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Towards normalization of soybean somatic embryo maturation

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Abstract Soybean (*Glycine max* L. Merrill) somatic embryos have been useful for assaying seed-specific traits prior to plant recovery. Such traits could be assessed more accurately if somatic embryos more closely mimicked seed development. Amino acid supplements, carbon source, and abscisic acid and basal salt formulations were tested in an effort to modify existing soybean embryogenesis histodifferentiation/maturation media to further normalize the development of soybean somatic embryos. The resultant liquid medium, referred to as soybean histodifferentiation and maturation medium (SHaM), consists of FNL basal salts, 3% sucrose, 3% sorbitol, filter-sterilized 30 m*M* glutamine and 1 m*M* methionine. SHaM-derived somatic embryos are more similar to seed in terms of protein and fatty acid/lipid composition, and conversion ability, than somatic embryos obtained from traditional soybean histodifferentiation and maturation media.

Keywords *Glycine max* · Somatic embryogenesis · Glutamine · Methionine · Oil

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Introduction

Soybean [*Glycine max* (L.) Merrill] has become one of the world's most important crops due to the high content of oil and protein in its seed. Therefore, soybean genomics efforts are focusing on seed traits (Stacey et al. 2004). The ability to use transgenic soybean somatic embryos for the efficient screening of seed-specific transgene expression has long been recognized (Cho et al. 1995; Liu et al. 1996; Mazur et al. 1999) and demonstrated for both oil (Cahoon et al. 1999; 2000; Marillia et al. 2002) and protein (Herman et al. 2003) traits. An extension of this concept is the use of soybean somatic embryos for reverse genetics approaches targeted towards seed-specific traits.

The greatest limitation to the widespread use of somatic embryos for the analysis of seed-specific transgene expression or reverse genetics is that somatic embryos do not always parallel the development of their zygotic counterparts. Zimmerman (1993) long ago noted that somatic embryogenesis tended to deviate from zygotic embryogenesis in the later stages of development, primarily the maturation stage. This stage would be the most relevant to any genomics program using somatic embryos as a tool to study either seed development or the accumulation of storage reserves.

From research done on alfalfa (*Medicago sativa* L.), the legume in which somatic embryogenesis has been characterized to the greatest extent, storage reserve content is correlated with conversion ability and somatic embryo size (Lai and McKersie 1994b; Fujii et al. 1990). The lack of proper storage reserves appears to be the single greatest factor affecting seedling vigor (Lai et al. 1995).

It has been pointed out that developmental shortcomings of somatic embryos are most likely due to inadequate tissue culture protocols or the composition of culture media (Merkle et al. 1994). This investigation was initiated to further optimize a medium for histodifferentiation and maturation of soybean somatic embryos, with the goal of obtaining higher conversion rates in the shortest time, with embryos that more closely resemble seed in terms of storage reserves.

Materials and methods

Plant material and culture conditions

Repetitive somatic embryos were induced from aseptically isolated immature cotyledons by placement on MSD40 medium and subsequently maintained on MSD20 as previously described (Bailey et al. 1993; Walker and Parrott 2001). Three varieties were used: 'Jack,' 'Benning' and 'PI 417138'. 'Jack' was used for the initial evaluation of all treatments, and the other two varieties were used to verify that the optimal treatment was also applicable to other genotypes (Experiment 5). Embryos were induced, maintained and matured under a light intensity of 10 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes and a 23-h photoperiod at 25°C. For treatments using liquid medium, 10 mg globular-stage embryos per culture vessel were used as starting material for the maturation medium treatments described below. Clusters of embryos were gently separated by pressing against the side of a baffled 125-ml Erlenmeyer flask containing 35 ml medium. Liquid cultures were continuously shaken at 130 rpm.

All experiments involved modifications to the FNLS3S3 soybean embryo maturation medium of Walker and Parrott (2001), which contains FN Lite macro salts (Samoylov et al. 1998a), Murashige-Skoog (MS; Murashige and Skoog 1962) micro salts, B5 vitamins, 1 g l^{-1} L-asparagine, 3% sucrose (Samoylov et al. 1998b) and 3% sorbitol. After 30 days in any given medium treatment, the response of embryos was assessed on the basis of four parameters: 1) the number of cotyledonary-stage embryos recovered from 10 mg starting material; 2) the average weight (in milligrams) of the resulting cotyledonary-stage embryos; 3) the proportion of initial embryos that matured to the cotyledonary stage and subsequently successfully germinated, transferred to soil and the first trifoliolate leaf expanded—represented as percent conversion; 4) the average number of days taken by the embryos to pass through all the stages to a plant in soil (Bailey et al. 1993). To assess the ability to convert into a plant (parameters 3 and 4), 40 embryos from each treatment underwent a week-long desiccation period after removal from maturation medium and were then placed on MSO germination medium (Bailey et al. 1993). All chemicals were tissue culture grade and were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com).

