Theor Appl Genet (1990) 80:785-790

THEORETICAL AND APPLIED GENETICS

© Springer-Verlag 1990

Induction of triploid *Citrus* plants from endosperm calli in vitro *

F.G. Gmitter, Jr. [1], **X.B. Ling** [2] **and X.X. Deng** [3]

University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850, USA

Received April 5, 1990; Accepted May 25, 1990
Communicated by P. Maliga

Summary. Triploid hybrid *Citrus* plants were regenerated by somatic embryogenesis in vitro from endosperm-derived calli. A sequence of media formulations was used to induce and support proliferation of primary callus from endosperm, to induce embryogenesis from primary callus, and to allow embryo development leading to viable plantlets. Calli were induced from cellular endosperm of *Citrus sinensis* (sweet orange), *C. Xparadisi* (grapefruit), and *C. grandis* (pummelo) excised 12-14 weeks post-anthesis. Induction of embryogenesis from sweet orange and pummelo primary calli required gibberellic acid and double mineral nutrient concentrations. Embryogenesis was not induced from grapefruit calli in these experiments. Only sweet orange embryos developed sufficiently to allow plant regeneration. Triploid axillary buds were minigrafted onto etiolated diploid rootstock seedlings in vitro in order to transfer triploid regenerants to soil and the external environment. Triploidy ($2n = 3x = 27$) was observed consistently in all phases of regeneration and in recovered plants. These results demonstrate that triploid hybrid plant recovery from *Citrus* endosperm can overcome barriers to sexual hybridization resulting from apomixis.

Key words: *Citrus* – Endosperm – Triploid – In vitro embryogenesis – Plant breeding

Introduction

Facultative apomixis via nucellar polyembryony is a characteristic common to most of the economically important *Citrus* species and scion cultivars, with few exceptions (Cameron and Frost 1968), and it significantly impedes progress toward cultivar development and genetic improvement (Soost and Cameron 1975). Most *Citrus* scion cultivars have originated as either chance seedlings or bud sport mutations exhibiting desirable improvements (Hodgson 1968). Zygotic seedlings are produced by monoembryonic types such as citron (*Citrus medica* L.), pummelo (*Citrus grandis* [L.] Osb.), and a few mandarin (*Citrus reticulata* Blanco) selections and hybrids (Soost and Cameron 1975); however, most of the monoembryonic selections are characterized also by commercially unacceptable numbers of seed per fruit. *Citrus* breeders have searched for methods that will allow sexual recombination but result in progeny with nearly seedless fruit. The production of triploid *Citrus* plants via interploid hybridization is one possible approach (Soost and Cameron 1969). Two seedless triploid pummelo × grapefruit (*Citrus Xparadisi* Macf.) hybrids, originating from 2x-4x hybridization, have been released (Soost and Cameron 1980, 1985).

The endosperm of most diploid angiosperms, including *Citrus*, is triploid hybrid tissue that arises from the fertilization of the two polar nuclei of the embryo sac by one sperm nucleus of pollen origin (Frost and Soost 1968). These nuclei are products of genetic recombination and segregation, so each endosperm is genetically unique, even among facultative apomicts like *Citrus*. Recovery of triploid plants from in vitro endosperm cultures following controlled pollination could be a useful breeding strategy for vegetatively propagated, perennial fruit crops for which seediness is both undesirable and unnecessary

---

* Florida Agricultural Experiment Station Journal Series No. R-00627

[1] To whom correspondence should be addressed

[2] Current address: South China College of Tropical Crops, Danxin County, Hainan, People's Republic of China

[3] Current address: Citrus Research Institute, Huazhong Agricultural University, Wuhan, Hubei, People's Republic of China

because of parthenocarpy. Triploid embryoids or plantlets have been successfully recovered in very few fruit tree genera, including *Actinidia* (Gui et al. 1982), *Citrus* (Wang and Chang 1978), *Malus* (Mu and Liu 1979), *Prunus* (Liu and Liu 1980), and *Pyrus* (Zhao 1983), following embryogenesis or organogenesis in vitro. Endosperm-derived *Citrus* and *Actinidia* plantlets were triploid ($2n = 3x = 27$ or 87, respectively). However, mixed cell populations observed in root tips of *Malus* and *Pyrus* regenerants included diploid, triploid, and aneuploid cells. None of the putative *Citrus* triploids were successfully transferred to soil or field plots for evaluation (C. J. Chang, personal communication). There have been no other reports of successful plant regeneration from endosperm culture of *Citrus*. The following report describes the successful production and verification of viable triploid *Citrus* plants from in vitro endosperm culture.

