

SOMATIC EMBRYOID INDUCTION FROM BRAZILIAN SOYBEAN CULTIVARS

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ABSTRACT

Somatic embryoids were regenerated and converted to plantlets from the immature zygotic cotyledons of Brazilian soybean cultivars IAS-5 and COBB. Young soybean pods with seeds 4 to 6 mm in length were used. Fifty to 150 pairs of cotyledon explants were cultured per cultivar per experiment. The culture procedure was divided into induction, maturation and conversion stages. The induction medium contained Murashige and Skoog (MS) medium with 30 mg/l 2,4-D, 3% sucrose, and 0.6% agar. MS medium with 10% sucrose, 0.5% activated charcoal, and 0.8% agar was used at the maturation stage. The conversion medium contained Schenk and Hildebrandt (SH) medium with 1% sucrose and 0.6% agar. An average of 2.4 embryoids was obtained from each cotyledon explant pair, of which 59.5% reached maturity with 58.7% mature embryoids converted into plantlets from cv. IAS-5. The same values for cv. COBB were 2.6, 31.4, and 90.9%, respectively. This resulted in 0.9 and 0.75 converted plantlets from each pair of cotyledon explants for cv. IAS-5 and COBB, respectively.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a crop species that is difficult to manipulate with modern *in vitro* culture and genetic engineering procedures. Finer and Nagasawa (1988) succeeded in establishing an embryogenic suspension system which was used efficiently in genetic transformation via microprojectile bombardment (Finer and McMullen, 1991). This suspension culture was derived from clusters of globular,

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secondary somatic embryos which were induced from the cotyledons of immature zygotic embryos (Finer, 1988). The capacity of somatic embryo regeneration from immature zygotic soybean cotyledons was first reported by Lippmann and Lippmann (1984) and complete protocols were developed and improved by Lazzeri *et al.* (1985), Ranch *et al.* (1985, 1986), Finer and Nagasawa (1988), and Buchheim *et al.* (1989). This represents the most productive means of *in vitro* soybean cloning.

The induction of somatic embryos from Brazilian soybean cultivars was first reported by Ferreira *et al.* (1990), based on a protocol developed by Ranch *et al.* (1986). We have based our study on a more refined procedure from Ranch's laboratory (Buchheim *et al.*, 1989) to induce somatic embryos from Brazilian soybean cultivars. For comparison purposes, one of the two cultivars we selected, "IAS-5", was also one of the two cultivars studied by Ferreira *et al.* (1990).

MATERIALS AND METHODS

Two Brazilian soybean cultivars, "IAS-5" and "COBB", were used in this study. The experiments on cv. IAS-5 were conducted twice. The procedure of Buchheim *et al.* (1989) was adopted with minor modifications. Young soybean pods with seeds 4 to 6 mm in length were harvested from greenhouse grown plants and used within 24 hours. Pods were disinfected for two minutes in 70% ethanol followed by 20 minutes in diluted commercial bleach (1% sodium hypochlorite), with a trace amount of Tween. After three changes of sterile, distilled water, the seeds were dissected under a dissecting microscope in a laminar flow hood. Excised cotyledon pairs were transferred onto the induction medium with the abaxial surface in contact with the culture medium. There were 10 cotyledon explant pairs in each 10-cm glass Petri dish (Figure 1A). The number of cotyledon pairs per cultivar per experiment varied from 50 to 150.

The culture procedure was divided into induction, maturation, and conversion stages. The induction medium contained MS medium (Murashige and Skoog, 1962) with 30 mg/l 2,4-D, 3% sucrose, and 0.6% agar. Culture dishes at the induction stage were wrapped in one layer of brown paper and incubated at $26 \pm 1^{\circ}\text{C}$ under a 16-h photoperiod of approximately 3 klx fluorescent light. The same incubation conditions were used in all three stages. After two months incubation, the somatic embryos from the induction medium were excised, individually for the larger ones or in clusters for the smaller ones, from the cotyledon tissues and transferred onto the maturation medium. Glass Petri dishes wrapped in brown paper containing MS medium with 10% sucrose, 0.5% activated charcoal, and 0.8% agar were used at the maturation stage. After 45 days incubation, the mature, dormant embryos were transferred onto the conversion medium containing SH medium (Schenk and Hildebrandt, 1972), with 1% sucrose and 0.6% agar in 150-ml glass bottles.

