Characterization of Sweet Clover Necrotic Mosaic Virus

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ABSTRACT

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A previously undescribed virus was isolated from sweet clover (*Melilotus officinalis*) plants showing systemic mosaic associated with ringspots and veinal necrosis. It was readily sap transmissible to 16 of 25 species tested including both leguminous and nonleguminous plants. Sap or purified preparations contained polyhedral particles 35 nm in diameter that sedimented at a velocity of 123 S as a single component. The virus particles contained a bipartite genome with RNA molecular weights (M_r) of 1.35 $\times 10^6$ and 0.55×10^6 daltons and a single species of coat protein with an M_r of 38,000 daltons. Amorphous inclusions were observed in the cytoplasm of infected epidermal cells. The virus resembled red clover necrotic mosaic virus and clover primary leaf necrosis in particle morphology. However, it was clearly distinguished by host range, molecular weights of RNA and protein, and serological properties. The name sweet clover necrotic mosaic virus is proposed for the virus.

Additional key words: dianthovirus, divided genome virus, legume virus.

In Alberta, sweet clover is a fast-growing legume that is valuable for soil improvement, silage production, and nutritious pasturage. It is useful also as a source of nectar and pollen for the honey industry. Sweet clover, a biennial crop, is used in short rotations and is particularly well-adapted to the drier areas of western Canada where it is fairly free from serious disease problems (7).

In 1979, during a field survey in the Athabasca area, Alberta, Canada, a polyhedral virus was isolated from a yellow-blossomed sweet clover plant (*Melilotus officinalis* (L.) Lam.) showing ringspot and systemic veinal necrosis associated with mosaic. The occurrence and some properties of the virus were reported (9). This paper reports the characterization of the virus.

MATERIALS AND METHODS

Virus source. The virus was originally isolated from sweet clover subjected to several single lesion transfers in *Phaseolus vulgaris* L. 'Red Kidney' and further multiplied in the same host. Infected leaves were harvested 5 days after inoculation and kept frozen at −60 C until required.

Other viruses were obtained from the following sources: red clover necrotic mosaic virus (RCNMV-SW) from B. Gerhardson, Swedish University of Agricultural Sciences, Uppsala, Sweden (5,6); RCNMV-E from R. T. Plumb, Rothamsted Experimental Station, Harpenden, England (2); clover primary leaf necrosis virus (CPLNV) from H. W. J. Ragetli, Research Station, Agriculture Canada, Vancouver (20); carnation ringspot virus (CRSV), tobacco and tomato ringspot viruses (TRSV, TomRSV) from R. Stace-Smith, Research Station, Agriculture Canada, Vancouver. The viruses were multiplied in Red Kidney bean for further use in serological studies.

Growth conditions. All plants were grown in 12-cm-diameter clay pots containing an autoclaved mixture of loam, sand, and peat (3:2:1, v/v). Unless otherwise stated, the plants were grown in a glasshouse at 25 ± 2 C.

Host range. Crude sap of infected bean leaves at a 1/10 dilution served as inoculum in host range tests. The plants tested included 25 species representing 19 genera and eight families. At least 12 plants of each species were used and the test was repeated at least three times with uninoculated controls. Both inoculated and uninoculated leaves were used for virus recovery tests on *Chenopodium amaranticolor* Coste & Reyn and/or by enzyme-linked immunosorbent assay (ELISA).

Light microscopy. Lower epidermal strips of *Vigna unguiculata* (L.) Walp. 'Early Ramshorn' primary leaves from healthy plants and infected plants 5 days after inoculation were immersed in 1% Phloxine B solution prepared in cellosolve (3). After 15 min, the strips were washed and mounted in tap water and examined with a light microscope.

