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OPTIMIZATION OF SOMATIC EMBRYOGENESIS AND EMBRYO GERMINATION IN SOYBEAN

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SUMMARY

Somatic embryos of soybean [*Glycine max* (L.) Merr.] are induced on immature cotyledons explanted onto a medium containing moderately high levels of auxin. Germinability of embryos is related to morphologic normality, and both are reduced by excessive exposure to auxin during the induction process. Shoot meristem development was improved by reducing exposure of cotyledonary explants from 30 to 10 to 14 d on 10 mg/liter α-naphthaleneacetic acid (NAA). A 3-d exposure was sufficient to induce embryos, and embryo frequency was not significantly increased by exposures to NAA for more than 1 wk. Embryo frequency was enhanced, however, by transfer after 9 d to fresh medium containing 10 mg/liter NAA. Germination of morphologically normal embryos was achieved without growth regulators, after maturation for 1 mo. on hormone-free medium and desiccation for 1 wk in a sealed, dry container.

Key words: Glycine max; tissue culture; regeneration; desiccation.

INTRODUCTION

The soybean [*Glycine max* (L.) Merr.] is the most important source of vegetable oil and protein in the world (11), but has proven difficult to manipulate in tissue culture. Lack of a reliable system for de novo regeneration has hampered the application of techniques such as somaclonal variation and recombinant DNA methods of gene transfer, which have been proposed as supplements to conventional breeding methods for improvement of oil and protein quality, and enhancement of resistance to pests and diseases (5,23).

Adventive regeneration occurs via somatic embryogenesis when cotyledons from immature soybean seeds are exposed to moderately high levels of auxin. Lippman and Lippman (17) obtained somatic embryos on cotyledons exposed to 0.5 to 1.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D), but were unable to obtain plants. Most subsequent studies used 2,4-D at 5 to 40 mg/liter or α-naphthaleneacetic acid (NAA) at 0.8 to 10 mg/liter to obtain embryos capable of germination (1,4,9,14, 19–21,24).

Although somatic embryos can be induced on either 2,4-D or NAA, the resulting embryos differ. Embryos induced on NAA arise from the distal submarginal regions of the cotyledons (10). Embryos induced on 2,4-D develop mainly from older, central areas of the cotyledons and frequently show abnormalities of the shoot apex, vascular system and cotyledons (10). NAA-induced embryos show near normal physiology, whereas 2,4-D-induced embryos fail to accumulate seed lectin and storage protein subunits (2).

Quality of somatic embryos. Somatic embryos with the most normal morphologies are the easiest to convert into plants (15). Even embryos induced on NAA, however, frequently have poorly developed meristems and tend to be difficult to germinate. Any treatment that increases embryo normality should increase the proportion of somatic embryos that can be converted into plants. Lazzeri et al. (16) found a synergistic interaction between levels of NAA and sucrose in the induction medium, such that embryo normality increased as NAA increased and sucrose decreased, with optimum concentrations at about 10 mg/liter NAA and 1.5% sucrose.

Moderately high auxin levels seem to be essential for efficient induction of somatic embryogenesis in soybeans, but auxins are also known to inhibit the meristematic development of somatic embryos (8). Consequently, limited periods of exposure of explanted cotyledons to NAA were evaluated as a means of overcoming the deleterious effects on embryo quality of prolonged exposure to auxin.

Germination of somatic embryos. Germination of soybean somatic embryos into plants has been generally inefficient, although various approaches have been used. Lazzeri et al. (14) used a maturation-germination medium supplemented with 0.15 mg/liter NAA and 0.33 mg/liter each of 6-benzylaminopurine (BA), kinetin, and zeatin. Germinating embryos were then transferred to hormone-free HPN medium (15) to obtain plants. Ranch et al. (20) used liquid medium supplemented with abscisic acid (ABA) and activated charcoal to mature somatic embryos, and then germinated them on medium supplemented with 0.6 μ*M* indole-3-butyric acid (IBA). Activated charcoal was included to reduce auxin carryover effects. Ghazi et al. (4) obtained plants from embryos by using GA_3 at 0.104 mg/liter, or zeatin at 0.110 mg/liter. Hammatt and Davey (9) desiccated mature somatic embryos in sterile pertri dishes until they had reached 40 to 50% of their original size. After desiccation, up to 30% of embryos were capable of germination. This approach is consistent with the observation that desiccation of soybean zygotic embryos is necessary for their germination (22).

