Plant Cell, Tissue and Organ Culture **64:** 55–62, 2001.

Effect of polyethylene glycol and sugar alcohols on soybean somatic embryo germination and conversion

David R. Walker & Wayne A. Parrott*
*Department of Crop & Soil Sciences, The University of Georgia, Athens, GA 30602-7272, USA (*requests for offprints; Fax: 706-542-0914; E-mail: wparrott@uga.edu)*

Received 30 September 1999; accepted in revised form 3 January 2001

Key words: embryo maturation, *Glycine max*, mannitol, osmotica, somatic embryogenesis, sorbitol

Abstract

Polyethylene glycol 4000, mannitol and sorbitol were tested as supplements to a liquid Finer and Nagasawa medium-based histodifferentiation/maturation medium, FNL0S3, for soybean (*Glycine max* L. Merrill) somatic embryos of 'Jack' and F138 or 'Fayette'. Significant differences were found among types and levels of osmotica for their influence on quantity of mature embryos recovered, and on germination (root and shoot emergence) and conversion of embryos into plants. Supplementation of FNL0S3 with 5% polyethylene glycol or 1.5% sorbitol improved germination frequencies without limiting embryo histodifferentiation. Supplementation with 3% sorbitol resulted in a 9-fold increase in germination frequencies and a 13-fold increase in conversion frequencies of 'Fayette' and 'Jack' embryos. However, these improvements were accompanied by a significant, 22% reduction in fresh weight of mature embryos. Overall, 3% sorbitol was found to be the best of the osmotic supplements tested, resulting in 51 ± 5% conversion frequency (mean ± SE), as compared to 4 ± 1% in the control. Supplementation of FNL0S3 with 3% mannitol did not improve embryo maturation.

Abbreviations: FN – Finer and Nagasawa (1988); H/M – histodifferentiation/maturation; MS – Murashige and Skoog (1962); PEG – polyethylene glycol

Introduction

Repetitive embryogenic cultures provide dense concentrations of mitotic, totipotent cells useful as target tissues for soybean [*Glycine max* (L.) Merr.] genetic transformation via particle bombardment (Finer and McMullen, 1991; Trick et al., 1997). While much progress has been made in developing and refining protocols for the initiation (Ranch et al., 1985), maintenance (Finer and Nagasawa, 1988; Bailey et al., 1993a), and conversion (Bailey et al., 1993b; Samoylov et al., 1998b) of soybean somatic embryo cultures, the frequencies at which these embryos initiate and complete conversion into plants are well below that typical of zygotic embryos (Parrott et al., 1988; Komatsuda et al., 1992). Partial desiccation of mature somatic embryos improves conversion frequencies (Hammatt and Davey, 1987), but efforts to better mimic the developmental environment of zygotic embryos should further improve the maturation of somatic embryos (Finkelstein and Crouch, 1986).

Factors which determine the ability of embryos to convert into plants include the synthesis and accumulation of storage compounds, especially storage proteins, and the acquisition of desiccation tolerance (Blackman et al., 1992; Kermode, 1995). The importance of water relations in controlling embryo maturation was proposed by Fischer et al. (1987), and has been supported by evidence from both embryo culture experiments (Xu et al., 1990) and *in situ* studies (Saab and Obendorf, 1989). Attempts to simulate the *in vivo* environment through modification of the composition of histodifferentiation/maturation (H/M) media used for somatic embryos showed increased storage compound levels and desiccation tolerance (Finkelstein and Crouch, 1986; Xu et al., 1990). Studies in soybean (Egli, 1990), alfalfa (Xu et al., 1990) and rapeseed (Finkelstein and Crouch, 1986) have shown that em-

bryo maturation is frequently associated with a low osmotic potential in tissues or medium surrounding the embryo.

Samoylov et al. (1998b) developed a liquid H/M medium, FNL0S3, to expedite the recovery of fertile soybean plants. Compared to the two-step, MS0M6AC/MS0M6 solid media H/M protocol described by Bailey et al. (1993a), the use of FNL0S3 resulted in a 3- to 4-fold increase in the number of mature, coyledon-stage embryos recovered per mg of embryogenic tissue. The use of FNL0S3 also shortened the length of time required for histodifferentiation and maturation from eight weeks to three weeks. However, germination frequencies of embryos matured on FNL0S3 were generally lower than those of embryos matured on MS0M6AC and MS0M6 media.

