Growth of Immature Peach Embryos in Response to Media, Ovule Support Method, and Ovule Perforation

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Abstract. The influence of medium formulation, methods of ovule support, and ovule perforation on in vitro growth of immature peach [*Prunus persica* (L.) Batsch] embryos (4.0 to 8.7 mm long) was investigated. Maximum embryo dry weights were attained with vermiculite support, ovule perforation, and 10% sucrose in the medium. At high sucrose levels, all three tested media (Monnier, modified Murashige and Skoog, or modified Stewart and Hsu medium) performed similarly. Adding IAA and kinetin to culture media did not enhance embryo growth. Using the vermiculite support system, small embryos of early maturing peach cultivars, obtained at fruit maturity, were cultured successfully and used as maternal parents in breeding programs. Chemical name used: indole acetic acid (IAA).

Seeds from early maturing peach cultivars that have fruit development periods (FDP) of <75 days fail to germinate due to small embryo size (Lesley and Bonner, 1952). A peach embryo's ability to germinate is correlated with its size (Kester and Hesse, 1955). Although embryo length and fresh weight are frequently used as size measurements (Kester and Hesse, 1955; Ramming, 1990), embryo dry weight is a more reliable indicator of maturity and viability. Ramming (1990) reported that the minimum size for successful embryo culture using Smith, Bailey, and Hough (1969) medium with 3% sucrose was 10-mm-long embryo and 60% ovule fill (embryo length ÷ ovule length × 100). This finding has been confirmed in our laboratory with 20 peach genotypes (unpublished data). The equivalent embryo (ovule) dry weight and percent dry weight (embryo dry weight ÷ embryo fresh weight × 100) were 20 mg (38 mg) and 9%

(10%), respectively. Embryos larger than these germinated more reliably (Kester and Hesse, 1955; Ramming, 1990). Germination failure precludes using early maturing peach genotypes as female parents in breeding programs. Although in ovule embryo culture has been used to promote immature (<5 mm) peach embryo growth (Ramming, 1990), success has been low (32%), and improved techniques and culture media are needed. Possible alterations to enhance embryo in vitro development include adding hormones, changing the basic nutrient mixture, altering the ovule support method, and perforating the ovule.

MS (Murashige and Skoog, 1962), SH (Stewart and Hsu, 1977), and Monnier (1976) media have been used for in ovule culture of immature embryos (Monnier, 1976; Ramming, 1985; Stewart and Hsu, 1977). Ramming (1985) reported that SH was better than MS for small (<10 mm) peach embryo culture. In addition, Pinto et al. (1993) reported that SH and Monnier were useful for enhancing the small peach embryo growth, but the media were not compared directly to each other. Successful in ovule culture of small peach embryos has been reported at medium (6%) to high (10% to 12%) sucrose concentrations (Pinto et al., 1993; Ramming, 1985), but little is known about the interaction between the media and sucrose level. Although the exogenous growth regulators kinetin and IAA have promoted growth of some immature embryos (Raghavan, 1966; Rangan, 1984), little work has been done with peach in this regard (Ramming, 1990). Pinto et al. (1993) found that using exogenous growth regulators was not effective for peach embryo culture.

The in vitro method of ovule support affects peach embryo growth (Fernandez et al., 1991; Ramming, 1985; Stewart and Hsu, 1977). Peach embryos grow better on filter paper

support (FPS) than when immersed in liquid medium (Ramming, 1985); however, paper supports preparations are time consuming. Recently, a vermiculite system has been described as useful (Pinto et al., 1993), but its effectiveness in encouraging embryo growth was not compared to the FPS system.

Ovule culture techniques and manipulations promote embryo development in several crops including *Vitis*, *Cuphea*, and *Hordeum* (Fernandez et al., 1991; Mathias et al., 1990; Ziebur and Brink, 1951). Cutting open the ovule can enhance embryo development (Fernandez et al., 1991; Mathias et al., 1990), presumably due to improved nutrient exchange between the medium and the embryo. Ovule manipulations, such as perforation, increase water and nutrient permeability, thus promoting embryo growth (Pinto et al., 1993). Our objectives were to evaluate the effects of medium formulations, ovule support methods, and ovule manipulations on the growth of small (<10 mm long), immature peach embryos.

