In Vitro Cell. Dev. Biol.—Plant 33:13–19, January 1997

1071-2690/97 $05.00 + 0.00

EFFECT OF EXPLANT ORIENTATION, pH, SOLIDIFYING AGENT AND WOUNDING ON INITIATION OF SOYBEAN SOMATIC EMBRYOS

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(Received 2 April 1996; accepted 22 August 1996; editor D. C. W. Brown)

SUMMARY

Several methods have been developed to obtain somatic embryos of soybean. We report here a new procedure that results in high frequency somatic embryo initiation in a short period of time. Somatic embryos were induced from immature cotyledons of the cultivars "Jack," "Thorne," "Resnik," and "Chapman." Immature cotyledons were cultured on a medium containing MS salts, B_5 vitamins, 6% sucrose, and 40 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Culture modifications included: orientation of the explants (adaxial or abaxial side of the cotyledon in contact with the medium), adjustment of medium pH (5.7 or 7.0), wounding of cotyledons with scalpel blades, inclusion of ethylene modulators, and use of Noble agar or Gelrite™ as the solidifying agent. The treatment that resulted in the highest embryo induction across the cultivars consisted of abaxial side of the explant facing the medium, pH 7.0 and 0.2% Gelrite™. "Jack" was the most responsive cultivar showing the first embryos as early as 14 d after culture. After 21 d, an average of 44 embryos per cotyledon was obtained with this cultivar. The inclusion of silver nitrate ($AgNO_3$) in the culture medium did not enhance the number of primary somatic embryos induced per cotyledon, but the addition of 15 μM $AgNO_3$ did result in a faster production of secondary embryos using the cultivar "Jack." Wounding of the explants with a scalpel resulted in an earlier induction of somatic embryos. Embryo initials were first observed after only 7 d. Histological examination of cultured cotyledons indicated that the somatic embryos originated from the subepidermal tissues and were of multicellular origin. This somatic embryo induction procedure could be useful for direct transformation work and permits the production of embryogenic tissue within 2 wk.

Key words: somatic embryogenesis; *Glycine max*; soybean; pH effect; Gelrite™; wounding.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] can be regenerated via either shoot morphogenesis (Barwale et al., 1986; Wright et al., 1986) or somatic embryogenesis (Ranch et al., 1985; Barwale et al., 1986); both methods of regeneration can be utilized for genetic transformation work. Tissues undergoing shoot morphogenesis have been transformed using *Agrobacterium* (Hinchee et al., 1988) and particle bombardment (McCabe et al., 1988), while proliferative embryogenic cultures have only been transformed via particle bombardment (Finer and McMullen, 1991; Sato et al., 1993; Parrott et al., 1994). In spite of all these reports of soybean transformation, the methods are far from routine. A more useful and efficient system for soybean transformation may rely on a new system where regeneration is direct and rapid.

One soybean tissue culture system that has not been extensively evaluated in transformation studies is induction of somatic embryos from immature cotyledons. Although proliferative embryogenic cultures provide a suitable target tissue for transformation, the time and labor required for establishment of these cultures can be great. Induced embryos, on the other hand, form directly from the explant in as little as a few weeks and may be suitable for direct transformation using either *Agrobacterium* or the particle gun. The current difficulty with the use of somatic embryo initiation for soybean transformation work is the inefficiency of the induction process.

We report here the efficient induction of soybean somatic embryos with the proper combination of explant orientation, pH, solidifying agent, and wounding of the explant. Immature cotyledon explants, plated with the abaxial surface in contact with a medium containing Gelrite™ (pH 7.0) and wounded with a scalpel blade, formed embryos within 2 wk of culture. A maximum average of 44 somatic embryos were obtained after 21 d of culture using the cultivar "Jack." The influence of wounding on the rate and site of embryo initiation is also reported. Lastly, the effects of ethylene modulators on induction of somatic embryogenesis in soybean were evaluated.

