FEATURE

The Use of Embryo Culture in Fruit Breeding

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Background

The first successful systematic culture of plant embryos on an artificial medium of defined chemical composition was by E. Hanning in 1904 with mature embryos of two crucifers (Narayanaswami and Norstog, 1964). Then, in 1929, Laibach (Maheshwari, 1966) pointed out the potential application of embryo culture in plant breeding to rescue embryos from interspecific hybrids that otherwise fail due to embryo abortion. Tukey's (1933b) culture of cherry embryos in 1933 was a milestone in embryo culture of fruit crops. His medium and procedure has been widely used and even applied to other crops, such as cucurbits (Whitaker and Davis, 1962). Using this procedure, Blake (1939) was the first to employ culture in a peach breeding program.

LaRue (Narayanaswami and Norstog, 1964) successfully cultured and grew small (0.5-mm-long) embryos of many genera as early as 1936 with the addition of inorganic salts, sugar, yeast extract, and indolebutyric acid to the medium. However, embryos in the globular stage and smaller are very difficult to culture successfully. Maheshwari (1958) demonstrated by use of in ovule embryo culture that it is possible to grow a four-celled proembryo of *Papaver somniferum* L. to maturity. The successful growth of ovule-cultured embryos depends on their stage of maturity at the time of culture, genotype, culture medium, and culture environment.

The developing embryo is a dynamic system that has changing requirements as it ma-

tures. The smaller the embryo, the more complex the medium required. Raghavan (1976) states in his review of embryo culture that there are heterotrophic and autotrophic stages in embryo development. The embryo in the heterotrophic stage of development is smaller than in the autotrophic stage and usually requires the presence of growth regulators to allow for proper development. The autotrophic stage begins at about the late heart stage and development of such an embryo does not depend on exogenous sources of growth regulators, thus making it more amenable to in vitro culture.

Embryo culture is useful when there is poor embryo development or abortion. Embryo abortion occurs in early ripening genotypes of *Prunus* where the flesh matures before seed maturity (Tukey, 1933a; Tukey and Lee, 1937), precluding their use as females in a breeding program. The poor germination of seeds from early ripening grapes is probably due to poor embryo development. Embryos have been rescued from early ripening *Prunus* (Tukey, 1934) and *Vitis* (Ramming and Emershad, 1984) cultivars, thus allowing their use as females. Many seedless grape cultivars are stenospemic (Stout, 1936), i.e., they are characterized by cessation of embryo development after fertilization and failure of seed development. Embryos have been rescued and grown into plants from stenospemic seedless grapes (Emershad and Ramming, 1984; Spiegel-Roy et al., 1985), allowing growth of progeny resulting from the hybridization of seedless with seedless grapes. The production of interspecific hybrids is useful for the transfer of desirable genes from wild to cultivated species. In many cases, wide crosses between species are difficult to produce because of many factors that act as barriers. Postzygotic barriers such as endosperm abortion are a common occur-

rence, but they have been overcome through the use of embryo rescue and many interspecific hybrids have been produced (Collins and Grosser, 1984). Embryo rescue has also been used to overcome postzygotic barriers during self- and cross-incompatibility (Fassliotus, 1977; Cox et al., 1960; Fridriksson and Bolton, 1963).

Embryo culture has been used for rapid seed germination tests of peach (Tukey, 1944; Flemion, 1936) and other woody plants (Flemion, 1941, 1948); for production of virus-free citrus plants through culture of nucellar embryos (Bitters et al., 1970, 1972); and for studying the developmental and growth requirements of embryos (Steward and Hsu, 1977). Embryo culture has also been used to shorten breeding cycles in apple (Nickell, 1951) and iris (Randolph, 1945), where effective germination was reduced from years to months by overcoming dormancy. The possibility also exists for using embryo culture as a screening procedure. Landa et al. in 1980 (Collins and Grosser, 1984) investigated the possibility of selecting mutants with high amino acid content. Pollen might also be screened for temperature tolerance (Zamir and Vallejos, 1983) before in vitro fertilization of ovules. Embryo culture has been reviewed by Sanders and Ziebur (1963), Narayanaswami and Norstog (1964), Maheshwari (1966), Raghavan (1966, 1976, 1977, 1980), Bhojwani and Razdan (1983), and Collins and Grosser (1984). One of the few reviews of ovule culture was by Rangan (1984). This review will cover the use of embryo rescue for two fruit crops, *Prunus* and *Vitis*.

