Characteristics of an isolate of white clover mosaic virus prevalent in Eastern Ontario[1]

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A virus found frequently in red clover plants in Ottawa area was identified as white clover mosaic (WCMV) by determining its host range, some physicochemical properties and serological reaction against WCMV-antiserum obtained from two different sources. Electron microscopic examination of leaf-dip and purified virus preparations showed flexuous filamentous particles with a modal length of 500 nm. Sedimentation coefficient ($S_{20,w}$) of the virus was 117 S, buoyant density 1.298 g/cm^3, extinction coefficient 3.1, and A260/A280 ratio 1.24. An antiserum prepared against the Ottawa isolate of WCMV reacted specifically with the virus in purified preparations as well as in clarified extracts of infected clover plants in microprecipitin, precipitin-ring and agar gel diffusion tests.

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Un virus souvent present dans les plants de trèfle rouge de la region d'Ottawa a ete identifie comme etant le virus de la mosa'ique du trefle blanc. Ceci a ete établi en determinant sa specificite d'hdte, quelques proprietees physiochimiques et sa reaction serologique envers deux antiserums obtenus a partir de sources differentes. L'examen au microscope electronique de preparations de feuilles trempées et de virus purifié a montré des particules filamenteuses flexueuses d'une longueur modale de 500 nm. Le coefficient de sedimentation ($S_{20,w}$) du virus est de 117 S, sa densité de sedimentation 1.298 g/cm^3, son coefficient de disparition 3.1, et le rapport A260/A280 1.24. Un antiserum, prepare contre l'isolat viral en provenance d'Ottawa, a reagi specifiquement avec le virus present dans des preparations purifiees de même que dans des extraits clarifies de plants de trefle infectes soumis à des tests serologiques de microprecipitation, de precipitation en anneau et de diffusion sur agar.

Introduction

Red clover (*Trifolium pratense* L.) is an important forage crop in Canada and its seed production averages about 4.5 million kg/year (6). White clover mosaic virus (WCMV), which occurs naturally in red clover, is widely distributed. Pratt (7) surveyed the clover fields in eastern Canada and reported that pea streak virus (PSV) was most prevalent followed by red clover vein mosaic (RCVMV), bean yellow mosaic (BYMV) and pea mosaic (PMV) viruses. Although WCMV was found in several provinces of eastern Canada, its incidence was not estimated. Also, the Canadian isolates of WCMV have not yet been fully characterized.

This paper reports host range, purification, serology and some physicochemical properties of WCMV isolated from red clover. Incidence of some filamentous legume viruses, including WCMV in red clover also was determined.

Materials and methods

During the summer of 1979, red clover fields in the Ottawa area were surveyed and 28 plants showing virus-like symptoms were collected. Twenty-two virus isolates from these samples were established in the greenhouse in red clover plants through sap inoculation. Preliminary examination of infected plants by the leaf-dip method showed filamentous virus particles in 20 isolates. Several of these virus isolates produced local lesions on *Gomphrena globosa* L. and one of them, hereafter referred to as Ottawa isolate, was propagated in red clover from a single lesion and was later identified as WCMV - a potexvirus.

Most plant species used were grown from seeds obtained from the U.S. National Seed Storage Laboratory (Fort Collins, Colorado), as recommended by the International Working Group on legume viruses to standardize their host range (4). To deterine the stability of the virus in *vitro,* the inoculum was prepared by grinding infected red clover leaves with an equal volume (w/v) of 0.01 M potassium phosphate buffer, pH 7.0, and passing the crude extract through a double layer of cheese cloth. Plants were inoculated with the extract by rubbing the upper surface of leaves that had been dusted with carborundum (600-mesh). The inoculated plants were kept in a greenhouse at 22-24°C with supplemental light for a 16-h day. Infectivity assays were made by counting local lesions on G. *globosa*. The virus was purified from 8-day old diseased pea plants (*Pisus sativum* L. cv. Lincoln) by a method similar to the one reported for carlaviruses (12). The procedure involves homogenization of frozen tissue in a phosphate-citrate buffer (0.0165 M sodium phosphate and 0.0018 M sodium citrate, pH 8.9), clarification of the extracts with $CaPO_4$, precipitaion of the virus with polyethylene glycol (PEG), differential and sucrose density gradient centrifugation. The only modifications made in this procedure were that the tissue was homogenized in the presence of chloroform (1 ml for each g of tissue) and the PEG-precipitated virus suspension was centrifuged on a 25% sucrose-cushion instead of two sequential centrifugations on 20 and 30% sucrose-cushion. Unless stated otherwise, purified virus pellets were suspended in the phosphate-citrate buffer.

