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. Han-ning in 1904 with mature embryos of two crucifers (Narayanaswami and Norstog, 1964). Then, in 1929, Laibach (Mahesh-vari, 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 (Narayanswami and Norstog, 1964) successfully cultured and grew small (0.5- 0.75 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 ovule embryo cul-ture that it is possible to grow a four-celled proembryo of Papaver somniferum L. to matur-ity. The successful growth of ovule-cul-tured 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 matures. The smaller the embryo, the more complex the medium required. Raghavan (1976a) states in his review of embryo culture that there are heterotropic and autotropic stages in embryo development. The embryo in the heterotropic stage of development is smaller than in the autotropic stage and usually requires the presence of growth reg-ulators to allow for proper development. The autotropic 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 ame-nable 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 Pru-nus (Tukey, 1934) and Vitis (Ramming and Emershad, 1984) cultivars, thus allowing their use as females. Many seedless grape culti-vars are stenospermic (Stout, 1936), i.e., they are characterized by cessation of embryo de-velopment after fertilization and failure of seed development. Embryos have been res-cued and grown into plants from stenosper-mocarpic 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 diffi-cult to produce because of many factors that act as barriers. Postzygotic barriers such as endosperm abortion are a common occur-

Results achieved

Peach embryo culture. After Tukey success-fully cultured cherry embryos (1933b),
others applied it in their breeding programs. Blake (1939) used the procedure and Hough (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 = 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 ovaries 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.

<table>
<thead>
<tr>
<th>Parent No.</th>
<th>Year cultured</th>
<th>Parent (1975)</th>
<th>Earliest-ripening selected seedling (May 1979)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV9-164</td>
<td>1975</td>
<td>6 June</td>
<td>10</td>
</tr>
<tr>
<td>Springgold</td>
<td>1976</td>
<td>21 May</td>
<td>10</td>
</tr>
<tr>
<td>Springcist</td>
<td>1976</td>
<td>29 May</td>
<td>10</td>
</tr>
<tr>
<td>PV9-164</td>
<td>1976</td>
<td>4 June</td>
<td>8</td>
</tr>
<tr>
<td>Armking</td>
<td>1976</td>
<td>7 June</td>
<td>14</td>
</tr>
<tr>
<td>Arm Queen</td>
<td>1976</td>
<td>10 June</td>
<td>21</td>
</tr>
</tbody>
</table>

*All open pollinated.

