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Plant Regeneration from Embryogenic Suspension Cultures of Soybean (*Glycine max* L. Merrill)

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Abstract

In order to establish efficient plant regeneration from embryogenic suspension cultures of soybean, *Glycine max* L, we examined the effects of auxin type and concentration, cytokinin type and concentration, and amino acid type and concentration on the growth of embryogenic clumps from induced callus, and the effect of desiccation of mature somatic embryos obtained from these clumps on the frequency of somatic embryo germination. Embryogenic callus was induced from the edge of the cotyledons cultured on MS medium containing 6% sucrose, 40 mg/L 2,4-D, 0.2% gelrite and pH 5.7. The growth of embryogenic clumps was best in early-staged, embryogenic callus that was placed in suspension culture of MS medium containing 5 mg/L 2,4-D and 0.5 mg/L asparagine. Single somatic embryos were isolated from the clumps and plated on the same medium for maturation. When the mature single somatic embryos were desiccated for 96 h, somatic embryo germination came up to approximately 90%. The plantlets germinated after embryos desiccation for 2 weeks were transfered to MS medium containing 3% sucrose, 0.2% gelrite and pH 5.7.

Introduction

Soybean (*Glycine max* L. Merrill) can be regenerated via either shoot differentiation or somatic embryogenesis (Barwele et al., 1986). Compared with another species, it required higher concentration of 2,4-D (Finer, 1988; Finer and Nagasawa, 1989; Wright et al., 1991; Choi et al., 1994; Ponappa et al., 1999). Plant regeneration via shoot differentiation has been achieved from cotyledonary node (Barwele et al., 1986; Wright et al., 1986) and primary leaf tissue (Wright et al., 1987; Kim et al., 1990). Plant regeneration has also been reported to be achieved via somatic embryogenesis from the immature cotyledons of developing seeds (Lazzeri et al., 1985; Liu et al., 1992; Anna and Waclaw, 1994; Rajasekaran and Pellow, 1997; Kim et al., 2000), the intact zygotic embryo (Ranch et al., 1985; Buchheim et al., 1989) and the excised embryo axis (Christianson et al., 1983). In the above studies, somatic embryos were formed directly from explants. However, the number of somatic embryos formed from explants was limited and the competent cells which develop into embryos could not be maintained by subculture.

Such problems might be solved by using embryogenic suspension cultures. For example, Christianson et al. (1983) 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. After that, Finer and Nagasawa (1989) reported the development of an embryogenic suspension culture obtained from embryogenic tissue at an early ontogenetic from immature cotyledons and was used to initiate embryogenic suspension cultures.

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The germination frequency of soybean somatic embryos is generally very low. A number of variables have been investigated in an attempt to improve the germination frequency of somatic embryos such as desiccation (or partial drying) (Parrott et al., 1988; Buchheim et al., 1989; Durham and Parrott; 1992). Desiccation had little effect unless embryo development was adequate, i.e. 5 mm in length. Indicating that somatic embryos had to be mature for breakage of dormancy. The importance of water loss in the attainment of seed germination capacity has well been documented for both monocotyledons and dicotyledons (Kermode, 1990).

The objectives of this paper are to establish efficient plant regeneration from embryogenic suspension cultures of soybean (*Glycine max* L. Merrill), and to investigate the effect of desiccation of mature somatic embryos obtained from the embryogenic clumps on the frequency of somatic embryo germination.

Materials and Methods

Induction of embryogenic callus

Soybean plants (*Glycine max* L. Merrill. cv. Jack) were grown in a greenhouse. Immature pods containing embryos approximately 4 mm in length were harvested 2 to 3 weeks after flowering. Pods were surface sterilized by immersion in a 20% solution of commercial bleach containing 0.05% Tween-20 for 20 minutes. Following 3 rinses in sterile, distilled water, the immature seeds were excised. The cotyledons were removed for culture as described by Lazzeri et al. (1985). Cotyledons were placed on MS medium (Murashige and Skoog, 1962) containing 6% sucrose, 40 mg/L 2,4-D, 0.2% gelrite and pH 5.7 and cultured at 25℃ under 16/8h photoperiod with a light intensity of 30 $\mu EM^{-2}s^{-1}$. Somatic embryos and embryogenic callus induced from cotyledon were subcultured every 4 week on this medium.

