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Repetitive somatic embryogenesis in peanut cotyledon cultures by continual exposure to 2,4-D

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Abstract

Somatic embryos from immature cotyledons in peanut (*Arachis hypogaea*) were initiated on media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). Over 90% primary embryogenesis and 41–46% repetitive embryogenesis were obtained 12 weeks after initiation by maintaining embryogenic cultures on medium containing 20 mg l^{-1} 2,4-D. Maintenance of cultures on medium with 30 or 40 mg l^{-1} 2,4-D resulted in lower primary and secondary embryogenesis, and proliferation of nonembryogenic callus. Transfer of embryogenic cultures to a secondary medium with 10 or 20 mg l^{-1} 2,4-D significantly enhanced secondary embryogenesis compared to basal medium without the growth regulator. The use of Murashige & Skoog versus Finer's media had no significant effect on embryogenesis (85–95%), repetitive embryogenesis (11–37%) or mean embryo number. Secondary embryogenesis was also maintained for over one year by repeated subculture of isolated somatic embryos on medium with 20 mg l^{-1} 2,4-D.

Abbreviations: B5 – Gamborg et al. medium (Gamborg et al. 1968), 2,4-D – 2,4-dichlorophenoxyacetic acid, FN – Finer & Nagasawa medium (Finer & Nagasawa 1968), MS – Murashige & Skoog medium (Murashige & Skoog 1962)

Introduction

When developing a regeneration system to be used in gene transformation, desirable characteristics include reliability and high frequency. A major advantage of developing a somatic embryogenic system is the potential for cultures to undergo secondary embryogenesis, i.e., to repetitively produce somatic embryos from developing somatic embryos or embryogenic callus. Repetitive embryogenesis allows for continued, high frequency embryogenesis and long term regeneration of transformed individuals. This ability can also be used in gene transformation selection protocols, where repetitive embryogenesis can be used to eliminate transgenic chimerics (Mathews et al. 1992).

In somatic embryogenic cultures derived from immature peanut (*Arachis hypogaea* L.) cotyledons, maintenance on medium without growth regulators resulted in a decline of repetitive embryogenesis by 8 to 12 weeks after initiation due to germination or callusing of embryos (Baker & Wetzstein 1994). The presence of an auxin may be necessary to maintain repetitive embryogenesis. Low levels of auxin in solid medium maintained secondary embryo formation for an undisclosed time in immature embryo axes (Hazra et al. 1989), in immature zygotic embryos for 150 days after initiation (Sellars et al. 1990) and in immature leaflets for 56 to 84 days after initiation (Baker & Wetzstein 1992). Durham & Parrott (1992) observed that 90% of primary somatic embryos produced secondary embryos in liquid culture containing auxin; embryogenic capacity was maintained for over a year, but plant formation was limited. The objective of this study was to evaluate means to enhance repetitive

embryogenesis and maintain plant formation in peanut immature cotyledon cultures. The 2,4-D levels were varied in both induction and secondary media, and two basic medium formulations (MS and FN) were compared. The effect of removing individual embryos from proliferating cultures during subculture was also assessed.

Materials and methods

Immature pegs were collected from green house-grown peanut plants of cv. AT127. Pegs were soaked for 1 h in soapy water (about 5 drops of Micro, Internat. Prod. Corp., Burlington, NJ, per liter), then transferred to 95% ethanol for 2 min, followed by treatment in 3.1 % NaOCl (60% (v/v) commercial bleach with 0.5% NaOCl) with 2-3 drops per liter of Tween-20 for 10 min, then rinsed three times with sterilized water. Because plant growth varied with time of year and indeterminate flowering made tagging individual flowers impractical, days after pollination was not a reliable method for explant selection. A visual classification based on cotyledon development was used: aseptically dissected embryos were used in which cotyledons were flat, translucent, elongating with a visible embryo axis, and with a slight longitudinal depression on the adaxial surface. Cotyledons were excised and placed abaxial side in contact with 20 ml of initiation (induction) medium, which was dispensed in test tubes (150 × 25 mm) with Magenta (Magenta Corp., Chicago, IL) enclosures. Explants were then transferred from test tubes to Petri dishes (100 × 15 mm) in which 3 explants were placed per Petri dish on media as specified below. Explants were transferred at monthly intervals to fresh secondary medium.

