Incidence and Geographic Distribution of Sweet Clover Necrotic Mosaic Virus in Alberta

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ABSTRACT

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During a survey period from 1979 to 1983, sweet clover necrotic mosaic virus (SCNMV), a member of the dianthovirus group, was found widespread in the central and northern areas of Alberta where major soil types are black soil and dark gray soil, respectively. In these areas, SCNMV occurred predominantly on sweet clover; the only exception was a SCNMV variant found on alfalfa (*Medicago sativa*). The virus was not detected in alsike clover (*Trifolium hybridum*), red clover (*T. pratense*), white clover (*T. repens*), and crown vetch (*Coronilla varia*) growing in the areas where the virus was prevalent. Root and leaf infection occurred when sweet clover seedlings were dip-inoculated in a virus suspension or inoculated by pouring virus inoculum around the seedlings. The presence of *Olpidium brassicae* zoospores in the inocula caused no significant differences in SCNMV infection rates. Sweet clover weevil (*Sitona cylindricollis*) was found infesting both SCNMV-infected and uninfected sweet clovers (*Melilotus officinalis* and *M. alba*). When virus-free *S. cylindricollis* was fed on SCNMV-infected sweet clover for 5–10 min, the virus was detected in the head, body, and feces. However, weevil transmission experiments to young sweet clover seedlings were unsuccessful.

Sweet clover is an introduced species that was reported growing in North America as early as 1739. Its value as a forage crop has been recognized since 1875. It is a fast-growing legume that is valuable for land reclamation and soil improvement, hay and silage production, and nutritious pasturage. It is also useful as a source of nectar and pollen for honeybees and is important for the production of high-quality honey (5). Sweet clover, a biennial crop in short rotations, is well adapted to the dry conditions of western Canada. It is winter-hardy and productive, especially on fertile, well-drained clay and clay-loam soils. However, it has also adapted successfully to sandy loams and heavy clay loams as well as Gray Luvisol soils. It grows best on neutral or alkaline soils and is one of the best legumes to grow on highly alkaline soils (1).

In 1979, an unidentified virus was discovered infecting yellow-blossomed sweet clover plants (*Melilotus officinalis* Lam.) in the Athabasca area (10) and was later found elsewhere in Alberta infecting *M. alba* Medik. Diseased plants showed ringspot and systemic veinal necrosis associated with mosaic, and virus infection resulted in pronounced reduction in foliage growth. The causal virus was characterized and named sweet clover necrotic mosaic virus (SCNMV) (10). In this paper, the results of investigations on virus distribution in Alberta and on virus transmission are reported.

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MATERIALS AND METHODS

Virus. The virus used as an immunogen 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 or in *Nicotiana clevelandii* Gray.

Test plants. For routine assay of field specimens, about 50–60 samples were tested at a time. *Chenopodium amaranticolor* Coste & Reyn., *Gomphrena globosa* L., and *P. vulgaris* L. 'Red Kidney' were used for virus recovery and identification of the virus.

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

Collection of field samples. We collected the samples at the edge of the field and in the crop stand by walking in a crisscross manner. Samples were also collected from sweet clover plants growing on the road shoulders and banks. Young shoots with a few leaves were cut and placed in a plastic bag. A series sample number was assigned and a record made of the exact location and symptoms observed. The samples were tested within 1–2 days of sampling by sap inoculation as well as by enzyme-linked immunosorbent assay (ELISA). In certain instances, particularly when sweet clover plants infected with SCNMV were growing together with alsike clover (*Trifolium hybridum* L.), red clover, and/or white clover (*T. repens* L.), a block of soil containing the plants was removed with minimum disturbance. The plants were grown in the greenhouse for further observation and testing.

ELISA. Antiserum to SCNMV was produced as reported previously (11). Basic procedures for ELISA were the same as those reported earlier (3). The absorbance values were read at 405 nm on a Titertek Multiskan (Flow Laboratories, Mississauga, Ontario).

Vector transmission. For *Olpidium* transmission, a virus-free culture of *Olpidium brassicae* (Wor.) Dang. established previously (6) was used in this investigation. Methods of maintaining and testing the *Olpidium* culture and test plant seedlings were the same as those described previously (7). Dip- or pour-inoculations with virus suspensions (1 mg/ml), in the presence (1×10^6 zoospores per milliliter) or in the absence of zoospores, were made using 10-day-old sweet clover seedlings. Assays of both inoculated and uninoculated seedlings were performed by ELISA 1, 2, and 3 wk after inoculation, using root and leaf samples. At the same time, root samples were examined microscopically for *Olpidium* infection. For insect transmission, sweet clover weevils (*Sitona cylindricollis* Fåhraeus) were collected in the field. After a fasting period of at least 3 days, they were kept on healthy sweet clover plants. In certain experiments, the weevils were used directly after the fasting period. Assays for SCNMV were performed by ELISA, using extracts of test plants and insects.

