Improvement of Shoot-tip Grafting *in vitro* for Virus-free Citrus[1]

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Abstract. A 30 to 50% frequency of successful grafts was obtained by using 2-week-old dark grown seedlings as rootstocks and 0.14 to 0.18 mm long shoot tips as scions. The shoot tip was inserted into an inverted-T made at the top of the decapitated rootstock epicotyl. Most scion cultivars gave satisfactory grafts on 'Troyer' citrange, whereas lemon, lime and citron yielded successful grafts only on 'Rough' lemon. The grafted plants were allowed to develop *in vitro* under 16 hr daily exposure to 1000 lux Grow Lux illumination and were provided with a nutrient solution containing a high concentration (7.5%) of sucrose. The best source of shoot tips was the flush from defoliated branches of field trees or glasshouse plants. It was also possible to use shoot tips from flushes arising in excised lateral buds cultured *in vitro*. Grafted plants were transplantable to soil 5 to 8 weeks after grafting with over 95% survival. Preliminary data indicated recovery of cultivars freed from tristeza and psorosis viruses, stubborn spiroplasma and exocortis viroid. Pathogen-free plants showed no reversion to the juvenile phase.

Infection by viruses and related pathogens is receiving increasing recognition as a significant factor in the productivity of tree crops. Declines of vigor, yield, and quality are being attributed more and more to these disease agents. Severe infections have resulted in the exclusion of some cultivars from commercial usage. Fortunately, methods are available to recover pathogen-free plants and the principal of these have been reviewed (13). Thermotherapy has been employed to provide budwood that is free of certain viruses (4, 16). Shoot-tip cultures have been used successfully with many herbaceous crops, but this technique is not yet applicable to tree genera (8, 12, 13). In the Rutaceae, plants arising from embryogenesis of the nucellus, either *in vivo* or *in vitro*, have been shown to be free of most pathogenic viruses (2, 19). Unfortunately, such plants show reversion to the juvenile state and the associated undesirable qualities are manifested for many years.

Preliminary studies suggested that certain pathogens that are difficult to eliminate by thermotherapy, e.g., citrus exocortis and stubborn, might be eliminated by a process of shoot-tip grafting *in vitro* (12). Moreover, the plants obtained by this method were observed to bypass the juvenile state. The procedure consisted of placing a 0.15 mm long shoot tip excised from an infected tree onto a decapitated rootstock seedling under aseptic conditions. The extent of grafting success was low, usually about 10%, and the effect of the procedure on recovery of plants free from viruses and virus-like pathogens was untested. We therefore evaluated several variables that might influence grafting success, and tested significant numbers of grafted plants for presence of diverse pathogens.

Materials and Methods

Characteristics Associated With the Rootstock

Aseptic germination of seeds. Seeds were peeled (both seed-coats were removed), wrapped in small squares of cheese-cloth in groups of 10 seeds per sheet, disinfested by immersing in a 0.5% sodium hypochlorite solution (commercial bleach diluted 1:9, v:v, with water + 0.1% Tween 20 wetting agent) for 10 min, and rinsed 3 times with autoclaved distilled water. The germination medium was the plant cell culture salt solution of Murashige and Skoog (14), solidified with 1% Difco Bacto-agar. The pH of the medium was set initially at 5.7 ± 0.1. The medium was distributed in 25-ml aliquots in 25 x 150-mm culture tubes and the tubes were capped with polypropylene closures (Bellco Kap-uts). The medium was sterilized by autoclaving at 121°C for 15 min. One seed was sown per tube and germination was allowed to occur at constant 27°C.

Factors influencing the behavior of the rootstock seedling. Seedlings that had been obtained in constant darkness were compared with those obtained under 16 hr daily illumination with 1000 lux Sylvania Gro Lux light. Seedlings that were 1, 2, 3 and 4 weeks old, and others with and without cotyledons and with and without root tips were used as rootstocks.

Cultivars employed as rootstocks. Poncirus trifoliata (L.) Raft. x *Citrus sinensis* (L.) Osbeck, 'Troyer' citrange, was used in most

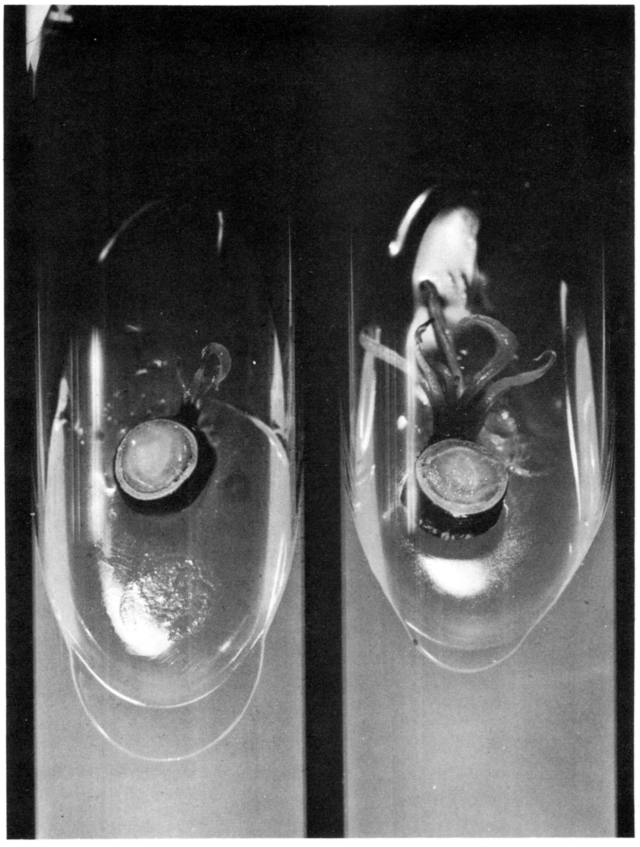

Fig. 1. Cultured lateral buds of citron showing development of shoots *in vitro*. Shoot tips from these were used in grafts.

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Table 1. Citrus cultivars used as source of shoot tips and infections shown to be present by indexing.

Cultivar	Infection
C. sinensis (L.) Osbeck	
'Robertson' navel orange	Excortis, psorosis
'Pera' sweet orange	None
'Cadenera Fina' sweet orange	Exocortis, tristeza
'Cadenera de Carcagente' sweet orange	Exocortis, concave gum
'Madam Vinous' sweet orange	Stubborn, psorosis, infectious variegation, seedling yellows-tristeza
'Frost' Valencia orange	Stubborn, psorosis
'Frost' navel orange	Exocortis
'Koethen' sweet orange	Concave gum
'Pineapple' sweet orange	Psorosis
'Olinda' Valencia orange	Yellow vein
C. reticulata Blanco	
'Clementine Monreal' mandarin	Exocortis, concave gum
'Willow Leaf' mandarin	Exocortis, cachexia-xyloporosis
'Satsuma' mandarin	Infectious variegation
C. paradisi Macf.	
'Genetic Dwarf' grapefruit	Exocortis
'Redblush' grapefruit	Stubborn
C. limon (L.) Burm.	
'Ricote' lemon	Exocortis, cachexia-xyloporosis
'Mesero' lemon	Exocortis
'Santa Teresa' lemon	Exocortis
'Meyer' lemon	Tatter leaf-citrange stunt complex
'Prior Lisbon' lemon	Cachexia-xyloporosis
C. aurantifolia (Christm.) Swing.	
'Bearss' lime	Exocortis
C. medica L. var. *sarcodactylis* (Noot.) Swing.	
'Fingered' citron	Exocortis
C. reticulata Blanco × *C. sinensis* (L.) Osbeck	
'Temple' tangor	Exocortis
Fortunella japonica (Thumb.) Swing.	
'Marumi' kumquat	None (Graft incompatibility with 'Troyer' citrange)

experiments as the rootstock cultivar. *Citrus jambhiri* Lush. 'Rough' lemon was also used as rootstock in grafts involving lemon and citron shoot tips to explore differences associated with the rootstock cultivar because those 2 scion cultivars gave low success when grafted onto 'Troyer' citrange.

