

UNSTABLE INFECTIVITY AND ABUNDANT VIRAL RNAS ASSOCIATED WITH STRAWBERRY MOTTLE VIRUS

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Abstract

A not yet identified virus (SMoV) associated with the strawberry mottle syndrome was mechanically transmitted from infected *Fragaria vesca* UC-5 to *Chenopodium quinoa* and from *C. quinoa* to *Nicotiana benthamiana*, *N. occidentalis* 37B and *N. hesperis* 67A. *C. quinoa*, *N. benthamiana* and *N. occidentalis* 37B developed systemic mosaic symptoms, whereas *N. hesperis* 67A produced local lesions. In crude extracts of these plants, isometric particles 28 nm in diameter were observed, sometimes in arrays within tubules. However, the presence of these particles was always erratic. Purification of the intact isometric virus particles from *C. quinoa* or *N. occidentalis* 37B when leaves were maximally symptomatic was unsuccessful. The infectious agent appeared to be unstable *in vitro*, could not be separated from the plant material and inconsistent results were obtained no matter the purification strategy followed. *N. hesperis* 67A has been used as a local-lesion host to evaluate the increase of infectivity in *N. occidentalis* 37B. Maximal infectivity was reached a few days after the onset of the systemic symptoms. Purifications from this plant material gave inconsistent results also. While virus particles in these crude extracts were seldom present, dsRNA-like material was readily revealed by agarose-gel electrophoresis. Infectivity associated with extracts of SMoV-infected leaves of *C. quinoa* and *N. occidentalis* 37B was susceptible to low concentrations of RNase. Phenol extracts of infected leaves yielded greater infectivity than buffer extracts of comparable tissue. Infectivity seemed to be associated with ssRNA.

1. Introduction

Strawberry mottle, one of the most important and widespread diseases of the cultivated strawberry, has been associated with a virus transmissible by grafting and by aphids (mainly *Chaetosiphon fragaefolii* Cockerell) in a semi-persistent way (Frazier and Sylvester, 1960). The virus has also been successfully transferred, by mechanical inoculations or by aphids, to *Chenopodium quinoa* in several laboratories (Adams and Barbara, 1986; Frazier, 1968; Leone *et al.*, 1992; Polák and Bezpalcova, 1988; Hepp and Converse, 1990). Hepp and Converse (1990) also reported the back-transmission of strawberry mottle virus (SMoV) by aphids from *C. quinoa* to *Fragaria vesca*.

Although the symptomatology and the natural and experimental transmission of SMoV seem well understood, there is no consensus yet about its biophysical properties. Because of this, it has been hypothesized that discrepancies may be caused by differences among isolates of SMoV (Converse *et al.*, 1988). Isometric virus particles have been described by different authors, ranging from about 14 nm (Polák and Jokeš, 1992), 25-30 nm (Hepp and Converse, 1990; Kitajima *et al.*, 1971; Leone *et al.*, 1992), to about 37 nm in diameter

(Yoshikawa and Converse, 1991). Double-stranded RNA has been found both in *F. vesca* and *C. quinoa* infected by SMoV but different values have been reported for the two infection-specific bands detected (Adams and Barbara, 1986; Yoshikawa and Converse, 1991). Some other properties of SMoV have been reported by Yoshikawa and Converse (1991) who succeeded in partially purifying the virus from *C. quinoa*. However, because of the difficulties encountered so far in many laboratories in thoroughly purifying SMoV, the virus is still virtually uncharacterized and unidentified (Converse, 1992). This also hampers the detection of SMoV for rapid indexing of propagative material. As for the other major aphid-borne strawberry viruses, strawberry crinkle and strawberry mild yellow-edge virus (SCV and SMYEV), strawberry is currently indexed for SMoV by a time-consuming leaf-graft bioassay on indicator plants. Rapid and reliable detection methods are still needed for SCV, SMoV and SMYEV to replace this bioassay (Converse, 1992). To this end, any consistent information gained on the properties of SMoV will support the strategy to follow for the development of uniform detection methods for the aphid-borne strawberry viruses. In this paper we report some new information about biological and molecular properties of SMoV.

2. Materials and methods

2.1 Virus maintainance and passage to herbaceous hosts

Isolates 1134 and 1279 of SMoV were maintained in *Fragaria vesca* UC-5 plants, transferred to *C. quinoa* by mechanical inoculations and propagated in this plant in a temperature-controlled glasshouse at 20°C, with additional illumination during winter to make a total daylength of 16 h. All other herbaceous plants were also grown under the same conditions.

