In Vitro Cell. Dev. Biol.—Plant 36:65–68, January–February 2000

1054-5476/00 $10.00+0.00

VIRUS INFECTIONS REDUCE *IN VITRO* MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY‡

CHIH-WEI V. TSAO[1†], JOSEPH D. POSTMAN[2] AND BARBARA M. REED[2]*

[1]*Department of Horticulture, Oregon State University, Corvallis, Oregon 97331-7304*
[2]*USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd., Corvallis, Oregon 97333-2521*

(Received 25 June 1999; accepted 22 October 1999; editor M. R. Becwar)

SUMMARY

Virus-infected plants are often symptomless and may be inadvertently used as explant sources in tissue culture research. Our objective was to determine the effect of virus infection on micropropagation. We studied the effects of single and multiple infections of three common raspberry viruses on the *in vitro* culture of 'Malling Landmark' red raspberry (*Rubus idaeus* L.). Virus-infected raspberry plants were produced by leaf-graft inoculation from known-infected plants onto virus-free 'Malling Landmark'. Single-virus source plants were infected with either tobacco streak ilarvirus (TSV), tomato ringspot nepovirus (TomRSV), or raspberry bushy dwarf idaeovirus (RBDV) and were free of other viruses as determined by enzyme-linked immunosorbent assay (ELISA) and bioassay. Virus-free, single, and multiple virus-infected 'Malling Landmark' explants were initiated into culture and multiplied on Anderson's medium with 8.9 μM N^6-benzyladenine (BA). At the end of the multiplication tests, ELISA reconfirmed virus infections. *In vitro* multiplication of 'Malling Landmark' was significantly reduced by multiple infections, and multiplication of plants infected with all three viruses (RBDV + TomRSV + TSV) was less than half that of virus-free cultures. Shoot height and morphology of *in vitro* cultures were not influenced by virus infection. The greenhouse stock plant with the three-virus infection was stunted and yellow compared to the control and the other infected plants.

Key words: Rubus; micropropagation; raspberry bushy dwarf virus; tomato ringspot virus; tobacco streak virus.

INTRODUCTION

The virus status of explants for *in vitro* culture is often unknown, but may play an important role in micropropagation. Inconsistent results and death of plant materials in tissue culture experiments might be due to undetected virus infection. Many raspberry cultivars are susceptible to several virus diseases in the field, and sensitive cultivars may be killed or severely weakened by virus infection (Frazier, 1970). Latent infections on tolerant cultivars may shorten the planting life of a field through reduced yield and fruit quality (Converse, 1963). Raspberry bushy dwarf idaeovirus (RBDV) and tobacco streak ilarvirus (TSV) are distributed world-wide and RBDV is transmitted by pollen. RBDV is often symptomless in either naturally or experimentally infected red raspberries (Murant, 1987), however 'crumbly fruit' and a 'yellows' symptom may occur under some conditions in sensitive cultivars (Jones et al., 1982). Jones (1979) found that RBDV co-infected with black raspberry necrosis virus caused 'bushy dwarf' symptoms in the field. TSV-infected *Rubus* plants do not generally show foliar symptoms, but may produce a chlorotic leaf pattern, chlorotic ringspot, or line-pattern symptoms when infection occurs with other viruses (Stace-Smith, 1987).

Symptoms of tomato ringspot nepovirus (TomRSV), which is soil transmitted via nematodes, may be visible throughout the growing season in raspberry. New leaves may show yellow rings, line patterns, or a fine yellow vein chlorosis in the spring following the year of infection (Stace-Smith and Converse, 1987). Hot weather tends to reduce symptom severity. Stace-Smith (1987) indicated that infection with TomRSV in a complex with other viruses induced a fernleaf mosaic symptom not seen in single infections. Chronic TomRSV infection causes dwarfing and slow growth in the spring. Spring cane death may occur and surviving canes produce small leaves with early fall abscission (Freeman and Stace-Smith, 1968).