The objective of the current study was to investigate whether supplementation of FNL0S3 medium (Samoylov et al., 1998b) with an osmoticum would improve germination and conversion frequencies of soybean somatic embryos. Two general types of osmotica were evaluated:

- plasmolyzing (D-mannitol and D-sorbitol) and
- non-plasmolyzing (polyethylene glycol, or PEG 4000).

Both types of osmotica have been shown to enhance germination of embryos from a variety of different plant species (Finkelstein and Crouch, 1986; Attree et al., 1991).

Materials and methods

Plant material and culture conditions

Repetitive embryogenic cultures of 'Jack', 'Fayette', and F138, a line derived from a 'Fayette' × PI 417138 cross, were initiated from immature cotyledons cultured on MSD40, as described by Bailey et al. (1993a), and then temporarily maintained on MSD20 (Wright et al., 1991). Both media contained Murashige and Skoog (1962) basal salts, B5 vitamins, 30 g l^{-1} sucrose, and 2 g l^{-1} Gelrite. MSD40 contains 180.8 μM 2,4-dichlorophenoxyacetic acid (2,4-D), with a pH of 7.0, whereas MSD20 contains 90.4 μM 2,4-D, with a pH of 5.8. The light intensity for these cultures averaged 13 μmol m^{-2} s^{-1}, with a 23-h photoperiod provided by cool white fluorescent tubes. After two months on MSD20, embryogenic clusters were maintained in FN Lite (Samoylov et al., 1998a) for at least one month before being used in an experiment. Clusters of globular-stage embryos with a compact, nodular morphology were selected, and 100 mg of this tissue was used per replication of each treatment. Clusters 2–4 mm in diameter were gently crushed to produce smaller clusters, which were then distributed equally among the four or five flasks comprising a replication. Triple-baffled 125-ml Erlenmeyer flasks containing 35 ml of medium were capped with a Bellco silicone closure (Vineland, NJ), and maintained on a gyratory shaker (130 rpm) in 3–6 μmol photons m^{-2} s^{-1} of continuous cool-white fluorescent light at 25–27°C.

All treatment media were based on FNL0S3 [FN Lite macro salts (Samoylov et al., 1998a), MS micro salts, B5 vitamins, L-asparagine, and 3% sucrose; Samoylov et al., 1998b]. FLN0S3 was supplemented with various levels and types of osmotica as described below. Mature embryos were collected after three weeks in the treatment media and weighed. Twenty-five embryos from each flask were partially desiccated in a 100 × 15 mm Petri dish containing a ca. 1-cm^3 block of auxin-free medium. Plates were sealed with Nescofilm® (Karlan Res. Prod. Corp., Santa Rosa, CA), and kept on a shelf in 13 μmol photons m^{-2} s^{-1}, with a 23-h photoperiod, at 25–27 °C for one week. The responses of twenty embryos from each desiccation dish were evaluated by transferring ten embryos to each of two 100 × 20 mm Petri dishes containing MSO (MS basal salts, B5 vitamins, 1.5% sucrose, and 0.2% Gelrite, at pH 5.8). These cultures were maintained at 25–27 °C under 63 μmol photons m^{-2} s^{-1} cool-white fluorescent light, with a 23-h photoperiod. For each treatment, the responses of 80–100 embryos from each replication were evaluated. Embryo selection before and after desiccation was 'semi-random' in that no effort was made to choose the largest embryos, but pre-cotyledonary stage embryos, fasciated, and fused embryos were avoided. Over-desiccated embryos were also avoided. For all experiments, data were taken on fresh weight of mature embryos and on the frequency of germination and apparent conversion after three weeks on MSO. In the final study, frequency of actual conversion was also determined.

Germination, as used here, refers to both root and shoot development on an embryo with an intact hypocotyl (i.e., initiation of conversion to a plant), as described by Ranch et al. (1985). Conversion (called apparent conversion in the final study to distinguish it from actual conversion) describes the development of expanded trifoliolates and branched roots on an