Inoculum was prepared by grinding one part of infected leaf material in nine parts (w/v) of tap water without additives. Leaves of carborundum-dusted test plants were inoculated with finger tips. If no symptoms were observed in *Nicotiana benthamiana* and *N. occidentalis* 37B after 3 weeks, the main stem was pruned back in order to stimulate symptom expression on the newly developing lateral shoots.

Evaluation of the increase of the infectivity in *N. occidentalis* 37B was performed using *N. hesperis* 67A as a local lesion host. Leaf disks were excised with a cork-borer (0.5 cm diam.) from 20 *N. occidentalis* 37B, following the infectivity in the first three leaves showing systemic symptoms in order of appearance (two disks per plant per leaf). Disks corresponding to one specific leaf were pooled and ground with mortar and pestle adding 3 ml of tap water. The sap obtained was inoculated as mentioned above on at least three *N. hesperis* 67A.

2.2 Partial purification of SMoV

Leaves of *C. quinoa* or *N. occidentalis* 37B (at least 100 to 300 g per experiment), harvested when systemic symptoms were maximal, were passed through a Pollähne press with 3 vol./g of 30 mM phosphate buffer, pH 8.1, containing 1 mM dithiothreitol, 2.5 mM glutathione and 1.5% insoluble polyvinylpyrrolidone (w/v). The slurry obtained was submitted to two cycles of low-speed centrifugations (10000 and 15000 g, 10 min each). The sap was made 0.1 M with respect to NaCl and 1% with respect to polyethylene glycol, M_r 6000 (PEG 6000; w/v), and stirred for 15 min at 4°C. The suspension was centrifuged at low speed and the resulting pellet was suspended in 1/10 of the initial volume of 5 mM phosphate buffer, pH 8.1. This suspension was then made 0.2% with

respect to Triton X-100 and 4.5% with respect to PEG 6000 (w/v) and stirred for at least 15 min at 4°C. The suspension was centrifuged at low speed and the pellet was resuspended in three steps of at least 15 min, each consisting of 20 ml of 5 mM phosphate buffer, pH 8.1. After each resuspension-step, the suspension was centrifuged at low speed and the resulting supernatant was stored at 4°C. The three supernatants obtained were pooled and concentrated to 10 ml by ultrafiltration on a membrane with a cut-off of 1×10^6 M_r (Filtron) with a Minitan-S (Millipore Corporation) system. This concentrated sample was further purified by FPLC using a Sephacryl S-1000 gel-filtration column (2.6x94 cm; separation range 5×10^5 - $>10^8$ M_r). The column was equilibrated with 5 mM phosphate buffer, pH 8.1 and eluted at a flow rate of 0.7 ml/min. The run was performed at 4°C. Fractions (6 ml each) corresponding to the left shoulder of the void volume peak and known to contain infectivity (Leone *et al.*, 1992) were collected and concentrated to about 10 ml by ultrafiltration on a membrane with a cut-off of 1×10^6 M_r. This almost colourless sample was then subjected to isopycnic centrifugations in 40% (w/v) CsCl or Cs₂SO₄ or to preparative isoelectric focusing with a Rotofor (Bio-Rad) apparatus in the pH range 3.5-10. The infectivity was followed throughout the purification procedure by back-inoculations to *C. quinoa* plants when purifications were performed with *C. quinoa* or to *N. hesperis* 67A plants when purifications were performed with *N. occidentalis* 37B. Fractions were also examined for virus particles by electron microscopy.

2.3 Isolation of dsRNA

Double-stranded RNA was extracted from *N. occidentalis* 37B SMoV (isolate 1134)-infected or uninfected plants (5 g per extraction), following the method described by Jordan (1986) with slight modifications. Nucleic acids were then separated on a 1% agarose slab gel at 75 V for 2 h in 40 mM Tris, 40 mM sodium acetate, 1 mM EDTA, pH 8.0. EtBr was incorporated in the gels at a concentration of 0.5 µg/ml and bands were visualized by UV light.

