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In Vitro Responses of Adventitious Embryos of Two Polyembryonic *Eugenia* Species

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Abstract. **Ovules were excised from immature fruit of 2 naturally polyembryonic, tropical trees, *Eugenia jambos* and *E. malaccensis*. Immature, adventitious embryos were removed from the ovules and were cultured on modified Murashige and Skoog (MS) medium that contained either 0–10 mg/liter benzylaminopurine (BA) or 0–10 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D). Depending on the developmental stage of the adventitious embryos, proliferation of axillary buds occurred on media with 2–10 mg/liter BA, whereas germination occurred on lower BA concentrations or in the absence of growth regulators. Root formation only was induced on media containing 3–10 mg/liter 2,4-D. Embryogenic callus was formed from adventitious embryos from 1–2 cm fruitlets on 1–2 mg/liter 2,4-D. The 2 species responded in a similar manner.**

The main obstacles to successful *in vitro* regeneration of woody plants from cell, organ, and tissue cultures are the absence of juvenile tissues in mature trees and the difficulty of inducing juvenility in potential explants. In at least 172 plant families, the nucellus becomes enlarged during ovule development (9). Adventitious embryony occurs from the enlarged nucellus in at least 16 of these plant families (13). Kaur *et al.* (1) have demonstrated the high frequency of naturally occurring polyembryony in forest and fruit trees from the tropical rain forest of Southeast Asia, e.g., *Citrus, Eugenia* spp., *Garcinia mangostana, Lansium domesticum,* and *Mangifera indica*.

The potential of the nucellus or of nucellar-derived adventitious embryos as juvenile explants for *in vitro* studies was first described by Maheshwari and Rangaswamy (8). Using naturally polyembryonic *Citrus* as a model, they induced somatic embryogenesis from cultured ovules. Subsequently, techniques for regenerating somatic embryos from *Citrus* nucellar tissue and callus cultures were greatly refined. Rangan *et al.* (12) were able to stimulate somatic embryogenesis directly from the excised nucellus from monoembryonic *Citrus in vitro*.

Kochba and Spiegel-Roy (2) have reviewed the potential uses of cell and tissue culture for improving *Citrus*. The application of efficient mutant selection techniques, the recovery of somaclonal variants, and other approaches could have considerable impact on breeding programs involving all fruit trees with prolonged juvenile periods. Despite the obvious potential of the nucellus or nucellar-derived adventitious embryos for *in vitro* studies, there have been relatively few attempts to exploit these tissues.

The investigation of the *in vitro* potential of the nucellus from tropical fruit trees other than *Citrus* spp. was initiated by Litz *et al.* with naturally polyembryonic mango cultivars (6, 7), naturally polyembryonic jaboticaba (*Myrciaria cauliflora*) (4), and monoembryonic mango cultivars (5). Somatic embryogenesis was observed in callus derived from either the isolated nucellus (5) or from nucellus-derived adventitious embryos (4).

The purpose of this study has been to elucidate regenerative pathways from *in vitro* cultures of adventitious immature embryos of 2 naturally polyembryonic *Eugenia* (*Syzygium*) species, *E. jambos* L., the rose apple, and *E. malaccensis* Lam., the Malay apple. The genus *Eugenia* (*Syzygium*) contains several hundred species in the Old and New World, including many species of great economic importance. According to Pijl (11), polyembryony in *E. jambos* is nucellar, whereas the adventitious embryos in *E. malaccensis* are derived from the inner integument.

Immature fruitlets were collected from trees of *Eugenia jambos* and *E. malaccensis* in the plant germplasm collection of the Tropical Research and Education Center of the Univ. of Florida in Homestead. The size of fruitlets ranged from 0.4–2.0 cm in length. Following surface-sterilization in 1.05% sodium hypochlorite containing 2–3 drops of Tween-20 for 20–25 min, the fruitlets were rinsed briefly with 3 changes of sterile distilled water. Polyembryonic ovules were removed from the fruitlets. The embryo masses were cultured on a range of medium formulations in disposable, plastic, 57 mm diameter petri dishes that were sealed with Parafilm. There were 10–15 ml of medium per petri dish. One ovule was placed in each culture container. The MS medium (10) was modified by using half strength major salts plus (per liter) 60 g sucrose, 400 mg glutamine, 100 mg ascorbic acid, 8 g Difco Bacto agar, and 2,4-D or BA at concentrations of 0–10 mg. The pH of the media was adjusted to 5.7 prior to autoclaving at 120°C and 1 kg/cm^2 for 15 min. Cultures were maintained in a growth chamber at 25° and with a 16 hr photoperiod (24 μ mol m^{-2} sec^{-1}) provided by Agri Lite fluorescent tubes. Fruitlets were segregated according to size, e.g., 0.4–0.8 cm, 0.8–1.2 cm, 1.2–1.6 cm, and 1.6–2.0 cm. Four specimens from each size range were inoculated onto each growth regulator concentration. The experiment was repeated once. Data have been compiled from all observations.

