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In Vitro Somatic Embryogenesis from Nucellar Callus of Monoembryonic Mango

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Abstract. **Slowly growing calli were induced *in vitro* from nucellar explants excised from fertilized ovules of immature 4.0–4.8 cm long monoembryonic mango fruitlets. The medium consisted of Murashige and Skoog formulation that had been modified accordingly: half strength major salts, 60 g/liter sucrose, 400 mg/liter glutamine, 100 mg/liter ascorbic acid, 1.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D), and 8 g/liter Difco Bacto agar. Somatic embryogenesis occurred from callus on this medium and following subculture to medium without growth regulators. Maturation and limited germination of somatic embryos occurred in the absence of growth regulators.**

Conventional breeding approaches with woody, perennial fruit crops have been complicated by generational cyles as long as 6 or 7 years, and by the absence of useful genetic markers. The advantages of efficient regeneration of tropical, perennial fruit trees from cell and tissue cultures in crop improvement programs have been reviewed by Kochba and Spiegal-Roy (3). The propagation *in vitro* of superior, disease-indexed selections that are otherwise hard to propagate clonally would have an immediate effect on the production of many tree crops. Mutant selection, the recovery of horticulturally useful somaclonal variants from cell and protoplast cultures, and the use of recombinant DNA may alter the breeding strategies for many tropical fruit crops. Unfortunately, the application of cell culture techniques to the improvement of woody, crop plants has been limited due to the absence of methods of regeneration from tissues of mature origin.

Maheshwari and Rangaswamy (9) demonstrated the practicality of using ovule culture to stimulate *in vitro* somatic embryogenesis from the nucellus of naturally polyembryonic *Citrus*. Subsequently, the induction of somatic embryogenesis from the excised nucellus from ovules of polyembryonic *Citrus* species was reported (13, 15). Rangan *et al.* (12) induced somatic embryogenesis directly from nucellar explants derived from monoembryonic *Citrus* species.

Rangaswamy (14) indicated that unusual nucellar growth or adventitious embryo formation from the nucellus occurs naturally in at least 16 plant families. Although the morphogenetic potential of the nucellus has been apparent for several years, there have been only a few successful attempts to exploit this tissue for *in vitro* studies involving woody plants. *In vitro* somatic embryogenesis has been described from nucellar explants of *Vitis vinifera* L. (10), *Ribes rubrum* L. (16) and *Myrciaria cauliflora* D.C. Berg. (6).

The mango, *Mangifera indica* L., is one of the most important fruit crops of the world. The annual production of mangos is exceeded only by grapes, *Musa* (bananas and plantains), *Citrus,* and apples (2). Litz *et al.* (7) indicated the potential for ovule culture to produce large numbers of somatic embryos from the nucellus of naturally polyembryonic mango cultivars. Later, they were able to stimulate callus from globular, adventitious, nucellar embryos on a medium containing 1–2 mg/liter 2,4-dichlorophenoxyacetic acid, and to obtain large numbers of somatic embryos, particularly in a liquid medium (8). All of the important mango cultivars in the United States and India are naturally monoembryonic. Therefore, the purpose of this study was to develop a system for the efficient induction of somatic embryogenesis from nucellar explants of monoembryonic mango cultivars.

Immature mango fruitlets were collected from several different monoembryonic cultivars in the Univ. of Florida Tropical Research and Education Center plant germplasm collection. Fruitlet length ranged from 0.7 to 7.0 cm. Following surface sterilization for 20 min. in 1% sodium hypochlorite to which 2–3 drops of Tween 20 were added, the fruitlets were washed with 3 changes of sterile, distilled water. The length of each fruitlet was determined before dissection, and the length of the zygotic embryo within each ovule also was determined prior to its removal. Ovules were removed aseptically from fruitlets and were bisected longitudinally to produce symmetrically equal halves. The nucellus was excised carefully from the ovules, and was transferred to sterile, modified Murashige and Skoog (MS) medium (11) in 120 mm petri dishes. The zygotic embryo was discarded.

Table 1. *In vitro* effect of 1 mg/liter 2,4-D on callus and somatic embryo induction from nucellar explants of mango.[z]

Cultivar	Cultures forming callus (%)	Callus cultures forming embryos (%)
Brooks	14	0
Golden Brooks	18	0
Irwin	60	40.0
Keitt	8	0
Kent	6	0
Ruby	52	23.1
Sensation	4	0
Tommy Atkins	36	11.1
Van Dyke	10	0

[z]Fifty sterile cultures were established from the same number of fruitlets for each cultivar. Size of discarded zygote ranged from 0.8 to 1.1 cm in length.

