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Authors

D'Onghia, A. M.
Carimi, F.
de Pasquale, F.
et al.

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Somatic Embryogenesis from Style and Stigma Cultures Eliminates *Citrus tristeza virus* (CTV) and *Citrus variegation virus* (CVV)

A. M. D'Onghia, F. Carimi, F. De Pasquale, S. Fiore,
K. Djelouah, and G. P. Martelli

ABSTRACT. *Citrus tristeza virus* (CTV) and *Citrus variegation virus* (CVV) were eliminated by somatic embryogenesis from style and stigma cultures from different infected citrus species. The initial explants and the formed callus were ELISA-positive, whereas no regenerated plantlets were infected by the biological indexing and serological assays carried out over a 3-yr period. Juvenile characters (thorns) disappeared from some branches of most genotypes during the first year of growth and apparently normal fruiting began 3 yr after culture initiation.

Index words. *Citrus tristeza virus*, *Citrus variegation virus*, sanitation, somatic embryos, embryogenic callus, stigma, style.

Somatic embryogenesis from stigma and style is a recently developed technique which successfully eliminated *Citrus psorosis virus* (CPsV), oak leaf pattern diseases (concave gum, cristacortis, impietratura) and viroids (4, 5). Therefore, its application for the elimination of *Citrus tristeza virus* (CTV) and *Citrus variegation virus* (CVV), two important viruses belonging to different genera, *Closterovirus* and *Ilarvirus*, was desirable in order to improve the efficiency of sanitation protocols, not

only in terms of sanitation rate, but also in terms of reduced time for healthy stocks production.

Several infected genotypes of different origin (Table 1) were chosen from a citrus collection, which had been extensively tested by biological indexing and ELISA for the presence of CTV and CVV (unpublished data). One of the three infected sources was always the species used as the specific indicator for the disease being considered.

Closed flowers were collected from each citrus genotype and freshly

TABLE 1
LIST OF *CITRUS TRISTEZA VIRUS* AND *CITRUS VARIEGATION VIRUS*-INFECTED CITRUS GENOTYPES REGENERATED BY SOMATIC EMBRYOGENESIS FROM STIGMA AND STYLE

Species/Cultivars	Infected plants no.	Origin	Regenerated plants no.
CTV:			
Washington navel orange	1	Palestine	18
Valencia orange	1	Palestine	12
Shamouti orange	1	Lebanon	8
Common mandarin	1	Lebanon	5
Meyer lemon	1	China	19
Common grapefruit	1	Albania	4
Kumquat	1	Italy	4
Mexican lime	1	Italy	15
CVV:			
Valencia late orange	1	Italy	14
Lemon thornless	1	Italy	22
Dweet tangor	1	Italy	18
Etrog citron	1	Italy	8

used for *in vitro* culture. The same explants were also ELISA tested for the presence of CTV and CVV.

Following the protocol of D'Onghia et al. (4), flowers were collected before opening and were surface-sterilized by immersion for 5 min in ethanol (70% v/v in water), 15 min in 2% sodium hypochlorite (w/v in water). Stigmas and styles were excised aseptically from flowers, and placed vertically onto Murashige and Skoog (6) semi-solid (7 g/l agar) medium (MS) containing sucrose (146 μ M), malt extract (0.5 g/l) and BAP (13 μ M). The pH of the medium was adjusted to 5.7 ± 0.1 with 0.5 M KOH before autoclaving at 121°C for 15 min. Explants and calluses were subcultured onto fresh medium at 30-day intervals and maintained in a climate chamber at $25 \pm 1^\circ\text{C}$ under a 16 h day length, and a photosynthetic photon flux of 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ Osram cool-white 18 W fluorescent lamps. Somatic embryos (2-3 mm in diameter) were isolated from embryogenic cultures (Fig. 1) and germination was attempted in hormone-free MS medium.



Fig. 1. Citrus embryos from style and stigma cultures.

A minimum of 4-5 different embryogenic explants for each genotype were produced. Plantlets regenerated *in vitro* (Fig. 2) were grafted onto 6 mo-old sour orange seedlings (3).