Characteristics Associated with the Scion

Size of shoot tips. The scion dimensions examined were the shoot apical meristem dome alone and the meristem plus 2, 4, or 6 leaf primordia.

Source of excised shoot tips. The shoot tips were obtained from a) actively growing new shoots on field and glasshouse grown plants, b) axillary buds, and c) flushes arising in axillary buds that had been cultured *in vitro* (Fig. 1).

Sometimes shoot tips were obtainable from field trees that were naturally in flush. Flushing, however, is ordinarily season-associated and suitable plant material is not always available. This inconvenience was overcome by establishing potted plants of desired cultivars in the glasshouse and inducing flushes by defoliating entire plants. Shoots that were 3 cm or shorter were used in order to avoid shoot tips that were abscising or otherwise degenerating. Terminals 1 cm long were pinched off, stripped of larger leaves, wrapped in small squares of cheesecloth, and surface sterilized by soaking in 0.25% sodium hypochlorite solution (commercial bleach diluted 20-fold and containing 0.1% Tween 20) for 5 min. The disinfected tissues were rinsed 3 times with autoclaved distilled water, and their shoot tips were excised and used as scions.

The lateral buds that served as a second source of shoot tips were obtained from glasshouse-grown plants. Young twig sections were stripped of their leaves and thorns, cut into shorter sections containing 2 to 3 axillary buds, washed with 95% ethanol followed by tap water to remove dust, and disinfected with 0.5% sodium hypochlorite solution for 10 min. The sections were rinsed 3 times with autoclaved water and the quiescent shoot tips were excised from each bud and employed in grafts.

To induce flushes in axillary buds *in vitro* (the third source of shoot tips), nodal sections containing lateral buds were first sterilized in 0.5% sodium hypochlorite solution. One or more bud scales were then removed, the stem piece was reduced to a smaller size, and the tissue was disinfected further in 0.25% hypochlorite solution. An explant containing 1 lateral bud was planted per nutrient tube. The medium used initially contained the inorganic salts according to the plant cell culture formula of Murashige and Skoog (14), 85 mg/l $NaH_2PO \cdot H_2O$, 3% sucrose, 0.4 mg/l thiamine hydrochloride, 100 mg/l *i*-inositol, 40 mg/l adenine sulfate·$2H_2O$, 1 g/l Difco Bacto Casamino acids, and 1% Difco Bacto-agar. The initial medium pH was set at 5.7. Aliquots of 25 ml of medium were distributed in 25 × 150-mm nutrient tubes and sterilized. The cultures were maintained under 16 hr daily exposure to 1000-lux illumination from Sylvania Gro Lux lamps and at constant 27°C. Slant cultures were employed to provide maximum exposure to light. The shoots that arose in these cultures (Fig. 1) were utilized as sources of scion shoot tips. In order to achieve consistency in the frequency of axillary buds that flushed and in the number and length of the shoots, some of the conditions associated with the *in vitro* process were investigated. They included light intensity and nutrient medium requirements. Twenty cultures were utilized per experimental treatment.

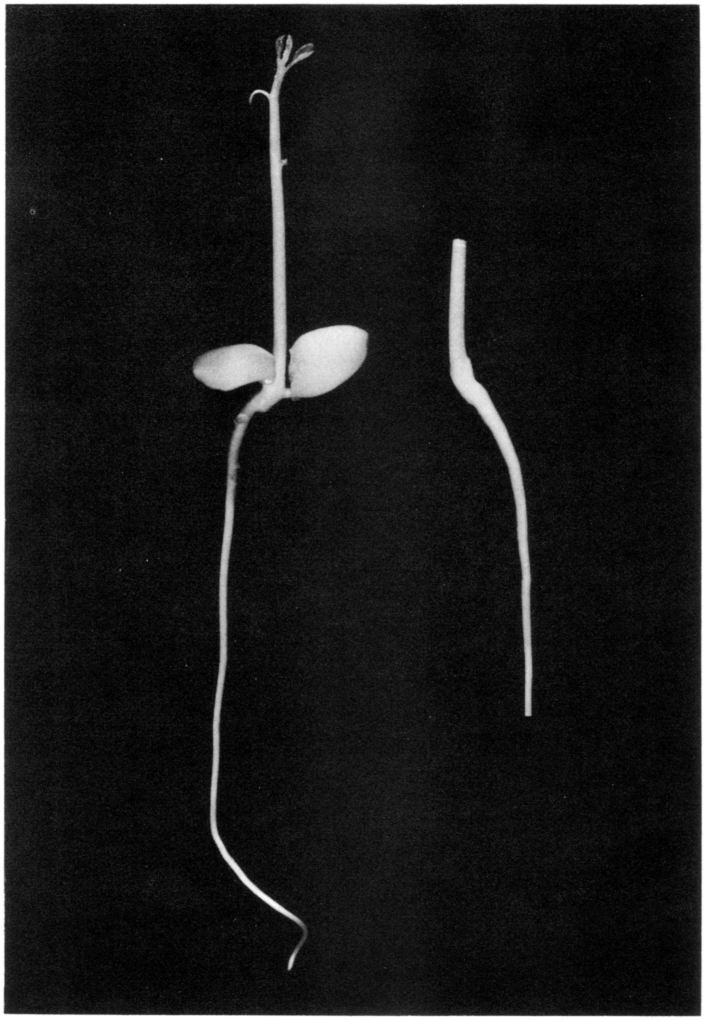

Fig. 2. Seedling and rootstock used for *in vitro* grafting of citrus. Left: 2-week-old 'Troyer' citrange seedling; right: seedling rootstock prior to grafting with epicotyl decapitated, cotyledons and root tip severed, and inverted-T incision (not visible in Fig.) made at top end of decapitated epicotyl.

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Cultivars used as scions and pathogens present. Field trees were used as source material for 'Cadenera Fina' and 'Pera' sweet oranges. The remaining cultivars were glasshouse grown potted plants containing 1 or more pathogens (Table 1). All source material had been indexed for the presence of pathogens. The viruses found were psorosis, tristeza, seedling yellows-tristeza, concave gum, infectious variegation, yellow vein, cachexia-xyloporosis and the tatterleaf-citrange stunt complex. Stubborn disease caused by *Spiroplasma citri* was found in certain cultivars and exocortis, a viroid of a low molecular wt RNA, was present in many cultivars.

