Elimination of Apple Chlorotic Leafspot Virus from Apple Shoot Cultures by Ribavirin

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ABSTRACT

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Apple shoot cultures systemically infected with apple chlorotic leafspot virus were grown for two periods of about 4 wk each on Murashige and Skoog medium to which the virus inhibitor ribavirin had been added at 10, 20, 40, or 80 μM. Sequential indexing showed that all treated shoots were virus-free after the first and second treatment periods. The resulting rooted shoots remained virus-free during subsequent transfers to ribavirin-free medium, to greenhouse conditions, and to the field. Phytotoxicity was observed in treatments with 40 and 80 μM ribavirin. Twenty cultured shoots treated with the sugar-free ribavirin base remained infected, whereas 20 of 137 untreated control cultures became virus-free. Results indicate that ribavirin treatment of cultured shoots is a reliable and simple method for eliminating apple chlorotic leafspot virus from infected apple.

Additional key word: chemotherapy

Foliar application of ribavirin (Virazole), a guanosine analog, has been shown previously to inhibit the replication of apple chlorotic leafspot virus (CLSV) in *Chenopodium quinoa* (Willd.) and of other viruses in various herbaceous plants (2,3,5,6,15). Lerch (12) and Shepard (17) observed similar but incomplete inhibition of potato virus in shoot and protoplast cultures; Shepard (17) and Cassells and Long (1) carried this one step further and produced virus-free potato plants from infected shoot cultures.

This study was undertaken to determine whether chemotherapy with ribavirin could be used to eliminate CLSV from infected *Malus pumila* Mill. 'Winter Banana' and whether virus-free trees could be obtained this way. The sugar-free triazole base of ribavirin, which can be produced at much lower cost than ribavirin itself, was similarly tested, although it had been found ineffective when applied as a foliar spray to herbaceous plants (A. J. Hansen, *unpublished*).

MATERIALS AND METHODS

CLSV-infected shoot cultures were established from excised Winter Banana tips 2–5 mm long that had been forced for 7 days. Previous indexing had shown that the mother tree was systemically infected with CLSV. Cultures were grown on Murashige and Skoog's (MS) medium (13,14) to which 5 μM of benzyl adenine had been added, following the technique described by Lane and McDougald (10,11). As soon as enough shoots had developed to initiate the experiments, individual shoots were excised from the mother cultures and transferred randomly to treatment media to which filter-sterilized ribavirin or triazole base had been added. All cultures were grown in controlled-environment chambers with a 16-hr simulated day length (5,000 lux supplied by fluorescent lights) and day/night temperatures of 25/21 C.

The virus status of the explants was evaluated when cultures were initiated. The outer leaflets from shoot tips were ground in 1:5 (w/v) of 1% nicotine alkaloid buffer (pH 9.0) and the resulting inoculum was applied to four fully expanded leaves of Carborundum-dusted *C. quinoa*, the local lesion host for CLSV (7). Any tip found free of virus was discarded. Mother cultures of infected shoots were maintained by periodic transfer to fresh basal medium without ribavirin. At the time of each transfer, basal leaves and stems were removed and indexed for CLSV as described. Cultures that gave negative indexing results were discarded. Treatments were carried out in three series, each consisting of two concentrations as follows: 1) 40 and 80 μM ribavirin; 2) 10 and 20 μM ribavirin; and 3) 40 and 80 μM ribavirin base. For each treatment, 10 replicate shoot tips 1–2 cm long were grown in individual vessels containing 10 ml of medium each. After two subculture periods of 3–4 wk on the treatment medium, the shoots of group 3 (ribavirin base) were discarded after indexing and those of groups 1 (high ribavirin) and 2 (low ribavirin) were subcultured twice on a basal medium to induce faster shoot growth and provide enough shoots for rooting. By this time, no maternal tissue remained. About 30 shoots from each treatment were then individually transferred to test tubes containing 10 ml of half-strength MS medium to which 10 μM naphthalene acetic acid had been added. As soon as root initials formed, the plantlets were transferred to sterile potting medium consisting of perlite-peat (1:1) and placed in a mist bed. Established plants were later transplanted into sterile greenhouse soil and kept with the indicator plants in an insect-proof greenhouse at 20–22 C with a minimal day length of 16 hr. After 3–5 mo, the young apple trees were transplanted into a nursery row in the field, where they were indexed by sap check to *C. quinoa* and by budding with the standard Russian seedling indicator.