Results achieved

Peach embryo culture. After Tukey successfully cultured cherry embryos (1933 b),

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others applied it in their breeding programs. Blake (1939) used the procedure and Hough improve on the medium and procedure (Smith et al., 1969). Embryos with 75% fill (PF_i) could be rescued with this procedure. Peach embryo culture was started in 1975 at the Horticultural Crops Research Laboratory, Fresno, Calif., using Smith, Bailey, and Hough (SBH) medium (Smith et al., 1969) and procedures to develop progeny from early ripening genotypes and increase the chances of developing good early ripening cultivars. The technique of Smith et al. (1969) of cubing the fruit to remove all skin, surface-sterilizing with phenol, cracking the pit, surface-sterilizing the seed with merthiolate, and rinsing three times with distilled water was very time consuming and could be toxic to people. Since techniques and procedures used in breeding programs must be efficient, simple, and able to process many embryos, the

above procedure was simplified. Our procedure, in Fresno, for embryo culture consisted of cracking the fruit and pit open to remove the seed and then flaming the seed in the laminar flow hood before extracting the embryo and placing it into the culture medium. Care must be taken to prevent damage during flaming. Damage occurs to the integument during flaming and the uptake of nutrients is reduced during ovule culture of very small embryos; hence, an alternative sterilization procedure for ovule culture was developed. The fruit is surface-sterilized with 70% ethanol for 30 sec, then with 0.525% Na hypochlorite for 5 min before it is cut open under sterile conditions and the seed is placed directly in the culture medium. Care must be exercised so the seed is not damaged or taken from open pits that generally are contaminated. Embryos from soft fruit have a greater chance of becoming contaminated

because of free-flowing juice.

The first population, from six early ripening genotypes, having 70- to 72-day fruit development periods, were developed using SBH medium. Without embryo culture, plants could not be developed from these genotypes. The procedure allowed the selection of genotypes ripening 10 to 30 days earlier than the parents from these populations (Table 1). Embryo development in these very early ripening selections was less than in the parents; hence, the SBH medium was inadequate to support their growth. A more-defined medium was developed for the successful culture of these genotypes. Murashige and Skoog (MS) (1962) medium with the addition of 1240 mg potassium succinate and 584.6 mg L-glutamine per liter and 3% sucrose allowed embryos as small as 5 mm long (PF_i = 25) to be grown successfully. This was first used in the breeding program in 1980. All embryos longer than 10 mm are routinely cultured on SBH and those 5 to 10 mm long on MS. The recovery of viable plants from ovules and embryos has ranged from 41% to 79% since 1976. Contamination is only a problem if inexperienced and untrained people perform the embryo culture. The successful use of embryo culture in our peach and nectarine breeding program from 1975 to 1987 is shown in Table 2. Embryos <10 mm long are more difficult to grow successfully even though they are grown on a more complex medium (Table 3). Only 32% of such embryos produced plants, com-

Table 1. Ripening dates of peach and nectarine cultivars used as parents and the earliest-maturing seedlings selected from their progeny.

Parent*	Year cultured	Date ripe	
		Parent (1979)	Earliest-ripening selected seedling (May 1979)
FV9-164	1975	4 June	5
Springold	1976	21 May	10
Springcrest	1976	29 May	10
FV9-164	1976	4 June	8
Armking	1976	7 June	14
Arm Queen	1976	10 June	21

*All open pollinated.

Table 2. Number of ovules and embryos cultured, and percentage of seedlings that were planted in the greenhouse and field, contaminated, or had small embryos that did not grow, and number of seedlings selected and propagated in the peach and nectarine breeding program for 1975-87.

