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Lonicera latent virus, a new carlavirus serologically related to poplar mosaic virus: some properties and inactivation in vivo by heat treatment

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Abstract

A virus with elongate particles (656 nm) was isolated from several *Lonicera* species. This virus, apparently belonging to the carlavirus group, is serologically distantly related to shallot latent virus and closely related to poplar mosaic virus. The inability to infect poplar and two other hosts of poplar mosaic virus characterizes the virus from *Lonicera* as a new virus which was named *Lonicera* latent virus.

The virus was easily sap-transmissible but was not transmitted by *Myzus persicae*.

Dilution end-point was about 10^{-3}, thermal inactivation between 65 °C and 80 °C and ageing in vitro 1–6 days.

Heat treatment, combined with tip-rooting appeared to be a good method to eliminate the virus from several *Lonicera* species and cultivars.

Additional keywords: Nicotiana megalosiphon, virus adaptation, yellow vein mosaic of *Lonicera japonica*.

Introduction

Several species of *Lonicera* are well known ornamentals and many species and cultivars are grown on a commercial scale. Most of them are propagated vegetatively which favours the occurrence of viruses. Lihnell (1951) reported the isolation of cucumber mosaic virus from *Lonicera periclymenum* showing ringspot symptoms. Schmelzer (1962/63) proved by grafting that the yellow vein mosaic typical of *L. japonica* cv. Reticulata (syn. var. *aureoreticulata*), is caused by an infectious agent and thus confirmed earlier reports on graft transmission of this phenomenon (Woods and DuBuy, 1943; Corp, 1949). When preliminary indexing *Lonicera* cultivars in order to find virus-free stock material we isolated a virus from several species and cultivars. Studies on host range, physical properties, purification and serology of this virus, are the main subject of this paper.

Materials and methods

Virus isolation. The type isolate M was isolated from *L. japonica* cv. Reticulata growing in a private garden in Wageningen. Other isolates were found in several species and

cultivars from different nurseries. Isolates of poplar mosaic virus (PMV) were from mosaic-diseased 'Florence Biondi' (FB3) and 'Robusta' (Robusta LH) poplar trees. They are described elsewhere in more detail (Van der Meer et al., 1980). Test plants were grown in 12 cm diam. pots in a glasshouse at 20–24 °C. Additional light was given in the period between the end of October and the end of March.

Inoculum from *Lonicera* for sap transmission experiments was prepared by macerating young leaves or just expanding buds in a 1% aqueous nicotine solution (w/v 1/5). Carborundum 500 mesh was used as an abrasive. When transmitting the virus between herbaceous hosts no nicotine was used, whereas with inoculation of woody hosts nicotine was added to the inoculum.

Virus propagation. For antiserum production the type isolate M was propagated in *Nicotiana megalosiphon* in an insect-proof, temperature-controlled glasshouse at about 20 °C. Inoculated and systemically infected leaves were harvested c. two weeks after inoculation.

Virus purification. Leaf material, buffer and organic solvents were chilled at 3 °C. Homogenizing was in a Waring blender in 0.1 M tris buffer adjusted to pH 9 with 0.1 M citric acid and containing 0.02 M sodium diethyldithiocarbamate and 0.01 M sodium thioglycolate (tris, pH 9). The first sediment obtained after precipitation of the virus with polyethylene glycol was also resuspended in this buffer. In all other cases 0.1 M tris, adjusted to pH 8 with 0.1 M citric acid (tris, pH 8) without reducing agents was used.

Unless otherwise stated centrifugation at low speed was at 6000 rev/min for 10 min in a Sorvall RC2-B centrifuge, the type of rotor used depending on the quantity of material. Centrifugation at high speed to sediment the virus was in a Beckman ultracentrifuge for 1.5 h at 25000 rev/min, or, when the virus was in sucrose solution, for 3 h at 25000 rev/min in rotor 30.

CsCl-gradient centrifugation was in a Beckman SW41 rotor, spun for 16 h at 30000 rev/min. In each of the six tubes 0.85 ml of virus suspension, obtained from c. 35 g of leaf material, was mixed with 2.4 ml of a CsCl solution in distilled water containing 0.623 g/ml. The tubes were filled up with paraffin oil. Virus zones at about 14–17 mm from the bottom of the tubes were isolated by puncturing the bottom. CsCl was removed by dialysis against tris buffer, pH 8.