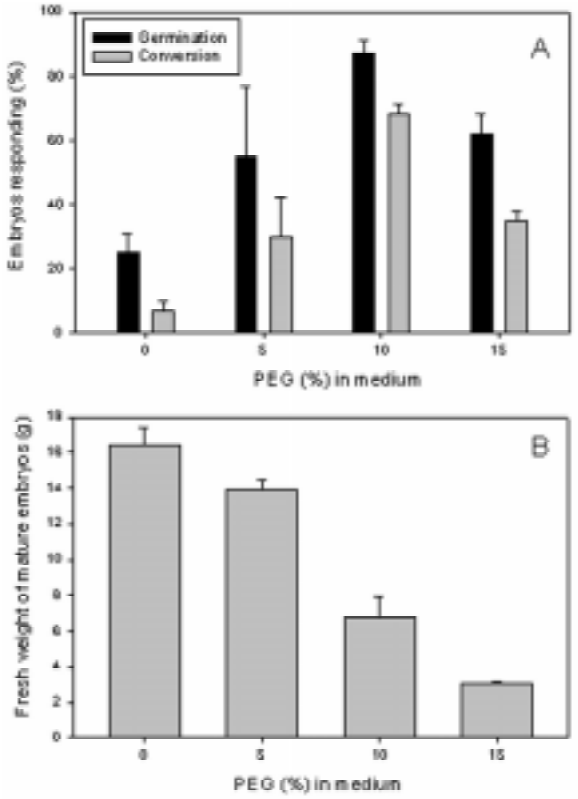

Figure 1. Response of 'Jack' somatic embyos matured in liquid FNL0S3 histodifferentiation/maturation medium supplemented with 0–15% PEG 4000. (*A*) Percent germination and conversion (untransformed means ± SE) of embryos from the four media tested. (*B*) Fresh weight of mature embryos recovered from 0.10 g of embryogenic tissue after 2 weeks.

embryo *in vitro*. Actual conversion refers to survival following transfer to soil.

Effects of a non-plasmolyzing osmoticum

Two separate experiments were conducted to determine what effect supplementing FNL0S3 with PEG 4000 (Fluka Chemie AG, Buchs, Switzerland) would have on germination and conversion of somatic embryos. In one experiment, 'Jack' embryos were differentiated and matured in FNL0S3 supplemented with either 0, 5, 10, 15 or 20% PEG 4000. There were three replications, with five flasks per replication. In a second experiment, 'Jack' embryos were differentiated and matured in FNL0S3 supplemented with either 0, 1, 2, 3, 4, or 5% PEG 4000. There were four replications and four flasks per replication.

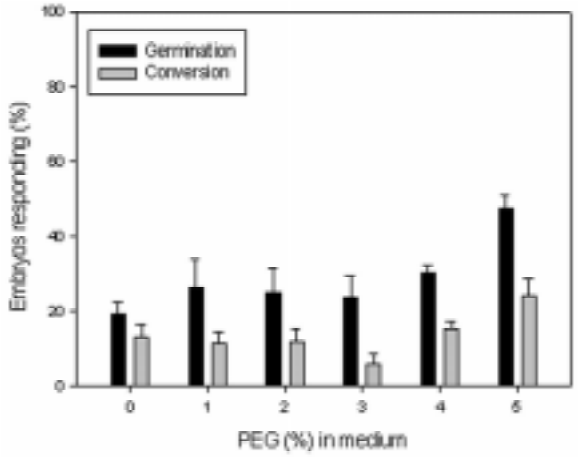

Figure 2. Effect of FNL0S3 supplementation with 0–5% PEG on germination and conversion frequencies of 'Jack' somatic embryos (means ± SE).

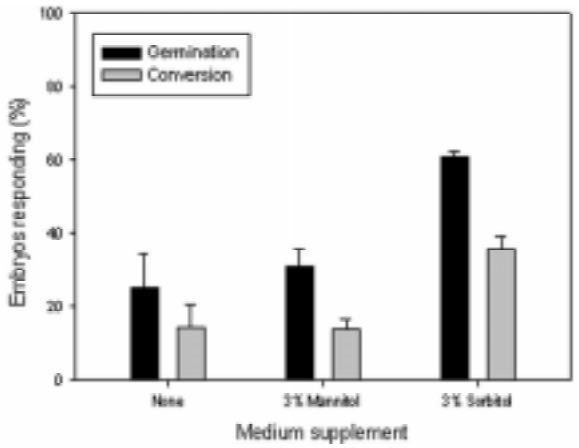

Figure 3. Effect of FNL0S3 supplementation with 3% mannitol or sorbitol on germination and conversion frequencies of 'Jack' somatic embryos (means ± SE).