Year	No. Ovules	No. Embryos	Percentage of both organelles				No. seedlings	
			To greenhouse*	To field	Contaminated	Small	Selected	Propagated
1975	---	1075	3	3	42	55	11	7
1976	---	2571	58	45	1	29	102	24
1977	---	2066	56	40	4	33	47	14
1978	---	4214	79	72	12	8	53	23
1979	---	6277	67	54	15	14	54	15
1980*	---	8129	54	32	22	9	49	11
1981 ^y	1419	6058	58	30	8	32	25	10
1982*	2140	5420	52	10	20	28	21	11
1983	5080	1281	41 (34, 70)	26	11	35	41	12
1984	3072	2102	38 (20, 64)	33	15	24	74	25
1985	1543	798	53 (37, 86)	50	4	25	31	12
1986	935	586	41 (30, 58)	36	8	41	6	---
1987	908	1184	66 (37, 89)	58	5	13	---	---

*MS and SBH media used and seed sterilized by flaming, starting year indicated.

^yOvules cultured on cotton support in petri dish. Planted ovules as seed in greenhouse only in year indicated.

*Ovules cultured on SH medium and sub-cultured in test tubes as embryos starting in year indicated.

*Numerals in parentheses: percentage of ovules and of embryos, respectively, planted in the greenhouse.

^ySeedlings not fruiting.

Table 3. Number of peach ovules and embryos cultured on MS or SBH medium from 1983 to 1987 and the percent planted in the greenhouse.

Year	Ovule culture		Embryo sub-cultured from ovule culture				Embryo culture			
	No.	No. viable embryos	MS* (No.)	Planted (%)	SBH* (No.)	Planted (%)	MS* (No.)	Planted (%)	SBH* (No.)	Planted (%)
1983	5080	70	2325	35	1240	73	226	6	1055	84
1984	3072	50	713	26	839	78	360	35	1753	86
1985	1543	67	694	36	338	92	115	48	683	92
1986	935	76	495	21	220	80	887	21	261	57
1987	908	72	419	35	239	81	127	50	1057	93

*Embryo length determined whether embryos were cultured on MS or SBH medium. Embryos <10 mm long were cultured on MS and those ≥10 mm on SBH medium.

pared to 78% for larger embryos.

Some recent selections ripen so early that their embryos are only 1 mm long at fruit maturity. The use of these selections as females required improvements in media and procedures. The cotton ovule culture medium of Stewart (1979) was tested for *Prunus* (Ramming, 1985), and it provided the necessary environment for the development of embryos initially 1.0 to 5.0 mm long. Cotyledons are well defined at this stage, and sucrose concentration was found to be more critical to embryo development and subsequent growth than cytokinins, auxins,

or additional organic addenda. Sucrose at 6% to 8% during ovule culture was optimum for embryo fresh and dry weight accumulation and germination (Figs. 1 and 2). Precocious germination is prevented during ovule culture by the integuments, allowing the embryos to increase in fresh and dry weight within the integuments. At this time, the embryo is using the nucellus and endosperm as well as the nutrient medium for growth. Embryos were excised after 2 weeks of ovule culture and sub-cultured at various sucrose levels during stratification at 0.5C. Embryo development continued during stratification,

a % Sucrose during initial culture

b % Sucrose during initial culture

Fig. 1. Fresh weight (a) and dry weight (b) of 'Springgold' peach embryos after 2 weeks of ovule culture and 45 days of stratification (strat.) at 0.5C at various sucrose levels. Embryo size was 2.1 mm at beginning of ovule culture. Vertical bars represent 95% confidence interval (n = 20).

Fig. 2. The number of 'Springgold' peach embryos that germinated after 2 weeks of ovule culture and 45 days stratification (0.5C) at various sucrose levels (n = 20).

increasing in fresh weight by 35% to 129% (Fig. 1). Lower sucrose levels (0% and 2%) at this time were found to be the most beneficial for increase in fresh and dry weight and germination (Figs. 1 and 2). When ovule culture was first used in 1981, the embryos were not excised or subculture, but treated like mature seeds during stratification and germination. A higher percentage of plants developed when the embryos were excised after the initial 2 weeks of ovule culture and then subculture on either MS or SBH during the stratification period than when they were treated like mature seed. This superior procedure has been used since 1983.

We have made many selections from seedlings produced by embryo culture methods. Two cultivars—Goldcrest peach and Mayfire nectarine—have resulted from embryo culture and are among the earliest of the commercially grown cultivars in California (Ramming and Tanner, 1987a, 1987b).