For sucrose-gradient centrifugation, the virus from the six tubes of the CsCl-gradients after dialysis was layered on top of sucrose gradients from 10–40% (w/v) in six tubes of a Beckman SW27 rotor. This was spun for 1.5 h at 24000 rev/min. The gradients were prepared linear with volume, using an LKB-Ultrograd gradient mixer. Virus zones were isolated with an ISCO density-gradient fractionator. Virus zones in sucrose were diluted 1:1 with distilled water or buffer before concentrating.

Portions of 200 g of leaf material were homogenized in 300 ml of tris buffer, pH 9, 60 ml of carbon tetrachloride and 60 ml of chloroform. The homogenate was centrifuged at low speed. While stirring, polyethylene glycol 6000 was added to the supernatant to make a 6% (w/v) solution and stirring was continued for 1 h. The suspension was then centrifuged for 15 min at 10000 rev/min. The sediment obtained was resuspended in 140 ml of tris buffer, pH 9, stirred for 1 h and centrifuged at low speed. Now the supernatant was centrifuged at high speed and the virus sediment obtained resuspen-

ded in 5.5 ml of tris buffer, pH 8. After stirring for 1 h the suspension was centrifuged at low speed and the resulting supernatant subjected to CsCl-gradient centrifugation. After isolation and dialysis of virus zones, the material was centrifuged on a sucrose gradient. Virus zones from the latter were injected intravenously into a rabbit without further treatment, or they were concentrated to 2 ml, mixed with 2 ml of glycerol and stored at –20 °C for intramuscular injection.

Antiserum preparation was by intravenous and intramuscular injection of a rabbit. For the latter virus preparations were emulsified with an equal volume of Freund's incomplete adjuvant. Two intravenous injections, given with a three-day interval were followed by an intramuscular injection, two weeks later. Per injection the virus from c. 100 g of leaf material was administered. Four weeks after the intramuscular injection 1 ml of concentrated virus was injected intravenously.

Two antisera to poplar mosaic virus (PMV) from Italy, with titres of 512 (A52 I) and 2048 (A56 II) in the slide precipitin test, were kindly provided by Dr E. Luisoni, Torino, Italy. An antiserum to a Dutch isolate of PMV with unknown titre and prepared by Berg (1964) was supplied by Ir D. H. M. van Slogteren, Bulb Research Centre, Lisse. Other antisera, among which one to PMV-FB3 (Van der Meer et al., 1980) were from own stock.

Serological testing to determine antiserum titres and for virus identification was performed with the micro-precipitin test under paraffin oil, using series of four-fold dilutions of antiserum as well as of antigen preparations. As test antigens sap centrifuged at low speed, purified preparations and partially purified preparations were used. For the latter 3 g of leaves were homogenized in 10 ml of tris buffer pH 8, and the homogenate was centrifuged at low speed. The supernatant was then centrifuged for $1\frac{1}{2}$ h at 20000 rev/min in a Sorvall RC2-B centrifuge and the sediment resuspended in 1 ml of tris buffer, pH 8. After centrifugation at low speed the supernatant was used for serological testing.

Electron microscopy. Chop preparations of *N. megalosiphon* infected with isolate M and of *N. tabacum* infected with TMV were prepared in 2% aqueous phosphotungstic acid adjusted with KOH to pH 6.5. Preparations with TMV were photographed alternately with preparations containing isolate M. Particles were measured in photographic negatives, using a low-power binocular microscope with objective lens X 1 and a micrometer eyepiece X 12.5, and from enlarged photographic prints of these negatives.

To calculate particle lengths, for every series of photographs the average length expressed in micrometers (from the negatives) or in mm (from the prints) was calculated and compared with the average length of TMV particles. The average length of TMV was assumed to be 300 nm.

Results

Isolation from Lonicera and symptoms on Lonicera

The virus could easily be transmitted mechanically from *Lonicera* species to test plants during the whole growing season. On *N. megalosiphon* often large numbers of local

lesions developed, indicating a high virus concentration in the inoculum. With inocula from *L. japonica* always few or no local lesions were obtained, indicating either a low virus concentration or the presence of virus inhibitors in this species. The virus was isolated from the following species and cultivars: *L.* × *americana; L.* × *brownii* cvs Dropmore Scarlet, Fuchsioides and Punicea; *L. caprifolium; L.* × *heckrottii* cv. Goldflame; *L. henryi; L. japonica* cvs Halliana and Reticulata; *L. periclymenum* cvs Belgica, Belgica Select and Serotina; *L.* × *telmanniana.*

Infected *Lonicera* plants in the glasshouse occasionally showed a reddish brown discoloration of secondary veins which was only visible on the underside of some leaves. Healthy plants never showed this symptom. Except for *L. japonica* cv. Reticulata most infected plants growing outdoors did not show symptoms, whereas from the few plants with symptoms also other viruses were isolated. Cucumber mosaic virus was isolated from one *L. periclymenum* with rings and line patterns and three other plants with mosaic symptoms appeared to be infected with a hitherto unidentified isometric virus (Van der Meer and Huttinga, unpublished).

