

## Some factors affecting somatic embryogenesis efficiency in soybean (*Glycine max* (L.) Merr.)

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### Abstract

Selected factors affecting somatic embryogenesis efficiency have been studied, namely genotype, explant type and its orientation in the medium, different basal media, different auxins for somatic embryo induction, and two ways of donor plant cultivation. The key role is played by genotype and auxin used, the minimum effect was observed due to basal media. In the series of subsequent experiments we have found the best combination of individual factors as follows: cv. Altona, 10  $\mu$ M 2,4-D, L2 basal medium, central part of immature cotyledon as initial explant oriented by adaxial side down on the agar medium, and field grown donor plants. This combination exhibited 100 % embryogenic explants with  $5.43 \pm 0.65$  somatic embryos per explant, *i.e.* somatic embryogenesis efficiency 5.43.

### Introduction

Rapid progress has been achieved in grain legume *de novo* regeneration during the last few years. The most advanced results have been obtained in soybean regeneration *via* somatic embryogenesis (for review see Barwale and Widholm 1990). These results allowed the use of regeneration protocols for somaclonal variation studies (Barwale and Widholm 1987, Hildebrand *et al.* 1989), transformation experiments and transgenic plant construction in soybean (Hinchee *et al.* 1990). The efficiency of the regeneration system directly affects the number of somaclones or transformants produced and therefore attempts have been made to study factors influencing somatic embryogenesis (Lazzeri *et al.* 1987a, b, 1988, Hartweck *et al.* 1988, Parrott *et al.* 1989, Komatsuda and Ohyama 1988).

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*Abbreviations:* picloram - 4-amino-3,5,6-trichloropicolinic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA -  $\alpha$ -naphthaleneacetic acid; dicamba - 3,6-dichloro-*o*-anisic acid; L2-medium - medium after Phillips and Collins 1979; MSB-medium - medium with mineral salts after Murashige and Skoog 1962, vitamins after Gamborg *et al.* 1968, SE - somatic embryogenesis, SEE - somatic embryogenesis efficiency.

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We have successfully induced somatic embryos in soybean cvs. Sluna, Maple Arrow and HM-AS-84 in previous experiments (Novák *et al.* 1987), but the efficiency of somatic embryogenesis was relatively low. In the experiments described below we have attempted to find the optimum combination of selected factors affecting soybean somatic embryogenesis which will substantially increase efficiency of this regeneration protocol.

## Material and methods

**Experiment I (1988) - Screening soybean genotypes for somatic embryogenesis capacity:** 21 soybean genotypes (cultivars and breeding lines) were used in the experiments (Fig. 3). Plants were grown in a greenhouse without artificial illumination (sowing dates: February and April) and in the field (April) at Šumperk (North-West Moravia). Immature cotyledons (4 to 7 mm in length) were isolated in May, June and July (greenhouse-grown plants) and in July and August (field-grown plants), depending on the flowering time and subsequent pod development of individual cultivars and lines. Surface sterilization of the pods and embryo isolation was done by the same method as in Novák *et al.* (1987). The embryo axis was removed as well as the part of the cotyledon adjacent to embryo axis. Cotyledonary explants were placed abaxial side down on agar L2-medium supplemented with 10  $\mu\text{M}$  2,4-D or 10  $\mu\text{M}$  picloram or 100  $\mu\text{M}$  NAA. The pH of the media was adjusted prior to autoclaving (100 kPa, 121  $^{\circ}\text{C}$ ) to 5.8. Explants were cultured in 250  $\text{cm}^3$  Erlenmeyer flasks (6 to 8 cotyledons per flask) containing 50  $\text{cm}^3$  of medium. One experimental variant (genotype  $\times$  sowing date  $\times$  auxin) consisted of 12 to 24 cotyledons. Cultures were maintained at  $25 \pm 2/20 \pm 2$   $^{\circ}\text{C}$  day/night, 16 h photoperiod with irradiance 20.4  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (cool-white fluorescent tubes). The following parameters were evaluated after 8 to 10 weeks of culture in each experimental variant: (1) embryogenic explants (%) - A, (2) the average number of somatic embryos per responding cotyledons, (3) the average number of somatic embryos per cultured cotyledons - B, (4) somatic embryogenesis efficiency (SEE =  $A \times B/100$ ; Lazzeri *et al.* 1987a, b).

