In Vitro Factors During Ovule Culture Affect Development and Conversion of Immature Peach and Nectarine Embryos

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Additional index words. embryo rescue, *Prunus*, breeding, stone fruit

Abstract. Various in vitro conditions for culture of ovules prior to extraction and culture of immature embryos of peach [*Prunus persica* (L.) Batsch] and nectarine [*Prunus persica* (L.) Batsch var. *nucipersica* Schneid.] were investigated. Culture vessels consisting of test tubes, petri dishes, and polycarbonate jars were tested along with various types of support and nutrient media. Agar support was superior to liquid media with filter paper supports. Agar produced the largest embryos with 90% to 93% being converted into plants compared to liquid with only 1% to 12% embryo conversion. The best ovule orientation and support was with the micropyle down and pushed halfway into an agar-gelled medium. In experiments two and three, test tubes with vertical ovule orientation (micropyle end of ovule pushed into agar) produced larger embryos, the largest plants and the greatest percentage of embryos that converted into plants (60% and 91%). Petri dish treatments were less successful in embryo conversion than test tubes and polycarbonate jars. The addition of activated charcoal (AC) to an agar-gelled medium produced significantly larger embryos with a similar conversion rate. The addition of an agar-gelled medium to culture vessels reduces preparation time compared to filter paper supports, and placing each ovule within a test tube eliminates cross contamination, making immature embryo culture more successful.

Seeds of peach and nectarine fruit that ripen in <90 d are considered immature. During this period of rapid fruit enlargement and ripening, the embryos within these seeds have not had sufficient time to attain full size and mature physiologically. Embryo abortion usually occurs during the period of fruit softening. These seeds when planted in soil will not germinate; consequently early-ripening genotypes are limited for use as male parents in breeding programs. Therefore, when developing early-ripening progenies with conventional breeding methods, breeders have used only mid- to late-season cultivars that develop mature seeds as female parents. When early-ripening genotypes are hybridized with mid- to late-season genotypes, only a small percentage of the hybrid population develops as early-ripening progeny, making the development of new cultivars slow and inefficient. Breeding efficiency can be increased when embryo culture is utilized, whereby both parents are early-ripening, resulting in greater percentages of hybrids that are as early or earlier than their parents.

Blake (1939) was the first peach breeder to utilize the embryo rescue technique developed by Tukey (1934). Ramming (1990), refining the embryo culture techniques developed by Smith et al. (1969), reported the advancement of progeny ripe dates by 10 to 38 d earlier than their parents. Plant development from embryos >10 mm ranged from 57% to 93%. However,

only 6% to 50% of embryos <10 mm developed into plants on a modified Murashige and Skoog (1962) medium. Subsequently 98% and 100% germination with 100% survival was obtained from peach and nectarine embryos 5 to 6 mm in length respectively, when cultured on Woody Plant medium (Emershad and Ramming, 1994).

Currently the earliest ripening peach and nectarine germplasm has embryo lengths measuring <1 mm when the fruit are ripe. Embryo rescue techniques have been inadequate for development and germination of these embryos. Ramming (1985) utilized the cotton ovule culture technique reported by Stewart and Hsu (1977, 1978) to obtain plants from 1-mm embryos. This procedure utilizes a liquid medium with a filter paper support. Combining the above procedure with embryo culture allowed up to 35% conversion of embryos into plants (Ramming, 1990). In our efforts to improve ovule culture for the recovery of plants from immature embryos, we present results in this report about various media, type of ovule support, orientation, and culture vessel type.