MATERIALS AND METHODS

Plants of soybean [*Glycine max* (L.) Merrill] cultivars Jack, Thorne, Resnik, and Chapman were grown in the greenhouse under 14h/10h photoperiod at 28° C. Immature pods containing embryos that were approximately 4 mm in length were harvested 2 to 3 wk after flowering. Pods were surface sterilized by immersion in a solution of 20% (vol/vol) commercial bleach and 0.01% (vol/vol) Tween 20 for 20 min followed by three rinses in sterile water. The

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TABLE 1

THE EFFECT OF MEDIA AND CULTURE CONDITIONS ON SOMATIC EMBRYOGENESIS OF THREE SOYBEAN CULTIVARS AFTER 21 D*

	Solidifying Agent							
	Agar				Gelrite™			
pH	5.7		7.0		5.7		7.0	
Orientation	AB**	AD**	AB	AD	AB	AD	AB	AD
Jack	2.5 bc***	1.2 c	8.5 b	3.1 bc	8.8 b	2.6 bc	44.2 a	5.0 bc
Thorne	1.0 bc	0.7 bc	5.9 ab	2.2 bc	2.5 bc	3.3 c	12.7 a	3.8 bc
Resnik	1.6 bc	0.05 c	4.4 ab	0.8 bc	0.6 c	0.2 c	9.0 a	0 c

* Average number of somatic embryos per cotyledon from three replications with 10 explants each.

** AB - adaxial side facing the medium; AD - adaxial side facine the medium.

*** Means in each row followed by the same letters are not significantly different at the 0.05 level according to Fisher's Least Significant Difference (LSD's) test.

immature cotyledons were excised from the seeds and placed on 40T6S medium (Finer, 1988) containing MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), 6% sucrose, and 40 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). All cultures were maintained at 28° C under 16h/8h photoperiod with a light intensity of 30 $\mu Em^{-2}s^{-1}$.

Excised cotyledons were plated either adaxial or abaxial surface in contact with the medium using a medium pH of 5.7 or 7.0 (before autoclaving) with either 0.8% Noble agar or 0.2% Gelrite™ (Merck & Co., Rahway, NJ) as the solidifying agent. Each Petri dish representing 1 replication contained 10 explants. Treatments consisted of a minimum of three replications.

Wounding. Cotyledonary explants were uniformly wounded along their long axis with a "3-blade scalpel" consisting of three pieces of razor blade oriented in parallel (1 mm apart) and attached to the base of a plastic syringe plunger. The wounds were made to a depth of approximately 1 mm. Wounded cotyledons were cultured on D40 medium (40T6S medium containing 0.2% Gelrite™, pH 7.0). All explants were oriented with the abaxial side facing the medium. The cultivars used in this experiment were "Jack" and "Resnik."

Ethylene modulators. $AgNO_3$ was filter sterilized and added to D40 medium at 15, 30, 45, and 60 μM. To evaluate other ethylene modulators, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) at 30 μM and ethylene antagonists aminoethoxyvinylglycine (AVG) at 5 μM, cobalt chloride ($CoCl_2$) at 5 μM, salicylic acid (SA) at 5 μM, and silver nitrate ($AgNO_3$) at 15 μM were added to the cooled medium following filter sterilization. All explants were placed with the abaxial side facing the medium.

Initiation of suspension or proliferative semisolid cultures. Cultured immature cotyledons were either placed in liquid FN medium (Finer and Nagasawa, 1988) after 0, 2, 7, 14, 21, and 28 d of culture on D40 medium or transferred to semisolid proliferative D20 medium (D40 with 20 mg/l 2,4-D and 3% sucrose) after 14, 21, and 28 d. For embryo proliferation on D20 medium, the effects of using pH 5.8 and 7.0 were also evaluated. Experiments were performed with four replications of 3 and 10 embryogenic cotyledons each, for liquid and semisolid cultures, respectively.

Histology. Wounded and nonwounded cotyledons cultured on D40 medium were collected at 0, 7, 14, and 21 d following culture initiation. For scanning electron microscopy, cotyledons were fixed in 0.2 *M* potassium phosphate buffer (pH 7.4) containing 3% glutaraldehyde, 2% paraformaldehyde, and 1.5% acrolein for 2 h at room temperature. Samples were then dehydrated in an ethanol series (50–100% ethanol at 10 min each), critical point dried, sputter coated with platinum, and viewed on an ISI-40 scanning electron microscope. For light microscopy, cotyledons were fixed in 0.2 *M* potassium phosphate buffer (pH 7.4) containing 3% glutaraldehyde, 2% paraformaldehyde, and 1.5% acrolein, rinsed three times in 0.1 *M* phosphate buffer, postfixed with 1% osmium tetroxide, stained with uranyl acetate, dehydrated in an ethanol series, and embedded in Spurr's resin (Spurr, 1969) as described earlier (Finer, 1988). Tissue was sectioned to 0.75 μm on a JB-4 microtome and the sections were stained with toluidine blue for viewing.