The Grafting Procedure

The procedure as initially utilized was as follows: a 2-week-old rootstock seedling was decapitated, leaving 1 to 1.5 cm of the epicotyl; the root was shortened to 4 to 6 cm and the cotyledons and their axillary buds were removed (Fig. 2). A shoot tip composed of the apical meristem and subjacent tissue to include 3 leaf primordia was isolated from the desired source, using a razor blade sliver attached to a Beaver surgical handle as a scalpel, and transferred to the top cut surface of the decapitated epicotyl. These steps of shoot-tip excision and transfer to rootstock were carried out aseptically and with the aid of a dissecting microscope. To minimize possible transfer of pathogens from infected to uninfected tissues, separate sets of dissecting instruments were used in handling the rootstock and the scion, and the scalpel used in excising the shoot tip was dipped in a 1% sodium hypochlorite solution between cuts. Dipping of instruments in the hypochlorite solution has been shown to be very effective in inactivating the exocortis viroid (20).

Various methods of placement of the scion tip onto the seedling rootstock were investigated (Fig. 3). In some cases, the shoot tip was placed on the top cut surface of the decapitated epicotyl; in others the scion was inserted in an inverted-T incision. When the placement involved the top cut surface of the decapitated epicotyl, the shoot tip was placed either in contact with the cortex (Fig. 3a), the vascular ring (Fig. 3b), or the pith (Fig. 3c). With insertions into the inverted-T incision, the location of the T incision along the length of the epicotyl and the location of the shoot tip within the incision was considered. In one case the inverted-T incision was made at the point of decapitation of the epicotyl, and in another at a region 3 to 5 mm above the juncture with the root. The shoot tip was placed in the incision with its basal cut surface in contact with either the rootstock's cortical surface, exposed by the horizontal cut (Fig. 3e), or the cambial region of the seedling (Fig. 3d). In contacts that involved the cambial region, the shoot tip was oriented perpendicularly to the vertical axis of the rootstock seedling (similar to standard budding procedure).

Maintenance of the Grafted Plants in vitro

The nutrient medium. A comparison was made of the effectiveness of Hoagland's solution and of Murashige and Skoog's plant cell culture salt formulation. The latter was found to be equally effective, if not superior; accordingly, it was used as the reference formula. The medium in its initial form also contained 100 mg/l *i*-inositol, 4% sucrose, 0.2 mg/l thiamine·hydrochloride, 1 mg/l pyridoxine·hydrochloride, and 1 mg/l nicotinic acid. Its pH was set at 5.7 ± 0.1 prior to autoclaving. The medium was distributed into 25 x 150-mm culture tubes in 25-ml aliquots. A supportive platform, made from a folded 9-cm circle of Whatman No. 50 filter paper, was placed in the nutrient solution (Fig. 4). The platform was perforated in its center for insertion of the root portion of the rootstock. The tubes were capped with Bellco Kap-uts and the medium was autoclaved at 121°C for 15 min. Further improvements in the nutrient formulation were obtained by adding these substances: N^6-benzyladenine (BA), 0, 0.001, 0.01, 0.1, 1.0, and 10.1 mg/l; indole-3-acetic acid (IAA), 0, 0.01, 0.1, 1.0, and 10.1 mg/l; sucrose, 0, 2.5, 5.0, 7.5, and 10.0%; and the B-vitamins thiamine hydrochloride, pyridoxine hydrochloride, and nicotinic acid in 0, 1, 3, and 10 times their initial concentrations. Tests of growth substances also consisted of dipping the top of the rootstock seedling, after decapitation but before grafting, for 5 sec in these solutions: 0, 0.01, 0.1, and 1.0 mg/l BA or 0 and 1.0 mg/l indole-3-butyric acid (IBA). Frequently the grafts showed scion shoot tips that were alive but remained quiescent even after 3 months, in such cases, a drop of 20 mg/l gibberellin A_3 (GA_3) solution was placed onto the shoot tips in attempts to induce their growth. The GA_3 solution was sterilized by Millipore filtration before use.

Care of the grafted plants in vitro. The grafted plants were kept upright and exposed 16 hr daily to 1000-lux illumination, provided by Sylvania Gro Lux lamps. A constant temperature of 27°C was standard. Variations in the light intensity were tested and they included 0, 300, 1000, 3000, and 10,000 lux. The grafted plants were

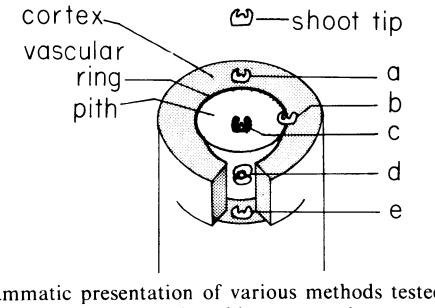

Fig. 3. Diagrammatic presentation of various methods tested in positioning shoot-tip scion onto decapitated seedling rootstock.

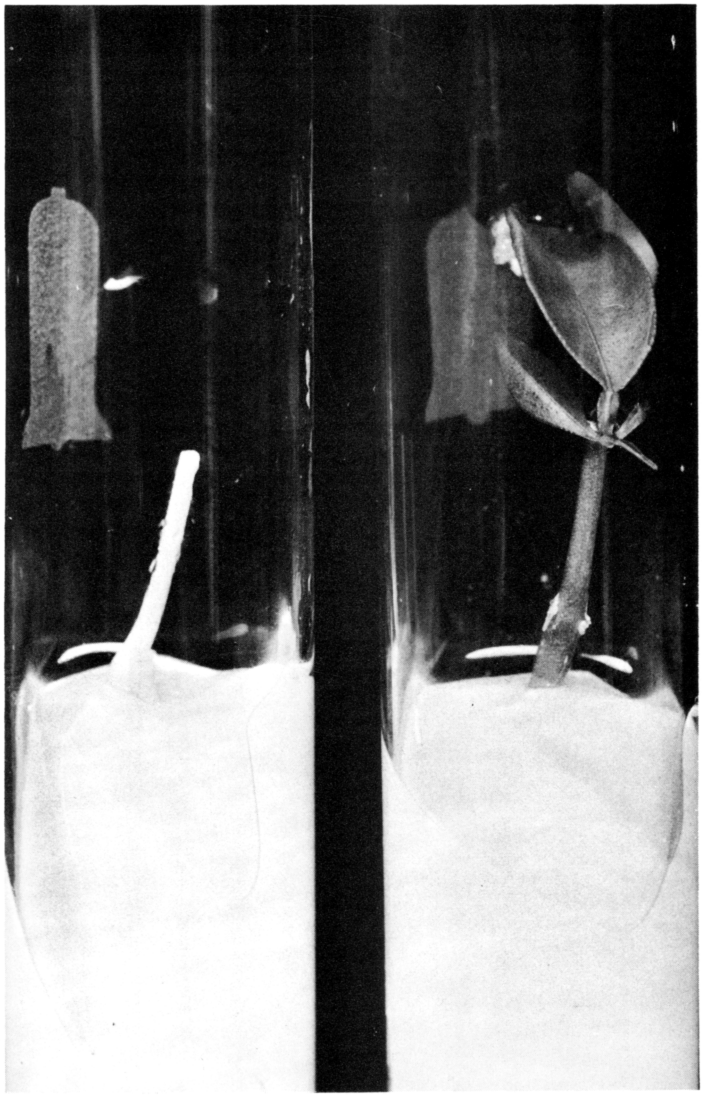

Fig. 4. Freshly prepared graft, left, and resultant plant after 1 month, right, of 'Madam Vinous' sweet orange.

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observed periodically using a dissecting microscope, and adventitious shoots arising from the rootstocks (Fig. 5) were removed aseptically with a pair of surgical scissors.

