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Repetitive somatic embryogenesis from peanut cultures in liquid medium

Richard E. Durham and Wayne A. Parrott

Department of Agronomy, The University of Georgia, Athens, GA 30602, USA

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Summary. A regeneration system based on repetitive somatic embryogenesis was developed for peanut (*Arachis hypogaea* L.). Embryogenic suspension cultures were initiated using individual somatic embryos induced from immature cotyledons cultured on a modified Murashige and Skoog medium containing 40 mg/l 2,4-D for 30 days. After transfer to a modified MS liquid medium, the somatic embryos produced masses of secondary and tertiary embryos which continued to proliferate following manual separation and subculture of the embryogenic clumps. The cultures exhibited exponential growth, and have been maintained for over one year without apparent loss of embryogenic potential. Further embryo development, germination, and conversion were achieved by placing embryo clumps onto hormone-free, solid medium. The inclusion of a desiccation period during embryo development enhanced conversion four-fold. Plants have been established in soil and appear to be phenotypically normal.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: 1-naphthaleneacetic acid; BA: 6-benzylaminopurine; MSO: Modified Murashige and Skoog basal medium; EM: embryogenic masses.

Key words: Tissue culture Regeneration - *Arachis hypogaea*

INTRODUCTION

Plant regeneration from cultured tissues of peanut has been reported sporadically for nearly 15 years. Most of the earlier reports described regeneration via organogenesis from a wide variety of sources including anthers, cotyledons, epicotyls, hypocotyls, leaves, and embryos (for review see Parrott et al. 1991a). Many of the later reports have utilized somatic embryogenesis for plant regeneration. Explants giving rise to somatic embryos have included immature cotyledons (Ozias-Akins, 1989; Sellars et al., 1990), immature zygotic embryo axes (Hazra et al., 1989), and young leaflets (Baker and Wetzstein, in press). A wide variety of auxin types and concentrations, e.g. 2,4-D (0.5-40.0 mg/l), NAA (1.0-30 mg/l), and picloram (0.001-0.02 mg/l) have been utilized for the induction of somatic embryogenesis. Secondary embryogenesis has been observed in many of these studies (Hazra et al., 1989; Sellars et al., 1990; and Baker and Wetzstein, in press), but there have been no reports of establishing long-term, repetitive embryogenic cultures of peanut. Repetitive embryogenic cultures of peanut would be particularly useful for regeneration and transformation studies because it can be technically difficult and time consuming to obtain immature zygotic embryos, which thus far have been the source of the most responsive explants for somatic embryogenesis.

Repetitive embryogenic cultures have been established for soybean (*Glycine max* (L.) Merr.) using a simple protocol of inducing somatic embryogenesis from immature cotyledons exposed to 40 mg/l 2,4-D and transferring the resulting embryos to liquid 10A40N medium (MS-based medium) containing an altered NO_3:NH_4 ratio, asparagine, and 5 mg/l 2,4-D (Finer and Nagasawa, 1988). The purpose of this research was to establish embryogenic cultures of peanut by a similar approach and maintain long-term cultures capable of regenerating plants.

MATERIALS AND METHODS

Plant material. Immature pegs of peanut cultivar AT127 (AgriTech Seeds, Ashburn, GA) were harvested from plants grown in a greenhouse soil bed containing Garden on a Spot artificial medium (Gold Kist, Atlanta, GA) and sand (2:1 ratio) under a 14 hr photoperiod, and night temperature ≥20°C. The pegs were hand-rinsed to remove adhering soil, washed in soapy water for one hour, rinsed in 95% ethanol for 2 min, sterilized in 60% commercial bleach (3.15 % NaClO) for 15 minutes, and rinsed three times in sterile water.

Correspondence to: R. E. Durham

Embryo induction and establishment of liquid cultures. The embryonic axes and adjoining cotyledonary tissues were removed from 10-12 mm immature zygotic embryos. The remaining cotyledonary tissue was placed on solid medium with the abaxial side in contact with the medium. The medium consisted of Murashige and Skoog (1962) salts, B5 vitamins (Gamborg et al. 1968), 40 mg/l 2,4-D, 3 % sucrose, 0.2 % Gelrite (Merck and Co. Rathway, NJ), and pH adjusted to 5.8 prior to autoclaving. Solid cultures were maintained in 100 X 20 mm polystyrene petri dishes in a growth room (24 ± 1 °C, 23 hr photoperiod, 87 $\mu Em^{-2}s^{-1}$ cool-white fluorescent lighting). After 30 days, individual somatic embryos were removed from the cotyledons and placed into liquid 10A40N medium (Finer and Nagasawa, 1988). For culture initiation, 4-5 somatic embryos, 3-5 mm long, were placed in 125 ml Erlenmeyer flasks, each containing 35 ml of medium. The cultures were agitated on an orbital shaker at 125 rpm. Growth room conditions were as described above, except for the light intensity which was 22 $\mu Em^{-2}s^{-1}$. The cultures were routinely subcultured every two weeks and the clumps of embryos were manually separated with a scalpel to approximately 5 mm in diameter. The most proliferative clumps were used to inoculate new cultures.

