

## Development of an embryogenic suspension culture of soybean (*Glycine max* Merrill.)

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**Abstract.** A rapidly growing, maintainable, embryogenic suspension culture of *Glycine max* L. Merrill. has been generated. The culture consisted almost entirely of clumps of proliferating globular embryos with very little nonembryogenic tissues. The number and size of somatic embryo clumps were used to quantify growth of embryogenic tissues under various conditions. Initiation and proliferation of this embryogenic suspension culture were dependent on the inoculum, method of subculture, and composition of the subculture medium. Twenty to 50 mg of highly embryogenic, early-staged soybean tissue were inoculated into 35 ml of liquid culture medium containing  $5 \text{ mg l}^{-1}$  2,4-D and either 15 mM glutamine or preferably 5 mM asparagine. Suspension cultures were subcultured at the same inoculum density every 4 weeks. The embryos matured and germinated following placement on solid media, resulting in consistent plant regeneration.

### Introduction

Plant tissue culture systems have been developed for soybean (*Glycine max* L. Merrill.) which utilize either shoot morphogenesis [2, 16, 17] or somatic embryogenesis [4, 12] as the route for regeneration. For shoot morphogenesis, either cotyledonary node [2, 16] or primary leaf tissue [17] was used to obtain cultures which formed shoots when placed on a medium containing benzyladenine. For somatic embryogenesis, the starting material was the immature zygotic embryo. The explant was either the intact zygotic embryo [12], the excised embryo axis [4], or the excised cotyledons [8].

Christianson et al. [4] were the first to report regeneration of soybean. Their report described a system in which embryogenic callus tissue was initially obtained from the zygotic embryo axis and maintained by recurrent selection. One piece of embryogenic callus, which was covered with small

embryoids, was used to initiate an embryogenic suspension culture. Replacement of the 2 nitrogen salts in Murashige and Skoog (MS) [10] salts with 20 mM ammonium citrate (N-amended medium) was necessary for the establishment of both the embryogenic callus culture and the embryogenic suspension culture. Even though this was the earliest report of regeneration of soybean, there have been no subsequent reports on this or any other soybean embryogenic suspension culture system. The methods for initiation and maintenance of the system described by Christianson et al. [4] are very different from those described in this report.

An embryogenic suspension culture system for soybean is beneficial for selection work and possibly genetic transformation. Due to the high medium-to-tissue contact, selection systems are more rigorous and growth is faster than tissue on solid media. In addition, soybean plant tissues which can be transformed by *Agrobacterium* [11] are not currently capable of regeneration. Either nonregenerable tissue is transformed or the transformation process diminishes the regeneration capacity of that tissue. Protoplast transformation systems have been reported for soybean using both *Agrobacterium* [3] and naked DNA [9] but the source tissue and the protoplast-derived tissues were not regenerable. Embryogenic suspension cultures have been shown to be a superior source of regenerable protoplasts in other systems [18]. An embryogenic suspension culture system of soybean may be the best source of regenerable, transformable protoplasts.

In this present report, highly embryogenic soybean tissue, at an early ontogenetic stage ('early-staged'), was used to initiate embryogenic suspension cultures of soybean. A low inoculum density was beneficial for both initiation and continued maintenance. The suspension culture medium was optimized for growth of embryogenic tissue with beneficial effects from amino acid additions. Plants were regenerated by sequential transfers of the suspension culture tissue on hormone-free solidified media.

## Materials and methods

### *Initiation of embryogenic callus*

Soybean plants (*Glycine max* L. Merrill. cv. Fayette) were grown in greenhouses under natural lighting from April to October and supplemental metal halide lighting (with a 12/12 h light/dark photoperiod) from November to March. Seven to 14 days after flowering, pods were removed and the immature seeds excised. Immature seeds were surface-sterilized for 20 min in

a 20% solution of commercial bleach containing 0.05% Tween-20. Following 3 rinses in sterile, distilled water, the cotyledons were removed from the seed for culture according to Lazzeri et al. [8].