Transfer of plants to soil. Plants that showed at least 2 expanded leaves in the scion shoot tip (Fig. 4), usually 3 to 5 weeks after grafting, were further cultured *in vitro* under a higher light intensity, 10,000 lux. After another 2 weeks they were transplanted into 10 cm pots containing a soil mixture previously developed for citrus (15). They were fertilized once with the Murashige and Skoog salt solution at transplant time. The pots were enclosed in polyethylene bags to minimize moisture loss and shaded with cheesecloth. The bags were opened 1 week later and after a second week the bags and cheesecloth were removed and the plants were allowed to continue growth under standard glasshouse conditions.

Testing Plants for Pathogens

Evidence for the presence of pathogens in plants derived through the *in vitro* grafting procedure was obtained by: a) observing the grafted plants of sweet orange or mandarin which are self-indicating for symptoms of psorosis and concave gum, and b) indexing, i.e., studying the response of specifically sensitive citrus seedlings after graft-inoculation with tissues obtained from *in vitro* grafted plants (5). The tissue used for virus transmission from smaller plants consisted of rectangular leaf pieces (3). The tissues used for larger plants were buds or bark pieces. Specific indicators included *C. aurantifolia* (Christm.) Swing. 'West Indian' lime to detect tristeza or seedling yellow-tristeza virus, various seedlings of sweet orange or *C. reticulata* Blanco x *C. sinensis* (L.) Osbeck 'Dweet' tangor to detect concave gum or psorosis virus and *C. medica* L. 'Arizona 861' citron for detection of the exocortis viroid. Grafted plants from stubborn-infected shoots of a number of sweet orange or grapefruit cultivars (Table 1) were placed in a warm temperature glasshouse and observed for 9–12 months for evidence of stubborn.

All plants from which scion shoot tips had been obtained were indexed for virus content at the time the shoot tips were excised for grafting, and periodically thereafter. These served as controls for all indexing of plants derived from *in vitro* grafts.

Indexing for the presence of stubborn was performed at the time of shoot tip collections. Two small leaves directly below the shoot tip were collected and rectangular leaf pieces (3) were graft-inoculated to indicator seedlings of 'Madam Vinous' or 'Hinkley' sweet orange, or *C. reticulata* Blanco x *C. paradisi* Macf. 'Sexton' tangelo. In this manner the incidence of stubborn present in leaves below the shoot tips could be compared with that of the self-indicating grafts derived from the shoot tips.

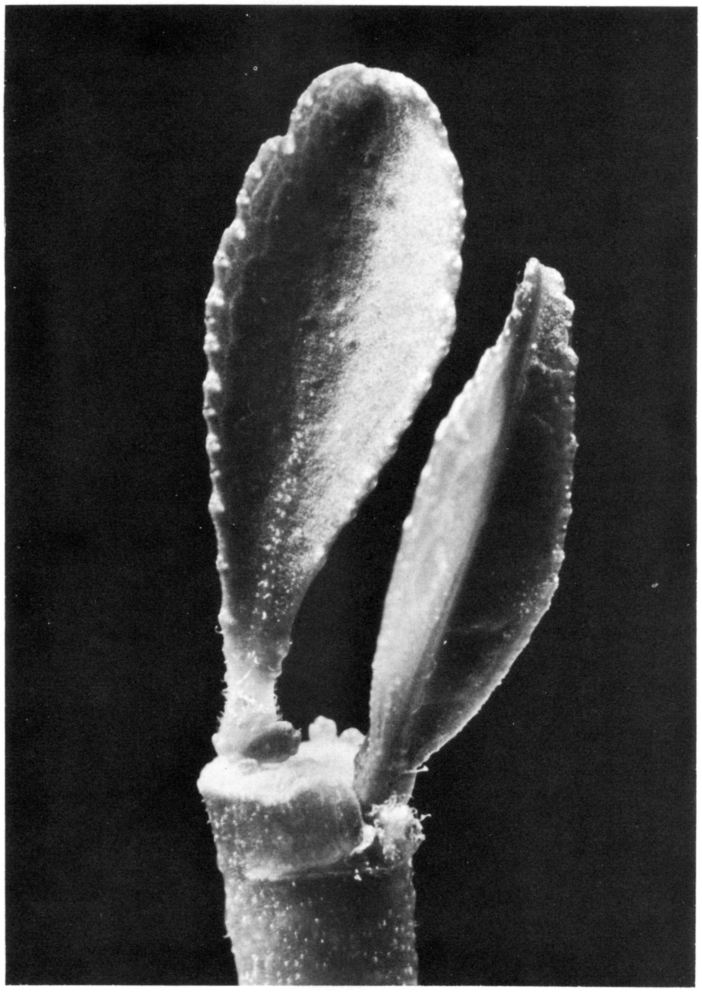

Fig. 5. Enlarged view about 10 ×), showing leaf arising from 'Meyer' lemon scion shoot tip (right) placed in inverted-T incision of rootstock, together with that from an adventitious bud that differentiated from rootstock tissue (left).

Results and Discussion

Characteristics Associated With the Rootstock

The frequency of successful grafts was greater if the rootstock seedlings were from seeds germinated in darkness. These seedlings gave 37.5% successful grafts, whereas those obtained under 16 hr daily exposure to 1000-lux illumination yielded only 2.7%. The dark-grown seedlings were etiolated, lacked expanded leaves and were taller than those obtained under light. Herman and Hess (7) observed that the regeneration of roots in bean epicotyl was better if cuttings were taken from etiolated rather than green seedlings. They noted less tissue differentiation in the etiolated epicotyl and inferred that there was an inverse relationship between degree of tissue differentiation and capacity for regeneration. Whether this applies to our citrus grafts is unproven. Regardless of reason, the choice of the *in vitro* grafting procedure is to use etiolated seedlings as rootstocks.

The age of the seedlings also played a significant role (Table 2). The highest percentage of successful grafts was obtained when 2-week-old seedlings were used as rootstocks. Younger seedlings, as well as those older than 2 weeks, were inferior. The unsuccessful grafts on older seedlings showed greater proportions of scion shoot tips that had dried, turned brown, and died; whereas those involving younger seedlings revealed shoot tips that had become quiescent and buried in callus produced by the rootstock tissue. The higher incidence of browning and drying of shoot tips indicates that perhaps the graft failures with older seedlings were related to moisture inadequacy; failures on the younger seedlings, on the other hand, seemed to be associated with precocious callus formation.

No difference in the degree of grafting success resulted between seedlings with intact cotyledons, and those with their cotyledons removed. However, when cotyledons were present, their axillary buds sprouted. It was necessary to remove these lateral shoots, since they appeared to depress growth of the grafted shoot tips. Removal of the root tip, its purpose being to facilitate handling of the grafted plant,

Table 2. Influence of age of 'Troyer' citrange seedlings at the time of grafting on percent of successful shoot-tip grafts *in vitro*. Scion shoot tip: 'Robertson' navel orange.

	Age of seedlings (Weeks after sowing)			
	1	2	3	4
% successful grafts	2.5	37.5	17.5	7.5
% unsuccessful grafts with dead shoot tips	15.4	32.0	51.5	75.7
% unsuccessful grafts with shoot tips buried in callus	84.6	68.0	48.5	24.3

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did not alter grafting success. No difference was observed between rootstocks with intact and decapitated roots. Accordingly, as standard practice the cotyledons and root tips were removed, the latter resulting in the root being shortened to 4 to 6 cm.