3. Results

3.1 Herbaceous hosts

We succeeded in transmitting by mechanical inoculations two isolates of SMoV, routinely maintained in *C. quinoa*, from this plant to *N. benthamiana*. Infected *N. benthamiana* plants first developed a very mild systemic vein mosaic. After repeated mechanical propagations in *N. benthamiana*, symptoms expression increased within several months to reach, under optimal conditions, a maximum at about 7 days after inoculation. The availability of *N. benthamiana* infected plants permitted a ready passage of SMoV from *N. benthamiana* to *N. occidentalis* 37B and *N. hesperis* 67A plants. *Nicotiana occidentalis* 37B responded to the infection by producing chlorotic local lesions on the inoculated leaves within 5 days after inoculation and systemic vein mosaic on the new developing leaves. Systemic symptoms reached a maximum about 2 weeks after inoculation. *Nicotiana hesperis* 67A reacted by producing necrotic local lesions on the inoculated leaves within 5 days after inoculation. The lesions sometimes became also systemic. When symptoms were severe, all *Nicotiana* plants also showed dwarfing.

While transmission of SMoV from *C. quinoa* to *C. quinoa* and between *Nicotiana* spp. was reliable, the transmission from *C. quinoa* to *Nicotiana* spp. was erratic. Confirmation that the virus in *Nicotiana* spp. was the same as that in *C. quinoa* was obtained by back-inoculations to *C. quinoa*, which developed the typical symptoms associated with SMoV

infections. Also, the isometric virus-like particles (ca 28 nm in diameter) described by Leone *et al.* (1992) were found in crude extracts of all experimental hosts. These particles were found sometimes in arrays within tubules (Fig. 1). However, their occurrence was always erratic and, when present, they were observed in very low amounts.

3.2 Purification of SMOV or dsRNA-like material

Attempts to purify SMOV were made using systemically infected leaves of *C. quinoa* or *N. occidentalis* 37B when symptom development was maximal (about 7 days after inoculation for *C. quinoa*; about 14 days after inoculation for *N. occidentalis* 37B). The techniques used were aimed at retaining infectivity, since the only way to evaluate the purification procedure was by means of back-inoculations.

Irespective of the plant used for purification or of the procedure followed, inconsistent results were obtained. Working with *C. quinoa*, the infectivity sometimes could be still found after the isoelectric focusing step and eluted in fractions with an isoelectric point (pI) between 5 and 6. However, no virus-like particles could be observed in these samples and plant contaminants were still present. After isopycnic centrifugations in CsCl or Cs₂SO₄, infectivity was completely lost with or without removal of the salt by extensive dialysis in 5 mM phosphate buffer, pH 8.1.

With *N. occidentalis* 37B the infectivity was always lost after the gel-filtration step, despite that the amount of starting leaf material used for purification was two to three times more than with *C. quinoa*. No improvements were obtained when discontinuous-gradient (20-60% sucrose) and rate-zonal (10-40% sucrose gradient) centrifugations were used alone or in combination with the techniques described in Materials and Methods. Extractions as reported by Yoshikawa and Converse (1991) also resulted in immediate loss of infectivity. In general, infectivity could only be concentrated, no matter the procedure employed, but could not be separated from the plant material.

The inconsistency of the results observed during the purification trials was found to be associated with an inconsistency of the infectivity in the starting plant material. This was studied by following the infectivity of the *N. occidentalis* 37B plants used for the purification trials for 16 successive weeks, employing *N. hesperis* 67A as a local lesion host (Fig. 2). Despite that growth and extraction conditions were maintained standard, the variability of the plant material was evident.

In order to improve the results we evaluated the increase of the infectivity in *N. occidentalis* 37B (Fig. 3). Maximal infectivity was found at 10-11 days after inoculation and did not correspond to the maximal expression of symptoms. Purifications attempted from leaves harvested when infectivity was maximal also gave inconsistent results. Furthermore, virus-like particles were seldom present in crude extracts from plants at this stage.

However, when ten-day-old *N. occidentalis* 37B material was analysed for the presence of dsRNA, two bands of about 6.3 and 7.8 kbp were readily detected by agarose-gel electrophoresis in infected but not in healthy plants (Fig. 4).

3.3 Properties of the SMOV-associated infectivity

The ease by which dsRNA-like material could be detected in a few grams of infected plants prompted us to investigate the properties of the SMOV-associated infectivity. Figure 5 shows the effect of RNase A₁ on the infectivity of SMOV and tomato mosaic virus (ToMV) in leaf extracts of *N. occidentalis* 37B. While the infectivity of ToMV was not affected at any of the concentrations of RNase tested, the SMOV-associated infectivity was found to be susceptible to RNase. The experiment was repeated using two different

isolates of SMoV in *C. quinoa* (Fig. 6). The pattern for the two isolates showed some differences, but for both, infectivity was completely lost at the highest RNase concentration.

