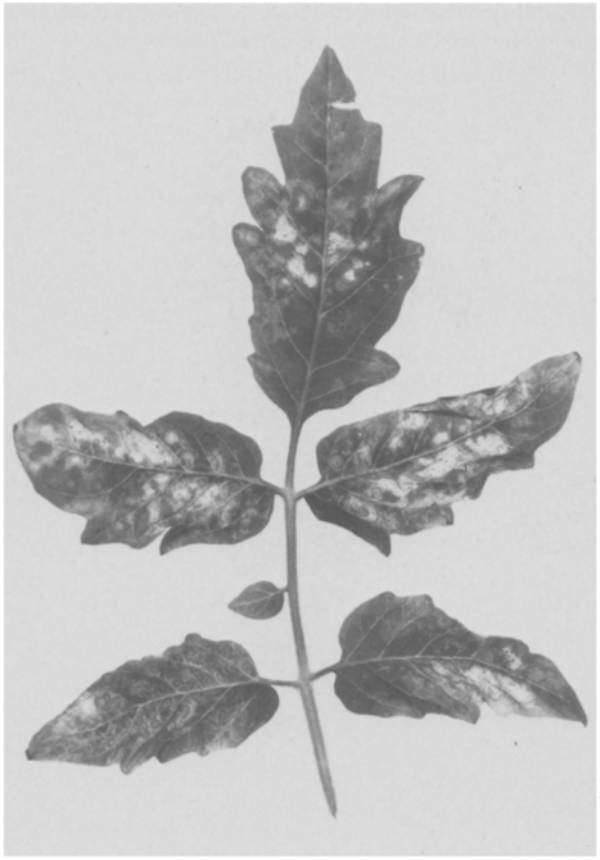

Fig. 2. Leaf of *Lycopersicon esculentum* with (concentric) local ringspots and necrotic lesions upon inoculation with the virus from *Limonium latifolium*.

Fig. 2. Blad van Lycopersicon esculentum met (concentrische) lokale kringvlekken en necrotische lesies na inoculatie met het virus uit Limonium latifolium.

Nicotiana tabacum 'White Burley'. Many irregularly shaped necrotic local lesions and veinal necrosis; usually symptomless systemic infection.
Petunia hybrida. Dark green discoloration of the inoculated leaves; faint systemic mottle.
Phaseolus vulgaris 'Bataaf'. Numerous white necrotic pin-point local lesions; no systemic infection.
Pisum sativum 'Koroza'. Brown or reddish brown necrotic local lesions; no systemic infection.
Solanum melongena 'Lange Violette'. Yellow mottle, line pattern and veinal necrosis in the inoculated leaves; no systemic infection.
Vicia faba 'Driemaal Wit'. No infection.
Vigna unguiculata 'Blackeye'. Small, dark brown necrotic local lesions; no systemic infection.
Zinnia elegans 'Californische Reuzen'. Pale chlorotic (ring)spots in the inoculated leaves and brownish discoloration of the veins; systemic chlorotic (ring)spots and veinal necrosis.

Mechanical inoculation of Limonium. Eighteen days after inoculation with a puri-

Fig. 3. Leaves of *Nicotiana clevelandii* with systemic line patterns, bright yellow vein-banding and malformation upon inoculation with the virus from *Limonium latifolium*.

Fig. 3. Bladeren van Nicotiana clevelandii met systemisch figuurbont, heldergeel nerfbandmozaïek en misvorming na inoculatie met het virus uit Limonium latifolium.

fied virus suspension 2 out of 6 seedlings of *L. latifolium* showed bright yellow ringspots and line pattern on the inoculated leaves. Four weeks later another plant from the same batch showed the above symptoms and after another three weeks the fourth one. The symptoms were similar to those in the original diseased plants in the garden of the PD. In another batch of six *L. latifolium* seedlings inoculated three weeks later than those in the first batch only one plant showed the typical disease symptoms (118 days after inoculation). No symptoms were observed in the inoculated plants of *L. dumosum* 'Zilverwit' and *L. vulgare*.

Persistence of infectivity in crude sap. Crude sap from infected *N. clevelandii* leaves was infective after dilution with water to 10^{-6} but not 10^{-7}. The thermal inactivation point was 75-80 °C,and at 20°C infectivity was retained for 55 days but not for 60 days.

Virus purification. Virus purification according to Lister and Bracker (1969) was a rather lengthy procedure and gave low yields (1 mg of virus per 150 g of leaf material). Therefore, the ether-carbon tetrachloride methode described by Huttinga (1972) was tried. The yield was about 10 times higher but the virus particles in the purified preparation showed a high degree of breakage so that they could not be used for

length measurements. The PEG method of Huttinga (1972) gave a lower yield than the second method (1 mg of virus per 40 g of leaf material) but the virus particles showed little breakage (Fig. 4). The yield might, however, be improved by centrifugation in the zonal rotor for a shorter period as the separation in the gradient was not optimal after 5 h of centrifugation, a substantial amount of the virus being down in the gradient and mixed with plant material.

