Using Dithiouracil and Ribavirin to Eliminate Cymbidium Mosaic Virus during Micropropagation of 'Uniwai Mist' Dendrobium Orchid

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SUMMARY. Our objective was to develop a virucide-based method to eliminate cymbidium mosaic virus from infected dendrobium protocorm-like bodies during micropropagation. We tested two virucides—dithiouracil and ribavirin. The best treatments for recovery of virus-negative tissue and subsequent plant development were 0.1 mM ribavirin or 0.2 mM dithiouracil in liquid medium for 5 or 6 weeks followed by culture on solid medium with 0.1 mM ribavirin or 0.1 mM dithiouracil, respectively. Most propagules transferred to solid medium were virus-free at 17 weeks on ribavirin, with up to 100% free of virus after 32 to 38 weeks on ribavirin or dithiouracil. Virus loss among protocorm-like bodies cultured without virucides ranged from 0% to 37%. Most plants remained free of virus 2.5 years after chemotherapy and flowered normally.

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Orchids are a globally important floricultural crop and are threatened by the mechanically transmitted potex virus cymbidium mosaic virus (CyMV). Floral disease symptoms include necrosis, color break, and flower distortion, yielding unsaleable cut flowers and potted plants. CyMV can also reduce plant vigor and cause irregular blooming, thereby resulting in lost income to the grower (Lawson, 1995; Wisler, 1989).

Many chemical compounds have been tested with plants in attempts to find a virucide that can eliminate virus infection *in vitro* (Hansen, 1989). Two compounds that have shown promising results are the nucleic acid base analogues ribavirin (Virazole; Viratek, Inc., Costa Mesa, Calif.) and dithiouracil (Dawson, 1984; Dawson and Boyd, 1987; Griffiths et al., 1990). In clonal propagation of orchid, ribavirin has been used with varying success to eradicate CyMV from cymbidium, mokara, and dendrobium orchids. Albouy et al. (1988) obtained 90% virus-free plantlets using 0.10 mM ribavirin in solid culture medium with infected protocorm-like bodies (PLBs) of cymbidium. Plants remained free of virus after 2 years in glasshouse culture. Treatment of plantlets and PLBs of thin sections of mokara with 0.04 to 0.08 mM ribavirin in liquid culture yielded a majority of tissues free of CyMV (Lim et al., 1993). Loi et al. (1991) reported CyMV elimination from 'Sonia' dendrobium in 50% to 100% of shoot tips and 25% to 50% of callus using 0.02 to 0.16 mM ribavirin in liquid medium with subsequent plant development on solid medium lacking ribavirin. The use of dithiouracil has not been reported in orchids.

This paper describes development of a protocol to eliminate CyMV from PLBs of 'Uniwai Mist' dendrobium using dithiouracil and ribavirin incorporated into liquid and agar-solidified tissue culture media during micropropagation.

Materials and methods

TISSUE CULTURE. Two strategies tested included 1) chemotherapy during tissue proliferation in liquid culture and 2) chemotherapy during proliferation as well as plantlet development on solid medium. CyMV-infected PLBs of *Dendrobium* Jaquelyn Thomas 'Uniwai Mist', a white cut-

flower cultivar prone to virus symptom expression, were used. PLBs are asexually derived spherical, meristematic structures that, at early stages, closely resemble protocorms formed by germinating seed (Bhojwani and Razdan, 1983; Morel, 1960). PLBs of 'Uniwai Mist' proliferated in Vacin and Went (VW) (Vacin and Went, 1949) liquid medium, pH 4.5, modified to contain 15% (v/v) coconut water and 57 mg·L⁻¹ iron chelate (Sequestrene 330 Fe; gift of D. Hartmann). PLBs were 2 to 5 mm in diameter at the onset of experiments. After the liquid culture phase, tissues grew on VW medium modified with 15% (v/v) coconut water, 75 g·L⁻¹ banana and solidified with 7.5 g·L⁻¹ agar (United States Biochemical Corp., Cleveland) for plant development. Liquid cultures (50 mL per 125-mL flask) were maintained at 26 ± 2 °C on a gyratory shaker at 110 rpm and subcultured weekly. Solid cultures (100 mL per polycarbonate box from G & B Orchids, Vista, Calif.) were maintained at 21 ± 2 °C with infrequent subcultures. Both cultures received 16-h days (16–21 μmol·m⁻²·s⁻¹; 40 W cool-white and Gro-lux Sylvania fluorescent lamps, Danvers, Mass.) and 8-h nights. Light readings were made at plant level.