Incidence of viruses in the *Rubus* accessions received at the National Clonal Germplasm Repository (NCGR) in Corvallis, OR, ranges from 0.31% of TomRSV (637 accessions tested), 2.76% of TSV (724 accessions), and 5.77% of RBDV (745 accessions) (J. D. Postman, unpublished). It is known that virus-infected tissue can have reduced *in vitro* growth compared to non-infected tissue. De Vries-Paterson et al. (1992) first reported the effects of single and two-virus infections in reducing asparagus *in vitro* root development, survival in culture, and the fresh and dry weights of micropropagated plants. *In vitro* shoot-tip culture alone, without other virus-elimination treatments, resulted in only 8.6% virus-free asparagus clones. Establishment of *in vitro* sugarcane cultures was more successful from virus-free material than from material showing

*Author to whom correspondence should be addressed: Email reedbm@bcc.orst.edu

†Part of a thesis submitted by C.-W.V.T. in partial fulfilment of the requirements for the MS degree.

‡The use of trade names in this publication does not imply endorsement by the U.S. Department of Agriculture or Oregon State University.

TABLE 1

VIRUS COMBINATIONS AND NUMBER OF BOXES (BLOCKS) USED FOR 'MALLING LANDMARK' RASPBERRY *IN VITRO* MULTIPLICATION TESTS

	No. of boxes[a] (culture period)			
Infecting virus (treatment)	1 (5–7 mo.)	2 (9–11 mo.)	3 (13–15 mo.)	4 (17–19 mo.)
Control (virus-free plant)				
TSV (tobacco streak virus)				
RBDV (raspberry bushy dwarf virus)				
TomRSV (tomato ringspot virus)				
RBDV + TomRSV				
RBDV + TSV				
RBDV + TomRSV + TSV				

[a] Ten shoots per box.

symptoms of maize streak virus (Peros et al., 1990). Most explants used for tissue culture research are not tested for virus infection and are not produced from heat-treated meristems. Combined heat treatment and meristem-culture are successfully used to initiate virus-free raspberry cultures (Pyott and Converse, 1981).

There are no reports on the effects of viruses on raspberry shoot cultures. 'Malling Landmark' was selected for this study because it shows mosaic symptoms, is used as a virus indicator plant, and can be multiplied *in vitro*. Virus-infected greenhouse-grown stocks, in addition to well-established virus-indexed 'Malling Landmark' raspberry plants were available at NCGR. The purpose of this study was to determine the effects of single and multiple virus infections on the *in vitro* propagation of 'Malling Landmark' raspberry.

Materials and Methods

Plant materials and virus cultures. Plants used in this study were grown from root cuttings made from a virus-free clone of *Rubus idaeus* L. cv. Malling Landmark raspberry (NCGR accession CRUB-96.001). This plant tested negative for tobacco ringspot virus (TobRSV), tomato ringspot virus (TomRSV), and tobacco streak virus (TSV) by standard double-antibody-sandwich ELISA (Clark and Adams, 1977) and for raspberry bushy dwarf virus (RBDV) by triple-antibody ELISA using a monoclonal antibody to decorate trapped virus followed by alkaline-phosphatase conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). This plant also tested negative for mechanically transmitted viruses using *Chenopodium quinoa* Willd. as the assay host, and for graft-transmitted viruses using *Rubus occidentalis* L. as the assay host with standard bioassays for *Rubus* viruses (Converse, 1987).

Rubus plants infected with assorted viruses are maintained as virus stock cultures at NCGR-Corvallis (Postman, 1998). *Rubus* plants infected with raspberry bushy dwarf (CRUB-9008), tomato ringspot (CRUB-9011), and tobacco streak viruses (CRUB-9003) were used as inoculum sources. These single-virus source plants were free of other viruses as determined by ELISA and by bioassay (unpublished NCGR virus indexing records). We chose three common raspberry viruses (TomRSV, RBDV, and TSV) for this study because antisera against these viruses were available for subsequent ELISA analysis. Antiserum PVAS-239 against TomRSV was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Dr. R. Martin (USDA-ARS, Corvallis, OR) provided polyclonal and monoclonal antisera against RBDV, and polyclonal antiserum against TSV.

