Severe Isolate of Alfalfa Mosaic Virus and Its Impact on Alfalfa Cultivars Grown in Alberta

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ABSTRACT

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Five isolates of alfalfa mosaic virus (AMV) occurring in Canada were classified as one mild, three moderate, and one severe type by host range and symptomatology on seven species, but they were indistinguishable by serology and morphology. Four alfalfa cultivars commonly grown in Alberta, Canada, along with a breeding line were tested in the greenhouse and growth chamber for their responses to inoculation with the severe isolate, A-515, both in the seedling stage and during repeated cuttings and regrowth. Their responses to sap inoculation in the seedling stage were not necessarily reflected in their capabilities to support AMV spread to previously noninfected plants by repeated cuttings. In cultivar Apica, which was the most resistant cultivar in the seedling stage, repeated cuttings progressively increased the number of infected plants. After the ninth cutting, only 7% of these plants remained noninfected. Cultivar Anchor was four times as susceptible to sap inoculation as Apica in the seedling stage; however, 33% of the inoculated Anchor plants remained virus-free after receiving the same treatment. The cultivars also differed in their ability to sustain AMV multiplication during regrowth as measured by enzyme-linked immunosorbent assay.

Alfalfa is the most important forage legume in North America, and among the several viruses affecting this crop, alfalfa mosaic virus (AMV) is probably the most common. AMV is seedborne, and primary infections in the field usually result from infected seed (10,11,14,22). Subsequent transmission is by several vector aphid species (7). Effects of AMV infection on alfalfa are variable, ranging from masked infection to mild mottle to bright yellow foliar mosaic (7,9,13). Development of visible symptoms is often most frequent during the early stages of growth in the spring and during regrowth under cool, cloudy weather conditions. Apparent recovery or masking is common during shoot maturation in the summer.

Theoretically, alfalfa can be infected mechanically with AMV by repeated cuttings during various stages of its growth. In the field, it is usually subjected to frequent cutting and regrowth cycles. It is important to know how such treatment may influence susceptibility to virus infection, secondary spread of the virus to previously noninfected plants within the crop, and survival of AMV-infected alfalfa. These aspects have not been studied previously. AMV infection may reduce fresh weight and/or dry weight in plants grown from seed (1,23) or from clonal cuttings (6,8,10,15,20). However, yield comparisons done in the past for plants grown from seed have often been made between plants considered "healthy" when not artificially inoculated and "infected" when sap-inoculated with AMV (23). Such comparisons may not have been appropriate because a proportion of "healthy" plants may have been asymptomatically infected by seedborne AMV or may have become infected by aphid transmission or by mechanical inoculation during cutting without necessarily developing visible symptoms. Although the frequency of AMV infection of alfalfa by sap-inoculation is variable, the proportion of infected plants after inoculation has not always been determined.

The purposes of this investigation were to characterize certain AMV isolates occurring in Canada and to determine the responses of alfalfa cultivars commonly grown in Alberta to AMV infection during repeated cuttings under growth chamber and greenhouse conditions where the presence of AMV was monitored periodically by enzyme-linked immunosorbent assay (ELISA) for each control and inoculated plant.

MATERIALS AND METHODS

Plant material. Seeds of the various alfalfa cultivars were obtained from the following sources: Anchor, Apica, Blazer, Vista, and breeding line WL-221 from the Research Station, Agriculture Canada, Lethbridge, Alberta; Anchor, Angus, Saranac, and Valor from the Lakehead Forage Association; Angus, Ceres, and Rambler from the Pembina Forage Association; and breeding line Br-III-80, a selection from the cultivar Beaver, from P. D. Walton, University of Alberta.

The test plants were grown in a sterilized soil mixture (loam, peat, sand; 3:2:1, v/v/v) in clay or plastic pots in the greenhouse at average temperatures of 22–25 C or in growth chambers with controlled light (12 hr/day at 11,000 lux) and temperature (26 C). During winter, supplementary illumination with cool-

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beam fluorescent light and incandescent light at an intensity of 28,000 lux at bench level was provided to maintain a 16-hr daylight period in the greenhouse. Alfalfa seedlings started from seed were tested for AMV by ELISA, and only AMV-free seedlings were used for inoculation experiments.

Alfalfa seedling inoculation. Eight cultivars and a breeding line (WL-221) were selected on the basis of low incidence of AMV in the seed from seed tests by ELISA. For seedling tests, seeds were germinated in sterilized sand-water cultures and subsequently grown 4 wk in half-strength Hoagland's nutrient solution (17). The seedlings were then transplanted into the soil mixture for further culture. During a period of 3–6 wk after germination, they were tested three times in the one- to three-, six-, and eight-leaf stages for AMV by ELISA.