Stock solutions of dithiouracil (2,4-dithiopyrimidine; Sigma Chemical Co., St. Louis) and ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Viratek) were added to autoclaved VW medium. Stocks were added to completely cooled (i.e., room temperature) liquid medium and to partially cooled (i.e., warm to the touch) solid medium before solidification. Stocks were not filter sterilized.

VIRUS DETERMINATION. Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) in Immulon 2 ELISA plates (Dynatech, Chantilly, Va.) was performed according to Clark and Adams (1977) using rabbit anti-CyMV polyclonal antibodies conjugated with alkaline phosphatase (Hu et al., 1993). Tissue extracts were prepared at a 1:10 (w/v) dilution. Duplicate wells were used per sample. Absorbance values at 405 nm were obtained using a microplate reader (model 450; Bio-Rad, Hercules, Calif.) at the times of 0.5, 1, 2, and once between 18 and 24 h (overnight) after initiation of ELISA plate incubation. Individual ELISA values were compared to known CyMV-positive and CyMV-negative tissues included

Table 1. Frequency of virus elimination from tissue of dendrobium 'Uniwai Mist' after chemotherapy over 5 weeks in liquid proliferation medium, based on enzyme-linked immunosorbent assay (ELISA) performed at intervals after transfer to plant development medium lacking virucide.

Virucide treatment	No. CyMV-negative* plants per total sampled on plant development medium		
	18 weeks ^y	29 weeks ^x	74–76 weeks ^z
None—VW ^w medium only	0/4 (0%)	14/34 (41%)	57/100 (57%)
Ribavirin, 0.1 mM	1/5 (20%)	24/39 (62%)	59/75 (78%)*
Dithiouracil, 0.2 mM	4/5 (80%)	21/40 (53%)	43/49 (88%)*

*Based on ELISA values <0.05 absorbance at 405 nm after a 2-h incubation.

^ySamples were caulogenic protocorm-like body (PLB) clumps. Statistical analysis not conducted due to small sample size.

^zSamples were leaves of individual plants.

^wVW = Vacin and Went medium.

*Significant at $P < 0.005$, based on the chi-square test in a 2×2 contingency table of virucide and no virucide treatments.

on each plate. Values were considered negative if the mean of the replicate wells was <0.05, or was similar to the negative control, for the 2 h and overnight readings (Hu et al., 1993).

LIQUID CULTURE CHEMOTHERAPY. PLBs infected with CyMV were proliferated for 5 weeks in liquid VW culture medium containing virucides and transferred subsequently to solid VW medium lacking virucides. The following range of concentrations was used in individual flasks: dithiouracil, 0.05, 0.15, and 0.2 mM; and ribavirin, 0.01, 0.05, 0.08, 0.1, 0.5, and 0.75 mM. The concentration ranges were determined by a previous replicated growth study (K. Porter, unpublished data). Five control flasks of PLBs were cultured in VW medium lacking virucides. PLBs (initially 5 g fresh mass, corresponding to about 2000 PLBs per flask) were blotted and weighed during weekly subcultures. All tissue, including that which was brown or necrotic, was retained. At the end of 5 weeks, PLBs were placed on solid VW medium for plantlet development and observed for color and percent survival. The frequency of virus elimination was determined by ELISA tests, conducted at the time of transfer to solid medium and 18, 29, and 74 to 76 weeks thereafter, and analyzed by the chi-square test in a 2×2 contingency table of virucide and nonvirucide treatments. Few samples were taken at early time points to maximize tissue available for plant formation.