The induction media were a modified MS medium with B5 vitamins, 3% (w/v) sucrose, and 0.4% (w/v) Gel-Gro (ICN Biochemicals, Division of ICN Biomedicals, Inc.); pH was adjusted to 5.8. Media were autoclaved at 121°C for 20 min. In Experiment I, cotyledons were cultured continuously on a medium containing 90.5 μM (20 mg l^{-1}), 135.7 μM (30 mg l^{-1}), or 181 μM (40 mg l^{-1}) 2,4-D. In Experiment II, cotyledons were cultured on induction medium with 90.5 μM (20 mg l^{-1}) or 181 μM (40 mg l^{-1}) 2,4-D for 30 days, then transferred to secondary medium with 0 μM (0 mg l^{-1}), 45.2 μM (10 mg l^{-1}), or 90.5 μM (20 mg l^{-1}) 2,4-D. In Experiment III, cotyledons were cultured on either MS or FN media containing 90.5 μM (20 mg l^{-1}) 2,4-D for 30 days. Explants then were transferred to the same medium with 0 μM (0 mg l^{-1}) or 90.5 μM (20 mg l^{-1}) 2,4-D. Replication numbers for Experiments I–III are included in Tables 1–3.

In Experiment IV, individual somatic embryos were isolated from cultures and placed in direct contact with medium containing 90.5 μM (20 mg l^{-1}). Embryos were collected from explants which were induced on 22.6 μM (5 mg l^{-1}) or 45.2 μM (10 mg l^{-1}) 2,4-D, and transferred to secondary medium without auxin for 8 weeks. Alternatively, embryos were removed from cultures after the completion of Experiment II, i.e., those induced on medium with 90.5 or 181, μM 2,4-D (20 or 40 mg l^{-1}) and cultured for 12 weeks secondary medium with 45.2 or 90 μM 2,4-D (10 or 20 mg l^{-1}). Embryo morphology was noted prior to isolation and transfer using the system developed by Wetzstein & Baker (1993). Roots on embryos exhibiting unwanted germination were excised and discarded; the embryo proper was cut into 2–5 mm long pieces before being placed on medium. Cultures were transferred monthly to fresh medium. There were between 20–30 embryos per treatment and these studies were repeated twice. Experiments I–IV were conducted in darkness at 26°C.

Data were collected on the number of immature cotyledons exhibiting primary and repetitive somatic embryogenesis, and the number of somatic embryos formed on each explant every 2 weeks after completion of the induction period (30 days) for Experiments I–III. Data were analyzed using the GLM procedure of the statistical analysis system SAS (v. 6.03. SAS Institute Inc.). Mean separations for mean number of embryos were performed after transforming embryo count data using square root of (embryo count + 0.5). For Experiment IV, the occurrence of repetitive embryogenesis was evaluated every 2 weeks.

Results and discussion

Experiment I

When cotyledons were cultured continuously on medium with 2,4-D, primary somatic embryogenesis was evident at all 2,4-D levels tested (Table 1). Percent primary embryogenesis dramatically decreased and mean embryo number per explant was significantly reduced at higher 2,4-D levels. Repetitive embryogenesis was observed in 17–43% of the explants for each 2,4-D level at 12 weeks after initiation, with a lower occurrence at high 2,4-D concentrations. Precocious (premature)

germination occurred in less than 5% of the embryos in all of the 2,4-D levels. However, continual exposure to 30 or 40 mg l^{-1} 2,4-D caused explants to produce excessive amounts of nonembryogenic callus (data not shown) which was absent on medium with 20 mg l^{-1} 2,4-D. Baker & Wetzstein (1994) likewise obtained high levels of embryogenesis (85%) with cotyledon explants which were initiated on 20 mg l^{-1} 2,4-D for 30 days then transferred to hormone free medium. However, repetitive embryogenesis was only 4% at 8 weeks after induction, and precocious germination was prevalent. In the present study, continued exposure to 2,4-D enhanced repetitive embryogenesis. However, media with concentrations of 2,4-D at 30 and 40 mg l^{-1} were supraoptimal, in that explants exhibited more browning, and both primary and secondary embryogenesis decreased compared to media with 20 mg l^{-1}. This same browning was observed by Baker &Wetzstein (1994) with cotyledons exposed to 40 mg l^{-1} 2,4-D for 30 days, which browned between 2–3 weeks after initiation. Browning of cultures associated with higher than 30 µM 2,4-D levels occurred with coconut (*Cocos nucifera* L.) (Karunaratne & Periyapperuma 1989).

