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Use of *in vitro* Cultures of *Daphne cneorum* L. for the Western Detection of Daphne Virus X[1]

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Abstract

In vitro cultures of *Daphne cneorum* L. were successfully used as test samples for the detection of the viral coat protein of daphne virus X (DVX) by Western analysis. This powerful serological technique could thus be used where viral antigen concentrations may be very low, such as in the early screening of *in vitro*-cultured "heat therapy" tips from virus elimination programs. Western analysis was conducted on randomly selected *D. cneorum* plants from a commercial nursery and on *in vitro* cultures from a commercial micropropagation lab. All the plants and cultures tested were DVX-infected, supporting the view that this ornamental shrub may be universally infected with DVX.

Index words: tissue culture, serology, antibodies, electrophoresis

Significance to the Nursery Industry

The suitability of *in vitro* cultures of *D. cneorum* as samples in Western analysis for DVX, demonstrated in this report, should be of interest to daphne micropropagators concerned with the quality of their product, and should be of particular importance to personnel engaged in plant health improvement or certification programs. The procedure described in this report should permit an early screening of *in vitro*-cultured "heat therapy" tips, thereby reducing the expenditure of valuable resources on cultures which are still virus-infected. The feasibility of directly testing *in vitro* cultures eliminates the need for regenerating plantlets from the cultures prior to virus testing. This should result in substantial savings in both labor and time and should accelerate the release of virus-free daphne to Industry. The use of *in vitro* cultures as samples for Western analysis should permit the detection of other viral pathogens in a wide variety of crops, provided the corresponding antisera are available.

Introduction

North American growers are becoming increasingly aware of the value of propagating virus-free plants, especially when addressing export markets. One procedure which is widely utilized for the production of virus-free plants consists of subjecting virus-infected plants to a heat therapy regime, removing the growing tips from the heat-treated plants after various treatment periods and culturing these "heat therapy" tips *in vitro*. Plantlets are then regenerated from the *in vitro* cultures and maintained in the greenhouse for several months before they are tested for the presence of virus. The conventional method for detecting DVX consists of rub-inoculating homogenates of leaves from the plant to be tested into herbaceous diagnostic species and monitoring the inoculated plants for the development of disease symptoms. The delay between plantlet regeneration and virus testing reflects the concern that some "heat therapy" tips might contain a concentration of virus particles too low to be detected by this bio-assay. Maintaining the plantlets in the greenhouse for a few months before herbaceous testing is thought to permit the virus concentrations to increase to levels which are detectable by this method.

Daphne cneorum L. is a member of the family Thymelaeaceae. This evergreen shrub from the mountains of Middle and Southern Europe (9) is highly valued due to its hardiness, compact growth and very fragrant carmine-pink flowers. All *D. cneorum* plants tested to date have been found to be infected with a potexvirus, daphne virus X (DVX), which is spread by vegetative propagation (5, 6; A.W. Chiko and S.E. Godkin, *unpublished*). DVX infection is not associated with any obvious symptoms (6). However, no report has yet been published comparing the growth rates or vigor of DVX-infected and virus-free *D. cneorum*.

In order to allow a more efficient use of the resources of plant health regulatory labs and to accelerate the release of virus-free daphne, an alternative DVX detection procedure was sought. The procedure needed to be DVX-specific, very sensitive, more rapid than herbaceous testing and directly applicable in the early screening of *in vitro*-cultured "heat therapy" tips. Accordingly, the use of *in vitro* cultures of *D. cneorum* as sample material for the detection of DVX was evaluated in conjunction with Western analysis, one of the most powerful serological techniques available for the detection of virus coat protein. Various commercial laboratories offer micropropagated daphne for sale, and these facilities also require a rapid, sensitive and labor-efficient method of evaluating and monitoring the virus-disease status of their cultures. This communication reports on the suitability of *in vitro* cultures of *D. cneorum* as samples for the detection of DVX by Western analysis. The results obtained when the procedure was applied to randomly selected *D. cneorum* from two commercial facilities are also reported.

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Materials and Methods

The first stage of this experiment consisted of validating the use of Western analysis for the detection of DVX in daphne. This was accomplished by: 1) verifying by herbaceous testing and immunosorbent electron microscopy (ISEM) that the *D. cneorum* specimens used were DVX-infected, and 2) conducting a Western analysis of the DVX-infected specimens to confirm that the major protein band detected corresponded in size to the DVX coat protein. The second stage consisted of determining whether *in vitro* cultures initiated from DVX-infected *D. cneorum* specimens were suitable as samples for Western analysis. Finally, randomly selected *in vitro* cultures provided by a commercial micropropagation lab were tested for DVX by Western analysis.