Virus-free plants from dithiouracil chemotherapy were transferred to the greenhouse about 1 year after subculture to solid medium. After 6 months, 45 individual plants were transplanted from community pots into 6.3-cm-diameter clay pots. Virus-free plants from ribavirin chemotherapy were

transferred to the greenhouse about 1.5 years after subculture to solid medium. After about 5 months, 56 individual plants were potted into 6.3-cm-diameter clay pots. Forty-three virus-free control plants were transferred to the greenhouse and potted individually at the same time as the plants treated with ribavirin. Thirty plants from each treatment were tested for CyMV 2.5 years after chemotherapy (1 to 1.5 years in the greenhouse) and observed for normal growth and flowering. No precautions were taken against manual transfer of virus during greenhouse handling.

LIQUID AND SOLID CULTURE CHEMOTHERAPY. Virus-infected PLBs (5 g initial per flask) were proliferated in four flasks each per compound with 0.2 mM dithiouracil, 0.1 mM ribavirin, or no virucide for 6 weeks. Cultures were then transferred to corresponding solid medium with dithiouracil concentrations of 0.05, 0.1, 0.15, or 0.2 mM; ribavirin concentrations of 0.05, 0.08, 0.1, or 0.5 mM; or no virucides, respectively. ELISA tests and chi-square analysis were conducted on tissues sampled from all flasks at the time of subculture to solid medium and again after 36 to 38 weeks. Thirteen virus-free plants transferred *ex vitro* from the 0.2 mM liquid/0.1 mM solid dithiouracil chemotherapy were transplanted after 10 months into 6.3-cm-diameter clay pots and tested for CyMV.

The best treatments from these two approaches were repeated to verify utility for virus elimination. PLBs (5 g initial per flask) were cultured, in triplicate, in 0.2 mM dithiouracil or 0.1 mM ribavirin for 5 or 6 weeks, then transferred onto solid medium with 0.1 mM dithiouracil or 0.1 mM ribavirin, respectively. Individual shoots or

caulogenic PLB clumps were sampled arbitrarily to assess frequency of virus elimination with the chi-square test at 17 weeks and at 32 to 36 weeks after transfer to solid medium.

Results and discussion

LIQUID CULTURE CHEMOTHERAPY. PLBs of 'Uniwai Mist' in VW medium lacking virucides had a fresh mass doubling time of 2 weeks and remained bright green during the 5 weeks in liquid culture. Use of virucides inhibited PLB growth and increased tissue necrosis at increasing concentrations. Dithiouracil at 0.15 mM produced small, pale green PLBs with a doubling time of 4 weeks, while 0.2 mM dithiouracil produced cultures with 10% brown, necrotic PLBs and a doubling time beyond 5 weeks. Fresh weight doubling time was between 2 and 2.5 weeks for the 0.01, 0.05, and 0.08 mM ribavirin treatments, similar to controls, with <5% necrosis after 5 weeks. PLB from the 0.1 mM ribavirin treatment were relatively small and dark green, with a 3 week doubling time and 10% necrosis after 5 weeks. The 0.5 and 0.75 mM ribavirin treatments produced soft, dead tissue within 6 to 14 days.

ELISA results at transfer to solid medium, for pooled samples of five 0.3 to 0.7 g PLBs each, showed that 0.2 mM dithiouracil either eliminated the virus or lowered the concentration to an undetectable level ($A_{405} = 0.020$; $A_{405} = 0.013$ for CyMV-negative controls; $A_{405} = 0.391$ for CyMV-positive controls). Samples from treatments of 0.05 and 0.15 mM dithiouracil, all concentrations of ribavirin, and all nontreated controls remained CyMV-positive, with 2 h A_{405} values ranging from 0.270 to 0.422.

