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Abogado

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Doctora

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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GERMAN ARIAS JARAMILLO, identificado con la cédula de ciudadanía No. 14.993.364 de Cali, y tarjeta profesional de abogado No. 18.842 del C.S. de la J., actuando como Apoderado del Centro Internacional de Agricultura Tropical – CIAT y de la Corporación BIOTEC, dentro del trámite de la patente sobre “Proceso para la propagación clonal *in vitro* de plantas de guanábana (*Annona muricata* L.), empleando microinjertación cíclica”, doy respuesta al oficio de la referencia de la siguiente forma:

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Los artículos que enviamos con esta respuesta al oficio de la referencia son los siguientes:

- a) Murashige T et al. A Technique of shoot apex grafting and its utilization towards recovering virus free Citrus clones. Hortscience 7 (1972): 118:119
- b) Navarro L; et al. Improvement of Shoot-tip grafting in vitro for virus free Citrus. J.Amer. Soc. Hort Sci 100 (5,1975): 471 – 479
- c) Jonard R et al. In Vitro Micrografting and its application to fruit science. Scientia Hort. 20 (1983) 147 – 159.

Los artículos que anexamos, solicitados por la Superintendencia de Industria y Comercio, dentro del trámite de la referencia, no afectan los requisitos de patentabilidad de la invención que se solicita, por las siguientes razones:

PRIMERO.- Al contrario de las especies vegetales herbáceas (aquellas que no producen tallos lignificados), la propagación *in vitro* de numerosas especies leñosas, en las que se pueden incluir especies de árboles frutales o forestales, no ha sido posible a nivel comercial.

Los problemas que se presentan al intentar aplicar metodologías convencionales del cultivo *in vitro* a estas especies, han sido enumerados y discutidos en detalle en la solicitud de la patente. En la especie leñosa para la cual se está solicitando la patente, el guanábano, a pesar de numerosos intentos, tampoco ha sido posible el desarrollo de metodologías de propagación masiva *in vitro*. La invención que se desea patentar incluye

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no solo una sino un conjunto de innovaciones que hace posible por primera vez la propagación *in vitro* a nivel comercial de clones de guanábano.

SEGUNDO.- Principalmente dos modificaciones de las metodologías convencionales hacen que el procedimiento de la solicitud haya resultado en el desarrollo de una metodología aplicable a nivel comercial como se indica en las páginas 15 y 16 de la solicitud de la patente, así:

- a) El uso de puntas cónicas para forzar un contacto directo y estrecho entre los tejidos del microexplante y los del patrón, que permitió incrementar considerablemente la eficiencia de la microinjertación;
- b) La aplicación del concepto de microinjertación cíclica *in vitro* como herramienta de propagación vegetativa de yemas.

TERCERO.- La Superintendencia de Industria y Comercio indica que *"cualquier persona versada en la técnica de microinjertación estaría motivada a emplear las nuevas yemas formadas por el microexplante para la producción de nuevos microinjertos, y así lograr una mayor multiplicación clonal"*. Esto, para resaltar que el proceso que se quiere patentar *"podría constituir el resultado de ensayos sistemáticos de la actividad investigativa"* y que resulta obvio para un investigador en el campo de la propagación clonal.

Alcanzar una mayor propagación clonal, sin embargo, no es suficiente para desarrollar metodologías de propagación aplicables a nivel comercial, puesto que si estas no son eficientes, como ha sucedido hasta el momento, los costos de aplicar las metodologías de cultivo *in vitro* en la propagación del guanábano serían tan altos que esta actividad no podría competir con otras técnicas de propagación. No resulta pues, obvio aplicar el concepto de microinjertación cíclica en la propagación clonal masiva si es que el investigador versado en técnicas de propagación no cuenta con metodologías eficientes de microinjertación. Estas últimas constituyen igualmente una de las innovaciones de la solicitud de la patente.

Con respecto a la argumentación de que un proceso patentado de propagación en papa, que involucra la microinjertación de yemas, constituye una anterioridad y puede afectar la patentabilidad del método desarrollado, nuestra opinión es que esto sería válido si la técnica de microinjertación empleada en papa fuera similar a la descrita en la solicitud de la patente, lo que no es el caso. Tal como se presenta en el ejemplo 1 con el uso de metodologías de injertación como las aplicadas en papa, que no incluyen el uso de puntas cónicas que protejan la yema microinjertada de la desecación, una innovación de la presente solicitud, solo se obtienen eficiencias de prendimiento tan bajas que no permiten la aplicación de la microinjertación como método de propagación.

PETICIÓN

Con fundamento en los argumentos expuestos anteriormente, solicitamos el reconocimiento de la patente de la referencia.

Atentamente,

GERMAN ARIAS JARAMILLO

Apoderado

C.C. No. 14.993.364 de Cali

T.P. No. 18.842 del C.S. de la J.

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A Technique of Shoot Apex Grafting and Its Utilization Towards Recovering Virus-free *Citrus* Clones¹

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Abstract. Shoot apices, excised from *Citrus* cultivars of known virus content, were successfully grafted *in vitro* onto disease-free rootstock seedlings and some virus-free plants were obtained. The prolonged juvenile phase which characterizes disease-free nucellar lines was bypassed by this procedure.

Morel and Martin (4) first demonstrated that virus-free plants of *Dahlia* could be recovered from infected plants through shoot apex culture. This technique, commonly referred to by the misnomer "meristem culture," has been applied routinely since to reproduce virus-free plants of diverse species (3). With this technique, stem apices 0.2-0.5 mm long are excised and rooted under aseptic conditions. The stem apex is composed of the apical meristem and a few subjacent primordial leaves, is often free of pathogens and is culturable in relatively simple nutrient media. Exposure of the source plant to warm temp (heat treatment) for prescribed periods lessens the possibility of pathogen transmission.

With *Citrus*, heat-treating has been satisfactory in providing bud-wood free of certain viruses, e.g., tristeza, vein enation and psorosis (Roistacher, unpublished). However, it has proven ineffective for the exclusion of other pathogens, e.g., exocortis. Successful culture of the shoot apex or meristem of *Citrus*, like other tree species, has not been attained thus far. It is possible to maintain explants *in vitro* for several months, but with no significant development. Successful culture of

nucellar tissue to recover virus-free plants of diverse *Citrus* species and cultivars was reported previously (1, 5). Unfortunately, plants originating from nucellar tissue are juvenile and remain so for substantial periods. In view of the failure of shoot apex cultures of *Citrus*, and to avoid the juvenile phase while reestablishing disease-free plants, a technique has been developed by which the shoot apex is grafted *in vitro* onto a disease-free seedling rootstock. Successful application of this technique depends on the fact that *Citrus* seedlings are generally free of virus and on the assumption that the shoot apex is pathogen-free.

While the authors are able to report on a workable procedure which has been adequate for present needs they cannot as yet prescribe optimum conditions; experiments are in progress to this end.

The Rootstock: *Citrus* seedlings tend to produce adventitious shoots, particularly from sites of injury. Thus, it is desirable that the rootstock should contain a distinct morphological marker. The "Troyer" citrange (*Citrus sinensis* Osbeck x *Poncirus trifoliata* (L.) Raf., and the trifoliolate orange (*Poncirus trifoliata* (L.) Raf.), because of their trifoliate leaves, were selected as marker stocks. Both are highly polyembryonic and their seedlings are principally clonal. Rootstock seeds were obtained from fruits harvested in season from the cultivar collection at Riverside. The seeds were removed from the fruits, washed in tap water, heat-treated (immersed 10 min in water at 52°C), surface-dried at room temp for several hr, dusted with Arasan, placed in polyethylene bags, and stored at 5°C until used.

When used, the seeds were rewashed, peeled (both integuments removed), wrapped in cheesecloth and surface-sterilized by immersion in

sodium hypochlorite solution (Purex diluted 10 x) for 10 min, rinsed twice with autoclaved water, and sowed in autoclaved, moist vermiculite. A mixture of 4% finely ground vermiculite and 25 ml deionized distilled-water placed in 25 x 250 mm Pyrex culture tubes was used as the germination medium. The culture tubes were capped with stainless steel closures and autoclaved for 15 min at 121°C. Three seeds were sown per tube and germination took place in darkness at 27°C. After 3 weeks the seedlings were removed aseptically from the tubes, selected for uniformity, transferred to sterile petri dishes, and decapitated 3 cm above the cotyledons for grafting.

The shoot apex: Wherever possible the terminal portions (2-3 cm) of growing shoots on mature, bearing trees of known virus content were used as source of shoot apices. The terminals were pinched off their subtending older growths, placed in polyethylene bags, and brought to the laboratory. They were stripped of their leaves, wrapped in cheese-cloth, surface-sterilized by immersing 10 min in 10 x diluted Purex, rinsed twice with autoclaved water, and transferred to sterile petri dishes for excision of their apices.

Performing the graft: The rootstock seedlings were transferred individually to sterile petri dishes lined with sterile moist Whatman No. 50 filter paper. The moist filter paper served to retard desiccation of tissues during excision and grafting. The disinfected stem tip of the scion cultivar was placed in another sterile petri dish, also lined with moist filter paper. All foliar appendages, except for 4-6 of the youngest leaf primordia, were removed and discarded. The shoot apex, i.e., the meristem dome together with the remaining primordial leaves, was severed and transferred from the stem to the decapitated rootstock. The cut surface of the decapitated seedling was apparently sufficiently moist to achieve adherence between shoot apex and rootstock. In view of the small size of the shoot apex, it was necessary to devise special micro-scalpels out of pieces of razor blades to accomplish the excision. It was also necessary to perform the excision and the transfer to rootstock with the aid of a dissecting microscope. All manipulations were performed in a clean-air hood to minimize air-borne contamination. Moreover, in excising the shoot apex, the scalpel was dipped in dilute Purex between each cut to

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²The authors gratefully acknowledge financial support from the California-Arizona Citrus Advisory Board.

avoid possible spread of pathogens from infected to uninfected tissues.