The *in vitro* responses of adventitious embryos of the 2 *Eugenia* species were very

Table 1. *In vitro* effect of 2,4-D on *Eugenia jambos* immature adventitious embryos.[z,y]

Embryo stage (fruitlet size cm)	2,4-D concentration (mg/liter)										
	0	1	2	3	4	5	6	7	8	9	10
0.4–0.8	---	C	C	C	---	---	---	---	---	---	---
0.8–1.2	G	C	CES	C	---	C	---	---	---	CR	---
1.2–1.6	G	CS	CS	C	C	C	C	CR	---	CR	R
1.6–2.0	G	CS	CR	CR	---	R	---	CR	CR	CR	R

[z]G = germination; C = callus; E = direct embryogenesis; R = root formation; S = somatic embryogenesis from callus; no response = 3 dashes.
[y]Effect of 2,4-D on *E. malaccensis* immature embryos was indistinguishable from its effect on *E. jambos*.

Table 2. *In vitro* effect of BA on *Eugenia malaccensis* immature adventitious embryos.[z,y]

Embryo stage (fruitlet size cm)	BA concentration (mg/l)										
	0	1	2	3	4	5	6	7	8	9	10
0.4–0.8	---	---	---	---	---	---	---	---	---	---	---
0.8–1.2	---	G	G	---	---	A	---	A	---	---	---
1.2–1.6	G	G	G	GA	GA	GA	A	---	A	A	A
1.6–2.0	G	G	GA	GA	A	GA	---	---	A	A	A

[z]G = germination; A = axillary bud proliferation; no response = 3 dashes.
[y]Effect of BA on *E. jambos* immature embryos was indistinguishable from its effect on *E. malaccensis*.

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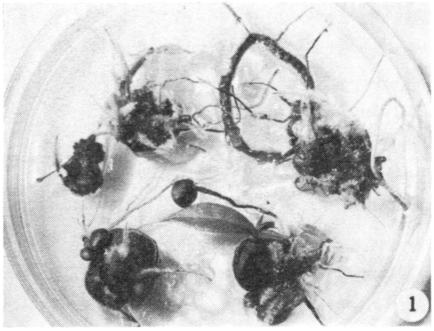

Fig. 1. Effect of high 2,4-D concentrations (10 mg/liter) on immature adventitious *E. jambos* embryos from 1.7 cm fruitlets. Extensive root formation.

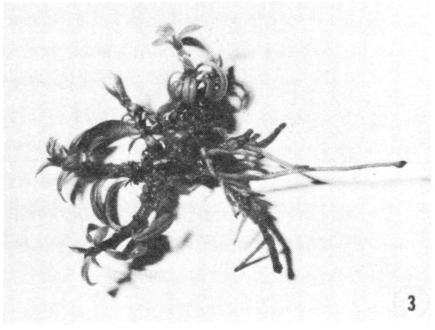

Fig. 3. Germination of adventitious *E. jambos* embryos on medium without plant growth regulators. Explant was derived from 1.8 cm fruitlet.

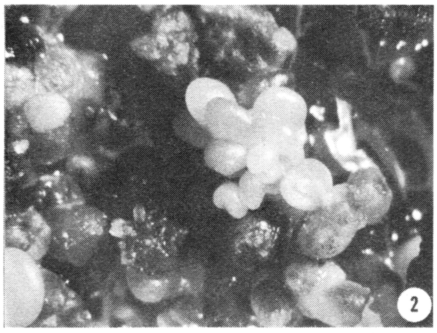

Fig. 2. Somatic embryogenesis from callus on medium with 1.0 mg/liter 2,4-D derived from immature adventitious embryos of *E. malaccensis*. Fruitlet size was 1.4 cm.