Materials and Methods

General procedures. The parental selections studied were 'Goldprince', P51-2, and B611505. The first two selections were chosen because they were early ripening peach plants. B611505 was used because many fruit with small embryos (4 to 8.7 mm long) were available.

All media were prepared with double distilled water, and the pH levels were adjusted to 5.7 ± 0.02 . The original MS medium was modified by adding ($\text{mg}\cdot\text{liter}^{-1}$) 16.2 $\text{MnSO}_4\cdot\text{H}_2\text{O}$ and 0.5 thiamine HCl and eliminating inositol. The SH medium was modified by replacing the NH_4NO_3 with NH_4Cl at 535 $\text{mg}\cdot\text{liter}^{-1}$ and increasing KNO_3 by 10-fold (5055 $\text{mg}\cdot\text{liter}^{-1}$). Monnier medium was prepared without modification (Monnier, 1976). Sucrose was added at 60 or 100 $\text{g}\cdot\text{liter}^{-1}$ for the first experiment and at 120 $\text{g}\cdot\text{liter}^{-1}$ for the others. Dram vials (9.4×2.2 cm; 37 ml) were used as culture vessels in all experiments. Embryos for all three experiments were cultured in darkness at $25 \pm 2^\circ\text{C}$ for 40 days. All fruit were surface-sterilized in 70% ethanol for 2 min and then in 20% (v/v) commercial bleach (1.05% sodium hypochlorite) for 5 min. The fruit were cracked and the ovules excised aseptically.

Support, media, and sucrose effects. Twelve treatments were used in a factorial design: three ovule support systems [FPS, gel-liquid support (GLS), and vermiculite support (VS)], two media (MS and SH), and two sucrose concentrations (6% and 10%). The ovules containing small embryos (8.7 ± 1.7 mm) were excised from B611505 fruit 70 days after full bloom (DAFB). Fruit were from the U.S. Dept. of Agriculture, Agricultural Research Service, Appalachian Fruit Research Station, Kearneysville, W.Va.

The FPS were prepared using Whatman filter paper no. 2 (5.5 cm) and 15 ml of medium. The GLS were prepared by combining 10 g Gelrite (Scott, Carson, Calif.), 1 g MgCl_2 ,

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and 1 liter double-distilled water; dispensing 15 ml/vial; autoclaving 15 min at 121°C; and cooling until the media solidified. Then, 0.9 ml sterilized liquid medium was poured on top of the solidified gel. For the VS treatment, 2.2 g (14 ml) of fine grade vermiculite and 9 ml of medium were placed in each vial before autoclaving. The excised ovules were laid (one per vial) on top of the FPS, placed in the thin layer of liquid medium on the GLS, or inserted vertically with the micropylar end down two-thirds deep into the wet VS. The lengths and fresh and dry weights of ovules and embryos were measured on a random 20-fruit sample on the day the experiment began (initial) and again for eight to 10 ovules from each treatment after 40 days in culture.

Monnier vs. SH media. The second experiment compared the embryo growth of perforated and intact P51-2 peach (embryo size 4 ± 1 mm at 65 DAFB) ovules cultured on Monnier or SH medium at three growth regulator levels (IAA at 0, 0.25, or 0.5 mg·liter⁻¹; medium that received IAA also received kinetin at 0.01 mg·liter⁻¹). In the third experiment, the growth of 'Goldprince' embryos (4 ± 0.8 mm at 55 DAFB) cultured on Monnier or SH medium was compared.

For the second and third experiments, the embryos were cultured in ovule on VS (2.2 g fine vermiculite plus 9 ml medium) using 12% sucrose in the medium. Before culturing, the prepared vials were autoclaved at 121°C for 20 min. For the ovule perforation (OP) treatment, excised ovules were perforated 12 times with a tearing needle around the micropylar end without damaging the embryos inside. The lengths of 10 embryos were measured at the beginning of the experiment, and the length of seven to 10 embryos were measured after 40 days in culture.

Statistical analysis. The SAS general linear model procedure was used to analyze the data as factorial for the first and second experiments; the third experiment was analyzed as a completely randomized design. The means were separated by Duncan's multiple range test (SAS Institute, 1985).