Materials and methods

Plant material

Attempts were made to induce proliferation in vitro from excised endosperm of polyembryonic 'Ridge Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.), 'Duncan' grapefruit (*C. Xparadisi*), and monoembryonic 'White Siamese' pummelo (*C. grandis*). Open-pollinated fruit were harvested from 6 to 16 weeks after anthesis (from May through July 1988) to determine the most suitable stage of endosperm development for in vitro response. Source trees were field grown at the Citrus Research and Education Center of the University of Florida in Lake Alfred, Florida. Fruit were surface sterilized with sodium hypochlorite (20% v:v) for 15 min, rinsed with autoclaved distilled water, and flamed with 95% ethanol. Unripened seeds were excised aseptically, washed free of adhering fruit tissue and juice with autoclaved distilled water, and dried with sterile absorbent paper. Outer and inner integuments were dissected and peeled carefully, and the nucellus and any visible embryos were discarded. In most cases, culture plates were inoculated with six endosperm each, and six plates were used for each treatment. Only whole, excised endosperm with little or no injury were used to initiate cultures.

Induction of callus proliferation and embryogenesis, plantlet production, and establishment in soil

MT medium (Murashige and Tucker 1969) was used in all phases of the regeneration protocol and amended as needed with adenine (Ad, 2 mg/l), casein hydrolysate (CH, 250–1000 mg/l), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1–2.0 mg/l), malt extract (ME, 500 mg/l), gibberellic acid (GA, 2–10 mg/l), and kinetin (KT, 5 mg/l). Media were solidified with agar (0.7%), pH 5.8, and autoclaved for 20 min (1.1 kg/cm^2). Various formulations of MT and amendments, including doubled MT inorganic component concentration (2MT), were tested for their ability to induce callus formation from endosperm, to induce globular embryogenic callus from primary callus, and to allow embryo development and plantlet production from proliferating calli. Cultures were grown in darkness at 24°–28°C to induce callus formation. After initiation and increase in darkness, all cultures were grown with a 16 h fluorescent light photoperiod.

Etiolated 15-day-old Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata* [L.] Raf.) seedlings, grown on paper bridge supports in glass tubes containing liquid MT medium with 0.5 g/l active charcoal, were used as rootstocks for in vitro minigrafting. The Carrizo seedlings were decapitated approximately 2 cm above the cotyledons, and young axillary buds from elongated endosperm-derived shoots were inserted into small vertical incisions made at the cut surface of the stock plants. Successful propagations were transferred into a synthetic soil mix and moved to a plant growth chamber for acclimation before transfer to the greenhouse.

Cytogenetic confirmation of ploidy level

Chromosome number was determined during all phases of the regeneration protocol including proliferating calli (7–10 days and 18 months after initiation); globular embryoids and cotyledonary embryos; young leaves, roots, and shoot tips produced on plantlets in vitro; and young leaves and axillary buds from actively growing plants in soil. All samples were stained using the ferric ammonium sulfate-hematoxylin procedure of Sass (1958) with modifications described below. Plant meristems were pretreated by 2–3 h immersion in a 20°C saturated aqueous *p*-dichlorobenzene solution and fixed in a 3:1 (v:v) ethanol: glacial acetic acid mixture for 24 h at 4°C. Callus tissue, embryos, and root tips were softened by hydrolysis in 25°C 5 *N* HCl for 8, 12, and 15 min, respectively. Rather than hydrolysis in HCl, young leaf or shoot meristems were macerated for 3 h at 25°C in a simplified enzyme cocktail preparation (Grosser and Chandler 1987) with only 6.0 m*M* MES (2[*N*-morpholino] ethane sulfonic acid) added.

Results and discussion

The efficient excision of uninjured endosperm required talent, diligence, and time. The flowering period of individual trees was 7–10 days, which thus dictated the time when endosperm were available in a suitable stage of development for culture initiation. Up to 10–11 weeks post-anthesis endosperm were in the free nuclear stage and could be distinguished from cellular endosperm by their liquid-to-soft texture, conspicuous central vacuole, and relative embryo size. Callus was not induced from free nuclear endosperm of the species studied. Endosperm became cellular between 11–14 weeks post-anthesis and were distinguished by their elastic texture, absence of central vacuoles in the endosperm, and embryo size (<20% of available seed volume) (Fig. 1). Cellular endosperm excised at 12–14 weeks post-anthesis were the most responsive. Cotyledonary embryos were observed generally at 14 weeks post-anthesis or later and occupied 50–100% of the available seed volume. Endosperm at this stage of development were rapidly consumed by the enlarging embryos, and callus was rarely induced.