'Troyer' citrange was satisfactory as a rootstock for sweet orange, mandarin, grapefruit, and kumquat shoot tips, but it was ineffective for lemon, citron and lime. A systematic test disclosed that 'Rough' lemon was superior to 'Troyer' citrange as the rootstock of 'Ricote' lemon and 'Fingered' citron shoot tips (Table 3). The need to examine the cultivar influence when selecting rootstocks for certain citrus species and cultivars is emphasized. The 'Troyer' citrange has an advantage of a trifoliate marker to facilitate identification of successful grafts. Adventitious shoots which arise from trifoliated rootstocks can be detected and removed, and their possible adverse influence on development of the grafted shoot tip is minimized. The absence of a suitable morphological marker, however, should not preclude the use of the *in vitro* grafting procedure.

Characteristics Associated With the Scion

The incidence of successful grafts is expected to rise with increasing size of the scion shoot tip, but it is also probable that the proportion of virus-free plants will decline. The scion size that is ultimately chosen must enable a realistic degree of grafting success and result in a reasonable number of pathogen-free plants. Only an insignificant proportion, 1.8%, of the grafts of the shoot apical meristem alone as scion resulted in successes (Table 4). The degree of success increased progressively as larger shoot tips were used, by including subjacent tissue and 2, 4, or 6 leaf primordia. Data at this writing are not available on the relationship between scion size and frequency of disease-free plants. As standard practice, however, a shoot tip composed of the apical meristem and subjacent tissue with 3 leaf primordia, measuring 0.14 to 0.18 mm in height, has been adopted.

Shoot tips obtained from flushes that were available in the field or in the glasshouse produced the highest percentage of successful grafts (Table 5). Considering other factors, the first choice as source of shoot tips is the actively growing shoot that occurs on the plant; excision was easiest from this source. The poorest source was the uncultured lateral buds because their shoot tips were most difficult to isolate; a considerable amount of tissue enveloped their shoot tips and substantial volumes of sap exuded during the excision process, adding to the already tedious procedure.

Although flushes *in vivo* gave the highest graft successes and furnished shoots from which shoot tips could be isolated most readily, their occurrence was season-dependent, except when potted plants were available and flushes could be induced in the glasshouse. The alternate source of shoot tips would be the flush of shoots resulting in cultured axillary buds. There are other benefits associated with this alternate source. Buds could be cultured in media containing antibiotic or anti-viral substances, thus minimizing further the concentration of pathogens in excised shoot tips. Oshima and Livingston (17) were able to increase the percentage of potato virus X-free shoot tips by culturing potato stem tips in a medium containing malachite green. Malachite green has been incorporated into the culture medium of citrus lateral buds; to date it has only been possible to establish the non-toxic range of concentrations. The degree to which this substance has reduced the virus content of the shoot tips is under test. A 30-mg/l concentration of malachite green appeared as the upper limit that is usable in the lateral bud culture medium. The use of axillary buds also provides a means by which plant material might be transported from a region or country to another more expediently than is possible by current procedures. Desired budwood could be fumigated and shipped to a tissue culture facility in the receiving region or country, the lateral buds could then be excised and cultured *in vitro*, and the shoot growth could be used as the source of shoot tips for grafting *in vitro* to obtain pathogen-free plants. All unused tissue should be destroyed by autoclaving. Plants derived from shoot-tip grafts would be tested under special quarantine facilities and destroyed if pathogens were found, or the plants in test-tubes could be shipped back to the country of origin for indexing.

Recognizing the above advantages, this investigation explored *in vitro* conditions which might influence flushing of lateral bud explants. The data for this report are expressed as total shoot development per lateral bud culture. They represent the combined values of number of shoots produced per bud and the length per shoot. Ordinarily each lateral bud produced more than 1 shoot; however, there was excellent correlation between total shoot growth and the number of shoots produced by a given bud.

The nutrient medium finally selected for the culture of citrus lateral buds is shown in Table 6. Flush development in the cultured buds was stimulated by BA (Fig. 6). The total growth of shoots increased progressively, up to a concentration of 1 mg/l of BA; higher concentrations, 3 and 10 mg/l, were inhibitory. Altman and Goren (1) recently reported that BA as well as another cytokinin, kinetin, caused

Table 3. Influence of the rootstock cultivar on grafting success of 'Ricote' lemon and 'Fingered' citron shoot tips.

Rootstock	% Successful grafts	
	'Ricote' lemon	'Fingered' citron
'Troyer' citrange	5.0	0.0
'Rough' lemon	27.5	20.0

Table 4. Influence of size of the excised shoot tip of 'Robertson' navel orange on the incidence of successful grafts on 'Troyer' citrange rootstock.

Scion size	Height of scion tissue (mm)	% Successful grafts
Apical meristem alone	0.05–0.06	1.8
Apical meristem and 2 leaf primordia	0.1–0.15	14.6
Apical meristem and 4 leaf primordia	0.2–0.3	34.6
Apical meristem and 6 leaf primordia	0.4–0.7	47.3

Table 5. Relationship between source of 'Robertson' navel orange shoot tips and incidence of successful grafts. Difficulty involved in the isolation of shoot tips from each source is reflected in number of grafts performed per hour.

Source of shoot tips	% Successful grafts	No. of grafts performed/hr
Flushes from potted plants	30–40	20
Flushes from lateral buds cultured *in vitro*	20–30	15
Freshly excised axillary buds	15–25	10

Table 6. Compositions of nutrient media used in the culture of axillary buds and of grafted citrus plants *in vitro*; initial pH of media, 5.7.

Constituent	Concentration in medium, mg/l	
	Axillary bud culture	Grafted plants
Mineral Salts	Murashige and Skoog formulation	Murashige and Skoog formulation
Organic Substances		
Sucrose	30,000.0	75,000.0
N^6-Benzyladenine	1.0	
Thiamine·HCl	0.4	0.2
i-Inositol	100.0	100.0
Pyridoxine·HCl		1.0
Nicotinic Acid		1.0
Adenine Sulfate·$2H_2O$	100.0	
Complex addenda		
Difco Bacto-Agar	10,000.0	

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the development of multiple shoots in cultures of citrus lateral buds *in vitro*. Our observation, therefore, confirms their finding. Similarly, Dutcher and Powell (6) described the development of more than 1 shoot in lateral bud cultures of the apple when the mediun contained BA, although this cytokinin was primarily observed to enhance callus formation.

Inasmuch as the conditions *in vitro* are inadequate for significant photosynthesis to occur, it was not unexpected that growth was absent in lateral bud cultures that lacked sucrose (Fig. 7). Flush development was enhanced progressively with increasing sucrose concentration in the medium, up to 3%; higher concentrations were somewhat repressive. The optimum concentration was 3%.

Adenine sulfate also promoted flushing in excised citrus buds. Increased flushing occurred in concentrations of 30 and 100 mg/l of the substance; a 300-mg/l adenine sulfate concentration was toxic (Fig. 8). Although not shown in the data, the length per shoot was observed to be disproportiontely enhanced in the presence of adenine sulfate.

The casein hydrolysate, Difco Bacto Casamino Acids, showed no significant beneficial effects. In fact, the concentration found in the initial medium, 1000 mg/l, might have been slightly toxic. Stimulation possibly occurred in a lower concentration of 300 mg/l. Indeed, no need for casein hydrolysate was demonstrated.