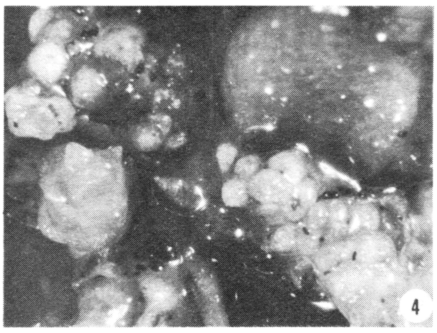

Fig. 4. Proliferation of axillary buds from immature adventitious embryos on medium containing of *E. jambos*. Explant derived from 1.2 cm fruitlet.

similar and were indistinguishable on the medium formulations used in this study (Tables 1, 2). Callus formation was evident after about 2 weeks on many of the adventitious embryos from all stages of fruitlet development, but only within the range of 1–5 mg/liter 2,4-D (Table 1). Callus was formed on adventitious embryo explants from 1–2 cm fruitlets on high concentrations of 2,4-D, but this callus usually was delayed and sometimes formed from roots. Somatic embryogenesis occasionally occurred directly from the cotyledons and from other explant tissues derived from 0.8–1.2 cm fruitlets after 1–2 months on medium with 2 mg/liter 2,4-D. Root formation only occurred from explants taken from 1–2 cm fruitlets on 5–10 mg/liter 2,4-D (Fig. 1); in addition, some callus was formed, although it was not embryogenic. The most immature explants did not survive on 4–10 mg/liter 2,4-D.

A loose and friable callus was induced from explants from 1.0–1.6 cm fruitlets on 1–2 mg/liter 2,4-D within 1–2 months. Somatic embryogenesis occurred from this callus 4–5 months after culturing (Fig. 2). The callus was cream-honey colored and originated primarily from the cotyledons of adventitious embryos. Somatic embryos passed through distinct developmental stages as they changed from white, globular embryos through rose-colored, intermediate stages to green at maturity. The cotyledons of mature embryos were thick, green and fleshy, and resembled embryos formed *in vivo*.

Explants from larger fruitlets (1.2–2.0 cm) germinated in the absence of growth regulators or on media containing 1–2 mg/liter BA. Typically, there were 3 or 4 plantlets from each ovule (Fig. 3), but there were occasionally 5 or more. All germinated plantlets were transferred successfully to soil.

The proliferation of axillary buds occurred from cultured explants derived from 1–2 cm fruitlets on 2–10 mg/liter BA. Typically, the proliferated shoots were oriented lengthwise on an enlarged ridge of tissue between the cotyledons (Fig. 4). In the presence of 1–5 mg/liter BA concentrations, shoot proliferation, and occasionally shoot growth and root formation occurred simultaneously. Branching occurred on the germinated shoots on media with 4–5 mg/liter BA.

Despite the differences in species and in the nature of polyembryony in the 2 *Eugenia* species, the *in vitro* responses of immature adventitious embryos from these species were indistinguishable. According to Pijl (11), the germination of polyembryonic *Eugenia* seeds is often uneven or incomplete. Many seeds form roots or shoots only, which Pijl attributed to incomplete development of the embryos. This incomplete development may be due to an imbalance in endogenous growth regulators, since high auxin concentrations *in vitro* stimulated root formation and inhibited shoot formation in the current study.

Kong and Rao (3) also observed enhanced root formation from internodal callus derived from monoembryonic *Eugenia grandis* seedlings on medium containing 10 mg/liter naphthaleneacetic acid. Multiple shoot formation occurred from explanted seedling nodes in the presence of 2–10 mg/liter BA. Somatic embryogenesis was not observed, although presumably adventitious shoots were induced from nodal callus on medium with 5 mg/liter BA. This inability to induce somatic embryogenesis may be due to their choice of auxin or their use of less juvenile tissues as primary explants.

The current study indicates the practicality for using 2 *in vitro* pathways for regeneration of *E. jambos* and *E. malaccensis*, i.e., shoot tip proliferation and somatic embryogenesis. Both of these species normally reproduce asexually by the formation of adventitious embryos, and are of minor economic importance. Consequently, there is no real advantage in using *in vitro* propagation systems for either of these plants. Other monoembryonic species within the genus *Eugenia*, however, are very important, e.g., clove (*E. aromaticum*), and are difficult or impossible to propagate vegetatively. Rangan *et al.* (12) demonstrated that monoembryonic *Citrus* species and cultivars could be regenerated *in vitro* directly from nucellar explants as readily as polyembryonic species. Litz (5) demonstrated that monoembryonic mango cultivars can be regenerated *in vitro* from nucellar callus. Thus, it should also be possible to induce somatic embryogenesis *in vitro* from nucellar or integumental explants of several monoembryonic *Eugenia* (*Syzygium*) species.

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