Euphytica **80:** 137–143, 1994.

New aspects of soybean somatic embryogenesis

Anna Nadolska-Orczyk & Waclaw Orczyk
Department of Plant Tissue Culture, Plant Breeding and Acclimatization Institute, Radzików, 05-870 Blonie, Poland

Received 10 March 1994; accepted 1 September 1994

Key words: somatic embryogenesis, *Glycine max*, cut cotyledons, polyamines, nitrogen ions, soybean

Abstract

Somatic embryo formation from immature cotyledons was improved in the following ways: by cutting into sections, supplementing culture media with spermine and using solid/liquid/solid type of culture. Cut cotyledons of the eight genotypes examined expressed a higher ability for somatic embryogenesis than whole cotyledons. Of the three polyamines tested, spermine considerably stimulated and putrescine slightly inhibited induction of somatic embryos. The ability of embryoid formation on medium with spermidine depended on the genotype. The solid/liquid/solid type of culture was better than the continuous solid culture. The best nitrogen ion content for the subculture of somatic embryos was 10 mM NH_4NO_3 and 30 mM KNO_3. The possibility of using these modifications in *Agrobacterium* transformation is discussed.

Introduction

There have been several reports concerning the regeneration of soybean plants from tissue cultures. Shoot organogenesis occurred from tissues such as cotyledonary nodes (Graybosch et al., 1987) and primary leaves of seedlings (Wright et al., 1987), while somatic embryogenesis occurred from immature embryos or cotyledons. Various studies examined the effects of genotype (Parrott et al., 1989; Komatsuda & Ohyama, 1988), growth regulators (Hammatt & Davey, 1987; Lazzeri et al., 1987a; Ranch et al., 1985), sucrose and growth regulators (Hartweck et al., 1988), basal media and hormones (Christou & Yang, 1989) or sugar, pH, light intensity and dissection of embryos as well as cotyledons (Lazzeri et al., 1987b) on induction of somatic embryos. Germination of such embryos and their transition to plantlets have also been described. The influence of growth regulators (Ranch et al., 1985) and sucrose together with growth regulators (Hartweck et al., 1988; Lazzeri et al., 1988; Komatsuda et al., 1991, 1992) was examined. Buchheim et al. (1989) reported that maturation of somatic embryos was achieved on medium containing activated charcoal with sucrose.

Looking forward method of soybean plant regeneration via somatic embrogenesis, for other, agronomically important genotypes (cultivars), several additional aspects were investigated. They included influence of polyamines, liquid medium, cotyledons injuring (cutting) and nitrogen ions on this process. Some modifications of culture conditions were made to adopt this method for Agrobacterium transformation.

Materials and methods

Plant material

The seeds of four cultivars: Polan (Sp), Aldana (Sa), Progres (Spr) and five breeding lines named: L4, L5, L6, L8, L9 were provided by Prof. Szyrmer from the Plant Breeding and Acclimatization Institute. Plants were grown in pots under a 16/8 light/dark photoperiod (350 $\mu M^{-2}s^{-1}$) at 22/16° C day/night temperature regime.

Pods containing immature seeds were collected and surface sterilized by immersing them in 70% alcohol followed by 20 min in sodium hypochlorite (7.5% v/v) and rinsed three times with sterilized distilled water.

Embryos from 3 to 6 mm long were removed from immature seeds. Their randomly selected cotyledons were placed in abaxial orientation up on agar solidified medium. Whole cotyledons were used only in the first experiment. Additionally, in the first and in the next experiments, cotyledons were cut into three pieces, perpendicular to their long axes. Four whole cotyledons or 12 pieces were cultured per Petri dish (80 mm in diameter), and each treatment included at least 20 cotyledons explants (3 × 20 cut pieces of cotyledons). The pieces from one cotyledon were placed next to each other on solid medium or were separated in liquid medium. They were scored as one cotyledon-explant. The number of replicates varied from two (Table 1, 3, 4, 5; Fig. 2) to five (Table 2).