Resistance tests of alfalfa cultivars by repeated cutting after AMV inoculation. Seedlings of four alfalfa cultivars and a breeding line (Br-III-80) were sap-inoculated with a severe isolate of AMV (A-515) at the three- to five-leaf stage. All plants were cut down to 5 cm above soil level every 30–45 days, usually when the growing shoots reached the flowering stage. After each cut, the regrowth of each plant was tested for AMV content by ELISA.

Virus isolates. Five AMV isolates were used for a comparative study. A-515, A-1, and A-2 were isolated from alfalfa grown in Alberta, whereas O-6A and O-34 were from alfalfa fields in southern Ontario (provided by Y. Paliwal, Plant Research Centre, Agriculture Canada, Ottawa, Ontario). The severe isolate A-515 was used as the inoculum for resistance studies. It was propagated in young plants of *Nicotiana tabacum* L. cv. White Burley, harvested 14 days after inoculation, and freeze-dried as stock inoculum for all experiments. AMV inocula were extracted from infected fresh leaves at a rate of 1:2 (w/v) in 0.025 M potassium phosphate buffer, pH 8.0, and diluted fivefold with the same buffer. AMV inocula from lyophilized leaves were prepared by extracting AMV at 1:20 (w/v) in buffer and further diluted fivefold with the same buffer. Purification of AMV for the production of antiserum was done as described previously (18).

ELISA. ELISA (5) was routinely used for assaying AMV with antiserum to AMV (A-515) as previously described (22). To determine the presence of AMV in seeds, individual seeds were ground in 0.8 ml of the extraction buffer (phosphate-buffered saline, pH 7.4). For the leaf test, two leaflets from the youngest well-developed leaf were taken, and in certain cases, inoculated leaves were also tested for AMV. About 40 mg of leaf tissue was ground in 0.8 ml of the extraction buffer. The clarified extracts from seeds and leaves were transferred in 200-μl aliquots to individual wells of microplates. An optimal dilution of 2 μg/ml of coating γ-globulin, and 1/2,000–1/3,000 dilutions of alkaline phosphatase-conjugated γ-globulin were used with *p*-nitrophenyl phosphate substrate. Reaction absorbance values were measured at 405 nm on a Titertek Multiskan (Flow Laboratories, Mississauga, Ontario). Samples were considered positive when the mean absorbance exceeded the mean value plus three times the standard deviation of healthy alfalfa sap control or the values were higher than 0.1 (about twice the highest absorbance value for virus-free samples used as an internal negative control). Samples yielding values higher than 0.5 were considered arbitrarily to be of high virus concentration.

Immunodiffusion test. Agar-gel double-diffusion tests were performed according to the method described by Ball (2). Both purified preparations and clarified juice were used as antigens. Clarified juice was obtained by treating the sap (extracted from infected leaf tissue in 0.5 volume of potassium phosphate buffer, pH 7.0, containing 0.04 M 2-mercaptoethanol) with 0.5 volume of chloroform and separating the aqueous phase by low-speed centrifugation. Controls included healthy plant sap against antiserum, infected sap against normal serum, and buffer against antiserum.

Electron microscopy. The procedures used for negative staining of AMV preparations were essentially the same as those previously described (16).

RESULTS

AMV incidence and identification of AMV isolates. The occurrence of AMV in Alberta was established during a 1968 field survey covering a total area of 3,332 ha of alfalfa (C. Hiruki, *unpublished*). Sampling was done at the edge of the field and in the crop stand by walking in a crisscross manner. AMV was detected in 19 of 220 samples collected. Systematic sampling of field specimens was resumed during 1979–1983. The virus was identified by serological reaction, particle morphology, and host reactions. Numbers of AMV-infected/numbers of samples tested were: 52/110 in 1979, 51/88 in 1980, 44/65 in 1981, 35/48 in 1982, and 13/25 in 1983. Isolate A-515 was originally isolated from alfalfa showing severe systemic yellow mosaic in a field of about 40 ha near Taber, Alberta, in 1981. About 20% of alfalfa stands in this field were estimated to be affected by similar symptoms, which were common in alfalfa after the first cutting. These symptoms, however, were not reproduced in greenhouse experiments using several alfalfa cultivars sap-inoculated with isolate A-515. In the greenhouse, A-515 caused green mosaic occasionally accompanied by necrotic spots of varying severity.