**Experiment II (1989) - Optimization of somatic embryogenesis process with responsive genotypes selected in 1988:** Cultivars Altona, Sluna, Evans, Touroy and UO-2-8 were used in Experiment II. Explant isolation was done in June (greenhouse) and August (field). Immature zygotic embryos were divided by the following method to obtain 7 types of initial explants: whole cotyledon (C), embryo axis (E), cotyledon divided by transverse section into apical ( $A_1$ ) and basal ( $B_1$ ) part, cotyledon divided by two transverse sections into apical ( $A_2$ ), central (M) and basal ( $B_2$ ) part. Only explant E contained organized meristems. Cotyledon explants were placed adaxial side down on agar medium.

Two basal media were used: L2 and MSB supplemented with 10  $\mu\text{M}$  2,4-D or 50  $\mu\text{M}$  NAA. Each experimental variant (genotype  $\times$  explant type  $\times$  basal medium  $\times$  auxin  $\times$  donor plant cultivation) included 8 to 10 explants. Only well defined somatic

embryos were evaluated (Fig. 1). The same parameters were recorded as in Exp. I, with addition of frequency of explants with morphologically normal somatic embryos, which is an important character connected with somatic embryo germination.



Fig. 1. Soybean somatic embryos (cv. Altona) on cotyledon explants after 10 weeks of culture on MSB-medium with  $10 \mu\text{M}$  2,4-D.

**Experiment III (1990) - The effect of explant position on agar medium:** In the third experiment the role of explant position on agar medium (adaxial versus abaxial) in somatic embryo formation was studied with cv. Altona, whole cotyledons and MSB medium supplemented with  $10 \mu\text{M}$  2,4-D or dicamba. The same parameters were evaluated as in Exp. II, with some additional characters (orientation of developing somatic embryos; callus and root formation) after 7 weeks in culture. Each experimental variant (auxin  $\times$  position on medium) included 25 to 60 explants in three replicates.

## Results

**Experiment I:** Of the three auxins tested 2,4-D was found to be the most efficient inducer of somatic embryogenesis in soybean, followed by NAA and picloram (Figs. 2 and 3). Therefore we took the data obtained with 2,4-D as a criterion for genotype evaluation (Fig. 3) and selection of proper material for further experiments. Application of picloram led to abundant callus formation on explanted cotyledons with infrequent somatic embryogenesis. Within 21 tested soybean cultivars and lines 20 showed some capacity for somatic embryogenesis (18 genotypes treated with 2,4-D, Fig. 3; 8 genotypes treated with NAA and 6 genotypes treated with picloram - data not shown). There were significant differences between genotypes in somatic embryogenesis efficiency (Fig. 3C). The embryogenic cultures were evaluated in the following subcultures on the same medium (2,4-D, NAA or picloram) with special

focus on good development and maturation of somatic embryos (unpublished data). In this respect cvs. Altona, Toury, Evans, Sluna and line UO-2-8 were considered as good models for further studies.

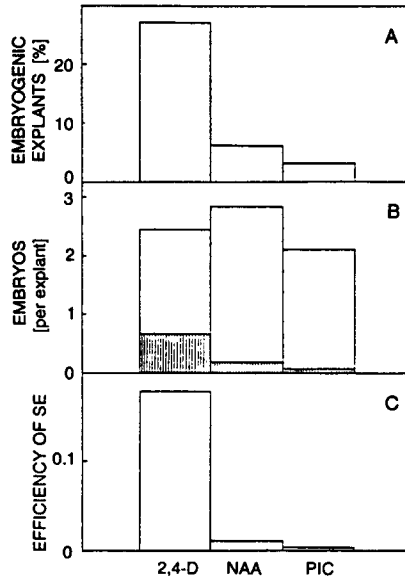


Fig. 2. Experiment I - Effect of three auxins on soybean somatic embryogenesis without respect to genotype (21 genotypes tested). L2 - medium with 10  $\mu\text{M}$  2,4-D or 10  $\mu\text{M}$  picloram or 100  $\mu\text{M}$  NAA; evaluated after 8 - 10 weeks of culture.

**Experiment II:** 15 experimental variants with SEE > 1.5 are listed in Table 1. In some combinations of selected factors the frequency of embryogenic explants reached 100 % with relatively high mean numbers of somatic embryos (3.25 to 5.43). From Table 1 the percentage of individual genotypes in 15 variants is evident; the dominant one being cv. Altona (53.3 %), on the same level is Toury and UO-2-8 (20 %). The above mentioned 15 variants contain no combination with NAA. A less evident tendency has been observed with basal medium, initial explant and donor plant cultivation.