Media and culture conditions

The basal culture medium containing MS salts and vitamins (Murashige & Skoog, 1962) supplemented with 5 mg l^{-1} 2,4-D and 3% sucrose, was used in primary culture. Additionally, in one experiment the polyamines: putrescine and spermidine at 10 mg l^{-1} and spermine at 10 and 50 mg l^{-1} concentrations (Table 3) were added to this medium. In this experiment explants were first put on MS2,4-D medium for four days of preliminary culture and next, on the media containing polyamines. For better examining the influence of polyamines and solid/liquid/solid type of culture on general response (including percent of regenerated cotyledons and mean number of embryoids per cotyledon) we estimated the coefficient of response according to the equation:

$$\frac{\text{percent of regenerated cotyledons} \times \text{mean no. of embryos}}{100\%} = \text{coef.resp.}$$

The influence of liquid culture (Table 4) was investigated over three days of treatment in liquid media, after two days of preliminary culture on solid medium. Media were solidified with 2 g l^{-1} Gelrite and autoclaved for 15 min at 121° C. Polyamines were sterilized by filtration. The pH of the media was adjusted to 5.8 before autoclaving.

In subculture experiments, MS and MS modified media with altered nitrogen source and nitrogen ion proportion were used. Nitrogen ion contents was as follow: 10 mM NH_4NO_3 + 30 mM KNO_3 (M1 and M3 medium), 1 mM $(NH_4)_2SO_4$ + 25 mM KNO^3 (M2 medium) and 21 mM NH_4NO_3 + 19 mM KNO_3 (MS1 medium). M3 basal medium was supplemented with 0.1 mg l^{-1} abscisic acid (ABA) and zeatin. M1, M2 and MS1 media contained the same growth regulators as M3 and additionally 0.1 mg l^{-1} IBA. Plantlets were developed on MS medium containing 1 mg l^{-1} GA_3.

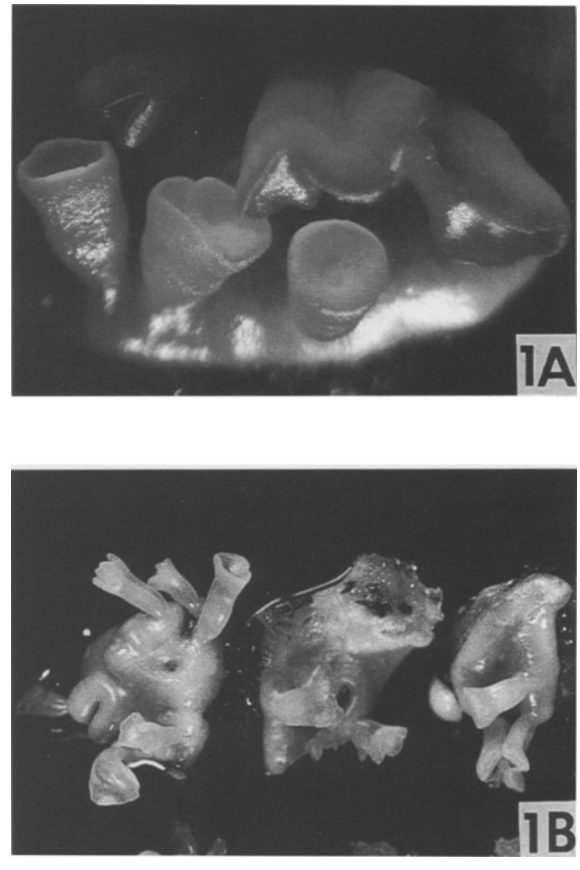

Fig. 1. Induction of somatic embryos along the wounded edge of cut cotyledons in Sa cultivar (A) and L9 line (B) after 4 weeks of culture.

Somatic embryos were classified into three size categories: I – < 2.9 mm, II – 3.0–5.9 mm, III – 6.0–10 mm length or they were measured individually.

