Improvement of Peach Embryo Culture Through Manipulation of Carbohydrate Source and pH

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Additional index words. tissue culture, MES, buffer, sugar, embryo rescue

Abstract. Carbohydrate source of peach [*Prunus persica* (L.) Batsch] embryo culture media affects embryo growth and survival. The first objective of this study was to determine the effect of five carbohydrates (fructose, glucose, maltose, sorbitol, and sucrose) in Woody Plant Medium (WPM) on the germination and survival of peach embryos in vitro. Fructose, glucose, maltose, and sucrose in WPM resulted in better embryo germination and survival than sorbitol. Fructose (2% and 3%) produced greater survival than all other carbohydrates tested in smaller embryos (<10% ovule dry weight). However, sucrose was better than all other carbohydrates tested in the larger embryos (≥10% ovule dry weight). In addition, large embryos (>10% ovule dry weight) on fructose at 1% combined with glucose, maltose, sorbitol, or sucrose at 1% had equivalent or higher survival than did those on either 1% or 2% sucrose in conjunction with the same carbohydrates. Embryo survival on different carbohydrates varied with genotype. The second objective of this study was to determine the effect of three levels of MES buffer (0.0 mM, 4.5 mM, and 9.0 mM) on medium pH stability and embryo survival. MES buffer at 0.0 mM and 4.5 mM concentration produced significantly better embryo survival than 9.0 mM. The pH stability was better at MES 9.0 mM, however survival decreased significantly. Chemical name used: [2-(N-morpholino)-ethane sulphonic acid] (MES)

Commercially productive, low-chill, early-ripening peach [*Prunus persica* (L.) Batsch] cultivars are important in the United States because they are marketed in April and May (Sherman and Rodriguez, 1987) when the supply of fresh peaches is low and the price is about twice that of peaches harvested in late June and July [U.S. Dept. of Agriculture (USDA), 1995].

A major problem with breeding early-ripening peach cultivars is the failure of seed germination. Aseptic embryo culture in a nutrient-rich environment can overcome this problem (Brooks and Hough, 1958). Sucrose (2% to 5%) is the most popular carbohydrate used with tissue culture (Brigden, 1994). However, other sources, such as fructose, glucose, maltose, and sorbitol, have proven more effective than sucrose in various tissue culture studies (Batty and Dunwell, 1989; Hammatt, 1993; Marino et al., 1993; Scozzoli and Pasini, 1992).

Embryo culture media used in peach breeding programs must produce acceptable survival on a wide range of early-ripening genotypes to be of practical use since it is not feasible to develop a different medium for each individual genotype. Although these genotypes ripen in April through mid June in Texas, previous work indicates that the most important factor in success of a medium is embryo maturity as measured by size or ovule percent dry weight (Bacon and Byrne, 1995; Burgos and Ledbetter,

1993; Kester and Hesse, 1955). At present, the most common procedure is to culture small embryos (5–10 mm) in Woody Plant Medium (WPM) (Lloyd and McCown, 1981), with 3% sucrose and larger embryos (>10 mm) in WPM with 2% sucrose (Chaparro and Sherman, 1994; Ramming, 1985; Rizzo et al., 1998).

The pH of tissue culture media can affect plant growth (Almehdi and Parfitt, 1986; Emershad and Ramming, 1994; Reeves et al., 1983). Plants effectively alter the pH of their culture medium in the normal process of growth by releasing H⁺ in response to cation adsorption (e.g., K⁺ or NH₄⁺) or by ammonium depletion in the medium (Bonga and Aderkas, 1992). Extreme pH differences close to the root surface may negatively affect the uptake of mineral nutrients (Frick and Mitchell, 1993), thus limiting growth and differentiation. MES is a promising pH buffer for use with embryo culture of peaches because it is biologically inert and non-interactive with medium ions (Bugbee and Salisbury, 1985).

The overall objectives of this study were to determine the effects of carbohydrate source and pH of embryo culture medium on germination and survival of immature peach embryos of varying sizes.

