heating area (pipes or heat exchangers) to compensate for the lower water temperature.

You must also calculate the initial cost of a heat pump. But considering today's high heating costs, we have found that a heat pump can pay for itself within a few years.

VIRUS ERADICATION THROUGH IN VITRO TECHNIQUES

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Abstract. *In vitro* heat therapy was successfully used in combination with shoot-tip culture to generate grapevine fanleaf virus-free *Vitis vinifera* 'Ranny Vira' but not grapevine leafroll-free *V. vinifera* 'Schuyler' or *V. piasezkii*. A combination of shoot tip culture and *in vitro* chemotherapy with either DHPA or vidarabine was ineffective in generating grapevine leafroll-free *V. vinifera* 'Limberger' while ribavirin appears more promising.

INTRODUCTION

Two important diseases of grapevines are grapevine fanleaf (GFL) and grapevine leafroll (GLR). Both have inflicted serious economic losses to the grape-growing industry. Consequently all new introductions of grapevines into Canada are indexed for these diseases at Saanichton as part of the national plant quarantine program.

New introductions found to be infected need to be cleaned up before they are released for commercial propagation. The conventional treatment consists of growing the infected plants in a heat therapy chamber, where they are subjected to a continuous heat treatment at 38°C. After 100 days 2 cm shoots are removed and grafted onto the appropriate virus-free woody indicator. The grafted indicator plants are then monitored for symptom expression. Only some 48% of the tips generated by this method are free of grapevine fanleaf virus (GFLV). With GLR the success rate is lower. At least part of the reason for the fact that the proportion of pathogen-free tips is relatively low may be that only large shoot tips can be taken from the heat-treated grapevines for grafting onto the woody indicators.

A simple solution to this aspect of the problem lies in combining heat therapy with tissue culture. Much smaller shoot tips can be excised from grapevine tissue cultures heat treated *in vitro* than from conventionally heat-treated plants,

thus making it more likely that the tip is virus-free. Additional advantages of this procedure are a considerable reduction in the space requirement, as each cultivar can be accommodated in a 125 ml Erlenmeyer flask, and a substantial saving in energy costs, as only a small controlled environment cabinet needs to be heated instead of a small room. A great deal of time can also be saved in releasing large numbers of plants to the industry. The shoot tips from the heat-treated cultures are used to initiate tissue cultures from which shoots are excised, rooted, potted, and indexed. Those cultures which are then identified as disease-free can be rapidly mass propagated *in vitro* to generate large numbers of clean stock plants. Subcultures can also be maintained at cool temperatures (8°C) as *in vitro* repository of virus-tested stock. The movement, both internationally and nationally, of such virus-tested clonal germplasm might also be greatly facilitated, as the transfer of *in vitro* cultures eliminates the risk of disseminating soil-borne pathogens.

A different *in vitro* technique for generating virus-free plants from virus-infected ones is the addition of antiviral drugs to the tissue culture medium. This approach offers all of the advantages mentioned above for *in vitro* heat therapy. In addition *in vitro* chemotherapy eliminates the heating requirements and thus offers an even greater reduction in energy costs.

The objectives of the present experiments were to determine: a) if *in vitro* heat therapy could be used, in combination with shoot tip culture, to generate disease-free grapevines from GFLV- or leafroll-infected plants and, b) if a combination of shoot tip culture and *in vitro* chemotherapy could be used to generate leafroll-free grapevines.

MATERIALS AND METHODS

Tissue culture. Media and procedures for culture initiation, proliferation and rooting were as previously published (20), except where otherwise noted.

***In vitro* heat therapy.** The grapevines used in this experiment were *V. vinifera* 'Ranny Vira' and 'Schuyler' and *V. piasezkii*. When indexed by the Plant Quarantine section the first was found to be infected with GFLV and the last two with grapevine leafroll. Tissue cultures of these grapevines at the stage of *in vitro* shoot proliferation were placed in Conviron chambers under the following heat and light regimes: 6 h at 39°C followed by 18 h at 22°C with a 16 h photoperiod at 18 $\mu Em^{-2}s^{-1}$ (approximately 1200 lux), the 39°C treatment beginning 6 h after the start of illumination. At selected intervals

shoot tips 2 mm in length were excised from the heat-treated cultures and used to initiate fresh cultures. These were then maintained at 22°C with a 16 h photoperiod (20). Shoots were excised from these cultures, rooted, and transferred to a mist chamber, and later to a greenhouse.