Phenol extracts of infected leaves, prepared in the same way as for the dsRNA extraction, yielded as much as or greater infectivity than extracts in 30 mM phosphate buffer, pH 8.1, with or without additional treatment with organic solvents (Table 1). The phenol-extracted infectivity was completely lost after treatment with RNase A₁ (Table 2). One RNase treatment was performed in the presence of 0.7 M NaCl in order to protect dsRNA (Schoen and Leone, this volume) and to test it for infectivity. Also this sample was not infective.

4. Conclusions

Erratic or inconsistent results found working with SMoV have been reported in the past by different authors (Adams and Barbara, 1986; Converse *et al.*, 1988; Converse, 1992). Our results working with two SMoV isolates showed that erraticness was mainly associated with its unstable infectivity, and this characteristic hampered purification and detection of intact particles. At the moment it is not known where these defective properties of SMoV originate. However, neither intact virus particles nor virus proteins could be obtained even employing very mild techniques that could be successfully applied for the purification of enzymes below the level of protein detectability (Leone *et al.*, 1990). In contrast to this, viral RNAs could be readily detected in SMoV-infected material. Two prominent, infection specific bands obtained after dsRNA extraction have been characterised as such by DNase/RNase treatments (Schoen and Leone, this volume). Infectivity was preserved by phenol extraction but was prone to abolishment by a treatment with RNase even when dsRNA was protected by high salt concentration in the reaction mixture. This suggests that a ssRNA, not dsRNA, was the primary infectious agent.

Until now all attempts to purify intact virus particles made in different laboratories have been unsuccessful (Adams and Barbara, 1986; Converse *et al.*, 1988; Leone *et al.*, 1992) or did not provide enough material for a thorough biological and molecular characterization of SMoV (Yoshikawa and Converse, 1991). Because of this, SMoV is still unidentified. Moreover, it has not been possible to raise an antiserum against it, thus hindering the development of a serological-based detection method. However, our results and those of other authors (Adams and Barbara, 1986; Yoshikawa and Converse, 1991) have demonstrated that dsRNA can be detected in infected plant material. As for practical applications, uniform detection methods are needed to replace the leaf-graft bioassay used for the three major aphid-borne strawberry viruses. The defective properties of SMoV described in this report affect the strategy to follow for the development of such methods. It is expected that future detection methods will be based especially on molecular biology, which already has occurred with SMYEV (Martin *et al.*, 1989).

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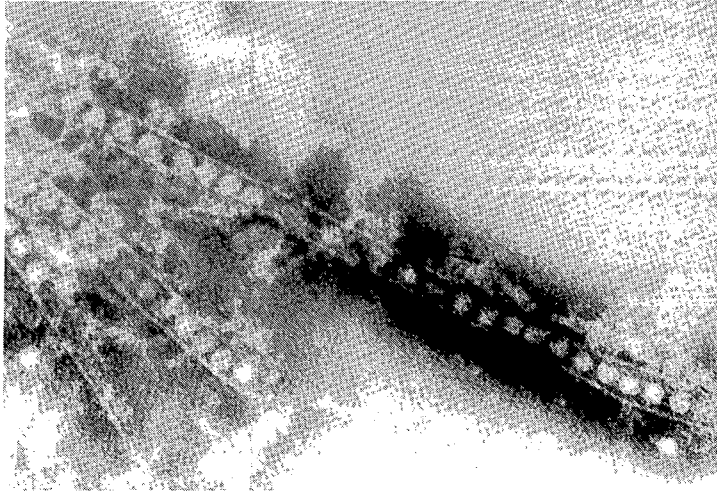


Figure 1. Electron micrograph of crude sap of *C. quinoa* infected with SMOV showing virus-like particles in array within tubules. Samples were stained with 2% potassium phosphotungstate in water. Bar represents 100 nm.