A pure isolate of A-515, established

Table 1. Differential host range of and typical symptoms caused by five alfalfa mosaic virus (AMV) isolates

Test plant	AMV isolates (symptom category)[a]				
	A-515 (severe)	O-6A (moderate)	O-34 (moderate)	A-1 (moderate)	A-2 (mild)
Nicotiana tabacum L. cv. White Burley	Nl,RSn/VC,Mo,NS[b]	ChlS/Mo	Vl/Mo	Vl/Mo	l/Mo
Phaseolus vulgaris L. cv. Red Kidney	NS,VN,Y/Y,NS	VC/ChlS	Nl,VN/–	NS,VN/–	ChlS,VN/Mo
Capsicum annuum L.	NS/Mo,Str,Ma	ChlS,NS/Mo,Ma	NS/Mo	NS/Mo,Ma	ChlS/Mo
Chenopodium quinoa Willd.	Nl/Mo,NS,Y,Ld	ChlS/Y	ChlS/Y	ChlS/Y	ChlS/–
C. amaranticolor Coste & Reyn.	Vl/VC,Ma	l/Mo	ChlS/ChlS	ChlS/–	–/–
Vigna unguiculata (L.) Walp. cv. Blackeye	Nl/VN	Nl/VC,VN	Vl/VC	Nl,VN/–	–/–
Cucumis sativus L. cv. Chicago Pickling	ChlS/–	–/–	–/–	–/–	–/–

[a]Severe, moderate, and mild are based on the responses of test plants in this investigation.
[b]Numerator refers to local symptoms and denominator refers to systemic symptoms. ChlS = chlorotic spots; l = latent infection; Ld = defoliation; Ma = leaf malformation; Mo = mosaic or mottle; Nl = necrotic lesions; NS = small necrotic spots or flecks; RSn = necrotic ringspots; Str = streak, or progressive leaf necrosis; VC = veinclearing; Vl = variable lesions; VN = vein necrosis; Y = general yellowing; and – = no infection, confirmed by return inoculation to *C. quinoa* and/or by ELISA.

through single-lesion isolation, was compared with four selected AMV isolates in serological reactions, virus particle morphology, and host reactions. In electron microscopy, all isolates typically consisted of bacilliform particles about 16–18 nm in diameter. Three major classes of particle length, i.e., 58–64, 43–48, and 34–37 nm, as well as small spherical forms, were observed in the preparations. In immunogel-diffusion tests, all isolates were serologically indistinguishable. In the host reaction study, five AMV isolates were tested on 18 plant species. Of the 18 species tested, the results from only seven species are presented in Table 1; symptoms on the remaining species were not differential. Three symptomatology types, severe, moderate, and mild, were distinguished. A-515 caused very severe symptoms on many species tested. The remaining four isolates, 0-6A, 0-34, A-1, and A-2, showed increasingly milder symptoms in the order of hosts listed; A-2 was the mildest.

Responses of alfalfa cultivars to AMV. The percentage of seedlings naturally infected with AMV was lower than the rate of seed infection when individual seeds and seedlings from the same seed lot, except for cultivars Ceres and Anchor from Lakehead, were tested for the presence of AMV by ELISA (Table 2). None of the infected seedlings showed visible symptoms at the time of the assay. A large percentage of the seeds planted did not germinate, and even some of those that did germinate were very weak and soon died. Cultivar source may be as important as the cultivars themselves in determining seed infection by AMV, as shown in the case of Anchor (Table 2). In a separate experiment, leaf samples for testing were taken separately from apical leaves and from inoculated leaves. In these tests, Apica was the most resistant and Br-III-80 the most susceptible to isolate A-515 of AMV on the basis of incidence of infection and relative virus content in infected plants (Table 3). Except for Apica, which had a low rate of infection in both seed and inoculated seedlings, there was little correlation between seed and seedling infection in the other cultivars. Br-III-80 had the highest rate of seed and seedling infection. An exception was Anchor, in which 44% of the plants inoculated became infected, although seed infection was extremely low (Table 3). However, only three of 20 infected plants of this cultivar showed high A_{405nm} values in ELISA, whereas much higher proportions of heavily AMV-infected plants were found in each of the remaining cultivars (Table 3).

