

Recovery of Citrus Selections Free of Several Viruses, Exocortis Viroid, and *Spiroplasma citri* by Shoot-Tip Grafting *in vitro*

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Murashige *et al.* (1972) developed a technique for grafting the small apical shoot of citrus to the top of a decapitated seedling grown *in vitro*. A few of these grafted plants, when indexed, were found freed of exocortis and stubborn pathogens. Also, Temple tangors derived by shoot-tip grafting *in vitro* (STG) were relatively thornless, flowered and fruited early, and appeared identical to the parent plants. Navarro, Roistacher, and Murashige (1975) improved the technique by testing various media formulas, different ways of placing the scion tip on the decapitated epicotyl, different rootstocks, light intensities, and different sources and sizes of the scion shoot-tip on graft success. Suc-

cessful grafts increased from less than 10 per cent to as high as 50 per cent and preliminary results suggested that *in vitro* grafted plants were freed of several citrus pathogens. This paper reports the final results of indexing over 400 plants derived by STG and consisting of various selections of sweet orange, mandarin, grapefruit, lemon, lime, citron, and tangor. Source plants were variously infected with viruses of tristeza (TV), seedling yellows-tristeza (SYTV), psorosis-A (PSV-A), concave gum (CGV), yellow vein (YVV), infectious variegation (IVV), cachexia, and tatterleaf (TLV), citrus exocortis viroid (CEV), and *Spiroplasma citri* (stubborn).

MATERIALS AND METHODS

Grafting technique. Figure 1 graphically outlines the technique of STG as recommended by Navarro *et al.* (1975). Shoots 3 cm or shorter were collected, carefully washed, disinfected in 0.25 per cent sodium hypochlorite, and rinsed three times in sterile distilled water. Then, using a razor blade sliver attached to a Beaver surgical handle, the small shoot tip consisting of the apical meristem and three leaf primordia was excised under a binocular microscope. Average length of shoot tips was 0.14 to 0.18 mm.

Rootstock seedlings, aseptically grown in the dark, were Troyer citrange for sweet orange, tangor, mandarin, or grapefruit scions and rough lemon for lemon, lime, or citron scions. The rootstock seedling was decapitated, leaving 1.0 to 1.5 cm portions of the epicotyl; the coty-

ledons and their subtended buds were removed and the root shortened to 4 to 6 cm. An inverted-T incision was made on the epicotyl, the vertical cut starting at the point of decapitation and extending 1 mm down the stem; a horizontal cut about 1 mm wide was made at the lower end of the incision. The incision was made through the cortex to the cambial region. The excised shoot-tip was placed in the incision with its basal cut surface in contact with the rootstock's cortical surface that had been exposed by the outward separation of the cortex from the stele (fig. 1-1). Grafted plants were allowed to develop aseptically in a nutrient solution containing inorganic salts according to the plant cell-culture formula of Murashige and Skoog (1962), 100 mg/l *i*-inositol, 0.2 mg/l thiamine hydro-

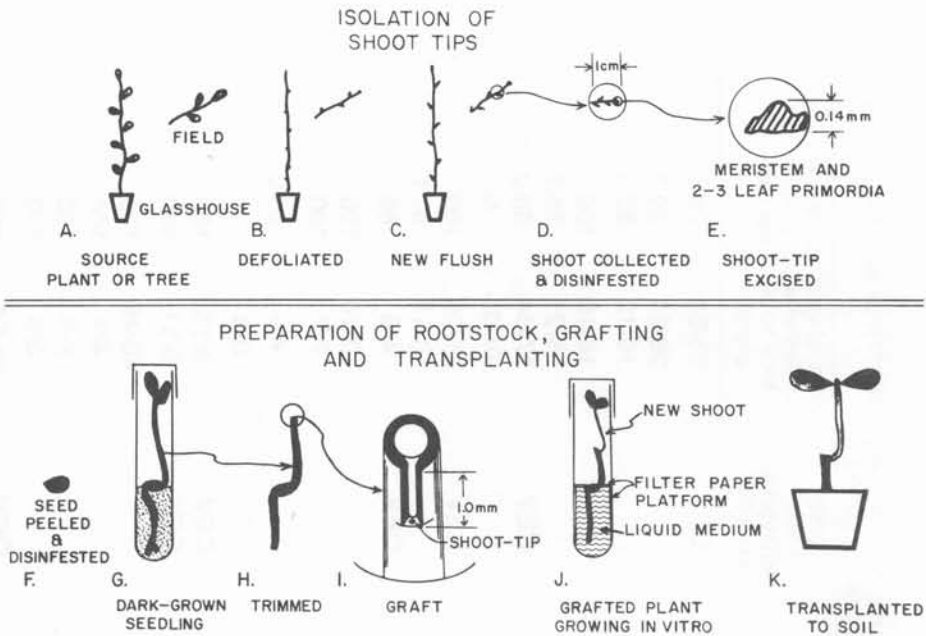


Fig. 1. Diagrammatic representation of the technique used in shoot-tip grafting *in vitro* to obtain grafted plants for virus indexing.

