Investigations on Establishing Virus-Free Citrus Plants Through Tissue Culture

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IN VIEW OF the difficulties encountered with citrus shoot apex culture to obtain virus-free plants, other methods have been sought to accomplish the same objectives. One of these, described herein, involves the isolation of the nucellus from the ovules of the mother plant and the stimulation of adventitious embryos therefrom, which will develop into plantlets. Another is that of micrografting, wherein the excised shoot apex is grafted onto a small virus-free seedling. The latter technique will be described in another manuscript.

The objective of this research was to determine whether or not adventive embryos could be initiated from the aseptically isolated nucellar tissue of either monoembryonic or polyembryonic types when cultured in vitro, and whether the embryos would give rise to virus-free plants. Comparisons were made with some seedless citrus cultivars whose ovules abort at an early stage of development. This report, therefore, contains results of experiments carried out over a 2½-year period and includes data on several species and hybrids of which the parent

trees are known to carry one or more recognized viruses.

Materials and Methods

In most cases the nucellus was isolated from ovules of immature fruit arising from flowers that had previously been pollinated with trifoliate orange pollen. The reasons for the controlled pollination were to stimulate ovule development and to provide a readily distinguishable marker so that any zygotically derived seedling could easily be differentiated from a nucellar seedling. Preliminary sampling procedures at weekly intervals indicated at what age the developing ovules in the immature fruit were suitable for culturing. The fruit were harvested from 70 to 130 days after pollination, depending on genetic source. At the proper stage of development, the hybrid embryo within the ovule is easily recognized by its position and the fact that it is the only embryo present. In citrus the nucellus is a relatively persistent and fleshy tissue located immediately beneath the seed coats enveloping the embryo sac. It is a large structure, which can be readily distinguished and isolated.

The fruit were brought into the laboratory, washed with tap water, and then surface sterilized by immersion in 10 per cent Purex for 20 min. A few drops of Tween 20 emulsifier were included in the disinfectant to promote wetting. Without rinsing, the fruit were cut open in a sterile transfer chamber, and the ovules were removed aseptically from the placental tissues. The zygotic embryo was removed under a dissecting microscope equipped with 10–20X magnification, and the nucellus was isolated. To accomplish this the immature ovule was held at the chalazal end with a pair of microforceps. A longitudinal incision from the chalazal end to the micropylar end of the ovule was made through the integuments with a sharp scalpel or dissecting needle. The integuments were peeled away to expose the nucellus. The nucellus was then split open; the endosperm and exposed zygotic embryo were separated and discarded. At the proper stage the endosperm contents were still liquid. The nucellus at this stage was still intact and did not show any signs of degeneration or of adventive embryo development. It was severed from where it was fused at the chalazal end and transferred to a nutrient tube. Only 1 nucellus was transplanted per tube. Forty replicate tubes were used for each experimental variable. The nucellus must be excised as rapidly as possible to prevent desiccation, but also with skill so as to minimize injury and insure the greatest percentage of successful cultures.

Preliminary experiments indicated that a modified Murashige-Skoog nutrient medium was satisfactory. It is constituted in mg/l as follows:

NH_4NO_3	1650	$Na_2MoO_4 \cdot 2H_2O$	0.25
KNO_3	1900	$FeSO_4 \cdot 7H_2O$	27.85
$MgSO_4 \cdot 7H_2O$	370	Na_2EDTA	37.25
$CaCl_2 \cdot 2H_2O$	440	Glycine	4.0
KH_2PO_4	170	Nicotinic acid	1.0
$MnSO_4 \cdot H_2O$	16.9	Pyridoxin · HCl	1.0
$ZnSO_4 \cdot 7H_2O$	8.6	Thiamin · HCl	0.2
$CuSO_4 \cdot 5H_2O$	0.025	i-Inositol	100
KI	0.83	Difco Bacto-Malt Extract	500
$CoCL_2 \cdot 6H_2O$	0.025	Sucrose	50,000
H_3BO_3	6.2	Difco Bacto-Agar	10,000

The pH of the medium was adjusted to 5.7. The agar and nutrients were dissolved by heating and the medium distributed into 25 × 150 mm pyrex culture tubes, 25 ml per tube, and capped with polypropylene tube closures. The nutrient tubes were then sterilized by autoclaving at 121°C for 15 min, then slanted before cooling.

The cultures were maintained at 27°C under 16 hr daily illumination at a low light intensity of approximately 1000 lux, provided by 20-W Gro-Lux lamps.