***In vitro* chemotherapy.** DHPA ((S)-9-(2,3-Dihydroxypropyladenine) or vidarabine (9-β-D-arabinofuranosyladenine) was added to grapevine initiation medium, prior to autoclaving. Cultures of *V. vinifera* 'Limberger' were initiated in these drug-containing media and were later transferred to drug-containing proliferation media. At various intervals 2 mm shoot tips were excised from the cultures and placed on drug-free grapevine initiation medium. When these subcultures reached the shoot proliferation stage, shoots were taken, rooted, and grown on as for the *in vitro* heat therapy experiment. Ribavirin (Virazole) (1-β-D-ribofuranosyl-1,2,4-trizole-3-carboxamide) was also tested as above except that in this case 2 cm long shoots were excised from *V. vinifera* 'Limberger' cultures actively proliferating in drug-free medium and were placed in ribavirin-containing proliferation medium. At various intervals, 2 mm shoot tips were excised and subsequently handled as above.

Indexing. Testing for the presence of GFLV was carried out by enzyme-linked immunosorbent assay (ELISA) (5) on tissue cultures initiated from the plants which had been regenerated after the *in vitro* heat treatments and which had been maintained in the greenhouse for approximately two years. Tissue cultures of GFLV- and leafroll-free *V. vinifera* 'Liemberger' were used as negative controls and cultures of GFLV-infected 'Ranny Vira' were used as positive controls. The plates were read on a Titertek Multiskan microplate reader (Flow Laboratories, Ont.) and samples giving an absorbance reading at 405 nm of at least twice the value of the negative control were considered positive for GFLV. Testing for leafroll was done by monitoring the plants for grapevine leafroll symptom expression. Readings were taken two years after the treatments were applied. In all cultivars tested, *V. vinifera* 'Schuyler', 'Limberg' and *V. piasezkii*, interveinal reddening accompanied with an angular downturn of the edges of the basal leaves were considered to indicate leafroll.

RESULTS

***In vitro* heat therapy.** A treatment duration as brief as 17 days was sufficient to generate GFLV-free *V. vinifera* 'Ranny Vira' although not all tips were virus-free (Table 1). All the tips obtained after 28 days of heat treatment gave rise to

GFLV-free plants. In contrast, this treatment was not successful in eliminating leafroll in either of the grapevine cultivars tested (Table 1). A treatment duration of 113 days yielded 2 tips of *V. vinifera* 'Schuyler' which appeared to be disease-free. However, further testing is required to confirm these results.

Table 1: Effect of *in vitro* heat treatment on the elimination of grapevine fanleaf or grapevine leafroll.[1]

Duration of treatment[2] (days)	Number of tips producing uninfected cultures/ number of tips taken		
	GFLV	GLR-1	GLR-2
17	1/2	--	0/1
21	--	0/2	--
28	2/2	0/1	0/1
37	--	0/2	--
39	--	0/1	--
40	3/3	--	0/2
47	--	0/1	--
62	--	0/3	--
66	--	0/1	--
72	--	0/2	--
77	--	0/1	--
82	--	0/3	--
85	--	0/1	--
99	--	0/1	--
112	--	--	0/1
113	--	2/3	--

[1] The GFLV-infected grapevine was *V. vinifera* 'Ranny Vira' and the leafroll -infected grapevines were *V. vinifera* 'Schuyler' (GLR-1) and *V. piasezkii* (GLR-2).

[2] Temperature cycle consisted of 6 h at 39°C followed by 18 h at 22°C, the high temperature being applied 6 h after the start of the light cycle. Light cycle was 18 $\mu Em^{-2}s^{-1}$ for 16 h followed by darkness for 8 h.