Experiment 1: Effect of different carbon source

Three percent maltose was substituted for 3% sucrose in the liquid FNLS3S3 maturation medium. Embryo differentiation and maturation were compared to that obtained in liquid FNLS3S3 maturation medium containing 3% sucrose. Since the molecular weight of both carbohydrates is very similar, no significant effect on the osmotic potential of the medium would be expected. The effects of sucrose have been extensively studied in soybean, and found to be important (Samoylov et al. 1998a).

Experiment 2: Effect of amino acid supplements

The need for asparagine in FNLS3S3 maturation medium was studied by evaluating histodifferentiation and maturation abilities of embryos grown in the following modifications of FNLS3S3: 1) without asparagine; or with asparagine replaced with 2) 6; 3) 30; 4) 60; or 5) 120 m*M* glutamine. All glutamine was filter-sterilized and added to the medium after autoclaving. The same treatments (2–5) were also performed using pyroglutamic acid instead of glutamine.

In a similar manner, the effect of methionine on embryo differentiation and maturation was initially studied by adding 2 m*M* methionine, then optimized by adding either 1, 3 or 5 m*M* to FNLS3S3 liquid maturation medium without asparagine and supplemented with the previously determined optimum level of 30 m*M* filter-sterilized glutamine. The effect of 5% stachyose was also evaluated, with or without the 30 m*M* glutamine.

Experiment 3: Effect of abscisic acid

The effect of abscisic acid (ABA) on soybean embryo maturation was studied by adding increasing amounts (10, 50, or 100 μ*M*) to liquid FNLS3S3 maturation medium supplemented with the previously determined optimum level of both 30 m*M* filter-sterilized glutamine and 1 m*M* methionine. ABA was added to the maturation medium at the end of the 30-day maturation phase. The effect of ABA was assessed 14 days later.

Experiment 4: Effect of basal salts

The effectiveness of two basal salts, namely MS and FN Lite macro salts, was compared using the factors found to influence liquid maturation medium, specifically 1) 6% maltose; 2) 3% sucrose and 3% sorbitol, and 3) the addition of both 30 m*M* glutamine and 2 m*M* methionine, using pairwise comparisons. Embryos were assessed for their ability to undergo histodifferentiation and maturation. Unlike the above mentioned liquid media, all media in this experiment were solidified with 2 g l^{-1} GelRite. In each case, 20 clusters (5 mg per cluster, each 2–3 mm in diameter) of globular-stage embryos were placed per Petri dish per replication for each medium treatment for 30 days. Embryos were then subcultured and assessed after a further 30 days.

Experiment 5: Applicability to additional genotypes

The liquid soybean embryo histodifferentiation and maturation medium developed in this study, namely FNLS3S3

without the 1 g l^{-1} asparagine in the original formula, but supplemented instead with 30 m*M* glutamine and 1 m*M* methionine, hereafter referred to as SHaM (soybean histodifferentiation and maturation), was tested for effectiveness with two other varieties, Benning and PI417138. Standard soybean somatic embryo histodifferentiation and maturation media were used for comparison: the solid MSM6 [MS salts, 6% maltose, 0.5% activated charcoal pH 5.8] and the liquid FNLS3S3 [FNL salts, 3% sucrose, 3% sorbitol, pH 5.8]. The newly modified medium was tested in both liquid and solid versions (2 g l^{-1} GelRite). To verify the fertility of the regenerated plants, five plants from each genotype in each treatment were allowed to flower and set seed.

Total soluble protein analysis

Total soluble protein was extracted from soybean seeds or embryos matured in either SHaM or MSM6 medium with an extraction buffer consisting of Tris-EDTA pH 7.4, and 0.1% sodium dodecyl sulphate (SDS) (Sambrook et al. 1989). Protein pellets were dissolved in sample buffer [50 m*M* Tris HCl, pH 6.8, 2% (w/v) SDS, 0.7 *M* β-mercaptoethanol, 0.1% bromphenol blue and 10% glycerol; Sambrook et al 1989] denatured at 95°C for 5 min and briefly centrifuged. Protein content was determined by Bradford (1976) assay. Thirty μg protein were loaded onto 12% SDS-PAGE gels and were electrophoretically separated. Protein gels were stained in 0.1% Coomassie Brilliant Blue R250 in 40% methanol, 10% acetic acid overnight, and de-stained for approximately 3 h in 40% methanol, 10% acetic acid.

Fatty acid content analysis

Single somatic embryos from cultures matured in MSM6 or SHaM medium were lyophilized and weighed in 13×100 mm screw cap glass test tubes. A measured amount of triheptadecanoin (http://www.nu-chekprep.com) dissolved in toluene was then added to each tube as an internal standard. An additional amount of toluene was added to the tubes to a final volume of 150 μl, along with 1 ml 2.5% (v/v) sulfuric acid in methanol. The tubes were capped under nitrogen and heated at 95°C for 2 h. Upon cooling, fatty acid methyl esters were extracted with the addition of 1 ml 1 *M* NaCl and 1 ml hexane. Following mixing and centrifugation, the hexane layer, containing fatty acid methyl esters, was analyzed with an Agilent 6890N gas chromatograph. Fatty acid methyl esters were resolved with a 30 m ×0.25 mm (inner diameter) INNOWax column (http://www.chem.agilent.com) and detected by flame ionization. The oven temperature was programmed from 185°C (1 min hold) to 230°C (3 min hold) at a rate of 7°C min^{-1}. Fatty acid content was determined by comparing the detector response of the total fatty acid methyl esters from the soybean embryos with that of methyl heptadecanoate from the internal standard. For analyses of the fatty acid content of mature seeds, the seed coat was removed from single seeds, and the seeds were then homogenized to a fine powder using a mortar and pestle. A portion of the powdered seed (approximately 20 mg) was transferred to a 13×100 mm screw cap tube, lyophilized and weighed. Fatty acid methyl esters were prepared and analyzed as described for somatic embryos.