Results

Globular embryos developed on the whole as well as the cut cotyledons. The percentage of explants forming somatic embryos and the number of embryos per cotyledon were higher in cut cotyledons in all tested genotypes (Table 1). In the case of whole cotyledons, somatic embryos were formed typically around the submarginal surface (Hartweck et al., 1988). How-

Table 1. The ability of cut and whole immature cotyledons of different lines and cultivars of soybean to form embryoids

Genotype	L4		L5		L6		L8		L9		Sp		Sa		Spr	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
cut	27/5.5		29.5.3		100/9.7		48/3.5		66/4.3		40/4.8		72/3.2		69/4.2	
whole	13/2.1		0.0		44/4.0		20/2.8		18/3.1		16/2.8		23/2.9		21/3.0	

1 – percentage of explants forming somatic embryos.
2 – mean number of embryos per one cotyledon.
Note: There were significant differences between cut and whole cotyledons of the genotypes in percentage of regenerated cotyledons and mean number of embryos according to LSD test.

Table 2. The ability of two lines and three cultivars of sectioned cotyledons of soybean to form embryoids in five replicated experiments

Genotype	No. of cotyledons	% of regenerated cotyledons		Mean no. of embryos per cot.	SD
		range	mean		
L8	97	37.9–87.5	58.8	4.45	3.27
L9	136	25.0–100.0	75.7	5.49	3.67
Sp	162	40.0–100.0	56.2	3.36	2.27
Sa	138	37.5–81.8	63.8	4.22	3.64
Spr	50	53.8–62.5	58.0	4.31	2.55

Note: There were no significant differences between genotypes in percentage of regenerated cotyledons and mean number of embryos according to LSD test.

Table 3. The influence of spermine, spermidine and putrescine on embryoid formation in primary culture of Sp, Spr cultivars and L9 line

Genotype/polyamide*	% of regenerated cotyledons	Mean no. of embryos	Coef. of resp.	SD
Sp/control	33.5	2.23	0.75	0.58
Sp/Put10	18.2	1.08	0.20	0.75
Sp/Spd10	33.3	2.25	0.75	1.62
Sp/Spm10	34.8	3.89	1.35	2.16
Sp/Spm50	42.6	2.75	1.17	1.26
Spr/control	37.6	2.18	0.82	1.09
Spr/Put10	46.8	2.34	1.09	1.59
Spr/Spd10	36.6	1.85	0.68	0.45
Spr/Spm10	56.9	3.87	2.20	2.39
Spr/Spm50	72.1	3.29	2.37	2.27
L9/control	76.5	3.38	2.58	2.39
L9/Put10	56.7	2.47	1.40	1.12
L9/Spd10	62.5	1.80	1.13	0.67
L9/Spm10	77.4	4.54	3.51	2.58

* Put10 – putrescine 10 mg l^{-1}; Spd10 – spermidine 10 mg l^{-1}; Spm10, Spm50 – spermine 10 or 50 mg l^{-1}, respectively.

Table 4. The effect of three day culture in liquid medium on embryoid formation in L9 line, Sp and Sa cultivar

Genotype	treatment	% of regenerated cotyledons	Mean no. of embryos	Coef. of resp.	SD
L9		85.5	4.29	3.66	2.43
L9	2/3*	100.0	6.00	6.00	2.32
Sp		50.3	6.40	3.22	4.88
Sp	2/3	75.0	4.17	3.13	1.72
Sa		15.2	3.33	0.51	1.53
Sa	2/3	25.7	3.67	0.94	1.53

* Two days of preculture on solid medium, three days of culture in liquid medium and 23 days of culture on solid medium.