***In vitro* chemotherapy.** DHPA at a concentration of 10 mgl^{-1} was not effective in eliminating leafroll in *V. vinifera* 'Limberger' (Table 2). While 1 out of 2, 1 out of 3 and 1 out of 3 tips taken at 86, 100 and 112 days, respectively, yielded plants which did not clearly show symptoms at the time of writing, the condition of these plants is such that they cannot be unequivocally considered leafroll-free. This view is supported by the results obtained with a concentration of 15 mgl^{-1} DHPA. The 3 tips taken after a treatment period of 86 days and that taken after 100 days all yielded plants which clearly show symptoms of grapevine leafroll. The tip taken at 112 days from the culture subjected to 15 mgl^{-1} DHPA yielded plants which are still of uncertain status.

Vidarabine, at a concentration of either 10 or 20 mgl^{-1}, was ineffective in generating leafroll-free shoot tips (Table 3).

A ribavirin treatment for 32 days at a concentration of 10 mgl^{-1} resulted in the production of tips which appear to be leafroll-free, based on symptom expression 2 years after the

treatment was applied (Table 4). Shorter treatment durations at higher concentrations of the drug gave similar results.

Table 2. Effect of *in vitro* chemotherapy with DHPA on the elimination of grapevine leafroll.[1]

Duration of treatment (days)	Number of tips producing uninfected cultures/ number of tips taken		
	Concentration (mg l^{-1})		
	0	10	15
50	0/1	--	0/1
67	0/3	--	0/3
69	0/4	0/3	0/3
81	0/2	--	0/2
86	0/2	1/2	0/3
100	0/2	1/3	0/1
112	0/2	1/3	1/1

[1] *V. vinifera* 'Limberger' was used.

Table 3. Effect of *in vitro* chemotherapy with vidarabine on the elimination of grapevine leafroll.[1]

Duration of treatment (days)	Number of tips producing uninfected cultures/ number of tips taken		
	Concentration (mg l^{-1})		
	0	10	20
59	0/3	0/2	0/1
64	--	0/1	1/1
71	1/1	0/2	--
76	--	0/1	0/1
85	0/2	0/1	--
90	--	0/1	--
99	0/1	0/1	--
104	0/1	0/1	--
114	--	0/1	--

[1] *V. vinifera* 'Limberger' was used.

Table 4. Effect of *in vitro* chemotherapy with ribavirin on the elimination of grapevine leafroll.[1]

Duration of treatment (days)	Number of tips producing uninfected cultures/ number of tips taken					
	Concentration (mg l^{-1})					
	0	10	15	30	45	60
14	--	0/3	--	0/2	--	--
18	--	0/1	--	2/3	1/1	1/1
26	--	--	--	--	--	1/1
32	0/1	2/3	1/2	1/1	1/1	--
43	1/1	1/3	2/2	1/1	--	1/1
59	--	2/2	1/1	2/2	2/3	1/1
101	1/1	--	--	2/2	1/1	--
115	--	--	--	1/1	--	--

[1] *V. vinifera* 'Limberger' was used.

DISCUSSION

Viruses are, by definition, obligate intracellular parasites. They depend for their multiplication on the biochemical machinery of the cells they infect. The virions, or virus particles, consist essentially of a nucleic acid genome surrounded by a shell composed entirely or partly of protein. This shell, or capsid, protects the virus genes against digestion by ubiquitous nucleases and is sometimes involved in the attachment of a virion to the outer membrane of a susceptible host cell. Although there are variations from one virus to the next, the following is a fairly comprehensive list of the events involved in virus multiplication: a) penetration of the viral nucleic acid or of the entire virion into the host cell, b) uncoating (removal of the protein shell surrounding the viral genes), c) expression of "early" genes, d) replication of the viral nucleic acid (the viral genome is used as a template in the manufacture of hundreds of copies of itself), e) expression of "late" genes, f) encapsidation (each copy of the viral genome becomes surrounded by a protein shell) and, g) release of the progeny virions. The newly manufactured virus particles can then infect more host cells, if the opportunity arises, and begin the process anew.