Results

Table 1 clearly shows that the developmental stage of the ovule influenced the successful initiation of adventive embryos and that the appropriate developmental stage varied with the cultivar. With Temple tangor no success was obtained until the tenth week after pollination; successful cultures were then obtained from the tenth through the eighteenth week. After the eighteenth week, the nucellus had degenerated. In contrast, the Robertson Navel, a normally seedless variety with abortive ovules, gave successful explants from the time of pollination until 6 weeks after pollination. Maximum success was obtained with nucellus isolated 6 weeks after pollination. Beyond 6 weeks in fruit development, the ovules had degenerated completely.

Table 2 lists the percentage of success of nucellus culture and the average number of embryos obtained per culture for 9 citrus cultivars. As a rule, more than 1 embryo was initiated from each successful nucellus explant. In other experiments it has been found that the embryos could be subcultured on the same nutrient medium to give rise to additional embryos.

Within 2–3 weeks the successful cultures were readily recognizable.

TABLE 1. RELATIONSHIP OF STAGE OF FRUIT DEVELOPMENT TO SUCCESS IN CULTURING THE NUCELLI OF TEMPLE TANGOR AND ROBERTSON NAVEL ORANGE

Weeks after pollination	Success of cultures (percentage)	
	Temple tangor	Robertson navel
0	0	10
2	0	17.5
4	0	12.5
6	0	75
8	0	
10	25	
12	30	
16	15	
18	15	
20	0	

Green specks appeared; after 4–5 weeks they gradually enlarged and differentiated into complete embryos. Seedlings 1–3 in. high were evident after 10–12 weeks. After the seedlings were 3 in. high or more, they were transplanted to soil media and placed in a greenhouse environment. In so doing, plant losses were high, due to excessive transpiration and desiccation. This problem was minimized by shading and maintaining a relatively high humidity. After 3–4 months in the greenhouse, plants ranged from 1 to 3 feet in height and were of an adequate size for plant materials to be removed for virus indexing procedures.

Seedlings of Robertson Navel were the first available for indexing. The mother tree carried several viruses, including those of exocortis and psorosis. The mother tree of the Temple tangor carried tristeza, vein-enation, and exocortis viruses. A preliminary check of the progeny seedlings by means of the Etrog citron test gave negative results for exocortis. Other indexing procedures indicated freedom from psorosis, tristeza, and vein-enation viruses, respectively. The preliminary results indicate that the viruses present in the mother plants were eliminated by cultivation of the nucelli.

TABLE 2. PERCENTAGE OF SUCCESS OF NUCELLAR EMBRYO INITIATION AND AVERAGE NUMBER OF EMBRYOS PER EXPLANT

Variety	Success (%)	Avg. no. embryos
Temple tangor	25	7.2
Clementine mandarin	10	2.0
Meyer lemon	15	7.3
Chandler shaddock	10	2.5
Ponderosa lemon	15	2.2
Khasi papeda	55	2.3
Citron (6 var.)	0	0
Robertson navel	75	7.2
Bearss lime	7	[a]

a. Not determined.

Discussion

For monoembryonic varieties of citrus, which have been impossible to free of virus by other means, the nucellus culture technique offers a practical approach. It is still far from being an ultimate solution because not all citrus varieties or species can be stimulated to develop adventive embryos under present cultural techniques. Not only are there differences in the ease of culturing adventitious embryos among the species and varieties examined, but some species such as the citron have so far not been cultured.

There is need to examine further the basic physiology underlying the embryo initiation process in order to make the technique more widely applicable. The medium routinely used in this investigation has simply been a medium that has been found satisfactory in other investigations

with citrus tissue culture, although it has been slightly modified. The addition of malt extract to the medium appears to be essential. The need for still other addenda is unknown.

Several embryos and plants can be obtained from a single nucellus transplant although the percentage of successful cultures for any species or variety is relatively low. The technique is still applicable because all one needs is a few virus-free plants for clonal propagation. It is desirable to have as many plants as possible inasmuch as genetic variants, including polyploids, have been known to occur among many plants propagated through tissue culture. There may be problems associated with virus-free plants derived from nucellus culture. The major one is a possible prolonged juvenility; it is not known what relationship exists between virus freedom and juvenility. Other problems involve the modification of present techniques. There is need to speed up the development of embryos into transplantable plants. There is need to improve the transplanting process to reduce mortality. Also it is not known whether the technique will exclude all viruses, such as that of stubborn.

It is not necessary to use marker pollen with monoembryonic citrus. It is important to recognize the proper stage of fruit development to detect the zygotic embryo and remove it at a suitable stage. This is easily achieved because the embryo is globular to heart-shaped and embedded in the nucellus tissue at the micropylar end from where it can easily be dislodged and discarded.