Additional inorganic phosphate in the medium also provided no benefit. The auxins, 2,4-D and IAA, caused only inhibition of flush development in axillary bud cultures and the gibberellin, GA_3, showed no beneficial effect. Altman and Goren (1) found that IAA inhibited growth in their bud cultures, but GA_3 stimulated the elongation of shoots that arose in the same culture. The inhibition of shoot growth in the excised bud by auxin is not unexpected, if it is assumed that auxin plays the same role in this system as it does in apical dominance *in vivo*. Similarly, the inhibition by exogenous GA_3 is also not unexpected, since this substance is known to accentuate the auxin inhibition of lateral bud emergence in apical dominance.

Dutcher and Powell (6) found that liquid media were superior to agar-solidified nutrient formulations in cultures of apple buds. Liquid media, whether provided in the stationary state or agitated slowly at 1 rpm on a rotating drum, inhibited growth of excised citrus buds completely.

The light intensity of the culture environment played a significant role in the development of flushes in citrus buds *in vitro*. A linear increase in the total shoot growth per bud occurred with increases in light intensity (Fig. 9). The increase in flush development was due largely to an increase in the average length per shoot and not to the number of shoots per bud. The best intensity was found to be the highest presently examined, or 10,000 lux. This intensity also resulted in significant expansion of leaves, a feature not observed at the lower light intensities. The growth of apple buds *in vitro* has also been reported to be promoted substantially by high light intensities (6). However, the apple differed in behavior from citrus in that the former showed no growth in total darkness, whereas some growth of citrus occurred even in complete darkness. The light did not initiate growth of citrus buds, but simply enhanced their rate of growth.

We obtained successful grafts *in vitro* with many of the commercial cultivars of citrus. With 'Troyer' citrange as the seedling rootstock,

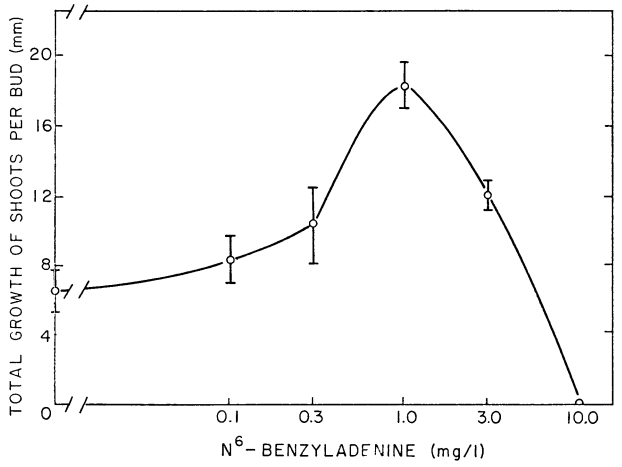

Fig. 6. Effects of BA concentration on shoot development in cultured lateral buds of 'Robertson' navel orange. Verticle lines indicate standard errors of means.

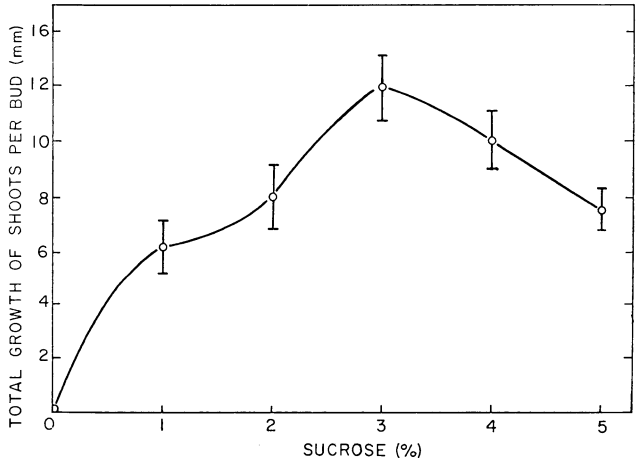

Fig. 7. Effects of sucrose concentration on shoot development in cultured lateral buds of 'Robertson' navel orange.

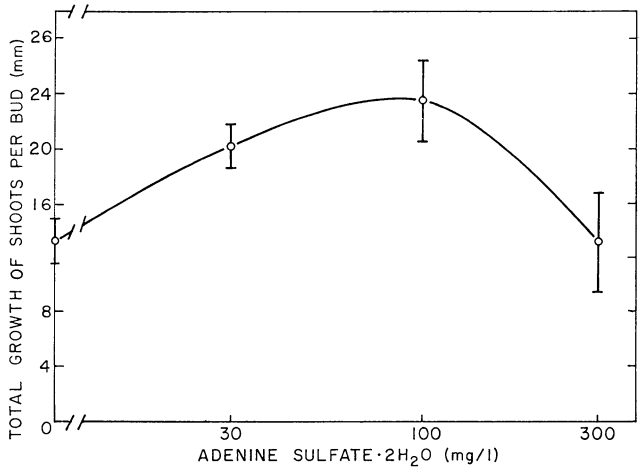

Fig. 8. Influence of adenine sulfate concentration on shoot development in excised 'Robertson' navel orange lateral buds *in vitro*.

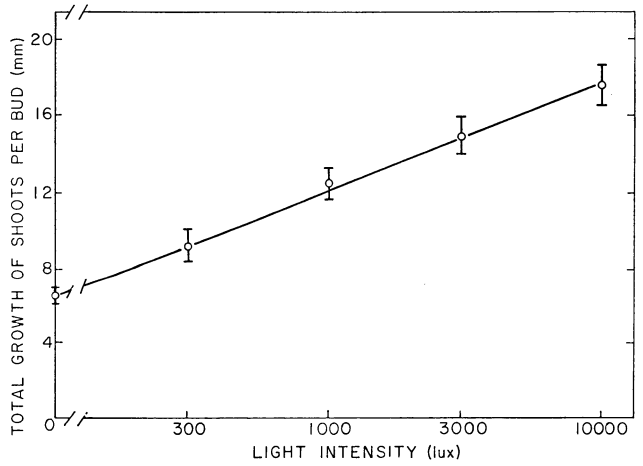

Fig. 9. Effect of illumination on shoot development in excised 'Robertson' navel orange lateral buds cultured *in vitro*.

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equal degrees of success were obtained with shoot tips of sweet orange, mandarin, grapefruit, and kumquat. Grafting successes were usually 30 to 50%, and sometimes as high as 90%. In contrast, shoot tips obtained from lemon, lime, and citron grafted poorly onto 'Troyer'. Usually all grafts of these scion-rootstock combinations failed; successful grafts were obtained when 'Rough' lemon was used as the rootstock.

Performing the Graft

The method of placement of the excised shoot tip onto the rootstock seedling significantly influenced the degree of grafting success. As apparent in Table 7, the highest success was obtained when the shoot tip was placed either over the vascular ring at the top cut surface of the decapitated epicotyl (Fig. 3b), or within the inverted-T incision made at the top of the decapitated epicotyl (Fig. 3e). The experiment was repeated many times without significant difference between the 2 methods. The method involving the inverted-T incision was adopted as standard because adventitious shoots from the rootstock seemed to arise most readily from tissues exposed at the top of the decapitated epicotyl, particularly the vascular ring. Adventitious shoots were not observed with significant frequency from within the inverted-T incision. In the absence of a morphological marker it would be difficult to distinguish between the adventitiously arising shoot and the shoot that develops from a successful graft. Even with a morphological marker, the distinction might not be possible until development had progressed substantially. Acceptable frequencies of successful grafts were obtained also when the shoot tip was placed either on the cortex (Fig. 3a) or on the pith (Fig. 3c) exposed at the top of the decapitated epicotyl. Placement of the shoot tip within the inverted-T incision, but with its basal cut surface in contact with the stele tissue (Fig. 3d), gave low grafting success, in contrast with standard nursery practice of budding citrus (18). The effectiveness of the inverted-T incision appeared to diminish significantly when the incision was made lower down the epicotyl, or 3 to 4 mm from the root.