Establishment of virus-infected 'Malling Landmark'. Daughter plants of virus-free 'Malling Landmark' were inoculated by leaf grafting (Frazier, 1974) from each of the three virus cultures individually and in all possible combinations to establish plants with single, double, and triple virus infections. Inoculated 'Malling Landmark' plants were assayed by ELISA several months after inoculation, and again during subsequent growing seasons. Single plants were selected, based on ELISA results, which were infected with individual viruses as well as with multiple infections. An uninoculated 'Malling Landmark' plant was used as a healthy control.

Establishment of in vitro cultures. Nodal cuttings (~3.0 cm) of virus-free and infected 'Malling Landmark' were collected in May, 1997 from greenhouse-grown plants. All but newly forming leaves were removed; explants were washed under running water for 5 min, surface-disinfected in a 10% bleach solution (0.525% sodium hypochlorite) with 0.1 ml l^{-1} of Tween 20 (Sigma) on a rotary shaker for 10 min, and rinsed twice in sterile water (5 min each).

All materials were initiated in 16 × 100-mm tubes of contaminant detection medium, half-strength MS liquid medium (Murashige and Skoog, 1962) with 3% sucrose, without growth regulators, adjusted to pH 6.9 (Reed et al., 1995). After 7 d shoots were transferred to Anderson's medium (Anderson, 1980) with 3% sucrose, 0.5 μM indole-3-butyric acid (IBA), 0.3 μM gibberellic acid (GA_3), and 8.9 μM N^6-benzyladenine (BA) (Reed, 1990). Medium was solidified with a combination of 0.35% agar (Difco granulated agar, Detroit, MI) and 0.145% Gelrite (Schweitzer-Hall, South Plainfield, NJ) and adjusted to pH 5.7 before autoclaving. Growth-room conditions were a 16-h photoperiod (cool white fluorescent illumination, 25 μmols m^{-2} s^{-1}) at 25°C. Plantlets were multiplied in GA7 Magenta boxes (Magenta Corp., Chicago, IL) and maintained with a 3-wk transfer cycle.

Virus indexing of in vitro cultures. Prior to the multiplication experiment at the fifth month in culture, two plantlets from each of three boxes for each culture and two young leaves from greenhouse stocks (one pot each) were randomly chosen for virus indexing. ELISA tests were repeated to confirm viral infections of experimental plant materials at the 9th and 17th months in culture.

Multiplication test. Multiplication tests were started after the fifth month in culture by placing 10 shoots (10 mm) in each box for the seven treatments. The four repeats of the experiment had different numbers of replicates (boxes), due to the availability of plant material (Table 1). All boxes were placed randomly in three plastic trays (blocks) side by side on the same shelf. Microshoots were multiplied and shoots 5 mm or longer were divided and transferred on a 3-wk cycle during each 12-wk experiment. Number and length (mm) of shoots were recorded.

Experimental design and statistical analysis. A randomized block design was used with uneven replicates (Magenta boxes) for each treatment within the four culture periods. Analyses followed the general linear model (GLM) procedure using SAS programming for the analysis of variance of virus treatment and culture period (SAS 6.12, 1989–1996; SAS Institute Inc., Cary, NC). The results of statistical analysis were considered significant at $P \leq 0.05$. Overall means include predicted values for missing data.

Results and Discussion

The virus-infected greenhouse-grown 'Malling Landmark' plants were symptomless and were indistinguishable from the controls, except for the plant infected with RBDV + TomRSV + TSV. This plant was stunted and had 'yellows' symptoms (data not shown). This is similar to growth under field conditions where virus symptom development is dependent on the virus or viruses present (Daubeny et al., 1982; Diekmann et al., 1994). The TomRSV-infected culture was initially very slow to multiply and was not

TABLE 2

MICROSHOOT PRODUCTION FROM VIRUS-INFECTED 'MALLING LANDMARK' RASPBERRY CULTURES DURING FOUR 12-WK CULTURE PERIODS

Treatment	Culture periods[a,b] 1 (5–7 mo)	2 (9–11 mo)		4 (17–19 mo)	Treatment means[c]
Control (virus-free plant)		Ax			
TSV (tobacco streak virus)		Axy			
RBDV (raspberry bushy dwarf virus)		Ay			
TomRSV(tomato ringspot virus)[d]					
RBDV + TomRSV		14.0 Ay			
RBDV + TSV		17.7 Ay			
RBDV + TomRSV + TSV		10.7 Az			
Culture period means	16.5 x	17.2 x	55.9 z	32.3 y	