Herbaceous testing. The greenhouse-maintained *D. cneorum* specimens used in this study were generously provided by a local nursery. Samples from each of six plants were ground in 0.5% bentonite plus 0.5% K_2HPO_4 (16), and the homogenate was rub-inoculated into Celite-dusted leaves of 4-week old *Nicotiana clevelandii* Gray seedlings. Uninoculated control *N. clevelandii* were maintained alongside the inoculated test plants. The herbaceous plants were maintained in a greenhouse at 20/18°C (68/64°F; day/night) minimum temperatures and monitored daily for the development of disease symptoms.

ISEM. Leaf discs from symptomatic *N. clevelandii* were ground in 0.01 M $MgCl_2$. Grids backed with Formvar-carbon films were sensitized by floating on droplets of a 1:1000 dilution of rabbit anti-DVX serum (provided by A.W. Chiko) in 0.06 M potassium phosphate buffer, pH 7.0 (6-PB), for 1 hour. The grids were transferred to droplets of the homogenate and virus particle trapping was allowed to take place for 1 hour. Grids were then transferred to droplets of a 1:100 dilution of serum in 6-PB for 40 min to decorate the trapped particles with antibodies. Grids were rinsed between steps with a gentle stream (about 1 ml) of 6-PB. Distilled water was used as a final rinse, prior to staining with 2% uranyl acetate. Grids were examined with a JEOL JEM-100C electron microscope.

In vitro culture. Shoots from DVX-infected *D. cneorum* were collected from greenhouse-maintained plants, surface sterilized by vigorous stirring for 15 min in 0.6% commercial sodium hypochlorite containing 0.1% Tween 20, and rinsed aseptically in sterile distilled water. The portion of the shoots infiltrated with sterilant was cut off and the remaining explants were placed, two per tube, in 25 × 150 mm culture tubes containing 15 ml medium, and capped with polypropylene closures (Bellco Kaputs). The culture initiation medium was "woody plant medium" (12) supplemented with 0.5 mg/l gibberellic acid (GA_3) and 1 mg/l N^6-benzylaminopurine (BAP). The cultures were transferred after 4 weeks into proliferation medium, identical in composition to the initiation medium, except that 2 mg/l N^6-[2-isopentenyl]adenine (2iP) was substituted for the GA_3 and BAP. Cultures were maintained at 20 ± 2°C (68 ± 4°F) with a 16-hour photoperiod at about 2000 lux provided by cool-white fluorescent tubes. Cultures were transferred to fresh proliferation medium every 4 weeks.

Electrophoresis and Western blotting. Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (10) was conducted on 0.75 mm-thick 15% polyacrylamide (29.2:0.8, acrylamide:bis-acrylamide) vertical gels with a 3% stacking gel in a Bio-Rad Mini Protean II system (Bio-Rad, Richmond, CA). Samples consisting of leaves from greenhouse-maintained *D. cneorum* or *in vitro* cultures of *D. cneorum* were ground in eight or four volumes (v/w), respectively, of 0.06 M Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.025% bromophenol blue and boiled for 5 min before they were loaded on the gel for electrophoresis. Prestained SDS-PAGE Standards (Bio-Rad) were also loaded on the gels. These standards are useful for monitoring the quality of electrophoretic transfers from SDS-PAGE gels to nitrocellulose membranes. Electrophoresis was at 8 mA per gel until the dye front moved through the stacking gel into the separating gel, and then at 25 mA per gel for approximately 45 min. The resolved proteins were then electroblotted onto nitrocellulose membranes (0.45 μm pore size; Schleicher and Schuell, Keene, NH) and subjected to Western analysis (8, 14) using, sequentially, rabbit anti-DVX serum, goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) and a substrate solution containing fast red TR salt and naphthol AS-MX phosphate (1).

Results and Discussion

DVX detection by Western analysis. Of the six *D. cneorum* specimens tested on *N. clevelandii*, five produced necrotic local lesions on the herbaceous host, followed by systemic chlorotic rings or mottle. The *N. clevelandii* inoculated with the sixth *D. cneorum* specimen remained free of disease symptoms. In ISEM tests of symptomatic *N. clevelandii*, virus particles trapped onto antiserum-sensitized grids were heavily decorated (Fig. 1). Such "decoration" tests using virus-specific antisera are diagnostic (15) and definitively identified the virus particles as DVX. Western analysis of tissue samples from the *D. cneorum* specimens shown to be virus-infected by herbaceous testing (Fig. 2A, lanes a, c–f) showed a major band with a relative mass (molecular weight; molecular mass) of about 26000 daltons. One dalton (Da), the unit of molecular mass, is by definition equal to the mass of one hydrogen atom. The size (molecular mass)