Proliferative embryogenic tissue of soybean was obtained according to Finer [5]. Cotyledon halves were placed on a medium containing MS salts [10], Gamborg's B5 vitamins [7], 6% sucrose,  $40 \text{ mg l}^{-1}$  2,4-D, and 0.8% agar (pH 5.7). Cultures were placed at  $28^\circ\text{C}$  with a 16/8 h light/dark photoperiod with a light intensity of  $30 \mu\text{Em}^{-2} \text{ s}^{-1}$ . Embryos and embryogenic tissue were subcultured every 4 to 6 weeks on this medium.

#### *Initiation of embryogenic suspension cultures*

Twenty to 50 mg of early-staged, highly embryogenic callus were placed into 35 ml of suspension culture medium contained in a 125 ml DeLong flask. Flasks were capped with stainless steel closures, sealed with parafilm, and agitated at 150 rpm. Suspension cultures were placed at  $28^\circ\text{C}$  with a 16/8 h light/dark photoperiod with a light intensity of  $30 \mu\text{Em}^{-2} \text{ s}^{-1}$ . The suspension culture medium (10A40N medium) initially consisted of modified MS salts [10], Gamborg's B5 vitamins [7],  $5 \text{ mg l}^{-1}$  2,4-D, 6% sucrose, and 15 mM glutamine (pH 5.7). The MS nitrogen was replaced with 10 mM  $\text{NH}_4\text{NO}_3$  and 30 mM  $\text{KNO}_3$  and the medium was sterilized by autoclaving. Suspension cultures were maintained at  $28^\circ\text{C}$  in the light with a 4 week subculture period. For subculture, 20 to 50 mg of embryogenic tissue were placed into 35 ml fresh 10A40N medium.

#### *Optimization of growth of embryogenic suspension cultures*

The suspension culture medium was initially formulated as described above. The following variables (outlined in Table 1) were tested in the following order and the medium was optimized in a step-wise manner: auxin, cytokinin, nitrogen substitution, amino acids. The contents of one flask, which contained a large quantity of proliferative embryogenic tissues, were used to inoculate all of the flasks in a given experiment. Flasks containing proliferative embryogenic tissues were selected by direct observation of the tissue in the flasks using an Olympus IMT-2 inverted microscope. Embryogenic tissues were characteristically yellow-brown in color, dense, and had a smooth surface. Three flasks were inoculated for each medium tested.

Embryogenic suspension cultures proliferated as clumps of globular embryos. Assuming that these clumps were perfect spheres and using the formula for calculating the volume of a sphere,  $4/3(\pi r^3)$ , an approximation

Table 1. Suspension culture media addenda/modifications.

Auxin	2,4-D	0.5, 1, 5, 10, 25 mg l <sup>-1</sup>
	2,4,5-T	0.5, 1, 5, 10, 25 mg l <sup>-1</sup>
	picloram	1, 5, 10, 25, 50 mg l <sup>-1</sup>
	dicamba	1, 5, 10, 25, 50 mg l <sup>-1</sup>
Cytokinin	BA	0.1, 1, 10 mg l <sup>-1</sup>
	kinetin	0.1, 1, 10 mg l <sup>-1</sup>
	2ip	0.1, 1, 10 mg l <sup>-1</sup>
Nitrogen	MS <sup>a</sup> nitrogen + 15 mM glutamine	
	B5 <sup>b</sup> nitrogen + 15 mM glutamine	
	10 mM NH <sub>4</sub> NO <sub>3</sub> + 30 mM KNO <sub>3</sub> + 15 mM glutamine	
	20 mM ammonium citrate ± 15 mM glutamine	
Amino acids	glutamine	5, 15, 50 mM
	proline	15, 50, 100, 300 mM
	alanine	15, 50, 100, 300 mM
	asparagine	1.5, 5, 15, 50 mM

<sup>a</sup> Murashige & Skoog [10]

<sup>b</sup> Gamborg et al. [7]

of packed cell volume (PCV) was made by counting the number and size (diameter) of embryogenic clumps. Nonembryogenic tissue, which could be visually distinguished from embryogenic tissue, was not included in these clump counts. Although the values obtained were approximations as the clumps were not perfect spheres, this was a simple, nondestructive method of measuring changes in packed cell volume (and therefore growth). A 28 µl PCV was calculated for the standard inoculum of two 3-mm clumps. Final clump counts were made after 4 weeks of growth.

