APPENDIX 3.

RIBAVIRIN 1993 YEAR-END REPORT

The use of Ribavirin for the elimination of virus from *in vitro* cultures of *Vitis vinifera* cv. Cabernet Sauvignon infected with grapevine fanleaf virus, grapevine leafroll associated viruses, and corky bark associated virus.

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Grapes, *Vitis vinifera* L., are the most widely grown and most valuable fruit crop in the world (Anonymous, 1985). Both rootstock and scion wood of grapes are clonally propagated which results in a high transmission rate of viruses from infected parent stock. Since grapes are long-lived perennials, even mild virus infections can result in significant reductions in vineyard productivity and profitability (Converse, 1985). Therefore, an essential part of grapevine virus disease control is the establishment and maintenance of virus-tested propagating stocks.

When possible, selections of desirable varieties or "clones" are made from sources which test negative for the presence of viruses. However, it is often times necessary to perform therapy to eliminate virus infections from valuable selections which are not available except from infected sources. Heat therapy was one of the first techniques used to eliminate grapevine viruses (Goheen and Luhn, 1973). More recently, tissue culture techniques including microtip culture (*), fragmented shoot tip culture (Barlass and Skene, 1982; Barlass and Skene, 1986) and regeneration

from callus (Goussard et al, 1990) have been used with some success in grapevine virus elimination programs. However, improvements in the efficiency of these techniques which increased either the rate of virus elimination or the rate of survival of selections subjected to treatments would by desirable.

One approach which has been successful in plant virus elimination (Hansen, 1989) programs has been the use of the antiviral chemotherapeutic ribavirin (1,- -D-ribofuramosyl-1,2,4-triazole-3-carboxamide) (Sidwell et al. 1972). Ribavirin apparently stops viral spread within plant tissue by inhibiting replication (Hansen, 1988). By growing explants on media containing varying concentrations of ribavirin, we should be able to obtain virus free newly growing tissue.

Previous work with ribavirin added to in-vitro cultures of grapevine infected with grapevine leafroll disease suggests that the drug has an effect on virus replication since application delayed the onset of disease symptoms (Stevenson and Monette, 1983). This paper reports on our efforts to develop a more effective approach to administering ribavirin to grapes undergoing virus elimination tissue culture therapy. Varying application rates were tested on V. vinifera 'Cabernet sauvignon' infected with two isolates of grapevine fanleaf virus (GFLV), three isolates of grapevine leafroll associated viruses (GLRaV), and an isolate of a grapevine corky bark associated virus (GCBaV).

MATERIALS AND METHODS

Preliminary experiments. Three different techniques were used to apply ribavirin to infected grape tissue. The first approach involved foliar application of 0, 10, 50, 200, and 800 ppm solutions twice weekly to dormant cuttings which were being rooted in sand with bottom heat. After 6-8 weeks sufficient growth had developed for 2-4 mm shoot tips to be taken and established as tissue culture explants. The second approach was to take 2-4 mm shoot tips and establish them directly on media which contained ribavirin at 0, 10, 50, 100, and 200 ppm. The third technique involved cutting single node cuttings (1-2 cm) of green growing canes (see below for additional details) and establishing them on media with ribavirin at 0, 1, 5, 10, 20, and 50 ppm; after the nodes had produced 2-4 cm of growth, 2-4 mm shoot tips were taken from these explants and established on standard tissue culture media.

Ribavirin as a chemotherapeutic to augment microtip propagation for the elimination of these viruses must be used in a simple, robust procedure to provide an improvement in current virus elimination technology. Problems were discovered with the first two approaches attempted. Foliar application was found to be time consuming and uniform applications were difficult to achieve as the cuttings grew. A long period of time was required before tissue was available to provide explants. The use of media made with varying concentrations of ribavirin was found to simplify the application to infected grape tissue. However, small shoot tips of 2-4 mm

developed poorly on media containing even low concentrations of the drug. Therefore, the third approach, in which nodes obtained from field grown vines were established on media containing ribavirin and the subcultured to normal media, was chosen for further study.