Materials & Methods

Expt. 1. Genotype and media effects on embryo development in cultured ovules and subsequent germination of cultured embryos. Fruit was harvested from nine peach and nectarine families from 55 to 70 d after bloom (DAB) (Table 1). After the stems had been removed, fruit was surface sterilized by agitating in 70%

ethanol for 1 min, washing in 0.525% sodium hypochlorite containing 0.01% Tween 20 for 10 min and rinsing with sterile distilled water. The fruit were cut open in a laminar flow hood with double bladed clippers dipped in 70% ethanol. Ovules were extracted, using aseptic techniques, and ten were placed within each cylindrical polycarbonate jar (95 × 65 mm) containing 30 mL of liquid medium with a 7-cm diameter #541 Whatman filter paper support (FPS), (Stewart and Hsu, 1978) or 30 mL of agar-gelled medium. Each jar was sealed with a screw top lid. The headspace volume, with the addition of the liquid or agar medium in the polycarbonate jar container, was 28.2 cm³/ovule. The media treatments were as follows: 1) Stewart and Hsu medium (SH) (Stewart and Hsu, 1978), 6% sucrose, FPS; 2) SH, 6% sucrose, 0.3% activated charcoal (AC), FPS; 3) SH, 6% sucrose, 0.65% agar; 4) SH, 6% sucrose, 0.3% AC, 0.65% agar; 5) SH, 10% sucrose, FPS; and 6) Quoirin and Lepoivre (1977) medium (QL), 6% sucrose, FPS. The pH was adjusted to 5.7 with 1 N HCl or 1 N NaOH. The funicular side of each ovule was pushed into the agar-gelled medium. One jar containing 10 ovules was cultured for each family and treatment, for a total of 60 ovules per family or 90 ovules per treatment. In addition, 10 embryos from each family were removed from ovules at the initiation of the experiment and their lengths recorded to determine an average initial embryo length for each family.

Embryos were removed from the ovules after incubation in the dark at 27 °C for 24 to 33 d and their lengths and weights recorded. Embryos were then transferred to test tubes (20 × 150 mm), containing 10 mL of Woody Plant medium (WP) (Lloyd and McCown, 1981) gelled with 0.65% agar plus 3% sucrose. Test tube closures were Permeable Membrane (PM) caps (Biomedical Polymers, Leominster, Mass.). Ovules cultured on agar-gelled media required soaking in sterile distilled water overnight to soften the testa and facilitate embryo removal. All embryos were pushed, radicle end down, halfway into the medium. Stratification for 60 d at 1.5 °C began immediately after transfer. After stratification, embryos were placed in a plant growth chamber at 20 °C under cool-white fluorescent lights with a 12-h photoperiod. Light intensity was $\approx 36.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After 1 week, embryos were transferred to a growth room at 25 °C under cool-white fluorescent lights with a 12-h photoperiod. Light energy was $\approx 58.4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Germination was recorded 3 weeks later. Fresh weights for each germinated seedling and its associated pair of cotyledons were recorded separately (Table 2).

An analysis of variance (ANOVA) was performed on data using Systat MGLH/GLM program (Wilkinson, 1989). Where significance of independent variables and their interactions occurred, a two-way ANOVA was performed along with a Tukey's honestly significantly different (HSD) multiple comparison test on pairs of means, developing a matrix of pairwise comparison probabilities.

Expt. 2. Effect of culture vessel and ovule

orientation on embryo development in cultured ovules and subsequent germination of cultured embryos. Open pollinated fruit from two nectarine families (K626-90 and P30-129) were harvested 67 DAB. Embryos were extracted from a representative sample and their embryo lengths recorded (Table 1). The remaining fruit were surface sterilized and cracked open as in Expt. 1. Ovules were aseptically removed and placed either into petri dishes (25 × 100 mm), each containing 30 mL of SH medium with 6% sucrose, 0.65% agar, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) or test tubes (20 × 150 mm) each containing 3 mL of the same medium. The headspace volumes in the petri dishes and test tubes were 15.7 cm³/ovule and 42.4 cm³/ovule, respectively. The medium volume placed within a test tube was equivalent to the average amount of medium available for each ovule in a petri dish (total medium volume/number of ovules). The pH was adjusted to 6.0 with 1 N HCl or NaOH. Ten ovules were placed within each petri dish and replicated four times for each treatment. The test tube treatment was replicated 40 times. Ovules were placed in four different orientations: 1) petri dish with ovule placed funiculus side into agar/horizontal orientation (PDFD/H); 2) petri dish with ovule placed funiculus side up/horizontal orientation (PDFU/H) (rotated 180° from treatment 1); 3) petri dish with ovule placed micropylar end into agar/vertical orientation (PDMD/V); 4) Test tube with ovule placed micropylar end into agar/vertical orientation (TTMD/V). Ovules in all the above treatments were pushed into agar half their length or width (except for treatment PDMD/V, which was one-fourth its length). Ovules in the above treatments were placed into a dark incubator at 27 °C for 30 d. Embryos were aseptically removed from cultured ovules after 30 d of incubation and their lengths and weights recorded. Embryos were transferred to test tubes containing 10 mL WP medium, 3% sucrose, 0.65% agar, 5 mM MES with a pH of 6.0. These test tubes were placed for 45 d into a dark cooler at 1.5 °C. The embryos were allowed to germinate, after chilling, inside a growth chamber at 20 °C under cool-white fluorescent lights (light intensity ≈36.5 μmol·m⁻²·s⁻¹), with a 12-h photoperiod. Germination was rated 2 weeks later. Embryos were considered to have germinated when the radicle was equal to or greater than half the length of its cotyledons. After 5 weeks, plants were taken out of test tubes, cotyledons removed and plant weights recorded. Plants were then transplanted to soil and survival was recorded.