Modified MS medium consisted of half strength major salts plus (per liter), 60 g sucrose, 100 mg ascorbic acid, 400 mg glutamine, 8 g Difco Bacto agar, and 0–10 mg of one of the following growth regulators: 2,4-D, naphthaleneacetic acid (NAA), benzyladenine (BA), or 2-isopentenyladenine (2iP). Embryogenic nucellar callus was subcultured on this medium but without any growth regulators to permit maturation of somatic embryos. Germination medium consisted of liquid, modified MS medium without growth regulators, and with 20% (v/v) coconut water or 400 mg/liter malt extract. The pH of the media was adjusted to 5.7 with 0.1 N HCl or KOH prior to autoclaving at 1.1 kg cm^{-2} and 120 C for 15 min. Cultures were maintained in a growth chamber that provided growing conditions of 24 μmol $m^{-2}s^{-1}$ light intensity from Agri Lite fluorescent tubes (16 hr photoperiod) and 25°.

The nucelli became dark shortly after culturing, although callus formation was unaffected. Callus was produced from nucellar explants on auxin-containing media 3–4 weeks after the cultures were established. Callus formed from nucellar explants on media containing 1–3 mg/liter 2,4-D and to a lesser extent on media containing 1–5 mg/liter NAA.

Table 2. Effect of ovule development on callus induction from nucellus of mango.[z]

Length of discarded zygote (cm)	Cultures forming callus on 2,4-D medium (%)
0	20
0.1	16
0.2	32
0.3	16
0.4	32
0.5	28
0.6	12
0.7	12
0.8	12
0.9	0
1.0	12
1.1	0
1.2	32
1.3	20
1.4	12
1.5	0

[z]Twenty-five sterile cultures were established from the same number of fruitlets for each cultivar.

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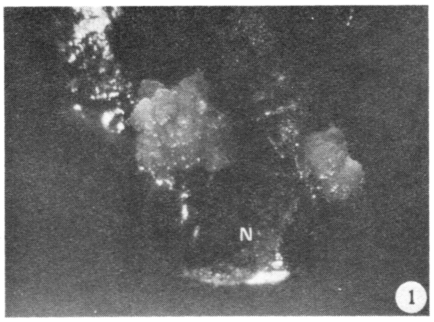

Fig. 1 Callus induction from 'Tommy Atkins' mango nucellar explants on MS medium with 1.0 mg/liter 2,4-D 3-4 weeks after culturing. N: nucellar explant.

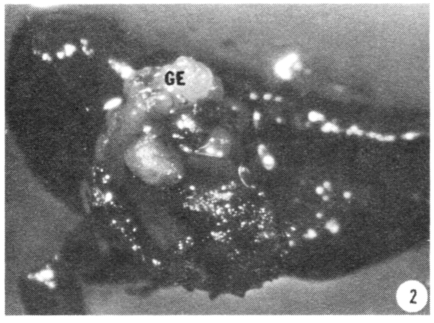

Fig. 2 Somatic embryogenesis from 'Tommy Atkins' mango nucellar callus on MS medium with 1.0 mg/liter 2,4-D 6 weeks after culturing. GE: globular somatic embryos.

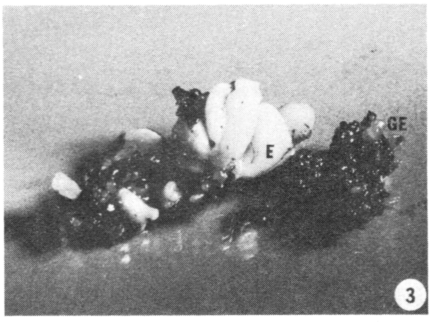

Fig. 3 Maturation of somatic embryos of 'Irwin' mango after subculture on modified MS medium without growth regulators. GE: globular somatic embryos; E: mature somatic embryo.

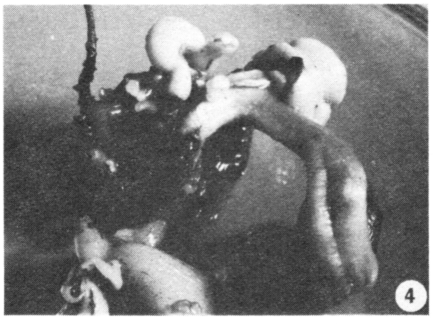

Fig. 4 Germination of somatic embryos of 'Ruby' mango in liquid modified MS medium without growth regulators.

