Incidence of Four Important Viral Pathogens in Canadian Vineyards

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ABSTRACT

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A national survey was conducted in Canada during 1994 and 1995 for four viral pathogens of quarantine significance in grapevine. A total of 11,417 samples, collected from 637 field sites, was tested for the presence of arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), and two viruses commonly associated with grapevine leafroll disease, grapevine leafroll associated virus types I (GLRaV-I) and III (GLRaV-III). Nationally, the incidence of nepovirus-infected samples was low, 0.53 and 0.25% for ArMV and GFLV, respectively, and higher for the two leafroll associated viruses, 1.67% for GLRaV-I and 10.8% for GLRaV-III. Nepoviruses were found only in samples of varieties of *Vitis vinifera* origin or hybrid crosses, whereas GLRaV-I and GLRaV-III were found in samples of all variety types. Among the varieties tested, GLRaV-III was found primarily in samples of hybrid (14.8%) or other (including the *Labrusca* varieties Concord, Niagara, and Elvira) (13.5%) origin, whereas GLRaV-I was found predominantly in samples of *V. vinifera* (4.05%) origin. The widespread occurrence of these viruses in Canada has resulted in a reevaluation of their current quarantine status.

As with many other countries, Canada implements phytosanitary restrictions on the importation of grapevine material in order to prohibit the entry of exotic diseases caused by viral, bacterial, fungal, and phytoplasmal agents. An important principle governing the maintenance of such quarantine control is that regulated pests must cause significant economic damage and must either not be present in the importing country or be present in limited distribution and be subject to official control measures. A recent national survey for four viral quarantine pathogens in grapevine was conducted by the Animal and Plant Health Directorate (APHD) of the Food Production and Inspection Branch (FPI) of Agriculture and Agri-Food Canada. The aim of this survey was to provide information about the overall prevalence of these viruses on a national level for plant quarantine purposes.

Tests were conducted to determine the incidence of two viruses belonging to the nepovirus group, grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV), and two viruses that are commonly associated with grapevine leafroll disease, grapevine leafroll associated viruses (GLRaV) types I and III.

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Fanleaf degeneration is the oldest known virus disease of *Vitis vinifera* L. Its name comes from the peculiar malformation of infected leaves, which exhibit widely opened petiolar sinuses and abnormally gathered primary veins, giving the leaf the appearance of an open fan (3). The impact of fanleaf degeneration varies with the tolerance of the cultivar to the virus. Sensitive cultivars are severely affected, showing progressive decline of the vines, low yields (up to 80% losses) and low fruit quality, shortened productive life of the vineyard, low proportion of graft take, reduced rooting ability of propagation material, and decreased tolerance to adverse climatic factors (7).

The causal agent of fanleaf degeneration, GFLV, is a member of the nepovirus group and is naturally transmitted from grape to grape by the longidorid nematodes *Xiphinema index* and *X. italiae* (4). A single brief feeding on an infected plant is sufficient to make nematodes viruliferous. *X. index* acquires GFLV from roots of infected vines and retains it for up to 8 months in the absence of host plants or up to 3 months when the nematode feeds on virus-immune host plants. GFLV cannot be disseminated over long distances by natural means because of the limited range of vector movement (no more than 1.3 to 1.5 m/year). Furthermore, although GFLV is pollenborne, it is not transmitted through grape seeds and has no natural weed hosts; thus the only natural reservoir for this virus is the grapevine itself (3). Long-distance spread is achieved chiefly by transfer of infected propagation material.

Several additional viruses, especially nepoviruses (including ArMV), are found in grapevines in central Europe and elsewhere. Some of these cause minor diseases in grapevines and other host plants. Others infect vines but do not produce well-defined symptoms of disease. As with GFLV, ArMV is naturally spread via nematode vectors in the genera *Xiphinema* and *Longidorus* (11), and is readily transmissible experimentally by sap inoculation to herbaceous test plants.

Grapevine leafroll is one of the most important and widespread diseases of grapevines, occurring in all grape-producing countries worldwide. Leafroll can reduce the growth and yield of many varieties, but its most undesirable effect is on fruit quality. Affected vines show an increased sensitivity to environmental stresses, a retardation of maturity of the grapes, and a sugar content that may be 25 to 50% lower than in comparable healthy vines (2). Symptoms include downward rolling of leaves and interveinal chlorosis. The symptoms spread outward from the vine trunk and become more intense as the season progresses. In the late fall, leaf laminae of dark-fruited varieties turn red, while the major veins remain green.