RESULTS

Sensitivity of virus assay methods. Before using ELISA for detecting SCNMV in field specimens, we established that under the present experimental conditions, ELISA was capable of detecting 10 ng of SCNMV antigen. In a comparison of results from inoculation tests and from ELISA, in more than 1,000 tests performed, there was complete agreement between them, except for one alfalfa isolate that was initially not detected by ELISA.

Virus distribution. SCNMV infection of sweet clover caused systemic necrosis associated with leaf distortion and mosaic, resulting in severe stunting of the whole plant. The distribution of SCNMV was investigated in six forage legume species grown in Alberta for various periods between 1979 and 1982 by bioassay and ELISA (Table 1). SCNMV

occurred predominantly on sweet clover, except for one SCNMV isolate on alfalfa (*Medicago sativa* L.) from the Barrhead area. The virus was not isolated from four other forage legume species, alsike clover, red clover, white clover, and crown vetch (*Coronilla varia* L.), growing in the areas where SCNMV was prevalent. In several tests in which red clover, white clover, and/or alsike clover plants were growing naturally with sweet clover plants infected with SCNMV, SCNMV was not detected in any legume plants other than sweet clover.

Virus stability. SCNMV is sap-transmissible and extremely stable with a longevity in air-dried leaf tissue up to 10 wk. The virus in sap was infectious to 10^{-6} but not at 10^{-7} when diluted in 0.01 M phosphate buffer (Na_2HPO_4-KH_2PO_4), pH 7.0. Virus infectivity was lost only when the virus in sap was heated to 95 C for 10 min. SCNMV was detected from soil around an experimentally infected plant and from drainage water collected from the pots containing SCNMV-infected plants in the greenhouse.

Virus transmission. Sweet clover seedlings individually grown in the incubator were first microscopically examined for *O. brassicae* infection and were assayed by ELISA for SCNMV infection in the roots and leaves. Although *O. brassicae* was not detected in the root 1 wk after inoculation, virus infection in the root was detected as early as 1 wk after inoculation by dipping or pouring (Table 2). Virus inoculation by pouring inoculum around the seedlings often resulted in infection rates higher than those by dip-inoculating sweet clover seedlings in the virus suspension. There was no significant difference between the inoculum with and without *Olpidium* zoospores. For insect transmission experiments, sweet clover weevils were allowed to acquire SCNMV during a feeding period of 5–10 min on infected sweet clover plants. After individual weevils were dissected, the head and body of each weevil were assayed separately by ELISA. SCNMV was detected in all heads (6/6, number of samples found positive/number of samples tested) and bodies (6/6) immediately after an acquisition feeding period of 5–10 min on infected plants. One hour after the acquisition feeding period, SCNMV was detected in 2/6 heads and 5/6 bodies. Eight hours after the acquisition feeding period, SCNMV was not detected in heads (0/6) or in bodies (0/6). In a separate test, six weevils were allowed to feed on healthy plants for 8 days after completing an acquisition feeding period of 48 hr. No virus transmission was observed. Later, we found that SCNMV was eliminated together with digested food in the form of feces within 2 hr of feeding on infected plants.

DISCUSSION

Alberta has four major soil climatic areas that must be considered in deciding kinds, crop combinations, and seeding ratios of forage crops to be grown in a given area (Fig. 1). Table 3 shows the distribution of SCNMV in relation to recommended forage legumes for each area. During a survey period from 1979 to 1982, SCNMV was found widespread in the central and northern areas of Alberta where major soil types are black soil, which is a Canadian term to describe soils with dark surface horizons of the black zone (20), and dark gray soil, which refers to an intrazonal group of imperfectly to poorly drained forested soils (20). Sweet clover is particularly recommended as an important component of a forage seed mixture for poorly structured dark gray soil. SCNMV is prevalent in this area. Table 1 shows that SCNMV occurred solely on sweet clover, with the exception of one SCNMV isolate on alfalfa. Recently, the alfalfa isolate was found to be a new pathogenic variant of SCNMV that is serologically distinct from the type culture of SCNMV (12). The fact that SCNMV was not isolated from other forage legume crops such as alsike clover, red clover, vetch, and white clover growing in the areas where SCNMV was prevalent suggests that SCNMV is very well adapted to sweet clover under field conditions in Alberta. In previous investigations (11), alfalfa, alsike clover, and white clover were not infected when sap-inoculated and maintained at 25 ± 2 C, whereas both *M. officinalis* and *M. alba* were infected with ease. White clover was also resistant to inoculation with RNA preparations isolated from SCNMV at greenhouse temperatures of 17 as well as 26 C (19).

As demonstrated earlier, SCNMV, a member of the dianthovirus group, contains a bipartite genome, and changes in its genome composition influence not only its symptomatology but also its host range (19). The fact that the alfalfa isolate is serologically distinct from the type culture of SCNMV appears to be an indication that serological diversity and host adaptation of SCNMV can occur in the field. Musil et al (18) recently reported the occurrence in Czechoslovakia of three serotypes of red clover necrotic mosaic virus (RCNMV), another member of the dianthovirus group, without definite geographical boundary in their distribution. Whether SCNMV occurs in many serotypes in Alberta is uncertain.