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Normal zygotic embryos of soybean will germinate in vitro without exogenous growth regulators. The aim of the present work was to reduce or eliminate the requirement for growth regulators beyond the initial embryo induction stage, and to increase the germination rate by simplification of maturation procedures or inclusion of a desiccation step or both.

Materials and Methods

Explant materials were obtained from soybean plants [*Glycine max* (L.) Merr.] grown in a greenhouse at ambient temperatures under natural lighting, supplemented when necessary to a 14-h photoperiod with illumination from high-pressure sodium lamps to maintain ideal flowering conditions. Plants were grown, five to a pot, in 10-in. pots using a 2:2:1 mixture of soil (Maury silt loam), Promix (Premier Brands, New Rochelle, NY), and sand. Plants were fertilized weekly with Peter's 20-20-20 fertilizer (W.R. Grace and Co., Fogelsville, PA). Cultivars J103 (Jaques Seed Co., Monroe, WI), Manchu, and Williams 82 were selected because of their excellent regeneration capacity. Pods were surface sterilized by immersion in 70% 2-propanol for 30 s, 25% commercial chlorine bleach (1.3% sodium hypochlorite) for 12 min, and three rinses in sterile water. Immature seeds, 3 to 5 mm in length, were excised from surface-sterilized pods. The end containing the embryonic axis was removed, and both cotyledons were pushed out of the seed coat and placed, abaxial side down, on N10 medium, consisting of Murashige and Skoog salts (18) (MS salts) B5 vitamins (3), 1.5% sucrose, 10 mg/liter NAA, 0.2% Gelrite (Kelco) as a solidifying agent, and adjusted to pH 5.8.

Quality of somatic embryos. To determine if somatic embryos could be obtained after exposure periods to an auxin shorter than the 1-mo. period used by all published protocols, excised cotyledons of the cultivar Manchu were exposed to NAA for either 8 to 20 d, after which the cotyledons were transferred to MS0 medium (as above, but with no NAA) for 22 and 10 d, respectively. Twenty cotyledons were placed on each plate. There were 10 plates (200 cotyledons) for each treatment. Plates were maintained at 25° C, with a 23-h photoperiod, and an average light intensity of 10 $\mu Em^{-2} \cdot s^{-1}$ provided by cool-white fluorescence tubes supplemented with 50-W incandescent bulbs. Thirty days after the initial explanting date, the resulting somatic embryos were removed from the explant cotyledons. Shoot meristem development of 100 embryos per treatment was evaluated using a 0 to 5 scale, where 0 = absence of meristem; 1 = rudimentary meristem, 2 to 4 = increasing degrees of meristem formation, and 5 = well-formed unifoliolate leaves.

To further evaluate the effect of exposure to NAA, immature cotyledons of the cultivar J103 were incubated on N10 medium for 3, 6, 9, 12, 15, and 30 d. At the end of the 3, 6, 9, 12, or 15 d exposures, the cotyledons were transferred to MSO medium for the remainder of the 30-d period. Treatments were blocked by day, such that one entire set of treatments was explanted on any given day. The number of somatic embryos per plated cotyledon was recorded, and the meristematic development of each embryo was evaluated using the 0 to 5 scale described above. This experiment was repeated with one modification of the protocol. In the first experiment, cotyledons in the 30-d treatment remained on the same N10 medium for 30 d, whereas in the second experiment, cotyledons in the 30-d treatment were transferred to fresh N10 medium after 9 d.

To clarify the effects of continued exposure to NAA beyond 9 d on the number of somatic embryos formed, 200 cotyledons of the cultivar J103 were placed on N10 medium as previously described. After 9 d, half of the cotyledons were transferred to fresh N10 medium. The number of somatic embryos per cotyledon was recorded 30 d after the cotyledons were initially explanted.

Germination of somatic embryos. Three media were initially compared for their ability to mature somatic embryos. These were the BKZN medium of Lazzeri et al. (14), the liquid LSG medium of Hsu and Obendorf (12), and LSG medium solidified with 0.2% Gelrite (Table 1). Cotyledons of cultivars Manchu and Williams 82 were induced on N10 medium for 30 d, after which the resulting somatic embryos were transferred to BKZN medium. After 30 d on BKZN medium, the somatic embryos were split into three groups (130 somatic embryos per repetition; 3 repetitions per treatment; the first 2 repetitions of Manchu embryos and the 3rd of Williams 82). One third of the embryos were transferred to fresh BKZN medium, another third to liquid LSG medium, and the remaining third to solid LSG medium for 30 d. After 30 d all embryos were transferred to HPN medium (14,15), and germination was recorded over a period of 3 mo. Germination was defined as elongation of the hypocotyl, development of a root system, and expansion of the primary leaves.

