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Soybean somatic embryogenesis: Effects of hormones and culture manipulations*

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Abstract. Somatic embryos were induced in cultures of immature soybean (*Glycine max* (L.) Merr) embryos, or isolated cotyledons on MS modified medium supplemented with NAA and 2,4-D, BAP and ABA. When NAA and 2,4-D were compared at similar concentrations (25 and 23 μM), 2,4-D produced larger number of somatic embryos, however, embryogenesis efficiency was improved in media containing NAA by using higher levels (100–150 μM) of the auxin. Somatic embryo morphology varied with auxin type: NAA-induced embryos more closely resembled zygotic embryos than did 2,4-D-induced embryos. Additions of BAP or ABA to auxin-containing media had either no effect or reduced embryo production, although ABA altered the morphology of 2,4-D-induced embryos. In media containing both NAA and 2,4-D, the latter was dominant in terms of embryo morphology. The effects of subculture frequency and of transfers between 2,4-D and NAA media were investigated: Subculture frequency influenced mainly the frequency of normal embryos, while preculture on 2,4-D increased subsequent embryogenesis efficiency on NAA medium but reduced the frequency of normal embryos.

Abbreviations. $\mu Em^{-2}s^{-1}$, microEinsteins per square meter per second; NAA, α-naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxy acetic acid; ABA, abscisic acid; BAP, benzylamino purine

Introduction

Much effort has been devoted to soybean tissue culture and to achieving plant regeneration in vitro (for review see [9]). While perennial *Glycine* species have been regenerated from complex explants [8, 10, 11, 23] and also from protoplasts [17, 18], until recently attempts to regenerate *G. max* had

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yielded only non-functional somatic embryos [4, 19]. Using immature embryos as explants, however, several instances of somatic embryogenesis [5, 14, 15] and whole plant regeneration [3, 12, 20] have now been reported. Regeneration from immature leaf tissue has also been reported [24]. These previous studies contain relatively little analysis of the major factors which affect somatic production and development. In particular, little attention has been given to the frequency of normal embryo production and to the numbers of embryos produced per responding culture.

The present study was made during the development of a soybean regeneration system [12] to investigate factors important to embryogenesis. In this paper we will discuss hormonal and culture manipulation effects, while in a companion paper we will discuss nutritional, physical and chemical factors [13].

Materials and methods

Two *G. max* genotypes were used in these studies: maturity group 00 cultivar McCall, (Dr. R.L. Bernard, University of Illinois), and maturity group II cultivar J103 (Jacques Seed Co., Wisconsin).

Plant growth, embryo isolation

Procedures for donor plant growth, pod sterilization and embryo isolation were as described previously [12]. Plants were grown in pots in a greenhouse, under natural light supplemented with 13 hrs artificial (high pressure sodium) light during winter months. Pods containing seeds of length 4.0 $\pm$ 1.0 mm were surface-sterilized by 30 sec immersion in 70% isopropyl alcohol, followed by 10 min in 25% Chlorox bleach and then two rinses in sterile water. Pods were opened and embryos isolated from the immature seeds [12]. In early experiments embryos were cultured whole, while in later experiments the embryonic axis was removed and the pair of isolated cotyledons plated side-by-side, as this procedure increased the efficiency of embryogenesis [13]. The type of explant used is specified in each table of results.

Media, culture conditions

The basal medium consisted of MS salts [16], B5 vitamins [6], 3% sucrose and 0.65% Phytoagar (Gibco) at pH 5.9 before autoclaving at 121 °C, 1.06 $Kg^{-1}cm^{-2}$ for 15 mins. Hormones were added to media before auto-

claving, with the exception of ABA, which was filter-sterilized and added to autoclaved medium. Ten embryos (or cotyledon pairs) were cultured per 30 ml of medium in 20 × 100 mm plastic dishes (Falcon), at 25 ± 3 °C under a 16 hr photoperiod of cool white fluorescent light ($\sim 20\,\mu Em^{-2}s^{-1}$). For convenience, in the text media with particular hormone contents are referred to in abbreviated form. Thus N50 and N150 denote media with 50 or 150 μM NAA, D23 medium with 23 μM 2,4-D, and so forth.