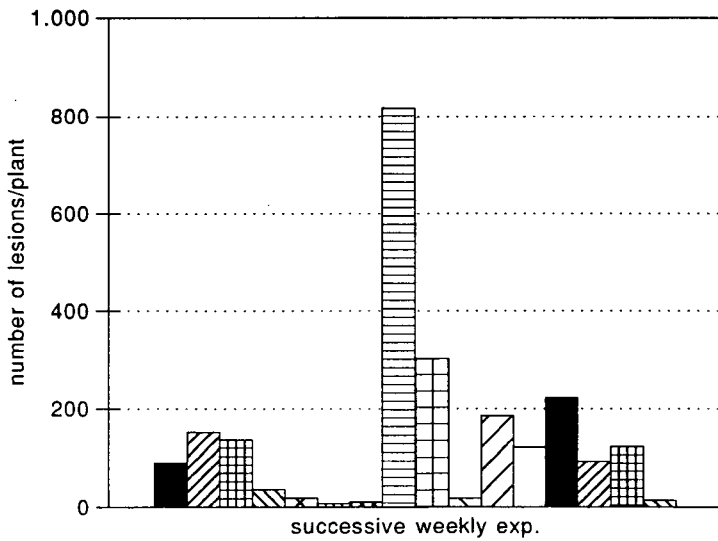


Figure 2. SMOV-associated infectivity (assessed by back-inoculations on *N. hesperis* 67A) in leaves of *N. occidentalis* 37B, 14 days after inoculation. The plants were used weekly for the purification trials.

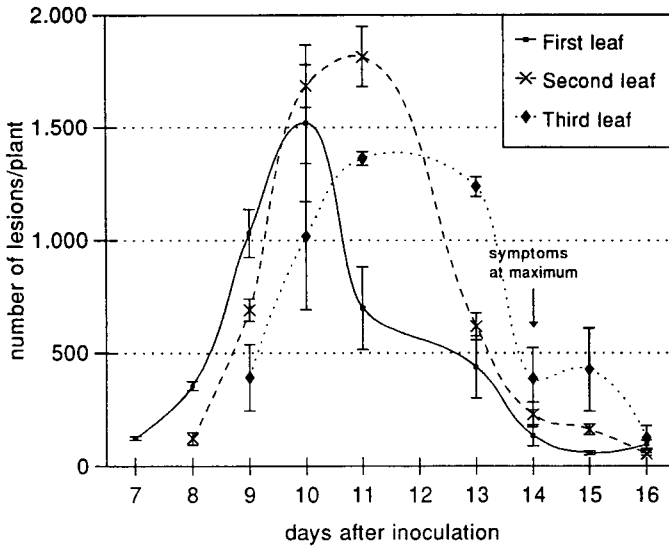


Figure 3. Evaluation of the increase of the SMoV-associated infectivity in *N. occidentalis* 37B, assessed by daily back-inoculations on *N. hesperis* 67A. The first three leaves showing systemic symptoms were followed, in order of appearance.

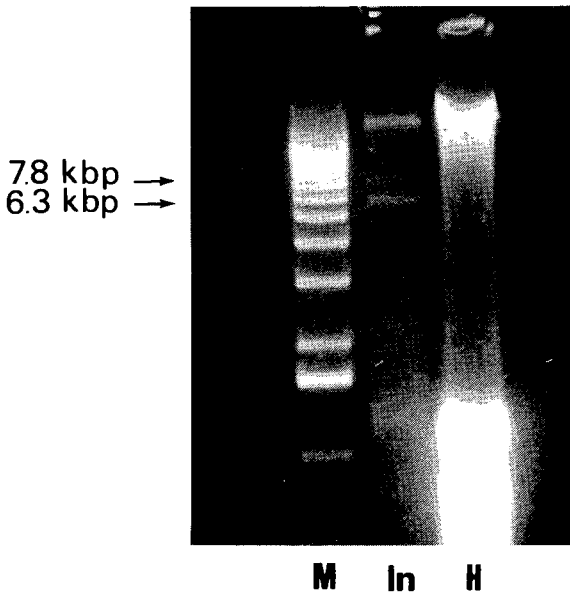


Figure 4. Double-stranded RNA-like material in *N. occidentalis* 37B infected with SMoV, 10 days after inoculation. From left to right: M = molecular markers; In = SMoV-infected *N. occidentalis* 37B; H = healthy *N. occidentalis* 37B. Sizes of dsRNA in kilobase pairs.