[a]Means in a column with different capital letters are significantly different at $P < 0.05$ by LSD.
[b]Means in a row with different small letters are significantly different at $P < 0.05$ by LSD.
[c]Treatment and culture period means include the predicted values for missing treatments generated by the General Linear Model of SAS.
[d]TomRSV was not included in the statistical analysis because it was not available in the first two culture periods. Predicted values for the missing data in the first two experiments of TomRSV were 30.6 and 31.3.

included in the first two experiments due to a shortage of plant material. ELISA tests confirmed that all *in vitro* plants remained infected with the expected viruses throughout the multiplication experiments and non-infected control plants remained virus free (data not shown).

There was a significant interaction between virus-infection treatment and the culture period ($P < 0.001$) for shoot multiplication. There were no differences among the seven treatments in the number of microshoots produced during the first and second culture periods (Table 2). All except the three-virus culture produced significantly ($P \leq 0.001$) more shoots in the third period (13–15 mo) than earlier, and shoot production declined slightly but not significantly in the fourth culture period. This is similar to the results of Jin et al. (1992) who noted irregular multiplication of *Rubus* shoots during extended culture. Multiplication of the three-virus culture was not significantly different over the four culture periods. These results indicate the need for repetition of experiments over time to alleviate differences caused by variable multiplication rates.

Overall treatment means showed significantly less multiplication by shoots infected with two or three viruses compared to the control and two of the single-virus infections (Table 2). There were no significant differences in multiplication among the treatments for the first two culture periods. All but the three-virus culture significantly increased multiplication during the third period and differences in multiplication by treatment became apparent. The double and triple infections resulted in less multiplication than the controls or the single virus infections for both the third and fourth culture periods. The mean multiplication of the control, TSV and TomRSV-infected cultures were high for the last two culture periods and were not significantly different ($P \leq 0.05$).

Shoot lengths among virus treatments and the virus-free 'Malling Landmark' culture were not significantly different in this study ($P < 0.05$) (data not shown). Most other studies of *in vitro* cultured plants also show no significant differences in shoot length with virus-infected or non-infected shoots. Potato cv. Igor infected with potato virus Y^{NTN} accumulates jasmonic acid in the roots, and roots are significantly shorter, but there are no significant differences in the average length of infected and healthy potato plantlets (Petrovic et al., 1997). Fresh and dry weights of infected potato shoots and roots are reduced but no *in vitro* shoot symptoms appear, such as the stunting, necrosis, and mosaic patterns normally observed on field plants (De Vries-Paterson et al., 1992; Petrovic et al., 1997). The only case of virus and virus-like agents affecting both the number and size of shoots produced is in nodal citrus-stem segments (Greno et al., 1988).

Growers and researchers working with blackberry and raspberry cultures as well as other plant species should be aware of the problems involved with using virus-infected stock plants. Viruses contaminating *in vitro* cultures may have significant impacts on multiplication. This study showed the effects of three common raspberry viruses on 'Malling Landmark' shoot cultures. These viruses are often symptomless on *Rubus* stock plants and *in vitro* cultures, but other plants or other viruses may produce definite *in vitro* symptoms, such as streak symptoms in *in vitro* sugarcane infected with maize streak virus (Peros et al., 1990). Although NCGR greenhouse stock plants infected with all three viruses showed little apparent growth inhibition, *in vitro* cultures with two or three viruses multiplied more slowly than controls or single virus-infected cultures. Shoot elongation was not impacted in this study. The elimination of viruses from stock plants can make a significant difference in experimental results from multiplication studies and would likely impact yield from commercial micropropagation.

ACKNOWLEDGEMENT

We appreciate the assistance with data analysis by Dr. David Thomas of the Oregon State University, Department of Statistics.

REFERENCES

Anderson, W. C. Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *Rubus occidentalis*. Acta Hortic. 112:13–20; 1980.

Clark, M. F.; Adams, A. N. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475–483; 1977.