Maintenance of grafted plants: The grafted plants were transferred to sterile culture tubes (25 x 150 mm Pyrex) containing 10 ml Hoagland's solution. A circle of 9-cm Whatman No. 50 filter paper, folded in the form of a stool and perforated in its center, was placed in the solution to serve as support. The root of the rootstock was inserted through the perforation in the filter paper platform. The tubes were capped with polypropylene closures. The cultures were placed upright under low intensity artificial illumination (16-hr daily exposure to 1000 lux light from 20-W Sylvania Gro Lux Lamps) and maintained at constant 27°C.

Following aseptic precautions, the tubes were uncapped periodically and any shoots showing trifoliate leaves were removed with a pair of elongated surgical scissors. At 1-month intervals the cultures showing successful grafts were transferred to fresh nutrient tubes to maintain vigorous growth. After approx 5 months the grafted plants were large enough to be transferred to soil. The plants were potted in autoclaved soil, watered with Hoagland's solution, covered with polyethylene bags to retard desiccation, and grown in the laboratory under higher intensity illumination (16-hr daily exposure to circa 4000 lux Gro Lux light). The plants were irrigated with Hoagland's solution as necessary. After 1-2 months, the plants were removed from the laboratory to a quarantine greenhouse and further cultured for virus indexing. Virus indexing followed standardized procedures (2).

Successful grafts have now been accomplished with several species and hybrids of *Citrus*, including 'Temple' tangor, 'Eureka' lemon, 'Washington' naval orange, 'Satsuma' mandarin, 'Marsh' grapefruit, and 'Cuban' shaddock. The incidence of success has been low, ranging from 5% in some experiments to 40% in others. Nevertheless, the final outcome has been adequate. Virus indexing tests, now completed for exocortis, have disclosed 4 of 6 'Temple' tangor and 1 of 2 'Eureka' lemon budlings to be free of the pathogen. The indexing tests for other virus diseases remain largely incomplete at this writing. In one case, a budling derived from a tree infected with both tristeza and vein enation was indexed and found negative for tristeza, but positive for vein enation.

A distinctive feature of the virus-free plants obtained through shoot apex grafts is that they have bypassed the juvenile phase typical of plants obtained through nucellus culture. Grafted 'Temple' tangor plants 1½ year-old are free of thorns and already flowering. In



Fig. 1. Stages in the development of a virus-free 'Temple' tangor plant from the shoot apex grafted *in vitro* onto a 'Troyer' citrange seedling. A. Excised shoot apex, about 117X magnified. B. Decapitated 'Troyer' seedling with freshly grafted 'Temple' tangor shoot apex on left and successful graft after few months on right. C. Virus-free plant established from shoot apex graft on left and from nucellus culture on right. D. Close-up of branches of plants in C. Note flowering and freedom from thorns in plant derived from shoot apex, in contrast to plant originating in nucellus on right.

contrast, 2-year-old nucellar-derived plants of the same hybrid are extremely thorny and still vegetative (Fig. 1).

Studies in progress are aimed at improving the incidence of graft success and the probability of pathogen-free citrus. Size of the excised shoot apex will influence both grafting success and pathogen exclusion. Unfortunately, increasing the size of the apex will enhance grafting, but will increase the hazard of disease transmission. Heat treatment and etiolation of source plants are expected to further reduce presence of virus in the excised shoot apex. Improvements in care of the rootstock, perhaps by supplementing the nutrient solution with sugar, vitamins, and growth substances, should extend grafting success. The method of insertion of the shoot apex on the rootstock may be important. A T-incision on the lateral flanks of the stem of the rootstock, exposing more cambial surface, probably would be a better place to insert the shoot apex, rather than the cut surface of the decapitated stem.

A major limitation of the technique of shoot apex grafting is the requirement of exceptional manual dexterity. Also, the technique applies only to instances where the virus in question is not transmitted through weeds used for the understock.

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3345 Jans 3045 Improvement of Shoot-tip Grafting *in vitro* for Virus-free Citrus^{1,59}

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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 Gro 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 pooled (both seed-coats were removed), wrapped in small squares of cheesecloth 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.) Raf. & *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
<i>C. sinensis</i> (L.) Osbeck	
'Robertson' sweet orange	Escaortia, psorosis
'Pera' sweet orange	None
'Cadmosa Flm' sweet orange	Escaortia, tristem
'Cadmosa de Carapente' sweet orange	Escaortia, concave gum
'Madam Vinous' sweet orange	Stubborn, psorosis, infectious variegation, seedling yellow-tristem
'Front' Valencia orange	Stubborn, psorosis
'Front' navel orange	Escaortia
'Koshas' sweet orange	Concave gum
'Pineapple' sweet orange	Psorosis
'Oltide' Valencia orange	Yellow vein
<i>C. reticulata</i> Blanco	
'Clementine Monreal' mandarin	Escaortia, concave gum
'Willow Leaf' mandarin	Escaortia, cactus-like-tytoporosis
'Setuna' mandarin	Infectious variegation
<i>C. paradisi</i> Macf.	
'Genetic Dwarf' grapefruit	Escaortia
'Redblush' grapefruit	Stubborn
<i>C. limon</i> (L.) Burm.	
'Ricote' lemon	Escaortia, cactus-like-tytoporosis
'Mozuro' lemon	Escaortia
'Santa Teresa' lemon	Escaortia
'Meyer' lemon	Taller leaf-citrus stunt complex
'Prior Lihon' lemon	Cactus-like-tytoporosis
<i>C. aurantiifolia</i> (Christm.) Swing.	
'Dears' lime	Escaortia
<i>C. medica</i> L. var. <i>sarcocaulis</i> (Noot.) Swing.	
'Fingered' citron	Escaortia
<i>C. reticulata</i> Blanco x <i>C. sinensis</i> (L.) Osbeck	
'Temple' tangor	Escaortia
<i>Forsyelia japonica</i> (Thunb.) 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. Terminalia 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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3% sucrose, 0.4 mg/l thiamine hydrochloride, 100 mg/l l-inositol, 40 mg/l adenine sulfate $\cdot 2\text{H}_2\text{O}$, 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 x 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.

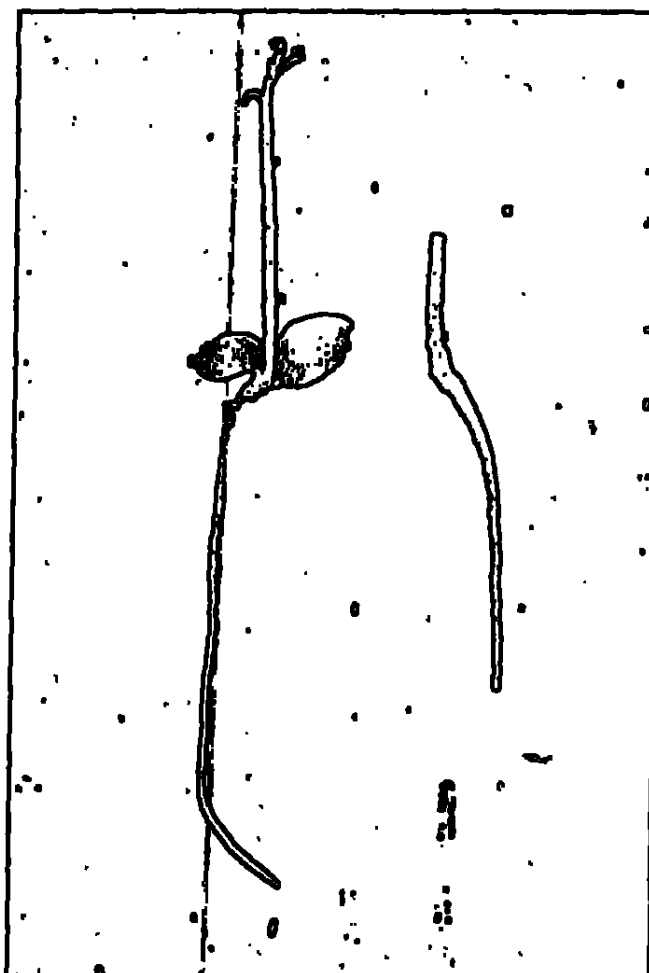


Fig. 2. Seedling and rootstock used for *in vitro* grafting of citrus. Left: 2-week-old 'Troyer' citrange seedling; right: seedling rootstock prior to grafting with epicotyl decapitated, cotyledons and root tip severed, and inverted-T incision (not visible in Fig.) made at top end of decapitated epicotyl.

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 tristeza, tristeza, seedling yellows-tristeza, concave gum, infectious variegation, yellow vein, crotch-xylem porosis and the tatterleaf-strange stunt complex. Stubborn disease caused by *Spiriplasma 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. 3d), or the cambial region of the seedling (Fig. 3e). 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 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: N⁶-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 A₃ (GA₃) solution was placed onto the shoot tips in attempts to induce their growth. The GA₃ 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

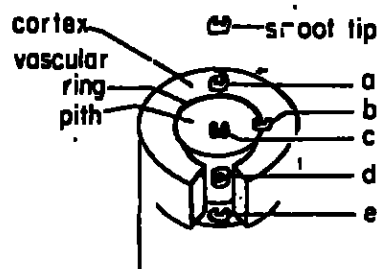


Fig. 3. Diagrammatic presentation of various methods tested in positioning shoot-tip scion onto decapitated seedling rootstock.