Initiation and growth of embryogenic suspension cultures

Early-staged, highly embryogenic callus was placed in 35 mL of suspension culture medium contained in a 125 mL Erlenmeyer flask in order to initiate suspension cultures. Suspension cultures were established after 2 months culture and maintained in a proliferating state by subculturing every 14 day. Flasks were agitated at 150 rpm. Suspension cultures were placed at 25℃ under 16/8 h photoperiod. The basal medium of suspension culture was MS medium containing 3% sucrose, 5 mg/L 2,4-D, 0.5 mg/L asparagine and pH 5.7. For subculture, 30 to 50 mg of embryogenic callus was placed in 35 mL fresh suspension culture medium.

Factors studied

The following factors were investigated as individual experiments : (1) Auxin type and concentration - the effect of 2,4-D, NAA, Dicambar or Picloram, each at 0.5, 1, 5, 10, 20, 40 mg/L was evaluated. (2) Cytokinin type and concentration - the effect of kinetin, BA or 2ip, each at 0.5, 1, 2, 5 mg/L was evaluated. (3) Amino acid type and concentration - the effect of glutamine, proline, alanine or asparagine, each at 0.1, 0.5, 1, 2, 5 mg/L was evaluated. In each case, 20 replications per treatment were used. The growth of embryogenic clumps was measured after 3 weeks culture.

Plant regeneration

For somatic embryo development, the clumps of globular somatic embryos were removed from suspension cultures and transferred to MS medium containing 3% sucrose, 0.2% gelrite and pH 5.7. After 4 weeks culture, the single somatic embryos were isolated from the clumps and plated on the same medium for somatic embryo maturation for additional 4 weeks. The mature single somatic embryos were desiccated in a dry 100 mm sterile petri dish each for 24, 48, 72 or 96 h. Each petri dish contained 20 somatic embryos with a combined fresh weight (FW) of 1.2-1.5 g. The percent of fresh weight loss[100 × (FW before desiccation - FW after desiccation)/FW before desiccation] was measured after each of the desiccation treatments. The desiccated and non-desiccated somatic embryos were transfered to MS medium containing 3% sucrose, 0.2% gelrite and pH 5.7 for germation. All cultures were maintained at 25℃ with 16/8 h photoperiod. Plantlets were planted in pots filled with 1:1:1 mixture of vermiculite, perlite and peat moss. Pots were covered with plastic boxes to ensure high humidity for 1 week. After additional one week, the plants were moved to the greenhouse.

Results and Discussion

Induction of embryogenic callus

Embryogenic callus and somatic embryos were first observed to be arising from the edge of the cotyledons after 10 to 14 day culture (Figure 1A, arrows). This embryogenic callus was light yellow and was very slow

growing. Because nonembryogenic callus did not proliferate on this medium, it was not necessary to preferentially subculture embryogenic callus as used by Christianson et al. (1983). The somatic embryos did not develop into the cotyledonary stage on the solid induction medium. With increased culture time, the cotyledons became brown and friable, and early-staged, globular embryo clumps were formed.