Any treatment designed to generate virus-free growing points in virus-infected plants must consequently interfere with one of the above events while still allowing normal plant metabolism to proceed relatively unhindered. Meristem culture and shoot tip culture (a shoot tip being defined as the apical meristem plus one or more leaf primordia) have been shown to be of great value in eradicating certain virus diseases in plants. One of the first instances was the production of virus-free dahlias in 1952 (15). Numerous reports have since been published of excellent results obtained using these methods (16,22). The success of the procedure is generally attributed to the uneven distribution of the virus within the plants (12), the meristematic areas often being virus-free. In these instances the meristematic area at the shoot tip may, through rapid division, move away from the infected cells faster than the progeny virus particles can migrate toward it. Alternatively, the success of the method may be due to some inactivating factor produced by the explant or to the effect of some constituent of the culture medium on the virus (10). The block in the multiplication strategy of the virus, according to the above theories, might take place at the extracellular stage. An intracellular block to virus replication may also be operating, according to the suggestion that the excision of small tips temporarily disorganizes the growth processes of the cells near the meristematic dome and that host cell enzymes required for

one or more steps of viral replication become unavailable (14). Virus eradication using shoot tip culture would thus be more efficient the smaller the explant and the lower the virus concentration in the tips (22).

Certain viruses cannot be eradicated simply by meristem or shoot tip culture. Their rates of multiplication and migration are such that they can keep up with shoot tip growth. In these instances, where virions are present in the area immediately next to the apical dome or possibly within it, the plant needs some outside assistance in producing virus-free tips. Conventional heat therapy techniques have provided the solution in many cases and heat therapy combined with meristem or shoot tip culture has offered the additional advantage that very small tips could be taken from the heat-treated plant material. The data presented in this report indicate that 2 mm shoot tips excised from heat-treated grapevine tissue cultures are free from GFLV. This is in agreement with a recent report (1). The observation by those authors that GFLV is not eliminated simply by shoot tip culture, without heat treatment, was also confirmed in our laboratory. The rationale for indexing tissue cultures rather than plants maintained in the greenhouse is that the cultures are not subject to re-infection or infection with other pathogens and that routine spraying against various pests is not required. Furthermore, GFLV titers in tissue-cultured grapevines are as high as or higher than those in freshly opened leaves of the plant from which the cultures were initiated (unpublished data). Because shoot tip culture *per se* does not eliminate GFLV (1), the success obtained in the present *in vitro* heat therapy experiment can be ascribed to the heat treatment itself rather than to metabolic disruption resulting from cell injury during explant excision. It is possible that the "RNA-dependent RNA polymerase" responsible for the manufacture of multiple copies of the GFLV genome, or some other virus-specific enzyme, may be completely inhibited at 39°C while the enzymes responsible for cell growth and division in the grapevine meristematic tissues may be only slightly inhibited. Knowledge of the exact mechanism of GFLV elimination through *in vitro* heat therapy will have to await a better understanding of the molecular biology of GFLV multiplication.

While the results presented here on the elimination of GFLV by *in vitro* heat therapy agree with those of a recently published report (1), our results concerning the elimination of grapevine leafroll by this method are at variance with that report. None of the tips taken from our heat-treated leafroll-infected grapevine tissue cultures yielded plants which were clearly leafroll-free. It is not clear from the previous report

how long the authors exposed their *in vitro* cultures to their heat therapy regime. Possibly the duration of treatment was longer than our 113 days. The authors also used shoot apices 1 mm in length while we used 2 mm shoot tips. This latter consideration and their use of different grapevine cultivars may be important in accounting for the differences between their results and ours. We are assuming that the leafroll disease initially present in their grapevines was the same as that present in ours. Any discussion of grapevine leafroll disease elimination is necessarily highly speculative, however, as the causative agent(s) has not yet been definitely identified. While it has been variously reported to be a potyvirus (21) or a closterovirus (7,17), no conclusive evidence has yet been presented that any specific type of virus can be regarded as the sole causal agent of grapevine leafroll (3). This disease is obviously complex and, until highly sensitive biochemical or immunochemical procedures become available to replace symptom expression as a diagnostic tool, the process of indexing to determine the success of eradication treatments must be considered to yield only tentative results. While symptom expression is useful in determining that a given treatment was not successful, more rigorous tests are needed to support a claim that the disease has been eliminated.