GLRaV-I and GLRaV-III are phloem-limited, nonmechanically transmissible closteroviruses that are most often associated with grapevine leafroll disease. The precise etiology of leafroll disease has yet to be determined, and progress in this area has been hampered due to the apparent association of a number of different viruses, usually as mixed infections, with varying disease symptoms and the inability to mechanically transmit the disease agent(s) to woody indicators (recently reviewed by Bovey and Martelli, 1992 [1]). To date, seven different viruses have been found to be associated with leafroll, and there is evidence that more will be discovered.

Grapevine leafroll associated viruses are most often transmitted through propagation of an infected mother plant. An extremely low rate of natural spread of leafroll viruses from infected to healthy vines growing nearby has been observed in California (10), and higher rates have been reported from other grape-growing regions of the world (5). Several species of mealybug have been shown to transmit leafroll associated viruses under experimental condi-

tions, but the significance of this mode of spread has yet to be determined under normal cultural conditions.

Canada has imported commercial quantities of grapevines since the early 1970s subject to strict import guidelines implemented by Agriculture and Agri-Food Canada in an effort to mitigate quarantine pest concerns. Current policy requires all imported vines to be free of nepoviruses (ArMV, GFLV), grapevine leafroll associated viruses (GLRaV-I, III), and a number of other pathogenic agents, including those responsible for corky bark disease and *Flavescence dorée*. The national grapevine survey, the results of which are presented in this report, has formed a basis for reviewing existing grapevine import policies.

MATERIALS AND METHODS

Survey design and sample collection. The survey was national in scope with the goal of randomly sampling all known vineyards and nurseries in British Columbia, Ontario, Quebec, and Nova Scotia at a rate of 2.5 plants per hectare. This level of sampling was selected to provide an overall detection limit for infection of ≤0.05% with 95% confidence, assuming a normal random distribution of disease and diagnostic tests that were 100% sensitive. Samples of dormant grapevine canes were collected during the late fall and winter of 1994, and testing was performed at three APHD-FPI laboratories (Centre for Plant Health, Sidney, BC; Health of Animals Laboratory, Guelph, ON; and Centre for Animal and Plant Health, Charlottetown, PEI), with the Centre for Plant Health acting as the reference laboratory.

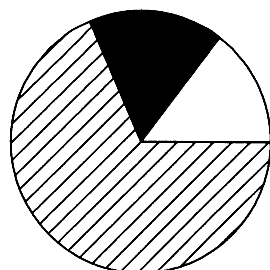
Testing protocol. The initial screening for all four viruses, ArMV, GFLV, GLRaV-I, and GLRaV-III, was conducted by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using bark scrapings from dormant canes. Serological reagents for the testing included rabbit polyclonal antisera for ArMV, GFLV (courtesy of L. Stobbs, Vineland Research Station, and P. Ellis, Pacific Agricultural Research Centre), and GLRaV-I (Bioreba, Inc., 3702 W. Sample St., South Bend, IN), and a monoclonal antibody specific for GLRaV-III (D. Gonsalves, Cornell University, Geneva, NY). Immunoglobulins were purified by fast protein liquid chromatography (FPLC, Pharmacia Biotech, Inc., 500 Morgan Blvd., Baie d'Urfé, Quebec), and alkaline phosphatase conjugates were prepared using a single-step glutaraldehyde condensation reaction as previously described (6). All samples found to be either positive or suspect were retested by ELISA, and in the case of GFLV and GLRaV-III, additional confirmatory testing was performed using a reverse transcriptase-polymerase chain reaction (RT-PCR) amplification technique. Additional confirmatory tests for GLRaV-I included Western immunoblot labeling and were performed by P. Monette, Centre for Plant Health.

DAS-ELISA. Bark scrapings from dormant grapevine canes were prepared using a modified pencil sharpener and stored frozen at –70°C until needed. Samples of bark scrapings (0.5 to 1.0 g) were homogenized with 5.0 ml of 0.5 M Tris-HCl, pH 8.2, 0.15 M NaCl, 2% PVP, 1% PEG-6000, and 0.05% Tween-20 using a Homex grinder (Bioreba); and 100-µl volumes were incubated for 16 to 24 h at 4°C in microtiter plate wells previously treated with 100 µl of purified rabbit IgG (1.0 µg/ml in 50 mM sodium carbonate, pH 9.6; O/N 4°C) specific for each of the viruses tested. Microtiter plate wells were then rinsed extensively with water and sequentially incubated with 100 µl of the respective alkaline phosphatase conjugated IgG for each virus being tested diluted in ELISA blocking buffer (Dulbecco's phosphate-buffered saline containing 2% PVP, 0.2% chicken egg albumin, 0.05% Tween-20) (90 min at 23°C) and *p*-nitrophenyl phosphate substrate (0.5 mg/ml in 10% diethanolamine, pH 9.8). The absorbance at 405 nm of each well was measured using a Dynatech MR5000 microplate reader interfaced with an IBM/PC compatible microcomputer. All samples were assayed in duplicate for each of the viruses tested, and results were judged to be suspect or positive if the mean absorbance (405 nm) was greater than 5× the average reading of a known healthy control. All suspect or positive samples were confirmed by the original testing laboratory as well as by the reference laboratory using DAS-ELISA, and a portion of these samples was also verified using an alternate technique such as RT-PCR.