Lipid composition analysis

Lipids were extracted from soybean somatic embryos essentially as described by Bligh and Dyer (1959). Soybean embryos (0.5 g fresh weight) were homogenized in 2 ml methanol:chloroform (2:1 v/v) in a glass screw cap test tube by use of a glass stirring rod. Following 30 min of incubation in this solvent, 1 ml chloroform and 1.4 ml water were added. The sample was mixed thoroughly and spun for 10 min in a clinical centrifuge (500 *g*) to effect a two phase separation. The recovered organic layer was dried under nitrogen and resuspended in 250 μl chloroform:methanol (6:1 v/v). A 1 μl aliquot of the lipid extract from MSM6- and SHaM-derived embryos was applied to a silica gel 60 thin layer chromatography (TLC) plate (http://www.merck.com). Lipids were resolved by incubation of the TLC plate in a mobile phase consisting of heptane:ethyl ether:acetic acid (60:40:1 v/v/v). Lipid classes were detected by incubation of the dried plate in iodine vapor.

For measurement of the relative content of triacylglycerols and polar lipids (primarily phospholipids) in embryos, lipid classes were resolved by TLC as described above. Triacylglycerols and polar lipids were identified by light staining with iodine vapor. Silica gel from zones of the TLC plate containing the lipid classes were scraped into a 13×100 mm glass screw cap test tube containing 1 ml 2.5% (v/v) sulfuric acid in methanol and a measured amount of triheptadecanoin. The tubes were then capped under nitrogen and incubated at 95°C for 45 min. Upon cooling, fatty acid methyl esters were recovered and analyzed by gas chromatography as described above.

Statistical analyses

Experiments 1 and 5 had three and five replicates performed, respectively. In these experiments, all four parameters were tested in all replicates, making statistical analysis for all values possible. For experiments 2–4, three replicates were performed to evaluate parameters 1 and 2 as described above, yet only a subset of 40 embryos per treatment were carried through to the plant stage (parameters 3 and 4) making the size of the data set for these latter variables for those experiments insufficient for statistical analysis. Means and significance were determined by one way analysis of variance (ANOVA) using PC-SAS (http://www.sas.com). Comparisons between the mean values were made by the least significant difference (LSD).

Results and discussion

Currently, there are two media and two protocols for the histodifferentiation and maturation of soybean somatic embryos. The first of these, MSM6 (Bailey et al. 1993), is a two-step process, whereby embryos are first placed on solidified MS basal salts supplemented with 6% maltose and 0.5% activated charcoal. After 30 days, embryos are transferred to the same medium, but without the charcoal. The second medium was initially termed FNLS3, and consisted of basal Finer and Nagasawa "Lite" (FNL) salts supplemented with 3% sucrose (Samoylov et al 1998b). Its main advantage was its ability to produce large numbers of somatic embryos from very little tissue in a short amount of time, but it suffered from the fact that very few embryos would convert into plants (Samoylov et al. 1998b). This limitation was partially solved through the addition of 3% sorbitol to the medium (Walker and Parrott 2001), now called FNLS3S3 medium.

Experiment 1: Effect of different carbon source

Carbon source is critical for embryo health, and requirements appear to differ between solid and liquid media. While maltose has commonly been used in solid medium, in liquid medium it has been reported to be detrimental for somatic embryos of alfalfa (Anandarajah and McKersie 1990) and soybean (Samoylov et al. 1998a). Reports of optimal sucrose concentration range from 2.5% (Lippmann and Lippmann 1993) to 5% (Saravitz and Raper 1995). Since maltose has been the carbon source of choice in solid maturation media, the effect of using maltose as a carbon source in liquid maturation medium was tested.

Overall the use of maltose instead of sucrose in maturation media resulted in smaller, and fewer, embryos per milligram of initial tissue (Table 1). However, the conversion rate of embryos differentiated on maltose was twice as high compared to those obtained on sucrose. Thus, it is possible to obtain 61% more plants per milligram of initial tissue (5.6 vs 3.4) when maltose is used instead of sucrose. Nevertheless, embryos grown on maltose required 27% more time to reach physiological maturity, negating any real advantage in the use of maltose over sucrose. Hence, sucrose was the carbon source of choice in all subsequent optimization of soybean histodifferentiation and maturation medium experiments.