Expt. 3. Effect of culture vessel and ovule orientation on embryo development in cultured ovules and subsequent germination of cultured embryos. Open pollinated fruit from two nectarine families (P29-80 and P59-29) were harvested 56 and 63 DAB, respectively. Embryos were extracted from a representative sample and their embryo lengths recorded. The remaining fruit were surface sterilized and ovules aseptically removed as previously described. Ovules were placed either into petri dishes (25 × 100 mm), cylindrical polycarbonate jars (65 × 95 mm) each containing 30 mL

of SH medium, 6% sucrose, 5 mM MES and 0.65% agar or test tubes (20 × 150 mm) each containing 3 mL of the same medium. Ten ovules were placed within each petri dish or polycarbonate jar and replicated four times for each treatment. The test tube treatment was replicated 40 times. Ovules were placed in four treatments: 1) test tube cultured with micropylar end pushed into agar/vertical orientation; (TTMD/V); 2) petri dish with funiculus side pushed into agar/horizontal orientation (PDFD/H); 3) polycarbonate jar with funiculus side pushed into agar/horizontal orientation (PJFD/H); 4) polycarbonate jar with micropylar end pushed into agar/vertical orientation (PJMD/V). Ovules, in all the above treatments, were pushed into the agar half their length or width. The above treatments were placed into a dark incubator at 27 °C for 30 d. Embryos, after 30 d of incubation, were aseptically removed from ovules and their weights recorded (Table 4). Embryos were then transferred to test tubes containing 10 mL WP medium, 3% sucrose, 0.65% agar, and 5 mM MES. These test tubes were placed for 60 d into a dark cooler at 1.5 °C. After 60 d, embryos were placed inside a growth chamber at 20 °C, under cool-white fluorescent lights (light intensity ≈36.5 μmol·m⁻²·s⁻¹) with a 12-h photoperiod. Germination was rated 18 d later. After an additional week, plants were taken out of test tubes, cotyledons removed and plant weights recorded (Table 4).

In Expts. 2 and 3, ANOVA was performed on data using the general linear modeling program in Super ANOVA (Abacus Concepts, Berkeley, Calif.). Where significance of independent variables occurred, a two-way ANOVA was performed along with a Tukey's HSD multiple comparison test on pairs of means, developing a matrix of pairwise comparison probabilities.

Results

Expt. 1. Genotype and media effects on embryo development in cultured ovules and subsequent germination of cultured embryos. The embryos enlarged during ovule culture for all nine families. There was significant difference among families for embryo enlargement during ovule culture. Families 8 and 9 had significantly greater embryo lengths than any other family with a 48% and 56% increase over initial embryo length, respectively. Families 1, 2, and 7 had the smallest embryo length after ≈1 month of ovule culture and were not significantly different from each other, but had increased over initial embryo length by ≈5 mm. Families with smaller initial embryo lengths (0.5 to 1.5 mm initial size range) had a greater percent increase in length than those within the 3 to 6 mm initial size range (Tables 1 and 2). The correlation of initial embryo length with embryo length and embryo FW after the ovule culture period was $r = 0.90$ (Sig. Probability = 0.0008) and 0.75 (Sig. Probability = 0.0197) respectively, supporting the above observation. Families 5, 6, and 8 had the greatest embryo weight after ≈30 d of ovule culture (Table 2). The germination rate was comparable (30%