Individual caulogenic clumps of

Table 2. Frequency of virus elimination from tissue of dendrobium 'Uniwai Mist' after chemotherapy of 6 weeks in liquid^a and 36 to 38 weeks on solid culture media.

Virucide in solid medium (mM)	No. CyMV-negative ^c plants per total sampled
None—VW ^x medium only	1/30 (3%)
Ribavirin	
0.10	4/4 (100%) [*]
Dithiouracil ^w	
0.05	18/30 (60%) [*]
0.10	42/42 (100%) [*]
0.15	6/8 (75%) [*]
0.20	4/8 (50%) ^{v*}

^aIn 0.1 mM ribavirin, 0.2 mM dithiouracil, or no virucide.

^bBased on enzyme-linked immunosorbent assay (ELISA) values <0.05 absorbance at 405 nm after a 2-h incubation.

^cVW = Vacin and Went medium.

^wLow-density plating.

^vSamples from one clump of tissue.

^{*}Significant at $P < 0.001$, based on the chi-square test in a 2×2 contingency table of virucide and no virucide treatments.

PLBs from 0.1 mM ribavirin (the highest concentration of ribavirin where PLBs survived), 0.2 mM dithiouracil, and controls in VW medium were tested for virus 18 weeks after removal from chemotherapy (Table 1). All controls contained virus with the 2 h absorbance reading averages ranging from 0.67 to 0.99. For the ribavirin treatment, one of five samples was virus-free ($A_{405} = -0.022$; $A_{405} = -0.015$ to -0.027 for the negative controls). Four of five 0.2-mM dithiouracil-treated samples were CyMV-free ($A_{405} = -0.022$ to -0.027). CyMV was detectable in the CyMV-positive control dilutions of 1:10 ($A_{405} = 0.823$) down to 1:1000 ($A_{405} = 0.070$).

To maximize tissue available for organogenesis, only a few samples were tested initially for virus. Thus, while the samples reflected only a small part of the population at the time of transfer to solid medium and at 18 weeks after transfer, the ELISA results indicated that some tissues became free of CyMV while other tissues retained virus after a 5-week liquid chemotherapy treatment.

At 29 weeks post-treatment, green plantlets 1 to 2 cm tall were obtained from treatments of 0.05, 0.08, and 0.1 mM ribavirin, 0.05 and 0.2 mM dithiouracil, and no virucide, with a total of 180 to 288 per treatment. Initial loss of PLB during chemotherapy appeared independent of subsequent vigorous growth of surviving tissue, as seen in the controls.

ELISA tests performed at 29 weeks post-treatment showed that many individual plantlets from dithiouracil and ribavirin treatments

were negative for CyMV; sampling at 74 to 76 weeks post-treatment showed these frequencies to differ significantly from that observed among plantlets that received no chemotherapy (Table 1). It is not entirely unexpected that virus was eliminated spontaneously in some control tissues (Table 1), given that 'Uniwai Mist' PLBs are highly meristematic with a rapid doubling time for orchids and that virus loss from meristematic explants is reported for other orchids (Lim et al., 1993; Morel, 1960).

All 30 greenhouse-grown plants from dithiouracil and 87% (26/30) from ribavirin treatment remained free of virus 2.5 years after chemotherapy and appeared similar to virus-free control plants. Racemes with no noticeable irregularities were observed during flowering. Of the controls that had become virus-free spontaneously in vitro, 87% (26/30) remained negative for CyMV in the greenhouse. As this frequency is identical to that of the ribavirin-treated plants, it is highly likely that virus was reacquired by the infected plants during greenhouse handling (Hu et al., 1994).