Purification. Infected bean leaf tissue (stored frozen at −60 C) was homogenized in a Waring Blendor in cold 0.1 M phosphate buffer, pH 7.0, (1:2, w/v) containing 0.5% ascorbic acid. The homogenate was clarified by chloroform-butanol (1:1) and the virus was precipitated by polyethylene glycol (PEG) 6,000 and sodium chloride at 8% and 0.4% (w/v), respectively, followed by two cycles of differential centrifugation (high speed centrifugation at 108,000 *g* for 2 hr in a Beckman model L5-75 ultracentrifuge and low speed centrifugation at 9,000 *g* for 10 min in a Sorval model RC-2B centrifuge). Other viruses (RCNMV-E, CPLNV, TRSV, and TomRSV) were also purified by the above method. For further purification, linear 10–40% sucrose gradients were prepared in 0.025 M phosphate buffer, pH 7.0, with an ISCO model 570 gradient former. The virus was layered on each gradient, centrifuged at 64,000 *g* for 90 min in a Spinco 25.1 swinging bucket rotor, and fractionated with an ISCO model D density gradient fractionator. UV spectral analysis was performed with a Beckman DU-8 spectrophotometer.

Electron microscopy. Virus samples supported by carbon-coated (49 μm, 500-mesh) grids were examined with a Philips 200 transmission electron microscope (60 and 80 kV) after staining with 2% uranyl acetate or 2% potassium phosphotungstate, pH 7.0, containing 0.1% albumin. Tobacco mosaic virus (TMV) was included as an internal standard.

For ultrastructural study of infected cells, inoculated primary leaves of cultivar Red Kidney of *P. vulgaris* were sampled 1 wk after inoculation. Comparable healthy leaves served as a control. The sample tissues, cut in 2-mm squares, were fixed in an equivolume mixture of 3% formalin and 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at 4 C for 7 hr. The fixed materials were then washed and postfixed in 2% osmium tetroxide for 5 hr, dehydrated through a graded ethanol series, taken to propylene oxide, and embedded in Araldite. Sections were cut on a Reichert ultramicrotome using a diamond knife and stained with 2% aqueous uranyl acetate for 2 hr

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followed by lead citrate for 2–5 min.

Analytical ultracentrifugation. The sedimentation coefficient of the purified preparation ($S_{20,w}$) in 0.025 M phosphate buffer, pH 7.0, was determined with a Beckman model E ultracentrifuge equipped with schlieren optics. The values were corrected to infinite dilution.

Serology. Antiserum was produced to SCNMV and RCNMV-SW in 6-mo-old San Juan rabbits by administering three intramuscular injections in Freund's complete adjuvant, followed by two intravenous injections of 1 ml doses at 2 mg/ml and the rabbits were bled 1 wk after the final injection. The antiserum titre was 1/512 to 1/1,024 by ring interface test. ELISA was performed according to the previously described method (4). Ring interface and agar double diffusion tests were performed according to standard methods (1).

RNA extraction. Purified virus was mixed with an equal volume of extraction medium consisting of 0.05 M tris-HCl, pH 7.8, 0.005 M ethylenediaminetetraacetate, 0.05 M NaCl, 0.8% sodium dodecyl sulfate, and 0.1% bentonite. Water-saturated phenol was added to the mixture and shaken vigorously for 15 min at room temperature. The aqueous-phase was subsequently treated once with phenol and three times with ether. Ethanol-precipitated RNA