to 55%) for all families even though their initial embryo lengths ranged from 0.6 to 6.2 mm. The correlation of initial embryo length with the percentage of germination and the percentage of plants per embryos was $r = -0.63$ (Sig. Probability = 0.0689) and -0.31 (Sig. Probability = 0.4155), respectively, corresponding to the above observations. Family 4 had the smallest initial embryo length but the highest germination rate (Tables 1 and 2). The size of plants large enough to be transplanted to soil was also similar across the different families. However, family 7 was the poorest for all measurements made except for average cotyledon weight.

There were no significant interactions between families and treatments for embryo length, weight, and cotyledon weight. The length and weight of the extracted embryos from all the tested families were significantly larger when ovules were cultured on agar treatments (SH6SA and SH6SACA). Treatments SH6SA and SH6SACA were significantly different from each other with AC (treatment SH6SACA) having greater embryo length and weight. Embryos from ovules incubated on liquid media treatments (SH6SFPS, SH6SACFPS, SH10SFPS and QL6SFPS) did not enlarge sufficiently for many to be transplanted to soil. However, both agar treatments (SH6SA and SH6SACA) induced embryo development into plants large enough such that >90% could be transplanted to soil.

Expt. 2. Effect of culture vessel and ovule orientation on embryo development in cultured ovules and subsequent germination of cultured embryos. The embryos of the family P30-129 grew larger (length and weight) and produced more plants, even though the initial embryo length was smaller than that of K626-90 (Tables 1 and 3). Once transplanted to soil, the percentage of plant survival was similar for both families. Embryos from 30-d-old cultured ovules (6.3 to 8.9 mm length, 109 to 183 mg weight) were less than half the size of embryos from tree grown ovules the same age (12.4 to 14.9 mm length, 316 to 454 mg weight) (Tables 1 and 3). The various ovule orientation treatments within the two types of culture vessels resulted in similar embryo weights. In treatment PDMD/V, embryo length and weight values were consistently lower (Table 3). Although embryo germination was similar for the various treatments, plant weight in the TTMD/V treatment was significantly greater than the rest. When excised after stratification, more embryos from this treatment had a pearly white healthy appearance, resulting in the greatest plant survival (Table 3).

Expt. 3. Effect of culture vessel and ovule orientation on embryo development in cultured ovules and subsequent germination of cultured embryos. The two families under investigation differed for all recorded growth variables (Table 4). P52-29 had significantly greater embryo and plant weight, whereas P29-80 had significantly greater germination and percent of plants surviving (Table 4). Treatment TTMD/V had significantly larger embryos after the ovule culture period. Treatment PDFD/H consistently had the lowest values for all growth

Table 1. Parentage of families, culture date, initial embryo length and duration of culture period for experiments on embryo culture of *Prunus*.

Family	Parentage		Fruit type	Bloom date	Collection date	Days after pollination	Avg init emb len (mm)	Days in culture	After 1 month on tree	
	Female	Male							Avg emb len (mm)	Avg emb wt (mg)
Expt. 1										
1	P78-21	P30-129	NE ^z	3/1/1994	4/25/1994	55	1.0	24		
2	P78-21	P97-14	NE	3/1/1994	4/25/1994	55	1.4	25		
3	P78-21	K622-3	NE	3/1/1994	4/25/1994	55	1.3	27		
4	P45-141	P45-11	PE	3/1/1994	4/25/1994	55	0.6	27		
5	K622-3	P97-14	NE	3/2/1994	5/6/1994	65	3.4	29		
6	K622-3	P36-1	NE	3/3/1994	5/6/1994	64	3.1	29		
7	P30-129	D33-1	NE	2/28/1994	5/4/1994	65	1.9	29		
8	P45-142	P45-141	PE	3/3/1994	5/13/1994	71	6.2	33		
9	P45-142	P46-36	PE	3/4/1994	5/13/1994	70	5.6	30		
Expt. 2										
	K626-90	OP ^y	NE	2/19/1995	4/27/1995	67	1.0	30	14.9	454
	P30-129	OP	NE	2/19/1995	4/27/1995	67	0.6	30	12.4	316
Expt. 3										
	P29-80	OP	NE	3/5/1996	4/30/1996	56	1.8	30		
	P52-29	OP	NE	3/6/1996	5/8/1996	63	2.0	30		

