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INDIRECT SOMATIC EMBRYOGENESIS AND PLANTLET REGENERATION IN GROUNDNUT (*ARACHISHYPOGAEAL.*) THROUGH CELL CULTURE

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Abstu act : High frequency somatic embryos were produced from hypocotyl cell suspension cultures of *Arachihypogaea* L. (cvs. VR1-2 and TMV-7). Callus cultures were established on MS medium augmented with B, vitamins, 2,4 D, NAA and KIN They were subsequently transferred to MS liquid medium with same salts. vitamins hut with different growth regulator combination and further subcultured at two weeks intervals into fresh medium tor embryo development Upon transfer to MS liquid medium supplemented with growth regulators, more number of somatic embryos developed from the embryogenic cells. Different stages of embryos with varying frequencies were observed tn cell suspension cultures. The embryos were counted at each stage ot development after one month of culture. The 2,4-D addition was proved to he necessary to induce embryogenesis and embryo maturation. Matured embryos were subsequently germinated and produced plantlets. Regenerate were successfully transplanted to potting soil and grown lo maturity ill the field.

Key words : *Arachus hypogaea*, cell culture technology, embryos, plantlets, somatic embryogenesis, suspension culture.

INTRODUCTION

A basic pre-requisite for the application of cell and tissue culture technology to crop improvement is the ability to regenerate plants from callus or cell suspension cultures. Recently, somatic embryogenesis has attracted attention in plant biotechnology, because it can provide useful systems lo produce transgenic plants as well as to obtain materials for synseed technology. However, the majority of legumes still remain recalcitrant to various cultural treatments (Eapen and George, 1990). High numbers of regenerants can be obtained originating from a few or single cells which increase the likelihood of achieving transformed plants. However, the efficient conversion of somatic embryos into plants in groundnut remains a problem (Chengalrayan *et al.*, 1994).

The development of somatic embryos from sporophytic cells has been reported in a larger number of plant species. Somatic embryos should have bipolar structure with a simultaneous development of the shoot and root meristems and no vascular connection with mother tissue (Williams and Maheswaran, 1986). To be of practical value. somatic embryogenesis should culminate in the formation of plantlets. Organogenesis in groundnut, has been achieved by several authors (see Venkatachalam *er al.*, 1994; 1996). Recently, there has been a great deal of interest in *in vitro* regeneration of peanut (Baker and Wet/stein, 1995; Durham and Parrott, 1992; Ozias-Akins, 1989) via somatic embryogenesis. Since immature zygotic embryos and immature cotyledons are not always readily available, attempts have been made to regenerate plants from other explains including leaflets (Baker and Wet/stein, 1992; 1995; Chengalrayan *et al.*, 1994).

Unfortunately, there are certain severe limitations with somatic embryogenic systems using immature zygotic embryos and immature cotyledons as explants. These include the need to maintain excessive greenhouse planting and the difficulty in obtaining pla' . material at a correct developmental stage and potential seasonal variations. Isolation of sterile cultures from immature embryos of groundnut also possess a problem due to a high contamination

Table 1. Frequency of somatic embryogenesis in VR1-2 and TMV-7 groundnut cultivars grown in suspension at the end of 30 day. Embryogenesis was induced with 2,4-D (5.0 mg l^{-1}) and BAP (1.0 mg l^{-1})

Cultivars	Stages of embryos (percentage mean ±SE)			
	Globular	Heart-1	Torpedo	Dicotyledonary
VRI-2	22.5 1.71	34.5 ± 2.16	42.5±3.05	18.3±1.47
TMV-7	30.1±2.08	25.3±1.89	31.4±2.61	14.2±1.26

Each observation was calculated as a per cent of somatic embryos formed per culture (flask) and each was the average of 10-15 cultures

Table 2. Effect of various concentrations of 2,4-D and in combination with 1,0 mg I of BAP on somatic embryo induction

Hormone concentration (mg l^{-1}) 2,4-D	Frequency of somatic embryos (mean ±SD)	
	VRI-2	TMV-7
1,0	8.6±0.95c	5.4 0.81d
2.0	20,3 2, 14d	17.3±2.23c
3.0	36.2±2.81c	31.5±3.54b
4.0	49.5±3.74b	42.9±3.92a
5.0	62.5±4.25a	5i.4±4.10a

Each value was the average of 5-10 replicates. Moan separation using Duncan's New Multiple Range Test, means with different letters are significant at I per cent level

rate because of its underground fruiting habit, An alternative approach would be in developing an embryogenic system using other aseptic seedling explants. There are, however, no previous reports on induction and development of somatic embryos in cell suspension cultures in this oil seed crop (Venkatachalam, 19%).