Effects of repeated cutting of AMV-inoculated alfalfa plants on disease development and plant survival. The four selected alfalfa cultivars and a breeding line were inoculated with AMV (A-515) in the seedling stage and were subjected to nine cycles of cutting and regeneration in the greenhouse during a period of about 10 mo. The results showed that differences in susceptibility to sap inoculation with AMV among the cultivars of alfalfa in the seedling stage do not necessarily reflect similar differences in their capacity to support mechanical spread of AMV by repeated cuttings. Repeated cuttings considerably increased the number of virus-infected plants in Apica, although this cultivar showed high resistance to infection in the seedling stage. With the remaining three cultivars and the breeding line, there were also increases in the final numbers of infected plants by the same treatment. Considerable fluctuations in infection rate occurred during regrowth after several cuttings in Rambler, Anchor, and Br-III-80. In Anchor, as many as 33% of the inoculated plants that were subjected to nine cuttings and subsequent regrowth remained noninfected by AMV. In Rambler and Br-III-80, 15 and 25%, respectively, of those plants that were infected by AMV did not survive from the same treatment (Table 4), although all plants that remained noninfected thrived. The cultivars also differed considerably in their ability to support AMV multiplication in subsequent regrowth of the infected plants. In Apica, the treatment resulted in the establishment of mild infections and a tendency toward reduction of virus content in subsequent regrowth, as indicated by lower ELISA values. In this cultivar, 18% of the plants had a low AMV concentration. Similar low virus concentrations were also detected in small percentages in AMV-infected plants of cultivars Rambler, Blazer, and Anchor but not in the breeding line Br-III-80 (Table 4).

DISCUSSION

This investigation established that, in most alfalfa seed samples tested for AMV by ELISA, the percentage of seedlings found naturally infected with AMV was significantly lower than that of seed infection when individual seeds and seedlings from the same seed lot were used. Possible reasons for this difference are 1) the detrimental effect of AMV infections, which result in poor germination of AMV-infected seeds, and 2) different rates of the incidence of AMV in seed coat and embryo of alfalfa seed. Recently, it has been shown that the difference in detection levels by ELISA in seeds and seedlings is related to the high incidence of AMV in the seed coat and the relatively low incidence in the embryo (22). AMV detected in the seed coat appears insignificant as a source of

Table 2. Detection of seed-transmitted alfalfa mosaic virus (AMV) in alfalfa seed and seedlings both evaluated from the same seed lot by enzyme-linked immunosorbent assay

Cultivar or breeding line (origin)[a]	Infected seeds[b] (%)	Seeds planted (no.)	Seeds germinated (no.)	Number of AMV-infected plants at growth stages[c]			Plants infected (%)
				I	II	III	
Angus (Pembina)	1.7 (58)	70	22	0	0	0	0
Angus (Lakehead)	3.4 (232)	70	26	0	0	0	0
Ceres (Pembina)	1.7 (58)	70	36	0	1	1	6
Saranac (Lakehead)	17.0 (230)	80	42	0	0	2	5
Vista (Lethbridge)	0.4 (232)	80	42	0	0	0	0
Anchor (Lakehead)	12.7 (290)	80	36	2	2	2	17
Anchor (Lethbridge)	0.3 (290)	36	14	0	–[d]	0	0
Apica (Lethbridge)	0.4 (290)	72	42	0	–	0	0
Blazer (Lethbridge)	2.1 (232)	36	12	0	–	0	0
WL-221 (Lethbridge)	1.7 (58)	36	16	0	–	0	0
Valor (Lakehead)	6.8 (235)	52	32	0	–	0	0

[a] Pembina = Pembina Forage Association, Lakehead = Lakehead Forage Association, and Lethbridge = Canada Agriculture Lethbridge Research Station.

[b] Extracts of individual seeds were tested by double-sandwich ELISA. Numbers in parentheses refer to the number of alfalfa seeds individually tested for AMV by ELISA.

[c] I = one- to three-leaf stage, II = six-leaf stage, and III = eight-leaf stage.

[d] Not tested.

infection of emerging seedlings.

Because most of the established cultivars of alfalfa undergo continuous hybridization with germ plasms of different origins in the field (3,19), the diversity of reactions of alfalfa cultivars to AMV is not surprising. Each alfalfa cultivar is in reality a composite of plants of different genotypes (3). The different responses of alfalfa cultivars to AMV may also be caused by the diversity of AMV strains involved (10,12,21) as well as by environmental factors (4). For example, a severe AMV strain, which caused bright yellow mosaic and/or necrotic symptoms in infected alfalfa, induced the greatest reduction in forage yield (10). In this investigation, infection rates with the severe isolate of AMV (A-515) were relatively high for all the cultivars and the breeding line (Br-III-80) tested; the latter was the most susceptible and Apica was the least susceptible to infection with AMV (Table 3). An interesting fact is that, from a certain number of plants of each cultivar, the virus was detected only from inoculated leaves or only from young, apical leaves, which points to a considerable genetic variability in resistance to AMV among individual plants of each cultivar. This observation also suggests that although the frequency of the detection of AMV was usually high in apical leaves, testing for virus infection in inoculated leaves might also be important for proper evaluation of AMV resistance of particular alfalfa cultivars. Apica, which had a low percentage of AMV-infected seeds, also was more resistant to AMV infection than Br-III-80, which had a high rate of seed infection. An exception was Anchor, in which 44% of the plants inoculated became infected, although seed infection was extremely low. This discrepancy suggests that separate genes may control seed infection and plant infection.