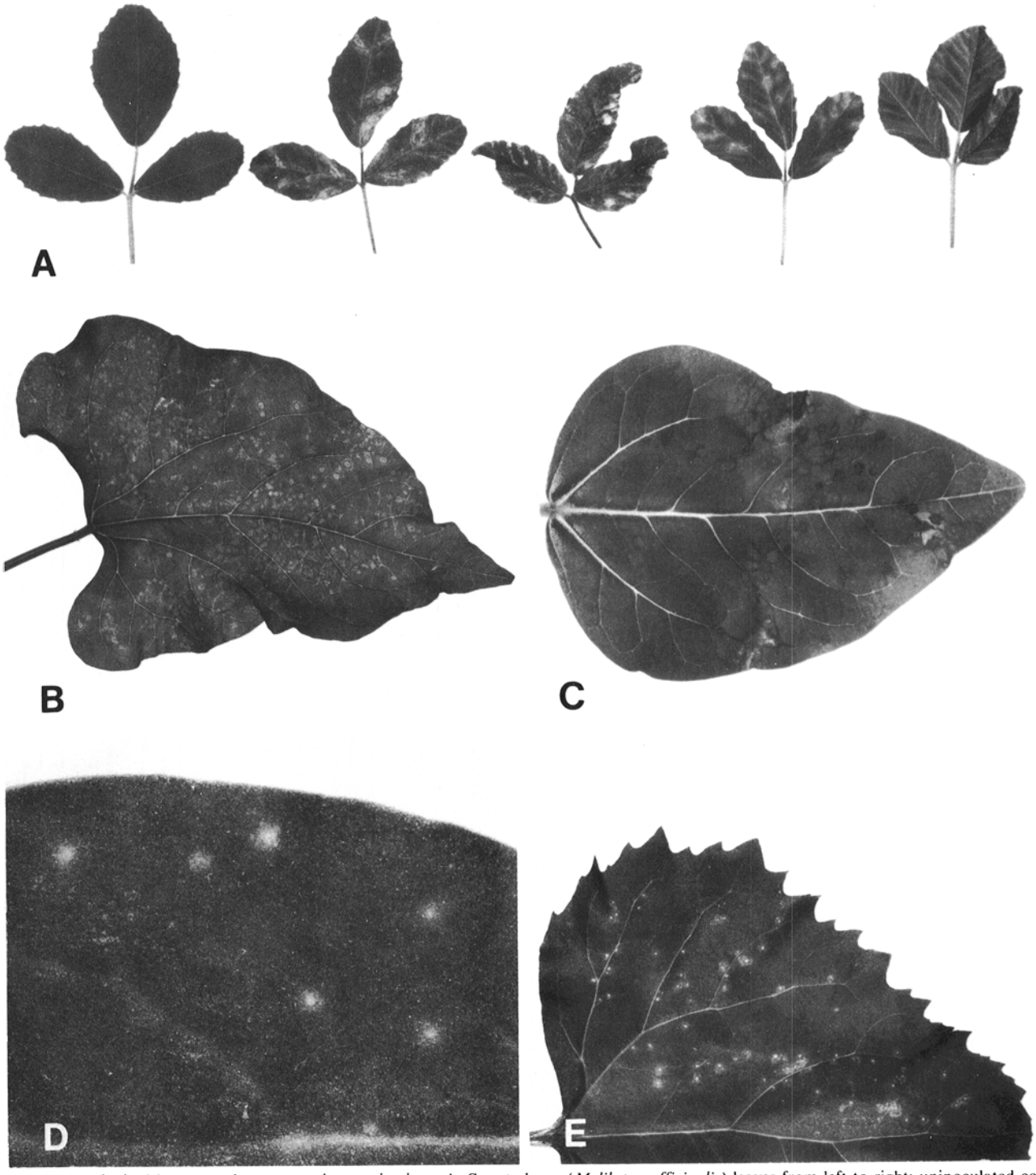

Fig. 1. Symptoms incited by sweet clover necrotic mosaic virus. **A,** Sweet clover (*Melilotus officinalis*) leaves from left to right; uninoculated control, inoculated leaf with ringspot and veinal necrosis symptoms, three systemically infected leaves showing veinclearing, mosaic, and veinal necrosis. Local necrotic lesions on **B,** *Phaseolus vulgaris* 'Red Kidney'; **C,** *Vigna unguiculata* 'Early Ramshorn'; **D,** *Cucumis sativus* 'National Pickling'; and **E,** *Chenopodium amaranticolor*.

was suspended in water containing 18% glycerol.