chloride, 1/mg/1 pyridoxine hydrochloride, 1 mg/1 nicotinic acid, and 7.5 per cent sucrose. Grafted plants grew *in vitro* for 3 to 5 weeks under 16 hours daily exposure to 1000 lux illumination and were transferred to 10,000 lux illumination for an additional 2 to 3 weeks. After this, plants were removed from their tubes and transplanted to 10-cm pots containing a modified UC potting mix for citrus (Nauer *et al.*, 1968). Potted plants were enclosed in polyethylene bags and shaded with cheesecloth. The bags were opened one week later and after a second week the bags and cheesecloth were removed. Navarro *et al.* (1975) reported 95 per cent survival of transplants from the test tube to soil.

Pathogen content of source plants.

The pathogens present and the origin of various source plants are recorded in table 1. Cadenera Fina, infected with TV and CEV, was a field tree probably of seedling origin. SYTV source trees 556 and 557 were Valencia and navel orange, respectively, and both indexed negative for PSV-A, CGV, and CEV, but induced

severe vein corking on leaves of West Indian lime and severe seedling yellows in rough lemon and Duncan grapefruit seedlings.

All four PSV-A sources indexed negative for TV and induced shock symptoms in sweet orange. When preinoculated sweet orange seedlings were challenged with lesion inoculum of PSV-A they were protected from development of lesions; all four were considered typical PSV-A. Shoots collected from Robertson navel orange were obtained from 18 budded plants in a glasshouse. All PSV-A sources when indexed were found negative for TV and vein enation virus (VEV) and rarely, if ever, induced oak-leaf patterns. The three CGV sources all induced typical oak-leaf patterns in sweet orange and Dweet tangor and never caused shock symptoms in these indicators. C-301 was a local source from Fawcett 571, whereas the other two sources were introductions from Spain and Sicily. When buds of these three CGV sources were grafted into sweet orange and later challenged with lesion inoculum of PSV-A there was

TABLE 1

RESULTS OF INDEXING FOR THE PRESENCE OF CITRUS PATHOGENS IN PLANTS DERIVED FROM GRAFTS OF *IN VITRO* SHOOT-TIPS OBTAINED FROM KNOWN INFECTED SOURCE PLANTS

Pathogen	Source plant of	Virus code no.	Origin	Other known pathogens in source	No. of virus free plants/ total no. of plants	Virus-free plants per cent
TV	Cadenera Fina	T-513	USA	CEV	33/33	100
SYTV	Madam Vinous	SY-556A	Hawaii		26/29	89.7
	Madam Vinous	SY-557	Hawaii		10/12	83.3
PSV-A	Pineapple orange	P-201A	China		16/16	100
	Madam Vinous	P-205A	USA		18/21	85.7
	Robertson navel	P-211	USA	CEV	17/34	50.0
	Stuttsman Valencia	P-209	USA		0/12	0
CGV	Cadenera	C-304	Spain	CEV	25/25	100
	Koethen	C-301	USA		10/11	91.7
	Clementine Monreale	C-305	Sicily	CEV	4/6	66.7
IVV	Satsuma	IV-401D	USA		10/10	100
	Madam Vinous	IV-401A	USA		7/7	100
YVV	Valencia	YV-920	USA		4/5	80.0
TLV	Meyer lemon	TL-100	China		0/8	0
Cachexia	Willowleaf	Ca-903	USA	CEV	0/55	100
	Ricote lemon	Ca-907	Sicily	CEV	0/11	100
<i>S. citri</i>	Madam Vinous	S-600	USA		46/46	100
	Navel orange	S-600	USA		4/4	100
	Valencia orange	S-600	USA		8/8	100
	Redblush grapefruit	S-600	USA		5/5	100
CEV	Cadenera	C-304	Spain	CGV	25/25	100

Cadenera Fina	T-513	USA	TV	33/33	100
Clementine Monreale	C-305	Sicily	CGV	6/6	100
Ricote lemon	Ca-907	Sicily	Cachexia	11/11	100
Temple tangor	E-814	USA		32/32	100
Santa Teresa lemon	E-818	Sicily		1/1	100
Genetic dwarf grapefruit	E-805	USA		3/3	100
Frost navel	E-803	USA		3/3	100
Bearss lime	E-805	USA		2/2	100
Sensitive citron	E-816	USA		1/1	100
Willowleaf	Ca-903	USA	Cachexia	54/55	98.2
Robertson navel	P-211	USA	PSV-A	24/26	92.3
Fingered citron	E-813	Hawaii		7/8	87.5

no protection, implying nonrelation to PSV-A (Roistacher and Calavan, 1965).