RESULTS AND DISCUSSION

Index results at the end of the first treatment period (4 wk on ribavirin medium) indicated that the 40- and 80-μM ribavirin treatments had been effective in eliminating CLSV from all shoots or had at least suppressed the virus to undetectable levels (Table 1). On the basis of these results, the second test (group 2) was initiated to determine whether concentrations of ribavirin lower than 40 μM would also be effective. Indexing showed that even with the lowest concentration tested (10 μM), all shoots were virus-free at the end of the first treatment period. The triazole base, on the other hand, had no discernible effect on the virus, because all 10 shoots (at two concentrations of base) remained infected. Treatment with the base was therefore discontinued.

Phytotoxic symptoms were evident in all 10 replicates of the 80-μM treatment and to a lesser degree in the 40-μM treatment. Stunting and poor growth were severe enough at 80 μM to prevent use of this treatment for practical purposes. Shoots in the 20- and 10-μM ribavirin treatments showed no apparent growth reduction but occasionally were mildly chlorotic. Apple shoots seem to be much less sensitive to ribavirin than potato shoots. The latter have been reported to be severely affected by concentrations as low as 10 μM (9).

Virus indexing conducted on all shoots at the beginning of the treatments showed that only 75% were CLSV-infected. This was expected because Fridlund (4) had shown that not all buds on infected 1-yr

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Table 1. Apple chlorotic leafspot virus (CLSV) indexing results of ribavirin-treated and untreated shoots and clumps

Indexing		Treatment (μM)						
		Controls	Ribavirin, group 1		Ribavirin, group 2		Ribavirin base, group 3	
Stage	Days from start	0	80	40	20	10	80	40
Subculture of mother cultures	21	9/10[a]	...	...	...	...	...	...
Treatment start	45	21/27	...	...	...	...	...	...
End of first treatment period	75	26/26	0/10	0/10	0/10	0/10	10/10	10/10
End of second treatment period	105	27/27	0/10	0/10	0/10	0/10	...	...
Shoot multiplication	135	30/42	0/16	0/20	0/6	0/7	...	...
Begin of rooting	165	4/5	0/9	0/8	...	...	...	...
Planted in field, index on *Chenopodium quinoa*	671	8/9	0/14	0/22	0/11	0/20	...	...
Field trees, index on Russian seedling	1,140	9/9	0/14	0/16	0/10	0/10	...	...

[a] Number of CLSV-infected samples/number of samples indexed. Cultures or shoots giving negative index results were discarded as soon as detected; treatments were conducted only with initially fully infected material. Shoots that became contaminated with fungi or bacteria were discarded and not tabulated.

apple bud sticks contain the virus.

Virus-free shoots resulting from ribavirin treatments were grown into young trees and planted in nursery rows in the field. Continued indexing has confirmed that these trees have remained free of CLSV. Because Secor and Nyland (16) have reported that rose ring mosaic seemingly eliminated by ribavirin injection can reappear 2 mo later, the posttreatment indexing time span is of particular significance. However, we consider a similar reappearance of CLSV in our treated trees to be highly unlikely because our shoot tips were not treated by injection but were continuously exposed to ribavirin for about 8 wk and because the trees have remained free of CLSV for 3 yr after the end of the treatment (Table 1).

The sugar-free triazole base of ribavirin was included in the tests because it has been shown to be the virus-inhibiting moiety of the molecule, at least for animal viruses (8,18), and because it is considerably cheaper. We had expected that more intimate and continuous contact between the base in the medium and the shoots might lead to a certain amount of uptake into the virus-carrying tissue. However, the results indicate that CLSV replication in the ribavirin base-treated shoots was not inhibited and that the sugar moiety is apparently needed for antiviral activity.