The effect of desiccation on germination of somatic embryos (9) was evaluated in another experiment. Somatic embryos of cultivars A. K. Harrow, Mandarin, Manchu, J103, McCall, Jilin 5, Wayne, and Shiro were maintained on MSO medium for 2 to 4 wk, after induction on N10 medium. Approximately half of the somatic embryos of each genotype were then transferred to empty, sterile pertri dishes which were subsequently sealed with Nescofilm. After 1 wk, all somatic embryos (desiccated and nondesiccated) were transferred to fresh MS0 medium. Desiccation induced rapid and uniform germination, permitting germination to be evaluated after 2 wk. Without desiccation, germination occurred sporadically over a period of several months.

Results and Discussion

Quality and Number of Somatic Embryos

Embryo quality. Embryos of the cultivar Manchu induced by an 8-d exposure to NAA at 10 mg/liter showed superior shoot meristem development compared to embryos induced by a 20-d treatment. The shorter exposure to NAA gave a mean shoot meristem develop-

TABLE 1

COMPARISON OF MATURATION MEDIA FOR SOYBEAN SOMATIC EMBRYOS

	Medium		
	BKZN	LSG	MSO
Salts	MS	LS	MS
Organics	B5	B5	B5
Sucrose, %	3	5	1.5
Glutamine, %		0.0913	—
Benzyladenine, mg/liter	0.017	—	—
Kinetin, mg/liter	0.017	—	—
Zeatin, mg/liter	0.017	—	—
Naphthalene acetic acid	0.05		

TABLE 2

EFFECT OF INCREASING EXPOSURE TO NAA ON THE SHOOT MERISTEM QUALITY OF J103 SOYBEAN SOMATIC EMBRYOS

Days on NAA	Days on MSO	Meristem Development Rating[a] Exp. I	Exp. II
3	27	0.56[b]	0.31[b]
6	24	0.81	0.34
9	21	0.32	0.17
12	18	1.18	0.25
15	15	0.75	
30	0	0.49	0.10

[a]Meristems were rated on a 0 to 5 scale, where 0 = no meristem; 1 = rudimentary meristem present; 5 = equivalent to meristem of a zygotic embryo.

[b]In experiment I, cotyledons remained on the same N10 medium for 30 d, whereas in experiment II, cotyledons in the 30 d treatment were transferred to fresh N10 medium after 9 d.

ment of 2.35, compared to 1.49 for the longer exposure. This difference was highly significant ($P < 0.0001$; t test).

The effects of auxin exposure time on the quality of somatic embryos of J103 are presented in Table 2. In the first experiment (I) there was a trend toward increasing meristem quality as exposure to NAA increased from 3 to 12 d ($r^2 = 0.995$; excluding the mean for the 9-d treatment), and a trend toward decreasing meristem quality as exposure to NAA increased from 12 to 30 d ($r^2 = 0.963$). In the second experiment (II) there was an overall decrease in meristem quality as exposure to NAA increased from 3 to 30 d ($r^2 = 0.70$). The two experiments were consistent in that increasing exposure of the excised cotyledons to NAA beyond 12 d was negatively correlated with normality of the shoot meristem of resulting somatic embryos.

Embryo number. A 3-d exposure to NAA was sufficient to induce somatic embryos (Table 3). The number of embryos continued to increase with increasing exposure to NAA until Day 12. Continued exposure beyond this time had either no effect (experiment II) or a deleterious effect (experiment I) on the number of embryos formed. The reduction of embryo number during continued exposure to NAA may not be due to NAA itself but to some other factor, such as medium depletion or the accumulation of toxins in the medium. Cotyledons transferred from N10 medium onto fresh N10 medium after 9 d produced significantly more somatic embryos than those

TABLE 3

EFFECT OF INCREASING EXPOSURE TO NAA ON THE INDUCTION FREQUENCY OF J103 SOYBEAN SOMATIC EMBRYOS

Days on NAA	Days on MSO	No. Embryos Induced per Cotyledon[a] Exp. I	Exp. II
3	27	0.56	0.95
6	24	0.26	2.78
9	21	0.45	2.92
12	18	0.57	2.36
15	15	0.49	—
30	0	0.34	3.16

[a]In experiment I cotyledons remained on the same N10 medium for 30 d, whereas in experiment II cotyledons in the 30-d treatment were transferred to fresh N10 medium after 9 d.