Variations of Wang's protocol for *Citrus* endosperm culture (Wang and Chang 1978) and alternative media that have been useful for embryogenesis from diploid nucellar calli [e.g, MT + ME (500 mg/l)] were compared. Wang and Chang (1978) induced endosperm calli on MT supplemented with 2,4-D (2 mg/l), BA (5 mg/l), and CH (1000 mg/l). Embryogenesis from callus was induced by transfer to MT supplemented with GA (2–15 mg/l);

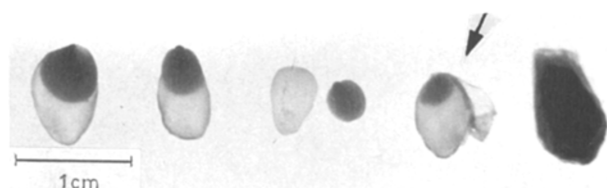

Fig. 1. Excised endosperm and embryos prior to media inoculation. The *arrow* indicates an example of the proper stage of maturity

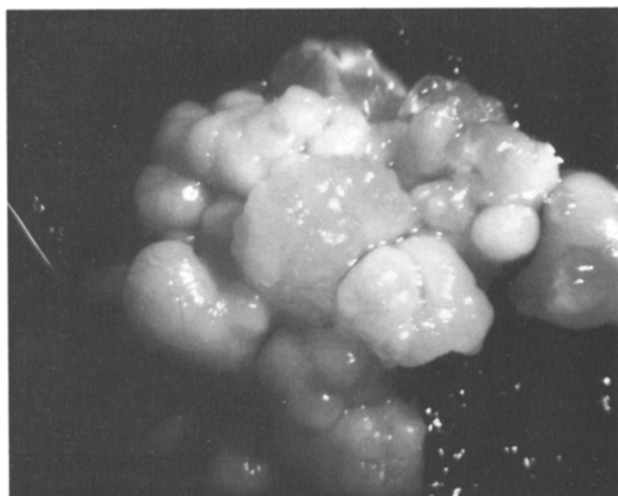

Fig. 2. Globular callus induced from primary endosperm callus

plantlet development occurred in the same medium. Observations of callus induction from 'Duncan' grapefruit endosperm indicated that the medium used by Wang to induce calli from pummelo and sweet orange was also effective for *C. Xparadisi* (Table 1). KT and ME added to Wang's recipe slightly increased the induction frequency. Endosperm did not respond on MT basal medium or MT with ME (500 mg/l). The response of pummelo and sweet orange endosperm to the media tested was similar to that of grapefruit (data not presented). Primary calli were induced in vitro from the endosperm of each species used, most frequently from the chalazal end, but occasionally from the mid-region also. Initial callus growth was generally soft and translucent, but calli became tougher, more rough in texture, and yellow within 6 weeks of initiation. Most calli were transferred to fresh medium with a lower BA concentration (0.25 mg/l) for increase in darkness. Slow-growing calli of each species frequently halted development after a few weeks.

All calli were transferred to light on either fresh induction medium (BA concentration: 0.25 mg/l), MT with ME (500 mg/l), or MT with GA (5–15 mg/l), each with 1 × or 2 × MT inorganic components. A transition from the amorphous to the globular callus form was observed only when 2 MT with GA was used for sweet orange and pummelo (Fig. 2); no further differentiation was observed

Table 1. Media influence on the frequency of callus induction from 'Duncan' grapefruit endosperm

Medium[a]	Endosperm cultured	Number responsive (%)
MT+BA+2,4-D	36	8 (22.2)
MT+BA+2,4-D+CH	36	17 (47.2)
MT+BA+2,4-D+CH+KT	48	15 (31.3)
MT+BA+2,4-D+CH+KT+ME	48	25 (52.1)

[a] Growth regulator concentrations (mg/l) were as follows: BA=5; 2,4-D=2; CH=1000; KT=5; ME=500

Table 2. Callus quality and cotyledonary embryo production from globular 'Pineapple' sweet orange calli

Medium[a]	Globular calli cultured	Callus quality/ embryo production[b]
MT+CH	6	*
MT+CH+BA	8	*
MT+CH+BA+GA	10	**
MT+CH+BA+GA+Ad	10	**
2MT+CH+GA[c]	12	**
2MT+CH+BA+GA	14	**–***
2MT+CH+BA+GA+Ad[d]	18	***