LIQUID AND SOLID CULTURE CHEMOTHERAPY. The frequency of virus elimination differed significantly between the virucide and no virucide treatments (Table 2). Virus-infected PLBs cultured in VW medium without chemotherapy produced an abundance of green plantlets, with 3% negative for CyMV when tested 36 to 38 weeks after transfer to solid medium. PLBs exposed to 0.05 and 0.1 mM dithiouracil in solid medium also produced many plants, but with 60% and 100% virus elimination, respec-

tively. Most tissues browned and did not form plantlets at dithiouracil concentrations of 0.15 and 0.2 mM. Similarly, plant formation was hindered severely by addition of ribavirin in solid medium; tissues did not survive treatments of 0.05, 0.08, or 0.5 mM ribavirin in solid medium. However, all samples taken of the few green vigorous shoots that formed on 0.1 mM ribavirin were negative for CyMV. Among the plants transferred subsequently to the greenhouse from the 0.2 mM dithiouracil liquid/0.1 mM dithiouracil solid treatment, all 13 remained free of virus after 10 months and displayed normal growth.

Repetition of the liquid and solid culture chemotherapy strategy confirmed elimination of virus by ribavirin. Plants obtained on solid medium with 0.1 mM ribavirin were up to 100% free of virus after 32 to 36 weeks and differed significantly from those cultured without chemotherapy (Table 3). Sampling at the earlier time point of 17 weeks showed a high percentage of plantlets were already negative for CyMV. Moreover, effectiveness of chemotherapy improved ($P < 0.01$) with exposure extended from 17 to 32 to 36 weeks during plant development, but not during PLB proliferation of 5 or 6 weeks.

However, ribavirin was detrimental to tissues of 'Uniwai Mist'. Despite improved plant recovery over the previous ribavirin trial, browning occurred among 30% to 64% of the tissue clumps per box and leaves on green shoots were only 0.5 cm long after 17 weeks in culture. In contrast, no tissue loss occurred among controls and plants were vigorous with leaves about 1 cm

Table 3. Frequency of virus elimination from tissue of dendrobium 'Uniwai Mist' after chemotherapy during culture in liquid then solid media, based on enzyme-linked immunosorbent assay (ELISA).

Virucide media (mM)	No. CyMV-negative ^a tissues per total sampled			
	After 17 weeks on solid medium		After 32–36 weeks on solid medium	
	5 weeks liquid	6 weeks liquid	5 weeks liquid	6 weeks liquid
None—VW ^b medium only	3/30 (10%)	0/24 (0%)	11/30 (37%)	4/30 (13%)
Ribavirin	24/30 (80%)*	19/24 (79%)*	28/28 (100%)*	27/28 (96%)*
0.1 liquid / 0.1 solid				
Dithiouracil	0/30 (0%)	0/24 (0%)	5/30 (17%)	7/30 (23%)
0.2 liquid/ 0.1 solid ^c				

^aBased on ELISA values <0.05 absorbance at 405 nm after a 2-h incubation.

^bVW = Vacin and Went medium.

^cHigh-density plating.

*Significant at $P < 0.001$, based on the chi-square test in a 2×2 contingency table of virucide and nonvirucide treatments.

long. Therefore, it is recommended that sufficient numbers of PLBs be used initially when starting chemotherapy with this virucide.

It was also important that tissues be in direct contact with virucidal medium, (i.e., under low density plating conditions), during plantlet development. Dense growth of tissues from dithiouracil treatment with no intervening subcultures resulted in many clumps that produced shoots not in contact with the virucide. While none of the clumps sampled from the uppermost layer of tissues in one box tested negative for CyMV at 17 weeks, 15% (4/26) of the clumps closest to the medium were free of virus. The low, overall effect of dithiouracil in this second trial (Table 3; maximum virus elimination of 23% versus 100% in first trial) is attributed to rapid exhaustion of medium and loss of direct contact with virucide due to high density plantlet growth. Tissues in the previous trial (Table 2) had been subcultured to fresh medium several times during plant growth to maintain a relatively low plating density.