^zNE/PE = nectarine/peach.^yOP = open pollinated.Table 2. Embryo size, germination, and plant growth for embryo culture of nine families of *Prunus* after ovules had been cultured on any of six media treatments in Expt. 1.

Family	Avg EL ^z (mm)	Avg EL ^y inc (%)	Avg EW ^x (mg)	Germ (%)	Avg cot wt ^v (mg)	Avg plt wt ^u (mg)	Plts/# embs ^t (%)
1	6.0 ab [*]	478	65 a	35 ab	374 a	484 a	34
2	6.3 abc	346	66 a	42 bc	288 a	385 a	42
3	7.0 cde	435	87 ab	50 c	321 a	499 a	40
4	6.6 bcd	1104	104 abc	55 c	386 ab	442 a	32
5	7.7 e	129	126 cd	34 ab	569 c	356 a	33
6	7.3 de	139	126 cd	31 ab	565 c	369 a	31
7	5.6 a	201	63 a	30 a	327 a	352 a	25
8	9.1 f	48	157 d	31 ab	655 c	404 a	31
9	8.7 f	56	110 bc	32 ab	530bc	460 a	32
<i>Treatment^f</i>							
SH6SFPS	5.0 a	85	22 a	3 a	*	*	0
SH6SACFPS	5.7 b	111	25 a	16 c	124 a	305 a	12
SH6SA	10.6 c	293	208 b	94 d	395 b	436 a	93
SH6SACA	11.7 d	333	282 c	92 d	536 b	422 a	90
SH10SFPS	5.4 ab	100	24 a	4 ab	94 a	227 a	2
QL6SFPS	5.8 b	115	37 a	13 bc	279 ab	534 a	1

^zAvg EL = average embryo length after 24 to 33 d of ovule culture.^y% Inc avg EL = percent increase in average embryo length during ovule culture.^xAvg EW = average embryo weight after 24 to 33 d of ovule culture.^v% Germ = percent germination, 3 weeks after stratification.^uAvg cot wt = average cotyledon weight, 3 weeks after stratification.^t% Plts/# embs = average plant weight, 3 weeks after stratification.^f% Plts/# embs = percent plants per number of embryos cultured.^{*}= means with the same letter are not significantly different (N = 60 by family, 90 by treat.) (P = 0.05) using Tukey's HSD multiples comparison test.^fTreatments: SH6SFPS = Stewart and Hsu, 6% sucrose, filter paper support; SH6SACFPS = Stewart and Hsu, 6% sucrose, activated charcoal filter paper support; SH6SA = Stewart and Hsu, 6% sucrose, agar; SH6SACA = Stewart and Hsu, 6% sucrose, activated charcoal, agar; SH10SFPS = Stewart and Hsu, 10% sucrose, filter paper support; QL6SFPS = Quoirin and Lepoive, 6% sucrose, filter paper support.

parameters measured. The test tube and polycarbonate jar treatments with the micropyle down produced the largest embryos and plants for all growth variables measured.

Discussion

Expt. 1. Genotype and media effects on embryo development in cultured ovules and subsequent germination of cultured embryos. Only polycarbonate jars with screw top lids were used for all treatments. The two best treatments (SH6SA and SH6SACA) had agar for support. Ovules placed on their sides on FPS allowed only a small surface area to come in contact with the medium. This may have inhibited media uptake resulting in poor

embryo development. Embryos excised from these treatments showed uneven cotyledon development. The cotyledon that had been against the filter paper was always larger. Pinto et al. (1994) working with *Prunus* showed that ovule orientation or positioning greatly influenced embryo development. When comparing vertically to horizontally grown ovules, vertical orientation had greater embryo weights. Santarem et al. (1997) and McClelland and Smith (1990) have shown that explant orientation in vitro influences its response to its surroundings. Agar medium allows the nutrients to be exposed to both cotyledons. The use of agar as a support is much easier and quicker to prepare, and allows earlier detection of contamination. As was previously reported (Emershad and

Ramming, 1994) the in ovulo cultured embryos within the 0.5- to 1.5-mm initial size range responded more favorably than the 3- to 6-mm initial size range.