Experiment 2: Effect of amino acid supplements

The nitrogen source is another critical component for proper embryo maturation. The original FN liquid medium (Finer and Nagasawa 1988) designed for soybean embryogenic cultures used 1 g l^{-1} asparagine (7.56 m*M*), which was maintained as a constant parameter during the development of FNL medium (Samoylov et al. 1998a). However, various reports on the culture of zygotic soybean embryos report that glutamine (Thompson et al. 1977) is a beneficial supplement. Glutamine is known to increase the size of cultured zygotic embryos (Lippmann and Lippmann 1993; Dyer et al. 1987), and to increase their synthesis of oil and protein storage reserves (Saravitz and Raper 1995). However, reports of optimal glutamine levels for soybean zygotic embryos range from 6 m*M* (Saravitz and Raper 1995) to 30 m*M* (Dyer et al. 1987). Likewise, pyroglutamic acid, formed by autoclaving glutamine (Lai and McKersie 1993; Lai et al. 1992), can increase embryo size in alfalfa (Lai and McKersie 1994a). Methionine supplements have also been reported to be helpful for growth stimulation (Coker et al. 1987).

A comparison was made between asparagine and glutamine at levels equivalent to 1 g l^{-1}. The results, as evident in Fig. 1a, showed that glutamine is better able than asparagine to promote larger cotyledonary-stage embryos. Due to the positive response with the addition of 1 g l^{-1} (6.84 m*M*) glutamine, a series of glutamine concentrations (6, 30, 60, 120 m*M*) was tested to optimize embryo maturation. The effects are shown pictorially in Fig. 1c and numerically in Table 2. The average mass of

Table 1 Effect of carbohydrate source on histodifferentiation in liquid medium. Means followed by the same letter within each column are not significantly different at the 5% level by LSD

Carbon source	Number of cotyledonary-stage embryos/10 mg tissue	Average weight (mg)/embryo	Percent conversion (%)	Average days to soil
Sucrose	114 a	26.0 a	39 b	30.0 b
Maltose	85 a	22.9 a	54 a	66.0 a

Table 2 Effect of amino acid supplements on histodifferentiation and maturation in liquid medium. Means followed by the same letter within each column are not significantly different at the 5% level by LSD

FNL0S3S3 +	Number of cotyledonary-stage embryos per 10 mg tissue	Average weight (mg)/embryo	Percent conversion	Average days to soil
7.5 m*M* Asn	132 a	11.7 bc	35	22.8
Without Asn	102 a	8.1 c	17	28.7
+6 m*M* Gln	152 a	16.2 b	20	25.7
+30 m*M* Gln	108 a	25.0 a	27	23.1
+60 m*M* Gln	107 a	38.5 a	30	19.9
+120 m*M* Gln	145 a	13.5 bc	17	23.1

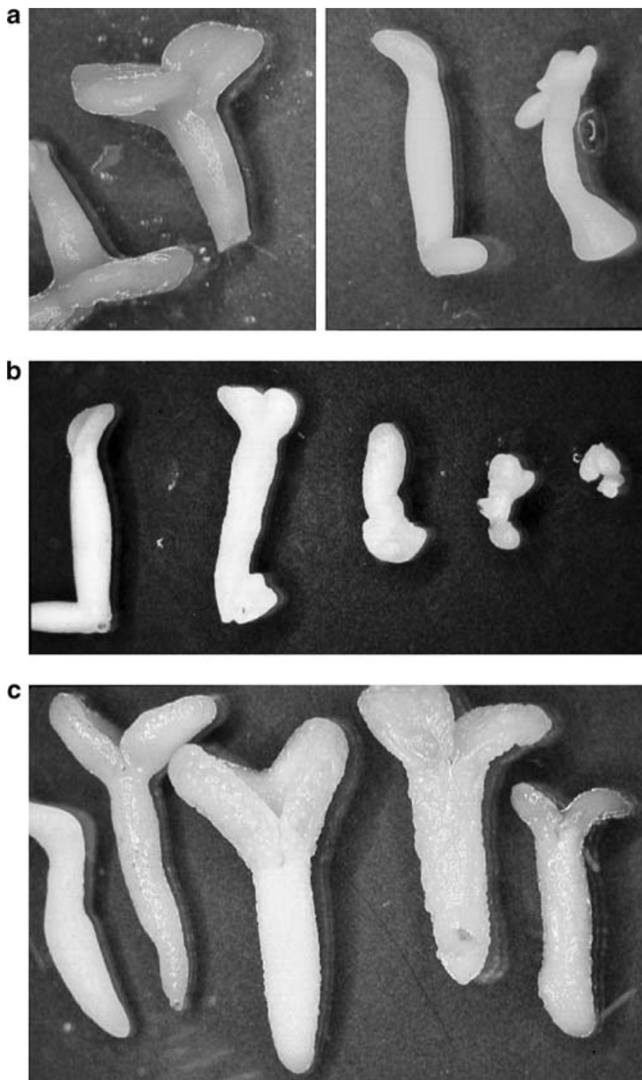

Fig. 1 **a** Representative cotyledonary-stage embryos from FNLS3S3 liquid medium supplemented with 1 g^{-1} filter-sterilized glutamine (*left*) or asparagine (*right*). **b** *Left* to *right*: 0, 6, 30, 60, and 120 m*M* pyroglutamic acid. **c** *Left* to *right*: 0, 6, 30, 60, and 120 m*M* filter-sterilized glutamine

cotyledonary-stage embryos was used as a measure of quality and thus as an indication of an increased likelihood of the ability of an embryo to germinate into a fertile plant. The 30 m*M* glutamine treatment was deemed optimum as it produced embryos that were significantly larger. In contrast, the presence of pyroglutamic acid was detrimental to embryo growth (Fig. 1b) at all concentrations.