[a] Growth regulator concentrations (mg/l) were as follows: CH=500; BA=0.25; GA=2; Ad=2
[b] Key: * = white callus, no embryos differentiated; ** = white and green calli, few embryos differentiated; *** = calli mostly green, many embryos differentiated with normal and abnormal morphology
[c] 2MT = 2 × concentration of MT inorganic components
[d] Three complete plantlets were produced on this medium

from grapefruit callus. Wang and Chang (1978) increased mineral nutrient and gibberellic acid concentrations in the medium to provide sufficient nutrition and stimulation for embryogenesis and development; our results confirm the necessity of this step.

Although enhanced nutrition and GA both were essential for globular callus induction and the stimulation of embryogenesis from primary calli, embryoid development was abnormal on medium supplemented with GA only, especially at concentrations greater than 10 mg/l. The development of embryos into viable plantlets on 2 MT with GA reported by Wang and Chang (1978) was not observed. Therefore, several media formulations were evaluated for their ability to support callus growth and continued embryo development leading to plantlet production. The best responses were observed on 2 MT with GA (2 mg/l) amended with CH (500 mg/l), BA (0.25 mg/l), and AD (2 mg/l) (Table 2). Sweet orange embryos reached the cotyledonary stage in this medium, and occasional root or shoot elongation was observed. Pummelo embryos did not develop beyond the globular stage on any

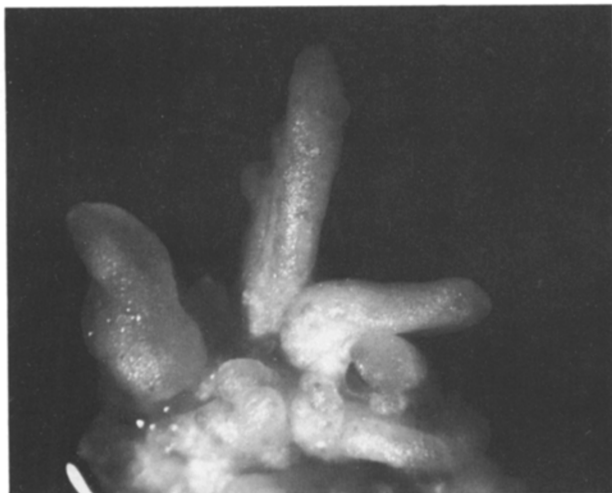

Fig. 3. Embryoid development from globular callus

Fig. 4. Minigrafted triploid scion on Carrizo citrange rootstock. *Arrow* indicates graft union

Fig. 5. Actively growing triploid *Citrus* hybrid scion with trifoliate leaves on Carrizo citrange rootstock

medium, however. Most embryos appeared abnormal with malformed cotyledons (Fig. 3). There were frequently several embryonic structures arising from a single callus that were fused and could be separated only by cutting. Embryos exhibiting polarity were transferred to MT or 2 MT with GA (5 mg/l) to induce germination, and some produced shoots or occasional proliferation, but root elongation was uncommon. Many shoots were thin, weak, and frequently died; a lower GA concentration may have resulted in more normal shoot development.

Although somatic embryogenesis occurred, and shoots and roots were produced by several different embryos, it was not possible to produce plants with complete shoot and root systems that survived in soil. The shoots that were produced did not respond to methods of in vitro shoot multiplication and adventitious root induction that have proven successful for a wide range of *Citrus* species and related genera (Grosser and Gmitter 1990). It was necessary to utilize in vitro minigrafting to recover actively growing plants in soil (Fig. 4). Four individual plants were recovered from eleven minigrafts. Subsequently, nine more minigrafts were performed, but the quality of available buds had declined and only one additional plant was obtained. One of the triploid scions (Fig. 5) had trifoliate leaves, demonstrating its hybrid origin with *P. trifoliata* as the most likely pollen parent. Triploid plants recovered thus far have been of variable vigor and vegetative morphology.

Ploidy level and chromosome number stability were observed throughout the regeneration process to monitor any chromosome number variation and to determine the ploidy levels of regenerated plants. Primary endosperm calli were triploid ($2n = 3x = 27$) for each species (Table 3). However, two types of primary 'Duncan' grapefruit callus were produced, one resembling sweet orange and pummelo endosperm calli and one resembling diploid embryogenic callus. Chromosome counts of the latter confirmed diploidy, and these calli were discarded. Nucellar tissue adhering to the endosperm at excision was likely the source of the diploid cell lines.