Symptoms on herbaceous hosts

N. megalosiphon showed many chlorotic lesions five or six days after inoculation, followed by a systemic vein yellowing (Fig. 1) and later by vein necrosis. *N. clevelandii* reacted with chlorotic lesions, followed by a systemic vein chlorosis. *N. glutinosa* showed faint flecks on inoculated leaves (Fig. 2) and a diffuse systemic mosaic or

Fig. 1. *Nicotiana megalosiphon* leaves with symptoms caused by *Lonicera* latent virus. Right: inoculated leaf. Left: first leaf with systemic symptoms.

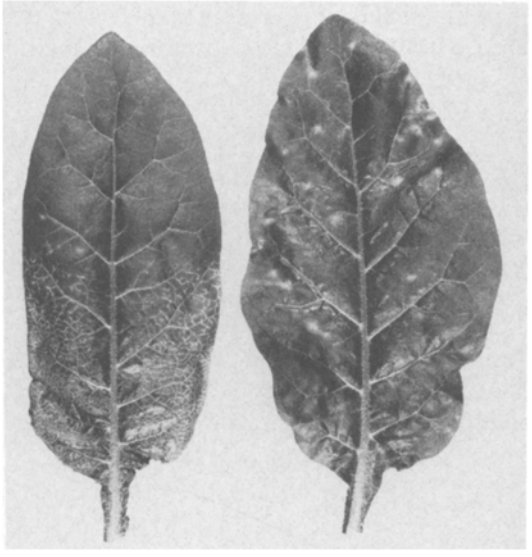

Fig. 1. Bladeren van N. megalosiphon met symptomen veroorzaakt door latent kamperfoelievirus. Rechts: geïnoculeerd blad. Links: systemische symptomen.

Fig. 2. Inoculated leaf of *Nicotinana glutinosa* with symptoms caused by *Lonicera* latent virus.

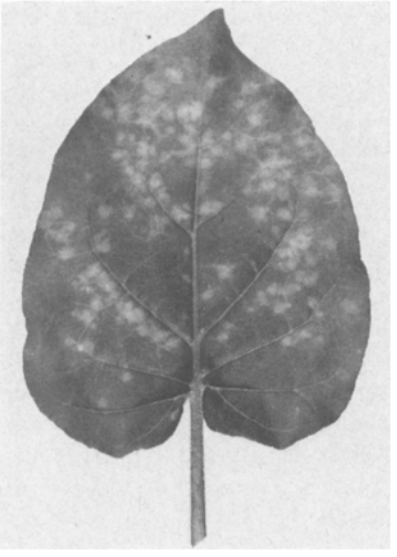

Fig. 2. Geïnoculeerd blad van N. glutinosa met symptomen veroorzaakt door latent kamperfoelievirus.

Fig. 3. *Chenopodium quinoa* leaves infected with a sub-isolate of *Lonicera* latent virus adapted to *C. quinoa*. Left: inoculated leaf. Right: first leaf with systemic symptoms.