Polyacrylamide gel electrophoresis. Virus RNA was analyzed on a 2.25% polyacrylamide slab gel by using tris-borate buffer system (19). Electrophoresis was carried out at 30 V per 12 cm gel for 2.5 hr at room temperature. Virus coat protein was analyzed on 10% polyacrylamide slab gels containing 0.1% SDS as described (17). Molecular weight (M_r) markers used for RNA were brome mosaic virus RNA 1 (1.09×10^6 daltons), RNA 2 (0.99×10^6 daltons), RNA 3 (0.7×10^6 daltons), and RNA 4 (0.28×10^6 daltons) as described (14) and TMV RNA (2.0×10^6 daltons). M_r markers for protein were: bovine plasma albumin (66,000 daltons), ovalbumin (45,000 daltons), porcine stomach mucosa pepsin (34,700 daltons), bovine pancreas trypsinogen (24,000 daltons), BMV capsid protein (20,000 daltons), and TMV capsid protein (17,500 daltons).

RESULTS

Symptoms on sweet clover. Sweet clover (*Melilotus alba* Desr. 'Denta' and *M. officinalis* 'Arctic') plants mechanically inoculated with SCNMV displayed chlorotic ringspot symptoms on inoculated leaves 5 days after inoculation. These were followed by systemic mottle, severe mosaic, leaf malformation, and veinal necrosis (Fig. 1A). A pronounced necrosis developed more frequently during winter than in summer months.

Host range. SCNMV infected 16 of 25 species tested which included both legumes and nonleguminous plant species (Table 1). Ringspot lesions were produced in the primary leaves of *P. vulgaris* 'Red Kidney' (Fig. 1B) and *V. unguiculata* 'Early Ramshorn' followed by systemic mosaic and veinal necrosis (Fig. 1C). *Cucumis sativus* reacted with local necrotic spots only (Fig. 1D). The virus incited distinct small local lesions in *C. amaranticolor* (Fig. 1E).

Light microscopy. Amorphous inclusions containing darkly stained granular material were observed in the cytoplasm of infected cowpea epidermal cells. In examinations of several epidermal strips, an average of 67% of cells contained these inclusions (Fig. 2A).

Purification. Purification with polyethylene glycol gave colorless virus preparations with an average yield of 0.3 mg/g of infected tissue. Purified virus had an absorption spectrum typical of nucleoprotein with a minimum absorbance at 240 nm and a maximum at 259 nm with a A_{260}/A_{280} ratio of 1.80.

Electron microscopy. Large amorphous aggregates consisting of spherical virus particles were observed in the infected cells (Fig. 2B). These were similar to the amorphous bodies observed in the light microscope. The tissue from uninoculated plants did not contain these aggregates. In the observation of purified virus preparations, 60% of 510 particles negatively stained with potassium phosphotungstate had an average diameter of 33 nm when TMV was used as an internal standard. A few of the particles were internally stained (Fig. 3A). Particles stained with uranyl acetate appeared as regular polyhedrons with angular outlines measuring 35 nm (Fig. 3B).

Analytical ultracentrifugation. The virus particles sedimented as a single component with a sedimentation coefficient ($S_{20,w}$) of 126 S. RCNMV-SW cocentrifuged as a control with an $S_{20,w}$ of 123 S.