Materials and Methods

Healthy, well-developed fruit of 10 genotypes ('Flordaking', 'Flordacrest', TX5B5, TX4392-6, 'Springprince', TX5393-1, 'Scarletpearl', TX4393-5, TX3189-1, and TX4D46W) was harvested from late April through late June, depending upon individual genotype maturation and stored at 4 °C ± 1 °C for a period of

<14 d before use. Standard sterilization and culture procedures were followed (Rizzo et al., 1998). Ovule/embryo size and ovule percent dry weight were determined prior to culturing with destructive measurements on a representative sample of 10 ovules per genotype.

Fruit was first surface sterilized. Under sterile conditions ovules were extracted, seed coats removed, and the embryos were placed into 25 × 95 mm Baxter shell vials containing 10 mL of WPM medium. Ten to 18 replications (embryos) per treatment were used in all experiments (n = 10 to 18). Racks of vials containing medium and embryos were sealed in large paper bags (randomly) and stratified in the dark at 4 °C for ≈10 weeks, after which time they were removed and placed under fluorescent lighting at ≈8.6 klux with a 16-h photoperiod at 28 ± 1 °C (1998) or 18 ± 2 °C (1999) to induce germination. After ≈3 weeks when the plants had both adequate shoot and root formation for survival out of culture, they were transferred to planting medium (Sunshine Mix #4, Sun Gro, Bellevue, Wash.) in 25 × 52 × 7 cm trays with humidity domes and grown under artificial fluorescent lighting at ≈8.6 klux with a 16-h photoperiod at 28 °C ± 1 °C. Humidity domes were removed after 2–3 d. After 2 to 3 weeks under lights in the laboratory, plants were transferred to mist benches in the greenhouse and left for ≈1 week. When the shoots were ≈10 cm long, they were planted into 5.1 × 6.4 × 25.4 cm Roottrainers (Spencer-Lemaire, Alberta, Canada). Germination data were recorded on the number of embryos that developed both adequate roots and shoots. Survival data was taken on the number of plants that survived transfer to Roottrainers in the greenhouse.

1998 carbohydrate source experiment. The basal medium was WPM nutrients and vitamins, (Lloyd and McCown, 1981) (pH 6.00 ± 0.01 and 4.5 mM MES added as a buffer). The effects of the five carbohydrates (fructose, glucose, maltose, sorbitol, and sucrose) at 2% and 3% (10 treatments) were examined on five peach genotypes ('Flordacrest', TX5B5, TX4392-6, 'Springprince', and three successive harvests at 1-week intervals of 'Flordaking') in a factorial experiment.

1999 fructose, sucrose, or both combination experiment. The effects of WPM containing either 1% fructose or 1% or 2% sucrose in conjunction with either 1% glucose, maltose, sorbitol, or sucrose (12 treatments) were examined on three peach genotypes ('Flordacrest', TX4392-6, and TX4D46W) in a factorial experiment.

1998 pH experiment. The effects of MES at three concentrations (0.0, 4.5, and 9.0 mM) and two pH levels (5.7 or 6.0) were examined on four genotypes ('Springprince', TX5393-1, 'Scarletpearl', and TX4393-5) with WPM containing 3% sucrose in a factorial experiment.

Survival data was taken in a binomial format (0 = dead and 1 = alive) ≈4 weeks after removal from stratification. Treatment effects in carbohydrate experiments (genotype, carbohydrate source, and carbohydrate concentration) and in pH experiments (genotype, pH level, and MES concentration) were analyzed

Received for publication 12 Feb. 2002. Accepted for publication 18 July 2002. The assistance of Kilsun Yoo in the analysis of sugar content in the media is gratefully acknowledged.

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as factorials using the SAS GLM and Duncan's multiple range test (SAS, 1985).

Results and Discussion

Since previous reports indicated that sucrose and fructose degraded upon autoclaving (Owen et al., 1991; Schenk et al., 1991), the carbohydrate concentrations were measured by liquid chromatography before and after autoclaving. Autoclaving was not found to substantially degrade any of the carbohydrates used (data not shown).

