REGENERATION OF SOYBEAN VIA EMBRYOGENIC SUSPENSION CULTURE

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ABSTRACT: In an attempt to establish an alternative plant regeneration system for soybean [*Glycine max* (L.) Merrill] cultivars used in Brazilian breeding programs, ten genotypes were tested for their embryogenic potential. Cotyledons were removed as explants from immature seeds harvested from field-grown plants. After 45 days on induction medium, the number of responding cotyledons and the number of somatic embryos per immature cotyledon were evaluated. The percentage of explants that produced somatic embryos varied from 1 to 70% among cultivars. The average number of somatic embryos produced per cotyledon pair ranged from 0.01 to 10.3 with a mean of 3.4. Suspension cultures were initiated with three *Agrobacterium tumefaciens* susceptible cultivars. Suspensions were successfully developed from Bragg and IAS5 cultivars. The packed cell volume, in one-month growth, increased 8.1 fold for Bragg and 3.5 fold for IAS5 and the fresh weight increased 6.6 and 2.8 fold, respectively. The cultivars differed for the analysed parameters. All tissue from each cultivar was transferred to the maturation medium and subsequently to the germination medium. The germination frequency was 45.7 and 54.9% for Bragg and IAS5, respectively. Plants were gradually exposed to ambient humidity over one week and then planted in soil. All plants yielded seeds in the greenhouse.
Key words: *Glycine max*, somatic embryogenesis, liquid medium, plant tissue culture

REGENERAÇÃO DE SOJA ATRAVÉS DE CULTURAS EMBRIOGÊNICAS EM SUSPENSÃO

RESUMO: Com o objetivo de estabelecer um sistema alternativo de regeneração de plantas para cultivares de soja [*Glycine max* (L.) Merrill] utilizadas em programas de melhoramento no Brasil, dez genótipos foram testados quanto ao seu potencial embriogênico. Os cotilédones utilizados como explantes foram excisados de sementes imaturas de plantas provindas do campo. Após 45 dias em meio de indução, o número de cotilédones embriogênicos e o número de embriões somáticos por cotilédone imaturo foram avaliados. A porcentagem de explantes que produziram embriões somáticos variou de 1 a 70% entre cultivares. O número médio de embriões somáticos produzidos por par de cotilédones variou de 0,01 a 10,3, com uma média de 3,4. Culturas em suspensão foram iniciadas a partir de três cultivares suscetíveis a *Agrobacterium tumefaciens*. Suspensões foram estabelecidas com sucesso para os cultivares Bragg e IAS5. Em um mês, o volume celular aumentou 8,1 vezes para Bragg e 3,5 vezes para IAS5 e o peso fresco aumentou 6,6 e 2,8 vezes, respectivamente. Todo o tecido de cada cultivar foi transferido para meio de maturação e, subseqüentemente, para meio de regeneração. A freqüência de germinação foi de 45,7 e 54,9% para Bragg e IAS5, respectivamente. Durante uma semana, as plantas foram expostas gradualmente à umidade do ambiente, sendo então plantadas em solo em casa de vegetação. Todas as plantas produziram sementes.
Palavras-chave: *Glycine max*, embriogênese somática, meio líquido, cultura de tecidos vegetais

INTRODUCTION

The success of soybean transformation has been limited due to the low efficiency of transformation methods. The major critical prerequisite for all transformation procedures is the ability in establishing and maintaining a culture with highly responsive plant tissues.

One of the most efficient methods for soybean regeneration is somatic embryogenesis, first described in 1983 (Christianson et al., 1983). After this, many reports of soybean somatic embryogenesis were published (Lippmann & Lippmann, 1984; Lazzeri et al., 1985; Ranch et al., 1986; Parrott et al., 1988). Nevertheless, most of the protocols were not successful for soybean transformation. In part, this limitation was due to the inability in producing a large number of embryos, which was overcome by applying high 2,4-D concentration during induction and proliferation of the somatic embryos (Ranch et al., 1986). On the other hand, transformation of the somatic embryos originated chimeric plants (Parrott et al., 1989), due to the