Culture assessment

Cultures were scored at 30 d. Four parameters were used to assess embryo response: I. Embryogenesis Frequency, = number of embryogenic cultures/ total cultures initiated (a "culture" is the tissue derived from a single zygotic embryo), II. Mean Embryo Number, = mean number of somatic embryos both normal and abnormal (see below for definition) per embryogenic culture. III. Efficiency, = Embryogenesis Frequency × Mean Embryo No., IV. Frequency of Normal Embryos, = number of cultures with normal embryos/total embryogenic cultures. In some experiments, the frequency of cultures producing adventitious roots was also recorded.

For assessment, embryos with distinct root and shoot poles and at least one defined cotyledons were classified as normal, while embryos lacking distinct shoot poles, without cotyledons or with fused cotyledons were classed as abnormal.

In all experiments, a minimum of 60 embryos was cultured per treatment, and generally $\geqslant 100$ embryos were used per treatment. In experiments where two genotypes were used, trends of response were always found to be very similar, so for simplicity, only data for one genotype is shown.

Experiments

The effects of various concentrations and combinations of hormones were tested by supplementing the basal medium as appropriate (see tables of results).

To determine the influence of subculture frequency on somatic embryogenesis cultures were initiated on medium containing 25 or 50 μM NAA and subcultured to fresh medium of the same composition at intervals of 5, 10 or 15 d. The total culture period was 30 d so cultures were transferred either five, two or one times, with control cultures having the standard incubation period.

Two experiments investigated the effects of either initial culture on high concentrations of NAA (100 or 150 μM), or of various periods on 23 μM

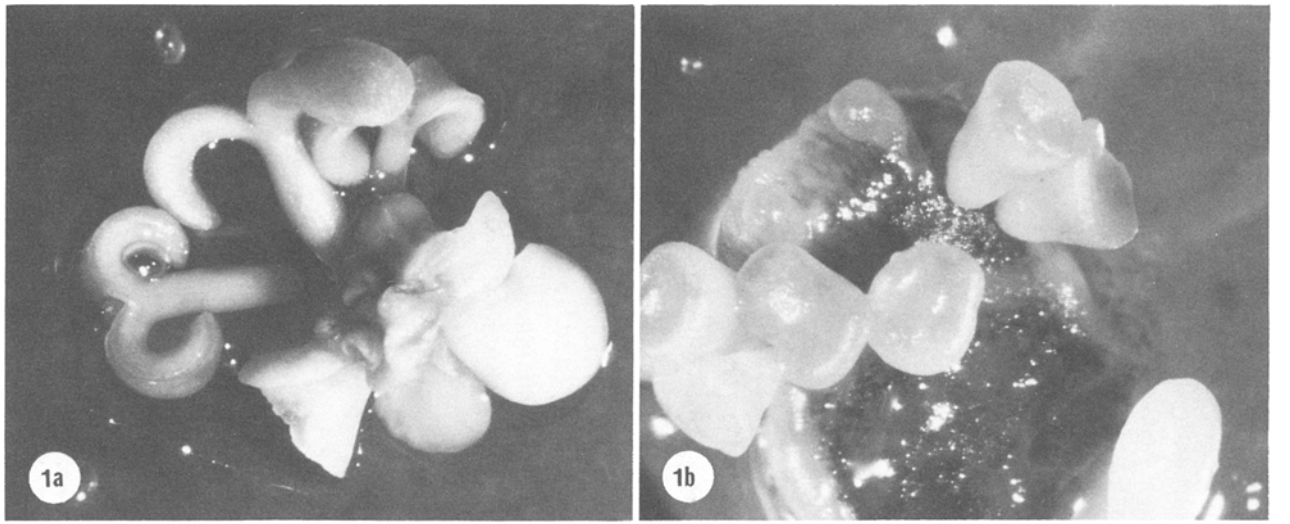

Fig. 1. Somatic embryo morphology. 1a. Normal somatic embryos, induced by 50 μM NAA, with distinct hypocotyls, normal cotyledons, and shoot apices. 1b. Abnormal somatic embryos, induced by 23 μM 2,4-D, with indistinct hypocotyls, poorly-defined or fused cotyledons, and no apparent shoot apices.