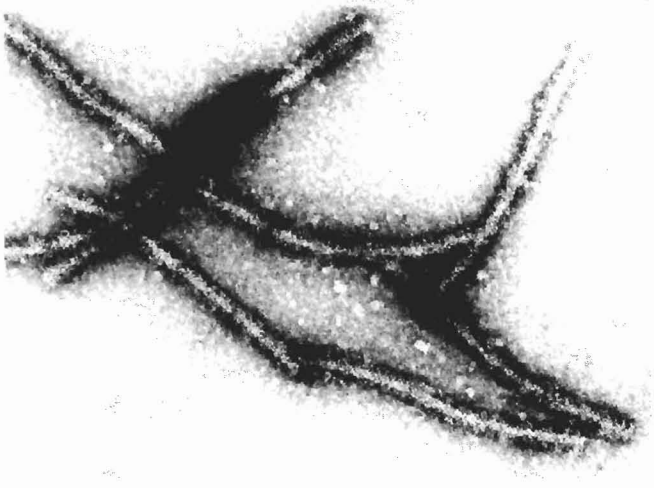

Fig. 1. Particles of DVX, from a symptomatic *Nicotiana clevelandii*, "decorated" with rabbit anti-DVX antibodies.

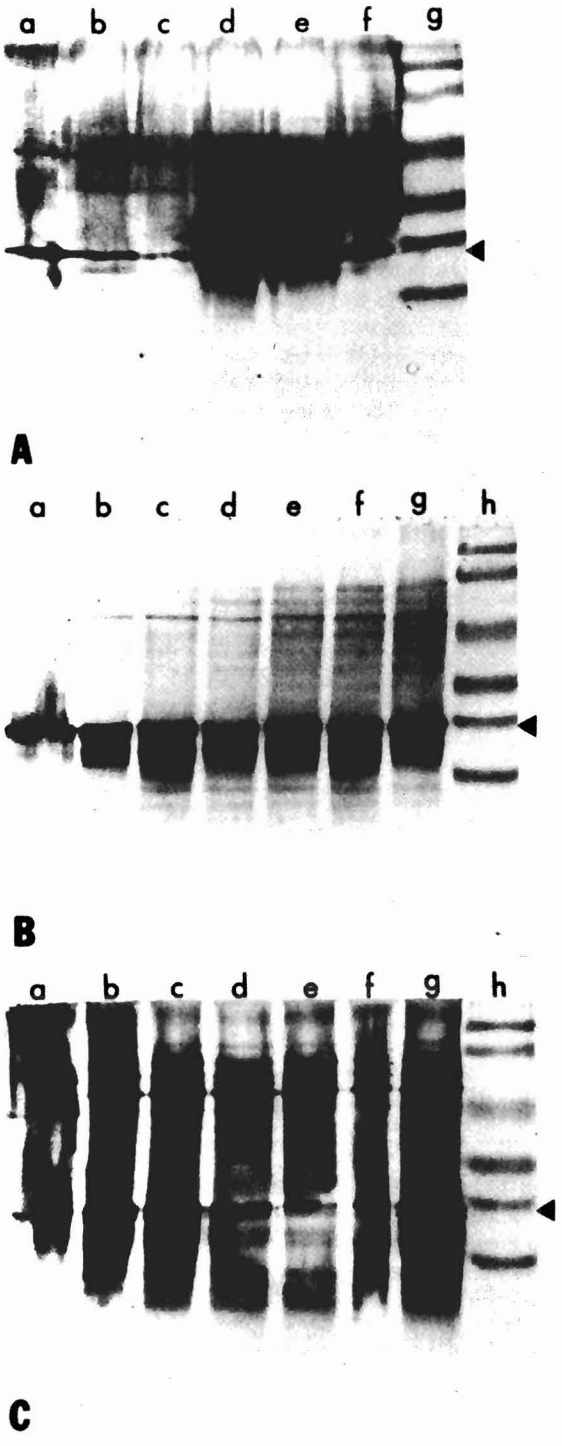

Fig. 2. Western analysis. A. Samples from 6 greenhouse-maintained *Daphne cneorum* (lanes a–f). All plants except that in lane b induced disease symptom expression in *Nicotiana clevelandii*. All plants, including that in lane b, contained the DVX coat protein (arrowhead). Lane g contains pre-stained SDS-PAGE (Bio-Rad) molecular weight standards: phosphorylase b (MW = 106000), bovine serum albumin (MW = 80000), ovalbumin (MW = 49500), carbonic anhydrase (MW = 32500), soybean trypsin inhibitor (MW = 27500) and lysozyme (MW = 18500). B. Samples from a greenhouse-maintained *D. cneorum* (lane a) and from 6 *in vitro* cultures of *D. cneorum* (lanes b–g). Samples from 6 other cultures gave identical results. Lane h contains molecular weight standards. C. Samples from a greenhouse-maintained *D. cneorum* (lanes b–g) provided by a commercial micropropagation lab. Samples from 6 other cultures gave identical results. Lane h contains molecular weight standards.

of proteins is generally expressed in these units, *i.e.* relative to the mass of the hydrogen atom. The size of the major band thus corresponded closely to the reported value of 23000 Da for the DVX coat protein (6). The *D. cneorum* specimen (lane b) which failed to produce symptoms on *N. clevelandii* also contained this band, suggesting that Western analysis was more reliable than testing on *N. clevelandii* for the detection of DVX.