multicellular origin of the primary embryos (Finer, 1988). Proliferation of secondary somatic embryos from single cells at or near the surface of the older primary embryos has the advantage to develop embryos of unicellular origin, which are a usefull target tissue for transformation, producing totally transgenic plants (Finer, 1988). These secondary somatic embryos can be proliferated either in liquid suspension culture medium (Finer & Nagasawa, 1988) or in solid proliferation medium (Santarém et al., 1997). Because of the highly proliferative nature of the embryogenic clumps in liquid medium, the suspension culture proved to be amenable to soybean transformation (Finer & McMullen, 1991; Parrott et al., 1994; Hadi et al., 1996; Liu et al., 1996).

Due to the agronomical importance of soybean for Brazil, second world producer, there is an interest in improving this crop by taking advantage of the powerful tools of recombinant DNA technology. However, the principal obstacle for soybean transformation and regeneration by somatic embryogenesis is the effect of genotypes on the response of tissues on induction (Komatsuda & Ohyama, 1988; Bailey et al., 1993; Tian et al., 1994), proliferation and regeneration media (Bailey et al., 1993; Santos et al., 1997). Thus, the screening for genotypes that are more responsive to induction and regeneration-competent is a prerequisite for an efficient transformation protocol. Here, we report differences among cultivars regarding induction, proliferation and regeneration of ten soybean cultivars using embryogenic suspension.

MATERIAL AND METHODS

Ten soybean cultivars (BR1, BR4, BR32, Bragg, CEP12, Davis, IAS4, IAS5, Ivorá and RS7) were used in this study. Young pods with immature seeds were harvested from field-grown plants and surface sterilized during 1 min in 70% ethanol and 15 min in 4% sodium hypochlorite containing Tween-20. Following four rinses in sterile, distilled water, immature seeds of 3-6 mm were excised and the cotyledons were removed as explants for culture. Cotyledon halves were placed with the abaxial side facing the modified D40 induction medium (Bailey et al., 1993), which contains MS salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 40 mg L^{-1} 2,4-D, 6% sucrose, 0.7% Gum Agar (Sigma), pH 5.8. One hundred pairs of cotyledons were cultured for each cultivar, 10 pairs in each plate. Cultures were maintained for 90 days at 25 ± 1°C with 16/8 h light/dark at a light intensity of 22.5 $\mu Em^{-2} s^{-1}$ for somatic embryo induction and production of secondary embryos.

Three soybean cultivars, BR4, Bragg and IAS5, were selected for evaluation of proliferation and regeneration capacities in liquid medium, due to their susceptibility to *Agrobacterium* infection (Droste et al., 1994), since later, proliferating embryogenic suspension cultures will be exposed to an integrated bombardment/*Agrobacterium* system for transformation. Two clumps of secondary somatic embryos at globular stage were placed into an 125 mL Erlenmeyer flask containing 30 mL of 10A40N liquid medium (Finer & Nagasawa, 1988), which consisted of MS salts modified by replacing nitrogen with 10 mM NH_4NO_3 and 30 mM KNO_3, B5 vitamins, 6% sucrose, 5 mg L^{-1} 2,4-D and 5 mM asparagine. The flasks were shaken at 25 ± 1°C with 16/8 h light/dark at a light intensity of 22.5 $\mu Em^{-2} s^{-1}$ for 30 days on an orbital shaker at 150 rpm. After this period, tissue was subcultured every 14 days with the same medium and incubation conditions by selecting only the yellow-green, dense and smooth surfaced embryogenic clumps at a low inoculum density of 2-7 clumps per flask. Cultures from BR4 cultivar showed turbidity, tissue necrosis and lack of friability. Thus, only suspension cultures of Bragg and IAS5 cultivars were maintained for a period of one year.

At the third month of culture, seven flasks from Bragg and IAS5 cultivars, with two embryogenic clumps each, were prepared for growth rate determination. Each clump had a diameter of 2-3 mm and the clumps of each flask had an approximated volume of 28 μL and weight of 15 ± 3 mg. Growth rates were calculated using an approximation of packed cell volume at the end of four-week growth.