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Sucrose density gradient columns containing the virus were analyzed with an ISCO model 185 fractionator coupled to a UA-2 absorbance monitor and a recorder. The absordance at 254 nm was recorded graphically resolving the ultraviolet absorbing material from any point within the gradient column. Purified virus and leaf-dip preparations were negatively stained with 2% phosphotungstic acid at pH 7.0 and examined in a Philips 300 electron microscope. At least 100 virus particles in leaf-dip preparations were measured to determine their modal length.

To calculate the virus extinction coefficient, the purified virus pellet was suspended in double-distilled water and U.V. absorption spectrum recorded. Weight of the virus was determined by placing 0.4 ml of virus suspension on a microscope cover glass and drying it at 105°C for 1 h. Sedimentation coefficient of the virus was determined in a Beckman Model E Analytical ultracentrifuge using Schlieren optics. The virus in the phosphate-citrate buffer was centrifuged at 28,000 rpm using An-D rotor at 20°C. The graphical method of Markham (5) was used to calculate the sedimentation coefficient. Buoyant density of the virus was determined by the CsC1 isopycnic centrifugation using a step gradient method (2). Southern bean mosaic virus (SBMV) was used as a reference. The visible zone was

Table 1. Host range of WCMV isolate from Ontario

Susceptible hosts		Non-susceptible plants
Test plant	Virus incubation period (days)	Test plant
Leguminoseae		Leguminoseae
Trifolium pratense L. cv. Kenland	13	*Phaseolus vulgaris* L.
T. repens L. cv. New Zealand	13	cvs. Early Blue Henderson
Melilotus alba Desr.	15	Kentucky
Medicago sativa L. cv. DuPuits	13	Pinto
Vicia faba L. cv. Bell Bean	6	
Vigna sinensis (Torner) Savi. cv. Blackeye	4	*Glycine max* (L.) Merr. cvs. Bragg Davis
Pisum sativum L.		Solanaceae
cvs. Alaska	8	
Progress #9	6	*Datura stramonium* L.
Thomas Laxton	6	cv. R. Fulton
Perfected Wales	6	*Petunia hybrida* Hort. Vilm-Andr.
Dark Skin Perfection	6	cv. King Henery
Lincoln	8	*Lycopersicon esculentum* Mill.
Phaseolus vulgaris L.		cv. Marglobe
cvs. Burpee's Brittle	7	Chenopodiaceae
Black Turtle Soup	7	*Chenopodium quinoa*
Bountiful	7	*C. album*
Contender	7	*Spinacea oleracea* L.
Great Northern	7	cv. Bloomsdale Long
Tendergreen	7	
Amaranthaceae		Cucurbitaceae
Gomphrena globosa L.	2	*Cucumis sativus* L. cv. Chicago pickling
Chenopodiaceae		
Chenopodium amaranticolor Coste and Reyn.	3	

removed and the buoyant density calculated from the refractive index of the sample containing the virus (8).

To prepare the antiserum, one ml of freshly purified preparation containing 2-2.5 mg virus was emulsified with an equal volume of Freund's incomplete adjuvant and administered intramuscularyinto the hind leg of a rabbit. Ten such injections were given at weekly intervals. Twelve days er the last injection, one additional injection of the virus suspension was given intravenously and five days later the rabbit was bled, serum separated by low speed centrifugation and stored in small lots at -20°C. Precipitin ring (13), microprecipitin (10) and agar gel double diffusion (9) tests were done as described earlier.

Six red clover fields located within 50 km around Ottawa were surveyed to determine the incidence of viruses. The number of healthy plants and those showing virus-like symptoms in each field were recorded by walking in W-fashion. Also plants showing virus-like symptoms were collected randomly from each field and the extract of each sample was tested against the antisera of WCMV (Ottawa isolate), BYMV, RCVMV, PSV and CYMV by the microprecipitin test. The extracts wre prepared by grinding leaf samples with an equal volume (w/v) of 0.05 M potassium phosphate buffer saline (pH 7.0, 0.85% NaCl) containing 0.5% mercaptoethanol, and the crude extract was passed through a double layer of cheese cloth. An equal volume of chloroform was then added to the filtrate and strirred well using a vortex shaker. The mixture was centrifuged at 12,000 *g* for 10 min and the supernatant used for serological tests.

Results and discussion

Nineteen plant species belonging to five different families were inoculated with the virus to determine its host range (Table 1). Only two plant species *Chenopodium amaranticolor* and *G, globosa,* outside the family Leguminoseae were found susceptible to the virus. Both of them produced local lesions while other susceptible plants developed systemic symptoms. The plants which did not show symptoms were checked for symptomless infection by assaying them on G. *flobosa.* None of these assays showed local lesions on the test plants. Infected red clover plants showed vein clearing at early stage of infection and later mosaic. Some of the plants with disease symptoms are shown in Figs. 1-3. Host range of the virus and symptoms produced are very similar to those reported for WCMV (1) with the exception that *Cucumis sativus* is not susceptible to the Ottawa virus isolate. Also, local lesions on G. *globosa* are produced only by one other WCMV isolate from California (1).