Table 5. The number (percentage) of L9 line somatic embryos on media containing different nitrogen ion compositions in three size categories after four weeks of subculture

Medium	No. of embryos	< 2.9 mm	3–5.9 mm	> 6 mm
M1	125	54 (43)	59 (47)	12 (10)
M2	114	73 (64)	33 (29)	8 (7)
M3	107	70 (65)	29 (27)	8 (8)
MS1	119	92 (77)	27 (23)	0

ever, induction of somatic embryos on cut cotyledons mainly occurred along the wounded edge (Fig. 1).

The regeneration capacity was high for all genotypes used (Table 2). The best one was L9 line with the highest percent of responding cotyledons (76%) and mean number of embryos per cotyledon (5.5). However, the range of the percentage of responding cotyledons was so wide and the standard deviation for the mean number of somatic embryos in five repeats of the experiment so high that the differences estimated statistically were not significant.

The effect of three polyamines: spermidine, spermine and putrescine were tested on two cultivars (Sp and Spr) and the L9 line (Table 3). According to the percent of regenerated cotyledons high concentration of spermine considerably increased somatic embryogenesis in Spr cultivar (72.1% comparing with 37.6%) and slightly in Sp cultivar (42.6% comparing with 33.5%). Mean number of embryos per explant as well as coefficient of response was higher on both 10 mg l^{-1} and 50 mg l^{-1} spermine medium. However, putrescine decreased the efficiency of embryogenesis in Sp cultivar and L9 line. The same level of response in the case of Sp or lower (Spr, L9) than the control, was obtained on the medium with spermidine.

The influence of continuous 4 weeks culture on solid medium versus a solid/liquid/solid culture (two days solid, three days liquid and the next 23 days solid) on the ability to produce somatic embryos were compared with three genotypes (Table 4). The percentage of cotyledons forming embryos was higher in all cases in the solid/liquid/solid (2/3/23 days) culture than in the continuous solid culture. Similarly, the number of somatic embryos was higher in L9 line and very little in Sa cultivar. Only Sp cultivar formed about 1/3 less embryos per explant on 2/3/23 type of culture comparing with solid medium.

Somatic embryos growing on media containing different nitrogen ion composition were classified into three categories, according to their length (Table 5). On the basis we could estimate their growth and maturation. The highest number (percent) of embryos in the second and third classes (from 3 mm to more than 6 mm), was obtained after subculture on M1 medium. In case of the other media, the numbers of embryos reaching these classes decreased from about 20% on M2 to 34% on MS1. The lowest number was obtained

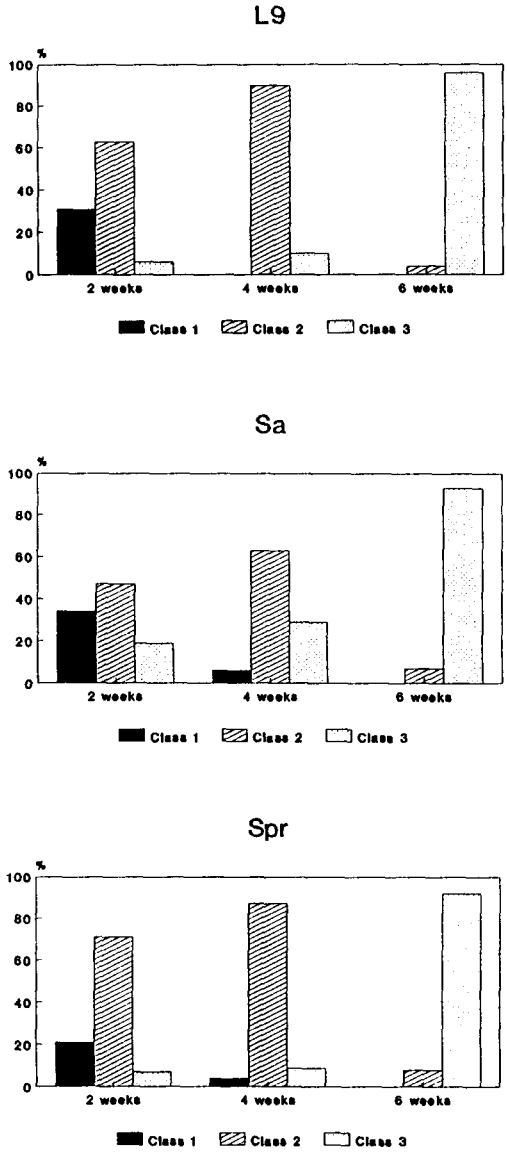

Fig. 2. The percentage of three classes of somatic embryos after 2, 4 and 6 weeks of subculture on M1 medium.