When AC was added to the SH6S media containing agar (SH6SACA), significantly larger embryo development was achieved. The role AC made in this difference may have been to increase post autoclave pH as well as absorbing inhibitory compounds such as polyphenols (Owen et al., 1991).

Expt. 2. Effect of culture vessel and ovule orientation on embryo development in cultured ovules and subsequent germination of cultured embryos. The shallow ovule depth in the petri dish with the micropyle down (treatment PDMD/V) resulted in reduced embryo length and weight. The test tube (treatment TTMD/V) with its vertical ovule orientation (micropyle end down) and greater headspace resulted in proper embryo development and appearance. The test tube closure may have also permitted more aeration than the parafilm wrap on the petri dishes. Researchers studying explant orientation, vessel size, aeration and gases within in vitro environments found all have an effect upon growth. Pinto et al. (1994) working with peach ovules found vertical placement of explant was more beneficial than media or sugar level. Laforge et al. (1990) mentions the gas diffusion rate into a vessel is a function of the type of closure. Since closures were different, the gas diffusion rates may have been different in our study. Demeester et al. (1995) found that growth declines in a more tightly closed container, and that the closure's gas exchange mechanism affected the vessels headspace composition. Auboiron et al. (1990) found that *Hevea calli* growing in confined conditions, produced large amounts of carbon dioxide and ethylene gases. Over time, excessive levels of these gases were shown to be inhibitory to callus growth and somatic embryo induction. Adkins et al. (1998) found that medium additives to control excessive levels of ethylene would benefit coconut and papaya callus growth, somatic embryo proliferation, maturation and germination. The smaller embryo size after 30 d of in vitro

Table 3. Embryo size, germination, plant growth, and survival for embryo cultures of two families after ovules had been cultured in four treatments in Expt. 2.

Family	Avg emb len ^z (mm)	Avg emb wt ^y (mg)	Germ ^x (%)	Avg Plt wt ^w (mg)	Plts ^v (%)	Plt surv ^u (%)
K626-90	6.3 a ¹	109 a	94 a	265 a	16 a	83 a
P30-129	8.9 b	183 b	99 a	279 a	68 b	81 a
Treatment ^t						
PDFD/H	9.0 b	178 a	100 a	225 a	23 a	71 a ¹
PDFU/H	9.0 b	179 a	100 a	231 a	49 bc	75 ab
PDMD/V	7.9 a	159 a	90 a	236 a	42 b	81 ab
TTMD/V	8.4 ab	174 a	100 a	356 b	60 c	89 b

^zAvg emb len = average embryo length after 30 d of ovule culture.

^yAvg emb wt = average embryo weight after 30 d of ovule culture.

^x% Germ = percent germination in test tube, 2 weeks after stratification.

^wAvg plt wt = average plant weight, 5 weeks after stratification.

^v% Plts = percent plants/10 ovules, 5 weeks after stratification.

^u% Plt surv = percent plant survival after establishment in soil.

¹Means with the same letter are not significantly different (N = 40) (P = 0.05) using Tukey's HSD multiple comparison test.

^tTreatments = PDFD/H: petri dish with funiculus down, agar/horizontal orientation; PDFU/H: petri dish with funiculus up, agar/horizontal orientation; PDMD/V: petri dish with micropyle down, agar/vertical orientation; TTMD/V: test tube with micropyle down, agar/vertical orientation.

¹Means with the same letter are not significantly different (P = 0.1) using Fisher's Protected LSD.

Table 4. Embryo size, germination, plant growth and percent plants after embryo culture of two families and four ovule treatments in Expt. 3.