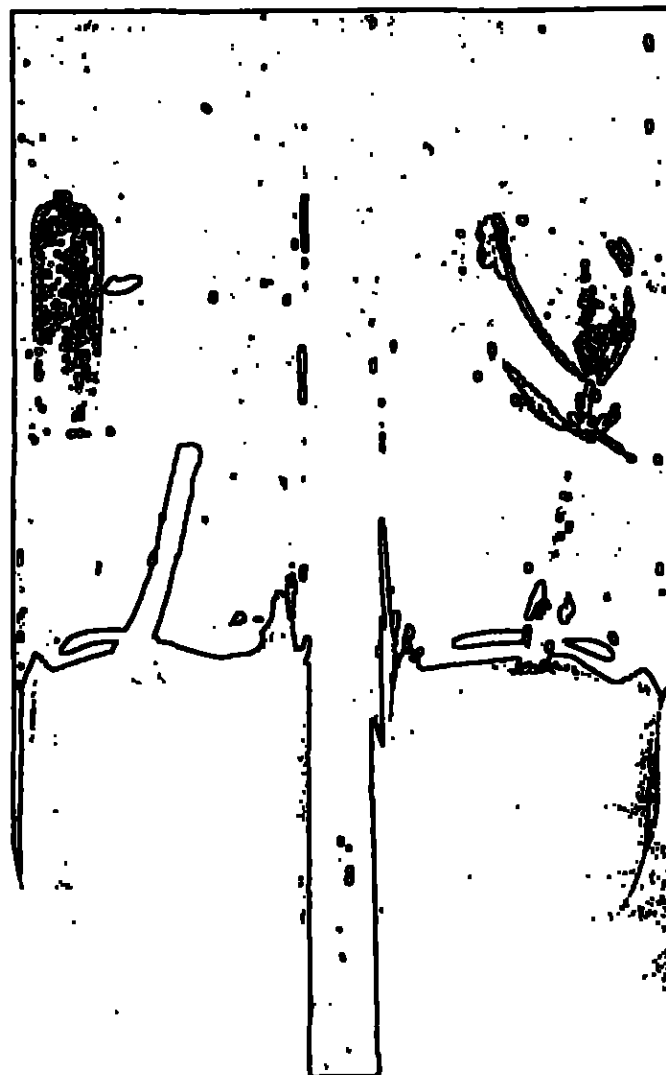


Fig. 4. Freshly prepared graft, left, and resultant plant after 1 month, right, of 'Madam Vinous' sweet orange.

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 *shenensis* (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 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 an 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* Muel. '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.

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

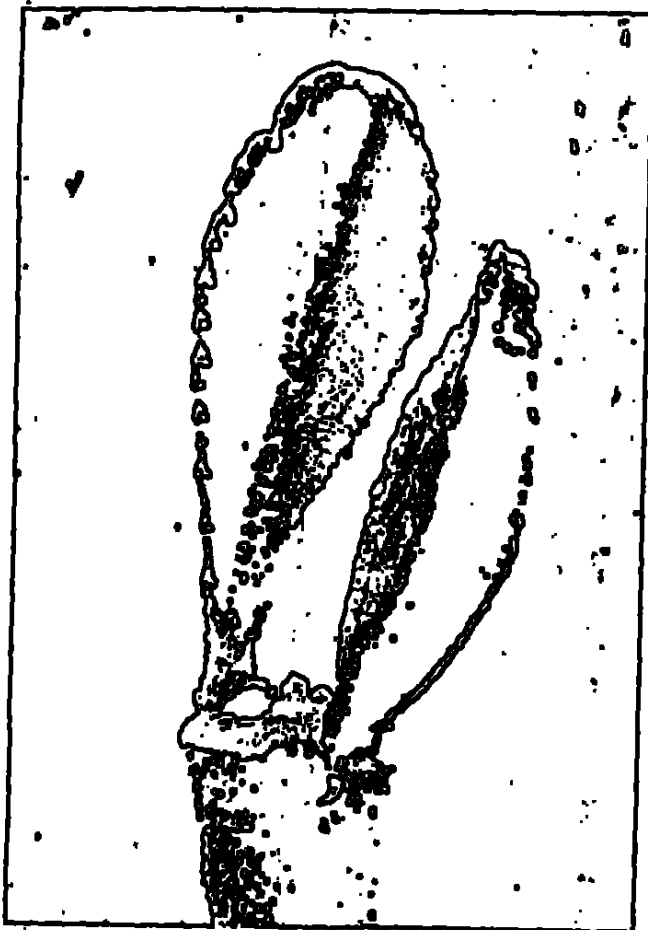


Fig. 5. Enlarged view about 10 x), showing leaf arising from 'Meyer' lemon scion shoot tip (right) placed in inverted-T incision of rootstock, together with that from an adventitious bud that differentiated from rootstock tissue (left).

did not alter grafting success. No difference was observed between rootstocks with intact and decapitated roots. Accordingly, us 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, 1.87, 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 furnish 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 maluchite green. Maluchite 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 maluchite 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 (11) recently reported that BA as well as another cytokinin, kinetin, caused

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 <i>in vitro</i>	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
L-Inositol	100.0	100.0
Pyridoxine-HCl		1.0
Nicotinic Acid		1.0
Adenine Sulfate-2H ₂ O	100.0	
Complex vitamins		
Difco Nucleo-Agar	10,000.0	

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

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 medium 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 disproportionately 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, GA₃, showed no beneficial effect. Altman and Goren (1) found that IAA inhibited

growth in their bud cultures, but GA₃ stimulated the elongation 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 GA₃ 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 as 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,

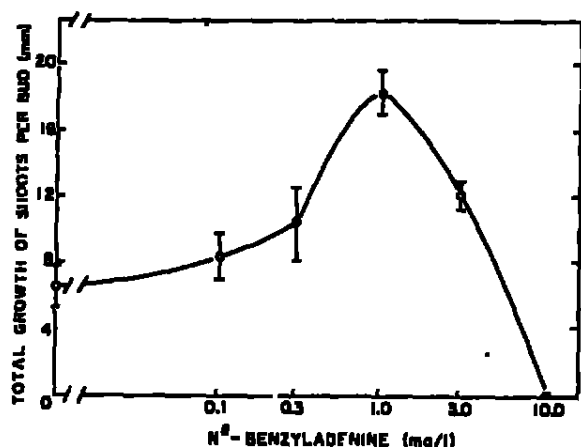


Fig. 6. Effects of BA concentration on shoot development in cultured lateral buds of 'Robertson' navel orange. Vertical lines indicate standard errors of means.

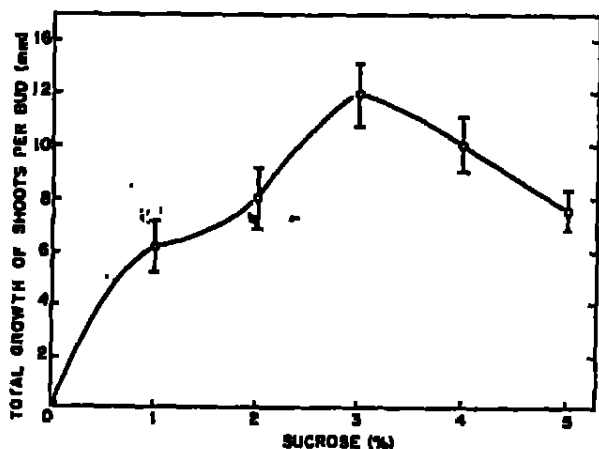


Fig. 7. Effects of sucrose concentration on shoot development in cultured lateral buds of 'Robertson' navel orange.

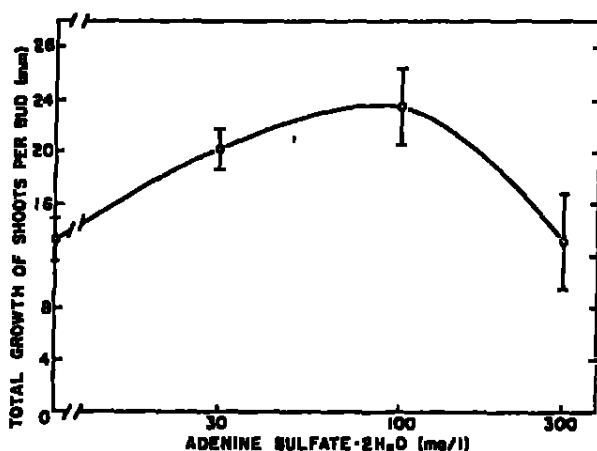


Fig. 8. Influence of adenine sulfate concentration on shoot development in excised 'Robertson' navel orange lateral buds *in vitro*.

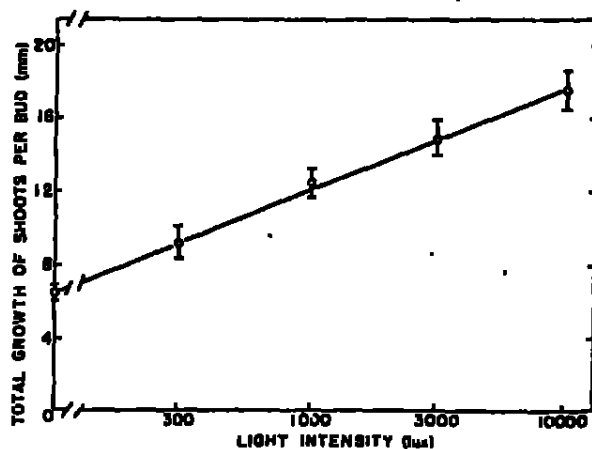


Fig. 9. Effect of illumination on shoot development in excised 'Robertson' navel orange lateral buds cultured *in vitro*.