The virus pellet obtained using the purification procedure recommended for carlaviruses (12) contained some host plant material. The modifications incorporated in this method provided purer virus preparationsin a shorter period. Electron micrographs of both purified virus preparations (Fig. 4) and leaf-dip preparations showed flexuous filamentous particles. Some end to end aggregation and a few broken virus particles were observed in the purified preparations. The modal length of the particles calculated from leaf-dip preparationswas 500 nm.

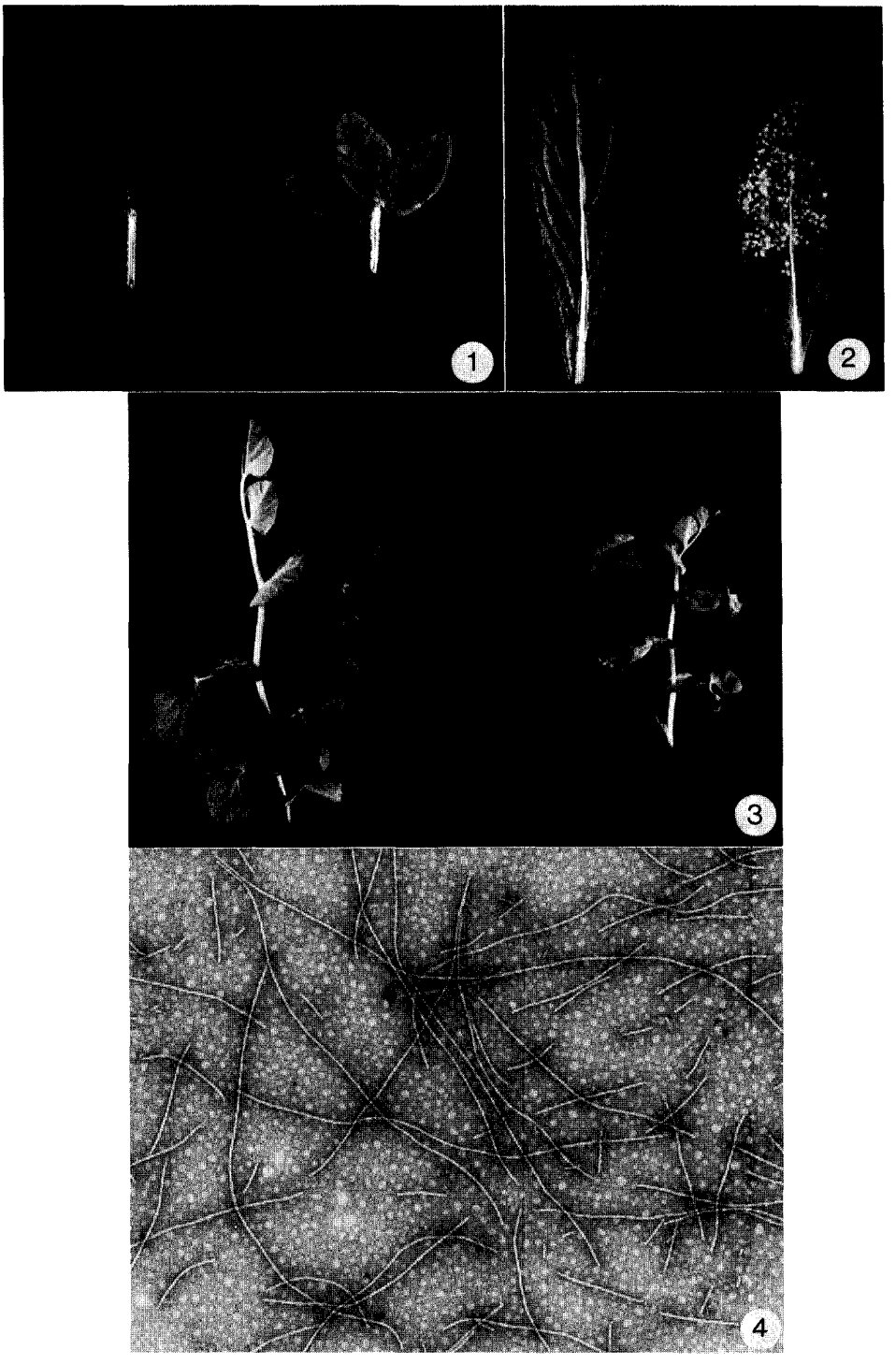
Table 2. Biophysical properties of WCMV

Property	Ottawa isolate	Other isolates
Thermal inactivation point	70–75°C	60°–80°C
Dilution-end point	10^{-5}–10^{-6}	10^{-5}–10^{-6}
Longevity in vitro	14–16 days	10–99 days
Sedimentation coefficient ($S_{20,w}$)	117 S	112–119 S
Extinction coefficient	3.1	3.6
Buoyant density	1.298	not reported
Particle modal length	500 nm	480 nm
A260/A280	1.24	1.20
References	Present study	(1, 3, 11)