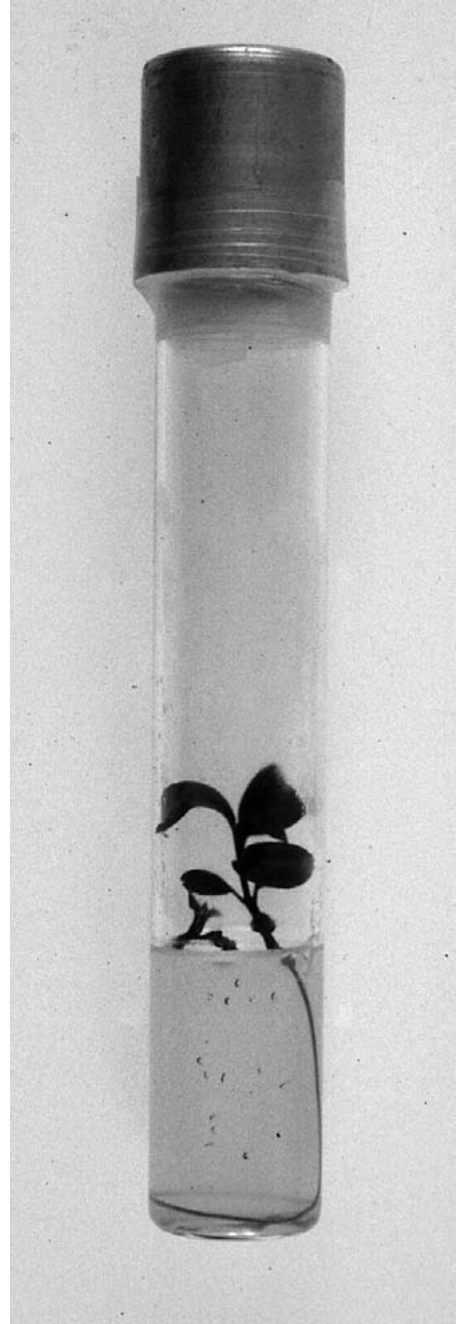


Fig. 2. Citrus plantlet growing in test tube.

Stigmas and styles, embryogenic calluses and regenerated plants were ELISA tested using commercial monoclonal antibodies raised against CTV and CVV (UCP-Morocco). All regenerated plants were serologically tested over a 24 mo period. Plants were also bud-grafted using woody indicators, Mexican lime and Eureka lemon for the detection of CTV and CVV, respectively, and kept at 22-24°C. Moreover, regenerated plants from CTV-infected Mexican lime and CVV-infected Etrog citron were maintained in the same conditions and observed for 1 yr.

Plants regenerated from stigma and style cultures were transferred to a greenhouse and in the field for evaluating their morphological and physiological traits.

All infected genotypes regenerated somatic embryos 2-5 mo after culture initiation. Regenerated plants exhibited initial juvenile growth, characterized by the presence of thorns on stems and branches. However, within 12 mo, the great majority of the plants developed thornless apical growth. As reported in previous works, plants multiplied from thornless portions of stems or branches by top grafting onto sour orange seedlings, completely lost juvenile characters.

Neither virus was detected in any of the regenerated plants (obtained from different embryogenic events) in any of the periodical ELISA tests, and they were appar-

ently still free of these viruses 2 yr after regeneration. No clear cut tristeza or variegation symptoms developed respectively on virus indicators grafted onto embryogenic plants, nor in the regenerated plants of Mexican lime and Etrog citron.

These very satisfactory results were obtained notwithstanding the fact that explants from donor plants and embryogenic callus lines were infected by CTV and CVV. Loss of juvenility in the early stages of growth confirms observations which reported the presence of fruit of lemon and sweet orange on thornless branches 3 yr after the embryogenic event (F. Carimi, F. De Pasquale, S. Fiore and A. M. D'Onghia, unpublished data).

Results obtained by the use of sensitive techniques such as graft transmission on woody indicators (7) and ELISA (1, 2) confirms previous studies that this technique eliminates virus infection from all regenerated plants (4, 5) and further shows that somatic embryogenesis from stigma and style, which utilizes explants of no ovular origin for obtaining true-to-type plants, is a very promising technique for the production of true-to-type and healthy citrus stocks.

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