Use of in vitro cultures as samples for Western analysis. Twelve randomly selected *in vitro* cultures of *D. cneorum*, initiated from specimens provided by a local nursery, were tested for DVX by Western analysis (Fig. 2B). All twelve contained a major band with a relative mass of about 26000 Da, indicating that all were DVX-infected. We observed during these analyses that the homogenates of *in vitro* cultures were much less viscous than those of samples from greenhouse-maintained *D. cneorum*. The lower viscosity of the homogenates from *in vitro* cultures made them easier to manipulate when loading the samples onto the gel. The bands detected in the Western analysis of the *in vitro* cultures (Fig. 2B) were also clearer than those seen in greenhouse-maintained *D. cneorum* (Fig. 2A). Similar results were obtained with another twelve *in vitro* cultures of *D. cneorum* (Fig. 2C), provided by a commercial micropropagation lab. All the commercially available *D. cneorum* tested were infected with DVX, in agreement with a previous report from New Zealand (5).

Western analysis is a very powerful serological technique which has been used for the detection of several plant viruses (8). The advantage of Western analysis, in comparison with enzyme-linked immunosorbent assay (ELISA), is that the former method provides information concerning the size of the molecules detected, while the latter does not. This information permits even greater confidence in the diagnosis obtained, when the size of the virus coat protein is known (8). The use of Western analysis is also preferable to ELISA when the antiserum contains, as do most polyclonal sera, antibodies directed against host plant components.

In vitro cultures have been used in the detection of numerous viruses by ELISA (2, 7, 13), but there has been no previous report of the use of *in vitro* cultures for the Western detection of viral coat protein. Our results clearly show that *in vitro* cultures can be used with this technique. Only limited studies have been conducted to date on the use of *in vitro* techniques with daphne. These have been concerned with micropropagation, with the regeneration and rooting of plantlets from tips excized from heat-treated plants (3, 4, 11), and with physiological questions (17, 18). This report demonstrates a further application of tissue culture techniques with daphne which should provide a valuable tool for plant health improvement programs. Our results clearly demonstrated that *in vitro* cultures could be used as samples for Western analysis in a fast and reliable method of DVX detection. This method should be directly applicable to the virus testing of ''heat therapy'' tips. It is reasonable to expect that other viruses infecting daphne or other plants could be similarly detected, using appropriate antisera.

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Air and Soil Temperatures and Fertilizer Level Affect Growth and Quality of *Epipremnum aureum* Bunt.[1]

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Abstract

Three minimum air temperatures (AT), 15.5°, 18.5°, and 21°C (60°, 65°, and 70°F), four constant soil temperatures (ST), 15.5°, 21°, 26.5°, and 32°C (60°, 70°, 80°, and 90°F), and three fertilizer rates (FR) 2.5, 4.2, and 5.9 g 19N-2.6P-10K (0.09, 0.15, and 0.21 oz 19-6-12) Osmocote/15 cm (6 in) pot/3 months were utilized on *Epipremnum aureum* 'Golden Pothos' during November–April in 1983–84, 1984–85, and 1985–86. Data collected included plant grade, leaf color grade, top fresh weight, root fresh weight and leaf surface area. Analyzed data were similar for all three experiments with AT × ST interactions significant for 4 of 5 measurements in 1984 and 1985 and for all 5 measurements in 1986. In general, as fertilizer rate increased, all measurements increased linearly with only root fresh weight not increasing significantly. Results from the 1986 experiment are used for discussion.

Index words: foliage plant, soil heating

Species used in this study: Golden pothos (*Epipremnum aureum* Bunt.)

Significance to the Nursery Industry

These experiments show that, even though plant appearance was generally best at highest AT (21°C/70°F) and ST (32°C/90°F), higher energy efficiency would result from using AT of 18.5°C (65°F) and ST of 26.5°C (80°F) without a significant loss in quality or salability of plants. Although ST of 26.5°C (80°F) reduced root weight, spending money to heat the soil is more economical than heating air.

Introduction

Most foliage plants have their origins in the tropics and require relatively high night temperatures to sustain rapid growth (11). Increases in costs of fossil fuels during the last decade have influenced producers to attempt energy conservation by lowering night and/or day temperatures. Problems of reduced growth rate and increased turnover time caused many producers to return to original temperature

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