Table 1. Effects of hormones on somatic embryogenesis (cv McCall, whole embryos cultured).

Hormone concn. (μM)	Embryogenesis frequency (A) (%)	Mean embryo no. (B)[a]	Efficiency (A × B)	Frequency of normal embryos (%)
NAA 25	29.3 ± 6.1	1.73 ± 0.21	0.51	27.3 ± 4.0
NAA 37.5	36.3 ± 5.5	2.24 ± 0.26	0.81	20.7 ± 6.2
NAA 50	32.9 ± 7.1	2.52 ± 0.41	0.83	26.1 ± 7.6
NAA 25, BAP 0.045	23.2 ± 6.3	1.69 ± 0.33	0.39	NR[b]
NAA 25, BAP 0.23	10.1 ± 4.1	1.71 ± 0.36	0.17	NR
NAA 25, ABA 0.38	11.8 ± 4.2	1.38 ± 0.18	0.16	NR
NAA 25, 2,4-D 0.23	24.0 ± 7.0	1.72 ± 0.41	0.41	16.7 ± 1.9
NAA 25, 2,4-DS 2.3	16.0 ± 5.5	1.67 ± 0.22	0.27	16.7 ± 1.7
NAA 25, 2,4-D 23	56.0 ± 6.2	3.69 ± 0.35	2.07	11.9 ± 3.3
2,4-D 23	60.0 ± 5.4	3.40 ± 0.35	2.04	12.6 ± 3.4
2,4-D 23, ABA 0.38	61.3 ± 7.2	2.85 ± 0.30	1.75	17.4 ± 5.0
2,4-D 23, ABA 3.8	41.4 ± 5.6	2.45 ± 0.29	1.02	3.4 ± 1.2

[a] In this and following tables, embryogenesis frequency, mean embryo no., and normal embryo frequency data are expressed as means and standard errors of the means.
[b] Not recorded.

2,4-D, before transfer to N50 medium. Data are presented only for the latter experiment, but both will be discussed.

The data here are derived from a long series of experiments. As important factors were identified the experimental system was modified, so comparisons of treatments may be made only within and not between experiments as all control treatments were not necessarily identical.

Results

Effects of auxins and hormone combinations

Somatic embryogenesis was dependent on the type and concentration of auxin in the medium (somatic embryos were never observed in the absence of exogenous auxin). Comparing NAA and 2,4-D at a similar concentration (23–25 μM), the latter was more potent for somatic embryo induction (Table 1), and gave the high efficiency value (efficiency is a measure of the yield of somatic embryos per culture).

There was a clear effect of auxin type on culture morphology. Somatic embryos induced by NAA had the most normal morphology (see Fig. 1a) although mono- and polycotyledonous embryos were common. 2,4-D-

Table 2. Effects of subculture frequency on somatic embryogenesis (cv McCall, whole embryos cultured).

Auxin concn. (μM)	Subculture frequency	Embryogenesis frequency (A) (%)	Mean embryo no. (B)	Efficiency (A × B)	Frequency of normal embryos (%)
NAA 25	5 d	55.6 ± 6.5	2.13 ± 0.21	1.18	3.3 ± 2.8
	10 d	53.3 ± 6.8	2.34 ± 0.21	1.29	9.4 ± 5.8
	15 d	50.0 ± 7.2	1.96 ± 0.22	0.98	0
	No subculture	46.7 ± 5.5	1.93 ± 0.17	0.90	3.6 ± 7.8
NAA 50	5 d	51.7 ± 6.7	2.00 ± 0.17	1.03	6.5 ± 4.3
	10 d	60.0 ± 3.0	1.70 ± 0.15	1.02	13.3 ± 6.7
	15 d	47.3 ± 4.4	2.23 ± 0.19	1.05	19.2 ± 4.5
	No subculture	48.0 ± 5.1	2.08 ± 0.22	1.00	16.7 ± 4.9

induced embryos were usually horn-shaped (see Fig. 1b) although leafy and fasciated structures were also seen. Cultures on NAA media produced little callus whereas 2,4-D invariably induced some callusing. Adventitious roots were usually formed on NAA media, but rarely on 2,4-D media.