The evaluation of individual factors in the framework of the whole experiment and their effect on frequency and efficiency of somatic embryogenesis is illustrated on Fig. 4. Non significant differences in SEE within 5 genotypes tested have been found (Fig. 4A) and similarly in Exp. I (Fig. 3C). These data demonstrate that selected genotypes have very similar somatic embryogenesis capacity. Cv. Altona has been repeatedly found as the most responsive cultivar with the highest proportion of explants containing morphologically normal somatic embryos.

The evaluation of growth regulators and basal media (Fig. 4B) showed significant difference between 2,4-D and NAA and minimum effect of two tested basal media. The proportion of explants with normal embryos was slightly higher on the media with NAA.

Table 1. Sequence of the best variants (explant × genotype × medium × donor plant) evaluated on the basis of parameter "somatic embryogenesis efficiency". Table includes values of efficiency > 1.5.

Genotype	Explant	Medium	Donor plant from	Embryogenic explants [%] - A	Embryos per responding explant	Embryos per cultured explant - B	Efficiency of SE A × B/100
Altona	M	L2	2,4-D	field	100.0	5.43 ± 0.65	5.43
Altona	M	MSB	2,4-D	greenhouse	100.0	4.25 ± 0.88	4.25
Altona	B2	L2	2,-4D	greenhouse	100.0	3.75 ± 0.63	3.75
Toury	B2	MSB	2,4-D	greenhouse	100.0	3.75 ± 0.75	3.75
Altona	C	MSB	2,4-D	greenhouse	75.0	4.33 ± 0.88	2.44
Altona	B1	MSB	2,4-D	greenhouse	90.9	3.22 ± 0.36	2.40
Altona	E	L2	2,4-D	field	100.0	2.29 ± 0.32	2.29
Sluna	E	L2	2,4-D	field	100.0	2.17 ± 0.65	2.17
UO-2-8	C	MSB	2,4-D	field	78.6	3.00 ± 0.49	2.07
Toury	C	L2	2,4-D	field	85.7	2.33 ± 0.61	1.96
UO-2-8	AI	L2	2,4-D	field	100.0	1.86 ± 0.23	1.86
UO-2-8	E	MSB	2,4-D	field	85.7	2.50 ± 0.56	1.83
Altona	C	L2	2,4-D	field	85.7	2.50 ± 0.56	1.83
Altona	AI	MSB	2,4-D	greenhouse	75.0	3.00 ± 0.66	1.83
Toury	C	MSB	2,4-D	field	85.7	2.42 ± 0.54	1.77

Table 2. The effect of explant orientation on agar MSB-medium supplemented with 10 µm 2,4-D or dicamba on soybean somatic embryogenesis. The whole immature cotyledons (cv. Altona) were placed adaxially and abaxially and evaluated after 7 weeks of culture. The data represents the mean of three replicates.

Auxin (10 µm)	Explant orientation	Callus formation*	Explants with roots [%]	Embryogenic explants [%] A	Explants with normal SE [%]	Embryos per cotyledons	Embryos per responding cotyl. - B	Efficiency of SE A × B/100
2,4-D	Adaxial	2.05 ± 0.07	17.42	80.30	39.39	3.08 ± 0.19	2.56 ± 0.19	2.06
2,4-D	Abaxial	2.20 ± 0.07	8.8	39.20	8.00	2.22 ± 0.18	0.87 ± 0.12	0.34
Dicamba	Adaxial	2.53 ± 0.06	-	12.80	2.40	1.69 ± 0.22	0.22 ± 0.06	0.03
Dicamba	Abaxial	2.24 ± 0.07	-	2.38	0.79	1.67 ± 0.67	0.04 ± 0.03	0.001

\* Evaluation scale: 0 - without callus, 1 - low intensity, 2 - medium intensity, 3 - high intensity

The effect of various types of initial explants is illustrated in Fig. 4C. Explants isolated from field-grown plants exhibited slightly better values of embryogenic explants and somatic embryogenesis efficiency (32.41 %, 0.246) than explants from greenhouse-grown plants (26.78 %, 0.145).