1998 carbohydrate source experiment. The genotypes tested were grouped into two categories. Small embryos (<10% ovule dry weight) were from peach genotypes TX5B5 (7.8% ovule dry weight, 10.8 mm embryo length), 'Flordaking' first harvest (8.3% ovule dry weight, 9.7 mm embryo length), and 'Flordaking second harvest' (9.8% ovule dry weight, 10.2 mm embryo length). Large embryos ($\geq 10\%$ ovule dry weight) were from peach genotypes 'Flordaking' third harvest (10.0% ovule dry weight, 10.4 mm embryo length), TX4392-6 (11.2% ovule dry weight, 11.8 mm embryo length), 'Springprince' (11.6% ovule dry weight, 11.9 mm embryo length), and 'Flordacrest' (11.9% ovule dry weight, 10.6 mm embryo length). Survival was influenced by genotype and carbohydrate source main effects as well as the genotype $\times$ carbohydrate source, carbohydrate source $\times$ carbohydrate concentration, and genotype $\times$ carbohydrate source $\times$ carbohydrate concentration interactions (Table 1).

Across all genotypes and carbohydrate treatments, immature peach embryos germinated, grew, and survived best with fructose, maltose, and sucrose. The use of sorbitol gave the lowest percent survival and glucose was intermediate (Table 2). Sorbitol may have resulted in poor survival because the embryos may not be able to efficiently metabolize sorbitol once the seed coat is removed, since most of the enzyme sorbitol oxidase, which converts sorbitol to glucose, is produced in the seed coat (Yamaki and Ryugo, 1986). Although sucrose is the most popular carbohydrate for tissue culture, it alone is not necessarily the best for peach embryo culture.

The genotype survival ranged from 25% for the earliest harvested 'Flordaking' embryos to 56% for 'Springprince' (data not shown). This is in part related to embryo size as indicated by the lower survival (25%) of the smallest 'Flordaking' embryos (first harvest) compared to the larger 'Flordaking' embryos that were harvested 1 and 2 weeks later (33% to 39%).

The interaction between carbohydrate source and the carbohydrate concentration was primarily due to differential survival of embryos on the two separate concentrations of maltose and sucrose (Table 2). Normally, 2% to 3% sucrose is used in peach embryo culture based upon embryo size with the lower carbohydrate concentration better for the larger embryo sizes (Chaparro and Sherman, 1994; Ramming, 1985; Rizzo et al., 1998). Further examination of the data by embryo size indicated that small embryo (<10% ovule dry

Table 1. Percent germination and survival of immature peach embryos influenced by genotype^a, carbohydrate source^b, and carbohydrate concentration^c in 1998.

Variation	df	Germination	Survival
Genotype	6	***	***
Carbohydrate source	4	***	***
Carbohydrate concentration	1	NS	NS
Genotype $\times$ carbohydrate source	24	***	**
Genotype $\times$ carbohydrate concn	6	**	NS
Carbohydrate source $\times$ carbohydrate concn	4	***	***
Genotype $\times$ carbohydrate source $\times$ carbohydrate concn	24	*	**
Total	1019		

^a'Springprince', n = 16; 'Flordacrest', n = 12; 'Flordaking', (three harvests) n = 10, 18, 18; TX4392-6, n = 16; TX5B5, n = 12 (n indicates the number of embryos from each genotype used with each treatment combination).

^bFructose, glucose, maltose, sorbitol, and sucrose.

^c2% and 3%.

NS*, **, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, 0.001, respectively.

Table 2. Survival of immature peach embryos related to carbohydrate source and interactions with carbohydrate concentration and embryo size in 1998. Combined data from 'Flordaking' (three harvests), TX5B5, 'Springprince', 'Flordacrest', and TX4392-6 were used.

	Carbohydrate source				
	Fructose	Glucose	Maltose	Sorbitol	Sucrose
Survival (%)	54 a ^z	40 b	45 ab	10 c	52 a
2% carbohydrate	54 ab	44 bc	32 c	12 d	64 a
3% carbohydrate	55 ab	38 c	61 a	9 d	43 bc
<10% ovule dry weight ^x	65 a	34 c	43 b	10 d	33 c
>10% ovule dry weight	48 b	46 b	49 b	10 d	67 a

^zDifferent letters following values indicate significant differences within rows according to Duncan's multiple range test ($P \leq 0.05$).