The comparison of three NAA concentrations, 25, 37.5 and 50 μM showed little difference in response, although 50 μM NAA gave the highest efficiency value Table 1. Medium containing 23 μM 2,4-D gave more than double the efficiency value of the most effective NAA medium, but with a low frequency of normal embryo production. In combinations of 25 μM NAA with 0.23, 2.3 or 23 μM 2,4-D, the two lower 2,4-D levels appeared to have little effect on embryogenesis efficiency, while the high level gave results very similar to those for 23 μM 2,4-D alone. Nevertheless, in each combination, 2,4-D appeared to be the dominant auxin in terms of somatic embryo morphology: even 0.23 μM 2,4-D markedly reduced the frequency of normal embryo production by comparison with N25 medium without 2,4-D.

Effects of cytokinins and ABA

In combination with 25 μM NAA, 0.044 μM BAP had little effect, while 0.22 μM BAP reduced embryogenesis frequency. The higher BAP concentration also stimulated friable callus production. ABA at 0.38 μM, in combination with 25 μM NAA, reduced embryogenesis in comparison with medium containing NAA alone. In combination with 23 μM 2,4-D, ABA at 0.38 μM, had little influence on embryogenesis efficiency but increased the frequency of normal embryos slightly over the control value (plain D23 medium). At 3.8 μM, however, ABA halved the embryogenesis efficiency and reduced normal embryo production. An occasional effect ($\sim$ 10% of

cultures) of ABA on the morphology of 2,4-D-induced somatic embryos was to make their cotyledons broad and leaf-like.

Subculture frequency effects

As simply increasing the NAA concentration in induction medium gave relatively small increases in embryogenesis efficiency (Table 1) the effects of subculture frequency (i.e. the provision of non-depleted medium) were examined (Table 2). Somatic embryo production showed no clear trend of response to subculture frequency in either N25 or N50 medium. The highest embryogenesis efficiency was recorded for subcultures on N25 medium. In N25 medium, the frequency of normal embryos was low throughout, while on N50 medium, the frequency was generally higher, but was reduced by the most frequent (5 d) subculture treatment.

Transfer experiments and high-NAA media

Among the 2,4-D — to — NAA transfer treatments, one day's exposure to 2,4-D reduced embryogenesis efficiency by comparison with the control (N50 30 d), but efficiency increased with the 3 d and 5 d 2,4-D treatments and then decreased with the 10 d treatment (Table 3). (In this experiment the embryogenesis frequency in the D23 30 d treatment was lower than routinely expected on this medium, although the mean embryo number was quite typical.) The highest frequencies of normal embryo production was seen in the absence of 2,4-D (N50, 30 d) or with 1 d culture on 2,4-D (D23 1 d, N50 29 d) and decreased with increasing exposure to 2,4-D. The single NAA — to — 2,4-D treatment gave the lowest efficiency value, and like the D23

Table 3. Effects of transfers between 2,4-D and NAA media on somatic embryogenesis (cv J103, isolated cotyledons cultured).

Treatment (medium/days)	Embryogenesis frequency (A) (%)	Mean embryo no. (B)	Efficiency (A × B)	Frequency of normal embryos (%)	Frequency of roots (%)
N50[a] 30 d	80.0 ± 6.6	3.18 ± 0.35	2.54	37.5 ± 3.9	54.6 ± 8.2
D23 1 d, N50 29 d	72.0 ± 5.3	2.56 ± 0.25	1.84	38.9 ± 5.2	80.0 ± 6.3
D23 3 d, N50 27 d	85.7 ± 5.2	3.67 ± 0.24	3.14	19.0 ± 6.8	79.6 ± 5.8
D23 5 d, N50 25 d	92.0 ± 4.4	3.89 ± 0.22	3.58	29.1 ± 7.2	72.0 ± 9.6
D23 10 d, N50 20 d	44.9 ± 6.3	3.23 ± 0.43	1.45	9.1 ± 2.7	0
D23 30 d	62.5 ± 7.9	3.48 ± 0.51	2.18	0	0
N50 5 d, D23 25 d	52.0 ± 9.2	2.19 ± 0.43	1.14	0	0

[a] See Materials and Methods for abbreviations for media.