Callus did not form on media containing either BA or 2iP or on medium without growth regulators.

Nucellar callus was slow growing, initially white or light green in color, and loose and friable in texture (Fig. 1). Callus became dark brown after a few days. Callus was induced from nucellar explants from apparently unfertilized 'Irwin' ovules and from ovules from which 0.1–1.3 cm long zygotic embryos had been removed. Nucellar explants from all monoembryonic mango cultivars that were studied produced callus, although the efficiency of callus induction varied on medium containing 2,4-D (Table 1). Explants from 'Ruby' and 'Irwin' ovules responded more favorably than explants from any of the other cultivars. Stage of ovule development seemed to have no effect on subsequent callus induction (Table 2). Callus induction was equally successful from the nucellus of ovules containing zygotic embryos up to 0.3 cm and from nucellus derived from relatively advanced ovules containing 1.2–1.4 cm zygotic embryos.

Somatic embryogenesis occurred from nucellar callus only on medium containing 1–2 mg/liter 2,4-D, about 5–7 weeks after explanting and 3–4 weeks after callus initiation (Fig. 2). Callus of only 3 cultivars, 'Tommy Atkins', 'Ruby', and 'Irwin' differentiated somatic embryos. A relatively low percentage of nucellar callus cultures was embryogenic, and this response was strongly cultivar-dependent (Table 1). Callus derived from 'Tommy Atkins' was less embryogenic than callus derived from 'Ruby' or 'Irwin'. Somatic embryos did not develop beyond the globular stage on medium containing 2,4-D. After subculture on medium without 2,4-D, advanced stages of somatic embryo development were observed (Fig. 3). The somatic embryos generally were normal in appearance, although some embryos had 3 or 4 cotyledons or demonstrated some degree of fasciation. Somatic embryogenesis continued to occur in cultures for several weeks on medium without 2,4-D, although many of these embryos were secondary, and developed directly from other somatic embryos.

Maturation of the somatic embryos frequently has been accompanied by gradual necrosis of the cotyledons and hypocotyls. Addition of malt extract, casein hydrolysate, and reducing agents were ineffective, although the addition of 20% (v/v) filter-sterilized coconut water appeared to delay, if not prevent, necrosis from occurring. Alteration of the physical nature of the growing conditions, e.g., light vs. dark or solid vs. liquid, had no effect on this developmental anomaly. Limited germination of somatic embryos has occurred (Fig. 4).

Conditions for induction of somatic embryogenesis from nucellar explants of polyembryonic (7,8) and monoembryonic mango cultivars have been demonstrated. Although *in vitro* somatic embryogenesis of monoembryonic *Citrus* occurred directly from the excised nucellus without an intermediate callus stage (12), monoembryonic mango cultivars were regenerated from nucellar callus. The efficiency of regeneration from nucellar callus of monoembryonic mangos differed with respect to cultivar. Similar observations have been made with *Citrus* cultivars (12). Unlike previous reports concerning the appearance of mango (8) and *Citrus* (9,13,15) callus, the nucellus-derived callus of this study cannot be described as pseudobulbil-like in appearance. The callus was loose and friable, but relatively slow growing, and therefore similar to the habituated 'Shamouti' *Citrus* callus first isolated and described by Kochba *et al.* (4).

Nucellus-derived plants generally are free of viruses and other disease-causing microorganisms, due to the absence of vascular connections between the surrounding maternal tissue and the nucellus (1). Some of the most devastating mango diseases, e.g., "decline" and "dieback", are caused by systemic fungal pathogens. Efficient recovery of monoembryonic mango plants from somatic embryos therefore would eliminate these systemic diseases and facilitate storage and international exchange of germplasm. Most important, however, is the potential for using *in vitro* procedures, such as mutant selection and somaclonal variation, for genetic improvement of perennial, tropical fruit trees such as the mango. Because of the constant disease and environmental stresses of tropical environments, catastrophic losses occur frequently in clonally propagated crops. Due to the long generational cycle of mangos, conventional plant breeding has not been responsive to crop-threatening situations. In fact, little is known about mango genetics. Somaclonal variation (5) has been observed in plants that have been derived from tissue cultures of other important crop species. Thus, the ability to regenerate polyembryonic and monoembryonic mango cultivars from callus may result in an increased frequency of horticulturally useful somatic mutations within the most important cultivars.