In conclusion, we validate effectiveness of ribavirin and describe use of a second compound—dithiouracil—in eliminating CyMV from orchids. While treatment of dendrobium PLBs with either of these compounds during proliferation in liquid medium increased the frequency of virus-free tissue, chemotherapy administered during proliferation and plant development (solid medium) stages increased eradication of virus up to 100%. The media combination of 0.2 mM dithiouracil liquid/0.1 mM dithiouracil solid or 0.1 mM ribavirin liquid/0.1

mM ribavirin solid yielded the best results. A rapidly growing culture in itself may lead to some virus eradication and, thus, would be ideal for maximizing the success of chemotherapy. Most plants obtained after chemotherapy remained free of virus for 2.5 years and flowered true to type, confirming results from greenhouse studies by Toussaint et al. (1993) and Albouy et al. (1988) with ribavirin-treated cymbidium PLBs, indicating that dithiouracil and ribavirin are nonmutagenic to plants of dendrobium 'Uniwai Mist'.

Literature cited

- Albouy, J., C. Flouzat, C. Kusaiak, and M. Tronchet. 1988. Eradication of orchid viruses by chemotherapy from in vitro cultures of Cymbidium. *Acta Hort.* 234:413–419.
- Bhojwani, S.S. and M.K. Razdan. 1983. Plant tissue culture: Theory and practice. Developments in crop science. Elsevier Sci., New York.
- Clark, M.F. and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475–483.
- Dawson, W.O. 1984. Effects of animal antiviral chemicals on plant viruses. *Phytopathology* 74:211–213.
- Dawson, W.O. and C. Boyd. 1987. Modifications of nucleic acid precursors that inhibit plant virus multiplication. *Phytopathology* 77:477–480.
- Griffiths, H.M., S.A. Slack, and J.H. Dodds. 1990. Effect of chemical and heat therapy on virus concentrations in in vitro potato plantlets. *Can. J. Bot.* 68:1515–1521.
- Hansen, A.J. 1989. Antiviral chemicals for plant disease control. *Critical Rev. Plant Sci.* 8:45–48.
- Hu, J.S., S. Ferreira, M. Wang, and M.Q. Xu. 1993. Detection of cymbidium mosaic virus, odontoglossum ringspot virus, tomato spotted wilt virus, and potyviruses infecting orchids in Hawaii. *Plant Dis.* 77:464–468.
- Hu, J.S., S. Ferreira, M.Q. Xu, M. Lu, M. Iha, E. Pflum, and M. Wang. 1994. Transmission, movement, and inactivation of cymbidium mosaic and odontoglossum ringspot viruses. *Plant Dis.* 78:633–636.
- Lawson, R.H. 1995. Viruses and their control, p. 74–104. In: *Orchid pests and diseases*. American Orchid Soc., West Palm Beach, Fla.
- Lim, S.T., S.M. Wong, and C.J. Goh. 1993. Elimination of cymbidium mosaic virus and odontoglossum ringspot virus from orchids by meristem culture and thin section culture with chemotherapy. *Ann. Applied Biol.* 122:289–297.
- Loi, J.S., S.M. Lee, L.T. Lam-Chan, S. Fan, and W.H. Yiang. 1991. Eradication of orchid viruses in Dendrobium Sonia using virazole. *Singapore J. Primary Industries* 19:16–22.
- Morel, G. M. 1960. Producing virus-free cymbidiums. *Amer. Orchid Soc. Bul.* 29:495–497.
- Toussaint, A., J. Kummert, C. Maroquin, A. Lebrun, and J. Roggemans. 1993. Use of virazole to eradicate odontoglossum ringspot virus from in vitro cultures of Cymbidium Sw. *Plant Cell Tissue Organ Cult.* 32:303–309.
- Vacin, E.F. and F.W. Went. 1949. Some pH changes in nutrient solutions. *Bot. Gaz.* 110:605–613.
- Wisler, G.C. 1989. How to control orchid viruses: The complete guidebook. Maupin House, Gainesville, Fla.