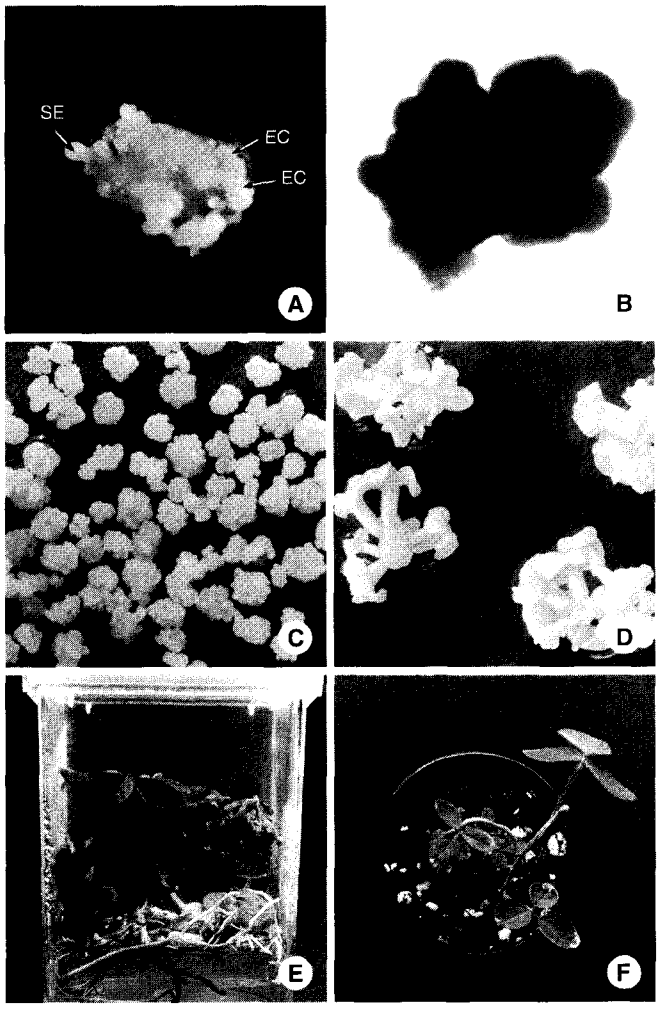

Figure 1. Somatic embryogenesis and plant regeneration of soybean. A; Embryogenic callus (EC) and somatic embryos (SE) from cotyledon explant (Bar=1 mm), B; Single clump from embryogenic suspension culture. Each lobe is a globular embryo (Bar=0.2 mm), C; Clumps of proliferating, globular embryos from embryogenic suspension culture (Bar=2 mm), D; Somatic embryos developed on gelrite-solidified medium (Bar=2 mm), E; Plantlets regenerated via somatic embryogenesis (Bar=1 cm), F; Well-established plant after transplanting in pot (Bar = 4 cm).

Initiation and growth of embryogenic suspension cultures

The embryogenic callus was finely dispersed due to the physical agitation of the liquid shake culture and was proliferated. Individual clumps of proliferating globular embryos were multi-lobed (Figure 1B) with each lobe representing an early-stage globular embryo. These clumps were characteristically yellow-green (Figure 1C). The clump size ranged from 0.6 to 7 mm with an average of 3.5 mm.

A. Effects of auxin type and concentration

Auxin (usually 2,4-D) has been applied to induce and proliferate the somatic embryo (Shoemaker et al., 1991; Liu et al., 1992; Ponappa et al., 1999; Kim et al., 2000), because 2,4-D, picloram and dicambar contains the most effective inducers of somatic embryogenesis. We examined the effect of auxins on the growth of embryogenic soybean suspension cultures. The embryo growth was greatly influenced by auxin type and concentration as shown in Table 1. At low concentration, all of the auxins promoted embryo development. At the high concentration of 2,4-D (20 mg/L), no growth of embryogenic suspension cultures

Table 1. Effect of auxins on growth of embryogenic soybean suspension cultures.

Auxins (mg/L)	Concentration (mg/L)	Fresh weight (mg±SE)
2.4-D	0.5	ED
	1	ED
	5	22.64±0.97
	10	10.57±0.62
	20	No growth
Picloram	0.5	ED
	1	ED
	5	ED
	10	ED
	20	ED
	40	7.01±1.03
Dicambar	0.5	ED
	1	ED
	5	ED
	10	ED
	20	ED
	40	9.30±0.62

ED: embryo development. The growth was measured after 3 weeks culture in MS medium.

was observed, suggesting that the concentration of 2,4-D (20 mg/L) was toxic to growth. Picloram and dicambar showed apparently lower-activity and promoted embryo proliferation only at very high concentration (40 mg/L). The concentration of 5 mg/L 2,4-D was the best concentration of auxins to embryo proliferation.