Results and Discussion

Support, media, and sucrose effects. The embryo and ovule increases in percent dry weight were influenced more by support systems than medium or sucrose concentration. Ovule length was not affected by the treatments (data not shown). The dry weight (percent) of the GLS embryos was significantly lower (3.3% to 6.2%) than that of embryos grown on either the VS (10.8% to 32.7%) or FPS (9.1% to 22.8%) systems (Tables 1 and 2). This result indicates that the increases in GLS embryo fresh weight were due to higher water content. The embryos from the GLS treatment were flaccid and appeared water-soaked, but those grown on the VS system were firm and white. According to Barrett-Lennard and Dracup (1988), an increase in air porosity in the culture medium prepared by breaking gelled medium into clumps promotes shoot growth of *Trifolium subterraneum* in vitro. Hence, the greater effectiveness of the VS system compared to the GLS system may have been due to higher gas diffusion from the support medium to the embryo. The GLS system had 0.9 ml compared to 9 ml medium in the VS system, which also may have influenced effectiveness.

The increase in dry and fresh weight of ovules and embryos on FPS was intermediate compared to those on the GLS and VS systems. Response to FPS was better than to GLS, confirming Ramming's (1985) findings that FPS is better than immersing embryos in liquid medium. The FPS system, however, was difficult to set up and generally inferior to the VS system in promoting embryo growth (dry weight, fresh weight, and length). In addition, the VS system that oriented the ovules vertically promoted uniform cotyledon growth; this growth contrasts the asymmetrical cotyledon growth from embryos cultured with the FPS and GLS systems, which oriented ovules horizontally.

Medium type and sucrose concentrations affected percent dry weight of embryos and ovules (Tables 1 and 2). Embryos cultured on

VS with SH at 6% or 10% sucrose or on MS plus 10% sucrose increased nearly two to three times more in embryo dry weight and percent embryo dry weight than those embryos cultured on GLS or FPS (Table 1). Although the dry weights of embryos on VS with either MS plus 10% sucrose or SH plus 6% or 10% sucrose were similar, those on SH plus 10% sucrose had the highest percent dry weights. The SH plus 6% or 10% sucrose medium with VS led to an average of 1.5-times higher percentage of embryo dry weight compared to the same media with FPS. An experiment comparing the effects of 10% and 12% sucrose on embryo and ovule growth (dry weight, fresh weight, and length) revealed no difference between the two (data not shown).

Ovule manipulations. P51-2 embryos were longer with OP than when left intact, regardless of medium and hormone concentrations used (Table 3). This result confirms previous work on smaller embryos (Pinto et al., 1993). Carbohydrates and other organic compounds enter the nucellus through diffusion (Murray, 1988; Yamaki and Ryugo, 1986). Working with small *Cuphea* embryos, Mathias et al. (1990) suggested that the seedcoat might limit this diffusion. The OP technique may have enhanced embryo growth in length by improving nutrient diffusion from the culture medium to the developing embryo or by direct tissue wounding. Embryo dry weights were not measured in this experiment because the embryos were cultured further in another experiment.

Media and hormone effects. Medium formulation had little effect on embryo growth with the selections that we tested. In the second experiment, Monnier and modified SH media equally promoted the growth in length of perforated 'Goldprince' and P51-2 embryos (Table 3). Exogenous hormones did not stimulate length growth of P51-2 embryos (4 mm long at 65 DAFB), confirming Pinto et al.'s (1993) results. Perhaps, this response was because embryos were hormone autotrophic or the hormone levels used were inappropriate.

Table 1. Ovule and embryo growth as affected by three support systems, two sucrose levels, and two media (Murashige and Skoog salts = MS or Stewart and Hsu salts = SH) compared with initial noncultured controls. Cultures were maintained in darkness for 40 days.