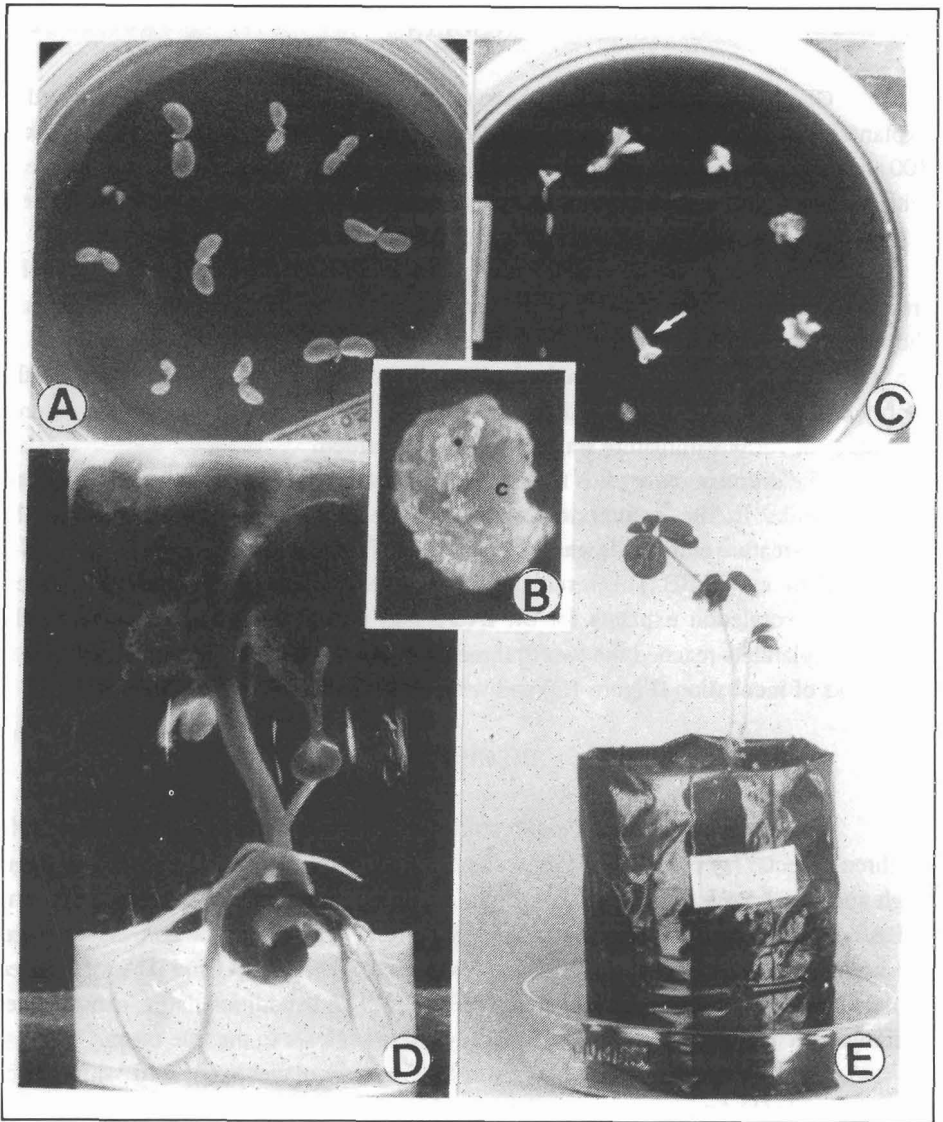


Figure 1 - (A) Cotyledon explants of immature soybean cv. IAS-5 placed on the embryoid induction medium in a 10-cm glass Petri dish; (B) Numerous somatic embryos (*) produced along the mid-rib of the adaxial surface of a cotyledon (c) explant of soybean cv. IAS-5 after one month incubation on the embryoid induction medium. Cotyledon length - 9 mm; (C) Dormant somatic embryos of soybean cv. IAS-5 developed from clusters of globular and heart-shaped embryoids after being placed on the maturation medium for one month in a 10-cm Petri dish. The arrow points to a mature embryoid; (D) A plantlet converted from a mature dormant embryo after being placed on the conversion medium for one month. Tube width - 2 cm; (E) A plantlet converted from a mature dormant embryoid and successfully planted out and acclimatized to the greenhouse conditions. Pot diameter - 13 cm.

