Induction and development of somatic embryos from cell suspension cultures of soybean*

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Abstract. *Glycine max* (L.) Merr. (soybean) and *Glycine soja* Sieb. and Zucc. cell suspension cultures were grown and used as inoculum sources for growing callus on agar-solidified nutrient media. Concentrations and chemical forms of the growth regulators in liquid and solidified media were altered in an attempt to achieve in vitro plant regeneration. Numerous embryoids, particularly of *G. soja*, were produced on basal nutrient media supplemented with 100 ppm casein hydrolysate, 0.1 μM abscisic acid, 2.25 μM 2, 4-dichlorophenoxyacetic acid, and 15 μM adenine or 0.46 μM kinetin. Often the roots of the embryoids elongated. This was enhanced in the presence of an inhibitor of gibberellin synthesis (1 to 20 μM Amo 1618). Callus recovered from a *G. soja* suspension culture produced one shoot structure when grown on a solid medium containing 0.2 μM Amo 1618 and 80 μM glutathione. The shoot structure consisted of two distinct buds, one producing two leaves. The shoot did not develop into a plant. Although regeneration of soybean plants was not achieved, these observations suggest that it may be achievable.

Introduction

Soybean, *Glycine max* (L.) Merr., is an important annual legume for the production of oil and protein from the seed. In vitro tissue and cell cultures are potentially useful tools in soybean-breeding programs, particularly for the selection of unique or mutant single cells, if cultures of the species were totipotent.

Legumes generally have not been amenable to in vitro manipulation and plant regeneration, and large-seeded legumes have been most recalcitrant [14]. Among these, soybean has been studied most extensively. Tissue and cell cultures of soybean grow vigorously [3, 6, 7, 10, 11, 16]. There is potential in the genus for regeneration of plants from cells by somatic embryogenesis, but embryoids have not been germinated [2]. Adventitious shoot formation has been achieved only with hypocotyl and epicotyl explants

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in primary culture [4, 8, 9, 15]. Tissue cultures of other large-seeded legumes have produced few plants [14]. A few small-seeded legumes have been much more responsive in culture [5], including alfalfa (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), and some of the clovers (*Trifolium* spp.).

The objective of the present investigation was to achieve plant regeneration of soybean tissue and cell cultures via somatic embryogenesis.

Materials and Methods

Experimental materials included *G. max* 'Calland,' 'Cutler-71,' 'Essex,' 'Forrest,' 'Northrup King B-216' (commercial cultivars); 'Norredo,' 'Sooty' (noncommercial, viny cultivars); *G. soja* P.I. 81762, P.I. 339731, P.I. 378683; and a *G. max* x *G. soja* 2nd backcross to *G. max* selection advanced to F_6 generation. *Glycine soja* seeds were scarified with medium-grade sandpaper. Seeds of each genotype were surface-sterilized by passage through 40% commercial bleach (2% sodium hypochlorite) for 10–20 min and rinsed twice in sterile distilled water. Seeds were germinated aseptically on agar-solidified SGL medium, described previously [5]. Approximately seven-day-old seedlings were used to provide hypocotyl and epicotyl tissue pieces for inoculation onto solidified L2 medium as described for callus initiation [12]. Healthy, friable callus was subcultured onto fresh L2 medium monthly.

Three- to 18-month-old callus was used to initiate cell suspension cultures by inoculating approximately 0.5 g tissue into 12.5 ml of SL2 medium [13] variations as described below. After one week, cell suspensions were brought to 25 ml with fresh medium. Cell suspensions were subcultured weekly by using 2–10 ml suspension to inoculate 25 ml fresh medium. Cell densities were maintained at an average of 1×10^6 cells/ml during suspension culture. Suspensions were passed through 75 μm sieves once during their history. Each suspension was exposed to only one of 25 liquid media. Suspension culture flasks (125-ml Erlenmeyers) were foam-stoppered and foil-capped. Cultures were rotated at 110 rpm under continuous fluorescent light ($\sim 100 \mu E\, m^{-2}\, s^{-1}$) at 25°C. Suspension cultures were used, after 1–18 months, to inoculate 260 variations of solidified L2 basal medium by pouring 2 ml suspension over approximately 40 ml medium in a 20 x 100 mm disposable Petri dish. The callus obtained was grown for up to three months without subculture onto fresh medium. The light and temperature conditions described above were used for callus growth. At least four replications of each genotype on each medium were evaluated.

The basal medium and growth regulator types and concentrations of SL2 and L2 media were varied. Basal medium variations included modifications of the total salts content, ammonium – nitrate ion ratios, organic nitrogen supplementation, total vitamin content, level of sucrose, and use of soluble starch. Growth regulator variations included modifications of the

type and concentration of auxin and cytokinin. The auxins evaluated were indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D), and 4-amino-3, 5, 6-trichloropicolinic acid (picloram). The cytokinins evaluated were adenine sulfate (ade), 6-furfurylaminopurine (kin), 6-benzylaminopurine (BA), and zeatin (trans isomer). Additions of the antiauxin 2, 3, 5-triiodobenzoic acid (TIBA), abscisic acid (ABA), glutathione (reduced form), gibberellic acid (GA_3), and the inhibitor of gibberellin biosynthesis 2-isopropyl-4-dimethyl-amino-5-methylphenyl-1-piperidine carboxylate methyl chloride (Amo 1618) were also evaluated.