Alfalfa could be infected in various stages of its growth by cutting tools freshly contaminated with AMV. This investigation shed some light on how repeated cutting influenced the susceptibility of different alfalfa genotypes to viral infection, the spread of AMV to previously noninfected plants within the crop, and the extent of survival of AMV-infected alfalfa plants. When alfalfa plants of different genotypes inoculated with AMV during the seedling stage were subjected to a series of cuttings and regrowth in the greenhouse, their differences in susceptibility to sap inoculation during the seedling stage were not necessarily reflected in their capabilities to support AMV spread to previously noninfected plants. Repeated cuttings considerably increased the number of virus-infected plants in Apica, which was highly resistant during the seedling stage. This fact suggests that AMV was transmitted from the infected to the noninfected plants during cutting and that young, regenerating tissue was susceptible to AMV infection. In the other three tested cultivars and the breeding line Br-III-80, there were also increases in the final numbers of infected plants, although there were considerable fluctuations in infection rate in the subsequent regrowth of Rambler, Anchor, and Br-III-80 after individual cuttings. In Anchor, which was four times as susceptible as Apica during the seedling stage, 33% of the inoculated plants remained virus-free after receiving the same treatment. This result suggests that AMV is not uniformly distributed in the shoots of Anchor. In the more susceptible cultivars, AMV infection contributed to the poor survival of infected plants after repeated cuttings.

The present investigation has provided a measure of the effect of AMV on alfalfa regrowth after repeated cuttings. This work has also shown that such cutting treatments may cause a gradual increase of AMV infection in the subsequent regrowths. The results highlight several factors that must be considered when

Table 3. Responses of alfalfa cultivars in the seedling stage of alfalfa mosaic virus (AMV)

Cultivar or breeding line (origin)	Infected seeds[a] (%)	Plants tested[b] (no.)	Number of samples infected when assaying			
			Inoculated leaves (I)	Apical leaves (A)	Both (I+A) leaves	Total
Apica (Lethbridge)	0.4 (290)	45	1	3	1	5 (4)[c]
Rambler (Pembina)	9.9 (290)	45	2	2	5	9 (6)
Blazer (Lethbridge)	2.1 (232)	45	6	5	7	18 (7)
Anchor (Lethbridge)	0.3 (290)	45	4	8	8	20 (3)
Br-III-80 (Univ. Alberta)	55.2 (232)	32	3	6	9	18 (12)

[a] Percentage of infected seeds detected by ELISA before seeds were germinated and virus-free plants were selected. Numbers in parentheses refer to the number of alfalfa seeds individually tested for AMV by ELISA.
[b] Alfalfa seedlings were inoculated with a severe isolate of AMV (A-515 isolate) at the three- to five-leaf stage.
[c] Numbers in parentheses refer to plants with high virus concentration, in which the mean ELISA absorbance value at 405 nm was higher than 0.5.

Table 4. Effects of repeated cutting on the mechanical spread of virus in alfalfa cultivars inoculated with alfalfa mosaic virus (AMV) in greenhouse studies[a]

Cultivar or breeding line (origin)	Plants tested (no.)	AMV-infected plants (%)					Infected plants with low virus concentration[b] (%)	Infected plants killed during repeated cutting (%)	Noninfected plants after nine cuts (%)
		Seedling stage	After three cuts	After five cuts	After eight cuts	After nine cuts			
Apica (Lethbridge)	44	11	25	68	77	91	18	2	7
Rambler (Pembina)	44	20	16	38	25	69	2	16	16
Blazer (Lethbridge)	45	40	38	51	53	78	5	9	13
Anchor (Lethbridge)	43	44	19	42	16	67	2	0	33
Br-III-80 (Univ. Alberta)	32	56	59	33	NT[c]	72	0	25	3

[a] Presence and relative concentration of AMV were determined by ELISA.
[b] Mean A_{405nm} values in ELISA between 0.1 and 0.5 after eight cuts.
[c] Not tested.

attempting to obtain an accurate estimate of AMV-induced yield reduction in alfalfa. An investigation on the effect of AMV infection on certain yield components of alfalfa cultivars and breeding lines is in progress.

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