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. 3a). 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.

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. Mali 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 GA₃ 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 nicotinic 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 7. Influence of method of placement of shoot tip onto rootstock seedling on incidence of successful grafts *in vitro*; scion cultivars: 'Cudenera de Caramante' and 'Pera' sweet oranges and 'Temple' tanger.

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

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.

% Successful grafts	% Sucrose in medium				
	0	2.5	5.0	7.5	10.0
	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

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 viroid in 31 plants of 'Cudeneri 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 Vinous', '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, 3-week-old aseptically 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



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.

its basal cut surface in contact with the rootstock's cortical surface that has been exposed by the outward separation of the cortex from the stele (Fig. 3c). 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 L-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.

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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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%.

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Resistance in Eggplant, *Solanum melongena* L., and Nontuber-Bearing *Solanum* Species to Carmine Spider Mite¹

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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. sisynthrifolium* 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 University⁴ were tested. Each accession or cultivar was seeded in fifty-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.

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⁴Cultivars Dumagete, Sinompiro, and Millionaire supplied by H. M. Wenger, Plant Breeder, Cornell University, Ithaca, NY.

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 21° ± 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 cellulocotton 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 *Solanum* 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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IN VITRO MICROGRAFTING AND ITS APPLICATIONS TO FRUIT SCIENCE

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ABSTRACT

Jonard, R., Hugard, J., Macheix, J.-J., Martinez, J., Mosella-Chancel, L., Poessel, J.L.
and Villemur, P., 1983. In vitro micrografting and its applications to fruit science.
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Improvements have been brought to in vitro micrografting. The breaking of dormancy by dipping the seeds of the rootstock in concentrated solutions of benzylaminopurine avoids having recourse to long stratification in the cold (60-120 days according to species). The initial pre-treatment of apices with cytokinin allows their development and facilitates later grafting. Moreover, adding DIECA to the cut sections avoids oxidation and, together with the insertion of a piece of agar between apex and rootstock, increases the success of micrografting. Finally, a special technique of in vivo micrografting of pre-treated apices on young plants has been developed.

Micrografting is commonly used to obtain peach trees free of Sharka and NRS viruses, and to study incompatibility mechanisms in heterografts, leading to an early diagnosis of incompatibilities.

Keywords: dormancy; micrografting; peach; stratification; virus; vitro; vivo.

INTRODUCTION

Following the first attempts of Ball (1946) and Moral and Martin (1952), the regeneration of complete plants from apex cultures has been successful for numerous herbaceous species (Quak, 1977). On the other hand, the application of this technique to woody plants is more difficult (Jones and Vine, 1968; Beaucheme, 1968), although a few satisfactory results have been obtained with different fruit trees, i.e. 38 different species or cultivars of *Prunus* (Boxus and Quohin, 1974; Quohin et al., 1977) and almond and almond-peach hybrids (Tabachnik and Kester, 1977). Furthermore, its application to apple trees has also been reported (Walkey, 1972; Abbott and Whiteley, 1976).

Numerous failures were noted following direct isolation of apices from

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woody plants, so Murashige et al. (1972) and Navarro et al. (1975) suggested the new technique of *in vitro* micrografting, first developed with different *Citrus* species. It consisted of grafting an excised apex on to a decapitated seedling as a rootstock.

Whether in association with heat treatment or not, the micrografting has led to the elimination of viruses in numerous cultivars of fruit trees. Furthermore, this technique has made it possible to obtain a better comprehension of the mechanisms of heterograft incompatibility; a phenomenon which affects the grafted plants by causing a significant reduction in the productivity of the tree combined with the appearance of physiological disorders, as frequently described (Herrero, 1951; Moosa, 1968).

Therefore, we have used this new technique not only to obtain woody virus-free plants in which apex culturing is difficult, but also to analyse incompatibility mechanisms in *Prunus* heterografts.

MATERIAL AND METHODS

Plant material. — Experiments have been conducted in Montpellier for several years on different species, mainly grafting of: (a) some *Citrus* (Lemon, Clementine and Bitter-Orange trees) on *Troyer Citrange*, a rootstock easily identified by its leaves; (b) apple trees (East-Malling rootstocks and 'Reinette du Canada') on *Malus Niedzwetzkiiana* (Alakief, 1978); (c) different *Prunus* (Mosella-Chancel, 1979; Martinez, 1979), *P. persica* Batsch ('Michellini' and 'Springtime'), on rootstocks whose seeds are phenotypically uniform (GF 305 INRA), *P. armeniaca* and *P. cerasifera*.

Initial technique of micrografting. Each apex (0.1–0.5 mm) was excised in rigorous aseptic conditions under a binocular microscope, then transferred on to the cambial zone of the section of a pre-decapitated seedling obtained from a sterile sowing. For some species, a long period of stratification (30–60 days at 3–4°C for *Prunus armeniaca*; 80–120 days for *P. persica*; 0–120 days for *P. cerasifera*) was necessary to break the dormancy of seeds (Dama-vandy, 1970). This cold treatment was conducted in darkness in aseptic conditions, either in culture tubes on a mineral solution (KNOP diluted 1/2) with agar (1%) and sucrose (4%) or in Petri dishes, several denuded and disinfected seeds being then arranged between layers of filter paper moistened with sterile distilled water.

In most cases, a leafy shoot regenerated from the apex. Many modifications have been applied to improve the technique and these will be discussed sub "Results" (Figs. 1 and 2).

Control of virus. — The presence of virus infection has been demonstrated by several methods:

directly on the rootstock GF 305, which is a polyvalent indicator (Bernhard et al., 1969);

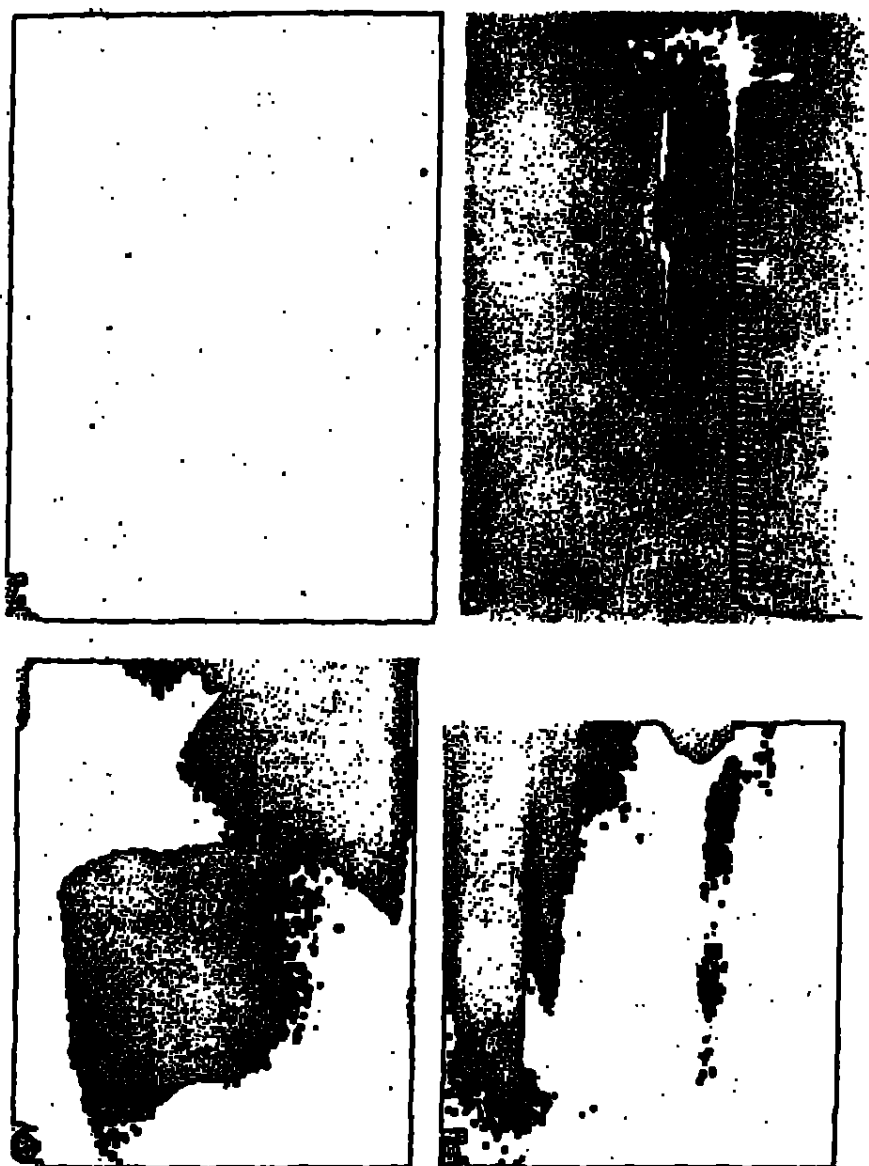


Fig. 1. Preparation of the decapitated seedling used as rootstock. A, entire seedling; B, after decapitation prior to grafting; C, rootstock: Lemon tree, scion: 'Troyer' Citrange; D, rootstock: Lemon tree, scion: Orange tree.



Fig. 2. *Malus pumila* Mill: development of leafy shoots from apices ('Rehette du Canada') grafted on to decapitated seedlings of 'Niedzwetakiens'. A and B, the stock seedling has been cut above the cotyledons; C, the stock seedling has been cut under the cotyledons. G = shoot developed from the apex, pg = rootstock seedling, et = cotyledons, Ca = proliferation callus, sp = filter paper support, M = liquid medium.

electron microscopy according to the leaf exudate method and the Enzyme-Linked Immunosorbent Assay (ELISA) (Clark et al., 1976);

inoculation of indicator herbaceous plants (e.g. *Cucumis sativus*) for the NRS Ilar type virus (Fulton, 1970).