30 d treatment, yielded no normal embryos. Adventitious root production was stimulated by 1–5 d exposure to 2,4-D, but no roots were produced in the longer 2,4-D treatments or in the NAA — to — 2,4-D transfer treatment.

In a second transfer experiment cultures were initiated on N50, N100 or N150 medium and were then transferred to fresh N50 medium after 5, 10 or 15 d, or were left on the original medium throughout the 30 d culture period. Embryogenesis frequency was similar among all treatments, but cultures on N100 and N150 media gave consistently higher mean embryo number values (N100, mean 4.07 ± 0.36, N150, mean 4.77 ± 0.40) than N50 medium (mean 3.62 ± 0.26), and the N150 30 d treatment induced the most normal embryos (40.5 ± 3.4%). The time of incubation before transfer had no consistent effect on embryogenesis efficiency. In the light of these results, the three media N50, N100 and N150 were further compared (Table 4). Throughout this experiment, the frequency of embryogenesis was high, but efficiency was again seen to increase at the elevated NAA concentrations, although the differences were less marked than in the previous experiment. The frequency of normal embryos was similar in the three treatments. Adventitious root production was comparatively more sensitive to auxin than embryogenesis, increasing by 28% over the NAA concentration range.

Discussion

The data here show the importance of exogenous auxin in regulating soybean somatic embryogenesis. Auxin type, concentration, and the timing and length of auxin treatment had specific effects on the process, both in terms of efficiency and of embryo morphology. Considering the efficiency of embryogenesis, media containing 2,4-D produced the largest numbers of somatic embryos, and when equivalent concentrations of 2,4-D and NAA (23 and 25 μM, respectively) were compared, the former was markedly more

Table 4. Effects of high concentrations of NAA on somatic embryogenesis (cv J103, isolated cotyledons cultured).

Auxin concn. (μM)	Embryogenesis frequency (A) (%)	Mean embryo no. (B)	Efficiency (A × B)	Frequency of normal embryos (%)	Frequency of roots (%)
NAA 50	85.0 ± 5.0	2.47 ± 0.18	2.10	35.3 ± 5.7	38.0 ± 3.7
NAA 100	96.3 ± 3.1	2.65 ± 0.17	2.55	34.6 ± 6.1	52.0 ± 6.5
NAA 150	93.2 ± 4.0	2.82 ± 0.17	2.63	38.2 ± 4.5	66.2 ± 4.0

active (Table 1). In order to induce consistently high numbers of somatic embryos on media containing NAA, considerably higher concentrations of the auxin (100 or 150 μM) were required (Table 4). Soybean cotyledon tissue has a high diffusive resistance [7], so high external concentrations of auxins may be required for "inductive" concentrations to develop within the explant. In other studies on soybean somatic embryogenesis, 2,4-D has been the most commonly-used auxin [5, 14, 15, 20], although only two studies have compared different auxins [3, 15]. Where different 2,4-D concentrations have been tested, the optima found have differed (5 μM [15], 22.5 μM [20]). Similarly, the increased somatic embryo production seen at high NAA concentrations in the present study contrasts with the optimum levels suggested by other workers (2.5 μM [15], 43 μM [3]). These discrepancies may stem from the use of different sucrose levels or of different explants (whole embryos versus isolated cotyledons), as both factors strongly affect somatic embryogenesis [13].

Somatic embryo induction from cotyledon tissue required only the provision of exogenous auxin. In treatments where either cytokinin (BAP) or ABA was also supplied, embryogenesis efficiency was either unaffected or reduced, depending on the supplement concentration. BAP stimulated the formation of friable callus, particularly at sites of damage, and this tissue never produced somatic embryos. An inhibitory effect of cytokinin in media containing 2,4-D has previously been reported [15]. ABA is known to be important in the regulation of soybean embryogenesis in vivo [1], and either stimulates or inhibits zygotic embryo growth and development in vitro, depending on embryo stage [2]. The present data suggest, however, that exogenous ABA inhibits auxin-induced somatic embryogenesis, although an effect on somatic embryo development was observed, in that cotyledons of some 2,4-D-induced embryos became leaf-like in the presence of 0.38 μM ABA.