^xThe smaller embryos (<10% ovule dry weight) were from 'Flordaking' (first two harvests), n = 10, 18 and TX5B5, n = 12. The larger embryos (>10% ovule dry weight) were from 'Flordaking' (third harvest), n = 18; 'Springprince', n = 16; 'Flordacrest', n = 12; and TX4392-6, n = 16 (n indicates the number of embryos from each genotype used with each treatment combination).

^yPercent dry weight was determined with destructive measurements on a representative sample of 10 ovules per genotype.

Fig. 1. Comparison of mean survival for immature peach embryos (all >10% ovule dry weight) over three genotypes (TX4D46W, n = 18; 'Flordacrest', n = 18; and TX4392-6, n = 18) on WPM containing either 1% fructose or 1% or 2% sucrose in combination with 1% glucose, maltose, sorbitol, or sucrose (1999 experiments). Different letters indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$).

weight) survival is better on fructose and large embryo (>10% ovule dry weight) survival is better on sucrose (Table 2). Whereas there are reports that genotype (Brigden, 1994), sugar carbohydrate type (Hammatt, 1993; Scozzoli and Pasini, 1992), and embryo size (Kester and Hesse, 1955; Ramming, 1990) may affect plant growth in vitro, this is the first report of a differential carbohydrate type effect related to embryo size in peach.

1999 fructose, sucrose, or both combination experiment. All genotypes used had relatively large embryos (>10% ovule dry weight). Their size ranged from 12.6% to 20.3% ovule dry weight and 13.0 to 13.3 mm embryo length. Carbohydrate concentration affected immature peach embryo survival (Fig. 1). One percent fructose in conjunction with 1% glucose or 1% maltose produced better embryo survival than either 2% or 3% sucrose, the most common carbohydrate concentrations used in peach embryo culture (Chaparro and Sherman, 1994; Ramming, 1985).

All fructose combinations (1% fructose with either glucose, maltose, sorbitol, or sucrose each at 1%) produced embryo survival greater than or equal to the sucrose combinations (1% and 2% sucrose with either glucose, maltose, sorbitol, or sucrose each at 1%) for immature peach embryos >10 mm in length (Fig. 1).

Although the genotype $\times$ carbohydrate source combination interaction was significant (Table 3), there were no clear trends. The interaction was accounted for by only 3 of the 12 carbohydrate combinations. This is important because, to be widely used, embryo culture must produce acceptable survival on a wide range of genotypes that produce immature embryos.

These experiments indicate that carbohydrate combinations—especially those including fructose—have the potential to produce higher embryo survival than the standard approach of using one carbohydrate source (sucrose) in the media.

1998 pH experiment. All genotypes used had relatively large embryos (>10% ovule dry weight). Their size ranged from 11.6% to 23.2% ovule dry weight and 11.9 to 14.7 mm embryo length. Although genotypic survival differed significantly (50% to 80%) as expected and MES concentration was also significant, no differences in survival were caused by the two pH levels (5.7 and 6.0) (Table 4). This was most likely due to the narrow pH range tested and was in line with previous studies on *Vitis* somatic embryos (pH range 5.0 to 6.0) in which pH had no effect on average number of surviving embryos (Emershad and Ramming, 1994) and on peach rootstock (pH range 5.2 to 5.8) which showed no pH effect on mortality, but agar medium at pH 5.8 was best for growth (Reeves et al., 1983).

The addition of MES at both the 4.5 mM and 9.0 mM concentrations stabilized medium pH to within 0.5 pH units over a 10-week period compared to a >1.0 pH unit change without the MES buffer. Previous studies (Bugbee and Salisbury, 1985; Harbage and Stimart, 1996; Owen et al., 1991) were short-lived compared

Table 3. Germination and survival of immature peach embryos cultured in vitro influenced by genotype^a and carbohydrate source combination^b in 1999.

Variation	df	Germination	Survival
Genotype	2	***	NS
Carbohydrate source combination	11	***	***
Genotype $\times$ carbohydrate source combination	22	**	**
Total	647		

^aFlordacrest, n = 18; TX4392-6, n = 18; TX4D46W, n = 18 (n indicates the number of embryos from each genotype used with each treatment combination).