RESULTS AND DISCUSSION

Improvements of the in vitro micrografting technique

- (1) For some species (*Prunus persica* 'GF 305', *P. armeniaca* and *P. Mahaleb*), the dormancy of the seed producing the seedlings used as rootstock was broken by a 24-h dipping in a solution of benzylaminopurin (200 mg l⁻¹) without any cold treatment (Roukas et al., 1980) (Table I).
- (2) A pre-treatment of the apices used as grafts enabled us to determine those which were able to develop. In such a modification, the excised apex was placed into a hemolysate tube on filter paper moistened by a mineral solution of Miller or Murashige and Skoog, supplemented with auxins and cytokinins. This treatment modified the "physio-

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TABLE I

Breaking dormancy of peach tree seeds ('GF 305') with a cytokinin (benzylaminopurin = BAP) and/or gibberellins (GA). Each batch included 50 seeds with their teguments; all the seedlings treated with BAP were at least 30 cm tall at the end of growth. Harrington formula for rate of sprouting: $\frac{N_1 T_1 + N_2 T_2 + \dots + N_n T_n}{N_1 + N_2 + \dots + N_n}$ where N_i = number of seeds sprouted at time T_i ; $N_1, \dots, N_n$ = number of seeds sprouted at times $T_1, \dots, T_n$

Treatments	Germination of seeds		% of seedlings with foliar aberrations
	%	Rate (days)	
Control: stratified 90 days at 5°C	96	2.0	2.1
Control: no stratification or other treatment	0	—	—
BAP 20 mg l ⁻¹	8	11.3	100
BAP 60 mg l ⁻¹	64	8.5	28
BAP 100 mg l ⁻¹	76	6.8	15.8
BAP 200 mg l ⁻¹	99.5	4.4	9.6
GA ₃ 100 mg l ⁻¹	8	7.6	100
GA ₃ + (28 + 72%) 100 mg l ⁻¹	8	7.3	75
BAP + GA ₃ + (28 + 72%) (200 + 100 mg l ⁻¹)	96	4.2	8.8
BAP + GA ₃ (200 + 100 mg l ⁻¹)	98	4.1	5.1

logical state" of the excised apex and led to a rapid development of leafy shoots, even from the smallest apices (0.1–0.2 mm), the direct grafting of which is generally difficult and ineffective. The grafting was carried out with small shoots, several mm long. An appreciable augmentation of successful graftings was obtained when a pretreatment was made with zeatin (Table II).

- (3) Oxidation and browning of the cut sections of either micrografts or rootstocks was avoided by adding DIECA (2 g l⁻¹) in the washing-medium and by putting droplets directly on to the tissues at the time of grafting. The nutrition of the micrografts was greatly improved by inserting, between the graft and the rootstock, either a piece of gelose with or without phytohormones (Alaklaf, 1978), or a drop of cytokinin (zeatin or BAP); thus, in the case of the peach, an application of zeatin (10⁻¹ mg l⁻¹) allowed a 44% success instead of 22% (Mosella-Chancel, 1979).
- (4) The Murashige and Skoog medium (without auxins) was the best for ensuring the further development of the micrografts provided that it was used with vermiculite, because the liquid alone or in the presence of agar forms asphyxic conditions for roots, with no formation of lateral roots (Martinez, 1979; Mosella-Chancel, 1979).
- (5) Thirty–60 days after grafting, the new plants were transplanted into pots containing equal parts of peat, vermiculite and leaf mould. This

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TABLE II

Effects of pre-treatments on the success of in vitro micrografting of peach trees. The medium of Murashige and Skoog was used for the pre-treatments and the cytokinin was added aseptically

Treatment of apex with secalin mg l ⁻¹	Hours	Number of grafts, N ₁	Number of living apices, N ₂	Number of developed plants, N ₃	% success N ₃ /N ₁
0		23	11	5	21.7
0.01	48	20	10	10	50
0.01	240	19	16	11	57.9
0.1	48	25	16	18	64
1	48	10	2	2	20

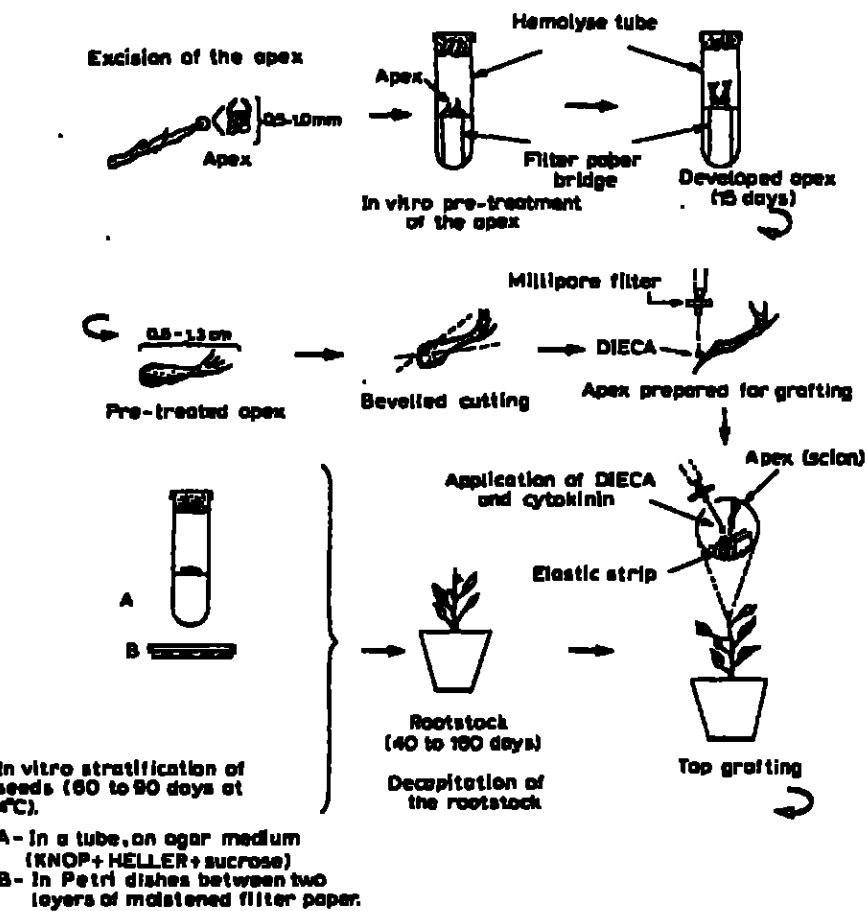


Fig. 3. In vivo micrografting of apices on to young plants cultivated in a greenhouse.

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Fig. 4. In vivo micrografting of pre-treated apex. A, realization of a top grafting. a = decapitating the shoot; b = a drop of DIECA (1.5 g l^{-1}) and zeatin or BAP (0.1 mg l^{-1}) is placed on the wounded zone; c = positioning of the pre-treated apex; d = applying an elastic strip to protect the grafted zone. B, top grafts. Development of the apex to give a leafy shoot 12 (e), 20 (f) and 31 (g) days after grafting. m = zone of micrografting.

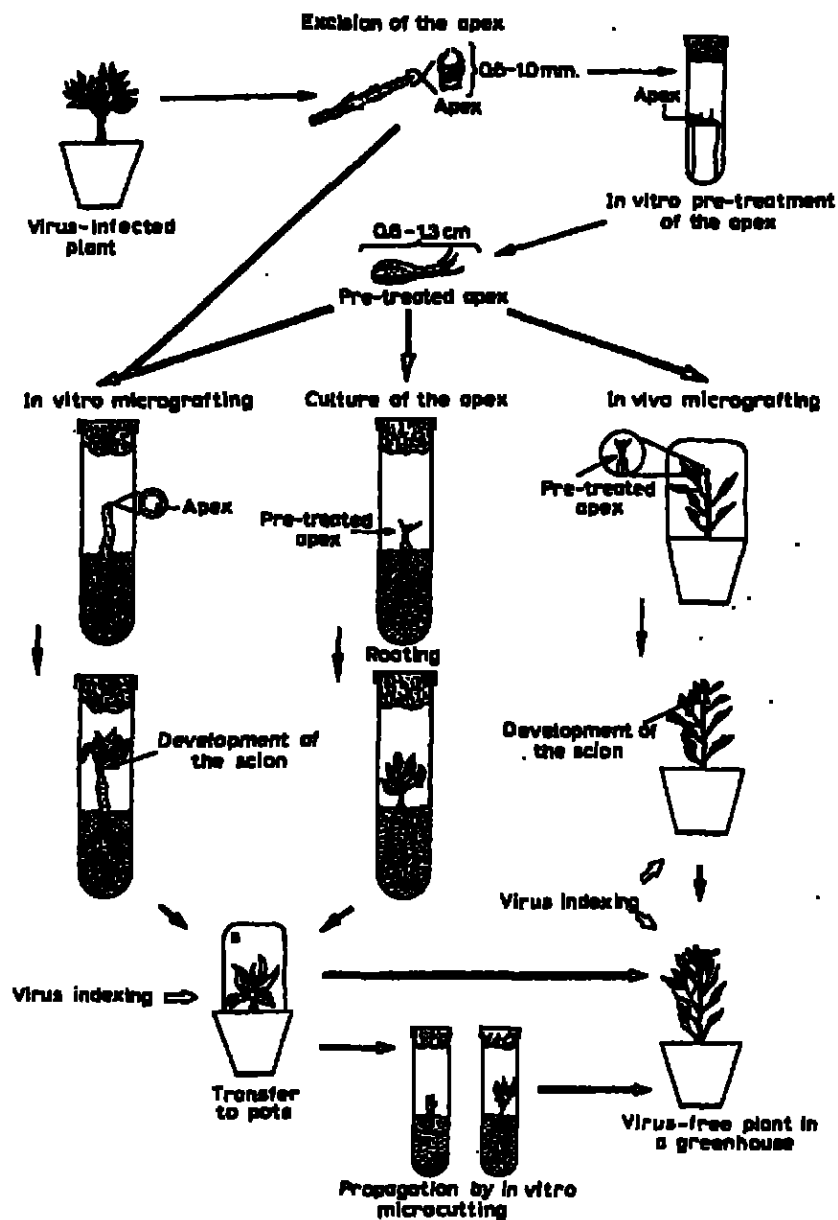


Fig. 5. General scheme showing the different modes of utilization of the apices of the peach tree for eliminating viruses.