A different approach to in vitro virus eradication in plants is to include in the nutrient medium a chemical with a demonstrated ability to prevent virus replication. In order to be useful in generating virus-free shoot tips, the chemical must be effective at a concentration which does not cause phytotoxicity.

The first compound tested in this study, DHPA, has been reported to inhibit the replication *in vitro* of several animal DNA and RNA viruses (6). This compound was relatively nontoxic to animal cell cultures. For example, a concentration of 600 μg ml^{-1} was required to reduce the proliferation of mouse L-929 cells by 50%. When cultures of *V. vinifera* 'Limberger' infected with grapevine leafroll were exposed to this drug, however, only a few survived the treatments with 30 mg l^{-1} or more (20). At concentrations as low as 10 mg l^{-1}, this drug inhibited shoot proliferation significantly and caused some mortality. The data in Table 2 indicate that most of the shoot tips produced at concentrations of 10 and 15 mg l^{-1} DHPA were still infected. The remainder are still of uncertain status.

Vidarabine is another nucleoside analog which has been shown to possess broad-spectrum antiviral activity *in vitro* (4). At the concentrations tested this compound was ineffective in generating leafroll-free shoot tips (Table 3). Concentrations higher than 20 mg l^{-1} tended to be phytotoxic.

The third compound tested, ribavirin, has been demonstrated to act against both animal and plant viruses (8,9,13). It has been used to regenerate virus-free plants from cucumber mosaic virus- and potato virus Y-infected tobacco explants (2) and has been shown to delay the onset of grapevine leafroll symptom expression (20). The data from our experiment combining ribavirin treatment and shoot tip culture suggest that this form of *in vitro* chemotherapy may yield leafroll-free grapevines (Table 4). Plants regenerated from the ribavirin-treated cultures have been grown in the greenhouse for approximately two years and several combinations of treatment duration and drug concentration presently appear to have yielded leafroll-free shoot tips, based on symptom expression. Because there is no herbaceous host which can be used for leafroll indexing, however, and in the absence of appropriate immunochemical test methods the present results must be interpreted with the utmost caution. It is possible that ribavirin simply exerted a virostatic effect. This, in combination with the excision of small shoot tips, might have resulted in tips containing very few infectious agent particles. Their concentration might eventually increase to a level inducing disease symptoms.

Relatively little progress has been made in the eradication of virus diseases through *in vitro* chemotherapy since the 1950s when potato tissue cultures were freed of potato virus X with malachite green (18) and tobacco tissue cultures were freed of potato virus Y with 2-thiouracil (11). The problem lies mainly in identifying compounds which can act selectively against the virus while leaving the host cell metabolism relatively unaffected. In the early days, the problem was compounded by the limited selection of therapeutic chemicals available. This situation has now greatly improved as more and more antiviral agents are being identified. In addition chemicals with antiviral activity are being intensively studied to determine which chemical moiety is responsible for the therapeutic properties. Such structure-function analyses often lead to the synthesis of artificial analogs with even greater antiviral activity than the parent compound.

It is unlikely that any single drug will prove to be capable of inhibiting all of the viruses causing serious economic losses in the major horticultural crops. It is more realistic to attempt to identify drugs which are each effective against one or a few economically important viruses. The broad-spectrum antivirals are the candidates which need to be screened first, because of their greater potential. The interferon system which operates in animal cells possesses very broad antiviral activity and a similar system may exist in plants (19). Whereas the interfer-

ons are extremely expensive products, polyinosinic acid•polycytidylic acid, a very potent inducer of interferon in animal systems, is much more affordable. Experiments are in progress at Saanichton to determine whether poly (I)•poly (C) treatments can be used in combination with shoot tip culture to generate virus-free grapevines.

There are numerous reasons, mentioned throughout the text, why *in vitro* virus eradication techniques should become important adjuncts to virus indexing and eradication programs. Tissue culture methods, already of great importance in the mass propagation of stock plants, would then have even further impact on commercial horticulture.

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