Although supplementation with 30 m*M* filter-sterilized glutamine was considered optimal for overall quality and size of mature embryos, it delayed physiological maturity by 1–2 weeks. A similar delay in maturation of soybean zygotic embryos due to the incorporation of glutamine has been reported (TeKrony et al. 1979). The result of adding increasing amounts of methionine (1, 3, or 5 m*M*) to FNL0S3S3 medium supplemented with 30 m*M* glutamine is shown in Table 3. There is a clear benefit to the addition of methionine, manifested primarily in percent conversion. Based on these findings, 1 m*M* methionine was added to all subsequent embryo histodifferentiation and maturation media.

Stachyose was tested, as it has been reported to be essential for proper desiccation tolerance in soybean (Blackman et al. 1992), and thus is important for physiological maturity. The one study carried out in alfalfa found stachyose levels to be lower during maturation in somatic embryos than in zygotic embryos (Horbowicz et al. 1995), suggesting that stachyose supplements might improve the proper maturation of soybean somatic embryos. However, there was no discernible effect due to stachyose with or without glutamine (data not shown), so it was omitted from further work.

Experiment 3: Effect of ABA

The addition of ABA was tested to see if it would further optimize the maturation medium, specifically to counteract the delay in maturation time that results from the addition of glutamine. In this work, the soybean somatic embryos treated with ABA appeared more yellow in color, usually an indication of physiological maturity (Tekrony et al. 1979), but ABA did not have a significant effect on any parameter evaluated (Table 4).

Tian and Brown (2000) previously reported that ABA promoted embryo development and maturation. The discrepancy in the apparent effects of ABA may lie in the timing of application. Tian and Brown (2000) applied ABA at the globular stage of development, while in this work ABA was applied during the maturation stage. Likewise, studies on microspore-derived embryos of *Brassica* found that treatments of 10 μ*M* ABA stimulated production of both protein (Wilen et al. 1990) and oil (Holbrook et al. 1992) reserves to levels comparable to those found within zygotic embryos. Yet other studies on *Brassica* microspore embryos found ABA to be ineffective or deleterious (Huang et al. 1991).

The use of osmotica in maturation media is a common element across plant species (Xu et al. 1990; Brisibe and Miyake 1994; Finkelstein and Somerville 1989). Proper osmotic pressure triggers the production of ABA, which in turn triggers physiological maturity, that is, the accumulation of storage reserves and the acquisition of desiccation tolerance in plant embryos (Brisibe and Miyake 1994; Kermode 1995), including cultured zygotic soybean embryos (Egli 1990; Blackman et al. 1995; 1991). The osmotic pressure of this developmental medium (Samoylov et al. 1998b) could have obviated the effect of exogenous applications of ABA.

Experiment 4: Effect of basal salts

Prior to this investigation, the two soybean histodifferentiation and maturation media used were the liquid FNLS3S3 and the two-step solid MSM6. As seen in Table 5, improvements to the efficiency of solid histodifferentiation

Table 3 Effect of methionine supplements on histodifferentiation and maturation in liquid medium. Means followed by the same letter within each column are not significantly different at the 5% level by LSD

FNL0S3S3G30 +	Number of cotyledonary-stage embryos per 10 mg tissue	Average weight (mg) per embryo	Percent conversion	Average days to soil
0 m*M* Met	115 a	36.28 a	27	27.6
1 m*M* Met	100 a	40.04 a	60	26.5
3 m*M* Met	94 a	32.85 a	40	26.0
5 m*M* Met	108 a	40.69 a	32	26.2

Table 4 The effect of abscisic acid (ABA) on histodifferentiation and maturation in liquid medium. Means followed by the same letter within each column are not significantly different at the 5% level by LSD test

FNL0S3S3G30M1 +	Number of cotyledonary-stage embryos per 10 mg tissue	Average weight (mg) per embryo	Percent conversion	Average days to soil
0 μ*M* ABA	133 a	23.07 a	67	24.1
10 μ*M* ABA	128 a	23.21 a	57	22.8
50 μ*M* ABA	117 a	26.34 a	60	23.5
100 μ*M* ABA	115 a	25.01 a	37	22.6