Although differing in degree, successful grafts were obtained in contacts between scion and rootstock which involved diverse tissues of the rootstock. This observation is generally in agreement with Mendel's (10, 11) statement that much of the tissue of the citrus rootstock is capable of producing callus and in effecting successful bud unions.

Table 7. Influence of method of placement of shoot tip onto rootstock seedling on incidence of successful grafts *in vitro*; scion cultivars: 'Cadenera de Carcagente' and 'Pera' sweet oranges and 'Temple' tangor.

Method of placement of scion	% Successful grafts
Shoot tip set on cortex surface in inverted-T incision; inverted-T at point of decapitation of epicotyl	45.0
Shoot tip set on cortex surface in inverted-T incision; inverted-T 3 to 4 mm above root	5.0
Shoot tip set on stele tissue in inverted-T incision; incision at top of the decapitated epicotyl; shoot-tip axis perpendicular to epicotyl axis	10.0
Shoot tip placed on stele tissue in inverted-T incision; incision 3 to 4 mm above root; shoot-tip axis perpendicular to epicotyl axis	15.0
Shoot tip placed on cortex exposed at top cut surface of decapitated epicotyl	37.5
Shoot tip set on pith exposed at top cut surface of decapitated epicotyl	35.0
Shoot tip placed on vascular ring tissue at top of decapitated epicotyl	50.0

Care of the Grafted Plants in vitro

An effective nutrient formula was developed for the grafted citrus plants *in vitro* (Table 6). Neither IAA nor BA influenced the degree of grafting success. IAA apparently stimulated lateral root initiation in concentrations of 1 and 10 mg/l. In contrast, BA in concentrations of 0.1 mg/l and higher inhibited root growth and caused the initiation of numerous buds in the root region of the rootstock. Moreover, grafts obtained in the presence of BA and characterized by development of adventitious shoots in the shoot region were able to survive transfer to soil. Applying either IBA or BA onto the top of the decapitated seedling prior to the placement of the scion shoot tip also failed to influence the extent of successful grafts. Maiti et al. (9) reported contrary results in conventional budding with grapefruit. They observed quick-dipping of scion buds in solutions of IBA to be beneficial.

The application of GA_3 solution to quiescent scion tips failed to induce elongation of the shoot tips.

The sucrose concentration of the nutrient medium of grafted plants played a significant role (Table 8 and Fig. 10). As evident in Table 8, the most successful grafts occurred in the medium containing 7.5% sucrose. There was also a progressive increase in the number of new leaves arising in shoot tips of the successful grafts with increasing sucrose concentration, up to 7.5%; a 10% sucrose concentration showed no additional benefit. Similarly, leaf size, as reflected in the length of leaves on successful grafts, was stimulated by increases in the sucrose concentration. Total leaf growth of the successfully grafted plants was thus affected favorably by increasing the sugar concentration from 2.5 to 7.5%. The production of new lateral roots was stimulated substantially by the higher sucrose concentration. All cultures grown at the 10% sucrose level produced new lateral roots and those with the 5 and 7.5% sucrose showed a significant number with new roots. On the basis of these data, the 7.5% sucrose concentration has been selected as optimum in the nutrient medium of citrus plants grafted *in vitro*.

The B-vitamins thiamine·hydrochloride, pyridoxine·hydrochloride, and nicotininc acid appeared to increase grafting success slightly. They were retained in the medium without change of concentrations.

The light intensity during growth of grafts *in vitro* did not appear critical, at least in the range of 300 to 10,000 lux; nevertheless, some light was required, inasmuch as no successful grafts resulted when the plants were placed under constant darkness. Accordingly, the 16-hr daily exposure to 1000-lux illumination from Sylvania Gro Lux lamps was retained as standard.

Table 8. Influence of sucrose concentration of the nutrient medium on percentage of successful grafts; scion cultivar: 'Madam Vinous' sweet orange; data recorded after 1 month.

	% Sucrose in medium				
	0	2.5	5.0	7.5	10.0
% Successful grafts	60	55	55	90	70
Number leaves/successful grafts	1.5 ± 0.7	1.4 ± 0.2	2.6 ± 0.3	3.0 ± 0.3	3.2 ± 0.2
Length of leaf on successful grafts (mm)	6.0 ± 1.1	7.5 ± 1.3	10.2 ± 1.4	11.8 ± 1.2	9.6 ± 1.2
Total leaf growth on successful grafts (mm)	9.0 ± 1.1	10.3 ± 2.2	26.8 ± 3.6	35.4 ± 3.6	30.7 ± 3.3
% cultures with new lateral roots	0	30	80	90	100

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Pathogen Content of Established Plants

Plants originating from the infected sources listed in Table 1 were transplanted to soil 5 to 8 weeks after *in vitro* grafting, with over 95% survival by our procedure. Preliminary results of indexing showed that a significant proportion of these plants were free from the citrus pathogens tested. Definitive data with respect to the virus content of the established plants are not yet available. They will require at least 2 indicator tests of each plant and the results for the longer term cachexia index will require at least one year or longer. Results of the first tests have indicated recovery of plants that are apparently free from psorosis and tristeza viruses, citrus stubborn spiroplasma, and exocortis viroid. The results of indexing for the presence of tristeza virus and exocortis virioid in 31 plants of 'Cadenera Fina' sweet orange showed that all were free of both pathogens. Initial indexing of 'Temple' tangor showed 5 of 6 plants free of exocortis; some of the pathogen-free plants flowered and set fruit within 11 months from grafting (Fig. 11), thus affirming that shoot-tip grafted plants do not revert to the juvenile state, as do disease-free plants recovered through nucellar embryogenesis, either *in vitro* or *in vivo* (12).

The first index tests of 'Robertson' navel orange plants derived from shoot-tip grafts disclosed that 36 of 92 or 39% were apparently free of psorosis virus, and that 77 out of 99 or 78% were negative for exocortis. Similarly, exocortis-free plants were obtained from previously infected plants of 'Clementine Monreal' mandarin, a 'genetic dwarf grapefruit, a 'Frost' navel orange and a 'Santa Teresa' lemon imported from Sicily.

The results of indexing for presence of stubborn showed 67% positive stubborn in the young leaves sampled directly below the shoot-tips used for grafting, whereas all 66 grafted plants of 'Madam Vinuous', 'Frost' Valencia, 'Frost' navel oranges and 'Red Blush' grapefruit were negative for stubborn.