### Regeneration

Clumps of globular somatic embryos were removed from suspension culture, plated on a regeneration medium containing MS salts [10], Gamborg's B5 vitamins [7], 3% sucrose, and 0.8% agar (pH 5.7), and incubated under the conditions used for initiation of embryogenic callus cultures. Embryos which did not germinate within 6–8 weeks were transferred to fresh medium. If the hypocotyl of the germinating soybean embryos became swollen, the shoot was excised and transferred to fresh regeneration medium for rooting. Plantlets from either intact somatic embryos or rooted, excised shoots were transferred to soil and covered with a beaker for a 1 week period. After an additional 1 week, the plants were moved to the greenhouse.

## Results and discussion

### *Callus induction from zygotic embryos*

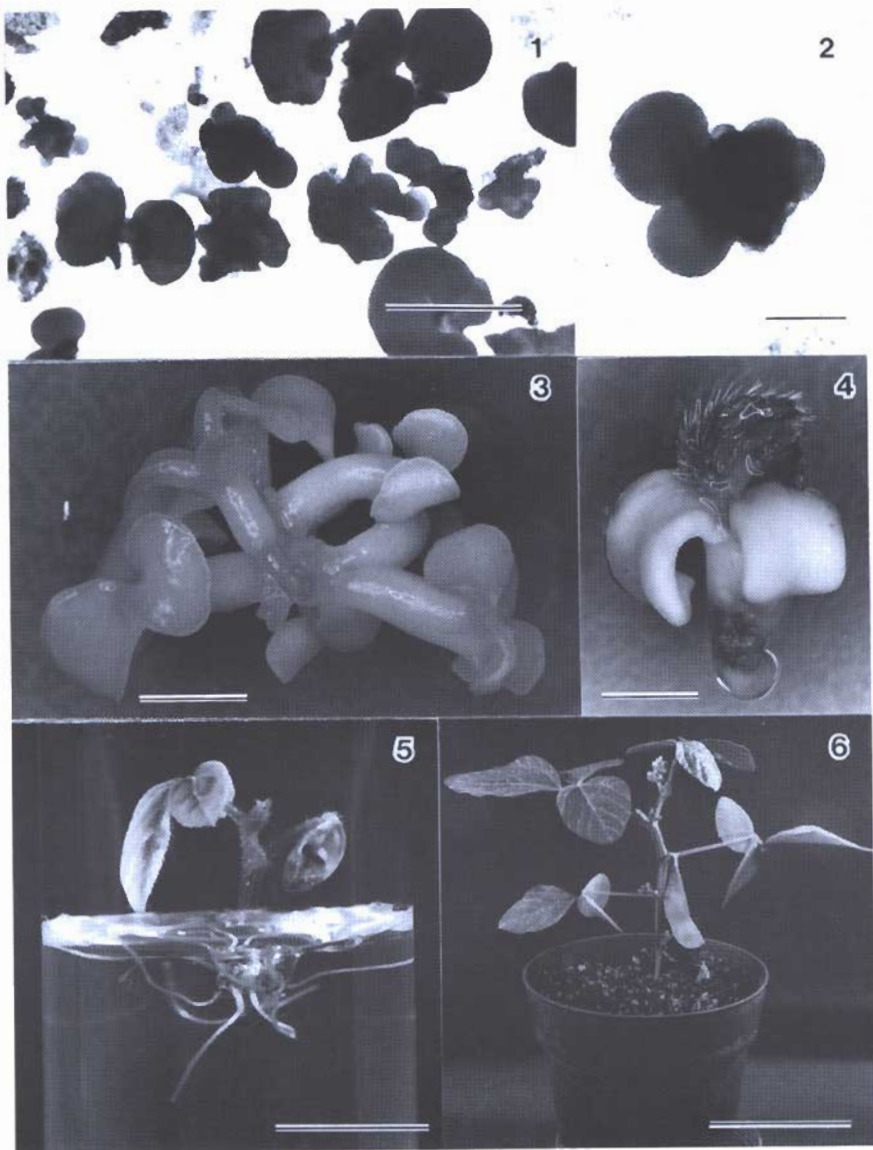
Somatic embryos were formed from excised cotyledons after 1–2 months of culture. The embryos were slow-growing and did not develop to the cotyledonary stage on the solid induction medium [5]. Nonembryogenic callus grew even slower on this medium and recurrent selection as used by Christianson et al. [4] was not necessary.