Results and Discussion

Cell suspension cultures of all soybean genotypes used in this investigation produced globular- and heart-staged somatic embryos. Younger cultures produced more somatic embryos than did older cultures. An example of a heart-staged somatic embryo grown in suspension culture is shown in Figure 1. Callus recovered from cell suspensions cultured on solidified media produced shoot buds on media with the same or similar compositions as those

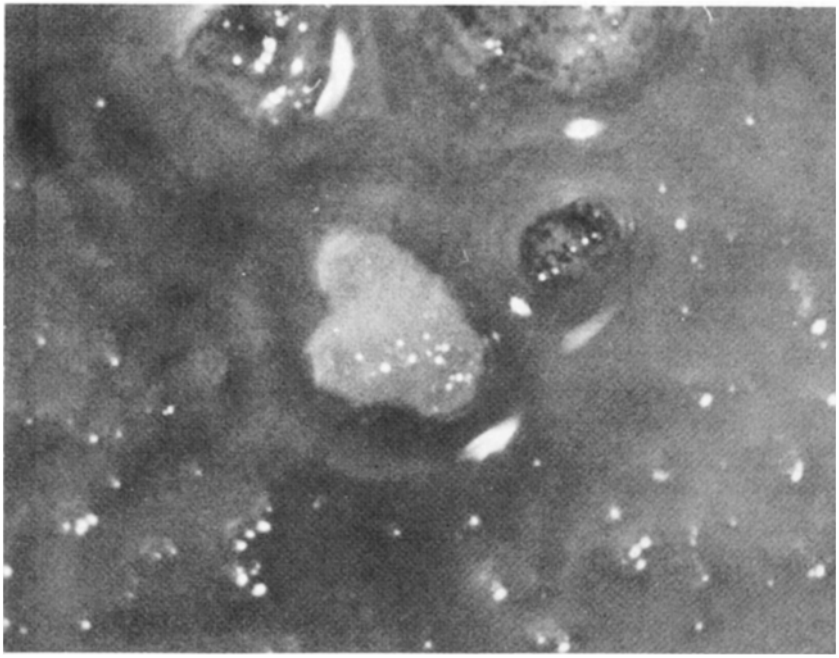

Figure 1. Heart-staged somatic embryo typical of those produced in cell suspension cultures of *Glycine max*; × 16.7.

used to produce somatic embryos in suspension culture. The basal media SL2 and L2 supported the induction of somatic embryogenesis or budding when supplemented with 100–200 ppm casein enzymic hydrolysate (CH) and growth regulators as described below.

The formation of somatic embryos and buds proceeded best using 0.45–2.25 μM 2, 4-D as the auxin. Other auxins were inferior to 2, 4-D but allowed the production of somatic embryos and buds. The antiauxin TIBA was not helpful. Ade (15 μM) or kin (4.6 nM–0.46 μM) were the best cytokinin treatments. The relative value of auxins and cytokinins for the formation of somatic embryos in one of the soybean lines is summarized in Table 1.

Table 1. Response of *G. soja* P.I. 81762 to auxins and cytokinins evaluated for the formation of somatic embryos in suspension culture

Growth regulator	Relative responses, 0–least to 6–best
TIBA, 20 nM–0.8 μM	0
IAA, 60 nM–11.4 μM	3
IBA, 25 nM–0.5 μM	4
NAA, 0.1–13.4 μM	4
Picloram, 5 nM–0.25 μM	4
2, 4-D, 45 nM–5 μM	6
2 μM 2, 4-D and 40 nM picloram	6
2 μM 2, 4-D and 50 nM IBA	6
Zeatin, 4.6–46 μM	0
BA, 44 nM–44 μM	3
Kin, 0.5 nM–10 μM	6
Ade, 3.5 μM–0.15 mM	6
15 μM ade and 0.46 μM kin	6

Previous work had shown 2.25 μM 2, 4-D and 0.46 μM kin to be the optimal auxin and cytokinin for somatic embryogenesis [2]. However, the occurrence of somatic embryos was found by us to be sporadic using these regimes.