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In Vitro Propagation and Growth of Hydrangea

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Abstract: ***Hydrangea macrophylla*** **Thunb. 'Merveille' can be propagated by tissue culture and the resulting plants acclimated to a greenhouse environment. Utilizing rapidly replicating cultures for continued propagation and slow replicating cultures for producing plants in a greenhouse is an effective method of increasing the overall tissue culture propagation rate. Planting out in a greenhouse by 30 July at the latitude of Lexington, Ky., provided adequate time to develop hydrangea plants that produced 3 or more flowers per plant by 15 Apr.**

Fewer hydrangea are grown as a florist pot plant today than in past decades. Weiler (6) considers the long cropping time, difficulty of transporting when in flower, and the large postgreenhouse demand for water to be major factors for the production decline. Some growers in the North frequently save stock plants for propagation. With the current high cost of greenhouse space, carry-over crops often are eliminated to accommodate a revenue producing crop. In addition, hydrangeas frequently are found to have virus diseases, especially hydrangea ringspot virus (5). Carrying diseased stock plants from year to year perpetuates the problem. In Europe, hydrangeas have been cultured aseptically to obtain stock plants free of virus, and subsequent propagation was by cuttings from these stock plants (1, 2, 5). Jones (3) has reported successful *in vitro* propagation and preliminary virus indexing of 5 of 10 hydrangea cultivars attempted; 'Merveille' hydrangea was not one of those tested. Commercial laboratories also have produced hydrangeas by *in vitro* propagation, but no reports of their studies are available.

Tissue culture propagation has the potential of producing large numbers of plantlets. Once the starting material is disease free, it can be maintained readily in aseptic culture. In addition, very little space is required in a tissue culture growth room to carry propagation stock from one year to the next. This study was undertaken to determine 1) if 'Merveille' hydrangea would respond to tissue culture propagation techniques, 2) if individual shoot isolates vary significantly in propagule production, and 3) if market quality pot plants could be produced in less than one year from plants propagated *in vitro*.

Stems were harvested on 28 June from a single 'Merveille' hydrangea growing outdoors at Lexington, Ky. The leaves were removed and the stems were immediately placed in 0.5% sodium hypochlorite solution for 10 min. Buds were cut from the stems and placed in fresh 0.5% sodium hypochlorite solution for 5 min, rinsed twice with sterile distilled water, and placed individually on 10 ml of culture medium in 25 × 150 mm culture tubes.

The basal medium used for all studies was Murashige and Skoog (MS) mineral salts (4) supplemented with the following (mg/liter): NaH_2PO_4, 170; *myo*-inositol, 100; thiamine·HCl, 0.4; Difco Bacto agar, 7000; and sucrose, 20,000. The initial isolation medium consisted of a basal medium with 0.1 mg NAA and 1 mg BA per liter. All media were adjusted to pH 5.7–5.8 before autoclaving. To obtain cultures free of microbial contamination, shoots were grown singly on 10 ml of basal medium with 1 mg/liter BA in 25 × 150 mm culture tubes. To study shoot production response to growth regulators, cultures were grown on 15 ml of basal medium with 4 levels of BA (0.2, 0.5, 1.0, 5.0 mg/liter) without auxin and also with 0.2 mg/liter of NAA. Ten replications were used for each treatment. Shoots were harvested 3 times at 35-day intervals.

To study shoot production as a function of individual shoot isolates, 20 random cul-

Table 1 Total shoot production of 20 randomly selected shoots through 5 harvests at 28-day intervals.[z]

Line no.	Total shoots
7	227 a
20	153 b
8	114 bc
14	102 cd
3	93 cd
12	90 cd
10	89 cd
15	81 cd
13	76 cd
9	72 cd
11	70 cd
2	69 cd
17	68 cd
1	66 cd
6	66 cd
16	66 cd
18	66 cd
5	62 cd
4	61 cd
19	50 d

[z]Shoot numbers followed by a different letter are significant at the $P = 0.05$ level as determined by Duncan's multiple range test.

Table 2. Mean number of nonflowering, flowering, and total stems per plant produced by tissue cultures 'Merveille' hydrangeas transplanted to 13 cm pots 30 July and transplanted to 10 cm pots 20 Aug.

Pot Size	Mean number of stems per pot[z]		
	Nonflowering	Flowering	Total
10 cm	3.3 A	1.0 B	4.3 B
13 cm	2.1 B	3.7 A	5.8 A

[z]Means within a column followed by a different letter are significantly different at $P = 0.01$ as determined by a single degree of freedom F test.

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