### *Embryogenic suspension culture initiation*

In early experiments, the embryogenic callus followed one of two routes in suspension culture depending on the medium, either development or proliferation. A 3–4 week incubation period was necessary to determine which of these two routes was being followed. Prior to the formulation of the 10A40N medium, development was most common. Development was defined as the enlargement and maturation of pre-existing embryos and did not result in the production of a maintainable suspension culture. As the pre-existing embryos became larger, they were sheared off of the clumps due to the physical agitation of the liquid shake culture. Microscopic examination was necessary to determine if the clumps in the suspension culture were developing embryos or masses of proliferating globular embryos (Fig. 1). The clumps of proliferating, globular embryos were characteristically yellow-green if viewed under a dissecting microscope and yellow-brown if viewed under an inverted microscope. The clump size ranged from 0.5 to 8 mm with an average of 4 mm. Individual clumps were multi-lobed (Fig. 2) with each lobe representing an early-stage globular embryo.

Although the embryos were apparently attached at their bases, preliminary histological studies indicated that the embryos arose from the apical surface of older embryos (J.J. Finer, unpubl.). This is similar to the apical proliferation reported for embryogenic tissue of soybean grown on the agar-solidified embryogenic callus initiation medium [5]. Surface proliferation gave rise to concentric layering of the proliferating embryos (until the clumps became large and broke apart). This suspension system may therefore be useful for DNA transformation work, where surface or apical tissues (which give rise to embryos) are more accessible for *Agrobacterium*-mediated or mechanical transformation.

The methods used to establish the soybean embryogenic suspension cultures in this report are substantially different from the methods described by Christianson et al. [4]. A different explant source was used to establish



*Figs. 1-6.* (1) Proliferative, embryogenic suspension culture of soybean at low magnification (bar = 1 mm). (2) Single clump from embryogenic suspension. Each lobe is a globular embryo (bar = 0.2 mm). (3) Embryo development on solid regeneration medium (bar = 2.5 mm). (4) Separated mature embryo with well-defined axes (bar = 4 mm). (5) Rooted shoot derived from somatic embryo (bar = 1 cm). (6) Regenerated plantlet from soybean somatic embryo (bar = 5 cm).

embryogenic callus and both the callus and suspension cultures were maintained on media which were very different from those described here. Although the embryogenic suspension cultures in both reports are clumpy, the system described in this present report has been optimized and the procedure is repeatable. Christianson et al. [4] initiated their suspension culture from one piece of callus and attempts were not made to optimize that suspension culture system.

#### *Growth and subculture*

A low inoculum was both convenient and beneficial for continued maintenance of the embryogenic suspension culture. This has also been reported for embryogenic suspension cultures of wild cotton [6]. Older cultures of embryogenic soybean could be poured into petri dishes and two 2–4 mm clumps placed in new liquid media. The inoculum was fairly uniform and many suspension cultures could be initiated using the tissue obtained from a single older culture. If a higher inoculum was used, or if the medium in a flask was partially replenished, the cultures would give rise to a non-embryogenic cell suspension. The nonembryogenic cells or cell clumps were fast-growing and could outgrow the slower-growing embryogenic tissue.