Effects of plasmolizing osmotica

Two different experiments were also conducted to evaluate the effect of FNL0S3 supplementation with D-mannitol and D-sorbitol on somatic embryo germination and conversion. In the first experiment, 'Jack' embryos were differentiated and matured in FNL0S3 supplemented with 3% D-mannitol or D-sorbitol, with FNL0S3 used as a control. Three replications with four flasks per replication were used. In the second experiment, the effects of supplementing FNL0S3 with either 0, 1.5%, or 3% sorbitol were evaluated. There were three replications, with five flasks per replication. Two genotypes were used: 'Jack' and F138, a line notable for its high conversion frequency.

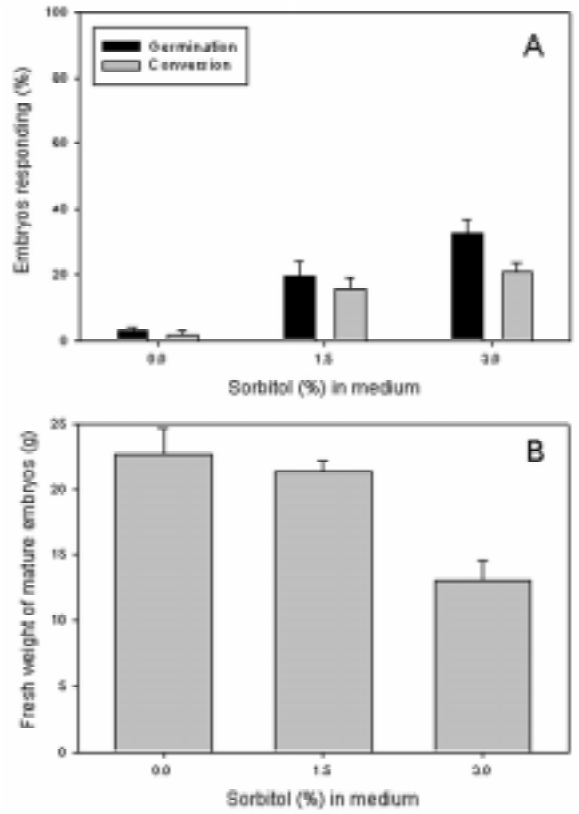

Figure 4. Effect of FNL0S3 supplemented with 0–3% sorbitol on germination and conversion frequencies of (*A*) 'Jack' and (*B*) F138 (means ± SE).

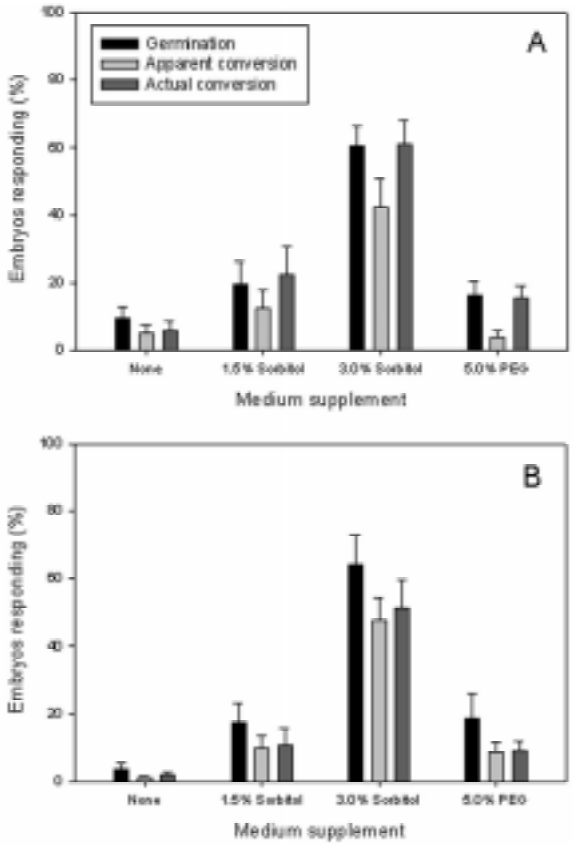

Figure 5. Effect of FNL0S3 supplemented with three different osmotic additions on germination, apparent conversion and actual conversion frequencies of (*A*) 'Fayette' and (*B*) 'Jack' (means ± SE).