Converse, R. H. Influence of heat-labile components of the raspberry mosaic complex on growth and yield of red raspberries. Phytopathology 53:1251–1254: 1963.

Converse, R. H., ed. Virus diseases of small fruits. USDA Agriculture Handbook No. 631; 1987.

Daubeny, H. A.; Freeman, J. A.; Stace-Smith, R. Effects of raspberry bushy dwarf virus on yield and cane growth in susceptible red raspberry cultivars. HortScience 17:645–647; 1982.

De Vries-Paterson, R. M.; Evans, T. A.; Stephens, C. T. The effect of asparagus virus infection on asparagus tissue culture. Plant Cell Tiss. Org. Cult. 31:31–35; 1992.

Diekmann, M.; Frison, E. A.; Putter, T. FAO/IPGRI technical guidelines for the safe movement of small fruit germplasm. Food and Agriculture Organization of the United States/International Plant Genetic Resources Institute, Rome; 1994.

Frazier, N. W., ed. Virus diseases of small fruits and grapevines: Berkeley, CA, University of California Division of Agricultural Science; 1970.

Frazier, N. W. Detection of graft-transmissible diseases in strawberry by a modified leaf grafting technique. Plant Dis. Rep. 58:28–31; 1974.

Freeman, J. A.; Stace-Smith, R. Effect of tomato ringspot virus on plant and fruit development of raspberries. Can. J. Plant Sci. 48:25–29; 1968.

Greno, V.; Navarro, L.; Duran-Vila, N. Influence of virus and virus-like agents on the development of citrus buds cultured *in vitro*. Plant Cell Tiss. Org. Cult. 15:113–124; 1988.

Jin, W.; Gu, Y.; Zhen, S. Z. *In vitro* propagation of *Rubus* species. Sci. Hortic. 49:335–340; 1992.

Jones, A. T. The effects of black raspberry necrosis and raspberry bushy dwarf viruses in Lloyd George raspberry and their involvement in raspberry bushy dwarf disease. J. Hortic. Sci. 54:267–272; 1979.

Jones, A. T.; Murant, A. F.; Jennings, D. L.; Wood, G. A. Association of raspberry bushy dwarf virus with raspberry yellows disease; reaction of *Rubus* species and cultivars, and the inheritance of resistance. Ann. Appl. Biol. 100:135–147; 1982.

Murant, A. F. Raspberry bushy dwarf. In: Converse, R. H., ed. Virus diseases of small fruits. Washington D.C.: United States Department of Agriculture, Agricultural Handbook No. 631; 1987:229–234.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473–479; 1962.

Peros, J. P.; Bonnel, E.; Reynaud, B. *In vitro* culture of sugarcane infected with maize streak virus (MSV). Plant Cell Tiss. Org. Cult. 23:145–149; 1990.

Petrovic, N.; Miersch, O.; Ravnikar, M.; Kovac, M. Potato virus Y^{NTN} alters the distribution and concentration of endogenous jasmonic acid in potato plants grown *in vitro*. Physiol. Mol. Plant Pathol. 50:237–244; 1997.

Postman, J. D. Temperate fruit germplasm borne pathogen collections at NCGR-Corvallis. Acta Hortic. 471:111–114; 1998.

Pyott, J. L.; Converse, R. H. *In vitro* propagation of heat-treated red raspberry clones. HortScience 3:308–309; 1981.

Reed, B. M. Multiplication of *Rubus* germplasm *in vitro*: a screen of 256 accessions. Fruit Var. J. 44:141–148; 1990.

Reed, B. M.; Buckley, P. M.; DeWilde, T. N. Detection and eradication of endophytic bacteria from micropropagated mint plants. In Vitro Cell. Dev. Biol. 31P:53–57; 1995.

Stace-Smith, R. Tobacco streak virus in *Rubus*. In: Converse, R. H., ed. Virus diseases of small fruits. Washington, D.C.: United States Department of Agriculture, Agricultural Handbook No. 631; 1987:235–239.

Stace-Smith, R.; Converse, R. H. Tomato ringspot virus in *Rubus*. In: Converse, R. H., ed. Virus diseases of small fruits. Washington, D.C.: United States Department of Agriculture, Agricultural Handbook No. 631; 1987:223–227.