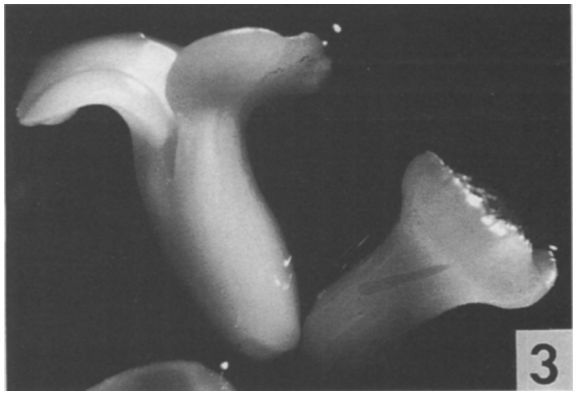

Fig. 3. Growth of L9 line somatic embryos on MS modified (M1) medium after two weeks of subculture.

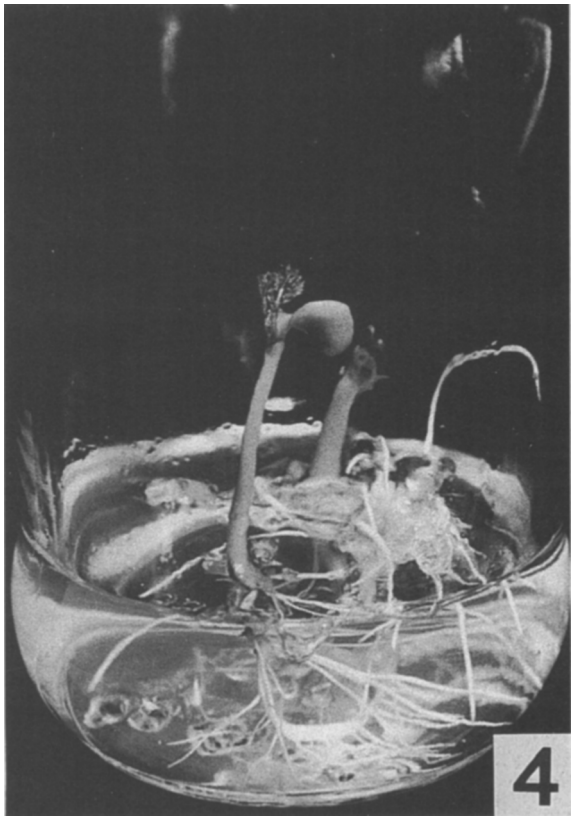

Fig. 4. Plantlets of L9 line developing on MS medium with 1 mg l^{-1} GA_3.

on MS medium that contained the same growth regulators as in M1 and M3. Subsequent culture for the following 2 and 4 weeks on M1 (Figs 2 and 3) showed that almost all embryos were longer than 6 mm. These embryos (longer than 6 mm) were able to continue their development to plantlets (Fig. 4) and plants. The effect was similar for all genotypes tested.

Discussion

The aim of the first experiment was to determine the possibility of obtaining somatic embryogenesis from

cut cotyledons, especially from their wounded part. Such wounding could make an opportunity for easier infection of plant cells by *Agrobacterium tumefaciens*. In the experiment, embryo formation was stimulated by cutting and it occurred on the wounded parts of cotyledon. The stimulation of embryogenesis by dissection of immature embryos or cotyledons in soybean was reported by Lazzeri et al. (1987b). However, the ways of dissection presented by the authors were different. They noticed that embryogenesis occurred mainly from intact tissue.