Influence of osmotica

The best media from the previous experiments were directly compared with one another and with unsupplemented FNL0S3 in the final study. 'Jack' and 'Fayette' globular-stage embryos were cultured in either FNL0S3, FNL0S3 + 1.5% sorbitol, FNL0S3 + 3% sorbitol, or FNL0S3 + 5% PEG 4000. There were four replications of five flasks each. Data were taken on mature embryo fresh weight, number of cotyledonary-stage embryos, germination, apparent conversion, and actual conversion (i.e., survival *ex vitro*). Actual conversion frequencies were determined by transplanting embryos classified as germinated and those with roots and a visible, but undeveloped, shoot apical meristem to a 1:1 mixture of sand and Fafard Mix #2 (Conrad Fafard Co., Agawam, MA). Converted embryos were gradually acclimated to ambient humidity, and percent survival was recorded after 19 days.

Data analyses

Each experiment was a randomized complete block design, and data were analyzed by analysis of variance using PC-SAS, version 6.10 (SAS Institute). Differences were declared significant based on Fisher's protected LSD ($p \leq 0.05$). Where necessary, germination and conversion frequency data were transformed (arcsin $\sqrt{percent}$) prior to analysis to correct for non-normality in the data set.

Results and discussion

Plasmolyzing osmotica, such as sugar alcohols, readily pass through the cell wall and cause temporary plasmolysis until their movement into the cytosol leads to osmotic recovery (Attree and Fowke, 1993). In contrast, PEG molecules are too large to move through the cell wall and do not cause plasmolysis.

Non-plasmolyzing osmotica are more effective in promoting somatic embryo maturation in some conifer species (Attre et al., 1991).

At the beginning of desiccation, most embryos had changed color from dark-green to yellowish-green, indicating physiological maturity and quiescence (Tekrony et al., 1979; Saab and Obendorf, 1989; Komatsuda et al., 1992). The rate and degree of desiccation of mature embryos is crucial to their subsequent ability to germinate (Obendorf et al., 1998), and the small block of semi-solid medium included in the desiccation plates served to modulate this process. Even small tears in the Nescofilm, however, permitted overdesiccation of embryos if not detected within one or two days. Over-desiccated embryos were brown in color and never germinated, so they were excluded from evaluations of germination and conversion frequencies.

Following germination, many embryos exhibited hypocotyl fracturing, whereby a transverse fracture or a region of dedifferentiated tissue developed across the hypocotyl, generally 3–6 mm below the cotyledons. Hypocotyl fracturing may result from tearing of the epidermis and collapse of internal tissue, similar to what Wakui et al. (1999) observed in *Brassica napus* embryos. Embryos with this condition were not counted as germinated or converted, though many had begun to germinate. Some hypocotyl fracturing was observed on embryos matured in all treatment media, but was less common among embryos matured in some media with osmotic supplements.

Initial evaluation of PEG

Globular-stage embryos transferred to a medium containing 20% PEG 4000 failed to histodifferentiate and became necrotic, so this treatment was dropped from the first experiment. All media with 5–15% PEG enhanced 'Jack' embryo conversion relative to unsupplemented FNL0S3 (Figure 1A). Embryos from FNL0S3 supplemented with 10% PEG exhibited the highest mean germination frequency (87%), though differences between treatments were not significant. Supplementation with 10% PEG resulted in 68% conversion, which was significantly higher ($p < 0.01$) than conversion frequencies from other treatments. Only about 7% of embryos matured in FNL0S3 converted, and both the 5% and 15% PEG media were superior (Figure 1A). Increasing the concentration of PEG in the medium caused a reduction in the fresh weight of mature embryos (Figure 1B). The mean fresh weights of embryos from FNL0S3 supplemented with 10–15% PEG were significantly lower ($p \leq 0.05$) than those of embryos from FNL0S3. None of the lower concentrations of PEG (1–5%) tested in the second experiment significantly decreased embryo mass, but 5% PEG was the only concentration which significantly improved embryo germination and conversion ($p < 0.01$) compared to the control (Figure 2). Supplementation with 5% PEG was therefore the best compromise between improved embryo conversion and decreased fresh weight of mature embryos.