Using the formula for the volume of a sphere, an inoculum of approximately 28  $\mu\text{l}$  PCV was calculated. After 30 days, this calculated PCV was approximately 1 ml (Tables 2–5). This represents a 35-fold increase in PCV. Although growth of soybean suspension cultures is not as rapid as other embryogenic liquid systems [15], this does represent a significant increase in growth rate over solid support systems for soybean [12].

#### *Medium optimization*

The effect of auxin type and concentration is shown in Table 2. At low levels, all of the auxins promoted embryo development. Embryo development in the presence of low auxin levels was abnormal and these embryos were not used for regeneration studies. At the highest level of 2,4-D and 2,4,5-T (25  $\text{mg l}^{-1}$ ), there was no growth of the embryogenic suspensions (Table 2). This level of these two auxins was toxic to growth under these conditions. Picloram and dicamba are apparently lower-activity auxins in this system and promote embryo proliferation only at very high levels. The auxin, 2,4-D, was maintained in the embryo proliferation medium at 5  $\text{mg l}^{-1}$  (Table 2).

Table 2. Effect of different auxin types and concentrations on growth of embryogenic soybean suspension cultures.

Auxin	Concentration (mg l <sup>-1</sup> )	Clump volume (ml)
2,4-D	0.5	D <sup>a</sup>
	1	D
	5	1.10 ± 0.39 <sup>b</sup>
	10	0.69 ± 0.38
	25	X <sup>c</sup>
2,4,5-T	0.5	D
	1	D
	5	0.82 ± 0.38
	10	0.24 ± 0.22
	25	X
Picloram	1	D
	5	D
	10	D
	25	D
	50	0.32 ± 0.55
Dicamba	1	D
	5	D
	10	D
	25	D
	50	0.44 ± 0.76

<sup>a</sup> Developing embryos.

<sup>b</sup> Standard deviation.

<sup>c</sup> No growth.

Table 3. Effect of added cytokinin on proliferation of embryogenic suspension cultures of soybean.

Cytokinin	Concentration (mg l <sup>-1</sup> )	Clump volume (ml)
0	—	1.66 ± 0.39
BA	0.1	2.12 ± 0.20
	1	1.59 ± 0.63
	10	1.50 ± 0.80
Kinetin	0.1	0.87 ± 0.51
	1	1.07 ± 0.64
	10	1.11 ± 0.64
2ip	0.1	1.58 ± 0.97
	1	0.90 ± 0.48
	10	0.68 ± 0.47

Table 3 shows the effect of added cytokinin on growth of the soybean embryogenic suspension. The lowest level of BA ( $0.1 \text{ mg l}^{-1}$ ) may have had a slight stimulatory effect on growth of embryogenic tissues. This slight enhancement of growth was overshadowed by the rapid proliferation of nonembryogenic tissues (nonembryogenic tissues were not included in PCV determinations) which was common in all of the cytokinin-containing media. In the absence of cytokinin, growth was still rapid and there was very little nonembryogenic tissue. As in other embryogenic systems [12], it was not necessary or beneficial to add cytokinins to the proliferation medium.

The effects of substitution of MS [10] nitrogen with other nitrogen sources are shown in Table 4. Although the average growth in the medium containing  $10 \text{ mM NH}_4\text{NO}_3 + 30 \text{ mM KNO}_3 + 15 \text{ mM}$  glutamine was slightly better than both B5 and MS nitrogen with  $15 \text{ mM}$  glutamine, the difference in growth between these three media was minimal. Substitution of  $20 \text{ mM}$  ammonium citrate  $\pm 15 \text{ mM}$  glutamine was toxic to the soybean embryogenic tissue (Table 4). In an earlier report [4], this substitution was essential for the establishment of embryogenic callus and suspension cultures of soybean. The tolerance of embryogenic tissue of soybean to ammonium citrate may be greater if embryogenic tissues were gradually exposed to this compound [Ranch, pers. comm.]. The callus system, which was initially used in the earlier report [4], was slow-growing due to medium contact on only one side of the tissue. That tissue in contact with the medium would buffer the tissue on the surface of the callus from immediate ammonium citrate exposure. The effects of exposure of tissue to a compound in a liquid suspension culture can be seen rapidly. The tissue is bathed in the compound and would likely be more sensitive to changes in media composition. The medium containing  $10 \text{ mM NH}_4\text{NO}_3 + 30 \text{ mM KNO}_3 + 15 \text{ mM}$  glutamine was maintained as standard.