Plant and virus sources. Field grown V. vinifera cv. Cabernet Sauvignon (Davis Foundation Plant Materials Service selection 05) from the Davis grapevine virus collection (Golino, 1992) was used for these experiments. The vines had been graft inoculated in 1989 with wood from vines which indexed positive for the respective viruses of interest. Each vine has subsequently tested ELISA positive for the appropriate virus. Explants were established from healthy vines and from vines infected with six different virus isolates: GFLV100, GFLV108, GLRaV101, GLRaV102, GLRaV106, GLRaV109, and GCBaV100. Young succulent shoots were harvested directly from the virus collection vineyard early in the day into plastic bags, held on ice, and used that day as a source of explant tissue.

Tissue Culture Conditions. Nodes were cut to 2 cm and surface sterilized in a solution of 30% commercial bleach for 15 minutes. They were then triple rinsed in sterile, distilled water under a hood and trimmed to approximately 1 cm while removing the bleach damaged tissue from each end. Finally they were placed in vials containing media at 0, 1, 5, 10, 20, or 50 ppm of ribavirin. The standard media to which the ribavirin was added was 1/2 strength Murashige and Skoog supplemented with 0.1 mg/l IAA, 0.05 mg/l

nicotinic acid, 0.01 mg/l thiamine, 0.05 mg/l pyridoxine, 10.0 mg/l myo-inositol, 0.20 mg/l glycine, 20 g/l sucrose, and 2.5 g/l agar, pH 5.7 0.2, autoclaved for 25 minutes at 121 C. Explants were then cultured in a Percival incubator (Model I-35 LLVL) at 25 1 C with a 16 hour day- 8 hour night.

Explants were allowed to grow until the shoot which developed from each node was 2-4 cm high. At this time, a microtip of 2-4 mm was excised from the shoot and established on normal media to grow until it was ready to be established in soil. In addition, for each virus isolate, a number of plants were established in soil from the original isolate to determine whether the virus had been maintained in that plant.

Tissue Culture Observations. Media containing 50 ppm ribavirin proved toxic. The roots were brown and stunted, there was very little shoot growth, and the tips taken from these explants generally failed to survive even when subcultured on normal media. The explants in 20 ppm ribavirin also had a high mortality rate. Interestingly, the explants on 5 ppm ribavirin seemed to fare better than healthy plants with no ribavirin. They were more vigorous in their growth with less media contamination.

Virus Detection. The indirect ELISA test was used to determine the presence or absence of virus in each plant involved in the experiment (Rowhani, 1992). For this test samples of tissue are taken from the portion of plant known to contain the highest virus

titer when virus is present, phloem tissue (petioles) for the grapevine leafroll associated viruses and grapevine corky bark associated virus, and the tips of plants for grapevine fanleaf virus. The plants were allowed to reach approximately 50 cm before samples were taken for testing. Tissue was homogenized in an alkaline buffer at a concentration of 1:10. The sample was loaded onto a polystyrene plate that was coated with $F(ab')_2$ fragments (partially digested antibodies fragments specific to each virus) for 90 min @ 37 C. Then IgG (whole antibodies specific to the virus), alkaline phosphatase (an enzyme), protein A (a conjugate of the enzyme) and healthy tissue [1:200] are incubated on top of the $F(ab')_2$ and virus (if present). If the virus is present a sort of sandwich forms of $F(ab')_2$ -virus-IgG-enzyme-conjugate. If virus that is specific to the antibody used is not present each of the other portions of the "sandwich" is washed away during rinsing steps between each new addition. The presence of the virus is revealed by a color change from the enzyme reaction that happens when the substrate p-nitrophenyl phosphate is added. The color change was calculated at 405 nm by a Kineticalc Bio-Tek Elisa reader. The absorbance taken was the average of 2 wells. Each test was preformed in triplicate. An absorbance of 3 times the healthy control was considered positive for the virus. Plants that tested negative will be retested in 6 mos. to verify that the negative result was not due to low virus titer. All of the initial testing is complete.

RESULTS

GFLV100 & 108

GFLV was lost during normal tissue culture manipulations for both isolates GFLV100 and GFLV108. Nodal explants should have tested positive since virus isn't eliminated but only inhibited by ribavirin treatment. However, out of 350 GFLV plants tested there were only 8 positives. The loss of GFLV in tissue culture is an interesting effect that has been noted by others (Walker, 1989). Therefore the fanleaf viruses were useless as indicators of ribavirin effectiveness.