Initial evaluation of plasmolzyzing osmotica

Inclusion of 3% mannitol failed to improve germination and conversion frequencies of 'Jack' embryos (Figure 3). Approximately 60% of embryos which matured in the medium containing mannitol exhibited unusual longitudinal cracks in the hypocotyl (i.e., perpendicular to the hypocotyl fractures typical of other embryos), which generally prevented conversion. Addition of 3% sorbitol resulted in a 2-fold increase in the germination and conversion frequencies, in comparison with unsupplemented FNL0S3 (Figure 3). The reason(s) for the superiority of sorbitol over mannitol is not known, but Mhaske et al. (1998) reported that 10.9% sorbitol promoted accumulation of triglycerides in peanut somatic embryos, whereas the same level of mannitol did not. Adding 3% of either sugar alcohol to FNL0S3 resulted in significant reductions in the mass of mature embryos, ranging from 24% (sorbitol) to 43% (mannitol). Similarly, mannitol used as an osmoticum has been found to reduce dry matter accumulation by seeds cultured *in vitro* (Egli, 1990).

In the subsequent study, 'Jack' embryos matured in FNL0S3 with 3% sorbitol had the highest germination frequencies, and conversion was improved with both 1.5% and 3% sorbitol, with no significant difference between these two concentrations (Figure 4A). Germination and conversion frequencies of 'Jack' embryos from all media were lower than those from the previous experiments, probably due to differences in the quality of the cell lines used. Supplementation with 3% sorbitol significantly reduced the fresh weight of 'Jack' embryos by 43% (Figure 4B) and that of F138 embryos by 26%. Supplementation with 1.5% sorbitol did not significantly reduce the fresh weight of 'Jack' embryos, but caused a significant 14% decrease in the mass of mature F138 embryos. Differences among treatment effects on germination of F138 were not significant based on analysis of transformed data.

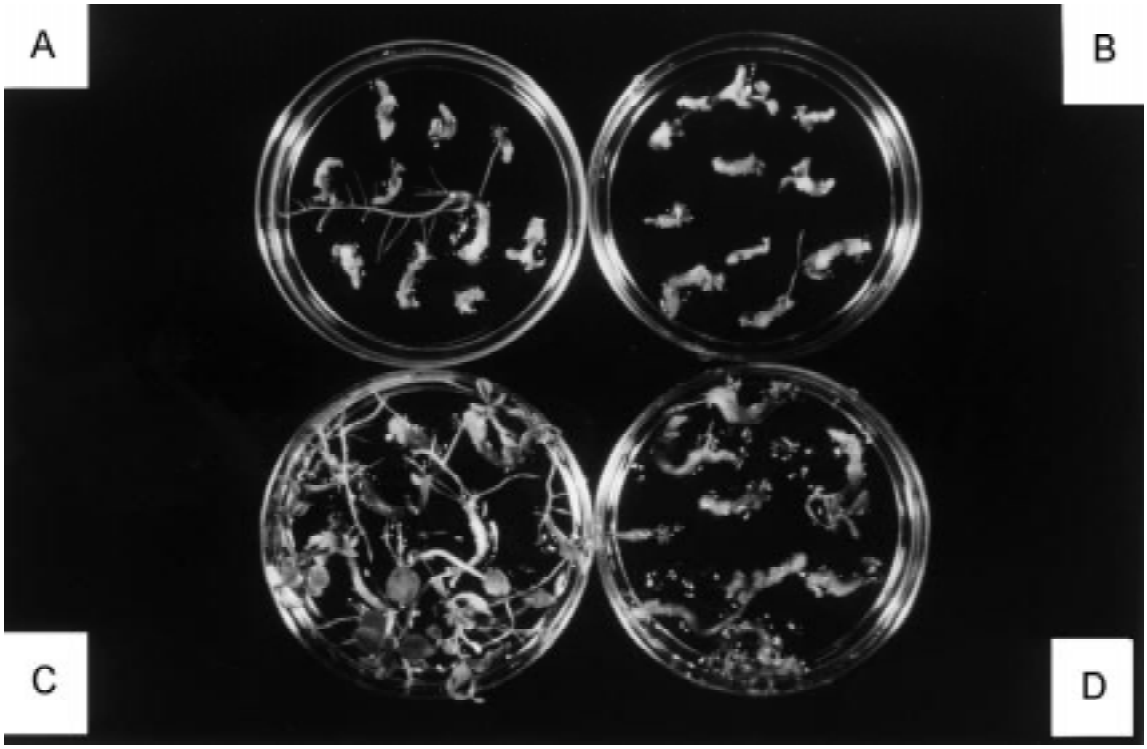

Figure 6. Photo of 'Jack' embryo responses to three different maturation media: (*A*) FNL0S3, (*B*) FNL0S3 + 1.5% sorbitol, (*C*) FNL0S3 + 3% sorbitol, and (*D*) FNL0S3 + 5% PEG.