Analysis of embryo induction. Primary somatic embryos were included in counts if they were clearly distinguishable as individual embryos on the surface of the cotyledons. Means of primary somatic embryos were calculated after 21 d on induction medium and analyzed by analysis of variance (ANOVA). Treatment means were separated using Fisher's Least Significant Difference ($\alpha = 0.05$).

Results and Discussion

Induction of somatic embryogenesis from immature cotyledons of soybean was influenced by explant orientation, pH, solidifying agent, and genotype. Embryo initiation was highest when the induction medium was adjusted to pH 7.0 and solidified with Gelrite™ and when the explants were cultured with the abaxial side facing the medium (Table 1). Among the three cultivars tested, "Jack" showed the highest embryogenic induction after 21 d in culture. The frequency of responding cotyledons was 100% and an average of 44.2 embryos per explant was obtained (Table 1). Using the medium and conditions described above, the first somatic embryos were observed arising from the edges of the excised cotyledons after 14 d in culture. After 21 d, the entire surface of the explant was covered with embryos (Fig. 1). In previous reports using different conditions, this level of induction was not observed and the average number of embryos per cotyledon was less than 11 (Lazzeri et al., 1987; Hartweck et al., 1988; Parrott et al., 1988). The frequency of embryo induction obtained with "Thorne" and "Resnik" also increased when pH 7.0 and Gelrite™ were used (Table 1). These results suggest a synergistic effect among pH, explant orientation, and gelling agent. The use of pH 7.0 has been reported for induction of somatic embryogenesis in soybean, but only four embryos per cotyledon were obtained in that

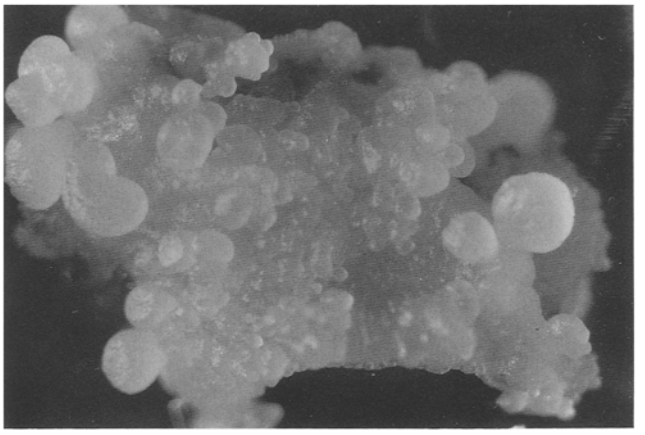

FIG. 1. Soybean somatic embryo induction after 21 d on D40 medium (×15.4).

TABLE 2

EFFECT OF WOUNDING ON SOMATIC EMBRYO INDUCTION OF TWO SOYBEAN CULTIVARS AFTER 21 D*

	Treatments	
Cultivars	Wounded	Nonwounded
Jack	36.0**a	14.7 a
Resnik	16.4 a	14.2 a

* Average number of somatic embryos per cotyledon from three replications with 10 explants each.

** Means in each row followed by the same letters are not significantly different at the 0.05 level according to Fisher's Least Significant Difference (LSD's) test.

study (Komatsuda and Ko, 1990). This result was probably due to the use of NAA (α-naphthaleneacetic acid) rather than 2,4-D for induction. Bailey et al. (1993) also used pH 7.0 for induction of somatic embryogenesis in soybean, but a comparison of different pHs was not made. The effect of pH on embryo induction could be related to auxin uptake in the cultured tissues. Edwards and Goldsmith (1980) reported a reduction in the rate of auxin uptake in maize coleoptiles at pH 7.0 compared to pH 5.8. The enhancement of embryo induction at pH 7.0 reported here for soybean may result from a slower and more gradual uptake of 2,4-D when using the induction medium containing relatively high levels of 2,4-D.

Explant orientation effects have been observed in shoot-producing cultures of several woody species where segments responded optimally when oriented horizontally and not vertically (McClelland and Smith, 1990). In *Lilium longiflorum*, explants cultured with their abaxial side in contact with the medium had a higher frequency of bulblet induction than those with the opposite orientation (Leshem et al., 1982). Effects of explant orientation have been reported in soybean (Hartweck et al., 1988; Buchheim et al., 1989). However, in that study, an average of only 11 somatic embryos was obtained when the explants were cultured with the abaxial side in contact with a medium containing 25 *mg/l* 2,4-D and solidified with Phytagar. However, the use or effect of other solidifying agents, wounding, or ethylene modulators was not reported.