Table 5 Comparison of two basal salts, carbon source, osmoticum and amino acid supplements on histodifferentiation and maturation in solid medium. Means followed by the same letter within each column are not significantly different at the 5% level by LSD. *MS* Murashige and Skoog macro salts, *FNL* Finer and Nagasawa Lite macro salts, *M6* 6% maltose, *S3S3* 3% sucrose + 3% sorbitol, *G30* 30 μ*M* glutamine, *M2* 2 μ*M* methionine

Medium	Number of cotyledonary-stage embryos per 10 mg	Average weight (mg) per embryo	Percent conversion	Average days to soil
MSM6	72 a	5.47 bc	20	24.5
MSS3S3	61 ab	4.78 bc	30	20.8
MSS3S3G30M2	65 ab	4.13 c	47	23.6
MSM6G30M2	50 ab	5.59 b	55	24.1
FNLM6	52 ab	5.62 b	25	23.5
FNLS3S3	51 ab	4.82 bc	35	24.5
FNLS3S3G30M2	57 ab	5.31 bc	57	22.6
FNLM6G30M2	46 b	7.93 a	35	23.7

Table 6 Comparison of modified histodifferentiation and maturation medium (SHaM) with MSM6 and FNLS3S3 in both liquid (*L*) and solid (*S*) phase across three genotypes: 'Benning', 'PI1417138', and 'Jack'. Means followed by the same letter within each column are not significantly different at 5% level by LSD

	Number of cotyledonary-stage embryos per 10 mg	Average weight (mg)/embryo	Percent conversion (%)	Average days to soil	Number plants/mg starting material
Benning					
FNLS3S3 (L)	156 a	9.7 d	31 c	28.3 a	4.8 ab
SHaM (L)	190 a	15.3 c	38 bc	25.3 a	7.2 a
MSM6 (S)	45 b	23.6 b	78 a	18.5 b	3.5 b
SHaM (S)	45 b	29.2 a	55 b	20.1 b	2.5 b
PI1417138					
FNLS3S3 (L)	138 b	4.9 c	56 ab	26.2 a	7.7 ab
SHaM (L)	201 a	11.5 b	50 b	25.3 a	10.1 a
MSM6 (S)	62 c	7.5 bc	72 ab	21.3 b	4.5 b
SHaM (S)	56 c	19.7 a	75 a	19.8 b	4.2 b
Jack					
FNLS3S3 (L)	180 a	9.3 c	19 b	25.6 a	3.4 ab
SHaM (L)	147 a	20.6 b	33 ab	23.9 a	4.9 a
MSM6 (S)	57 b	16.4 b	34 ab	21.8 a	1.9 b
SHaM (S)	56 b	30.6 a	60 a	23.1 a	3.4 ab

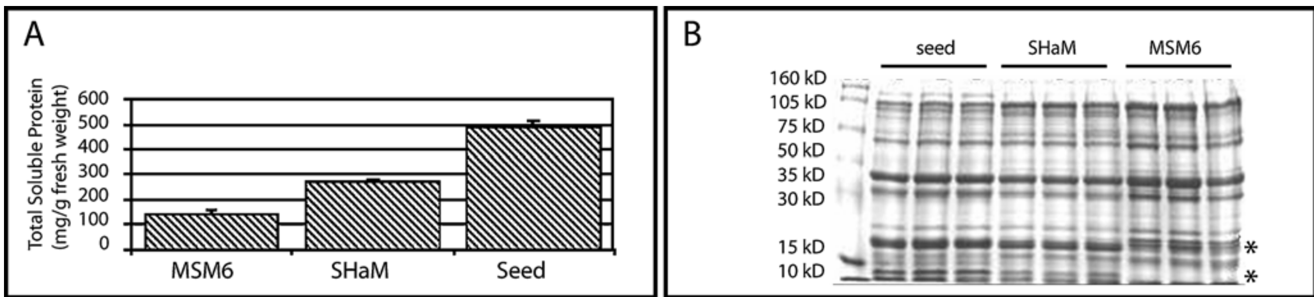

Fig. 2A, B Comparison of total soluble protein of soybean seeds, and embryos matured on either MSM6 solid medium or SHaM liquid medium. **A** Quantification of total soluble proteins from seeds, MSM6 embryos and SHaM embryos. Values represent the mean of 12 replicates (±SE). **B** Total protein profile of seeds (lanes *1–3*), embryos matured in SHaM medium (lanes *4–6*), and embryos matured in MSM6 medium (lanes *7–9*). * Notable differences in protein profile between embryos matured on MSM6 and seeds