Table 4. Effect of the nitrogen source replacement on growth of embryogenic suspension cultures of soybean.

Nitrogen source	Clump volume (ml)
MS <sup>a</sup> nitrogen + 15 mM glutamine	$0.63 \pm 0.35$
B5 <sup>b</sup> nitrogen + 15 mM glutamine	$0.80 \pm 0.22$
$10 \text{ mM NH}_4\text{NO}_3 + 30 \text{ mM KNO}_3 + 15 \text{ mM}$ glutamine	$1.07 \pm 0.41$
$20 \text{ mM NH}_4\text{citrate}$	X <sup>c</sup>
$20 \text{ mM NH}_4\text{citrate} + 15 \text{ mM}$ glutamine	X

<sup>a</sup> Murashige and Skoog [10]

<sup>b</sup> Gamborg et al. [7]

<sup>c</sup> No growth

Table 5. Effect of amino acid addition on growth of embryogenic suspension cultures of soybean.

Amino acid	Concentration (mM)	Clump volume (ml)
0	—	1.64 ± 0.37
Glutamine	5	2.33 ± 0.45
	15	2.31 ± 0.41
	50	1.19 ± 1.42
Proline	15	1.55 ± 0.65
	50	1.49 ± 0.16
	100	1.40 ± 0.56
	300	0.58 ± 0.43
Alanine	15	1.07 ± 0.06
	50	1.91 ± 0.41
	100	1.58 ± 0.87
	300	0.45 ± 0.28
Asparagine	1.5	1.28 ± 0.73
	5	2.09 ± 0.23
	15	1.17 ± 0.69
	50	0.54 ± 0.48

Amino acids have been shown to have dramatic effects on somatic embryogenesis [1, 14]. This is also the case with this soybean suspension culture (Table 5). Glutamine at 5 and 15 mM, alanine at 50 mM, and asparagine at 5 mM enhanced growth of embryogenic tissue in suspension cultures. Although all of these amino acid additions promoted growth, 5 mM asparagine was selected as best based on the quality of embryogenic tissue produced. The 10A40N medium was subsequently modified to contain 5 mM asparagine in place of 15 mM glutamine. Although all suspension cultures used in this present report were initiated in the glutamine-containing medium, the 10A40N medium containing 5 mM asparagine can also be used for initiation of embryogenic liquid cultures. Cultures initiated and maintained in media containing either glutamine or asparagine performed similarly (J.J. Finer, unpubl.). The differences between the effects of beneficial amino acids were often subtle. If glutamine or alanine was added to the medium, the embryogenic clumps contained small areas which were slightly necrotic. In media containing asparagine, there were no necrotic areas and the clumps were generally smaller and more numerous. The smaller clump size allowed simple transfer of the suspension cultures with a wide-mouth pipet. A small clump size is also desirable for both selection and protoplast isolation work where a high surface area-to-medium ratio is beneficial.

In this amino acid addition experiment (Table 5), some of the treatments gave rise to over 2 ml PCV. In the earlier experiments, there was only 1 ml PCV at the end of the experiment. Variation among and within treatments was common in all experiments (Tables 2-5) and visual selection of the flasks and the tissue within those flasks was important to keep this variation minimal. The inocula for one experiment were taken from one flask and were of uniform morphology and size. The variation among experiments was most likely due to differences in the physiological state of the inoculum.