RESULTS

Globular embryoids were observed on the adaxial surface of the cotyledon explants four weeks after induction incubation (Figure 1B). After two months incubation, 100 and 420 embryoids were obtained from cv. IAS-5 in experiment I and II, respectively, with an average of 2.4 embryoids per cotyledon pair. A total of 210 embryoids were obtained from cv. COBB, with an average of 2.6 embryoids per cotyledon pair.

The embryoids changed from green to milky, yellowish-white in color (resembling mature beans), partially dehydrated, and entered dormancy after 45 days in the maturation medium (Figure 1C). In the two cv. IAS-5 experiments, 69 and 50% of the embryoids, respectively, reached mature/near-mature stages and 31.4% of the embryoids of cv. COBB reached the same stage. The remaining embryoids entered dormancy at various immature stages and were discarded.

The dormant embryoids turned green and started to produce taproots after one to two weeks in the conversion medium. The conversion percentage for the mature/near-mature embryoids was 44.9 and 71.4% for the two experiments of cv. IAS-5 and 90.9% for cv. COBB. This resulted in an average of 0.9 and 0.75 converted plantlets per pair of cotyledon explants for cv. IAS-5 and COBB, respectively. Some of the converted plantlets reached the two to three trifolium stage after approximately four to six weeks of incubation (Figure 1D) and were transplanted into soil (Figure 1E).

DISCUSSION

The procedure followed in this study differed from that of Ferreira *et al.* (1990) in three aspects: (1) the multiplication stage was omitted. (2) At the maturation stage, high sucrose, hormone-free, solid medium was used, whereas low sucrose, IBA- and ABA-containing liquid medium was used by Ferreira *et al.* (3) At the conversion stage, the hormone-free medium with 1% sucrose was used, whereas GA₃ and IBA were added to the higher (3%) sucrose medium used by Ferreira *et al.* In addition, larger sample sizes were used in this study, which would likely yield data closer to the true mean.

This refined procedure had the following advantages compared with that of Ferreira *et al.*: (1) An improvement of the number of mature embryoids produced by each cotyledon explant pair from 1.7 to 2.4 on cv. IAS-5. (2) About 3.5 to 4 months time saved from explant dissecting to the time for planting. (3) The omission of the high 2,4-D containing multiplication stage, the complete omission of hormones in the maturation and conversion media, and the reduction of total *in vitro* incubation time, which are likely to reduce the frequency of mass chromosomal and genetic mutations.

We acknowledge that the rates of embryoid induction and plantlet production in Brazilian soybean cultivars are still low with this procedure. Our studies with U.S.

cultivars (Hu, unpublished data) indicated that significant improvements can be expected when the following modifications are adopted: (1) use of healthy, early season pods from field-grown plants; (2) increase of sucrose and 2,4-D concentrations at the induction stage (Finer, 1988); (3) air-drying of the mature embryoids before conversion (Buchheim *et al.*, 1989); (4) replacement of sucrose with maltose at the maturation stage and replacement of agar with Gelrite at the maturation and conversion stages (Finer and McMullen, 1991). We also discovered that the percentage of embryoids reaching the mature stage can be further improved by briefly activating the dormant immature embryoids (from the maturation stage) in the conversion medium for three to seven days, followed by transferring them back to the maturation medium for a month of further embryonic growth and dormancy induction (Hu, unpublished data).

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RESUMO

Embriões somáticos obtidos a partir de cotilédones zigóticos imaturos das cultivares brasileiras de soja IAS-5 e COBB foram regenerados e convertidos em plântulas. Foram utilizados legumes jovens com sementes de 4-6 mm de comprimento. Cinquenta a 150 explantes (= pares de cotilédones) foram cultivados por cultivar e por experimento. O procedimento de cultura foi dividido em três estádios: indução, maturação e conversão. O meio de indução continha o meio de Murashige and Skoog (MS) com 30 mg/l de 2,4-D, 3% de sacarose e 0,6% de ágar. O meio MS com 10% de sacarose, 0,5% de carvão ativado e 0,8% de ágar foi utilizado no estágio de maturação. O meio de conversão continha o meio de Schenk and Hildebrandt (SH) com 1% de sacarose e 0,6% de ágar. Foi obtida uma média de 2,4 embriões por explante, dos quais 59,5% alcançaram a maturidade, sendo que 58,7% dos embriões maturados foram convertidos em plântulas na cultivar IAS-5. Os valores respectivos para a cultivar COBB foram 2,6, 31,4 e 90,9%. Isto resultou em 0,9 e 0,75 plântulas convertidas por explante para a cultivar IAS-5 e COBB, respectivamente.

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