Indexing results of the untreated controls in all three groups allowed a rough estimate of the frequency of spontaneous virus loss from shoot cultures because all shoots used in the experiments were virus-infected at the beginning of the experiment. During the first two transfers, all untreated control cultures (26 and 27, respectively) remained infected, whereas 12 of 42 cultures were apparently virus-free after the third transfer, and one of five cultures was virus-free after the fourth transfer (Table 1). Uninfected shoots were detected in three of nine otherwise infected shoot clusters transferred during the experiments.

From a practical point of view, ribavirin treatment of infected shoot cultures seems to be a simple and effective way to eliminate CLSV from apple. Elimination of the virus from all treated shoots drastically reduces the need to index large numbers of trees after treatment. The method would presumably be similarly effective against CLSV in other hosts and against other ribavirin-susceptible viruses. This is the first report of elimination of sap-transmissible virus from a woody host by ribavirin.

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LITERATURE CITED

1. Cassells, A. C., and Long, R. D. 1980. The elimination of potato viruses X, Y, S and M in meristem and explant cultures of potato in the presence of virazole. Pot. Res. 25:165-173.
2. Cassells, A. C., and Long, R. D. 1982. The regeneration of virus-free plants from cucumber mosaic virus- and potato virus Y-infected tobacco explant cultures in the presence of virazole. Z. Naturforsch. 35c:350-351.
3. Fazio, D., de, Caner, J., and Vicente, M. 1978. Inhibitory effect of Virazole (ribavirin) on the replication of tomato white necrosis virus. Arch. Virol. 58:153-156.
4. Fridlund, P. R. 1982. Distribution of chlorotic leaf spot virus infected buds on various lengths of apple budsticks in successive years. Acta Hortic. 130:85-87.
5. Hansen, A. J. 1979. Inhibition of apple chlorotic leaf spot virus in *Chenopodium quinoa* by ribavirin. Plant Dis. Rep. 63:17-20.
6. Hansen, A. J. 1984. Effect of ribavirin on green ring mottle causal agent and necrotic ringspot virus in *Prunus* species. Plant Dis. 68:216-218.
7. Hansen, A. J., and Gilmer, R. M. 1976. Apple chlorotic leaf spot and other filamentous virus infections in Prunus. Pages 206-208 in: Virus Diseases and Non-infectious Disorders of Stoned Fruits in North America. U.S. Dep. Agric. Agric. Handb. 437. Washington, DC.
8. Harris, S., and Robins, R. K. 1980. Ribavirin: Structure and antiviral activity relationships. Pages 1-21 in: Ribavirin—A Broad-Spectrum Antiviral Agent. R. A. Smith and W. Kirkpatrick, eds. Academic Press, New York.
9. Klein, R. E., and Livingston, C. H. 1982. Eradication of potato virus X from potato by ribavirin treatment of cultured potato shoot tips. Am. Potato J. 59:359-365.
10. Lane, W. D. 1978. Regeneration of apple plants from shoot meristem-tips. Plant Sci. Lett. 13:281-285.
11. Lane, W. D., and McDougald, J. M. 1982. Shoot tissue culture of apple: Comparative response of five cultivars to cytokinin and auxin. Can. J. Plant Sci. 62:689-694.
12. Lerch, B. 1977. Inhibition of the biosynthesis of potato virus X by ribavirin. Phytopathol. Z. 89:44-49.
13. Murashige, T., and Barnhill-Jones, J. 1974. Callus and organ culture methods in virus disease therapy. Pages 207-222 in: International Symposium on Virus Diseases of Ornamental Plants. Int. Soc. Hortic. Sci. (The Hague), Netherlands.
14. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.
15. Schuster, G. 1976. Effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole) on the multiplication of systemic viruses in *Nicotiana tabacum* 'Samsun.' Inst. Tabakforschung 23:21-26.
16. Secor, G. A., and Nyland, G. 1978. Rose ring pattern: A component of the rose-mosaic complex. Phytopathology 68:1005-1010.
17. Shepard, J. F. 1977. Regeneration of plants from protoplasts of potato virus X-infected tobacco leaves. II. Influence of virazole on the frequency of infection. Virology 78:261-265.
18. Smith, R. A. 1980. Mechanisms of action of ribavirin. Pages 99-118 in: Ribavirin—A Broad-Spectrum Antiviral Agent. R. A. Smith and W. Kirkpatrick, eds. Academic Press, New York.