Table 3. Chromosome numbers observed during different stages of sweet orange, pummelo, and grapefruit regeneration

Cultivar	Primary calli		Globular[a] embryoids		Plantlets	
	No. cells	2n	No. cells	2n	No. plants	2n
'Ridge Pineapple' sweet orange	24	27	32	27	4	27
'White Siamese' pummelo	16	27	8	27	–	–
'Duncan' grapefruit-A[b]	34	27	–	–	–	–
'Duncan' grapefruit-B	12	18	–	–	–	–

[a] One and five globular embryos were sacrificed for ploidy determination from pummelo and sweet orange, respectively
[b] 'Duncan'-A callus resembled that from sweet orange or pummelo endosperm. 'Duncan'-B callus was produced from a single endosperm, but resembled diploid embryogenic callus from in vitro *Citrus* nucellar cultures

The number of chromosomes observed in the cells of globular embryoids, shoot and root tips from in vitro plantlets, and young leaf and axillary meristems from grafted plants in soil was 27 ($2n=3x=27$) (Table 3, Fig. 6). Mu and Liu (1979) observed variation in chromosome number in *Malus* endosperm and derived callus, as well as in root tip cells of recovered plants. Zhao (1983) observed mixoploidy among *Pyrus* endosperm regenerants. Heterogeneous endosperm cell populations from which these regenerants were derived likely caused the chromosome number variation. Diploid and triploid *Actinidia* plants were recovered from endosperm cultures (Gui et al. 1982). The chromosome number in the *Citrus* endosperm cultures was remarkably stable, however, in all phases of the regeneration protocol. In fact, embryogenic callus lines that continued to proliferate nearly 18 months post-induction have remained triploid and occasionally produce embryos that have proven to be triploid as well.

Several characteristics of triploid *Citrus* tissues were different from those of typical diploid *Citrus*. Plant regeneration from either endosperm or nucellar callus follows embryogenic pathways, but successful triploid regeneration required a more complex sequence of medium manipulation to induce embryogenesis. Nucellar callus can be initiated, proliferated, and plantlets produced on basal MT medium supplemented only with ME, whereas primary endosperm and globular callus died on such medium. Endosperm calli did become habituated 15 months post-induction. Triploid embryo development, morphology, and germination were more abnormal than usually observed among diploids. Finally, excised triploid shoots were unresponsive in media capable of inducing shoot proliferation or adventitious rooting from a wide genetic range of diploid *Citrus* and related genera.

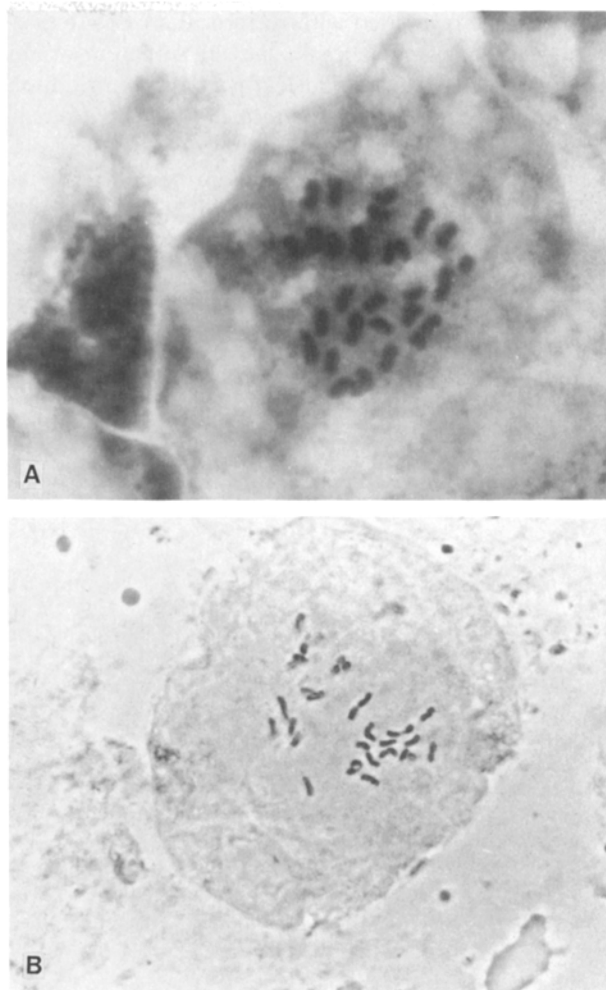

Fig. 6. Metaphase in cells from (**A**) globular callus and (**B**) a shoot meristem from a soil-grown plant showing $2n=3\times=27$ (magnification: $\times 1000$)