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transfer was always a delicate phase, especially for some species of *Prunus*. To avoid it, an original technique has been developed (Mosella-Chancel, 1979). Pre-treated apices (as described above) were directly grafted in aseptic conditions on young plants developed in a greenhouse (Fig. 3). An application of DIECA (1 g l^{-1}) and of a cytokinin ($10^{-1} \text{ mg l}^{-1}$) on to the cut sections had a favourable effect (Fig. 4). A comprehensive view of the procedures used, including the isolation of the apex, a difficult phase in woody plants, is summarised in Fig. 5.

Utilisations of micrografting in orchards

Two main approaches have been developed: the elimination of viruses in fruit trees for which the obtention of normal plants is difficult from directly excised apices, and the analysis of incompatibility mechanisms in heterografts.

Development of virus-free plants. — Apices of 0.4 mm were excised from the terminal shoot tips of peach trees ('GF 305 INRA') previously inoculated in a greenhouse either with the Sharka virus (Strain marcus; Marenaud, 1976), or with an Ilar-type virus (Strain NRS G; Marenaud et al., 1976). After a 15-day period of pre-treatment, each apex gave a 1-cm shoot which was grafted on to a 30-day-old peach tree ('GF 305') grown aseptically. Grafted plants were individually inserted into a plastic bag and held in controlled conditions (25°C , 5000 watts: 16 h/24h). Control of virus infection gave most encouraging data, even without the application of any heat treatment (Table III and Fig. 6).

TABLE III

Elimination of Sharka and NRS viruses by micrografting. For methods used, see text

	Apices excised from plants contaminated with the viruses	
	Sharka (strain M)	NRS (strain G)
Number of plants micrografted	29	14
Number of plants free of viruses	21	8
% of virus-free plants	72.4	57.1

Incompatibility mechanisms in heterografts. — Two types of incompatibilities have been studied, and compared to homografts as controls. The first was called "localised" (*Prunus armeniaca*/*Prunus cerasifera*), the second "translocated" (*Prunus persica*/*Prunus cerasifera* and *Prunus persica*/*Prunus armeniaca*). Under the best conditions, compatible homografts led to about 80% viable plants after transfer into a greenhouse. In all cases, the success of

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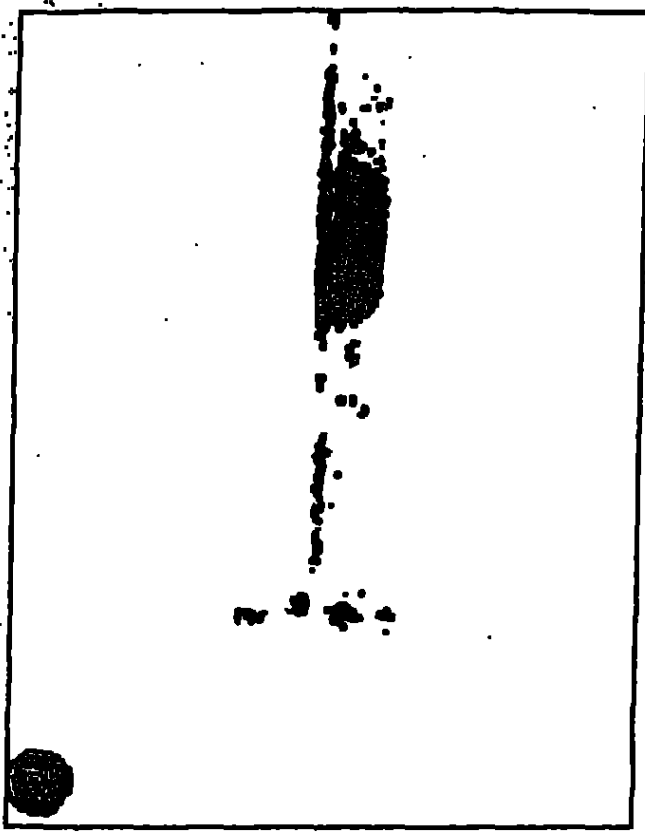


Fig. 6. Elimination of Sharka virus by in vitro micrografting. Left, virus-infected plants; right, virus-free plants, 6 months after grafting.

TABLE IV

Results of in vitro micrografting of *Prunus armeniaca* ('Polonais') apices on to myrobalan *Prunus cerasifera* seedlings as rootstock (localized incompatibility); total number of graftings: 160. The medium of Murashige and Skoog was used with added sucrose (3%) and auxin (NAA: 1 mg l⁻¹). The control homografts gave 80% viable plants before their transfer to the greenhouse

Days after micrografting	Number of apices having produced shoots	% of living grafts
7	138	86
14	101	63
21	93	58
28	84	52
35	22	20
42	12	7.5
60	0	0

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TABLE V

Development of heterografts (translocated incompatibility). All surviving plants showed necrotic symptoms, characteristics of incompatibility which led to a rapid death of grafted plants

	Type of grafts: scion/rootstock	
	<i>P. persica</i> / <i>P. uranica</i>	<i>P. parvica</i> / <i>P. cerasifera</i>
Number of in vitro micrografts	100	160
Leafy shoots developed after 20 days	32 (32%)	25 (15.6%)
Young plants sufficiently developed to be transferred to pots	30	25
Surviving plants, after 60 days in the greenhouse	6	2

grafting depended on the period of the year at which the apices were taken; the most favourable time being June. Biochemical analysis showed a correlation of -0.795 between the capacity of apices to be grafted and their peroxidase activity when they are taken off (Poessel et al., 1980). The first indications of localised incompatibility appeared as early as the 14th day, and the grafts decayed quickly without any further development (Table IV). With translocated incompatibility, some grafts may give viable plants but the early symptoms of incompatibility appeared at the time of transfer to pots, about 2 months after grafting (Table V). Although in vitro micro-grafting did not enable us to obtain new viable heterografts, it allowed the detection of early incompatibilities which only appear in the orchard after several years (Rogers and Beakbane, 1967).

CONCLUSION

In vitro micrografting of the shoot apex makes an important contribution to the improvement of orchards. Successive modifications of technique have allowed the detection of early incompatibilities and the establishment of fruit trees free of viruses. This is generally difficult for woody species when the apices are directly cultivated on a nutritive medium.

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES**

**EXPEDIENTE N° 01-35848
IPC A01H 4/00, 1/06
CONCEPTO TÉCNICO N° 937**

Como resultado del examen de fondo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se conceptúa que

1. DOCUMENTOS ESTUDIADOS:

Para el presente estudio se tendrá en cuenta:

- La descripción que obra en los Folios: 7 a 24
- Las reivindicaciones 1 a 7 (folios: 5 A 6).

2. OBJETO DE LA INVENCION

El objeto de la presente solicitud se refiere a un proceso de multiplicación clonal in vitro de plantas de guanabano que comprende los pasos de: a) Establecer un micro injerto a partir de (i) un microexplantado de árbol adulto con yemas preexistentes y (ii) una plántula obtenida de una semilla germinada in vitro. b) Cultivar el microinjerto cultivado en (a) en condiciones apropiadas para que se desarrolle la yema, c) utilizar las yemas formadas por el microexplantado para producir nuevos microinjertos sobre hipocotilos de plántulas de semillas germinadas in vitro y d) repetir los pasos (b) y (c) cuantas veces sea necesario hasta obtener el número deseado de microinjertos. Donde las etapas a) y c) implican la introducción del portainjerto (con el microexplantado ya insertado) en un utensilio cónico hueco presionando contra las paredes de éste de manera que los tejidos del microexplantado y del portainjerto queden en contacto estrecho y en el que el microinjerto se cultiva in vitro durante varios días hasta lograr la unión de los tejidos del microexplantado y del portainjerto.

El objeto de la solicitud definido anteriormente se clasificó en la IPC A01H 4/00, 1/06

3. ES UNA INVENCION

El artículo 15 de la Decisión 486 de la Comisión de la Comunidad Andina dispone que "No se considerarán invenciones:

- "a) los descubrimientos, las teorías científicas y los métodos matemáticos;
- b) el todo o parte de seres vivos tal como se encuentran en la naturaleza, los procesos biológicos naturales, el material biológico existente en la naturaleza o aquel que pueda ser aislado, inclusive genoma o germoplasma de cualquier ser vivo natural;
- c) las obras literarias y artísticas o cualquier otra protegida por el derecho de autor;
- d) los planes, reglas y métodos para el ejercicio de actividades intelectuales, juegos o actividades económico-comerciales;
- e) los programas de ordenadores o el soporte lógico, como tales; y,
- f) las formas de presentar información."