In this report, we describe a cell suspension culture system for induction of somatic embryos from hypocotyl explants. A distinct advantage of this system over previous somatic embryogenesis systems using immature and zygotic embryos and cotyledon explants is an assured year round availability of tissue. Problems of obtaining embryos and cotyledons at specific developmental stages are eliminated. Aseptic cultures without contamination are readily obtained using our methods described hereunder.

MATERIALS AND METHODS

Two cultivars of *Arachis hypogaea* L. VRI-2 and TMV-7 (obtained from Tamil Nadu Agricultural University, Coimbatore, India) were used in this study. The seeds were surface sterilized by immersion for 7 min in 0.1 % (W/V) mercuric chloride solution, rinsed several times in sterile distilled water and placed aseptically on the basal medium (MS) according to Murashige and Skoog (1962) with B_5 vitamins (Gamborg *et al.*, 1968), 3% (W/V) sucrose, 0.8% (W/V) agar. pH adjusted 5.8 with 0.1 *N* NaOH before being autoclaved at 121°C for 15 min. Seedlings were grown at 1500 lux at a photoperiod of 16 h/day and 24±2°C.

Hypocotyl explants were dissected out from 7-day-old seedlings and were incubated in 25 x 150 mm tubes containing 15 ml of the solid MS medium containing B_5 vitamins, 2,4-D (2.0 mg l^{-1}), NAA (2.0 mg l^{-1}), and KIN (0.5 mg l^{-1}) for callus initiation. The physical culture conditions were not modified. To start the suspension cultures, pieces of calli with an average weight of 500 mg, were transferred into 100 ml Erlenmeycr flasks containing 25 ml of MS liquid medium supplemented with different concentrations and combinations of 2,4-D (0-5.0 mg l^{-1}) and BAP (1,0 mg l^{-1}). The suspension cultures were maintained at 12 h photoperiod at 500 lux, 24±2°C on a rotatory shaker (100 rpm). Subcultures were carried out two weeks intervals by removal of

spent medium and adding 20 ml of the same fresh medium. After a mouth, the number of somatic embryos per culture was determined.. Samples of suspension cultures were taken at random at the end of 15 days of incubation in liquid medium and the number of somatic embryos were counted under a microscope. Counts were made from 10-15 different independent cultures and the percentage of somatic embryos calculated was based on the total number of embryos present in each culture. For the development of somatic embryos into plants, they were plated on *MS* solid medium supplemented with NAA (0.5 mg 1-1), BAP (1.0 mg 1-1} and GA3 (0.5 mg l-1). The regenerated plants were transferred to plastic cup containing red soil and sand in the ratio 1:1 and maintained in the controlled environment. After two weeks, they were shifted to field, grown well, flowered normally and set viable seeds.