Many articles have revealed the influence of genotype on *in vitro* response. In soybean, the differences in somatic embryogenesis from immature embryos were indicated (Ranch et al., 1985; Komatsuda & Ohyama, 1988). The ability of the immature cotyledons to form embryoids was determined by Parrott et al. (1989b) by means of variance analysis. The authors reported that the genotypes with the highest percentage of responding cotyledons also formed more somatic embryos per cotyledon. We could see a similar tendency in genotypes investigated in this work. However, in contrast to Parrott et al. (1989b) statistically estimated differences of embryogenic ability were not significant in our experiments.

The wide range of regeneration ability between repeats, in spite of precisely controlled culture conditions of plants and explants, revealed that there were other factors, closely connected with the physiological state of explants, influencing this trait. The importance of non-heritable components in tissue culture has been shown for various plants (Nadolska-Orczyk & Malepszy, 1989; Dunwell et al., 1987), but not for soybean. It seemed to be especially important for this type of explant, where the endogenous compounds could play important role in embryogenesis.

The influence of polyamines on somatic embryogenesis has been investigated mainly with carrot cultures as a model system (see review Mengoli & Bagni, 1992). Following a general trend in untreated cultures, spermine was increased during the transition from globular to heart stage of embryos, while putrescine was the major polyamine between heart and torpedo stage. In our experiment a supplement of spermine after four days of preliminary culture of cotyledons caused an increase in somatic embryo formation. Putrescine added at the same time of culture decreased or had no effect on the response. Further observations revealed that polyamines added to the primary culture influenced the subsequent growth of somatic embryos (data not shown). This experiment indicated that polyamines could have a strong effect on somatic embryogenesis in grain legumes.

The aim of the examination of the three day culture in liquid was to adapt this method to coculture with *Agrobacterium*. The experiment showed that culture on solid/liquid/solid medium is not only possible but also gave better rates of regeneration. Lazzeri et al. (1987a) reported that the production of somatic embryos in solid cultures, transferred several times (first after 5 days) to fresh medium, was similar to those maintained for the 30 days. Considering these results, we supposed that the increase of response in our experiment was not the result of triple transfer but the effect of culture in liquid medium and the time of the first transfer. In contrast to Lazzeri et al. (1987a), it was done after two days of preculture – during the period of somatic embryo initiation.

The basal medium used for growth of somatic embryos has been mainly MS (Christou & Yang, 1989; Buchheim et al., 1989; Lazzeri et al., 1987b) or B5 (Ranch et al., 1985; Hammatt & Davey, 1987). Neither MS nor B5 was effective enough in our experiments. Comparing nitrogen ion contents in MS modified media (Table 5), the best one for the first subculture of somatic embryos and subsequent growth for four weeks (Fig. 2) was the medium that contained 10 mM NH_4NO_3 and 30 mM KNO_3. The same ratio of ammonium to nitrate ions was effective for the subculture of lupin somatic embryos (Nadolska-Orczyk, 1992) as well as pea somatic embryos (1994). But, in the case of lupin, the NH_4/NO_3 ratio had to be increased to be effective (from 1:25 in primary to 1:3 in subculture) while, for soybean it had to be decreased (from 21:19 to 1:3). ABA and zeatin as well as IBA in small amounts, were effective growth regulators for soybean embryos. Other authors also applied IBA (Ranch et al., 1985) or IBA and ABA (Christou & Yang, 1989) but in different concentrations and compositions.

Acknowledgements

The authors are grateful to Miss L. Milkowska for her skillful technical assistance. This work was partially supported by Committee for Scientific Research (KBN), grant number 50339203.

References

Buchheim, J.A., S.M. Colburn & J.P. Ranch, 1989. Maturation of soybean somatic embryos and the transition to plantlet growth. Plant Physiol. 89: 769–775.