B. Effects of cytokinin type and concentration

We examined the effect of cytokinins on growth of embryogenic suspension cultures of soybean. As shown in Table 2, the growth of embryogenic suspension cultures was best in the lowest concentration (0.5 mg/L) of BA or 2ip and in the highest concentration (5 mg/L) of kinetin tested. This growth was more effective in BA-supplemented MS medium than in kinetin-supplemented one or 2ip-supplemented one. However, this enhancement of growth was overshadowed by the rapid proliferation of nonembryogenic callus (nonembryogenic callus was not included in fresh weight determinations). These data were similar to the previous results of Ranch et al. (1985) and Finer and Nagasawa (1989) that the supplement of cytokinins to the proliferation medium was not necessary or beneficial.

C. Effects of amino acid type and concentration

Amino acids have been shown to have dramatic effects on somatic embryogenesis (Finer and Nagasawa, 1989; Jorgensen, 1993; Wilhelm, 2000). The effect of amino acids on the growth of embryogenic suspension cultures of soybean was shown in Table 3. The growth of embryogenic suspension cultures was enhanced at 0.1 and 0.5 mg/L glutamine, 0.5 mg/L alanine and 0.5 mg/L asparagine. Although the addition of all of these amino acids promoted the growth, 0.5 mg/L asparagine was most effective for the growth of embryogenic clumps. If glutamine, proline or alanine was added to the medium, the embryogenic clumps contained small areas which were necrotic (black spots). In the 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 and may be suitable for transformation using the particle bombardment.

Table 2. Effect of cytokinins on growth of embryogenic soybean suspension cultures.

Cytokinins (mg/L)	Concentration (mg/L)	Fresh weight (mg±SE)
Kinetin	0.5	14.92±1.19
	1	19.01±1.47
	2	20.83±1.49
	5	23.27±1.53
BA	0.5	36.63±1.57
	1	25.72±1.51
	2	24.56±1.56
	5	21.37±0.98
2ip	0.5	31.42±2.39
	1	19.39±1.21
	2	15.17±1.15
	5	12.23±1.19

The growth was measured after 3 weeks culture in MS medium.

Table 3. Effect of amino acids on growth of embryogenic soybean suspension cultures.

Amino acids (mg/L)	Concentration (mg/L)	Fresh weight (mg±SE)
Glutamin	0.1	32.16±1.17
	0.5	31.88±1.08
	1	23.55±2.51
	2	16.42±3.72
	5	13.27±1.35
Proline	0.1	20.39±1.65
	0.5	19.56±0.36
	1	18.32±1.41
	2	14.32±2.57
	5	9.01±1.08
Alanine	0.1	14.77±0.15
	0.5	26.36±1.07
	1	21.81±2.28
	2	15.21±1.73
	5	8.23±1.72
Asparagine	0.1	19.67±1.91
	0.5	26.35±2.05
	1	18.15±1.97
	2	13.27±2.37
	5	9.45±1.38

The growth was measured after 3 weeks culture in MS medium supplemented with 5mg/L 2,4-D.

Plant regeneration

We grew the embryogenic clumps by suspension culture of embryogenic callus in MS medium containing 5 mg/L 2,4-D and 0.5 mg/L asparagine. For embryo development, clumps of globular somatic embryos were removed from the suspension culture medium and placed

on the gelrite-solidified MS medium containing 3% sucrose (Figure 1D). The germination frequency of somatic embryos was generally very low. The desiccation has been applied to enhance the frequency of somatic embryo germination as in other reports (Parrott et al., 1988; Buchheim et al., 1989; Durham and parrott, 1992; Wilhelm et al., 2000. We examined the relationship between fresh weight loss and the frequency of somatic embryo germination (hypocotyl elongation, radicle protrusion and root formation). As shown in Figure 2, the germination frequency showed positive correlation with fresh-weight loss. When desiccated for 96 h, the fresh-weight loss reached approximately 50%. The germination rate of 96-h-desiccated embryos was approximately 90%, while non-desiccated somatic embryos did not germinate. The germinated embryos became green and produced elongated hypocotyls with radicles (Figure 3). This result was similar to the Hammatt and Davey's report (1987) that somatic embryos were germinated by desiccating until they became to 40-50% of their original size. Rosenberg and Rinne (1988) and Ponappa et al. (1999) also reported the similar result that the desiccation of zygotic soybean embryos was necessary for their germination. Enhanced germination following desiccation of somatic embryos has been observed in several species, including soybean