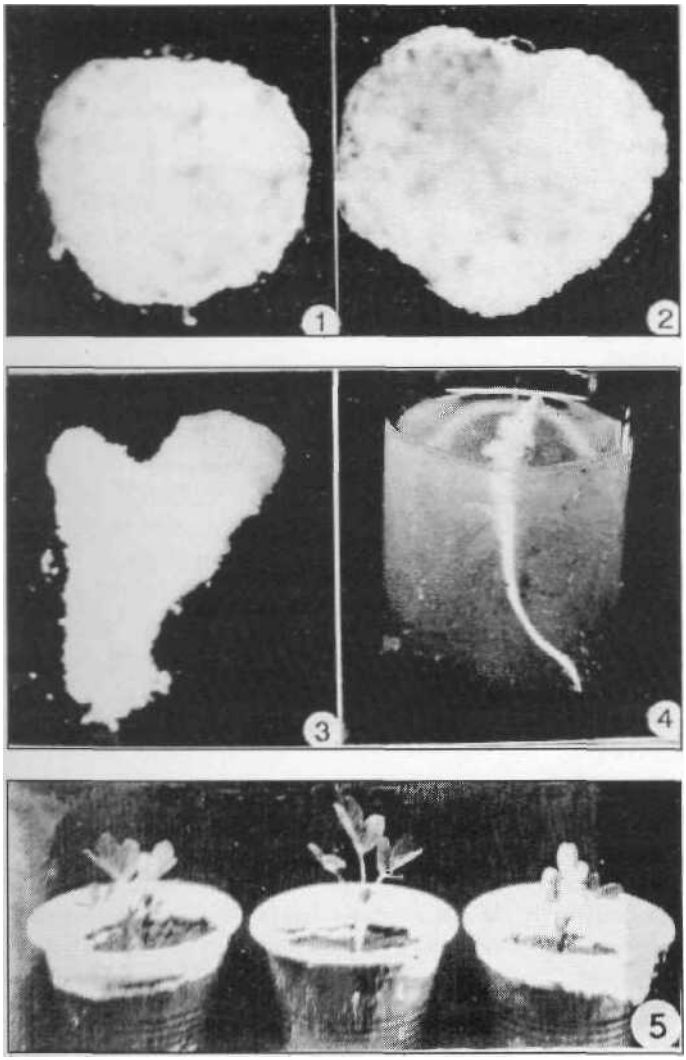

Plate I. Morphology of embryos in suspension culture, after the second subculture (1. Globular-shaped embryo 2. Heart-shaped embryo 3. Dicotyledonary-staged embryo 4. A small plantlel development from somatic embryo 5. Plantlcts raised via somatic embryogenesis growing in the plastic cups)

RESULTS AND DISCUSSION

Callus induction from hypocotyl explants

The present investigations have shown that it is possible to develop somatic embryogenesis in A. hypogaea from hypocotyl derived cell suspension cultures. Callus was initiated from hypocotyl explants within 10 days of culture. Vigorous callus proliferation was observed within 3 weeks. The calli obtained on MS medium were friable and pale green in colour. The high frequency of callus induction was observed on MS medium supplemented with 2,4-D (2.0 mg 1-1), NAA (2.0 mg l-1) and KIN (0.5 mg 1-1).

Initiation of cell suspension cultures and embryo development

Friable and fast growing calli obtained on the MS solid medium were transferred to MS liquid medium with an identical hormone composition. The dispersion of these calli produces a suspension composed of free cells, small clusters of 15-20 cells, and

irregular aggregates. Repeated subcultures on the same medium increased the cell and the cluster densities, hut the differentiation of embryos was not observed. After 6 weeks in the medium containing 2,4-D with or without other growth regulators, yielded the globular embryos (Plate 1) either isolated or in cluster. They can be easily identified from undifferentiated aggregates as judged by their texture and their colour. The developmental changes at more advanced stages, as heart- and torpedo-embryos were observed after second subculture. Further divisions in the cotyledonary regions produced embryos with two distinct cotyledons. After the fourth transfer to a fresh medium, the number of embryos in each stages is described in Table 1. The highest frequency of embryos was observed in VRI-2 (42.5%) than TMV-7 (31.4%) cultivar.

The next remarkable feature was the transfer of the embryogenic rissue to a liquid medium, a condition allowing to enhance the contact between the tissue and the medium. The embryo initiation does not occur in any specific subculture. For instance, induction can take place at any time, and different development stages appeared simultaneously. The suspension culture remained embryogenic after four transfers. Similar observations were reported in *Vigna* suspension cultures (Eapen and George (1990) and in sunflower (Prado and Berville, 1990), However, Kageyama *el al.* (1991) suggested that seven days seemed to be the optimum period in the initial medium for the development of somatic embryo with normal morphogenesis.

Embryo maturation

There were considerable differences in the morphology of embryos developing under the light conditions. Somatic embryos grown under a 12 h (light intensity 500 lux) photoperiod were greenish and bipolar structure in liquid culture. The factors involved in maturation of somatic embryos were also related to the liquid condition of the culture and the hormonal composition of the media. The transfer of embryos from the liquid media to the solid media with the same hormonal composition stopped the maturation process of the embryos at the stage achieved before. The embryos incubated on the solid media increased in size during the first week (Plate 1), but no further external morphological change was observed. Light has been reported to have both promoting and inhibiting effects on somatic embryogenesis under both 0 h photoperiod (Baker and Wetzstein, 1992) and a 16 h photoperiod (Baker *el. al.*, 1994, Sellars *et al.*, 1990). In our study, the effect of photoperiod was compared under different (12 h photoperiod) culture conditions. Under our growth conditions, 12 h photoperiod (light intensity 500 lux) was found to be the optimum cultural environment to obtain high frequency of embryos. Lazzeri *et al.* (1987) reported that the light intensity also influenced the soybean somatic embryogenesis.