The addition of ABA at 38 nM–0.19 μM increased the number of heart-staged somatic embryos and buds by fourfold (Table 2). ABA was previously

Table 2. Relative responses (0–least to 10–best) of two soybean lines on several media evaluated for the formation of somatic embryos

Additives to basal media SL2 and L2	*G. soja* P.I. 81762	*G. max* cv. Essex
(1): 2.5 μM 2, 4-D and 0.46 μM kin	4	1
(2): (1) plus 100 ppm CH	5	2
(3): (2) plus 10 nM–0.2 μM GA_3	3	0
(4): (2) plus 0.1 μM ABA	9	6
(5): (4) plus 20 nM GA_3	7	4
(6): (4) plus 0.1 μM Amo 1618	10	7
(7): (6) plus 60 μM–3 mM glutathione	10	7

shown to alter the developmental pattern but not the number of somatic embryos in plant cultures [1]. ABA may promote the development of globular-staged soybean embryos to the heart stage. GA_3 inhibited the growth of soybean cells and callus (Table 2). The use of 0.1 n*M*–0.1 μM Amo 1618 promoted soybean growth in vitro (Table 2). Amo 1618 improved the development of somatic embryos, but it did not increase the number of embryos formed. The addition of glutathione did not improve somatic embryogenesis (Table 2). Somatic embryogenesis in soybean cultures was reproducible using basal SL2 and L2 media supplemented with 100 ppm CH, 2.25 μM 2, 4-D, 0.1 μM ABA, 0.1 μM Amo 1618, and either 15 μM ade or 0.46 μM kin.

The different genotypes used in this investigation gave different numbers of embryoids and buds. This was observed previously with a different set of genotypes [2]. *Glycine soja* P.I. 81762 consistently produced advanced stages of embryos in suspension culture, some with well-formed cotyledons. The other *G. soja* genotypes and the advanced generation of the *G. max* x *G. soja* hybrid produced numerous embryos with rudimentary cotyledon development in suspension cultures. The two viny *G. max* genotypes often produced embryoids at the heart stage and at the torpedo stage, but these generally lacked well-developed cotyledons. All commercial *G. max* genotypes produced a few globular- and heart-staged embryoids. 'Essex,' 'Forrest,' and 'Cutler-71' produced embryoids in most experiments, while 'Northrup King B-216' and 'Calland' yielded a few embryoids in some experiments. 'Essex' was the best of the commercial types, while *G. soja* P.I. 81762 was the best of the genus. These two lines were compared for the formation of embryoids in an experiment summarized in Table 3. The ranking for

Table 3. The formation of somatic embryos by two soybean lines grown in suspension culture on SL2 basal medium containing 2, 4-D, kin, ade, ABA, Amo 1618, and CH

	G. soja P.I. 81762	*G. max* cv. Essex
No. weeks cultures observed	20	20
No. weeks somatic embryos observed	17	11
Range of somatic embryo development	Heart cotyledonary	Heart torpedo
Average no. somatic embryos per ml suspension culture	3	0.2

embryoid production in suspension culture was paralleled by the ranking for bud production in callus recovered from the suspension cultures.

Vigorously growing roots often occurred on embryoids in suspension and callus cultures. Root growth occurred to some degree on most of the media. Increasing the sucrose in the medium from 2.5% to 7.5%–12.5%

reduced the frequency of root growth and did not promote the development of embryoids. The use of 1–20 μM Amo 1618 with auxin, cytokinin, and ABA increased the amount of root growth.

One shoot structure, shown in Figure 2, was recovered in callus derived

Figure 2. Leafy shoot produced by *G. soja* callus derived from a cell suspension culture; × 12.

from a cell suspension during this investigation. Three-month-old callus of *G. soja* P.I. 339731, grown on standard semisolid L2 medium containing 0.25 μM picloram and 0.44 μM BA, was used to initiate a cell suspension on liquid SL2 basal medium containing 2.25 μM 2, 4-D, 15 μM ade, 7.6 nM ABA, 1 μM Amo 1618, and 100 ppm CH. A seven-month-old cell suspension was used to inoculate solidified L2 basal medium containing 2.25 μM 2, 4-D, 15 μM ade, 0.19 μM ABA, 10 nM Amo 1618, 80 μM glutathione, and 100 ppm CH. After two months on this medium, the callus produced a shoot structure that included two green leaves and an associated green bud. The leaves turned yellow-white after an additional month of culture on the same medium. The structure was then transferred to an L2 basal medium containing 22.2 μM BA, 50 nM IBA, 38 nM ABA, and 0.1 μM Amo 1618. The leaves turned green and one new leaflet appeared after two weeks on this medium. After four weeks, the shoot was transferred to another medium (0.44 μM BA,

50 n*M* IBA, 38 n*M* ABA, and 0.1 μM Amo 1618), but no further growth was obtained. Glutathione probably was not critical in this situation, based on other observations (Table 2). Both glutathione and ade were present in the media on which the single shoot developed. The possibility that these two compounds stimulate development of soybean buds and embryoids warrants further study.

This investigation has resulted in the first report of the formation of a shoot from a suspension-derived soybean culture. Although a plant was not obtained from the shoot structure, these observations suggest that regeneration of soybean plants from long-term cultures may be possible.

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