Embryo conversion. For embryo germination and conversion, embryogenic masses (EM) were removed from culture and placed onto solid MSO medium (salts and vitamins as previously described, but lacking growth regulators). After one month, the EM were manually separated and individual embryos transferred to MSO medium. After one additional month, a portion of the enlarged embryos were desiccated for 10 days in an empty petri dish and returned to MSO medium. Embryos not dessicated were maintained on MSO medium. Culture conditions were as described for solid cultures. Upon conversion (i.e., the development of both a shoot and a root) the plantlets were transferred to Magenta GA7 boxes (Magenta Corp., Chicago, IL) containing MSO medium for further growth. Once the root system was well established, the plants were transferred to Magenta boxes containing 5 cm pots filled with a sterilized mixture (1:1) of sand and Hyponex (Hyponex Corp., Marysville, OH) potting mix, and gradually acclimated to a lower humidity environment by progressively opening the lid of the container. Upon complete acclimatization, plants were transferred to the greenhouse and allowed to set seed.

Measurement of growth in liquid medium. Embryo growth rate in the liquid medium was evaluated by measuring the change in volume of the EM during a series of three biweekly subcultures. The volume of the EM was measured by recording liquid displacement in a sterile, 10 ml graduated cylinder. The study was initiated using cultures that had been continually subcultured in liquid medium for 8 to 10 months. Three flasks were used to initiate each experiment and the experiment was repeated five times. Growth data were analyzed using SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA).

Results and Discussion

Immature peanut cotyledons exposed to 40 mg/l 2,4-D produced an average of two somatic embryos per cotyledon (data not shown) which were used to initiate repetitively embryogenic cultures (Fig. 1). When placed in liquid medium, approximately 90% of the somatic embryos enlarged and produced secondary embryos from the primary embryo. These secondary embryos appeared to be arrested in the early torpedo stage of development and gave rise to additional rounds of embryogenesis until EM were formed, approximately two months after liquid culture initiation (Fig. 2). After continous culture for eight months or longer, embryo development in 30-40% of the peanut cultures appeared to be arrested at earlier stages of embryo development between the late globular or early heart stage (not shown). This phenomenon is similar to that reported for soybean embryogenic cultures (Finer and Nagasawa, 1988), except the embryos comprising the EM of soybean cultures were arrested at the globular stage of development. Although several modifications of this medium were evaluated (5A40N, having half the ammonium of 10A40N; 10A40N with 2X 2,4-D, having 10 mg/l rather than 5 mg/l 2,4-D; and 10A40N with 0.5 mg/l BA), none were superior to the original medium. The successful use of the 10A40N medium for repetitive somatic embryogenesis in both peanut and soybean suggests that it may also be suitable for studies involving other large-seeded legumes.

At each subculture, the EM were divided into clumps approximately 5 mm in diameter, and consisting of approximately 8-10 embryos. When left undivided for three subcultures or more, approximately 25% of the embryos within the masses germinated precociously, forming roots but no shoots. However, growth was uniform when the clumps remained 5-10 mm in diameter. Callus tissue was not observed, but the liquid medium became turbid over time, possibly due to the sloughing off of external cells from the EM. Liquid cultures have now been maintained for over one year without apparent loss of embryogenic capacity.

Embryo germination and conversion to plants were achieved by transferring EM to MSO medium, which permitted the growth, development, germination, and conversion of the somatic embryos. While embryos could be converted when maintained on MSO medium, the conversion frequency was low (approximately 4%). Other investigations have indicated that a desiccation period following somatic embryo development enhanced germination and conversion (Gray, 1989; Parrott et al. 1988). Inclusion of a desiccation period after embryo maturation resulted in a four-fold increase (from 3.8% to 15.3%) in the percentage of embryos which converted into plants. The 10 day desiccation period occurred approximately two months after transfer from liquid, germination occurred within one month of dessication, and shoot development occurred sporadically thereafter. Plantlets were obtained as early as four months after removal from liquid culture. Converted plants have been established in soil (Fig. 3) and are now being evaluated in the greenhouse. The plants appear to be phenotypically normal, and have flowered and set seed.

The growth potential of the peanut cultures was measured in cultures 8-10 months old and is depicted in Fig. 4. The growth was exponential, more than doubling in volume with every two-week subculture period. The growth curve was nearly an exact fit to a third-order regression ($R=1.00$) and closely fit a second-order regression ($R=0.99$, not shown). Since the morphology of the EM did not change as long as the masses were