Table 1. Summary of samples collected and tested per region

	BC[a]	ON	NS	PQ	Totals
Hectares	613	4,185	67	26	4,891
Field sites	131	480	19	7	637[b]
Growers	130	348	17	9	504
Samples collected	1,487	9,818	114	67	11,486
Samples tested	1,485	9,779	114	39	11,417

[a] Province abbreviations: British Columbia (BC), Ontario (ON), Quebec (PQ), Nova Scotia (NS).
[b] The discrepancy between the numbers of field sites and growers, especially in ON, reflects the fact that one grower may have more than one vineyard at different locations. For the purpose of the survey, the field site was the unit of concern.



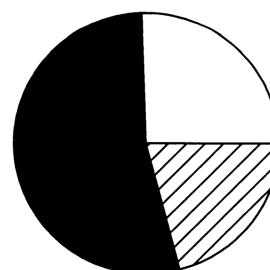


Fig. 1. Variety and type profile of samples collected from British Columbia and Ontario during the course of the national grapevine survey. Varieties were typed as either the species *Vitis vinifera,* hybrid crosses, or other varieties, including those of *Labrusca* origin.

RT-PCR amplification technique. In addition to confirmatory testing by DAS-ELISA, a number of the samples that were found positive for GFLV (20/31) or GLRaV-III (108/1,227) were retested using a RT-PCR technique. For detection of GFLV, oligonucleotide primers were chosen that resulted in the specific amplification of a 321-bp fragment corresponding to nucleotides 762 to 1,083 from the 3′ terminal region of RNA 2 (9). For GLRaV-III, DNA primers that resulted in the amplification of a 340-bp fragment (8) were chosen.

Samples of bark scrapings (0.5 to 1.0 g; previously frozen at −70°C) from dormant canes were homogenized with 5.0 ml of lysis buffer (4 M guanidinium isothiocyanate, 50 mM Tris-HCl, pH 7.4, and 10 mM EDTA) using a Homex grinder. Extracts containing viral RNA were prepared using RNeasy spin columns (Qiagen, Inc., 9600 DeSoto Ave., Chatsworth, CA) according to the manufacturers recommendations. For each sample, 5 µl of RNA extract was mixed with 1 µl of appropriate complementary primer (GFLV: 5′-CCAAAGTTGGTTTCCCAAGA-3′; GLRaV-III: 5′-ATTAACTTGACGGATGGCACAGC-3′; 10 µM) and annealed by incubating for 2 min at 95°C followed by 5 min at 6°C. Subsequently, 4 µl of RT master mix (125 mM Tris-HCl, pH 8.3, 100 mM KCl, 15 mM $MgCl_2$, BSA at 0.25 mg/ml, 25 mM dithiothreitol, 1.25 mM deoxynucleoside triphosphates [dNTPs], 1.25 U/µl Superscript-II reverse transcriptase [Gibco/BRL, Canadian Life Tech., P.O. Box 12098, Stn. A, Toronto, ON]) was added, and first-strand complementary DNA (cDNA) synthesis was performed by incubating for 45 min at 42°C.

For PCR amplification, 40 µl of PCR master mix (25 mM Tris-HCl, pH 8.3, 62.5 mM KCl, 2.5% sucrose, 0.125 mM cresol red, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 1.25 µM appropriate complementary primer, 1.25 µM appropriate homologous primer [GFLV: 5′-ACCGGATTGACGTGGGTGAT-3′; GLRaV-III: 5′-ATAAGCATTCGGGATGGACC-3′], and TAQ DNA polymerase [5 U/µl; Gibco/BRL] at 0.0625 U/µl) was added to each 10 µl of first-strand cDNA reaction mixture, overlaid with 25 µl of light mineral oil, and subjected to 35 cycles of amplification consisting of 15 s at 95°C (denaturation), 30 s at 58°C (annealing), and 45 s at 72°C (elongation) using a Stratagene Robocycler Gradient 9600 thermocycler.