The benefit of using Gelrite™ in place of agar as a gelling agent in media has been reported for several species (Huang et al., 1995). Adventitious embryogenesis was increased by using Gelrite™ instead of purified agar in *Mangifera indica* (De Wald et al., 1989) and *Oryza sativa* (Koetj et al., 1989). The modified procedure we have reported—adjusting the pH to 7.0 and using a high 2,4-D concentration and Gelrite™ as the solidifying agent, allowed an increase of the efficiency of soybean somatic embryo formation.

The wounding treatment did not increase the number of embryos formed and no difference was observed between the cultivars tested (Table 2). However, in wounded explants, somatic embryos were induced earlier than in the explants without wounding (Fig. 2 A–H). Nadolska-Orczyk and Orczyk (1994) reported an enhancement of somatic embryo induction in soybean tissue following wounding. However, the average number of embryos induced per wounded cotyledon in that study was less than six. A statistically significant difference between wounding versus nonwounding was not observed in our study due in part to the large variation observed among the treatments. This variation may have been caused by differences in greenhouse-grown donor plants throughout the different seasons. Although wounding did not increase the frequency of embryo induction in our study, it may be useful for *Agrobacterium*-mediated transformation work, where wounding of the tissue is most often beneficial. The response of the immature cotyledons to wounding could be related to the ethylene production by the tissue during culture. Wounding stimulates ethylene production by inducing formation of ACC synthase (Yang and Hoffman, 1984). Although the effects of ethylene modulators on somatic embryogenesis have been evaluated in several species (Biddington, 1992), they have not been evaluated in soybean. The effects of ethylene modulators on soybean embryogenesis are shown in Tables 3 and 4. The inclusion of $AgNO_3$ in the culture medium did not increase the number of primary embryos induced per cotyledon (Table 3), but the addition of 15 μM $AgNO_3$ resulted in a faster production of secondary embryos when the cultivar "Jack" was used (data not shown). Cotyledons cultured on media containing either 45 or 60 μM $AgNO_3$ produced somatic embryos that tended to develop rather than proliferate. The average number of embryos induced on medium containing AVG, ACC, $AgNO_3$, $CoCl_2$, and SA was not significantly different from the control (Table 4). In *Hordeum vulgare*, the addition of ACC and $AgNO_3$ to the medium also showed no stimulatory effects on somatic embryo induction (Evans and Batty, 1994). In *Medicago sativa* (Meijer and Brown, 1988), it was not possible to demonstrate a relationship between ethylene production and somatic embryogenesis. While $CoCl_2$ and $NiCl_2$ inhibited ethylene production and strongly retarded somatic embryo formation, AVG did not affect ethylene production, but embryo induction still declined. Although only one concentration of ACC, AVG, $CoCl_2$ and SA was evaluated in our study, these concentrations were considered optimal for other species (Vain et al., 1989; Chi et al., 1994). The use of ethylene as a growth regulator for the improvement of somatic embryo induction may require the establishment of optimum concentrations for each tissue or step during the process of soybean embryogenesis.

To determine if secondary embryo formation was advantageous prior to initiation of suspension or proliferative semisolid cultures, both types of cultures were initiated from explants taken at various times following initial culture. When liquid medium was used, no embryo formation or proliferation was observed from cotyledons taken 0, 2, or 7 d after culture initiation on D40 medium. No difference in embryo proliferation was observed when cotyledons containing induced embryos, taken 14, 21, or 28 d after culture on D40 medium were placed in liquid FN medium. Similar results were observed with proliferative semisolid cultures from explants taken 14, 21, or 28 d following plating of cotyledons on induction medium. In contrast to the effect of the pH on embryo initiation, cultures maintained on semisolid medium at pH 5.8 or pH 7.0 showed no difference in proliferation rates (data not shown). This shows that once embryogenesis has been induced in cells of immature cotyledons, it is not necessary to maintain the tissue under those same conditions needed for induction. Therefore, pH 5.8 was used for both liquid and semisolid proliferative cultures, which were initiated between 14 and 21 d after plating of cotyledons.