Christou, P. & N-S. Yang, 1989. Developmental aspects of soybean (*Glycine max*) somatic embryogenesis. Ann. Bot. 64: 225–234.

Dunwell, J.M., R.J. Francis & W. Powell, 1987. Anther culture of *Hordeum vulgare* L.: a genetic study of microspore callus production and differentiation. Theor. Appl. Genet. 74: 60–64.

Graybosch, R.A., M.E. Edge & X. Delannay, 1987. Somaclonal variation in soybean plants regenerated from cotyledonary node tissue culture system. Crop Sci. 27: 803–806.

Hammatt, N. & M.R. Davey, 1987. Somatic embryogenesis and plant regeneration from cultured zygotic embryos of soybean (*Glycine max* L. Merr.). J. Plant Physiol. 128: 219–226.

Hartweck, L.M., P.A. Lazzeri, D. Cui, G.B. Collins & E.G. Williams, 1988. Auxin-orientation effects on somatic embryogenesis from immature soybean cotyledons. *In Vitro* Cell Developmental Biology 24: 821–828.

Komatsuda, T., K. Kaneko & S. Oka, 1991. Genotype × sucrose interactions for somatic embryogenesis in soybean. Crop Sci. 31: 333–337.

Komatsuda, T., W. Lee & S. Oka, 1992. Maturation and germination of somatic embryos as affected by sucrose and plant growth regulators in soybeans *Glycine gracilis* Skvortz and *Glycine max* (L.) Merr. Plant Cell, Tissue and Organ Culture 28: 103–113.

Komatsuda, T. & K. Ohyama, 1988. Genotypes of high competence for somatic embryogenesis and plant regeneration in soybean *Glycine max*. Theor. Appl. Genet. 75: 695–700.

Lazzeri, P.A., D.F. Hildebrand & G.B. Collins, 1987a. Soybean somatic embryogenesis: Effects of hormones and culture manipulations. Plant Cell, Tissue and Organ Culture 10: 197–208.

Lazzeri, P.A., D.F. Hildebrand & G.B. Collins, 1987b. Soybean somatic embryogenesis: Effects of nutritional, physical and chemical factors. Plant Cell, Tissue and Organ Culture 10: 209–220.

Lazzeri, P.A., D.F. Hildebrand, J. Sunega, E.G. Williams & G.B. Collins, 1988. Soybean somatic embryogenesis: interactions between sucrose and auxin. Plant Cell Report 7: 517–520.

Mengoli, M. & N. Bagni, 1992. Polyamines and somatic embryogenesis in higher plants. Newsletter IAPTC 68: 2–8.

Nadolska-Orczyk, A. & S. Malepszy, 1989. *In vitro* culture of *Cucumis sativus* L. & Genes controlling plant regeneration. Theor. Appl. Genet. 78: 836–840.

Nadolska-Orczyk, A., 1992. Somatic embryogenesis of agriculturally important lupin species (*Lupinus angustifolius, L. albus, L. mutabilis*). Plant Cell, Tissue and Organ Culture 28: 19–25.

Nadolski-Orczyk, A., L. Milowska & W. Orczyk, 1994. Two ways of plant regeneration from immature cotyledons of pea. Acta Societatis Botanicorum Poloniae 63(2).

Parrott, W.A., E.G. Williams, D.F. Hildebrand & G.B. Colins, 1989. Effect of genotype on somatic embryogenesis from immature cotyledons of soybean. Plant Cell, Tissue and Organ Culture 16: 15–21.

Ranch, J.P., L. Oglesby & A.C. Zielinski, 1985. Plant regeneration from embryo-derived tissue cultures of soybeans. *In Vitro* Cellular and Developmental Biology 21: 653–658.

Wright, M.S., D.V. Ward, M.A. Hinchee, M.G. Carnes & R.J. Kaufman, 1987. Regeneration of soybean (*Glycine max* L. Merr.) from cultured primary leaf tissue. Plant Cell Report 6: 83–89.