Table 2. Number of ovaries 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.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. Ovules</th>
<th>No. Embryos</th>
<th>To greenhouse*</th>
<th>To field</th>
<th>Contaminated</th>
<th>Small</th>
<th>Selected</th>
<th>Propagated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1975</td>
<td>---</td>
<td>1075</td>
<td>5</td>
<td>3</td>
<td>42</td>
<td>55</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>1976</td>
<td>---</td>
<td>2271</td>
<td>58</td>
<td>45</td>
<td>1</td>
<td>29</td>
<td>102</td>
<td>24</td>
</tr>
<tr>
<td>1977</td>
<td>---</td>
<td>2266</td>
<td>56</td>
<td>40</td>
<td>4</td>
<td>44</td>
<td>47</td>
<td>14</td>
</tr>
<tr>
<td>1978</td>
<td>---</td>
<td>4214</td>
<td>79</td>
<td>72</td>
<td>12</td>
<td>8</td>
<td>53</td>
<td>24</td>
</tr>
<tr>
<td>1979</td>
<td>---</td>
<td>6277</td>
<td>67</td>
<td>54</td>
<td>12</td>
<td>14</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>1980†</td>
<td>---</td>
<td>8179</td>
<td>54</td>
<td>52</td>
<td>20</td>
<td>32</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>1981†</td>
<td>1419</td>
<td>6058</td>
<td>58</td>
<td>20</td>
<td>1</td>
<td>4</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>1982*</td>
<td>2140</td>
<td>5420</td>
<td>52</td>
<td>10</td>
<td>20</td>
<td>78</td>
<td>71</td>
<td>11</td>
</tr>
<tr>
<td>1983†</td>
<td>5188</td>
<td>1261</td>
<td>41 (34, 70)</td>
<td>26</td>
<td>11</td>
<td>35</td>
<td>41</td>
<td>12</td>
</tr>
<tr>
<td>1994</td>
<td>1572</td>
<td>2012</td>
<td>39 (20, 64)</td>
<td>26</td>
<td>13</td>
<td>24</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>1985</td>
<td>1243</td>
<td>798</td>
<td>53 (37, 86)</td>
<td>50</td>
<td>4</td>
<td>45</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>1986</td>
<td>955</td>
<td>566</td>
<td>41 (30, 58)</td>
<td>36</td>
<td>8</td>
<td>41</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>1987</td>
<td>908</td>
<td>1384</td>
<td>66 (31, 89)</td>
<td>58</td>
<td>5</td>
<td>13</td>
<td>6</td>
<td>13</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Ovary culture</th>
<th>Embryo sub-cultured from ovule culture</th>
<th>Embryo culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>No.</td>
<td>No. viable embryos</td>
</tr>
<tr>
<td>1983</td>
<td>5080</td>
<td>70</td>
</tr>
<tr>
<td>1984</td>
<td>3072</td>
<td>50</td>
</tr>
<tr>
<td>1985</td>
<td>1543</td>
<td>67</td>
</tr>
<tr>
<td>1986</td>
<td>935</td>
<td>76</td>
</tr>
<tr>
<td>1987</td>
<td>908</td>
<td>72</td>
</tr>
</tbody>
</table>

*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 fe-
nales required improvements in media and
procedures. The cotton ovule culture me-
dium 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 cul-
ture by the integuments, allowing the em-
byos to increase in fresh and dry weight
within the integuments. At this time, the em-
byo is using the nucellus and endosperm as
well as the nutrient medium for growth. Em-
bryos 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,
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 ben-
eficial 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 dur-
ing 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 meth-
ods. Two cultivars—Goldcrest peach and
Mayfire nectarine—have resulted from em-
byo culture and are among the earliest of
the commercially grown cultivars in Cali-
ifornia (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 stratifi-
cation, and no viable plants were produced.
Hormones during the initial ovule culture pe-
riod were necessary as for tomato, and levels
used were those described by Neal and To-
poleski (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 ap-
plied to grapes. We used modified White’s
macroelements medium with Norstog’s mi-
croelements and vitamins plus glycine and
casein hydrolysate (Emershad and Ram-
mimg, 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 in-
dividually in 20 × 150-mm test tubes, strat-

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...ized for 3 months at 0.5° C, 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 × 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 subcultured, 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 genotype-dependent, based on data from culturing the...
same genotypes for several years. Using ovule culture, many progeny have been developed from seedless × 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 × 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.

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

<table>
<thead>
<tr>
<th>Year</th>
<th>No. ovules cultured</th>
<th>Embryos developed</th>
<th>No. ovules with multiple embryos</th>
<th>Plants set in field</th>
<th>No. plants lost during acclimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>4,075</td>
<td>647</td>
<td>21</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>1984</td>
<td>8,859</td>
<td>3203</td>
<td>36</td>
<td>725</td>
<td>22</td>
</tr>
<tr>
<td>1985</td>
<td>16,942</td>
<td>3781</td>
<td>22</td>
<td>1602</td>
<td>42</td>
</tr>
<tr>
<td>1986</td>
<td>7,484</td>
<td>1124</td>
<td>15</td>
<td>341</td>
<td>30</td>
</tr>
<tr>
<td>1987</td>
<td>10,070</td>
<td>2737</td>
<td>25</td>
<td>388</td>
<td>69</td>
</tr>
</tbody>
</table>

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

*Number of multiples not determined.*

### Literature Cited


Emershard, R.L. and D.W. Ramming. 1982. In-


