Regeneration of Virus-Free Plants from Yellow-Green Areas and TMV-Induced Enations of *Nicotiana tomentosa*

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ABSTRACT

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TMV-infected *Nicotiana tomentosa* plants formed enations at a low frequency on the undersides of several systemically infected leaves. About 60% of the plantlets regenerated from enations were virus-free. Unexpectedly, about one-third of the plants regenerated from yellow-green areas were virus-free.

Additional key words: high-frequency regeneration, plant tissue culture.

The most common research host for tobacco mosaic virus (TMV) is *Nicotiana tabacum* L., which, when systemically infected, exhibits a mosaic of dark-green areas surrounded by a yellow-green background. In contrast, TMV-infected *N. paniculata* L. and *N. tomentosa* Ruiz + Pav. develop enations on the underside of infected leaves in addition to a mild mosaic. These outgrowths are dark green, leaflike in appearance, and vary in size and shape, extending downward as much as 0.5 cm. Some enations appear as small inverted cups. The outgrowths are structurally and morphologically similar to the leaves from which they develop. Enations appear only on leaves that develop after the appearance of systemic symptoms and never on inoculated leaves (2).

Murakishi and Carlson (4,5) confirmed that the dark-green areas are virus-free and that virus-free plants could be regenerated from these areas by using the virus-host combinations TMV/*N. tabacum*, TMV/*Petunia hybrida* L., and turnip yellow mosaic virus/*Brassica napus* L. The mechanism by which dark-green areas arise is unknown. I was interested in confirming Jensen's 1933 report (2) of TMV-induced enations on *N. paniculata* and *N. tomentosa* and in determining by tissue culture techniques whether TMV-induced enations are virus-free.

MATERIALS AND METHODS

N. paniculata and *N. tomentosa* seeds were obtained from L. Burk (Tobacco Research Laboratory, USDA, ARS, Oxford, NC 27565). Plants were grown in the greenhouse and leaves were inoculated with the type strain of TMV when the plants were 10–15 cm high. To obtain callus cultures, the leaves were surface sterilized by immersion for 15 min in 1% sodium hypochlorite containing 0.1% Triton X-100 and following that with three 10-min rinses in sterile distilled water. Small 1–3 mm^2 leaf sections were taken from the yellow-green areas (avoiding dark green areas) or from enations and placed on LS medium (3) containing IAA (indoleacetic acid concentrations ranging 0.3 to 3.0 mg/L) and kinetin (0.3 ml/L) or δ,δ-(dimethylallylamino)-purine (10.0 mg/L) and incubated under fluorescent light (3.7 kergs/cm^2/sec). After one month or more, calli were transferred to LS medium containing IAA (0.3 mg/L) and δ,δ-(dimethylallylamino)-purine (10.0 mg/L) to induce shoot formation and subsequently to LS medium lacking hormones which induced root formation. Plantlets were transferred to Styrofoam cups containing vermiculite until they were of sufficient size to be planted in the greenhouse. Explants were similarly prepared from uninfected *N. tomentosa*. To assay for TMV, tissue was ground in small amount of 0.1 M phosphate buffer, pH 9.0, containing 1.0% Celite and rubbed on leaves of *N. tabacum* 'Xanthi-nc.' Enations developed on TMV-infected *N. paniculata*, but this host proved difficult to maintain in our greenhouse and was therefore not studied further.

Approximately 50 *N. tomentosa* plants were grown from seed and inoculated with TMV. Three of the 50 plants developed enations on two or three systemically infected leaves about 1 mo after inoculation. Even though the enation-bearing plants continued to grow another meter in height and flowered, enations were never seen on subsequently formed leaves. Explants were prepared from yellow-green areas, enations, and uninfected tissues and cultured to regenerated plants. The mean number of days from initiation of explants to transfer to root induction medium was 65 days, 95 days, and 149 days for uninfected, yellow-green areas, and enations, respectively. The standard LS culture medium contained IAA (3.0 mg/L) and kinetin (0.3 mg/L); *N. tomentosa* explants formed callus more readily when kinetin was replaced with δ,δ-(dimethylallylamino)-purine at 10.0 mg/L (IAA concentration unchanged). Primary explants from yellow-green areas always bioassayed positive for TMV; ie, as shown by inoculation of *N.*

TABLE 1. Number of infected calli or plants obtained by culturing explants from TMV-induced enations and yellow-green areas of infected *N. tomentosa* leaves

Source of explant	Calli indexing positive for TMV/total no. of calli	Plants indexing positive for TMV/total no. of plants regenerated
Uninfected plants	0/41	0/29
Yellow-green areas	84/93 (9.7%)[a]	47/70 (32.8%)[a]
TMV-induced enations	3/8 (62.5%)	3/8 (62.5%)

[a] Percent of calli or plants that indexed virus-free.