Family	Avg emb wt ^z (mg)	Germ (%) ^y	Avg Plt wt ^x (mg)	Plts ^w (%)
P29-80	126 a ¹	90 b	902 a	90 b
P-52-59	198 b	80 a	1050 b	75 a
Treatment ^t				
TTMD/V	184 c	90 b	1062 b ¹	91 b
PDFD/H	125 a	70 a	842 a	61 a
PJFD/H	151 ab	90 b	902 a	91 b
PJMD/V	162 b	90 b	1024 b	88 b

^zAvg emb wt = average embryo weight after 30 d of ovule culture.

^y% Germ = percent germination, 18 d after stratification.

^xAvg plt wt = average plant weight, 25 d after stratification.

^w% Plts = percent plants/10 ovules.

¹Means with the same letter are not significantly different (N = 40) (P = 0.05) using Tukey's HSD multiple comparison test

^tTreatments = TTMD/V: test tube culture with micropyle end in agar/vertical; PDFU/H: petri dish culture with funiculus side down into agar/horizontal; PJFD/H: polycarbonate jar with funiculus side down into agar/horizontal; PJMD/V: polycarbonate jar with micropyle end down into agar/vertical.

¹Means with the same letter are not significantly different (P = 0.1) using Fisher's Protected LSD test

culture compared to in vivo growth (Table 1) demonstrates that all growth requirements for full embryo development are not being met.

Expt. 3. Effect of culture vessel and ovule orientation on embryo development in cultured ovules and subsequent germination of cultured embryos. Test tubes and polycarbonate jars containing ovules with vertical orientation with the micropylar end into agar produced the largest embryos and plants (Table 4) compared with horizontal ovule orientation. The horizontal orientation may affect embryo development by minimizing exposure to nutrients. In addition, the poorer embryo growth within petri dishes could be attributed to a reduced headspace volume and sealing with parafilm which would reduce aeration, thereby modifying headspace gas composition within the dish. The number of explants within different size vessels can affect growth, by increasing metabolic byproducts, which, if not properly dissipated could inhibit growth. Many researchers have reported on vessel size and headspace gas composition and how it affects plant growth. Monette (1983) and McClelland and Smith (1990) studying shoot tip cultures of various species

found that as the vessel size becomes larger, shoot tip proliferation increased. In addition, McClelland and Smith (1990) observed that if vessels were not sealed with parafilm that shoot quality was better. It was suggested that this may be due to better gas exchange within the vessel. Auboiron et al. (1990), Laforge et al. (1990) and Demeester et al. (1995) have shown that the size of the culture container and the closure device determine the headspace gas composition which influences growth of the plant material. Laforge et al. (1990) found that growth could be slightly enhanced in plantlets of asparagus and strawberries by proper gas exchange in miniature growth chambers. Auboiron et al. (1990) working with *Hevea* showed by avoiding the accumulation of excessive levels of gases such as ethylene and carbon dioxide, that somatic embryo induction would not be inhibited. It has been shown that vessels that differ in their gas exchange mechanism affect headspace composition. Demeester et al. (1995) working with *Prunus* shoot tips observed that as gas exchange slows down due to a more tightly closed container, the growth rate declined accordingly.

Conclusion

In our studies, test tubes and polycarbonate containers used in conjunction with agar were beneficial to ovule/embryo development. Agar permitted the proper positioning of the ovule increasing its surface area of contact allowing for more nutrient uptake. Test tubes and polycarbonate containers allowed for more media depth to permit proper ovule insertion and had greater headspace volume for possibly better gas composition than petri dishes. Perhaps to further improve *Prunus* embryo growth within the ovule, one could increase gas exchange within the culture vessel. Studies of interest would be comparing different vessel closures to increase gas exchange yet minimize water loss. To prevent the spread of contamination and eliminate explant interaction of in ovulo embryo cultures, one may use test tubes over polycarbonate jars. With the proper medium, support, vessel and closure, in ovulo embryo culture of *Prunus* can be enhanced. These findings, if utilized in *Prunus* breeding programs, should generate greater numbers of hybrid progenies accelerating the development of early-ripening selections and cultivars.

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