Cachexia virus present in a Willowleaf mandarin was obtained from Dr. L. G. Weathers as USDA code 6-6-6 and indexed negative for TV, VEV, PSV-A and CGV but positive for CEV. Cachexia was also present in a Ricote lemon, imported from Spain, which indexed negative for viruses in West Indian lime, sweet orange, calamondin, Eureka lemon, and *Citrus excelsa* but positive for CEV and cachexia.

All stubborn source plants had been inoculated with severe stubborn (E. C. Calavan code 276). This source indexed negative for PSV-A, CEV, CGV, VEV, and TV. An initial index for stubborn was made when young shoots were collected. Young leaves directly below the shoot tip were graft-inoculated to suitable indicators by the method of Calavan *et al.* (1972a) and transmitted the stubborn pathogen to 67 per cent of the indicator plants.

Seedlings of West Indian lime, rough lemon, and *C. macrophylla* all showed excellent yellow vein symptoms when inoculated with YVV (obtained from L. G. Weathers) combined with VEV (Weathers, 1961).

TLV was present in a Meyer lemon (Variety Improvement no. 141), which indexed negative for TV, SYTV, CEV, PSV-A, CGV and VEV.

There were 13 sources containing CEV (table 1). All induced severe epinasty in citron, except Willowleaf mandarin, Frost Navel (Variety Improvement no. 14), Bearss lime, and fingered citron which induced only mild reactions. A very sensitive citron was of special interest. This was a seedling of Arizona 861 which showed unusually pronounced symptoms after inoculation with a very mild isolate of CEV. We felt that if CEV could be eliminated from this seedling, a new more sensitive clonal indicator would be available for detecting mild isolates of CEV.

With certain exceptions, all shoots were collected from new growth obtained after denuding screenhouse or glasshouse grown plants (fig. 1-A to D). Exceptions were Robertson navel and Temple tangor

of which shoots were collected from the growing tips of a number of budded plants, and Cadenera fina sweet orange, of which shoots were from new spring growth on field trees.

Indexing procedures and conditions. All index plants were grown in a U.C. potting mix in 1-gallon containers in a glasshouse. Plants were fertilized with each watering and maintained in optimum growing condition free from insect pests. Inoculated plants were held in two rooms: a warm room ranging from 25° C minimum to 40° C maximum for indexing stubborn, cachexia and CEV and a normal room ranging from 18° C minimum to 31° C maximum for indexing most viruses.

Numbers and kinds of indicators used to index each shoot-tip-grafted plant were: two West Indian lime seedlings for TV and SYTV, five West Indian lime seedlings for YVV, with VEV added to enhance symptom development (Weathers 1961); two to three Arizona 861 citron scions on rough lemon for CEV and IVV; five *C. excelsa* plus five Troyer citrange

RESULTS

The results of indexing are given in table 1. With few exceptions the indexing of source plants resulted in 100 per cent infection.

All 63 plants originating from stubborn-infected shoots of four cultivars and observed for over one year under warm conditions were found negative for stubborn. Seven slower growing plants were indexed twice for *S. citri* by the method of Fudl-Allah *et al.* (1971) and found negative.

With rare exceptions, replicated indicator plants reacted as all positive or all negative. A number of inoculated seedlings which indexed negative for PSV-A or CGV, when challenged with inoculum of the original source material, accepted the challenge, indicating no cross-protection, and were considered free of mild strains of these two viruses.

Shoot-tip grafting *in vitro* was effective in avoiding TV, two isolates of SYTV, three of four isolates of PSV-A, all

seedlings for TLV; three Parsons Special Mandarin, budded on rough lemon for cachexia virus by the method of Roistacher *et al.* (1973); three Pineapple or Madam Vinous sweet orange or Dweet tangor seedlings for PSV-A in P-201, P-205 and P-209 and for all CGV sources; and two to three of the same indicators for PSV-A in Robertson navel orange.

Many of the shoot-tip-grafted plants were self-indicating. Those derived from sources infected with PSV-A, CGV, IVV or *S. citri* were held for one year and carefully observed for symptoms.