### *Regeneration*

Regeneration of plants did not require the addition of plant growth regulators as described in other reports [2, 12, 13]. For development, clumps of globular embryos were removed from the suspension culture medium and placed on an agar-solidified medium containing 3% sucrose (Fig. 3). Either the globular embryos were capable of producing their own hormones or there was sufficient carry-over of auxin from the suspension culture medium to support early embryo development.

Embryos, which developed on the clumps, were removed (Fig. 4) for germination. Mature somatic embryos did not have cotyledons as large as do zygotic embryos but they did possess well-defined hypocotyls and root and shoot axes (Fig. 4). Germination occurred following transfer to fresh medium. In approximately one out of three cases, there was balanced root and shoot growth but the hypocotyl became swollen. For this reason, shoots from these embryos were excised and rooted on fresh regeneration medium (Fig. 5). All of the excised shoots formed roots. The remaining germinating embryos were left intact. Plantlets from either intact somatic embryos or rooted, excised shoots were transferred to soil (Fig. 6). The regenerated plants generally flowered early and were fertile. Although the main purpose of this research was to establish and maintain soybean embryogenic suspension cultures, over 150 plants have been regenerated. This embryogenic suspension culture has been maintained for over 1 year with monthly subculture and no loss in embryogenic and regenerative capacities.

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## References

1. Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta* 164: 207-214
2. Barwale UB, Kerns HR, Widholm JM (1986) Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. *Planta* 167: 473-481
3. Baldes R, Moos M, Geider K (1987) Transformation of soybean protoplasts from permanent suspension cultures by cocultivation with cells of *Agrobacterium tumefaciens*. *Plant Mol Biol* 9: 135-145
4. Christianson ML, Warnick DA, Carlson PS (1983) A morphogenetically competent soybean suspension culture. *Science* 222: 632-634
5. Finer JJ (1988) Apical proliferation of embryogenic tissue of soybean (*Glycine max* (L.) Merrill). *Plant Cell Rep* (in press)
6. Finer JJ, Reilley AA, Smith RH (1987) Establishment of embryogenic suspension cultures of a wild relative of cotton (*Gossypium klotzschianum* Anders.). *In Vitro Cell Dev Biol* 23: 717-722
7. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50: 151-158
8. Lazzeri PA, Hildebrand DF, Collins GB (1985) A procedure for plant regeneration from immature cotyledon tissue of soybean. *Plant Mol Biol Rep* 3: 160-167
9. Lin W, Odell JT, Schreiner RM (1987) Soybean protoplast culture and direct gene uptake and expression by cultured soybean protoplasts. *Plant Physiol* 84: 856-861
10. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 5: 473-498
11. Owens LD, Cress D (1985) Genotypic variability of soybean response to *Agrobacterium* strains harboring the Ti or Ri plasmids. *Plant Physiol* 77: 87-94
12. Ranch JP, Oglesby L, Zielinski AC (1985) Plant regeneration from embryo-derived tissue cultures of soybean. *In Vitro Cell Dev Biol* 21: 653-658
13. Ranch JP, Oglesby L, Zielinski AC (1986) Plant regeneration from tissue cultures of soybean by somatic embryogenesis. In: IK Vasil (Ed) *Cell Culture and Somatic Cell Genetics of Plants*, Vol 3, pp 97-109. Academic Press Inc.
14. Stuart DA, Strickland SG (1984) Somatic embryogenesis from cell cultures of *Medicago sativa* L. II. The interaction of amino acids with ammonium. *Plant Sci Lett* 34: 175-181
15. Verma DC, Dougall (1977) Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. *Plant Physiol* 59: 81-85
16. Wright MS, Koehler SM, Hinchee MA, Carnes MG (1986) Plant regeneration by organogenesis in *Glycine max*. *Plant Cell Rep* 5: 150-154
17. Wright MS, Ward DV, Hinchee MA, Carnes MG, Kaufman RJ (1987) Regeneration of soybean (*Glycine max* L. Merr.) from cultured primary leaf tissue. *Plant Cell Rep* 6: 83-89
18. Yamada Y, Zhi-Qi Y, Ding-Tai T (1986) Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). *Plant Cell Rep* 5: 85-88