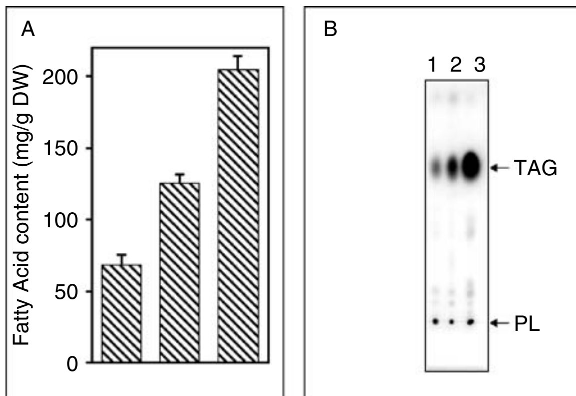

Fig. 3A, B Comparison of fatty acid content and lipid composition of soybean seeds, and embryos matured on either MSM6 solid medium or SHaM liquid medium. **A** Quantification of fatty acid content of mature seeds and MSM6- and SHaM-derived embryos. Values represent the mean of 15 replicates (±SE). **B** Thin layer chromatographic (TLC) analysis of lipid composition. An equal volume of lipid extract from 0.5 g fresh weight of MSM6- and SHaM-derived embryos was applied to the TLC plate and stained with iodine vapor. Lanes:*1* MSM6 embryos, *2* SHaM embryos, *3* mature seeds. *TAG* Triacylglycerols, *PL* polar lipids (primarily phospholipids)

and maturation medium were made by using the ingredients from the optimized liquid medium recipe—specifically FNLS3S3 supplemented with 30 m*M* glutamine and 2 m*M* methionine (Table 5). This experiment was already underway when the optimum concentration of methionine was determined to be 1 m*M*, which is why an educated estimate (2 m*M*) of the optimum concentration was used.

Experiment 5: Applicability to additional genotypes

Three varieties were used to evaluate the effectiveness of SHaM. The results in Table 6 show that, across all genotypes tested, the newly modified SHaM medium yielded better results. All solidified media consistently produced an order of magnitude fewer embryos per unit tissue and took an extra 2 weeks to complete the histodifferentiation and maturation process. Although conversion frequency tended to be higher in solidified media, liquid media compensated by producing more somatic embryos, such that the number of plants regenerated per milligram of starting tissue was consistently higher from liquid SHaM. All treatments were equal in terms of ability to produce fertile plants.

Analyses of storage reserves

The total amount of soluble protein per unit mass was significantly higher in SHaM embryos than in MSM6 embryos (Fig. 2a), making it possible for a number of protein analyses to be conducted with a relatively small amount of tissue. Furthermore, the overall soluble protein profile of SHaM embryos better resembled that of a seed than did MSM6 embryos (Fig. 2b). Likewise, both the fatty acid content (Fig. 3a) and lipid profile (Fig. 3b) of SHaM-embryos were improved over those from MSM6-derived embryos. The fatty acid content of SHaM embryos was found to be 125±7.0 $\mu g\ mg^{-1}$ dry weight, compared to 67.8±7.4 $\mu g\ mg^{-1}$ dry weight for MSM6 embryos, but still lower than the 205±9.0 $\mu g\ mg^{-1}$ dry weight found in seed. In a similar fashion, SHaM embryos, like seeds, were found to be enriched in triacylglycerols (TAG) and the relative TAG content is considerably greater in SHaM embryos than in MSM6 embryos. Specifically, the relative TAG content in the total lipid extract was found to be 95% in seeds, 88% in SHaM embryos and 70% in MSM6 embryos.

Conclusions

Previous results (Samoylov et al. 1998b; Walker and Parrott 2001) had shown the importance of osmoticum for proper soybean somatic embryo maturation. The present work shows that organic nitrogen supplements promote accumulation of storage reserves. This role may be of even greater importance for embryos, which normally store high levels of protein, as is the case with soybean. In terms of soluble protein, and lipid content and composition, SHaM-derived soybean somatic embryos are a better model for the seed than are MSM6-derived embryos. Thus, SHaM medium is useful for the study of

seed-specific traits. Nevertheless, as is evident from Figs. 2 and 3, even those embryos obtained from SHaM medium are not completely comparable to seed, in terms of both quantity and quality of the oil and protein. Future medium manipulations are still needed to produce somatic embryos that mimic their zygotic counterparts even more closely.

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References

Anandarajah K, McKersie BD (1990) Enhanced vigor of dry somatic embryos of *Medicago sativa* L. with increased sucrose. Plant Sci 71:261–266

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

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

Blackman SA, Obendorf RL, Leopold AC (1995) Desiccation tolerance in developing soybean seeds: the role of stress proteins. Physiol Plant 93:630–638

Blackman SA, Wettlaufer SH, Obendorf RL, Leopold AC (1991) Maturation proteins associated with desiccation tolerance in soybean. Plant Physiol 96:868–874

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brisibe EA, Miyake H (1994) Abscisic acid and high osmoticum regulation of development and storage reserve accumulation in sugarcane somatic embryos. Jpn J Crop Sci 63:689–698

Cahoon EB, Carlson TJ, Ripp KG, Schweiger BJ, Cook GA, Hall SE, Kinney AJ (1999) Biosynthetic origin of conjugated double bonds: production of fatty acid components of high-value drying oils in transgenic soybean embryos. Proc Natl Acad Sci USA 96:12935–12940

Cahoon EB, Marillia E-F, Stecca KL, Hall SE, Taylor DC, Kinney AJ (2000) Production of fatty acid components of meadowfoam oil in somatic soybean embryos. Plant Physiol 124:243–251

Cho M-J, Widholm JM, Vodkin LO (1995) Cassettes for seed-specific expression tested in transformed embryogenic cultures of soybean. Plant Mol Biol Rep 13:255–269