Serology. Ring interface tests indicated cross reaction titers of

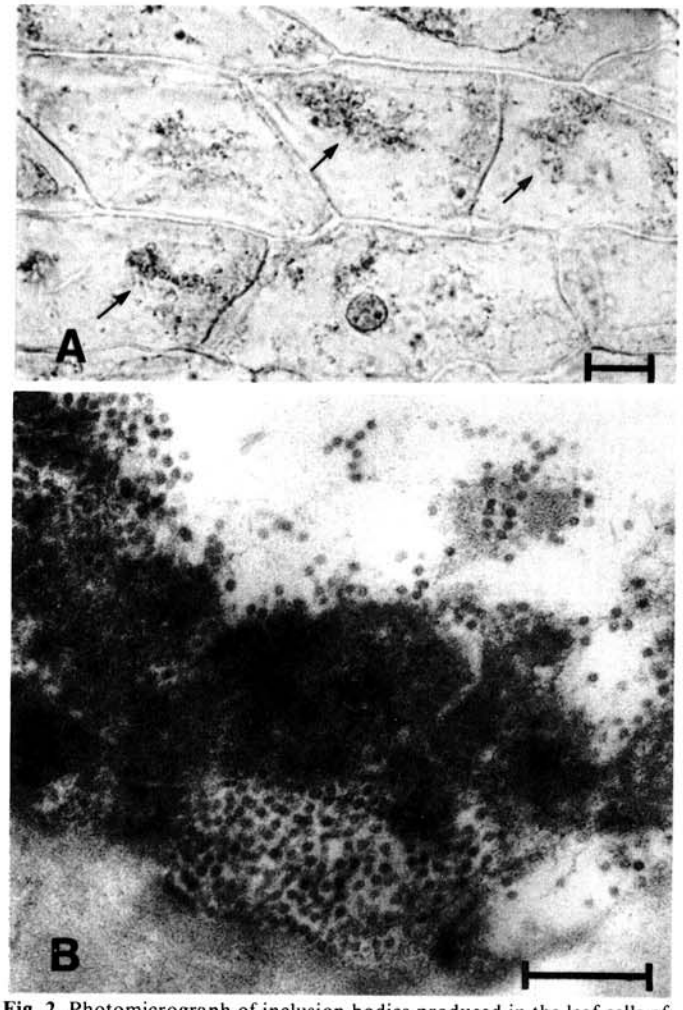

Fig. 2. Photomicrograph of inclusion bodies produced in the leaf cells of primary leaves. **A,** *Vigna unguiculata* 'Early Ramshorn' 5 days after inoculation with sweet clover necrotic mosaic virus (SCNMV). Arrows indicate darkly stained amorphous inclusion bodies. Bar represents 10 μm. **B,** *Phaseolus vulgaris* 'Red Kidney' 7 days after inoculation with SCNMV. Bar represents 200 nm.

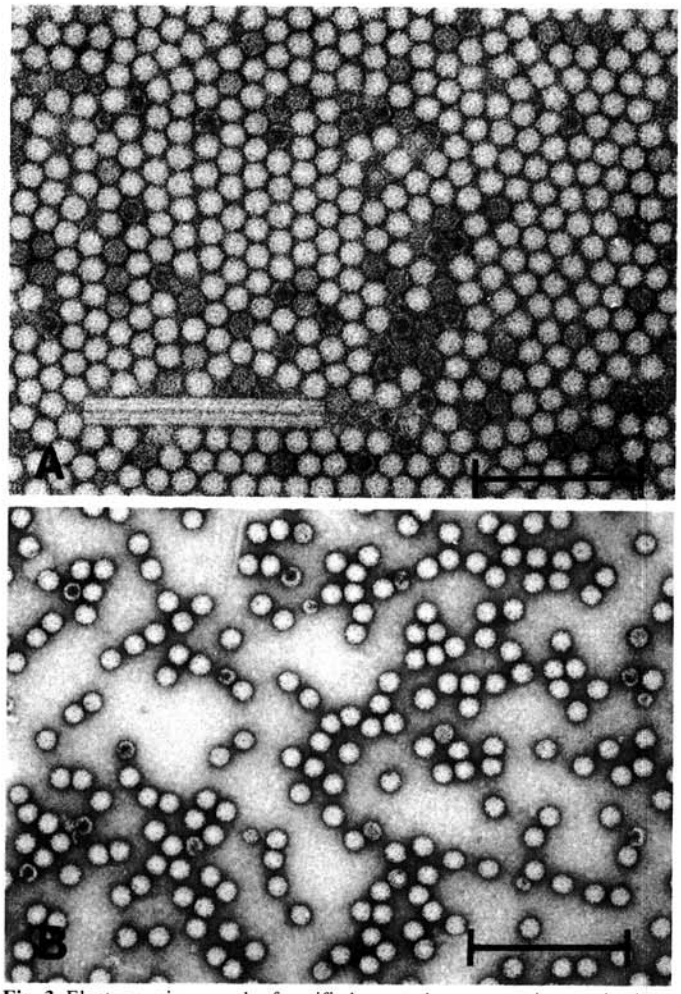

Fig. 3. Electron micrograph of purified sweet clover necrotic mosaic virus particles; **A,** stained with 2% potassium phosphotungstate. Note the stain penetration of some particles. **B,** Stained with 2% uranyl acetate. TMV was used as an internal standard. Bars represent 200 nm.