Experiment II

In Experiment II, the 2,4-D concentrations were modified in the induction and secondary media in an attempt to improve primary embryo induction and repetitive embryogenesis. From 83–100% of the cotyledons induced on 20 mg l^{-1} 2,4-D produced primary embryos on the secondary media used. The presence of 2,4-D in the secondary medium resulted in significantly greater repetitive embryogenesis and mean number of embryos per explant compared to secondary medium with no 2,4-D (Table 2). With cotyledons induced on medium with 40 mg l^{-1} 2,4-D, secondary medium had no effect on primary embryogenesis, secondary embryogenesis or mean number of embryos (Table 2). Overall, primary embryogenesis was significantly lower using 40 versus 20 mg l^{-1} (i.e., comparisons of the two induction media with secondary media pooled).

Precocious germination decreased from over 50% on 0 mg l^{-1} 2,4-D secondary medium, to about 30% and 5% on 10 and 20 mg l^{-1} 2,4-D secondary media, respectively for both induction media. Most embryos maintained on secondary media with 2,4-D were single with broadened hypocotyls or multiple fused embryos (Classes 4, 5, and 6, using the system of Wetzstein & Baker 1993). Previous studies have shown these embryo types to more prevalently exhibit repetitive embryogenesis.

Experiment III

There were no significant differences in primary embryogenesis or repetitive embryogenesis in overall comparisons between MS and FN media. When comparing 2,4-D levels within the secondary medium, repetitive embryogenesis and mean number of embryos per explant were higher in MS induction medium with 20 versus 0 mg l^{-1} 2,4-D (Table 3). This trend was not significant with FN medium. Regardless of medium formulation, embryo development was comparable to that described in Experiment II. In secondary medium with 20 mg l^{-1} 2,4-D, precocious germination was reduced to 3–7%, as compared to 28–33% with 0 mg l^{-1} 2,4-D secondary medium at 12 weeks after initiation (data not shown).

The total levels of nitrogen in MS and FN are similar, but of different compositions. Total nitrogen in MS is 841 mg l^{-1} (composed of 1900 mg l^{-1} KNO_3, and 1650 mg l^{-1} NH_4NO_3) and in FN is 753 mg l^{-1} (composed of 3030 mg l^{-1} KNO_3, 800 mg l^{-1} NH_4NO_3, and 2192 mg l^{-1} glutamine). These differences in amount and form of nitrogen did not appear to influence embryogenesis, in contrast to other species in which changes in medium formulation or nitrogen levels have increased primary embryogenesis. Stuart & Strickland (1984) with *Medicago sativa* L. showed increasing ammonium up to 25 mM increased embryogenesis and any further increase diminished embryogenesis. When ammonium or nitrate were the sole nitrogen source there was little embryogenesis in eggplant, *Solanum melongena* L. (Gleddie et al. 1983). A 2:1 ratio of nitrate to ammonium resulted in the highest frequency of primary embryogenesis. Induction of embryos reached a maximum efficiency at 2900 mg l^{-1} KNO_3 in *Asparagus cooperi* Bak. (Ghosh & Sen 1991).

Experiment IV

In other systems, somatic embryos isolated from the explant material have been a source of secondary embryos (Arcioni et al. 1990; Finer & Nagasawa 1988; Plata & Viéitez 1990; Stamp & Henshaw 1987; Tulecke & McGranahan 1985; Raj Bhansali et al. 1990; Maheswaran & Williams 1986). In the current study, isolating peanut somatic embryos during subculture on basal secondary medium failed to produce sec-

Table 1. Influence of continual exposure to 20, 30 or 40 mg l^{-1} 2,4-D on primary embryogenesis, repetitive embryogenesis, and number of embryos per explant at 12 weeks after initiation.[1]

2,4-D (mg l^{-1})	Rep. No.[1]	% Primary embryogenesis	% Repetitive embryogenesis	Mean # embryos per explant ± S.E.[3]
20	28	93 a	43 a	10.9 ± 1.4 a
30	38	63 b	32 ab	6.2 ± 1.3 b
40	35	37 c	17 b	4.0 ± 1.3 b

[1]Mean separation using Duncan's multiple range test, means with different letters are significant at the 10% level.
[2] Number of replications.