^b1% and 2% sucrose and 1% fructose in combination with 1% glucose, maltose, sorbitol, or sucrose.

NS, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

Table 4. Germination and survival of embryo cultured immature peach embryos influenced by genotype^a, pH level^b, and MES concentration^c in 1998.

Variation	df	Germination	Survival
Genotype	3	**	***
pH level	1	NS	NS
MES concn	2	***	***
Genotype $\times$ pH level	3	NS	NS
Genotype $\times$ MES concn	6	NS	NS
pH level $\times$ MES concn	2	NS	NS
Genotype $\times$ pH level $\times$ MES concn	6	NS	NS
Total	381		

^a'Springprince', n = 14; TX5393-1, n = 16; 'Scarletpearl', n = 16; TX4393-5, n = 16 (n indicates the number of embryos from each genotype used with each treatment combination).

^b5.7 or 6.0.

^c0.0, 4.5, or 9.0 mM concentrations.

NS, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

to the 10 weeks of cold storage required for peach embryo culture. It is not surprising that the drop in pH associated with this experiment was more pronounced (0.20 to 1.54 pH units compared to 0.03 to 0.35 pH units from those prior experiments).

Autoclaving significantly alters the pH of medium (Schenk et al., 1991). In peach-embryo culture experiments, autoclaving lowered the

pH of WPM without MES four to five times as much as medium with MES (pH change of 0.45 to 0.53 without compared to 0.10 to 0.11 with 9.0 mM) (Fig. 2).

In spite of the pH stabilization effects of MES (Fig. 2), the highest concentration (9.0 mM) was detrimental to embryo survival (Table 5). This detrimental effect was also shown with *Trifolium repens* L. (Rys and Phung, 1985), but

Fig. 2. MES concentration related to pH stability of Woody Plant Medium before autoclaving (AC), after AC, and after a 10-week period of cold storage (Cold) in 1998. (Beginning pH levels of 5.7 and 6.0 respectively). Fourteen to 16 peach embryos from each of four genotypes ('Springprince' n = 14, TX5393-1 n = 16, 'Scarletpearl' n = 16, and TX4393-5 n = 16) were used per treatment.

Table 5. Percent survival of immature peach embryos of four genotypes related to two pH levels and three MES concentrations in 1998.

	Genotype				Mean values ^z
	Springprince	TX5393-1	Scarletpearl	TX4393-5	
Embryo length (mm)	11.9	14.5	14.7	13.7	13.7
Ovule % dry weight ^y	11.6	17.0	23.2	13.5	16.3
% survival	50 b ^a	51b	52 b	80 a	58.3
pH 5.7	56 a ^w	58 a	58 a	76 a	61 a
pH 6.0	48 a	41 a	43 a	83 a	55 a
MES 0.0 mM	60 a	50 a	60 a	88 a	65 a
MES 4.5 mM	63 a	56 a	60 a	88 a	67 a
MES 9.0 mM	34 b	44 a	34 b	63 b	44 b

^zMean value of each row respectively

^yOvule percent dry weight was determined with destructive measurements on a representative sample of 10 ovules per genotype.

^aDifferent letters following values indicate significant differences within the row according to Duncan's multiple range test ($P \leq 0.05$).

^wDifferent letters following values indicate significant differences within columns according to Duncan's multiple range test ($P \leq 0.05$).

not with beans (*Phaseolus vulgaris* L.), corn (*Zea mays*, L.), lettuce (*Latucia sativum*, L.), and tomatoes (*Lycopersicon esculentum* Mill.) (Bugbee and Salisbury, 1985). Consequently, the effect appears related to species sensitivity, period of storage/exposure time, or both. Although promising as a pH buffer, MES is not recommended for immature peach embryo culture except at low concentrations.

These experiments indicate that for small embryos (<10.0% ovule dry weight) fructose (2% to 3%) is better than similar levels of glucose, maltose, sorbitol, or sucrose. For larger embryos (>10.0% ovule dry weight) no single treatment was better than all the rest, but sugar combinations of 1% fructose combined with other sugars (either glucose, maltose, sorbitol, or sucrose each at 1%) were better than or equal to sucrose alone or in combinations.

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