Variation in the efficiency of embryogenesis, whether in response to auxin type or concentration or to other factors generally reflected similar responses of the two constituent parameters: in that treatments which induced a high percentage of explants to become embryogenic also induced comparatively high numbers of embryos on these responding cultures.

Variation in soybean somatic embryo "quality" or morphology has been commented on previously [15, 20], but no previous study has reported the frequencies of normal and abnormal embryos. This parameter is, however, of importance to the application of embryogenesis systems, as the efficiency of conversion of embryos to plantlets often varies considerably with embryo normality [4, 21]. The major factor affecting soybean somatic embryo morphology is auxin type, although other physical and chemical factors are

also involved [13]. Embryos induced by NAA typically exhibit clear bipolarity, with distinct radicle and hypocotyl regions, well-defined cotyledons and a shoot apex visible from an early stage of development (see Fig. 1). The most frequent abnormalities seen in NAA-induced embryos are the loss of one or more cotyledons, and fusion to the parental cotyledon tissue. Embryos induced by 2,4-D are in general horn-shaped, with indistinct or fused cotyledons. The shoot apex is frequently underdeveloped in otherwise "mature" embryos. The differences in morphology between NAA- and 2,4-D induced embryos are reflected in their abilities to germinate. Embryos with normal morphology germinate readily, while abnormal embryos are recalcitrant and often require long periods of incubation or culture manipulations for germination [12, 20]. In alfalfa, another legume, somatic embryos induced by low 2,4-D concentrations have more normal morphology and seed storage protein profiles and have a higher frequency of conversion to plantlets than embryos induced by high 2,4-D concentrations [21]. Somatic embryos induced on media containing both NAA and 2,4-D had morphologies typical for embryos induced by 2,4-D. Even when the ratio of NAA to 2,4-D exceeded 100:1 (25:0.23 μM, Table 1) the latter was the dominant auxin in terms of embryo morphology and the frequency of normal embryos was markedly reduced, although this level of 2,4-D had little effect on embryogenesis efficiency.

Regarding the influence of subculture frequency, it is perhaps surprising that somatic embryo production was similar in cultures transferred several times to fresh medium and in those maintained on the original medium for the whole 30 d culture period. Nevertheless, this effect was observed with two different auxin concentrations (Table 2). This finding suggests either that depletion of medium components (or accumulation of deleterious compounds) are neither limiting nor promotive factors for embryo production, or that the major events which affect the process take place within the first five days of culture. The observation that in N50 medium a 5 d subculture interval reduced the frequency of normal embryos implies a separation between the processes of embryo induction and embryo development: the former being insensitive to the subculture treatments tested, the latter being influenced by the 5 d subculture treatment. Separation between the processes of embryo induction and development is supported by the previously-mentioned finding that, in combination with NAA, very low concentrations of 2,4-D had little effect on embryo production (induction) but did influence embryo morphology (development).

The efficiency of somatic embryogenesis on NAA medium was altered by preincubating explants on 2,4-D medium (Table 3). The period of preincubation appeared to be critical: 5 d exposure to 2,4-D gave the highest

efficiency value, while 1 d or 10 d exposure gave lower efficiency values than continuous culture on NAA medium. In alfalfa, the induction of organogenesis by the exposure of callus to 2,4-D was shown to be influenced strongly by the duration of culture on the induction medium. Organogenesis frequency was maximal with 3 or 4 d exposure to 2,4-D and declined sharply with longer or shorter 2,4-D treatments [22].

The data for the 2,4-D — to — NAA transfer experiment demonstrate a cumulative effect of 2,4-D on somatic embryo morphology: 1 d exposure to 2,4-D had no detectable effect, but longer exposures to 2,4-D progressively reduced the frequency of normal embryos. The finding that the 5 d NAA, 25 d 2,4-D treatment yielded no normal embryos suggests that embryos which start their development under normal inductive conditions are still susceptible to conversion to abnormal morphology by subsequent exposure to 2,4-D.

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