Table 2. The effect of induction media (20 or 40 mg l^{-1} 2,4-D) and secondary media (0, 10 or 20 mg l^{-1} 2,4-D) on primary embryogenesis, repetitive embryogenesis, and number of embryos per explant 12 weeks after initiation.[1]

Induction medium (2,4-D mg l^{-1})	2nd medium (2,4-D mg l^{-1})	Rep. no.[2]	% Primary embry.	% Repetitive embry.	Mean no. embryos per explant ± S.E.[1]
20	0	23	83 a	13 b	4.4 ± 0.7 b
	10	20	100 a	45 a	8.6 ± 0.9 a
	20	22	90 a	41 a	7.9 ± 1.3 a
40	0	22	68 a	14 a	5.7 ± 1.4 a
	10	22	59 a	32 a	6.4 ± 1.8 a
	20	23	61 a	35 a	5.7 ± 1.8 a

[1]Means separated with different letters within an induction medium are significant using Duncan's multiple range test at the 10% level.
[2]Number of replications.

Table 3. The effect of induction medium (MS or FN medium with 20 mg l^{-1} 2,4-D) and secondary medium (0 or 20 mg l^{-1} 2,4-D) on repetitive embryogenesis, and number of embryos per explant at 12 weeks after initiation.[1]

Induction medium (2,4-D mg l^{-1})	2nd medium (2,4-D mg l^{-1})	Rep no.[2]	% Primary embry.	% Repetitive embry.	Mean no. embryos per explant ± S.E.
MS	0	53	85 a	11 b	4.1 ± 0.5 a
	20	54	91 a	37 a	8.7 ± 1.0 b
FN	0	48	92 a	15 a	5.9 ± 0.8 a
	20	48	88 a	29 a	7.8 ± 1.1 a

[1]Mean separation done by Duncan's multiple range test at the 10% level. Different letters among an induction medium are significant.
[2]Number of replications.

ondary embryos, regardless of the medium used for embryo induction. Most embryos callused or turned brown. Embryo developmental class did not affect the response (data not given). When somatic embryos were isolated from cultures maintained on secondary medium with 10 or 20 mg l^{-1} 2,4-D, about 10% of the embryos produced secondary embryos. Embryos undergoing repetitive embryogenesis were of classes 4, 5, and 6 (broad single embryos or fused multiple embryos) using the system developed by Wetzstein & Baker (1993). These embryo classes have been shown to produce repetitive embryos when they were retained on the original explant. Germinated peanut embryos rarely produced any secondary embryos in contrast to that reported in cassava (*Manihot esculenta* Crantz) where germinated embryos produced more repetitive embryos than torpedo embryos (Raemakers et al. 1993).

Repetitive embryogenesis has been maintained for over a year by isolation and subculture of individual embryos on an auxin-containing medium (10 or 20 mg l^{-1} 2,4-D) with no detectable change in morphology. The embryos germinated and formed plants which have flowered and set seed. Continuous exposure of cultures to auxin increased the time required for embryo germination and plant production compared to embryos obtained from cultures maintained on basal medium (see Wetzstein & Baker 1993), i.e., 3–4 months versus 2 months; percentage of germination was unchanged (data not shown).

Previous reports indicate that loss of repetitive embryogenesis and precocious germination result if embryogenic cultures are maintained on basal medium (Baker & Wetzstein 1994). We have found that repetitive embryogenesis can be retained for at least 12 weeks after initiation by maintaining the cultures on an auxin-containing medium. The manner in which cultures are transferred also affects repetitiveness. Removal of individual embryos from adjoining tissues and placing them in direct contact with auxin-containing medium has resulted in continued repetitive embryogenesis for over one year without marked changes in morphology. Variables for further evaluation to improve repetitive embryogenesis include the timing and developmental stage for embryo isolation, and type of transfer medium. Such factors have influenced other systems (Stamp & Henshaw 1987; Raj Bhansali et al. 1990; Plata & Viéitez 1990).

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