Although the results of inoculation tests agreed well with those of ELISA, except with the alfalfa isolate, the possibility of serological diversity of SCNMV must be carefully investigated. Recently, 21 monoclonal hybridoma cell lines have been established (9). The use of resulting monoclonal antibodies for precise serological studies will definitely be useful as shown in the comparative studies of dianthoviruses (8,11).

With regard to vector transmission of

Table 1. Rates of occurrence of sweet clover necrotic mosaic virus in forage legumes in Alberta

Plants sampled	1979	1980	1981	1982	Total
Alfalfa	0/89[a]	0/20	1/19	0/33	1/161
Alsike clover	0/19	0/29	0/11	0/2	0/61
Red clover	0/2	0/31	0/31	0/16	0/80
Sweet clover	1/3	9/13	38/210	16/479	64/705
Crown vetch	···	0/1	···	···	0/1
White clover	0/5	···	0/1	···	0/6

[a] No. of samples infected/no. of samples tested.

Table 2. Infection of sweet clover (*Melilotus officinalis*) by sweet clover necrotic mosaic virus (SCNMV) and *Olpidium brassicae* zoospores

		Experiment							
		1		2			3		
				Virus			Virus		
Inoculum	Inoculation method	Virus in root	*Olpidium* in root	In root	In leaf	*Olpidium* in root	In root	In leaf	*Olpidium* in root
SCNMV only	Pour	10/10[a]	0/10[a]	10/10	0/10	0/10	9/10	1/10	0/10
Olpidium only	Pour	0/10	0/10	0/10	1/10	10/10	0/9	0/9	9/9
SCNMV + *Olpidium*	Dip	4/10	0/10	2/10	1/10	10/10	3/10	0/10	10/10
SCNMV + *Olpidium*	Pour	7/10	0/10	9/10	0/10	10/10	10/10	2/10	10/10
Uninoculated	···	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a] No. of seedlings infected by SCNMV or *Olpidium*/no. of seedlings inoculated.

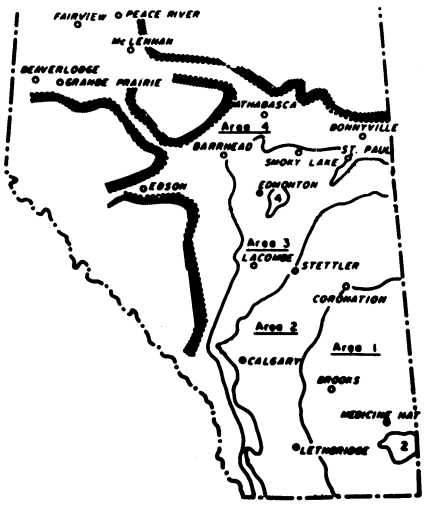

Fig. 1. Map of Alberta showing four soil climatic areas (according to Canadian terminology): 1 = brown soil, 2 = dark brown soil, 3 = black soil, and 4 = dark gray soil; heavy lines indicate areas currently considered nonagricultural land.

Table 3. Distribution of sweet clover necrotic mosaic virus in sweet clover in relation to major soil climatic areas in Alberta

Area[a]	1979	1980	1981	1982	Total	Forage crop[b]
1	...	...	...	0/12[c]	0/12	Alfalfa
2	...	...	...	0/13	0/13	Alfalfa Sweet clover
3	...	...	5/20	3/308	8/328	Alfalfa Sweet clover Bird's-foot trefoil Sainfoin
4	1/3	9/13	33/190	13/146	56/352	Alfalfa Sweet clover[d] Alsike clover Red clover Bird's-foot trefoil

[a] 1 = Brown soil, 2 = dark brown soil, 3 = black soil, and 4 = dark gray soil.
[b] Forage crop species recommended as suitable for the areas by Alberta Agriculture.
[c] No. of samples infected/no. of samples tested.
[d] Sweet clover is particularly recommended as an important component of forage seed mixture for poorly structured dark gray soil.

dianthoviruses, association of *Olpidium* species with RCNMV has been implicated (2,14). However, the results of this study clearly indicated that a tobacco isolate of *O. brassicae* is not involved as a vector of SCNMV (Table 2). Recent studies (4,15,16) indicated that lettuce isolates of *O. brassicae* do not serve as a vector of RCNMV.

Although *S. cylindricollis* is the major pest of sweet clover in Alberta, limited experiments did not yield evidence of SCNMV transmission by this weevil. Earlier studies (2) also concluded that several weevil species such as *S. lineatus* L., *Apion aestivum* Germ., *A. assimile* Kirb., *A. apricans* Hbst., and *A. aethiops* Hbst. did not transmit RCNMV.

Dianthoviruses, including SCNMV, generally reach high concentrations in infected plants and are exceedingly stable in vitro. High virus infectivity was found in soil drainage water not only in this investigation but also with carnation ringspot virus, the type member of the dianthovirus group (17), and it was reported as a source of secondary infection (13). SCNMV may well be spread also by machinery operation in the field because the longevity of the virus in vitro is extremely long.

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