After packed cell volume estimation, the entire tissue contents of the seven flasks from each cultivar were used in plantlet regeneration studies. To induce histodifferentiation, clumps of proliferating embryogenic tissue were placed on MSM6AC maturation medium (Bailey et al., 1993), containing MS salts, B5 vitamins, 6% maltose, 0.5% activated charchoal, 0.3% Phytagel™, pH 5.8. After four weeks, the embryos were individualized and subcultured on MSM6 medium (Finer & McMullen, 1991) for additional four weeks.

Histodifferentiated embryos were classified in eight morphological types, according to Buchheim et al. (1989). The milky-white to milky-yellow somatic embryos were placed in dry, sterile glass plates with up to 25 embryos per plate for two days to allow further desiccation. This desiccation step was found capable of improving conversion percentage (Parrott et al., 1988; Buchheim et al., 1989).

The partial dehydrated somatic embryos were first transferred into 100 mL glass plates (10 embryos/plate) with 25 mL MSO conversion medium, containing MS salts, B5 vitamins, 3% sucrose, 0.3% Phytagel™, pH 5.8. After root elongation, the embryos were transferred individually to 100 mL flasks, containing 15 mL of MSO medium. After reaching the two to three trifoliolate stage, approximately four weeks later, the converted plantlets were transferred to plastic cups containing a mixture of soil and carbonized rice hulls (3:1), covered with plastic film. Plants were gradually exposed to ambient humidity over one week and than planted in 1 kg pots with organic soil in greenhouse.

Data of percentage of embryogenic cotyledon pairs, number of somatic embryos per cotyledon pairs and number of somatic embryos of embryogenic cotyledon pairs were analysed using the Kruskal-Wallis' non parametric analysis of variance. Cultivars were then pairwise compared using ranks at the 0.05 significance level.

RESULTS AND DISCUSSION

The ten tested cultivars are listed in TABLE 1 along with the somatic embryo production for each cultivar 45 days after culture initiation. The embryogenic potential expressed by the percentage of cotyledon pairs that produced somatic embryos (Figure 1A) ranged from 1 to 70% with a mean of 46%. The average number of somatic embryos produced per cotyledon pair ranged from 0.01 to 10.3 with a mean of 3.4. When means were calculated based on embryogenic cotyledon pair, the average number of somatic embryos ranged from 0.1 to 14.5 with a mean of 6.0. Similarly large differences in induction percentages among cultivars were reported in the literature. Komatsuda & Ohyama (1988) obtained from 0 to 95% of induction from 26 Japanese cultivars, Komatsuda (1990) obtained from 11.7 to 100% induction from 36 selected world soybean genotypes and Tian et al. (1994) obtained from 18.5 to 97.5% induction from 20 long-day adapted genotypes. In the above three studies, the ranges were 0 to 8.9, 0.2 to 12.9, and 0.6 to 11.8 somatic embryos per cotyledon pair, respectively. Induction frequencies should be considered when starting an *in vitro* culture, choosing cultivars with high capacity of somatic embryo production.

Among the three selected *Agrobacterium* susceptible cultivars, embryogenic suspensions were successfully developed from Bragg and IAS5. They were maintained for a period of one year at 14-day subculture intervals with a low inoculum density. Embryogenic clumps of globular-stage are shown in Figure 1 B.

The growth rate of the above two embryogenic suspension cultures was determined and is presented in TABLE 2. The packed cell volume, in one-month growth, increased 8.1 fold for Bragg and 3.5 fold for IAS5 and the fresh weight increased 6.6 and 2.8 fold, respectively. These data suggest that a soybean genotype with low efficiency of somatic embryo induction may be capable of establishing proliferating embryogenic suspension culture. The Bragg cultivar, for example, initially produced only an average of 2.8 somatic embryos from each pair of cotyledon which was below the overall average of 3.4 somatic embryos. From this cultivar, a more efficient embryogenic suspension culture was established than the one from IAS5 cultivar which produced 4.6 somatic embryos per pair of cotyledon explants. Comparable observation was reported by Bailey et al. (1993), where genotype PI417138 was among the least inducible of all genotypes they had studied for the initial somatic embryo induction, but it had both the highest qualitative evaluation and the highest somatic embryo yield on suspension culture. So, both induction and proliferation capacities of each soybean cultivar have to be considerated to allow high regeneration frequencies.