The culture of zygotes could be useful for rescuing embryos from interspecific hybrids that abort due to postzygotic barriers and for zygotic screening techniques. In 1986, we induced embryos 7 to 10 days post-bloom to enlarge to ≈ 12 mm (300 mg). However, the embryos appeared to abort during stratification, and no viable plants were produced. Hormones during the initial ovule culture period were necessary as for tomato, and levels used were those described by Neal and Topoleski (1985). Additional work on *Prunus* is needed to be able to rescue zygotes.

Grape embryo culture. The seeds of many early ripening grapes do not germinate when they are stratified and planted in soil. These genotypes are valuable as females and as males to increase the chances of developing early ripening grape cultivars. Therefore, peach embryo culture techniques were applied to grapes. We used modified White's macroelements medium with Norstog's microelements and vitamins plus glycine and casein hydrolysate (Emershad and Ramming, 1984). Using this technique, we have improved initial germination from 0%-16% to 19%-24% and total plant production to 30%-32% (Table 4). Seeds are cultured individually in 20 $\times$ 150-mm test tubes, strat-

Table 4. Number of seed and sub-cultured embryos that germinated from three early ripening seeded grape genotypes as a result of embryo culture.

Genotypes	No. seed cultured	Seed germinated		No. embryos germinated ^z	Plants		No. plants in field ^y	Germination in petri dish ^x (%)
		No.	%		Total	%		
Early muscat	1079	204	18.9	142	346	32.1	190	0
C64-97	195	47	24.1	12	59	30.3	41	7
C66-151	1569	382	24.3	108	490	31.2	415	16

^xUngerminated seed was cut open, embryo excised, and sub-cultured. Germination based on number of plants planted in the greenhouse.

^yPlant difference due to loss during acclimation in the greenhouse.

^zSimulated germination in soil. Only seeds containing endosperm (sinker) were cultured.

Table 5. Number of early ripening grape seeds without endosperm (floaters) containing embryos and the number of plants obtained as a result of embryo culture.

Genotype	No. floaters	Floaters with embryos		No. plants developed
		No.	%	
Early muscat	221	56	25	7
C64-97	139	17	12	0
C66-151	424	141	33	11

Table 6. Number of grape seed from early ripening genotypes embryo-cultured and the percentage of plants developed for the breeding program compared to germination without embryo culture (EC).

Year	No. crosses	No. emasculations	Cultured seed		Planted in field		Germination ^x without EC (%)
			No. ^z	%	No.	% ^y	
1983	8	32,740	3213	9	656	20	6
1984	26	57,911	3209	5	1219	37	27
1985	40	69,813	4805	7	2192	53	11
1986	18	23,660	3165	13	1282	40	5

^xOnly seeds containing endosperm (sinker) were cultured.

^yThe percentage germinated was higher, but plants were lost during acclimation in the greenhouse.

^zEstimate of germination based on percentage germination in petri dishes and the percentage of each genotype making up seeds that were cultured.

ified for 3 months at 0.5C, and then allowed to germinate. Some seed germinate immediately and are planted directly in the greenhouse. Some small embryos come out of the seed and germinate directly on the medium and can be planted in the greenhouse when large enough. Embryos from the ungerminated seed will grow when the seed is cut open and the embryo extracted and sub-cultured. Even floaters (seed without endosperm) occasionally contain embryos that can be successfully rescued, although not efficiently enough for a breeding program (Table 5). Results of culturing crosses from early ripening seeded grapes for the breeding program as described above are shown in Table 6.

Every grape breeder's dream has been to interbreed stenospermic seedless grapes. This step holds the promise of eliminating the intermediate seeded female and the second generation for intermating or back crossing (Fig. 3). In addition to saving one generation of seedlings and ≈ 5 years; land, labor, fertilizer, and water are also saved. The genotypes can also be hybridized directly without being diluted by genes from a seeded female, making it easier to combine complementary traits. The proportion of seedless progeny should also be higher, requiring smaller populations to develop the same number of seedless seedlings as produced in seeded $\times$ seedless crosses.