Scanning electron microscopy (Fig. 2 *A–H*) and sectioning of tissue for light microscopy analysis (Fig. 3 *A–H*) were performed on wounded and nonwounded tissues in order to determine the timing and origin of the somatic embryos. In general, the wounded cotyledons responded much more rapidly than the nonwounded explants (Fig. 3 *B,F*). In wounded cotyledons, the first divisions in the tissue adjacent to the wound site occurred by Day 4 (data not shown). Cell proliferation was later observed throughout the cotyledonary tissue

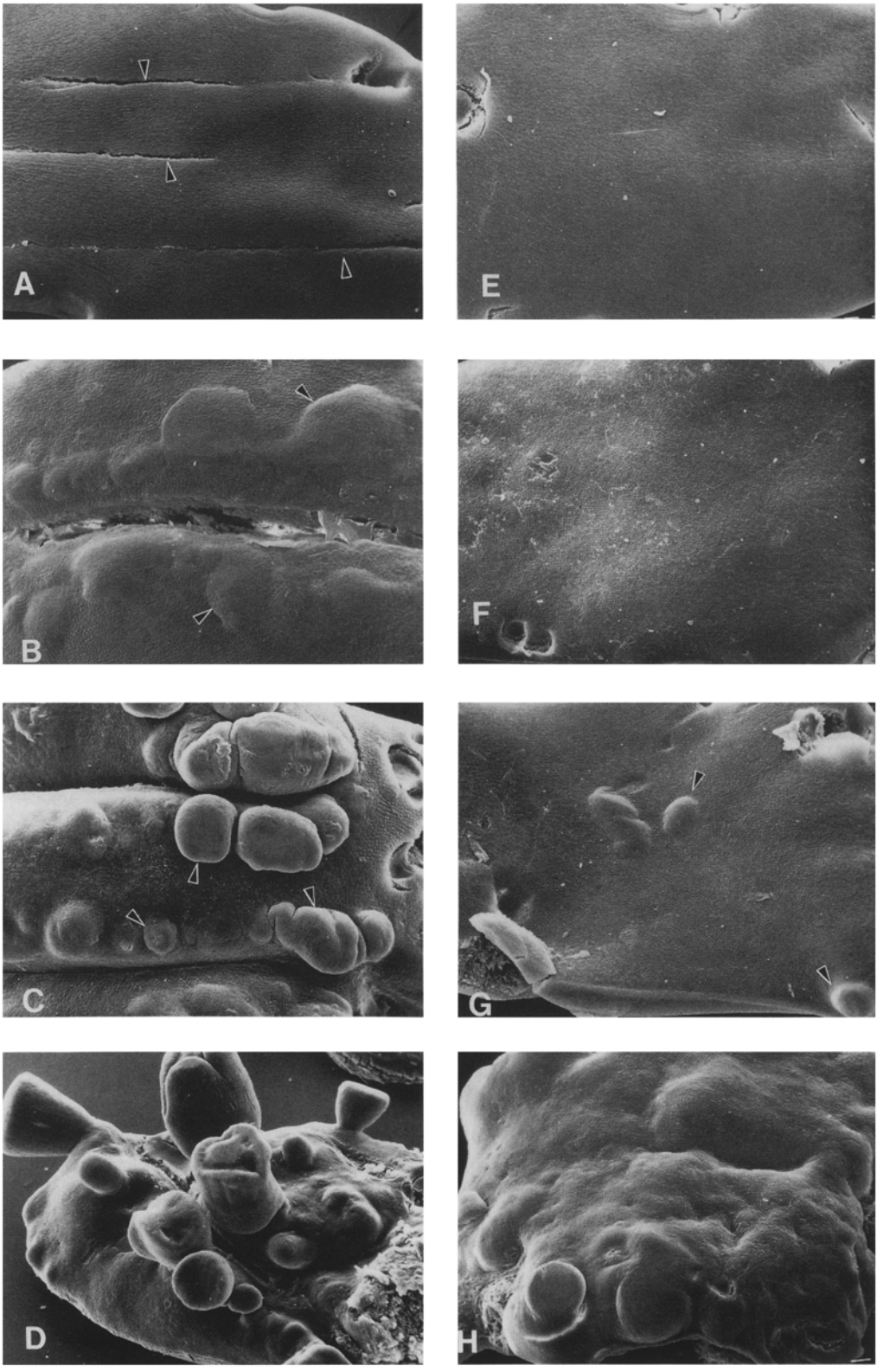

FIG. 2. Scanning electron microscopy of wounded and nonwounded immature cotyledons of soybean. (*A–D*) Wounded tissue. (*E–H*) Nonwounded tissue. (*A*) Day 0. *Arrows* indicate wound sites (×38). (*B*) Day 7. *Arrows* indicate the first somatic embryos (×41). (*C*) Day 14. *Arrows* indicate somatic embryos at the wound sites (×27). (*D*) Day 21 (×17). (*E*) Day 0 (×30). (*F*) Day 7 (×27). (*G*) Day 14. *Arrow* indicate the first somatic embryos (×31). (*H*) Day 21 (×27).