Teniendo en cuenta que el objeto de la solicitud en estudio es el definido en el numeral 2 de esta comunicación, se establece que dicho objeto no se encuentra dentro del alcance de las excepciones señaladas en el artículo 15 de la Decisión 486 de la Comisión de la Comunidad Andina, y por consiguiente, la materia reclamada en esta solicitud puede considerarse una invención.

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4. EXCEPCIONES DE PATENTABILIDAD

El artículo 20 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "No serán patentables:

- "a) las invenciones cuya explotación comercial en el territorio del País Miembro respectivo deba impedirse necesariamente para proteger el orden público o la moral. A estos efectos la explotación comercial de una invención no se considerará contraria al orden público o a la moral solo debido a la existencia de una disposición legal o administrativa que prohíba o que regule dicha explotación;
- "b) las invenciones cuya explotación comercial en el País Miembro respectivo deba impedirse necesariamente para proteger la salud o la vida de las personas o de los animales, o para preservar los vegetales o el medio ambiente. A estos efectos la explotación comercial de una invención no se considerará contraria a la salud o la vida de las personas, de los animales, o para la preservación de los vegetales o del medio ambiente sólo por razón de existir una disposición legal o administrativa que prohíba o que regule dicha explotación;
- "c) las plantas, los animales y los procedimientos esencialmente biológicos para la producción de plantas o animales que no sean procedimientos no biológicos o microbiológicos;
- "d) los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales."

Teniendo en cuenta que el objeto de la solicitud en estudio es la invención definida en el numeral 2 de esta comunicación, se establece que dicho objeto no se encuentra dentro del alcance de las excepciones de patentabilidad señaladas en el artículo 20 de la Decisión 486 de la Comisión de la Comunidad Andina, y por consiguiente, la invención reclamada en esta solicitud puede considerarse patentable.

5. DETERMINACIÓN DEL ESTADO DE LA TÉCNICA

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina que establece que "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40."

Consultadas las bases de datos y los archivos con que cuenta la entidad no se encontraron documentos que hubieran sido publicados o hubieran cumplido el plazo previsto en el artículo 40 antes 8 de mayo de 2001, fecha de presentación de la solicitud, que se relacionen estrictamente con un procedimiento de multiplicación clonal in vitro de plantas de guanábano comprendiendo los pasos de: a) Establecer un micro injerto a partir de (i) un microexplantado de árbol adulto con yemas preexistentes y (ii) una plántula obtenida de una semilla germinada in vitro. b) Cultivar el microinjerto cultivado en (a) en condiciones apropiadas para que se desarrolle la yema, c) utilizar las yemas formadas por el microexplantado para producir nuevos microinjertos sobre hipocotilos de plántulas de semillas germinadas in vitro y d) repetir los pasos (b) y (c) cuantas veces sea necesario hasta obtener el número deseado de microinjertos. Donde las etapas a) y c) implican la introducción del portainjerto (con el microexplantado ya insertado) en un utensilio cónico hueco presionando contra las paredes de éste de manera que los tejidos del microexplantado y del portainjerto queden en contacto estrecho y en el que el microinjerto se cultiva invitro durante varios días hasta lograr la unión de los tejidos del microexplantado y del portainjerto.

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Los documentos mencionados en el concepto técnico 232 y los anexados por el solicitante en respuesta a un requerimiento de ésta oficina, definen el estado de la técnica en general pero no afectan de manera contundente los requisitos de patentabilidad de la presente invención.

6. EVALUACIÓN DE LOS REQUISITOS DE PATENTABILIDAD

El artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina establece que: "Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial."

En cumplimiento del artículo 14 de la Decisión 486, se procedió a evaluar los requisitos novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

6.1. EVALUACIÓN DE LA NOVEDAD.

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Una invención se considerará nueva cuando no está comprendida en el estado de la técnica.

"El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.

"Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40."

Teniendo en cuenta el objeto de la invención definida en el numeral 2 de esta comunicación se realizó la búsqueda de anterioridades. Consultadas las bases de datos con que cuenta esta oficina no se encontraron documentos que formaran parte del estado de la técnica previo a la fecha de presentación y que trataran a un procedimiento para la multiplicación clonal invitro de plantas de guanábano comprendiendo los pasos de: a) Establecer un micro injerto a partir de (i) un microexplantado de árbol adulto con yemas preexistentes y (ii) una plántula obtenida de una semilla germinada in vitro. b) Cultivar el microinjerto cultivado en (a) en condiciones apropiadas para que se desarrolle la yema, c) utilizar las yemas formadas por el microexplantado para producir nuevos microinjeritos sobre hipocotilos de plántulas de semillas germinadas in vitro y d) repetir los pasos (b) y (c) cuantas veces sea necesario hasta obtener el número deseado de microinjeritos. Donde las etapas a) y c) implican la introducción del portainjerto (con el microexplantado ya insertado) en un utensilio cónico hueco presionando contra las paredes de éste de manera que los tejidos del microexplantado y del portainjerto queden en contacto estrecho y en el que el microinjerto se cultiva invitro durante varios días hasta lograr la unión de los tejidos del microexplantado y del portainjerto.

6.2. EVALUACIÓN DEL NIVEL INVENTIVO

El artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina dispone que "Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."

Basándonos en el objeto de la invención definido en el numeral 2 de esta comunicación se

establece que aún cuando en el estado de la técnica se encuentran reportes de la existencia de procedimientos para la multiplicación clonal invitro de plantas empleando microinjertación, ninguno de ellos representa procesos de alta eficiencia para la propagación de plantas leñosas como el guanabano, en las cuales la propagación masiva in vitro no ha sido posible, a diferencia de los resultados que se alcanzan en especies herbáceas. Por otra parte, en ninguno de dichos procedimientos se revela el empleo de puntas cónicas para forzar un contacto directo y estrecho entre los tejidos del microinjerto y los del patrón incrementando la eficiencia de la microinjertación; así como tampoco se divulgan la microinjertación cíclica in Vitro para la propagación vegetativa masiva de yemas.

Siendo que la invención no es obvia y no se deriva evidentemente del estado de la técnica, se considera que la invención cumple con el requisito de nivel inventivo definido en el artículo 18 de la Decisión 486.

6.3. EVALUACIÓN DE LA APLICACIÓN INDUSTRIAL

El artículo 19 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Se considerará que una invención es susceptible de aplicación industrial, cuando su objeto pueda ser producido o utilizado en cualquier tipo de industria, entendiéndose por industria la referida a cualquier actividad productiva, incluidos los servicios."

Partiendo del hecho de que el objeto de la invención es el que se define en el numeral 2 de esta comunicación, al ser empleado en empresas de papel, pigmentos o metal, se considera que dicho objeto es susceptible de aplicación industrial según el artículo 19 de la Decisión 486.

Por lo expuesto anteriormente, se recomienda conceder el objeto reclamado en las reivindicaciones 1 a 7 de los folios 5 a 6 de la solicitud en estudio, toda vez que cumple con los requisitos de novedad, nivel inventivo y aplicación industrial establecidos por el artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina.

EXAMINADOR TÉCNICO Código # 202063	JEFE DIVISIÓN NUEVAS CREACIONES	FECHA 2008-08-12
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Industria y Comercio
SUPERINTENDENCIA

REPUBLICA DE COLOMBIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN N° 17204

Por la cual se otorga una patente de invención

CERTIFICADO 28644

Rad. N° 01-35948

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

en ejercicio de sus facultades legales, en especial de las que se confirieron en el artículo 4, numeral 5 del decreto 2153 de 1992, y

CONSIDERANDO:

PRIMERO: Que mediante escrito radicado en esta Superintendencia con el número 01035948 del día 08 de mayo del 2001, se solicitó conceder patente para la invención contenida en el expediente de la referencia.

SEGUNDO: Que efectuado el examen de forma y cumplidas las exigencias legales, se ordenó publicar el extracto sin que se hubieran presentado observaciones.

TERCERO: Que realizado el examen de fondo se requirió al solicitante en los términos de los artículos 45 y 46 de la Decisión 486 de la Comisión de la Comunidad Andina, para que presentara respuesta a las observaciones de carácter técnico visibles a folios 48 a 53 del cuaderno administrativo, relacionadas con la patentabilidad o cumplimiento de los requisitos establecidos por esta Decisión, para la concesión de la patente.

CUARTO: Que el solicitante mediante escritos radicados bajo los números 01-035948-00008-0000 y 01-035948 00007-0000 del 24 de abril del 2006, atendió oportunamente el requerimiento formulado, presentando las aclaraciones respectivas en relación con las observaciones efectuadas. De igual manera allegó la documentación requerida en los folios 52 y 53, para dar cumplimiento en su totalidad a las observaciones efectuadas en el examen de fondo.

QUINTO: Que en virtud de lo dispuesto en el artículo 14 de la Decisión 486 expedida por la Comisión de La Comunidad Andina se otorgarán patentes "...para las invenciones sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial".

SEXTO: Que en el presente caso cumplen los requisitos indicados en el numeral anterior las reivindicaciones 1 a 7 contenidas en los folios 5 y 6, razón por la cual este Despacho encuentra procedente conceder para ella el privilegio de patente solicitado.



Industria y Comercio
SUPERINTENDENCIA

REPUBLICA DE COLOMBIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION ¹⁷²⁰⁴

Por la cual se otorga una patente de invención

CERTIFICADO ²⁸⁶⁴⁴

Rad. N° 01-35948

RESUELVE:

ARTICULO PRIMERO: Otorgar privilegio de patente de invención a la creación denominada:

**" PROCESO PARA LA PROPAGACION CLONAL IN VITRO DE PLANTAS DE
GUANABANO (ANNONA MURICATA L), EMPLEANDO LA MICROINJERTACION
CICLICA "**

Clasificación IPC: A 01 H 4/00, 1/06.