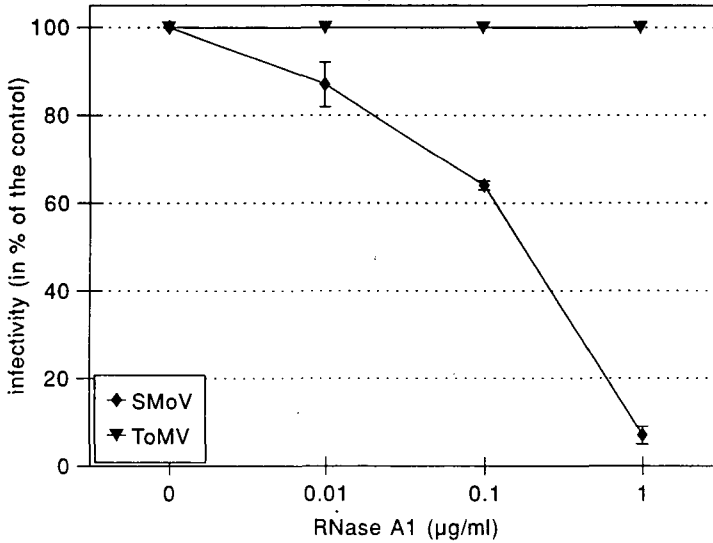


Figure 5. Effect of RNase A₁ on the infectivity of SMoV and ToMV in leaf extracts of *N. occidentalis* 37B (assessed by back-inoculations on *N. hesperis* 67A for SMoV and on *N. glutinosa* for ToMV). Leaf extracts in 30 mM phosphate buffer, pH 8.1, were incubated with the indicated concentration of RNase A₁ for 1 h at 20°C, precipitated with 6% PEG 6000 (w/v) and the pellets obtained after low-speed centrifugation were resuspended in phosphate buffer and inoculated.

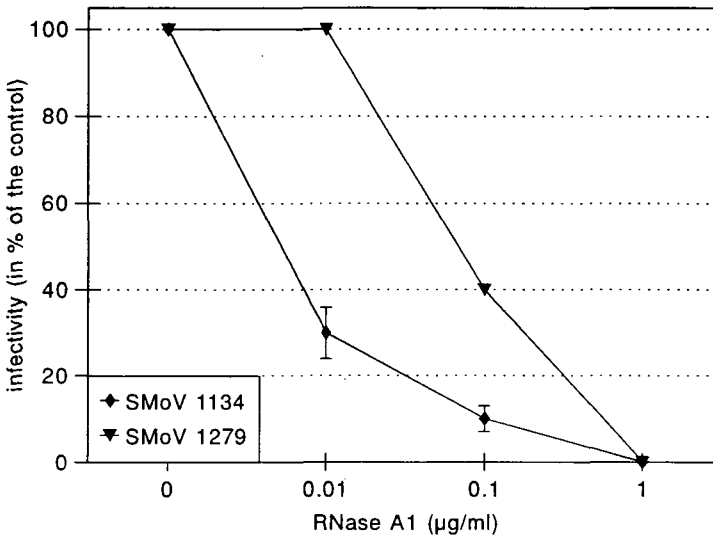


Figure 6. Effect of RNase A₁ on the SMoV-associated infectivity (isolate 1134 and 1279) in leaf extracts of *C. quinoa* (assessed by back-inoculations on *C. quinoa*). The leaf extracts were treated as indicated in Figure 5.

Table 1. Effect of organic solvents or phenol on the SMoV-associated infectivity in leaf extracts of *N. occidentalis* 37B and *C. quinoa*.

Treatment of extracts from:	SMoV-associated infectivity in:	
	<i>N. occidentalis</i> 37B	<i>C. quinoa</i> ^a
butanol, 8% ^c	0 ^b	0
chloroform ^c	0	0
butanol/chloroform, 1:1 ^c	3	0
phenol ^d	212	100

^a Valid both for SMoV isolate 1134 and 1279.

^b Values expressed in % of the non-treated extracts (100%), made in 30 mM phosphate buffer, pH 8.1.

^c The extracts were centrifuged at low speed and aliquots were treated with or without organic solvents as indicated for 1 h with stirring at 4°C. After low-speed centrifugation, the aqueous phases or the supernatants were centrifuged for 2 h at 50000 rpm in a Beckmann Ti70.1 rotor. Each pellet was resuspended in phosphate buffer and inoculated.

^d The extract was prepared as for the extraction of dsRNA and after the extraction with phenol, nucleic acids were precipitated by adding 1/10 vol. of sodium acetate, pH 5.6, and 2.5 vol. of 100% ethanol, centrifuged at low speed and the resulting pellet was dissolved in phosphate buffer and inoculated.

Table 2. The effect of RNase A₁ on the phenol-extracted infectivity of SMoV from leaf extracts of *N. occidentalis* 37B.

Treatment	SMoV-associated infectivity ^a
RNase ^b , 0.0 M NaCl	0
RNase ^b , 0.7 M NaCl	0
no RNase, 37°C	23
no RNase, 0°C	67

^a Values expressed in % of the non-treated phenol-extract (100%), made as described in Table 1.

^b Final concentration: 5 µg/ml.