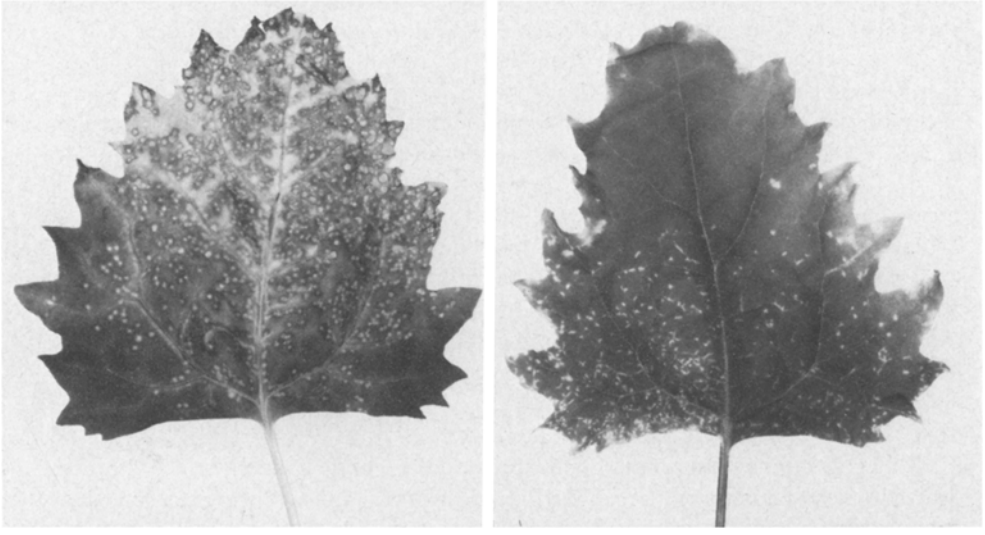

Fig. 3. Bladeren van C. quinoa geïnfecteerd met een sub-isolaat van latent kamperfoelievirus aangepast aan C. quinoa na meerdere passages door N. megalosiphon en C. quinoa. Links: geïnoculeerd blad. Rechts: eerste blad met systemische symptomen.

sometimes vein banding. No symptoms developed on *N. rustica* and *N. tabacum* cv. White Burley and only occasionally the virus could be detected in inoculated leaves of these hosts.

Chenopodium quinoa never showed symptoms when inoculated with sap from infected *Lonicera* species even with inocula that induced large numbers of lesions on *N. megalosiphon*. When *C. quinoa* was inoculated with sap from infected *N. megalosiphon* or *N. clevelandii*, however, limited numbers of small lesions developed on the inoculated leaves. Such lesions became visible when the inoculated leaves started yellowing because of ageing, which was about 14 days after inoculation. With further successive transmission from *C. quinoa* to *C. quinoa* many more lesions were obtained and in such tests some isolates caused also systemic symptoms (Fig. 3). In the same way local lesions could be produced on *C. amaranticolor*, but systemic symptoms never developed. When transmitted from *C. quinoa* back to *N. megalosiphon*, sub-isolates retained the ability to cause many lesions on *C. quinoa* after several transfers on *N. megalosiphon*.

Cucumis sativus reacted with a mild systemic vein clearing in some tests, but usually remained symptomless. It was always found to be systemically invaded by the virus.

Phaseolus vulgaris cv. Bataaf and *Vigna sinensis* did not develop symptoms with any of three different isolates and the virus could not be recovered from these hosts.

Aphid transmission

M. persicae from a colony on infected *N. megalosiphon* did not transmit isolate M to

any of 10 healthy *N. megalosiphon* in experiments with 50 aphids per test plant. Neither was any virus transmission detected when aphids from a virus-free colony were starved for 2 h before allowing them an acquisition feeding period of $\frac{1}{2}$ h on infected *N. megalosiphon*, using 10 aphids for each of 10 *N. megalosiphon* test plants.

Seed transmission
No virus could be detected in 80 seedlings from seeds obtained from infected plants of *L. periclymenum* when indexed on *N. megalosiphon.*

Persistence of infectivity in crude sap
Isolate M retained its infectivity for 24 h but not for 36 h at 20°C. Thermal inactivation was about 65° and dilution endpoint was a little over 10^{-3}. With two other isolates infectivity was retained for 36 and 144 h, respectively, the thermal inactivation points were 75°C and 80°C; the dilution end-point of both isolates was 10^{-4}. In all persistency experiments *N. megalosiphon* was used as donor plant and as test plant.

Retransmission of the type isolate to L. japonica
Repeated attempts with crude sap, to transmit the isolate M from infected *N. megalosiphon* to young plants of a green seedling clone of *L. japonica*, were unsuccessful. In a later experiment with purified virus one out of seven inoculated plants became infected as was shown by back-testing to *N. megalosiphon.* The infected plant did not show symptoms, indicating that the isolate M is not the cause of the yellow vein mosaic of the *L. japonica* cv. Reticulata source, from which this isolate was obtained. This vein

Table 1. Serological relationships between *Lonicera* isolates and PMV. Figures represent titres obtained in micro-precipitin tests.

Antigens	Antisera to					
	Lonicera M		PMV			
					Italy	
			Berg[4]	FB3	A52 I	A56 II
Lonicera M	16384	1024[3]		256	64	1024
Lonicera 1-4-1[1]	16384		1024	256		
Lonicera 5-1-2[2]	16384			256	64	1024
PMV-FB3	64			256		
PMV-Robusta LH	256			1024		
Shallot latent virus		64[3]				

[1] From *L.* × *heckrottii.*
[2] From *L.* × *brownii.*
[3] From Bos et al., 1978.
[4] See Berg, 1964.