Differences among treatments for conversion frequencies of F138 were also not significant. Combined analysis showed highly significant differences ($p < 0.01$) between genotypes and among treatments, but no genotype × medium interaction was detected.

Final assessment of best osmotic supplements

Supplementation of FNL0S3 with 3% sorbitol significantly ($p < 0.01$) decreased the fresh weight of mature 'Fayette' embryos relative to the unsupplemented control (16.8 g vs. 23.6 g), but resulted in a 6-fold increase in germination, apparent conversion, and actual conversion frequencies (Figure 5A). Adding 1.5% sorbitol or 5% PEG did not reduce embryo fresh weight, but also did not significantly improve germination or conversion in this study. The mean number of cotyledonary-stage 'Fayette' embryos recovered from 100 mg of globular-stage embryos ranged from 654 to 812 for different treatments, but these differences were not significant due to variation among replications within treatments.

Adding 3% sorbitol also significantly ($p < 0.01$) improved 'Jack' embryo germination, apparent conversion, and actual conversion above all other treatments (Figure 5B). Supplementation with either 1.5% sorbitol or 5% PEG to FNL0S3 resulted in significant, but smaller improvements in germination and apparent conversion, and the effects these supplements were not significantly different (Figure 5B). The average number of 'Jack' cotyledonary embryos obtained from 100 mg of globular-stage embryo clusters ranged from 795 to 864 for the four treatments, with no significant differences.

Analysis of the combined data for 'Jack' and 'Fayette' revealed a significant difference ($p \leq 0.05$) between the genotypes for total fresh weight, with the mean mass of 'Jack' cotyledonary embryos slightly higher than that of 'Fayette' embryos (22.9 g vs. 21.0 g). An equivalent number of embryos was obtained from the two genotypes. Significant differences ($p < 0.01$) between treatments were found for total fresh weight, germination, and both apparent and actual conversion frequencies.

Because of the inverse relationship between embryo conversion potential and the mass of mature embryos recovered, it is important to use supplementary osmotica at levels which afford a compromise between

improved embryo response and impediment of histodifferentiation. Although supplementation with 3% sorbitol caused a moderate decrease in fresh weight, there was still a 220-fold increase in weight during histodifferentiation and maturation, so the improvement in conversion frequency more than compensated for the decrease in weight relative to unsupplemented FNL0S3.

Saab and Obendorf (1989) monitored water and osmotic potentials in soybean embryo axis, cotyledon, seed coat, and pod tissues during periods of seed growth and maturation *in situ*, and found that changes in water relations were limited to the last 7 days of maturation, at which time sharp declines occurred in all of these tissues. It is possible that the introduction of a supplementary osmoticum to the H/D medium following histodifferentiation might improve embryo maturation without producing a negative effect on the number of cotyledonary-stage embryos recovered. However, this would add another step to the current protocol, which already allows recovery of hundreds of embryos from only 100 mg of highly embryogenic tissue.

Acknowledgements

This work was supported by state and Hatch monies allocated to the Georgia Agricultural Experiment Stations and by funding provided by the United Soybean Board and The Soybean Tissue Culture and Genetic Engineering Center. The authors would like to thank Adam Eidson and Judy Wu for their assistance with these experiments.

References

Attree SM & Fowke LC (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tiss. Org. Cult. 35: 1–35

Attree, SM, Moore D, Sawhney VR & Fowke LC (1991) Enhanced maturation and desiccation tolerance of white spruce (*Picea glauca* [Moench.] Voss) somatic embryos: Effects of a non-plasmolysing water stress and abscisic acid. Ann. Bot. 68: 519–525

Bailey MA, Boerma HR & Parrott WA (1993a) Genotype effects on proliferative embryogenesis and plant regeneration of soybean. In Vitro Cell. Dev. Biol. 29P: 102–108

Bailey MA, Boerma HR & Parrott WA (1993b) Genotype-specific optimization of plant regeneration from somatic embryos of soybean. Plant Sci. 93: 117–120

Blackman SA, Obendorf RL & Leopold AC (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiol. 100: 225–230