1/8 and in agar gel double diffusion tests precipitation lines developed up to a 1/16 dilution of SCNMV antiserum in the presence of RCNMV-SW, RCNMV-E and CPLNV (Table 2). CRSV did not react with SCNMV antiserum in similar tests. In ELISA, 1 μg of coating gamma globulin per milliliter and 1/3,000 dilution of the conjugate were found optimal in routine testing for SCNMV. As a positive control 1 μg/ml of virus was used in testing field samples and indicator plants. In reciprocal ELISA to determine the relationship between SCNMV and RCNMV-SW, gamma globulin and virus concentration up to 0.4 mg/ml were used. While homologous tests gave positive reactions as judged visually and by $A_{405\ nm}$ values, heterologous combinations were negative. Purified preparations of RCNMV-E, CPLNV, CRSV, TRSV, and TomRSV also gave negative results when tested by ELISA against SCNMV antibody.

Molecular weights of RNA and capsid protein. SCNMV, RCNMV-SW, and CPLNV all contained two pieces of RNA. Their molecular weights were slightly different: SCNMV-RNA 1, 1.35×10^6 daltons. SCNMV-RNA 2, 0.55×10^6 daltons; RCNMV-RNA 1, 1.45×10^6 daltons, RCNMV-RNA 2, 0.59×10^6 daltons; CPLNV-RNA 1, 1.50×10^6 daltons; and CPLNV-RNA 2, 0.60×10^6 daltons. Capsid proteins from the three viruses had the following M_r values: SCNMV protein, 38,000 daltons; RCNMV protein, 37,500 daltons; and CPLNV protein, 38,500 daltons.

DISCUSSION

Although SCNMV naturally occurs in both *M. alba* and *M. officinalis* and is widespread in the northwestern and central regions of Alberta, it has not been isolated from field-grown red clover, white clover, alsike clover, or vetch, which are commonly grown as forage crops in the area. The virus, however, appears to be particularly well-adapted to sweet clover. The virus was isolated only once in 161 tests from field-grown alfalfa that was mildly stunted. Cultivar Hungaropoly red clover, which was reported to be highly susceptible to RCNMV and produced severe systemic symptoms (2), was only locally susceptible to SCNMV and produced no symptoms (Table 1). Alfalfa, white clover, and alsike clover were resistant to SCNMV in similar tests (Table 1).

SCNMV resembles CRSV (12), RCNMV (8,10), and CPLNV (20) in particle morphology and sedimentation behavior, and is genetically related to them as determined by pseudorecombination tests (18). Thus, it clearly qualifies as a member of the newly described dianthovirus group (16). Moreover, like other dianthoviruses, it has a relatively wide host range, and can cause necrotic ringspots on many leguminous species. In spite of such similarities, SCNMV is serologically clearly distinguishable from other members of the dianthovirus group as it is not serologically related to CRSV and only distantly related to RCNMV (Table 2). CPLNV was previously reported as a new virus occurring on the Canadian Pacific coast (20) and now appears to be a strain of RCNMV (11). It also possesses only a remote serological relationship to SCNMV (Table 2). As reported in this paper and elsewhere (18), SCNMV has small (but significant) differences in M_r of RNA components and coat protein from those of CPLNV and RCNMV. SCNMV is also easily distinguishable from CPLNV and RCNMV by differences in host range and severe symptom expression on certain plant species (Table 1). For example, both *C. amaranticolor* and *C. quinoa* are highly susceptible to SCNMV (Table 1 and Fig. 1E). In contrast, these species were reportedly resistant to CPLNV (20). Likewise, *Nicotiana clevelandii* and *N. glutinosa*, both resistant to CPLNV (20), were systemically susceptible and produced mosaic symptoms after mechanical inoculation with SCNMV (Table 1). While SCNMV incited mosaic symptoms on *Tetragonia expansa* following the local lesions on inoculated leaves (Table 1), none of the RCNMV strains tested (except TpM-34 originally from Czechoslovakia) infected this species systemically (10). Although *Petunia hybrida* was resistant to SCNMV, RCNMV-SW infected it symptomlessly (5).