Although *in vitro* grafting requires much dexterity and special skills, the technique is expected to play a significant role in efforts to produce citrus clones that are free of many pathogens. Perhaps the procedure could be used in combination with thermotherapy to further enhance the probability of elimination of systemic pathogens. In the procedure developed from this study, 2-week-old asceptically and dark grown 'Troyer' citrange seedlings are used as rootstocks ('Rough' lemon may be preferred in some cases) and 0.14 to 0.18 mm long shoot tips as scions. The seedling to be used as rootstock is decapitated, leaving 1 to 1.5 cm portion of the epicotyl; the cotyledons and their subtending buds are removed; and the root is shortened to 4 to 6 cm. An inverted-T incision is made on the epicotyl, the vertical cut starting at the point of decapitation and extending 1 mm down the stem; the horizontal cut at the lower end of the incision is made about 1 mm wide. The incision is made through the cortex and reaches the cambial region of the stele. The shoot tip is placed in the incision with its basal cut surface in contact with the rootstocks's cortical surface that has been exposed by the outward separation of the cortex from the stele (Fig. 3e). The grafted plants are allowed to develop aseptically in a nutrient solution which contains inorganic salts according to the plant cell culture formula of Murashige and Skoog, 100 mg/l *i*-inositol, 0.2 mg/l thiamine·hydrochloride, 1 mg/l pyridoxine·hydrochloride, 1 mg/l nicotinic acid, and 7.5% sucrose. The best source of shoot tips is the actively growing shoots that can be induced on field trees or greenhouse plants by defoliating selected branches. Lateral buds of source trees can be cultured *in vitro* and used as a second source of shoot tips.

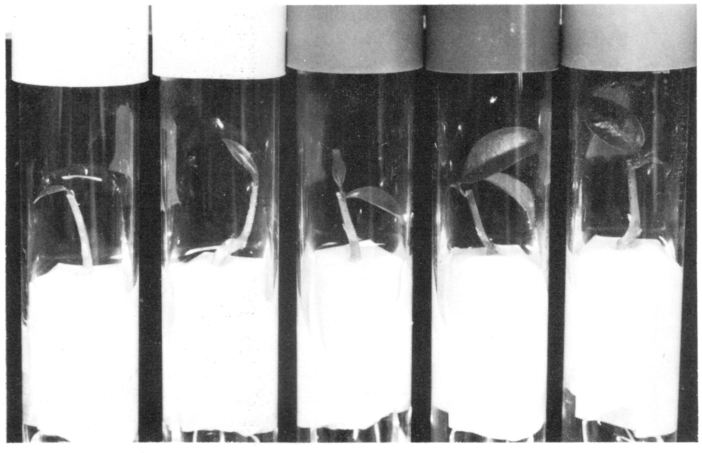

Fig. 10. Influence of the sucrose concentration of the nutrient solution on the development *in vitro* of grafts of 'Madam Vinous' sweet orange shoot tips on 'Troyer' citrange rootstocks. Left to right: 0, 2.5, 5.0, 7.5, and 10.0%.

Fig. 11. 'Temple' tangor plant recovered free of exocortis viroid through *in vitro* shoot-tip grafting procedure. Note fruit on plant that flowered 11 months after grafting.

The method of *in vitro* grafting of shoot apices excised from pathogen-infected plants onto seedling rootstocks might have more general applicability among diverse plants. Indeed, it should be tested with other woody genera, especially tree crops, in which meristem and shoot tip cultures or other methods of virus disease therapy have not been employed successfully.

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Resistance in Eggplant, *Solanum melongena* L., and Nontuber-Bearing *Solanum* Species to Carmine Spider Mite[1]

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Abstract. The Agricultural Research Service collection of eggplant (*Solanum melongena* L.) and related *Solanum* species from throughout the world was screened for resistance to the carmine spider mite *Tetranychus cinnabarinus* (Boisduval). Tolerance to mite feeding damage was found in *S. melongena* (P.I. 269663 and 269660), and antibiosis was found in *S. mammosum* L. (P.I. 245968), *S. sisymbrifolium* Lam. (P.I. 337597), and *S. pseudocapsicum* L. (P.I. 368425). P.I. accessions 245968, 368425, 269663, and 269660 were least preferred for feeding and oviposition.

Eggplant (*Solanum melongena* L.) is a minor self-pollinated vegetable crop in the U.S. with no more than 3,000 acres in production annually (8). However, in the Orient and Middle East, eggplant is cultivated as extensively as the tomato is in the U.S. (1). Eggplant is attacked by several arthropod pests including carmine spider mites *Tetranychus cinnabarinus* (Boisduval) (6). We therefore screened a representative collection of *Solanum* germplasm for resistance to this mite.

Materials and Methods

The entire Plant Introduction (P.I.) collection of eggplant (*S. melongena* and related species) maintained by the Plant Introduction Station at Experiment, GA and 3 cultivars obtained from Cornell University[4] were tested. Each accession or cultivar was seeded in Jiffy-7 peat pots, transferred to 4 inch clay pots containing a greenhouse soil mixture and fertilized every 3 weeks to maintain vigorous growth. Beltsville cultured strain of carmine spider mite was used in all tests and reared on lima beans.

a) Three hundred and forty five accessions of *S. melongena* and 12 accessions of related *Solanum* species were mass screened for susceptibility or resistance to the carmine mite; each entry was replicated 3 times (3 plants/entry). b) Selections from experiment 1 were retested with each entry replicated 5 or 7 times. c) Selected accessions and cultivars from experiment 2 were evaluated for antibiosis. d) Accessions and cultivars selected from antibiosis tests were subjected to leaf disk tests for feeding preference and oviposition in 2 tests, each replicated 9 times.

Jersey King, P.I. 143402, and P.I. 163264 were included in all tests as susceptible controls. Mass mite infestations were achieved in Experiments 1 and 2 by pinning one mite-infested bean trifoliate to each of the test entries. Injury was rated visually after 25 days, on a 1–9 scale with 1 equal to no damage and 9 equal to severe damage. In Experiment 3, five randomly selected adult female mites were placed in 22-mm-diameter cages, (9) that were attached to the top surface of the third or fourth leaf from the terminal of each test plant. The top surface was selected because of the ease in handling and transferring cages and mites. After 12–13 days, living mites and eggs (biomass) were recorded.

The test plants were 8 to 10 week-old greenhouse grown plants treated weekly with resmethrin and dichlorvos to prevent unwanted insect infestations. Pesticide treatments were terminated 5 days before testing. Experiments 1, 2, and 3 were conducted under natural photoperiods for the months of November through February at 23° ± 5°C and relative humidity was 54 ± 20%.

In Experiment 4, leaf disks (18 mm diam) were randomly cut from the test plants and arranged randomly (bottom side up) in a circle on moistened cellucotton in a 15-cm petri dish so mites could easily migrate from disk to disk. Five adult females were transferred from the stock colony to the center of each disk, and mite movement was recorded every 2 hr for the first 6 hr and at 20 and 24 hr. The preference test was conducted in a laboratory maintained at constant temperature (21° ± 2°C) and relative humidity (70% ± 10%) with 50-ft candle of illumination.

Results

Experiment 1. Of 345 *S. melongena* accessions tested in Experiment 1, 12 exhibited a moderate level of resistance and were saved for further testing. Two of the 12 accessions of the related *Solanium* species, P.I. 337597 and P.I. 245968, appeared nearly immune to mite damage (Table 1). The range of susceptibility and plant types of the *Solanum* species are illustrated in Fig. 1.

Experiment 2. Many of the *S. melongena* accessions selected for

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[4] Cultivars Dumaguete, Sinompiro, and Millionaire supplied by H. M. Munger, Plant Breeder, Cornell University, Ithaca, NY.

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