The purified virus suspensions obtained had an A_{260}/A_{280} absorption ratio varying from 1.13 to 1.16 (average 1.15).

Sedimentation coefficients. In the analytical centrifuge the virus sedimented as three components with average sedimentation coefficients at 20°C of 129, 161 and 206 S, respectively.

Fig. 4. Electron micrograph of a purified preparation of the virus from *Limonium latifolium*. Bar represents 100 nm.

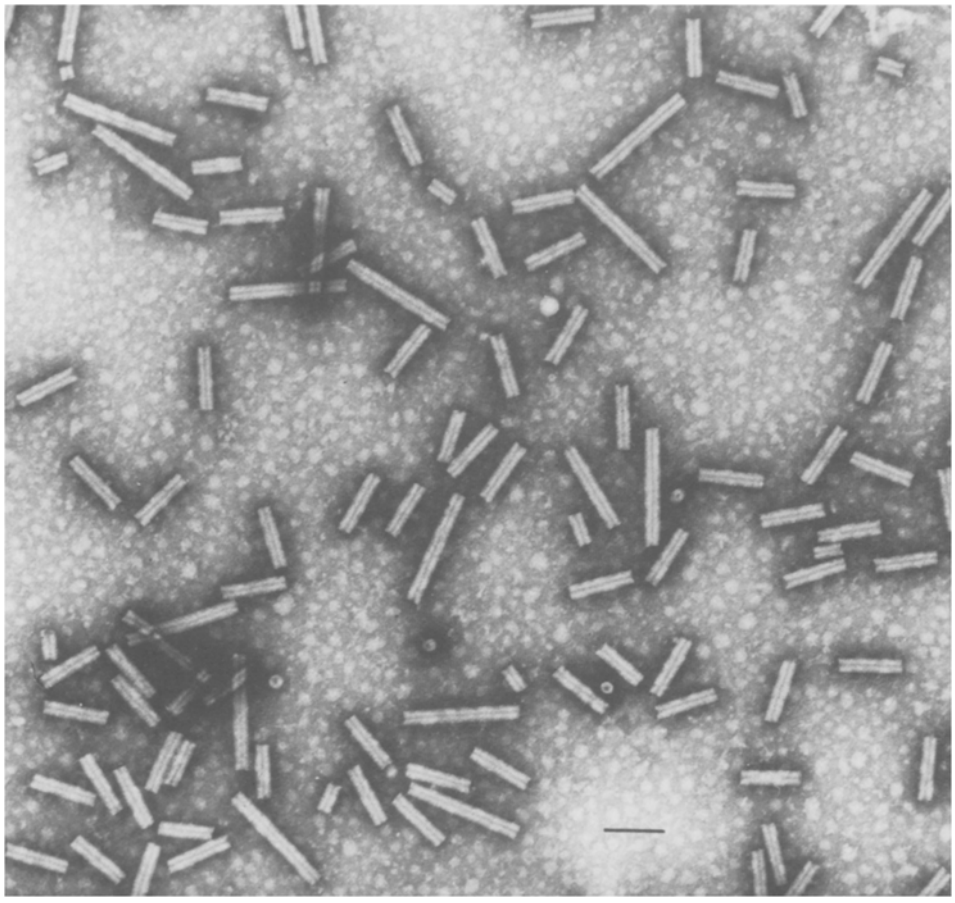

Fig. 4. Elektronenmicroscopische opname van een gezuiverd preparaat van het virus uit Limonium latifolium. De vergrotingsstreep geeft 100 nm weer.

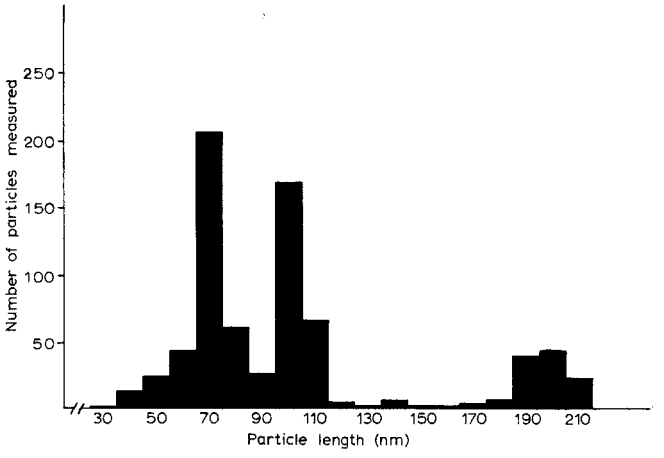

Fig. 5. Histogram of length of particles from a purified preparation of the virus from *Limonium latifolium.*

Fig. 5. Lengteverdeling van deeltjes in een gezuiverd preparaat van het virus uit Limonium latifolium.