Optical density tracings at 254 nm of density gradient column upon fractionation showed a single peak which coincided with the visible virus band and highest infectivity. Analytical centrifugation showed that the virus sedimented as a single component. The sedimentation coefficient ($S_{20,w}$) of the virus, in four suspensions containing 0.2 to 1.9 mg/ml virus, increased with dilution from 105 S to 115 S. The $S_{20,w}$ of the virus extrapolated to infinite dilution was 117 S. Centrifugation of purified virus in CsCl step gradient showed one sharp band. The buoyant density (ρ) of the virus was calculated to be 1.298 g/cm^3 in all three experiments. The ρ of SBMV, centrifuged with the Ottawa virus isolate in the same tube, came to 1,359 g/cm^3 which agrees with the reported value (8). Virus samples removed from he bands obtained after the different centrifugations mentioned above were infectious and the particles retained their normal morphology. The purified Ottawa virus isolate had an absorption spectrum typical of a filamentous virus with a maximum at 259 nm and a minimum at 247 nm. Extinction coefficient of the virus at 259 nm, determined by weighing the virus at different concentrations, was 3.1 ± 0.05.

Some in vitro properties of the virus isolate under investigation along with the values reported for other WCMV isolates are summarized in Table 2. These properties are in general agreement with other WCMV isolates but the extinction coefficient of the Ottawa virus isolate is lower than that reported only for Newzeland isolate of WCMV (3).

The titer of the antiserum, using 1 mg/ml of purified virus, was 1024 and 512 as determined by microprecipitin and precipitin ring tests, respectively. The minimum concentration of virus detectable by both tests was 0.4 mg/ml. Clarified extracts of the infected clover plants also gave a



Figs. 1-3. Symptoms produced by White clover mosaic virus: diseased leaves or plant are shown on the right and the healthy ones on the left. 1) Systemically infected red clover leaf showing mosaic. 2) *Gomphrena globosa* leaf showing local lesions 3 days after inoculation with the virus. 3) Systemically infected pea plant showing curling of leaves, mild necrosis and stunting.

Fig. 4. Electron micrograph showing flexuous filamentous particles from a purified preparation of the virus. X 37,200.

positive reaction in both tests. To obtain a positive reaction in immunodiffusion test, virus particles in the purified preparations as well as in the clarified extracts had to be disrupted by 3 cycles of freezing and thawing. Such virus preparations produced a thick precipitin band midway between the antiserum and antigen wells. To check for nonspecific reactions, concurrently with each experiment antiserum and preimmune serum were tested against clarified extracts of healthy clover plants, and purified virus against the preimmune serum. No positive reactions were observed in any of these tests.

To confirm the identity of the Ottawa isolate. purified virus preparations at 1 mg/ml were tested against undiluted antisera of WCMV (obtained from two sources), BYMV, PSV, RCVMV and clover yellow mosaic virus (CYMV). Positive reactions were observed only with WCMV-antiserum in all three types of serological tests.

Virus-like symptoms were observed on 17% (88/504) of red clover plants during the survey of six fields. The incidence of such plants in different fields varied from 16.6 to 23.3%. The clarified extracts from 346 randomly collected plants showing virus-like symptoms were tested serologically for the presence of certain filamentous viruses. WCMV had the highest incidence followed by BYMV, CYMV, PSV and RCVMV (Table 3). About 60% of the test plants were found to be infected with more than one virus.

Table 3. Incidence of some filamentous viruses infecting red clover in eastern Ontario

Field	No. of plants tested serologically[a]	No. of plant samples reacted positively against the antisera of[b]					No. of samples reacted negatively
		WCMV	BYMV	CYMV	PSV	RCVMV	
1	60	48	18	4	3	3	8
2	46	13	8	3	2	1	26
3	48	25	23	19	16	15	3
4	67	13	8	8	7	6	43
5	70	40	4	3	3	2	22
6	55	16	6	6	5	7	34
Total	346	155	67	43	36	34	136
%		44	19	12	10	9	39

[a]Clarified extracts of plants showing virus-like symptoms were tested by microprecipitin test.

[b]WCMW-white clover mosaic virus, BYMV-bean yellow mosaic virus, CYMV-clover yellow mosaic virus, PSV-pea streak virus, RCVMV-red clover vein mosaic virus. The antiserum against WCMV used was prepared during the present studies and others were obtained from different sources as mentioned in the acknowledgement.

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