Aliquots (20 µl) of PCR-amplified DNA were analyzed by electrophoresis through 1.8% agarose gels for 1.5 h in 1× TPE buffer (90 mM Tris-phosphate, pH 8.2, and 2 mM EDTA) at 75 V. Separated fragments were visualized using a UV transilluminator following staining with ethidium bromide (10 µg/ml).

RESULTS AND DISCUSSION

In Canada, the grapevine and associated wine industries have become a significant economic sector, accounting for greater than $C600 million annually in gross domestic product. The province of Ontario accounts for greater than 85% (approximately 4,185 ha) of the total commercial vineyard area, with a further 613 ha grown in British Columbia, primarily in the Okanagan valley region. The provinces of Quebec and Nova Scotia represent much smaller proportions, with approximately 26 and 67 ha, respectively. The goal of this survey was to provide a national perspective on the incidence of four regulated viruses causing serious diseases of grapevines. Toward this end, the survey sampling strategy aimed at collecting an average of 2.5 samples per hectare from each vineyard and nursery within the four primary grape-growing regions. In total, 11,486 samples were collected representing 637 field sites (504 different growers) nationwide (Table 1).

All samples were categorized as belonging to one of three major groups: (1) varieties belonging to the species *V. vinifera,* (2) hybrid crosses, and (3) other varieties, including those of *Labrusca* origin. A profile of the samples collected from British Columbia and Ontario showing the percentage of samples in each of these categories, as well as the predominant varieties and their relative abundance, is shown in Figure 1. In British Columbia, samples of *V. vinifera* comprised greater than 75% of the total, with the remainder being either hybrid (16.2%) or other (18.4%) varieties. This profile is different from that observed for Ontario, where other varieties (including *Labrusca* varieties such as Concord, Niagara, and Elvira) comprised 53.7% of the samples collected, with the remainder being almost equally divided between *V. vinifera* (20.9%) and hybrid (25.6%) varieties.

Nationally, the incidence of nepovirus-infected samples was found to be very low, averaging 0.53% for ArMV and 0.25% for GFLV, while the prevalence of samples found infected with GLRaV-I and -III was higher, 1.67 and 10.8%, respectively (Table 2). The presence of nepoviruses was detected only in samples of hybrid (1.6%) and *V. vinifera* (1.3%) varieties, while some level of infection with the leafroll associated viruses was found in samples of varieties from each group (Table 3). The highest incidence of GLRaV-I was found in samples of *V. vinifera* varieties (4.05%), with lower levels observed in samples of

Table 2. Aggregate summary of infected samples by region

	BC[a] (1,485)[b]	ON (9,779)	PQ (39)	NS (114)	Totals (11,417)
ArMV[c]	0.34% (5)	0.55% (54)	2.56% (1)	0	0.53% (60)
GFLV	0.06% (1)	0.32% (31)	0	0	0.25% (32)
GLRaV-I	1.28% (19)	1.75% (171)	0	0.87% (1)	1.67% (191)
GLRaV-III	2.15% (32)	12.2% (1,191)	5.12% (2)	1.75% (2)	10.8% (1,227)

[a] Province abbreviations: British Columbia (BC), Ontario (ON), Quebec (PQ), Nova Scotia (NS).
[b] Numbers in parentheses represent absolute numbers of samples tested per region or the number of samples found positive or suspect for each virus in each region.
[c] ArMV = arabis mosaic virus, GFLV = grapevine fanleaf virus, and GLRaV-I and -III = grapevine leafroll associated virus types I and III.

Table 3. Percentage of samples from each variety type found to be infected with one or more viruses

	British Columbia			Ontario			Total (including PQ and NS)		
	Hybrid	Other	*Vitis vinifera*	Hybrid	Other	*V. vinifera*	Hybrid	Other	*V. vinifera*
ArMV[a]	0.83% (2)[b]	0	0.27% (3)	1.44% (36)	0	0.88% (18)	1.39% (39)	0	0.66% (21)
GFLV	0	0	0.09% (1)	0.28% (7)	0	1.17% (24)	0.25% (7)	0	0.79% (25)
GLRaV-I	0	0	1.7% (19)	0.92% (23)	0.73% (38)	5.37% (110)	0.85% (24)	0.7% (38)	4.05% (129)
GLRaV-III	11.6% (28)	0	0.36% (4)	15.4% (385)	14.1% (736)	3.42% (70)	14.8% (415)	13.5% (738)	2.32% (74)

[a] ArMV = arabis mosaic virus, GFLV = grapevine fanleaf virus, and GLRaV-I and -III = grapevine leafroll associated virus types I and III.
[b] Numbers in parentheses represent the absolute numbers of samples found to be infected.

either hybrid (0.85%) or other (0.7%) categories. In contrast, samples of varieties of hybrid or other origin had significantly higher incidences of GLRaV-III infection (14.8 and 13.5%, respectively) than did samples of *V. vinifera* varieties (2.32%) (Table 3).