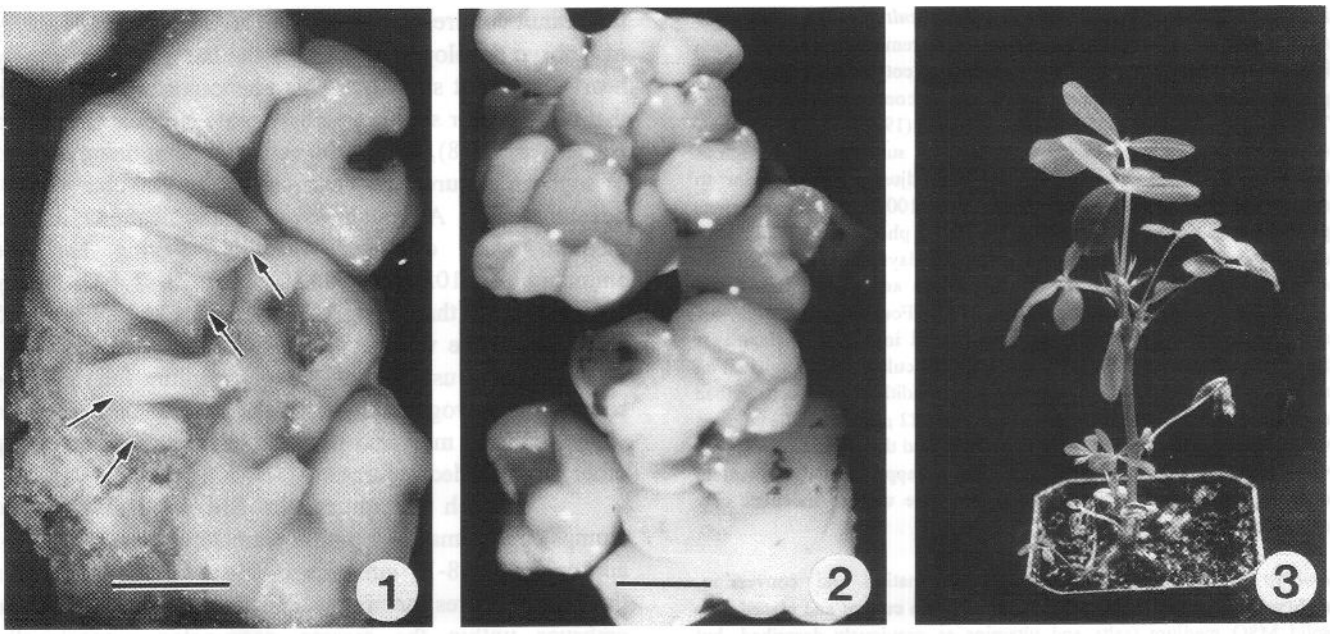

Fig.1. Primary somatic embryos forming on immature peanut cotyledons following 20 days of culture on medium with 40 mg/l 2,4-D. The embryos designated by arrows are of the type used to initiate repetitive embryogenic cultures in liquid medium. Actual embryos used to initiate liquid cultures had been growing on the medium for 30 days. Bar=5mm.

Fig.2. Clumps of peanut repetitive embryos harvested from liquid culture maintained for three months. Bar=5mm.

Fig.3. Peanut plant in 60 mm pot, regenerated from embryogenic suspension culture and established in soil.

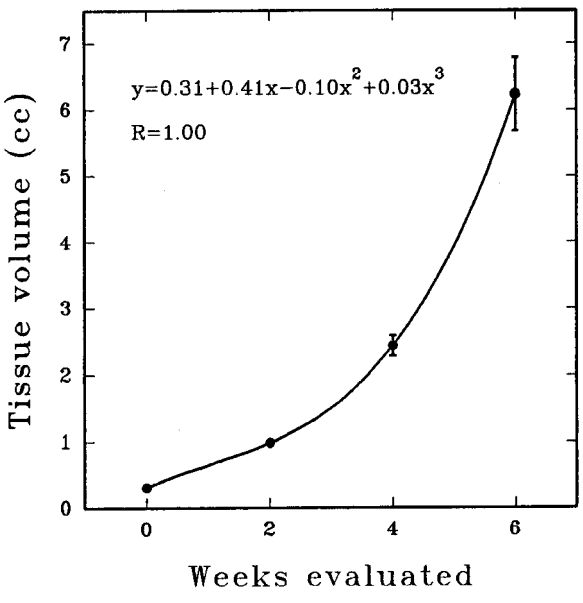

Fig.4. Growth of 8-10 month old repetitively embryogenic peanut cultures over a six week period. Vertical lines represent the standard error.

divided manually, the increase in volume of these masses directly correlates to an overall increase in embryo number.

Conclusion

The establishment of repetitively embryogenic cultures in liquid medium extends the information previously available on peanut somatic embryogenesis. It is now possible to regenerate plants on a large scale, while avoiding problems associated with the continuous initiation of cultures from fresh explants. In addition, repetitively embryogenic cultures have been especially useful for the transformation of cotton (Finer and McMullen, 1990), soybean (McMullen and Finer, 1991), maize (Gordon-Kamm et al., 1990), and citrus (Hidaka et al., 1990), and may similarly be useful in peanut. The availability of repetitively embryogenic cultures allows for sorting out of transformed versus non-transformed tissue in a selective medium following transformation and thus allows for the recovery of stably transgenic plants (McGranahan et al., 1988; Parrott et al., 1991b). After transformation, the nonchimeric somatic embryos obtained should be easily produced in quantity via repetitive somatic embryogenesis of the primary transformant.

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