GLRaV106

This leafroll virus type shows an increase in the percentage of plants negative for virus that corresponds to an increase in ribavirin concentration. The high number of nodal plants that were negative was unexpected. Possibly the virus titer was so low that it could not be detected. If that is still the case the test after 6 months will be positive. It's also possible that the chemotherapeutic acts strongly enough to inhibit replication on even the most immediate growth of the explant. See figure 1.

Healthy The healthy control acted as expected. All healthy plants were tested once for each virus type in this experiment. All tests were negative. See figure 2.

LR101 Ribavirin had no discernible affect on LR101. Nearly all plants tested were positive for virus regardless of the

concentration of ribavirin. See figure 3.

LR109 also appeared unaffected by ribavirin. That LR101 and LR109 should have the same results is expected since they both are categorized as leafroll type III (meaning they both react to the same type of leafroll antisera). See figure 4.

CB100 This corky bark associated virus type showed a corresponding increase in the percentage of virus negative plants that correlated to increasing concentrations of ribavirin. Along with a higher mortality rate at 50 ppm. See figure 5.

LR102 With this virus all 0 ppm plants were positive. The 1 & 5 ppm ribavirin had roughly equal positive and negative results, but at 10 & 20 ppm ribavirin the tips show a dramatic increase in the percent that are negative. See figure 6

WORK PLAN SEPT 93

Retest all the plants that originally tested negative with the exception of healthy control plants. Correlate all the data.

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LITERATURE CITED

Anonymous. 1985. Production Yearbook, vol. 38. Food and Agriculture organization of the United Nations (FAO), Rome.

Converse, R. H. 1985. Latent Viruses: harmful of harmless? Hort. Sci. 20:845-848.

Goheen, A. C. and Luhn, C. F. 1973. Heat inactivation of viruses in grapevines. Rivista di Patologia Vegetale 9 (Suppl.): 287-289

Golino, D. 1992. The Davis Grapevine Virus Collection. Am. J. Enol. Vit. 43: 43 (2): 200-205.

Hansen, A.J. 1988. Chemotherapy of Plant Virus Infections. pp 285-299 in: Applied Virology Research, Vol. 1. Ed: Kurstak, E., Marusyk, R., Muphy, F., and Van Regenmortel, M.H.V. Plenum Publishing Corp.

Rowhani, A. 1992. Use of F(ab') Antibody Fragment in ELISA for Detection of Grapevine Viruses. Am. J. Enol. Vit. 43: 43(1): 38-40.

Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T., and Robins, R.K. Broad-spectrum antiviral activity of virazole: 1- -D-ribofuramosyl-1,2,4-triazole-3-carboxamide. Science 177: 705-706.

Stevenson, J.H. and Monette, P.L. 1983. Delay of onset of leafroll symptom expression in Vitis vinifera 'Liemberger' from ribavirin treated in-vitro cultures. Can. J. Plant Sci. 63:557-560.

Streeter, D.G., Witkowski, J.T., Khare, G.P., Sidwell, R.W., Bauer, R.J., Robins, R.K., and Simon, L.N. 1973. Mechanisms of Action of 1- -D-ribofuramosyl-1,2,4-triazole-3-carboxamide (Virazole), A New Broad-spectrum Antiviral Agent. Proc. Nat. Acad. Sci. 70: 1174-1178.

Walker M.A. 1989. The Characterization of Resistance to Grapevine Fanleaf Virus in Vitis vinifera. PhD Thesis, University of California, Davis.

GLRaV106

[Ribavirin]		+ Elisa	- Elisa	% Negative	# Explants Started	%Survival Rate
0 ppm	node	6	14	70%	86	23%
	tip	11	9	45%	51	39%
1 ppm	node	9	7	44%	81	20%
	tip	14	7	35%	38	55%
5 ppm	node	8	10	56%	70	26%
	tip	12	25	68%	51	72%
10 ppm	node	4	11	73%	103	15%
	tip	3	21	88%	61	39%
20 ppm	node	2	14	88%	72	22%
	tip	1	16	94%	39	44%
50 ppm	node	0	10	100%	89	11%
	tip	0	1	100%	45	2%

Figure 1: Summary of Elisa results and survival rates for node and tip explants from grapevines with leafroll isolate 106 treated with ribavirin at concentrations from 0-50 ppm.