Plants from seedless genotypes were first produced by embryo culture in our laboratory in 1981 (Emershad and Ramming, 1982). Since the embryos ranged from four to 50 cells at the time of culture, they were impractical to culture directly as embryos. The ovule is cultured, allowing the embryo to enlarge directly in the integument, after which the embryo is excised and subculture, allowing it to germinate and grow into a plant. In 1983, ovules from 29 seedless genotypes with varying sizes of seed traces were cultured from open-pollinated fruit (Table 7). There exists a range of responses to culture, as measured by the percentage of ovules with embryos, which is not correlated to seed trace weight (correlation coefficient = 0.19). During culture, multiple embryos develop in some ovules, usually as a result of somatic embryogenesis from the zygotic embryo. This conclusion was reached based on position of multiple embryos attached to zygote and on fruiting seedlings. The amount of multiple embryogenesis seems to be genotypic-dependent, based on data from culturing the

Fig. 3. Method for hybridization of seedless genotypes using a seeded female as the genetic bridge compared to direct hybridization of seedless made possible by embryo culture.

Table 7. Number of ovules cultured, embryos developed, and size of embryos developed from seedless genotypes cultured in vitro in 1983.

Genotypes	Seed trace fresh wt (mg)	No. ovules cultured	Embryos developed		Average embryo size (mm)	No. ovules with multiple embryos
			No.	%		
C29-171	42.3	53	22	42	1.5	0
B31-164 CSUF	20.8	58	30	52	1.5	6
C35-33	17.6	60	24	40	1.9	0
C20-149	15.5	40	3	8	0.7	0
A74-61	15.2	64	16	25	1.3	8
B18-185	14.4	68	26	39	2.2	0
C85-82	11.7	64	37	58	2.1	7
A73-150	11.6	55	26	48	1.5	7
B46-112 Self	11.2	74	51	69	9.5	1
B1-68	9.8	37	14	38	0.6	0
B29-82	9.7	30	10	34	2.3	0
B44-2	9.2	62	16	26	2.3	0
C58-22	8.8	74	10	14	4.5	0
C88-89	8.8	55	13	24	2.0	2
P60-58	7.8	61	31	51	3.5	1
B74-198	7.7	60	44	74	1.4	10
C76-79	7.1	68	9	14	0.8	1
Flame Seedless	7.0	63	21	34	3.1	1
C18-36	6.8	52	1	2	0.1	0
C33-199	4.8	23	0	0	---	---
B5-15	4.7	60	23	39	1.4	0
C22-121	4.6	34	13	39	7.7	0
B73-167	4.4	61	24	40	2.7	4
C89-173	4.4	66	26	40	1.9	3
C84-116	4.3	46	13	29	0.8	0
Fiesta	3.3	57	23	41	0.8	2
C67-7	1.7	67	33	50	1.9	1
A8-2	1.6	23	0	0	---	---
Thompson Seedless	1.4	56	12	22	10.6	1

Table 8. Number of aborted grape seeds cultured, and embryos and plants developed from seedless x seedless crosses for the grape breeding program.

Year	No. ovules cultured	Embryos developed		No. ovules with multiple embryos	Plants ² set in field		No. plants lost during acclimation
		No.	%		No.	%	
1983	4,075	647	21	---	50	8	---
1984	8,859	3203	36	---	725	22	168
1985	16,942	3781	22	596	1602	42	142
1986	7,484	1124	15	160	341	30	69
1987	10,670	2737	25	388	276	10	---

²The percentage planted in the field is less than the actual number of plants developed due to plant loss during acclimation in the greenhouse.

³Number of multiples not determined.

Fig. 4. Percentage seedlings in each seed size class from seedless x seedless cross P79-101 x C32-68. Based on average seed trace weight for 1986 and 1987. Genotypes are generally classified as seedless if trace fresh weight is <25 mg.

same genotypes for several years. Using ovule culture, many progeny have been developed from seedless x seedless grape crosses (Table 8). The first family that fruited has shown 82% seedlessness (Fig. 4) instead of the 15% average (Loomis and Weinberger, 1979) found in seeded x seedless progeny (Fig. 4). Genotypes with aborted seeds that are so small they are barely visible with the naked eye (<1 mm long) were found in 23% of the seedlings. One such seedling had larger berries than either parent, which is very encouraging in the development of naturally large fruit in seedless grapes.

Embryo culture can be a useful tool in breeding fruit crops and we have explored only a few of these. In addition to being able to culture embryos before abortion, it would be extremely valuable to be able to perform in vitro pollination and fertilization. Crossing barriers could be eliminated and the potential to screen male and female gametes before hybridization might be possible.

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ESSENTIAL REFERENCES

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