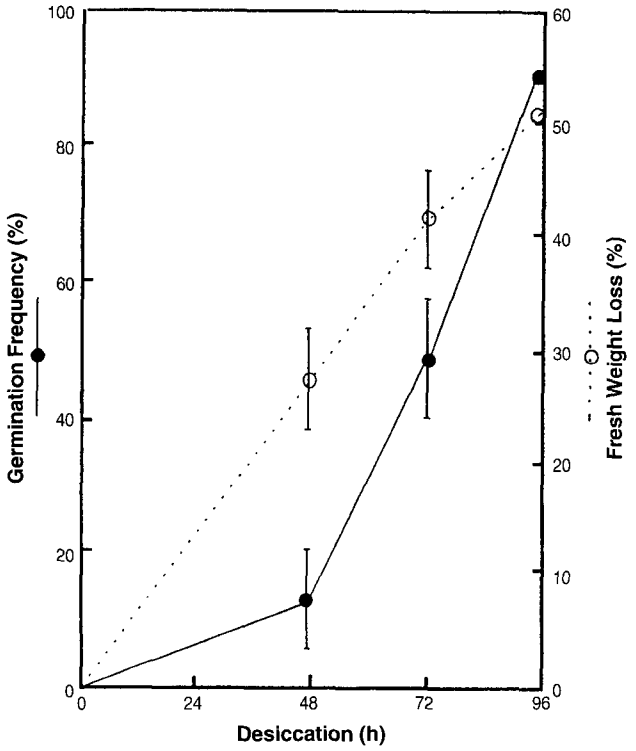

Figure 2. Relationship between fresh weight loss and germination frequency of soybean somatic embryos.

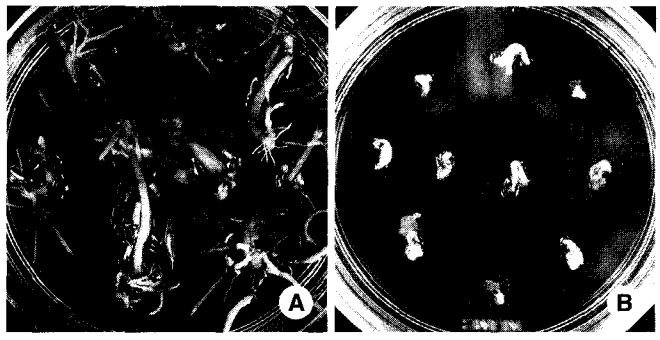

Figure 3. Morphological changes of desiccated and non-desiccated somatic embryos about 10 days after culture, A; 96 h-desiccated somatic embryos germinating on MS medium, B; Non-desiccated somatic embryos on the same medium.

(Parrott et al., 1988; Buchheim et al., 1989), grape (Gray, 1989), peanut (Durham and Parrott; 1992) and oak (Wilhelm, 2000). The desiccated somatic embryos were transferred and regenerated in MS medium containing 3% sucrose, 0.2% gelrite and pH 5.7 (Figure 1E). Figure 1F shows the plantlets 2 weeks after planting in pots filled with 1:1:1 mixture of vermiculate, perlite and peat moss.

In conclusion, we established the efficient embryogenic suspension culture condition of soybean. Embryogenic callus was best induced from the edge of the cotyledons cultured on MS medium containing 6% sucrose, 40 mg/L 2,4-D, 0.2% gelrite and pH 5.7. The embryogenic callus was cultured for production of embryogenic clumps in MS suspension medium containing 5 mg/L 2,4-D and 0.5 mg/L asparagine. We also established an efficient plant regeneration condition from embryogenic suspension cultures of soybean. When single somatic embryos were desiccated for 96 h, somatic embryo germination dramatically improved up to approximately 90%. The population of cells produced via the embryogenic suspension cultures is more uniform than in callus, and thus somatic embryo development can be partially synchronized. The suspension culture of soybean established in this study may contribute as good sources for efficient transformation via particle bombardment, physiological study and artificial seed production.

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