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tabacum cultivar Xanthi NC. At the time when calli would have been transferred to shoot induction medium, some of the calli were harvested for TMV indexing.

RESULTS

Calli derived from- and plants regenerated from- uninfected tissues were virus-free (Table 1). Approximately 10% of the calli derived from yellow-green areas were virus-free while 62.5% of the calli derived from TMV-induced enations were virus-free. Approximately one-third of the plants regenerated from yellow-green areas showed no viral symptoms and indexed virus-free. More than half of the plants regenerated from enations also proved to be virus-free (Table 1). All virus-free plants were susceptible to TMV. The small number of calli and plants derived from TMV-induced enations is a consequence of the low frequency of enations forming on infected leaves and the recalcitrance of these explants to form calli on standard LS medium.

DISCUSSION

The high frequency of regeneration of virus-free plants from yellow-green areas was unexpected since previous reports of studies in which *N. tabacum* was the host suggested that it was very unlikely that virus-free plants could be obtained from these areas by the techniques used in this study (4). Several workers have obtained virus-free plants of *N. tabacum* from virus-infected calli (1,6). Generally, the infected calli were grown many generations or physically manipulated in some manner before regeneration of virus-free plants was accomplished. Shepard (7) was able to regenerate virus-free plants after isolation of a population of mesophyll protoplasts from plants of *N. tabacum* systemically infected with potato virus X. Seven and a half percent of the regenerated plants were virus free.

The high frequency regeneration of virus-free plants from yellow-green areas of *N. tomentosa* could be explained by either of two mechanisms: 1) The yellow-green areas may have a significant number of cells that have escaped infection and the cell culturing procedure may selectively favor virus-free cells over virus-bearing cells. 2) Essentially all cells in intact leaves are infected, but the cell-culturing procedure may free cells of virus by inducing a physiological state unfavorable for virus multiplication.

This report confirms Jensen's 1933 observation (2) that TMV induces enations on *N. tomentosa* and *N. paniculata*. Jensen reported enation formation on every virus-infected plant while in this study only 6% of the TMV-infected plants developed enations. This difference may be due to the differences in TMV strains used as inoculum, genetic variation in the plants, and/or differences in growing conditions. Since only 6% of the TMV-infected plants developed enations, experiments to test the effect of different TMV strains on enation formation will require large numbers of plants. However, experiments are under way to determine whether clones of *N. tomentosa* can be isolated that form enations at higher frequencies. To obtain these clones, virus-free plants will be regenerated from enation-bearing plants. These TMV-free plants will be inoculated with TMV and the frequency at which enations form will be compared to that of plants grown from seeds.

These results suggest that yellow-green areas from some virus-host combinations may harbor virus-free cells and that using cell culture techniques to obtain "uncommon" hosts for studying virus-cell interactions may be productive.

LITERATURE CITED

1. Chandra, N., and Hildebrandt, A. C. 1967. Differentiation of plants from tobacco mosaic virus inclusion-bearing and inclusion-free single tobacco cells. Virology 31:414-421.
2. Jensen, J. H. 1933. Leaf enations resulting from tobacco mosaic infection in certain species of *Nicotiana* L. Contrib. Boyce Thompson Inst. 5:129-142.
3. Linsmaier, E. M., and Skoog, F. 1965. Organic growth requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.
4. Murakishi, H. H., and Carlson, P. S. 1976. Regeneration of virus-free plants from dark-green islands of tobacco mosaic virus-infected tobacco leaves. Phytopathology 66:931-932.
5. Murakishi, H. H., and Carlson, P. S. 1978. Regeneration of virus-free petunia, tobacco, and *Brassica* plants from green islands of mosaic-infected leaves. (Abstr.) In Vitro 15:192.
6. Omura, T., and Wakimoto, S. 1978. Differentiation ability of the tobacco mosaic virus-eliminated tobacco tissue cultures. Ann. Phytopathol. Soc. Jpn. 44:247-254.
7. Shepard, J. F. 1975. Regeneration of plants from protoplasts of potato virus X-infected tobacco leaves. Virology 66:492-501.