In comparing the two main grape-growing regions, Ontario and British Columbia, there was a marked variation in the overall incidence of GLRaV-III infected samples, 12.2 and 2.15%, respectively. This apparent difference was due in large part to the significantly different profile of varieties grown in each province (Fig. 1). For samples of hybrid origin from both regions, the incidence of GLRaV-III infection was comparable at 11.6 and 15.4% for British Columbia and Ontario, respectively (Table 3).

In British Columbia, all of the infected samples were either *V. vinifera* (47.4%) or hybrid (52.6%) varieties. No samples of the varieties categorized as "other" were found to be infected with either nepoviruses or leafroll associated viruses type I and III. The composition of infected samples in Ontario was noticeably different from that observed in British Columbia. Table grape varieties, such as Concord, Niagara, and Elvira, comprised 53.5% of the samples found to be infected with one or more viruses, while *V. vinifera* and hybrid varieties accounted for 15 and 31.2%, respectively, of the infected samples. This pattern was consistent with the overall variety composition of the samples collected in this province.

While the survey was not specifically designed to measure the incidence of these viruses within particular grape varieties, it was observed that generally the numbers of infected samples within particular varieties correlated with the relative abundance of that variety. It was, however, noteworthy that samples of Elvira, which comprised 3.5% of the samples tested from the "other" category, made up greater than 20% of the infected samples within this category, and that 87% of the Elvira samples tested from Ontario were infected with either GLRaV-I (three samples) or GLRaV-III (157 samples).

The distribution of infected samples varied significantly with particular viruses and between the two major grape-growing regions of Canada (Table 4). Due in large part to the very low overall incidence of samples infected with either ArMV or GFLV, the sites of infection were also well-contained and comprised only three field sites (2.3% of total) in British Columbia and 50 sites (10.4% of total) in Ontario. Generally, samples infected with either GLRaV-I or GLRaV-III were more widely distributed than those infected with nepoviruses. Within British Columbia, samples from 17 sites (12.9% of the total) were found to be infected with either GLRaV-I or GLRaV-III (GLRaV-I: 11 sites, GLRaV-III: 5 sites, both GLRaV-I and III: 1 site), with one of these sites accounting for 43% of the leafroll infected samples detected. Both GLRaV-I and GLRaV-III were significantly more widespread in Ontario. Taken together, samples infected with either of these two viruses were found on 286 (59.6%) of the 480 field locations.

The survey described in this report represents the first time a survey of truly national scope has been conducted for quarantine plant virus pests in Canada. The results are significant and will substantially influence the direction of grapevine import regulatory policy. While it is beyond the scope of this report to propose specific changes to plant-protection import policies, some comments may be appropriate.

The relatively high incidence of GLRaV-III infected samples in varieties of other than *V. vinifera* origin (e.g., hybrid crosses, *Labrusca* varieties such as Concord, Niagara, Elvira, and others), which have been present in Canada for a considerable time and for which movement between Canada and the United States has not been highly regulated, is significant and may imply that this virus is endemic within North America. The higher correlation between GLRaV-I infected samples and varieties of *V. vinifera* origin would be consistent with the notion that this virus has been introduced through the importation of grapevine propagation material from Europe and elsewhere. The results presented in this report do not support the continued regulation of GLRaV-I and GLRaV-III as quarantine pests, and it is the authors' view that future control of grapevine leafroll disease may best be accomplished through a mandatory industry-sponsored domestic certification scheme. The lower, albeit significant, incidences of ArMV and GFLV also raise important questions as to whether these viruses should retain a quarantine status or be dealt with through a certification program.

Table 4. Proportion of surveyed field sites containing virus-infected samples

	BC (131)[a]	ON (480)	Total (611) (including PQ and NS)
ArMV[b]	2.3%	7.7%	6.7%
	(3)	(37)	(41)
GFLV	0.8%	4.4%	3.6%
	(1)	(21)	(22)
GLRaV-I	9.2%	18.8%	16.9%
	(12)	(90)	(103)
GLRaV-III	4.6%	57.3%	46.6%
	(6)	(275)	(285)

[a] Numbers in parentheses represent the absolute number of field sites tested or found to contain samples infected with one or more of the viruses.

[b] ArMV = arabis mosaic virus, GFLV = grapevine fanleaf virus, and GLRaV-I and -III = grapevine leafroll associated virus types I and III.

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