Inoculation was done by grafting two buds or stem pieces to the stems of indicator plants and wrapping with polyethylene tape. Bud inoculum taken from the original source plants and grafted to suitable indicators served as positive controls for each test. Over 2,000 indicator plants were inoculated with scion tissue from shoot-tip-grafted plants, and over 450 indicator plants inoculated with tissue from source plants were used as positive controls. Indexing was carried out over a 2-year period.

three isolates of CGV, both isolates of IVV, YVV, cachexia virus, and all 13 isolates of CEV. The method did not avoid one isolate of PSV-A (P-209) from Valencia orange in 11 plants, nor TLV from Meyer lemon in 8 plants.

CEV was avoided in one STG propagation from an infected sensitive seedling of Arizona 861. This virus-free plant is now under test as a superior citron for indexing CEV.

STG separated some components of SYTV in three of five West Indian lime seedlings. There were also instances of separation of strains of CEV, and separation of CEV from PSV-A and CGV.

Observations of a number of shoot-tip-grafted plants of various cultivars of sweet orange, mandarin, grapefruit, lemon, lime, citron, and tangor over a 2-year period have confirmed them to be comparable, in growth and flowering characteristics, to plants produced by traditional bud-grafting propagation

methods. None has shown the juvenile growth and flowering characteristics typical of nucellar seedlings. Many plants

have flowered and set fruit in the glasshouse.

DISCUSSION

Pathogen-free citrus selections have been obtained by nucellar embryony, nucellar tissue culture, thermotherapy, clonal selection, indexing, and now by STG. Plants derived by nucellar embryony (Weathers and Calavan, 1959) or nucellar tissue culture (Bitters *et al.*, 1972) are excessively vigorous, thorny, late bearing, and have fruit which may vary in quality and likeness from the original. Also, some viruses may pass through seed (Bridges *et al.*, 1965). Thermotherapy has successfully eliminated TV, SYTV, PSV-A, CGV, IVV, TLV, and VEV from citrus plants, but has failed to eliminate CEV, YVV, cachexia virus, and Dweet mottle virus (Calavan *et al.*, 1972b; Roistacher and Calavan, 1972). Many cultivars have been found free of viruses by selection and extensive indexing as in the California Citrus Variety Improvement Program (Reuther *et al.*, 1972) and similar programs. Our results show that CEV, cachexia virus, and *S. citri* can now be routinely eliminated from citrus by STG.

Virus-free plants derived by STG can be made available for budwood increase within one year of grafting. Such buds should produce trees with fruit and growth characteristics identical to the source trees from which they were derived. Vigor and field potential should be superior to those of infected plants.

The use of STG for production of virus-free plants of old citrus budlines opens the way for a more efficient and less time-consuming means of developing citrus improvement programs in countries where most citrus is virus-infected. Such a program is outlined by Navarro (1976) in this volume.

Methods available for recovery of pathogen-free plants by cell and organ culture were reviewed by Murashige and Jones (1974). Many of these methods employ shoot-tip culture *in vitro* with herbaceous plants but have not proven

applicable to trees. We now believe that the method should be tested for various trees for elimination of viruses.

The reason why shoot tips are sometimes free of pathogens is not known. There is some evidence that shoot tips have less virus content. Limasset *et al.* (1949) showed that tobacco mosaic virus decreased with decreasing leaf age and was nil in the shoot-tip region. This observation has been confirmed with other pathogens and plants. Hollings and Stone (1965) observed that carnation ringspot virus was absent in the extreme shoot tips, and Kassanis (1975) was unable to detect potato paracrinkle S and X viruses in the potato shoot apical meristem. This suggests that many pathogens do not move readily into the protoxylem or protophloem elements of apical meristems.

This technique can now be used for separation of viruses. We were able to separate PSV-A and CGV from CEV, and Navarro *et al.* (1976) showed that shoots excised from axillary buds would readily separate CEV from PSV-A. Components of SYTV were segregated by STG and gave results similar to those obtained by Desjardins *et al.* (1959) using thermotherapy. TLV virus was not segregated into two viruses by STG, providing further evidence that TLV and citrange stunt virus may be the same (Garnsey, 1974). It would be of interest to test for separation for PSV-A and CGV by this technique. Indirect evidence presented here suggests that the three isolates of CGV which were readily eliminated in shoot-tip grafts might have been separated from certain PSV-A isolates. This technique might be tried to separate the viruses of impietratura or cristicortis from the component inducing oak-leaf patterns, which are apparently always present.

An improved indicator for detecting mild isolates of CEV has emerged from these experiments. This makes it possible to eliminate viruses from very sensitive infected gametic indicator seedlings.

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