Different biological vectors have been reported or implicated in natural transmission of dianthoviruses. For example, CRSV is transmitted by certain nematodes (13) and RCNMV by an *Olpidium* sp. (15). Other workers observed the close association of an *Olpidium* sp. with soil transmission of RCNMV, but provided no direct evidence that the fungus is specifically the vector of the virus (2,6). Insect transmission tests of RCNMV with six species of weevils and two species of aphids were negative (2). Further studies of the apparently restricted natural distribution of SCNMV may reveal some interesting new knowledge about virus transmission by vectors. Further work is needed to provide information on the mode of transmission of SCNMV in the field. The name sweet clover necrotic mosaic virus is proposed for the virus described in this paper.

TABLE 1. Host range and symptomatology of sweet clover necrotic mosaic virus

Family	Species	Symptoms[a] Local	Systemic
Aizoaceae	*Tetragonia expansa*	N	M
Amaranthaceae	*Gomphrena globosa*	N	–
Chenopodiaceae	*Chenopodium amaranticolor*	N	–
	C. quinoa 'Selection C'	N	–
	Beta vulgaris	–	–
Compositae	*Zinnia elegans* 'Red Buttons'	–	–
Cucurbitaceae	*Cucumis sativus* 'National Pickling'	N	–
Leguminoceae	*Melilotus alba* 'Denta'	R	M, Vnc
	M. officinalis 'Arctic'	R	M, Vnc
	Phaseolus vulgaris 'Red Kidney'	R	M, Vnc
	Lathyrus adoratus	–	–
	Medicago sativa	–	–
	Pisum sativum 'Alaska'	R	–
	Trifolium hybridum 'Hungaropoly'	–	–
	T. pratense	–*	–
	T. repens	–	–
	Vicia faba 'Broad Windsor'	R	–
	Vigna unguiculata 'Early Ramshorn'	R	Vnc
Solanaceae	*Datura stramonium*	–	–
	Lycopersicon esculentum 'Earliana'	–	–
	Nicotiana clevelandii	N	M
	N. glutinosa	–*	M
	N. tabacum 'Bright Yellow'	–*	–
	N. tabacum 'Havana 425'	N	–
	Petunia hybrida 'Fire Chief'	–	–
Umbelliferae	*Petroselinum crispum*	–	–

[a] Coded symptom descriptions: M, mosaic; N, necrotic lesions; R, necrotic ringspot; Vnc, veinal necrosis; –, no symptoms and virus was not recovered; –*, no symptoms but virus was recovered by infectivity assay and/or by ELISA.

TABLE 2. Serological relationship between sweet clover necrotic mosaic virus (SCNMV) and other dianthoviruses and non-dianthoviruses

Virus	Antiserum	Ring interface test	Agar gel diffusion test	ELISA
SCNMV	SCNMV	1/1,024	1/512	+[a]
SCNMV	RCNMV-SW[c]	1/8	1/16	–[b]
RCNMV-SW	RCNMV-SW	1/512	1/256	+[a]
RCNMV-SW	SCNMV	1/8	1/16	–
RCNMV-E[d]	SCNMV	1/8	1/16	–
CPLNV[e]	SCNMV	1/8	1/16	–
CRSV[f]	SCNMV	–[b]	–	–
TRSV[g]	SCNMV	–	–	–
TomRSV[h]	SCNMV	–	–	–

[a] The reaction was positive up to 10 ng/ml of virus.
[b] Indicates negative results.
[c] Red clover necrotic mosaic virus, Swedish isolate.
[d] Red clover necrotic mosaic virus, English isolate.
[e] Clover primary leaf necrosis virus.
[f] Carnation ringspot virus.
[g] Tobacco ringspot virus.
[h] Tomato ringspot virus.

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