This research has demonstrated the feasibility of recovering triploid hybrid plants via in vitro embryogenesis of endosperm-derived callus of sweet orange. Improvement in the rate of successful plant recovery is necessary before this method can be used for creating triploid families for selection. Culture conditions that may allow triploid plant regeneration from endosperm of *Citrus* species other than *C. sinensis* should be defined, including media formulation and sequence, and temperature and light effects. Genetic effects from different pollen parents on regeneration capability should be investigated.

The potential genetic benefits for *Citrus* cultivar improvement programs possible with this approach to hybridization justify continued research and development. Polyembryonic parents with minimal fertility could be hybridized to create progeny families for selection. In effect, the use of reciprocal crosses with desired parents would double the number of nuclear hybrid combina-

tions possible compared with reciprocal crosses to produce zygotic progeny, because the female parent would provide two-thirds of the nuclear genomic contribution to endosperm hybrids. The selection of prospective plants for hybridization directed toward improved, seedless *Citrus* scion cultivars could be based on the genetic input desired and not on genotypes for apomixis.

Acknowledgements. The authors thank C. Cai, D. Chen, S. Huang, and M.K. Wendell for technical assistance, T. Zito for photographic contributions, and B. Thompson for manuscript preparation. This work was supported in part by a grant from the United States Department of Agriculture (86-CRCR-1-2002).

References

Cameron JW, Frost HB (1968) Genetics, breeding, and nucellar embryony. In: Reuther W, Batchelor LD, Webber HJ (eds) The citrus industry, vol. II. University of California Press, Berkeley, Calif, pp 325–370

Frost HB, Soost RK (1968) Seed reproduction: development of gametes and embryos. In: Reuther W, Webber HJ, Batchelor LD (eds) The citrus industry, vol. II. University of California Press, Berkeley, Calif, pp 290–324

Grosser JW, Chandler JL (1987) Aseptic isolation of leaf protoplasts from *Citrus*, *Ponicrus*, *Citrus* × *Ponicrus* hybrids and *Severinia* for use in somatic hybridization experiments. Sci Hortic 31:253–257

Grosser JW, Gmitter FG, Jr (1990) Protoplast fusion and citrus improvement. In: Janick J (ed) Plant breeding reviews, vol. 8. Timber Press, Portland pp 339–374

Gui YL, Gu SR, Xu TY (1982) Studies on morphological differentiation of endosperm plantlets of Chinese gooseberry in vitro (in Chinese with English summary). Acta Bot Sinica 24:216–221

Hodgson RW (1968) Horticultural varieties of citrus. In: Reuther W, Batchelor LD, Webber HJ (eds) The citrus industry, vol. I. University of California Press, Berkeley, CA, pp 431–591

Liu SQ, Liu JQ (1980) Callus induction and embryo formation in endosperm culture of *Prunus persica* (in Chinese with English summary). Acta Bot Sinica 22:198–199

Mu SK, Liu SQ (1979) Studies on initiation of apple endosperm callus and variation of chromosomal ploidy of callus cell of endosperm (in Chinese with English summary). Acta Bot Sinica 21:309–311

Murashige T, Tucker DPH (1969) Growth factor requirements of citrus tissue culture. Proc First Int Citrus Symp 3:1155–1161

Sass JE (1958) Botanical microtechnique. Iowa State University Press, Ames, pp 73–75

Soost RK, Cameron JW (1969) Tree and fruit characters of *Citrus* triploids from tetraploid by diploid crosses. Hilgardia 39:569–579

Soost RK, Cameron JW (1975) Citrus. In: Janick J, Moore JN (eds) Advances in fruit breeding. Purdue University Press, West Lafayette, pp 507–540

Soost RK, Cameron JW (1980) 'Oroblanco', a triploid pummelo-grapefruit hybrid. HortScience 15:667–669

Soost RK, Cameron JW (1985) 'Melogold', a triploid pummelo-grapefruit hybrid. HortScience 20:1134–1135

Wang D, Chang CJ (1978) Triploid citrus plantlet from endosperm culture (in Chinese). Sci Sinica 21:822–827

Zhao HX (1983) Plant regeneration from endosperm culture of pear (in Chinese). Chinese Bull Bot 1:38–39