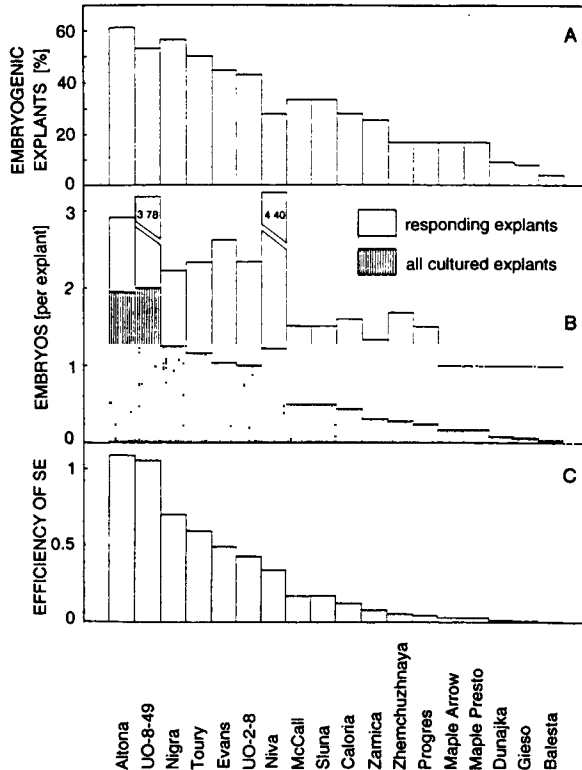


Fig. 3. Experiment I - Screening soybean genotypes for somatic embryogenesis capacity. Genotypes are ranked according to somatic embryogenesis efficiency. The data represent two replicates (greenhouse- and field-grown donor plants) of variants with 10  $\mu$ M 2,4-D; evaluated after 8 - 10 weeks of culture. Genotypes Fiskeby V, ISZ-10 and UO-7-39-2-1 exhibited no response.

**Experiment III:** The position of explant (whole cotyledon) on the surface of agar medium evidently affected the capacity for somatic embryogenesis. The cotyledons placed adaxial side down exhibited higher frequency of embryogenic explants as well as mean number of somatic embryos as compared to explants oriented abaxial side down (Table 2) both with 2,4-D and dicamba supplemented media. Dicamba is significantly less effective than 2,4-D.

Nevertheless, the somatic embryos originated preferentially from adaxial side of the explanted cotyledons within both positions tested (Fig. 5).