Coker GTI, Garbow JR, Schaefer J (1987) ^{15}N and ^{13}C NMR determination of methionine metabolism in developing soybean cotyledons. Plant Physiol 83:698–702

Dyer DJ, Cotterman CD, Cotterman JC (1987) Comparison of in situ and in vitro regulation of soybean seed growth and development. Plant Physiol 84:298–303

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

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

Finkelstein R, Somerville C (1989) Abscisic acid or high osmoticum promote accumulation of long-chain fatty acids in developing embryos of *Brassica napus*. Plant Sci 61:213–217

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

Herman EM, Helm RM, Jung R, Kinney AJ (2003) Genetic modification removes an immunodominant allergen from soybean. Plant Physiol 132:36–43

Holbrook LA, Magus JR, Taylor DC (1992) Abscisic acid inductin of elongase activity, biosynthesis and accumulation of very long chain monounsaturated fatty acids and oil body proteins in microspore-derived embryos of *Brassica napus* L. cv. Reston. Plant Sci 84:99–115

Horbowicz M, Obendorf RL, McKersie BD, Viands DR (1995) Soluble saccharides and cyclitols in alfalfa (*Medicago sativa* L.) somatic embryos, leaflets, and mature seeds. Plant Sci 109:191–198

Huang B, Bird S, Kemble R, Miki B, Keller W (1991) Plant regeneration from microspore-derived embryos of *Brassica napus*: effect of embryo age, culture temperature, osmotic pressure, and abscisic acid. In Vitro Cell Dev Biol Plant 27P:28–31

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

Lai F-M, Lecouteux CG, McKersie BD (1995) Germination of alfalfa (*Medicago sativa* L.) seeds and desiccated somatic embryos I. Mobilization of storage reserves. J Plant Physiol 145:507–513

Lai F-M, McKersie BD (1993) Effect of nutrition on maturation of alfalfa (*Medicago sativa* L.) somatic embryos. Plant Sci 91:87–95

Lai F-M, McKersie BD (1994b) Regulation of starch and protein accumulation in alfalfa (*Medicago sativa* L.) somatic embryos. Plant Sci 100:211–219

Lai F-M, McKersie BD (1994a) Regulation of storage protein synthesis by nitrogen and sulfur nutrients in alfalfa (*Medicago sativa* L.) somatic embryos. Plant Sci 103:209–221

Lai F, Senaratna T, McKersie BD (1992) Glutamine enhances storage protein synthesis in *Medicago sativa* L. somatic embryos. Plant Sci 87:69–77

Lippmann B, Lippmann G (1993) Soybean embryo culture: factors influencing plant recovery from isolated embryos. Plant Cell Tissue Organ Cult 32:83–90

Liu W, Torisky RS, McAllister KP, Avdiushko S, Hildebrand DF, Collins GB (1996) Somatic embryo cycling: evaluation of a novel transformation and assay system for seed-specific gene expression in soybean. Plant Cell Tissue Organ Cult 47:33–42

Marillia EF, Giblin EM, Covello PS, Taylor DC (2002) Expression of meadowfoam Des5 and FAE1 genes in yeast and in transgenic soybean somatic embryos, and their roles in fatty acid modification. Plant Physiol Biochem 40:821–828

Mazur B, Krebbers E, Tingey S (1999) Gene discovery and product development for grain quality traits. Science 285:372–375

Merkle SA, Parrott WA, Flinn BS (1994) Morphogenic aspects of somatic embryogenesis. In: Thorpe TA (ed) Somatic embryogenesis. CRC, Boca Raton, Fla., pp 155–203

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

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning—a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

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

Samoylov VM, Tucker DM, Parrott WA (1998b) Soybean [*Glycine max* (L.) Merrill] embryogenic cultures: the role of sucrose and total nitrogen content on proliferation. In Vitro Cell Dev Biol Plant 34:8–13

Saravitz CH, Raper CD Jr (1995) Responses to sucrose and glutamine by soybean embryos grown in vitro. Physiol Plant 93:799–805

Stacey G, Vodkin L, Parrott WA, Shoemaker RC (2004) National science foundation-sponsored workshop report. Draft plan for soybean genomics. Plant Physiol 135:59–70

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

Thompson JF, Madison JT, Muenster A-ME (1977) In vitro culture of immature cotyledons of soya bean (*Glycine max* L. Merr.). Ann Bot 41:29–39

Tian LN, Brown DCW (2000) Improvement of soybean somatic embryo development and maturation by abscisic acid treatment. Can J Plant Sci 80:721–276

Wilen RW, Mandel RM, Pharis RP, Holbrook LA, Moloney MM (1990) Effects of abscisic acid and high osmoticum on storage protein gene expression in microspore embryos of *Brassica napus*. Plant Physiol 94:875–881

Walker DR, Parrott WA (2001) Effect of polyethylene glycol and sugar alcohols on soybean somatic embryo germination and conversion. Plant Cell Tissue Organ Cult 64:55–62

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

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5:1411–1423