At the end of the first month on the maturation medium, the somatic embryos grew from globular stage to the histodifferentiated stage with well developed cotyledons and axis (Figure 1C). The somatic embryos expressed no obvious further growth during the second month incubation but their color changed from green to milky-white or milky-yellow. From the original seven flasks

TABLE 1 - Somatic embryo induction from immature cotyledon explant pairs of ten soybean cultivars.

Cultivar	Number of cotyledon pairs	Embryogenic explants	Somatic embryos / explant pair	Somatic embryos / embryogenic pair
		%	mean ± S.D.	mean ± S.D.
RS7	100	70 a[1,2]	10.3 ± 1.8 a	14.5 ± 2.3 a
BR1	94	63 ab	3.8 ± 0.6 ab	6.3 ± 0.9 ab
IAS5	98	50 abc	4.6 ± 1.1 abc	8.0 ± 1.6 ab
CEP12	88	52 abc	4.3 ± 1.1 abcd	8.4 ± 2.0 ab
Ivorá	93	46 bc	3.1 ± 0.7 bcd	6.1 ± 1.2 ab
Davis	91	59 abc	2.3 ± 0.6 bcd	3.6 ± 0.8 b
Bragg	96	47 abc	82.8 ± 0.9 bcd	4.3 ± 1.2 b
BR4	84	36 c	1.4 ± 0.3 cd	3.6 ± 0.7 b
IAS4	85	35 d	1.4 ± 0.5 de	3.7 ± 1.2 b
BR32	100	1 d	0.01 ± 0.01 e	0.1 ± 0.1 c
Mean ± S.D.	92.9 ± 5.8	46.0 ± 19.2	3.4 ± 2.8	6.0 ± 3.9
Kruskal-Wallis H		42.11	47.13	43.03

[1]Means and S.D. of observed values.
[2]Multiple comparisons test: different letters in the same column indicate significant differences at 0.05.

of Bragg cultivar, a total of 564 somatic embryos were obtained (TABLE 3) with 76.6% abnormal ones (as described by Buchheim et al., 1989). For IAS5 cultivar, 93 somatic embryos were obtained with 78.5% of them showing abnormality.

Although a high percentage of the resultant somatic embryos from the suspension cultures was of abnormal types, conversion data indicated that a large percentage of them were capable of germinating into normal plants as long as shoot apex were present on the embryos. After a two-day air desiccation, 45.7% (258) of the somatic embryos from the original seven flasks of Bragg cultivar converted into plants in MSO medium with 79.1% plants derived from the abnormal somatic embryos (TABLE 3). For IAS5 cultivar, 54.9% (51) converted with 78.4% from the abnormal somatic embryos. High abnormality percentages were obtained as a rule, rather than exception, among soybean somatic embryo production reports (Buchheim et al., 1989; Bailey et al., 1993; Finer, 1995; Santos et al., 1997). Plants from both cultivars were successfully transferred to soil and yielded seeds in the greenhouse (Figure 1 D).

This is the first report for regeneration of soybean cultivars in Brazil using suspension culture of somatic embryos. The availability of a proliferative system opens up the possibility of using embryogenic tissue as a target for particle bombardment and *Agrobacterium*-mediated transformation. Embryogenic tissue is generally very responsive to transformation (Finer & McMullen, 1990; Fromm et al., 1990), and embryogenic soybean is no exception (Finer & McMullen, 1991; Parrott et al., 1994; Hadi et al., 1996; Liu et al., 1996). Another advantage of using embryogenic suspension cultures is that chimeric plants should not be recovered if embryos are allowed to proliferate under selection for a period of time after transformation. The liquid medium allows the proliferation of secondary embryos from apical tissues of older somatic embryos, permitting generation of large amounts of uniform transformed embryogenic tissue and the recovery of solid transgenic fertile plants.

TABLE 2 - One-month growth of embryogenic suspension cultures of two soybean cultivars. (Initial volume: 28 μL; initial weight: 15 ± 3 mg).