Buoyant density. The suspension of virus purified according to the ether-carbon tetrachloride method had buoyant densities of 1.301 and 1.306 g/ml, that of virus purified according to the PEG method 1.311 and 1.319 g/ml.

Electron microscopy. The number of virus particles in crude sap preparations of *L. latifolium* leaves with line patterns was very small. A purified virus suspension contained particles of three predominant lengths (Fig. 5). The normal lengths of the virus particles in the three peaks were determined as 70, 102 and 194 nm, respectively.

Serology. The antiserum to the purified virus had a homologous titre of 512 in microprecipitin tests. Purified preparations of the virus tested against antisera to TRV-CAM, TRV-PRN, TRV-Italy 5, TRV-Italy 6, TRV-Lisse, TRV-L 20 and PEBV in microprecipitin tests gave a positive reaction with antiserum to TRV-CAM only (heterologous titre of 8), whereas the same purified preparations tested against the same antisera minus TRV-CAM reacted positively with antiserum to TRV-PRN in gel diffusion tests. In the latter tests crude sap from tobacco plants infected with TRV-Lab, TRV-P(MF) and TRV-P(SB) reacted positively with antiserum to the virus from *L. latifolium.*

Discussion

From the results of host range studies, persistence of infectivity in crude sap, particle morphology, 260/280 absorbance ratio, and serological tests it is clear that the virus which causes line pattern in *L. latifolium* is TRV (TRN-Lim).

Schmelzer (1957) reported systemic infection after inoculation of a Dutch isolate of TRV onto seedlings of *L. latifolium* and *L. suworowi,* but he did not give a description of the symptoms in the former species (the symptoms in *L. suworowi* consisted of necrotic lesions, ringspots and arched necrosis on the inoculated leaves followed by systemic white necrosis, mosaic, crinkling and curling of the leaves).

According to the comparative symptomatology of TRV strains from an Oregon source as presented by Lister and Bracker (1969) TRV-Lim resembles their yellow

strain of TRV in its reaction on *N. clevelandii*, but the symptoms in *N. glutinosa, N. megalosiphon, N. tabacum* and *P. hybrida* were more reminiscent of those caused by their unstable variants of TRV.

Classification of TRV-Lim into one of the serotypes of TRV described by Harrison and Woods (1966) is impossible as our isolate gave a serological reaction with both antiserum to TRV-PRN which belongs to serotype I whose members were found in Europe, and antiserum to TRV-CAM, originally from Brazil (serotype III).

Although it is known that a number of TRV isolates contain particles with three different modal lengths such as, for instance, some isolates from Europe and the U.S.A. (Harrison and Woods, 1966), two isolates from pepper in California (Semancik, 1966) and an isolate from peony in New Zealand (Jones and Young, 1978), the three different particle lengths established for TRV-Lim need not be characteristic of this virus. It is conceivable that the isolate contained two variants of TRV with different lengths of their short particles. The two different buoyant densities observed might point into the same direction although the number of experiments was too small to attach much value to the differences found. Cooper and Mayo (1972) reported that the bulk cultures of both TRV-CAM and TRV-PRN contained multiple buoyant density components although the long and short particles of each of these isolates had the same buoyant density in cesium chloride.

The three sedimentation coefficients determined in our experiments corresponded reasonably well to the three particle lengths found.

Acknowledgments

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Samenvatting

Figuurbont op Limonium latifolium veroorzaakt door tabaksratelvirus

Tabaksratelvirus (TRV) werd geïsoleerd uit *Limonium latifolium* planten die heldergeel of rood figuurbont op de bladeren vertoonden. Er werd aangetoond dat dit virus, aangeduid als TRV-Lim, de ziekteverwekker was. De reacties van dit virus op *Nicotiana clevelandii* deden denken aan die van een gele stam van TRV afkomstig uit Oregon (VS), maar de symptomen op *N. glutinosa, N. megalosiphon, N. tabacum* en *Petunia hybrida* vertoonden meer gelijkenis met die welke veroorzaakt worden door de zogenaamde onstabiele varianten van TRV. De verdunningsgrens was 10^{-6}-10^{-7}, de inactiveringstemperatuur 75-80°C en de houdbaarheid in vitro 55-60 dagen. De gezuiverde virussuspensie bevatte deeltjes met drie normale lengtes, nl. 70, 102 en 194 nm. Het virus sedimenteerde als drie componenten met gemiddelde sedimentatiecoëfficiënten van respectievelijk 129, 161 en 206 S. In gezuiverde suspensie vertoonde TRV-Lim twee verschillende zweefdichtheden. Het virus was serologisch verwant aan TRV-isolaten uit Europa en Brazilië.

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Address

Laboratorium voor Virologie, Binnenhaven 11, 6709PD Wageningen, the Netherlands.