Tabel 1. Serologische verwantschappen tussen Lonicera isolaten en PMV. De getallen geven de titers weer verkregen in de micro-precipitatietoets.

mosaic, however, appeared easily graft-transmissible to plants of the green seedling clone, which confirmed earlier reports (Schmelzer, 1962/63).

Electron microscopy. The average length of 192 particles measured from negatives was 658 nm and of 154 particles measured from reprints 654 nm.

Serology. Four weeks after the intramuscular injection the antiserum prepared had a titre of 1024 to purified isolate M. Two weeks after the last intravenous injection the titre was 4096.

In many of the serological tests non-specific reactions occurred. This was especially true when clarified extracts from *N. clevelandii*, *N. megalosiphon*, or *C. quinoa* were tested and sometimes also with preparations that were subjected to one cycle of differential centrifugation. The data mentioned below and in Table 1 are from experiments in which there was no evidence of such non-specific reactions. With these a clear serological relationship was demonstrated between *Lonicera* isolates, poplar mosaic virus and shallot latent virus. Antisera to red clover vein mosaic virus and pea streak virus did not react with *Lonicera* virus (isolate 1-4-1).

No or only very small serological differences were detected between isolates of *Lonicera* virus that had been cultivated in *C. quinoa* and the same isolates that had been kept continuously on *N. megalosiphon*, using the antiserum to isolate M (not passed through *C. quinoa*).

Transmission attempts to poplar

In two experiments isolate M and isolate 1-4-1 were inoculated from infected *N. megalosiphon*, *N. clevelandii* and *C. quinoa* to 33 healthy, 15 cm long plants of *Populus* × *euamericana* cv. Zeeland growing in pots in a glasshouse. Later three similar poplars were inoculated with sap of *N. clevelandii* infected with isolate 5-1-2, an isolate always causing large numbers of local lesions on the *Nicotiana* test plants. None of these 36 inoculated poplars developed symptoms and no virus could be detected when testing them repeatedly on *N. megalosiphon* until one year after inoculation. All of the 12 poplars inoculated in the same way, with PMV isolates FB3 and Robusta LH, however, showed symptoms within two months after inoculation and the virus could be easily transmitted from them to *N. megalosiphon*. Eight control plants remained free of symptoms and tests on *N. megalosiphon* were negative.

Comparison of Lonicera virus and PMV on other hosts

When inoculating PMV isolates FB3 and Robusta LH and *Lonicera* isolates 5-1-2 and M each to 13 *L. periclymenum* seedlings, one, three, six and none, respectively, became infected, as was demonstrated by back-testing to *N. megalosiphon*. Some plants infected with the PMV isolate Robusta LH, as well as some plants infected with the 5-1-2 isolate from *Lonicera*, showed a reddish brown discoloration of the veins in some leaves.

Symptoms caused by PMV isolates on *Nicotiana* species, *Chenopodium* species and cucumber were similar to those caused by *Lonicera* isolates, except that the systemic symptoms on *N. clevelandii* caused by PMV were more severe than those caused by isolates from *Lonicera*.

Although inocula from poplar induced up to 200 local lesions per leaf in *N.*

megalosiphon, such inocula never induced symptoms in *C. quinoa*. Inoculations from *N. megalosiphon* to *C. quinoa*, however, resulted in infection of *C. quinoa* in the same way as mentioned for *Lonicera* isolates.

PMV caused local lesions on *V. sinensis* en *P. vulgaris* whereas *Lonicera* isolates M, 1-4-1 and 5-1-2 did not cause symptoms on these hosts and could not be recovered from inoculated plants.

Inactivation in vivo by heat treatment

To produce virus-free stock material of *Lonicera*, infected plants of several species and cultivars were heat treated for six to eight weeks at 37°C (16 h) day temperature and 25°C (8 h) night temperature. After such treatment 1 cm long tips were rooted in a mixture of sand and peat and tested at different intervals on *N. megalosiphon* until one year after treatment. In this way virus-free plants were obtained of *L.* × *heckrottii*, *L.* × *heckrottii* cv. Goldflame, *L. japonica* cv. Halliana, *L.* × *telmanniana*, *L.* × *brownii* cv. Punicea and *L.* × *americana*. The *L. periclymenum* cvs Serotina and Belgica Select appeared rather sensitive to high temperature and no virus-free plants of these cultivars have so far been obtained.