Flask number	Bragg		IAS5	
	Volume	Weight	Volume	Weight
	μL	mg	μL	mg
1	145.1	72.5	81.2	53.8
2	274.8	158.8	96.7	52.1
3	267.4	87.8	62.1	23.0
4	270.4	104.3	114.0	52.5
5	143.5	52.7	97.3	32.9
6	193.5	91.4	151.7	49.5
7	298.0	127.7	72.3	34.4
Mean ± S.D.	227.5 ± 65.4	99.3 ± 35.3	96.5 ± 29.9	42.6 ± 12.3

TABLE 3 - Conversion of plants obtained from suspension cultures of somatic embryos from Bragg and IAS5 cultivars.

Form	Bragg		IAS5	
	Maturated embryos	Converted embryos	Maturated embryos	Converted embryos
	%			
NORMAL				
Dicotyledonous	23.4	20.9	21.5	21.6
ABNORMAL				
Monocotyledonous	14.6	15.6	34.7	19.1
Polycotyledonous	15.5	5.5	11.4	3.5
Fused cotyledons	11.7	34.1	3.5	31.3
Trumpet	11.7	4.3	11.4	6.9
Fasciated	10.7	13.7	11.4	12.4
Long hypocotyl,				
vestigial cotyledon	2.9	3.1	1.7	3.6
Proximal diaxial fusion	9.5	2.8	4.4	1.6
Total % of abnormal	76.6	79.1	78.5	78.4
Number of embryos	564	258	93	51

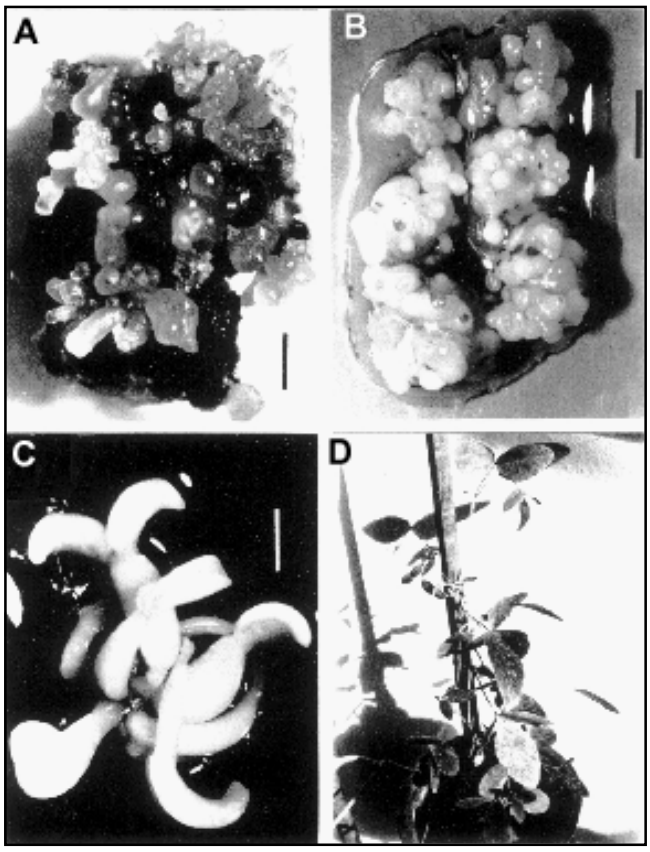

Figure 1 - (A) Somatic embryo induction in soybean Bragg cultivar after 90 days of incubation on the D40 medium. Note the secondary somatic embryos proliferated from the apical portions of the primary embryos. Bar = 1 mm. (B) Clumps of globular embryos from the suspension culture of soybean Bragg cultivar. Bar = 2 mm. (C) A cluster of mature somatic embryos from a tissue clump of the embryogenic suspension culture of soybean Bragg cultivar after one month incubation on the maturation (M6MAC) medium. Bar = 2 mm. (D) A seed producing mature soybean plant of IAS5 cultivar regenerated from the embryogenic suspension culture. Pot diameter = 14 cm.

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