TABLE 4

EFFECT OF THREE MATURATION MEDIA ON THE PERCENT GERMINATION AFTER 3 MO. OF SOMATIC EMBRYOS OF SOYBEAN CVS. MANCHU AND WILLIAMS 82

Repetition	Cultivar	Percent Germination on Maturation Medium LSG Solid	BKZN	LSG Liquid
1	Manchu	54.6	11.5	3.6
2	Manchu	50.0	28.0	12.0
3	Williams 82	45.0	21.6	5.0
Average		50.2	20.39	6.9

that remained on the same medium throughout the 30-d culture period (2.71 vs. 1.20; $P = 0.003$, t test). A 12- to 14-d exposure of cotyledons to NAA results in a good compromise between embryo number and embryo quality.

Germination of somatic embryos. Of the three maturation media evaluated in the first experiment, solidified LSG medium gave the highest germination percentages and liquid LSG medium the lowest (Table 4). The mean germination percentages for the treatments differed significantly, as determined by analysis of variance. Embryos matured on BKZN medium developed abnormally swollen hypocotyls, whereas embryos matured in liquid LSG were frequently injured by agitation of the cultures. The most rapid and uniform germination was achieved after desiccation of somatic embryos matured on MSO medium for 4 wk (Table 5). Desiccation produced soil-ready seedlings within 2 to 3 wk after embryos were returned to MSO medium.

Embryos had to reach a minimum age before they were able to survive desiccation. Most seemed to develop the physiologic ability to survive desiccation and germinate upon rehydration when cultured for 4 wk on MSO medium after removal from the explant tissues. These observations are in accordance with those of Gray et al. (6), who found that the developmental stage of orchardgrass somatic embryos influenced their ability to withstand desiccation and to germinate upon rehydration. They are in contrast, however, with the observations of Kitto and Janick (13), who found that carrot somatic embryos could survive desiccation only when first encapsulated in water-soluble plastic resin. Desiccation

TABLE 5

PERCENT GERMINATION OF SOMATIC EMBRYOS OF SEVEN SOYBEAN GENOTYPES ASSESSED 2 WK AFTER DESICCATED SOMATIC EMBRYOS WERE RETURNED TO MSO MEDIUM

Genotype	Percent Germination After: n[a]	Desiccation	n[a]	No Dessication
A.K. Harrow	39	97	9	0
Mandarin	10	100	10	0
Jilin 5	30	80	16	0
Wayne	32	47	39	0
McCall	70	38	87	1
J103	463	35	619	5
Manchu	23	26	11	0

[a]n = Total number of somatic embryos.

of orchardgrass somatic embryos decreased the number that germinated into plants, but desiccation of grape somatic embryos enhanced their ability to produce plants (7). These studies on carrot, orchardgrass, and grape embryos differed from the present study, however, in that they did not incorporate a maturation period between the removal of the embryo from its source tissue and the onset of desiccation.

Ranch et al. (21) reported the use of a maturation medium containing 0.5% activated charcoal and 10% sucrose. Embryos were then transferred to hormone-free MS medium until germination occurred. The high osmoticum of this maturation medium may have helped achieve a degree of desiccation within the embryos. LSG medium also has a relatively high osmotic potential and may have a similar desiccating effect.

Previous work indicated a need for cytokinins during embryo germination, and led to the development of BKZN medium (14,15). However, the cytokinin was presumably required to reverse the effects on the shoot apex of an excessively long auxin treatment during induction. Apparently, growth regulators are not necessary to convert normal somatic embryos into plants. Embryos must attain a minimum state of maturity and a physiologic state permitting germination. The necessary growth can be achieved by placing embryos on hormone-free MS salts with 1.5% sucrose (MSO) for 4 weeks after they have been removed from the explant tissue. A physiological state permitting germination can be rapidly induced by desiccating the somatic embryo in a dry, sterile petri dish for one week.

Soybean embryogenesis is now a simple process, requiring only one basal medium. MSO medium (MS salts, B5 vitamins, 1.5% sucrose, 0.2% Gelrite, pH 5.8) supplemented with 10 mg/liter NAA is utilized as an induction medium for 2 wk. After 2 wk the explanted cotyledons are transferred to MSO for a futher 2 wk while somatic embryos appear. The somatic embryos are removed from the cotyledons and placed on fresh MSO for 1 mo., after which they are placed in an empty petri dish for 1 wk. The desiccated embryos are then returned to MSO until they germinate. It is now possible to obtain soil-ready seedlings 2 to 3 mo. after the immature cotyledons are explanted. This represents a major increase in efficiency over the period of up to 9 mo. required to achieve lower germination rates in other studies.

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