Tests on *N. megalosiphon*, until 18 months after treatment, showed that several heat treated cuttings of *L. japonica* cv. Reticulata were free of the described 656 nm virus. However, heat treatment did not have any effect on the yellow vein mosaic of this cultivar.

Discussion

Unlike other purification methods tried and not mentioned here, the method described gave good yields and relatively little aggregation. However, non-specific aggregation did occur in several of the serological experiments performed. The data mentioned are from tests in which there was no evidence of such non-specific aggregation. So in these tests the antiserum titres were hardly influenced by non-specific aggregation. Thus the tests clearly show that the *Lonicera* virus is serologically closely related to PMV from the Netherlands as well as from Italy. The viruses could be distinghuished serologically with the antiserum to *Lonicera* isolate M. Besides serologically, the *Lonicera* virus also resembles PMV in its liability to aggregate (Biddle and Tinsley, 1971).

The calculated particles length of 656 nm for the *Lonicera* virus lies clearly within the range of that of the carlaviruses (Fenner, 1976) and is almost equal to the length of 661 nm which we found for PMV (Van der Meer et al., 1980).

Based on particle length and results of serological tests only, the virus could be considered as a strain of PMV. However, the inability of three different isolates to infect poplar, French bean and cowpea marks the *Lonicera* virus as a pathogenically different virus. Because the virus is latent in *Lonicera* we propose to name it *Lonicera* latent virus (LLV). This name has been used already in a note by Brunt and Thomas (1975) for a possibly identical virus from *L. periclymenum* with particles of 650 nm.

The distant serological relationship with shallot latent virus (Bos et al., 1978) links LLV with other viruses of the carlavirus group, while the serological relationship between LLV and PMV provides the hitherto missing link between PMV and other carlaviruses (Luisoni et al., 1976).

The 'acquired' ability of both PMV and LLV to cause symptoms on *C. quinoa* after

passage through *Nicotiana* species and *C. quinoa*, needs further investigation. It seems related to the adaptation phenomenon of a bean yellow mosaic virus strain from *Gladiolus* to *Vicia faba*, described by Koenig (1976). Preliminary tests so far failed to detect serological differences between isolates kept on *Nicotiana* species and sub-isolates adapted to *C. quinoa*.

Back-transmission of LLV to *L. japonica* proved that LLV is not the cause of the typical yellow vein mosaic in *L. japonica* cv. Reticulata. The inability of elimination by heat treatment suggests that this phenomenon is caused by a virus unrelated to LLV, or by another infectious entity. Recently this has been comfirmed by Osaki et al. (1979) who proved that *Lonicera* yellow vein mosaic is caused by a strain of tobacco leaf curl virus.

Inactivation of LLV by heat treatment provides a method to obtain virus-free stock material of *Lonicera* species and thus may help to prevent the national and international distribution of viruses. The clean stock material will be incorporated in the certification scheme in the Netherlands (Van der Meer, 1974; Meijneke, 1974; Elzenga, 1974).

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Samenvatting

Latent kamperfoelievirus, een nieuw carlavirus: enkele eigenschappen en inactivering in vivo door warmtebehandeling

In verschillende soorten en cultivars van het geslacht *Lonicera* (kamperfoelie) blijkt een virus voor te komen dat gemakkelijk door sapinoculatie kan worden overgebracht op kruidachtige planten.

Een tegen gezuiverd virus bereid antiserum had een titer van ca. 4096. Er kon mee worden aangetoond dat het virus van kamperfoelie serologisch nauw verwant is met populieremozaïekvirus (Tabel 1). Het virus van kamperfoelie is echter niet in staat om populier, *Phaseolus vulgaris* 'Bataaf' en *Vigna sinensis* te infecteren en wordt mede daarom als een afzonderlijk virus beschouwd. Het wordt aangeduid als latent kamperfoelievirus (*Lonicera* latent virus) en behoort evenals populieremozaïekvirus tot de carlavirusgroep (aardappelvirus-S-groep).

Het virus blijkt vrij gemakkelijk te kunnen worden geëlimineerd door besmette kamperfoelieplanten gedurende ongeveer zes weken een warmtebehandeling (37°C) te geven en daarna de uiterste toppen (1 cm) te stekken. Van verschillende cultivars werd op deze wijze virusvrij uitgangsmateriaal verkregen.

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