Medium	Sucrose concn (%)	Ovule support system ^a	Embryo				Ovule		
			Length (mm)	Fresh wt (mg)	Dry wt (mg)	% Dry wt ^b	Fresh wt (mg)	Dry wt (mg)	% Dry wt
Initial ^c			8.7 d ^w	96 f	8.0 d	8.4 ef	614 bc	60.0 ef	9.8 ef
MS	6	FPS	11.2 bc	174 d-f	16.0 cd	9.1 ef	528 b-e	64.4 bc	11.7 de
	6	GLS	12.6 ab	296 a-d	11.9 d	3.7 g	702 ab	35.8 ed	5.4 gh
	6	VS	11.8 b	235 b-e	25.2 b-d	10.8 e	433 c-f	60.3 bc	14.0 d
	10	FPS	7.6 d	69 f	11.6 d	15.9 cd	344 ef	63.1 bc	18.7 c
	10	GLS	12.0 b	250 b-e	11.0 d	4.2 g	708 ab	39.0 de	6.0 gh
	10	VS	12.7 ab	355 ab	63.3 a	19.0 c	573 b-d	107.0 a	19.9 c
SH	6	FPS	10.9 bc	239 b-e	34.8 b	14.8 d	414 d-f	69.6 b	17.0 c
	6	GLS	12.3 ab	304 a-c	10.8 d	3.3 g	805 a	35.3 e	4.4 h
	6	VS	11.1 bc	293 a-d	57.4 a	19.3 c	597 b-d	118.4 a	19.8 c
	10	FPS	9.5 cd	135 ef	32.2 bc	22.8 b	309 f	73.2 b	23.5 b
	10	GLS	14.3 a	402 a	23.0 b-d	6.2 fg	681 ab	50.0 b-d	7.5 fg
	10	VS	11.2 bc	186 c-f	59.8 a	32.7 a	354 ef	100.6 a	30.5 a

^aFPS = filter paper support, GLS = gelrite support, VS = vermiculite support.

^b% Dry wt = dry weight as a percentage of fresh weight.

^cEmbryos measured the day the experiment started.

^wMeans separations within columns by Duncan's multiple range test at $P \leq 0.05$ ($n = 8$ to 10).

Table 2. Analysis of variance for growth of ovules and embryos on three support systems and two media compared to initial, noncultured controls.

Source of variation	Embryo			Ovule	
	Length	Fresh wt	Dry wt	Fresh wt	Dry wt
Ovule support (O)	**	**	**	**	**
Media (M)	NS	NS	**	NS	**
Sucrose level (S)	NS	NS	**	*	**
O × M	NS	*	**	NS	**
O × S	**	NS	**	NS	**
M × S	NS	NS	*	*	*
O × M × S	NS	**	NS	*	NS

NS, *, ** Nonsignificant or significant at $P \leq 0.05$ or 0.01 , respectively.

Table 3. Final length of small peach embryos cultured in ovule as affected by media, IAA, and ovule perforation on vermiculite support.

Parental selection ^y	Ovule perforation	Embryo length (mm)					
		Medium					
		Monnier			SH ^z		
		IAA (mg·liter ⁻¹)			IAA (mg·liter ⁻¹)		
		0	0.25	0.50	0	0.25	0.50
Goldprince ^x	+	11.1 a ^w	---	---	11.0 a	---	---
P51-2 ^v	+	11.4 ab	9.8 b	13.1 a	10.4 b	10.2 b	9.8 b
	-	6.0 c	5.3 c	5.1 c	6.1 c	5.7 c	5.6 c

^zSH = modified Stewart and Hsu medium, all three treatments contained 0.01 mg kinetin/liter and 12% sucrose.

^y'Goldprince' (4 ± 0.8 mm) and P51-2 (4 ± 1.0 mm) embryos were 55 and 65 days old, respectively, when cultures were initiated.

^xAnalysis of variance for 'Goldprince' embryo length showed medium effects were nonsignificant.

^wWithin each genotype, means separation by Duncan's multiple range test at $P \leq 0.05$.

^vAnalysis of variance showed P51-2 embryo length growth to be nonsignificant, ovule perforation treatment significant at $P \leq 0.01$, and medium-hormone treatment and interactions nonsignificant.

The VS method improved small embryo (4 to 8.7 mm) growth relative to FPS or GLS, while reducing the labor needed for embryo rescue. In general, the ovule support system and ovule manipulation techniques were more effective than media formulations for stimulating in vitro growth of immature embryos of early ripening peach cultivars. The optimum system for 'Goldprince', P51-2, and B611505 was VS support plus OP with 10% sucrose and either SH, Monnier, or MS medium. Adding IAA and kinetin to the culture media did not stimulate embryo growth.

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