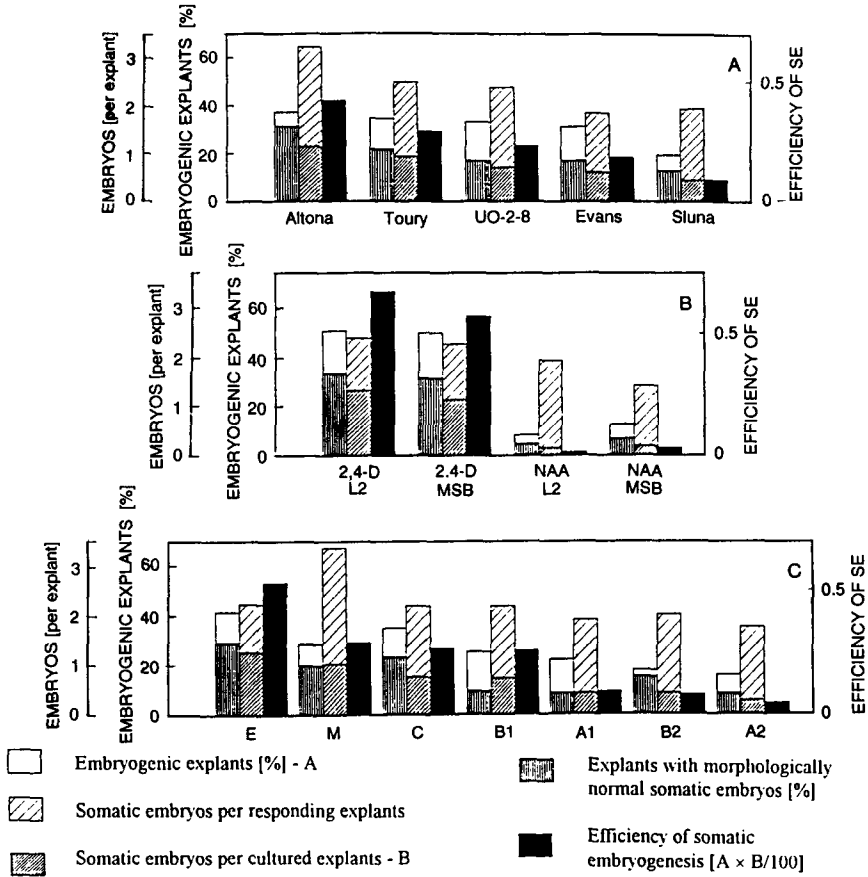


Fig. 4. Experiment II - Soybean somatic embryogenesis. (A) Effect of genotype without respect to initial explant and medium. (B) Effect of auxin and basal medium without respect to genotype and initial explant. (C) Effect of initial explant without respect to genotype and medium. Evaluated after 8 - 10 weeks of culture.

## Discussion

Evaluation of four auxins (2,4-D, NAA, picloram and dicamba) showed that 2,4-D induced the highest frequency of embryogenic explants as well as the highest number of somatic embryos per explant in all experiments (Fig. 2, 4B, Tables 1 and 2). As compared to other authors we have obtained poor results with NAA (Lazzeri *et al.* 1985, 1987a, b, Tétu *et al.* 1987, Barwale *et al.* 1986, Komatsuda and Ohyama 1988, Hartweck *et al.* 1988) and dicamba (Christou and Yang 1989). NAA was also found ineffective in inducing direct somatic embryogenesis from the immature embryo axis of peanut (Hazra *et al.* 1989). Practically negligible efficiency of somatic embryogenesis was exhibited by picloram (Fig. 2C). Only with the use of 2,4-D or NAA we have obtained good development and maturation of somatic embryos (Griga

1990). Application of picloram and dicamba led to intensive callus formation and also recalling of somatic embryos. A frequently observed feature of explants cultured in the presence of 2,4-D was the parallel formation of somatic embryos, callus and roots on the same explant. A similar situation was observed with NAA, but the callus formation was minimum and root formation was extremely expressed (see also Hartweck *et al.* 1988). The cultures on dicamba and picloram supplemented media exhibited an absolute absence of rhizogenesis.

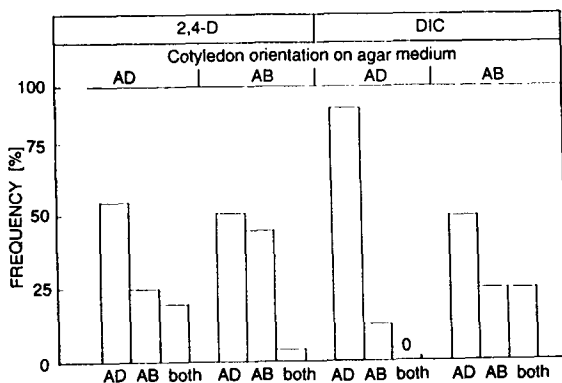


Fig. 5. Experiment III - Effect of explant orientation on agar medium on soybean somatic embryogenesis. Proportion of explants [%] with somatic embryos developing from adaxial (AD), abaxial (AB) or both sides of cultured cotyledons. Whole immature cotyledons (cv. Altona) were placed abaxial or adaxial side down on MSB-medium supplemented with 10  $\mu$ M 2,4-D or dicamba. Evaluated after 7 weeks of culture. The data represent the mean of three replications.

The concentration of 10  $\mu$ M 2,4-D used in our experiments in induction media seemed to be quite sufficient for somatic embryo initiation, though the 2,4-D levels routinely used by other authors were rather higher (22.6 to 113  $\mu$ M; Ranch *et al.* 1985, 1986, Buchheim *et al.* 1989, Lazzeri *et al.* 1985, Ghazi *et al.* 1986, Hartweck *et al.* 1988). Even 452  $\mu$ M 2,4-D could induce morphologically normal somatic embryos in soybean (Ranch *et al.* 1986) and 180  $\mu$ M was effective for induction of embryogenic callus and establishing well growing soybean embryogenic suspension cultures (Finer 1988, Finer and Nagasawa 1988). Nevertheless, we have obtained 100 % embryogenic explants with the use of 10  $\mu$ M 2,4-D in some cases (Table 1). Comparison of low and high 2,4-D concentrations (Ranch *et al.* 1986) suggested that low 2,4-D level (*e.g.* 5  $\mu$ M) was sufficient to induce embryogenic tissue, but not sufficient for proper ontogenic development of induced somatic embryos. Thus, development of such somatic embryos resulted in many morphological abnormalities.