Embryo germination and plantlet development

In order to obtain germination of the somatic embryos and to prevent them from turning into callus, they were transferred to germination medium. After a week of culture on germination medium, somatic embryos started to develop into plantlets. The number of germinating somatic embryos and plantlet development were found to be best on medium containing 0.5 mg l^{-1} NAA, 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} GA_3 (plate 1). Most of the somatic embryos developed into plantlets so rapidly that they could be transferred to MS basal medium after two weeks. Regenerated plantlets were transferred to plastic cups containing soil. Although green plantlets could be transplanted to field directly, the survival rate could be increased by culturing in controlled temperature for a week before transplanting into the field.

Our results show different genotype abilities to initiate calli and producing shoots and roots, regardless of the medium composition. The genotype also affects the regeneration capacity in the liquid media. The advance in the development process was different according to the tested genotypes, considering four embryo stages: globular, heart, torpedo and dicotyledonary stage. Witrzens *et al.* (1988) have found that the response to the culture conditions in sunflower from somatic explants is influenced by the genotype. Espinasse and Lay (1989) and Prado and Bervile (1990) established callus formation from sunflower embryos in early stages and shoot regeneration was rarely observed from these embryos (depending upon the embryo genotype). Eapen and George (1990) obtained plantlets at a tow frequency from somatic embryos of two *Vigna* genotypes and one genotype did not respond, although recallused at a later stage. Eapen and George (1993) and Ozias-Akins (1989) observed significant differences among peanut genotypes for somatic embryo formation, subculture and plant regeneration. Nevertheless, in the data presented by Dunbar and Pittman (1992) concerning organogenesis from leaf explants, 13 *Arachis* species showed no significant differences.

Effect of growth regulators

When transferred from the solid MS medium to the liquid medium, the calli derived from both the genotypes were able to produce embryos until the cotyledonary stage. 2,4-D in combination with 1,0 mg l^{-1} BAP was found to be the most favourable for induction of embryos. But the high frequency of somatic embryos was observed on MS medium supplemented with 5.0 mg l^{-1} 2,4-D; 1.0 mg l^{-1} BAP in VRI-2 (62.5%) than TMV-7 (51.4%) cultivar (Table 2). It is generally recognized that the embryogenic induction by 2,4-D is common effect of this growth regulator. 2,4-D has proved to be useful to obtain a somatic embryogenic response in a number of species. In contrast, this regulator inhibits embryo development in *Corylus avellana* L. (Perez *et al.*, 1986) and some genotypes of *Dacus carota* (Borkid *et al.*, 1986). This growth regulator was successfully used to induce embryo formation from peanut on solid medium (Chengalrayan *et al.*, 1994, Eapen and George, 1993, Ozias-Akins, 1989; Venkatachalam, 1997). In earlier studies, direct somatic embryogenesis was obtained following culture of various explants (except hypocotyl) of peanut on an induction medium containing both 2,4-D and BAP or KIN. In this study, it is clear that the addition of 2,4-D is necessary not only for the induction of embryos but also for their maturation. We have obtained more advanced stages for groundnut embryogenesis than observed earlier, in liquid culture with 5.0 me l^{-1} 2 4-D But, on other hand, the advance in the maturation process does not inhibit the induction of new embryos. In contrast, the continuous initiation of secondary somatic embryos from the primary embryos at different stages of development, is promoted by 2,4-D with BAP. This secondary embryogenesis is often associated with poor growth or suppression of the main embryogenic axis (Williams and Maheswaran, 1986).

In cell suspension culture conditions, advanced stages such as torpedo- and dicotyledonary stage were achieved. The undifferentiated tissue in proportion with the total mass tissue was reduced in this medium. Analysing the effect of each one of growth regulator on embryo maturation advancement, it is observed that GA_3 combined with 2,4-D and BAP, has a highly significant effect on both the genotypes of A. *hypogaea* L. However, the transfer of the cell suspensions to the MS solid medium where 2,4-D was the only growth regulator added, always resulted in necrosis during the first subculture. When BAP was present in the culture medium accompanying 2,4-D, the proportion of undifferentiated tissue

was reduced significantly in both the genotypes. In liquid media, BAP in the presence of the 2,4-D, always improved the embryo development. Eapen and George (1990) mentioned that BAP and GA_3 enhance the frequency of embryos in liquid culture in *Vigna* sp. In contrast, the incorporation of ABA, GA_3, z, BAP and KIN in the embryo induction medium (solid) was ineffective in improving the frequency of somatic embryo formation (Chengalrayan *et al.*, 1994). The absence of growth regulators caused necrosis of transferred tissues.

In conclusion, the cell culture technology in the present study may, improve somatic embryo development with normal morphogenesis in groundnut. is considered that the cell culture technology may he more efficient in cell selection for disease resistance, salt and drought tolerance and synchronization than the solid culture system.

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