Egli DM (1990) Seed water relations and the regulation of the duration of seed growth in soybean. J. Exp. Bot. 41: 243–248

Finer JJ & McMullen MD (1991) Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. In Vitro Cell. Dev. Biol. 27P: 175–182

Finer JJ & Nagasawa A (1988) Development of an embryogenic suspension culture of soybean [*Glycine max* (L.) Merrill]. Plant Cell Tiss. Org. Cult. 15: 125–136

Finkelstein RR & Crouch ML (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol. 81: 907–912

Fischer W, Bergfeld R & Schopfer P (1987) Induction of storage protein synthesis in embryos of mature plant seeds. Naturwissenschaften 74: 86–88

Fujii JAA, Slade D, Olsen R, Ruzin & Redenbaugh (1990) Alfalfa somatic embryo maturation and conversion to plants. Plant Sci. 72: 93–100

Hammatt N & Davey MR (1987) Somatic embryogenesis and plant regeneration from cultured zygotic embryos of soybean (*Glycine max* L. Merr.). J. Plant Physiol. 128: 219–226

Kermode A (1995) Regulatory mechanisms in the transition from seed development to germination: interactions between the embryo and the seed environment. In: Kigel J & Galili (eds) Seed Development and Germination (pp 273–332). Marcel Dekker, Inc., New York

Komatsuda T, Lee W & Oka S. (1992) Maturation and germination of somatic embryos as affected by sucrose and plant growth reguators in soybeans *Glycine gracilis* Skvortz and *Glycine max* (L.) Merr. Plant Cell Tiss. Org. Cult. 28: 103–113

Mhaske VB, Chengalrayan K & Hazra S (1998) Influence of osmotica and abscisic acid on triglyceride accumulation in peanut somatic embryos. Plant Cell Rep. 17: 742–746

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Obendorf RL, Dickerman AM, Pflum TM, Kacalanos MA & Smith ME (1998) Drying rate alters soluble carbohydrates, desiccation tolerance, and subsequent seedling growth of soybean (*Glycine max* L. Merrill) zygotic embryos during *in vitro* maturation. Plant Sci. 132: 1–12

Parrott WA, Dryden G, Vogt S, Hildebrand DF, Collins GB & Williams EG (1988) Optimization of somatic embryogenesis and embryo germination in soybean. In Vitro Cell. Dev. Biol. 24: 817–820

Ranch JP, Oglesby L & Zielinski AC (1985) Plant regeneration from embryo-derived tissue cultures of soybean. In Vitro Cell Dev. Biol. 21: 653–658

Saab IN & Obendorf RL (1989) Soybean seed water relations during *in situ* and *in vitro* growth and maturation. Plant Physiol. 89: 610–616

Samoylov VM, Tucker DM & Parrott WA (1998a) Soybean embryogenic cultures: the role of sucrose and nitrogen content on proliferation. In Vitro-Plant 34: 8–13

Samoylov VM, Tucker DM, Thibaud-Nissen F & Parrott WA (1998b) A liquid-medium-based protocol for rapid regeneration from embryogenic soybean cultures. Plant Cell Rep. 18: 49–54

SAS Institute Inc: SAS user's guide statistics, Release 6.03, Cary, NC (1988)

Stoop JMH, Williamson JD & Pharr DM (1996) Mannitol metabolism in plants: a method for coping with stress. Trends Plant Sci. 1: 139–144

TeKrony DM, Egli DB, Balles J, Pfeiffer T & Fellows RJ (1979) Physiological maturity in soybean. Agron. J. 71: 771–775

Trick HN, Dinkins RD, Santarem ER, Di R, Samoylov VM, Meurer C, Walker D, Parrott WA, Finer JJ & Collins GB (1997) Recent advances in soybean transformation. Plant Tiss. Cult. Biotechnol. 3: 9–26

Wakui K, Takahata Y & Kaizuma N (1999) Scanning electron microscopy of desiccation-tolerant and -sensitive microspore-derived embryos of *Brassica napus* L. Plant Cell Rep. 18: 595–600

Wright MS, Launis KL, Novitzky R, Duesing JH & Harms CT (1991) A simple method for the recovery of multiple fertile plants from individual somatic embryos of soybean [*Glycine max* (L.) Merrill]. In Vitro Cell. Dev. Biol. 27P: 153–157

Xu N, Coulter KM & Bewley JD (1990) Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. Planta 182: 382–390