The results demonstrated in Table 1 showed that certain combinations of experimental factors can significantly increase the efficiency of somatic embryogenesis. In this respect the interaction genotype  $\times$  auxin seems to be the most important. The effect of genotype on somatic embryogenesis in soybean was demonstrated by several authors (Parrot *et al.* 1989, Komatsuda and Ohyama 1988, Ranch *et al.* 1986, Komatsuda and Ko 1990) and it can be concluded that

responsiveness of commercial soybean cultivars is relatively good as compared to other grain legumes, e.g. *Pisum sativum* or *Vicia faba* (Kysely *et al.* 1987, Kysely and Jacobsen 1990, Lehming-Mertens and Jacobsen 1989, Griga *et al.* 1987, Stejskal and Griga 1992).

Some basic information has also been found about the effect of dissection of the initial explant, which is limited to date to immature zygotic embryo or its parts in soybean (Lazzeri *et al.* 1987b). In our experiments we have found no significant differences between dissected parts of zygotic embryo (Fig. 4C). Evaluating the large number of explants (*viz.* Materials and methods, Exp. II) we did not observe a substantial increase in the frequency of somatic embryogenesis after cotyledon dissection as Lazzeri *et al.* (1987b) did. Surprisingly, the embryo axis exhibited the best response in this experiment (Fig. 4C). However, in this type of explant the proliferation of somatic embryos as well as organized meristems (embryo apex, axillary meristems of cotyledons) takes place at the same time in initial culture. These meristems are frequently abnormal due to 2,4-D and it is very difficult to distinguish between true somatic embryos and malformed buds during the non-destructive way of evaluation. However, in successive subcultures the somatic embryogenesis is dominant way of *de novo* regeneration in these explants. Unfortunately there is very little or unclear evidence about the use of the embryo axes (or whole zygotic embryo) as the initial explants for somatic embryo induction in grain legumes. In some studies with soybean (Christianson *et al.* 1983, Novák *et al.* 1987) as well as pea and peanut (Kysely *et al.* 1987, Kysely and Jacobsen 1990, Hazra *et al.* 1989) whole zygotic embryos or embryo axes served as efficient explants for somatic embryogenesis. However, Lazzeri *et al.* (1987b) observed the lowest embryogenic frequency in whole soybean zygotic embryos within various embryo derived explants tested and the authors concluded that "the presence of embryonic axis suppresses embryogenesis from cotyledon tissue". Ranch *et al.* (1986) also stated that soybean somatic embryos never developed on the embryo axis and cotyledons were only responsive when excised from immature embryos and cultured independently. Thus the role of organized meristems of zygotic embryos in the process of somatic embryogenesis should be satisfactorily answered on the base of additional experiments.

Nevertheless, the dissection of zygotic embryo into more (5 to 6) parts and culturing them separately results in an approximately 10 fold increase in somatic embryo production as compared to culture of the whole (undamaged) zygotic embryo (Griga 1990).

The effect of explant orientation on agar medium as well as polarity of explant has been demonstrated in various plant species and explant types. In soybean a detailed study was reported by Hartweck *et al.* (1988) and Hopher *et al.* (1988). Our data (Table 2, Fig. 4) indicate very clear tendency - somatic embryos originate preferentially from adaxial surface of cotyledon; the contact of adaxial side with agar medium has an additive effect, which increases the total number of somatic embryos, including the number of morphologically normal ones. These findings are in contrast with results of Hopher *et al.* (1988), but the situation is similar to that in peanut (Ozias-Akins 1989). The localization of embryogenic initiation sites as well as

structural and physiological differences between cells and tissues of abaxial and adaxial parts of the cotyledon connected with auxin transport (Hartweck *et al.* 1988) seem to significantly affect the somatic embryogenesis process.

On the basis of results presented here we can recommend the most effective combination of individual factors studied as follows: cv. Altona, 10  $\mu$ M 2,4-D, L2-medium, central part of immature cotyledon oriented by adaxial side to the agar medium, and field grown donor plants (Table 1). However some other combinations (Table 1) give comparable results.

The protocol described above is applicable for somaclonal variation studies, transformation experiments and synthetic seed production in soybean.

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