HEALTHY

[Ribavirin]		+ Elisa	- Elisa	% Negative	# Explants Started	%Survival Rate
0 ppm	node	0	16	100%	72	22%
	tip	0	13	100%	23	56%
1 ppm	node	0	15	100%	72	21%
	tip	0	9	100%	26	35%
5 ppm	node	0	15	100%	72	21%
	tip	0	9	100%	38	24%
10 ppm	node	0	15	100%	72	21%
	tip	0	12	100%	15	80%
20 ppm	node	0	13	100%	72	18%
	tip	0	13	100%	27	59%
50 ppm	node	0	17	100%	71	24%
	tip	0	14	100%	25	56%

Figure 2: Summary of Elisa results and survival rates for healthy control node and tip explants tested for GFLV100 & 108, LR106, LR102, LR101, LR109, CB100. The explants were treated with 0-50 ppm ribavirin concentrations of ribavirin.

GLRaV101

[Ribavirin]		+ Elisa	- Elisa	%Negative	#Explants Started	%Survival Rate
0 ppm	nodes	15	0	0%	109	14%
	tips	14	1	6%	58	26%
1 ppm	nodes	16	0	0%	78	21%
	tips	13	1	7%	53	26%
5 ppm	nodes	17	0	0%	86	20%
	tips	17	1	6%	45	40%
10 ppm	nodes	16	1	6%	84	20%
	tips	15	1	6%	27	59%
20 ppm	nodes	16	0	0%	73	22%
	tips	2	1	50%	28	11%
50 ppm	nodes	0	0		68	0%
	tips	0	0		11	0%

Figure 3: Summary of Elisa and survival results for node and tip explants from grapevines with leafroll virus isolate 101 treated with 0-50 ppm ribavirin.

GLRaV109

[Ribavirin]		+ Elisa	- Elisa	%Negative	#Explants Started	%Survival Rate
0 ppm	node	5	0	0%	55	9%
	tip	10	1	9%	31	35%
1 ppm	node	3	0	0%	53	6%
	tip	10	0	0%	25	40%
5 ppm	node	8	0	0%	54	15%
	tip	13	2	13%	35	37%
10 ppm	node	10	0	0%	54	19%
	tip	8	0	0%	22	36%
20 ppm	node	2	0	0%	53	4%
	tip	8	0	0%	14	57%
50 ppm	node	3	0	0%	51	6%
	tip	0	1	100%	6	17%

Figure 4: Summary of Elisa and survival results of node and tip explants from grapevines with leafroll virus isolate 109 treated with 0-50 ppm ribavirin.

GCBaV100

[Ribavirin]		+ Elisa	- Elisa	%Negative	#Explants Started	%Survival Rate
0 ppm	node	10	5	33%	72	21%
	tip	7	6	46%	47	28%
1 ppm	node	11	3	21%	72	19%
	tip	6	2	25%	33	24%
5 ppm	node	14	4	22%	72	25%
	tip	8	6	43%	47	30%
10 ppm	node	15	5	25%	90	22%
	tip	7	8	53%	41	37%
20 ppm	node	5	8	62%	54	24%
	tip	7	7	50%	35	40%
50 ppm	node	0	3	100%	74	4%
		0	0		25	0%

Figure 5: Summary of Elisa and survival rates for node and tip explants from grapevines with corkybark virus isolate 100 treated with 0-50 ppm ribavirin.

GLRaV102

[Ribavirin]		+ Elisa	- Elisa	%Negative	#Explants Started	%Survival Rate
0 ppm	node	13	0	0%	97	13%
	tip	6	0	0%	37	16%
1 ppm	node	8	8	50%	79	20%
	tip	8	8	50%	41	39%
5 ppm	node	6	7	54%	78	17%
	tip	5	6	55%	31	35%
10 ppm	node	12	5	29%	53	32%
	tip	5	4	44%	16	56%
20 ppm	node	13	10	43%	55	42%
	tip	4	13	76%	22	77%
50 ppm	node	1	1	50%	67	3%
	tip	0	1	100%	6	17%

Figure 6: Summary of Elisa and survival results of node and tip explants from grapevines with leafroll virus isolate 102 treated with 0-50 ppm ribavirin.