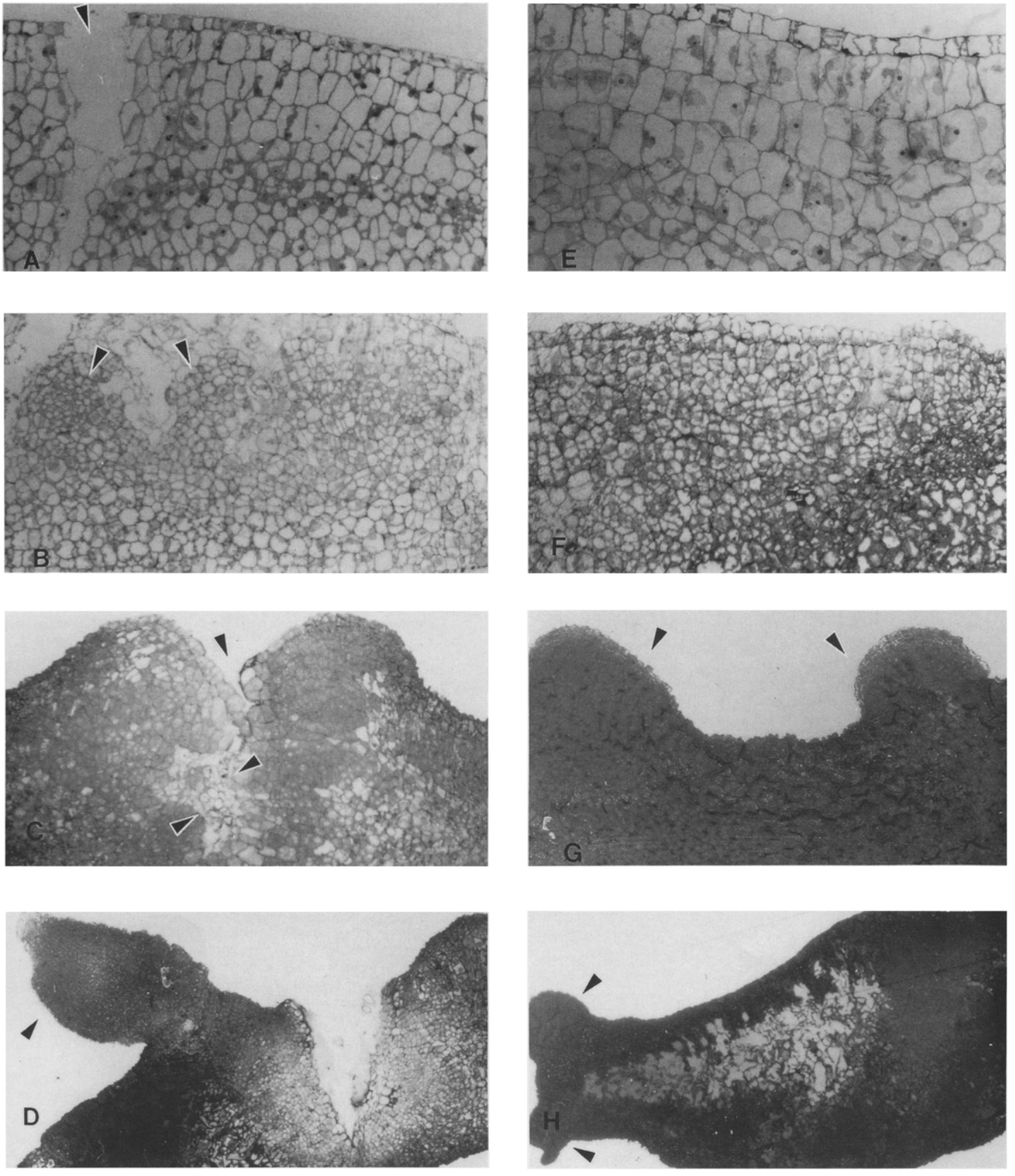

FIG. 3. Sections of cultured immature cotyledons of soybean. (*A–D*) Wounded tissue. (*E–H*) Nonwounded tissue. (*A*) Day 0. *Arrow* indicates wound site (×7.7). (*B*) Day 7. *Arrows* indicate meristematic areas (×8.1). (*C*) Day 14. *Arrows* indicate remnant of wound site (×8.1). (*D*) Day 21. *Arrows* indicates somatic embryo (×5.1). (*E*) Day 0. (×4.5). (*F*) Day 7 (×18.6). (*G*) Day 14. *Arrows* indicate somatic embryos in early stage of development. (×7.6). (*H*) Day 21. *Arrows* indicate somatic embryos from edge of cotyledon on adaxial and abaxial surfaces (×7.9).

TABLE 3

THE EFFECT OF $AgNO_3$ ON SOMATIC EMBRYOGENESIS INDUCTION OF TWO SOYBEAN CULTIVARS AFTER 21 D*

	Silver Nitrate (μM)				
Cultivars	0 (control)	15	30	45	60
Jack	15.2 ab**	23.1 a	13.4 b	13.0 b	11.7 b
Chapman	14.4 ab	13.1 b	13.6 b	12.2 b	10.4 b

*Average number of somatic embryos per cotyledon from three replications with 10 explants each.

** Means in each row followed by the same letters are not significantly different at the 0.05 level according to Fisher's Least Significant Difference (LSD's) test.

TABLE 4

EFFECT OF ETHYLENE MODULATORS ON SOMATIC EMBRYOGENESIS OF SOYBEAN, CULTIVAR "JACK"*

Ethylene Modulators	Average Number of Embryos/Cotyledon
Control	20.8 ab**
ACC (30 μM)	14.6 ab
AVG (5 μM)	29.5 a
$AgNO_3$ (15 μM)	20.4 ab
$CoCl_2$ (5 μM)	13.5 b
SA (5 μM)	9.9 b

*Average number of somatic embryos per cotyledon from three replications with 10 explants each.

** Means followed by the same letters are not significantly different at the 0.05 level according to Fisher's Least Significant Difference (LSD's) test.

(Fig. 3 *B*). The tissue that was damaged as a direct result of the wounding rapidly degenerated.

In nonwounded tissue, cell divisions were observed after 7 d in culture (Fig. 3 *F*). By this time point, in the wounded explants, rapid cell divisions were observed in the adaxial portion of the cotyledon and some meristematic areas were already formed (Fig. 3 *B*). With continued culture, somatic embryos were almost always observed adjacent to the wound site on the wounded explants by Day 14 (Figs. 2 *C* and 3 *C*). Prediction of the timing and origin of embryo initiation is useful for transformation studies, where it is important to target the proper tissue at the proper time. Some degree of organization was also observed on nonwounded tissue after 14 d of culture (Figs. 2 *G* and 3 *G*). Somatic embryos at different stages of development were observed after 21 d (Figs. 2 *D,H*; 3 *D,H*). Hepher et al. (1988) reported the formation of a superficial embryogenic tissue that was associated with the adaxial surface of soybean cotyledons. Hartweck et al. (1988) reported a multicellular origin from epidermal and subepidermal cells. Finer and McMullen (1991) showed single cell origin of soybean somatic embryos in proliferative liquid cultures. Our histological observations suggest that embryo induction in soybean may be from a small group of subepidermal cells and that wounding of the tissue results in earlier and more controlled production of somatic embryos.

The high efficiency of somatic embryo induction reported in this work is a result of the synergistic effect of pH, solidifying agent, concentration of 2,4-D, and wounding of the initial explants. The accelerated embryo formation using wounding allows the initiation of proliferative cultures as soon as 2 wk after placement of the cotyledons in culture.

Knowledge of the timing and origin of soybean somatic embryo induction may make this system suitable for *Agrobacterium*- and particle-bombardment-mediated transformation.

ACKNOWLEDGMENTS

The authors are grateful to Barbara Norris, Juliane Essig, and Lloyd Ringley, Jr., for technical assistance. We also thank Harold N. Trick for his help with scanning electron microscopy. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University/ Ohio Agricultural Research and Development Center, Rhône Poulenc and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Brazil. Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by OSU/OARDC, and also does not imply approval to the exclusion of other products that may also be suitable. OARDC Journal Article Number 43–96.

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