Reivindicación(es): 1 a 7 **Follo(s):** 5 y 6.

Titular(es): CORPORACION BIOTEC y CENTRO INTERNACIONAL DE
AGRICULTURA TROPICAL – CIAT.

Domicilio(s): Km. 17 recta Cali – Palmira, Valle del Cauca, Colombia.

Inventor(es): Álvaro Mejía, Nelson Royero, Jorge Cabra y William Roca.

Vigente desde: 08 de mayo del 2001 **Hasta:** 08 de mayo del 2021

ARTICULO SEGUNDO: El titular tendrá los derechos y las obligaciones establecidos en la Decisión 486 de la Comisión de la Comunidad Andina y su privilegio caducará en las condiciones prescritas por las disposiciones legales vigentes sobre propiedad industrial.

ARTICULO TERCERO: Entregar al titular copia auténtica de esta resolución y el certificado de patente, previa las anotaciones en los libros de registro.

ARTICULO CUARTO: Notificar el contenido de la presente resolución al doctor Germán Arias Jaramillo, o a quien haga sus veces, en su calidad de apoderado de la Corporación



Industria y Comercio
SUPERINTENDENCIA

REPUBLICA DE COLOMBIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN 17204

Por la cual se otorga una patente de invención

CERTIFICADO 28644

Rad. Nº 01-35948

Blotec y del Centro Internacional de Agricultura Tropical – CIAT., entregándole copia de la misma, advirtiéndole que contra ella procede el recurso de reposición ante el Superintendente de Industria y Comercio, del cual podrá hacer uso al momento de la notificación o dentro de los cinco 5 días hábiles siguientes a ella.

NOTIFÍQUESE Y CUMPLASE

Dada en Bogotá, D.C., a los **29 JAN. 2006**

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,

JAIRO RUBIO ESCOBAR

Doctor

GERMÁN ARIAS JARAMILLO

Km. 17 recta Cali – Palmira, Valle del Cauca. (Km. 2)

APORTE RECIBIDO POR VALOR DE
\$ 1/01 POR CONCEPTO
DE DERECHOS ADMINISTRATIVOS

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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RADICACIÓN: 1-35848

EDICTO No. 10813

LA SECRETARÍA GENERAL AD-HOC HACE SABER:

Que esta Superintendencia profirió Resolución No. 17204 de 28-JUN-2006. Que el contenido de la parte resolutive es como sigue: El Superintendente de Industria y Comercio.

RESUELVE

ARTICULO PRIMERO: Otorgar privilegio de patente de invención a la creación denominada:

**" PROCESO PARA LA PROPAGACION CLONAL IN VITRO DE PLANTAS DE
GUANABANO (ANNONA MURICATA L.), EMPLEANDO LA MICROINJERTACION
CICLICA "**

Clasificación IPC: A 01 H 4/00, 1/08.

Reivindicación(es): 1 a 7 **Folio(s):** 5 y 6.

Titular(es): CORPORACION BIOTEC y CENTRO INTERNACIONAL DE AGRICULTURA TROPICAL – CIAT.

Domicilio(s): Km. 17 recta Cali – Palmira, Valle del Cauca, Colombia.

Inventor(es): Álvaro Mejía, Nelson Royero, Jorge Cabra y William Roca.

Vigente desde: 08 de mayo del 2001 **Hasta:** 08 de mayo del 2021

ARTICULO SEGUNDO: El titular tendrá los derechos y las obligaciones establecidos en la Decisión 488 de la Comisión de la Comunidad Andina y su privilegio caducará en las condiciones prescritas por las disposiciones legales vigentes sobre propiedad Industrial.

ARTICULO TERCERO: Entregar al titular copia auténtica de esta resolución y el certificado de patente, previa las anotaciones en los libros de registro.

ARTICULO CUARTO: Notificar el contenido de la presente resolución al doctor Germán Arias Jaramillo, o a quien haga sus veces, en su calidad de apoderado de la Corporación

Biotec y del Centro Internacional de Agricultura Tropical – CIAT., entregándole copia de la misma, advirtiéndole que contra ella procede el recurso de reposición ante el Superintendente de Industria y Comercio, del cual podrá hacer uso al momento de la notificación o dentro de los cinco (5) días hábiles siguientes a ella.

NOTIFÍQUESE Y CUMPLASE

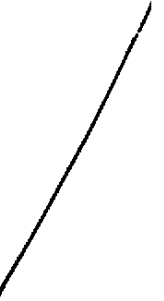
Dada en Bogotá, D.C., a los **29 JUN 2006**

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,


JAIME RUBIO ESCOBAR

Para notificar a los interesados, se fija el presente EDICTO en lugar visible al público en la SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO, por el término de (10) diez días hábiles contados a partir de las 8:30 a.m. de hoy.

Secretaría General Ad-Hoc 14-JUL-2006. Esta edicto se desflja hoy 28-JUL-2006 a las cinco (5:00 p.m.)
Secretaría General Ad-Hoc
EDICTO No. 10813



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GERMAN ARIAS JARAMILLO
Abogado

Palmira, 18 de abril de 2006

Doctora
ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Bogotá, D.C.

Asunto: Radicación 01-35948
Trámite 002
Evento 001
Actuación 430
Oficio 2098

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 01-035948-00008-0000 Fec: 2006-04-24 11:58:40
Plan: 2020 NIFICACIONES
Tm: 2 PATENTES Evt: 1 REGDEPOSITO
Act: 444 RESPUESTAREQUE Folios: 2

GERMAN ARIAS JARAMILLO, identificado con la cédula de ciudadanía No. 14.993.364 de Cali, y tarjeta profesional de abogado No. 18.842 del C.S. de la J., actuando como Apoderado del Centro Internacional de Agricultura Tropical – CIAT y de la Corporación BIOTEC, dentro del trámite de la patente sobre “Proceso para la propagación clonal *in vitro* de plantas de guanábana (*Annona muricata* L.), empleando microinjertación cíclica”, doy respuesta al oficio de la referencia de la siguiente forma:

PRIMERO.- Al contrario de las especies vegetales herbáceas (aquellas que no producen tallos lignificados), la propagación *in vitro* de numerosas especies leñosas, en las que se pueden incluir especies de árboles frutales o forestales, no ha sido posible a nivel comercial.

Los problemas que se presentan al intentar aplicar metodologías convencionales del cultivo *in vitro* a estas especies, han sido enumerados y discutidos en detalle en la solicitud de la patente. En la especie leñosa para la cual se está solicitando la patente, el guanábano, a pesar de numerosos intentos, tampoco ha sido posible el desarrollo de metodologías de propagación masiva *in vitro*. La invención que se desea patentar incluye no solo una sino un conjunto de innovaciones que hace posible por primera vez la propagación *in vitro* a nivel comercial de clones de guanábano.

SEGUNDO.- Principalmente dos modificaciones de las metodologías convencionales hacen que el procedimiento de la solicitud haya resultado en el desarrollo de una metodología aplicable a nivel comercial como se indica en las páginas 15 y 16 de la solicitud de la patente, así:

- a) El uso de puntas cónicas para forzar un contacto directo y estrecho entre los tejidos del microexplante y los del patrón, que permitió incrementar considerablemente la eficiencia de la microinjertación;
- b) La aplicación del concepto de microinjertación cíclica *in vitro* como herramienta de propagación vegetativa de yemas.

TERCERO.- La Superintendencia de Industria y Comercio indica que “cualquier persona versada en la técnica de microinjertación estaría motivada a emplear las nuevas yemas formadas por el microexplante para la producción de nuevos microinjertos, y así lograr una mayor multiplicación clonal”. Esto, para resaltar que el proceso que se quiere

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patentar "podría constituir el resultado de ensayos sistemáticos de la actividad investigativa" y que resulta obvio para un investigador en el campo de la propagación clonal.

Alcanzar una mayor propagación clonal, sin embargo, no es suficiente para desarrollar metodologías de propagación aplicables a nivel comercial, puesto que si estas no son eficientes, como ha sucedido hasta el momento, los costos de aplicar las metodologías de cultivo *in vitro* en la propagación del guanábano serían tan altos que esta actividad no podría competir con otras técnicas de propagación. No resulta pues, obvio aplicar el concepto de microinjertación cíclica en la propagación clonal masiva si es que el investigador versado en técnicas de propagación no cuenta con metodologías eficientes de microinjertación. Estas últimas constituyen igualmente una de las innovaciones de la solicitud de la patente.

Con respecto a la argumentación de que un proceso patentado de propagación en papa, que involucra la microinjertación de yemas, constituye una anterioridad y puede afectar la patentabilidad del método desarrollado, nuestra opinión es que esto sería válido si la técnica de microinjertación empleada en papa fuera similar a la descrita en la solicitud de la patente, lo que no es el caso. Tal como se presenta en el ejemplo 1 con el uso de metodologías de injertación como las aplicadas en papa, que no incluyen el uso de puntas cónicas que protejan la yema microinjertada de la desecación, una innovación de la presente solicitud, solo se obtienen eficiencias de prendimiento tan bajas que no permiten la aplicación de la microinjertación como método de propagación.

PETICIÓN

Con fundamento en los argumentos expuestos anteriormente, solicitamos el reconocimiento de la patente de la referencia.

Atentamente,

GERMAN ARIAS JARAMILLO

Apoderado

C.C. No. 14.993.364 de Cali

T.P. No. 18.842 del C.S. de la J.



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Santiago de Cali, 21 ABR 2006
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