MICROPROPAGATION AND PROTOPLAST CULTURE OF PEANUT (*ARACHIS HYPOGAEA* L)

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ABSTRACT

Multiple shoot bud formation was obtained in six varieties of *Arachis hypogaea* by using conditioned cotyledonary nodal segments. Germination of seeds in the presence of a cytokinin and isolating and culturing the cotyledonary segments from such conditioned seedlings on a medium enriched with BAP stimulated shoot bud formation. *In vitro* flower development was also observed in some instances. Complete plants were produced by rooting the differentiated shoot buds. Protoplasts isolated from shoot cultures underwent divisions and formed colonies and callus. However no regeneration occurred in the callus derived from protoplasts.

INTRODUCTION

INVESTIGATIONS on tissue culture of legumes have been on the increase in the recent past and successful plant regeneration has been reported in many genera[1]. The present communication deals with peanut (*Arachis hypogaea*) which is an important leguminous crop and a major source of food and oil in the semiarid tropics. This study describes the successful plant regeneration obtained from cotyledonary node segments as well as on isolation and culture of protoplasts from shoot cultures in six cultivated varieties of peanut.

MATERIALS AND METHODS

Six cultivars of *Arachis hypogaea* [TG-3, TG-9, TG-14, TG-17, TG-21 and TG-SI] were used in the present investigations. The seed material was obtained from Mutation Breeding Section, Biology and Agriculture Division, BARC, Bombay. Seeds were removed from the pods and submerged in ethanol for 1 minute and sterilized with 0.1% mercuric chloride solution for 5 min. Subsequently the seeds were washed thrice with autoclaved distilled water and germinated on a basal medium (BM) alone as well as on BM supplemented with phytohormones. Hypocotyl, epicotyl, axillary buds and cotyledons derived from 10-day-old seedlings served as explants. BM was comprised of inorganic salts of Murashige and Skoog[2], B-5 vitamins[3], and 3% sucrose with 0.8% agar (bacteriological grade, Sisco Laboratories). It was variously supplemented with IAA (indole-3-acetic acid), IBA (indole butyric acid), NAA (α-naphthalene acetic acid), NOA (β-naphthoxy acetic acid), BAP (benzyladenine), KIN (kinetin), 2, i-*p*(2, isopentyladenine) and ZEA (zeatin). The pH of the medium was adjusted to 5.8 before autoclaving. Each experiment consisted of 24 replicates and was repeated thrice. The cultures were incubated under continuous fluorescent light (1000 lux) at 25 + 2°C, with a relative humidity of 50–60%.

Mesophyll protoplasts were isolated from 30-day-old shoot cultures growing on MS[2] medium (figure 2A). The leaves were macerated in 0.3 M mannitol solution and kept for 10 min in this solution before incubating in enzyme mixture. Good yield of protoplasts was obtained after 4–5 hr, incubation in enzyme solution containing 2% cellulase R-10 'Onozuka', 1% macerozyme R-10 (Kinki Yakult) and 0.05% pectolyase Y23 (Seishim Pharmaceuticals, Tokyo) with 0.6 M mannitol as the osmotic stabilizer. After the incubation period, debris was removed by sieving the protoplast suspension through sieves (pore size 100 μm) followed by removal of enzyme solution by centrifuging at 40g for 5 min. The pellet was loaded on the top of 20.5% sucrose (Analar) solution and centrifuged for 10 min at 40 g. The floating protoplast suspension was pipetted out washed in 0.6 M mannitol solution and cultured in glass or plastic petridishes, 2 cm diameter containing 2 ml of several liquid culture media like B5[3], 8P[4] and V-47[5]. The density of the protoplasts was maintained at 10^5/ml medium.

RESULTS AND DISCUSSION

Seed germination was 100% in all the varieties except in TG17 where is was about 60%. Seeds germinated on cytokinin (BAP, KIN, ZEA, 2ip 1 mg/l) supplemented medium developed into seedlings which were more vigorous with a thick main root with numerous laterals and broader leaves in comparison to seedlings obtained from auxin-treated seeds. The seedlings obtained on auxin and cytokinin medium

formed the source material for organ culture experiments.

Explants of hypocotyl, epicotyl and axillary buds of all the varieties showed profuse callusing on MS supplemented with IAA or NAA (1 mg/l). Occasionally rooting was observed in the calli. Organization of roots in callus tissues (derived from fruit pericarp) of *Arachis hypogaea* has been reported by Rangaswamy *et al*[6]. Although the callus tissues obtained from various organs were grown on MS fortified with several combinations of auxins and cytokinins, none of them induced shoot differentiation. This in contrast to the observations of Mroginski *et al*[7] who reported callus development and shoot regeneration in young leaf explants of *Arachis hypogaea*.

Significant results were obtained in cultures of cotyledons having nodal segments. Multiple shoot buds differentiated from nodal parts of cotyledonary segments cultured on MS supplemented with cytokinins such as ZEA, BAP, KIN and 2, i-p. Among these BAP was most effective (figure 1A). The degree of shoot bud differentiation varied with different concentrations of BAP and with the different varieties. On an average about 10–12 shoot buds were differentiated from one cotyledonary node. In 5 out of 6 varieties, MS + BAP (1 mg/l) proved optimum for multiple shoot bud formation. These observations are summarised in table 1. Cotyledon explants devoid of nodes resulted in callus formation (figure 1B). In the present instance some of the buds differentiated on MS + BAP (1 mg/l) directly produced flowers *in vitro* (figure 1F), which is contrary to the observation made by Narasimhulu and Reddy[9], where they obtained flower bud formation unaccompanied by vegetative growth in deembryonated cotyledons of *Arachis* on Blaydes medium containing 0.5 mg/l BAP. They also observed the positive influence of IAA and NAA in combination with cytokinins on flowering of *Arachis*.

The regenerated shoots were isolated and transferred to a variety of root-inducing media. On MS medium (at half strength) supplemented with IAA (1 mg/l) 90–100% of shoot buds developed roots within 3 weeks (figure 1C, E). The roots were green, long and slender. Thus complete plants were obtained (figure 1D). The *in vitro* developed plantlets were successfully established in paper cups (figure 1G). No morphological abnormalities were observed in the regenerated plants.

The yield of protoplasts was more or less uniform in all tested varieties. An optimum number of protoplasts were released by the enzyme combination of cellulase 2% + macerozyme 1% + pectolyase 0.05%.

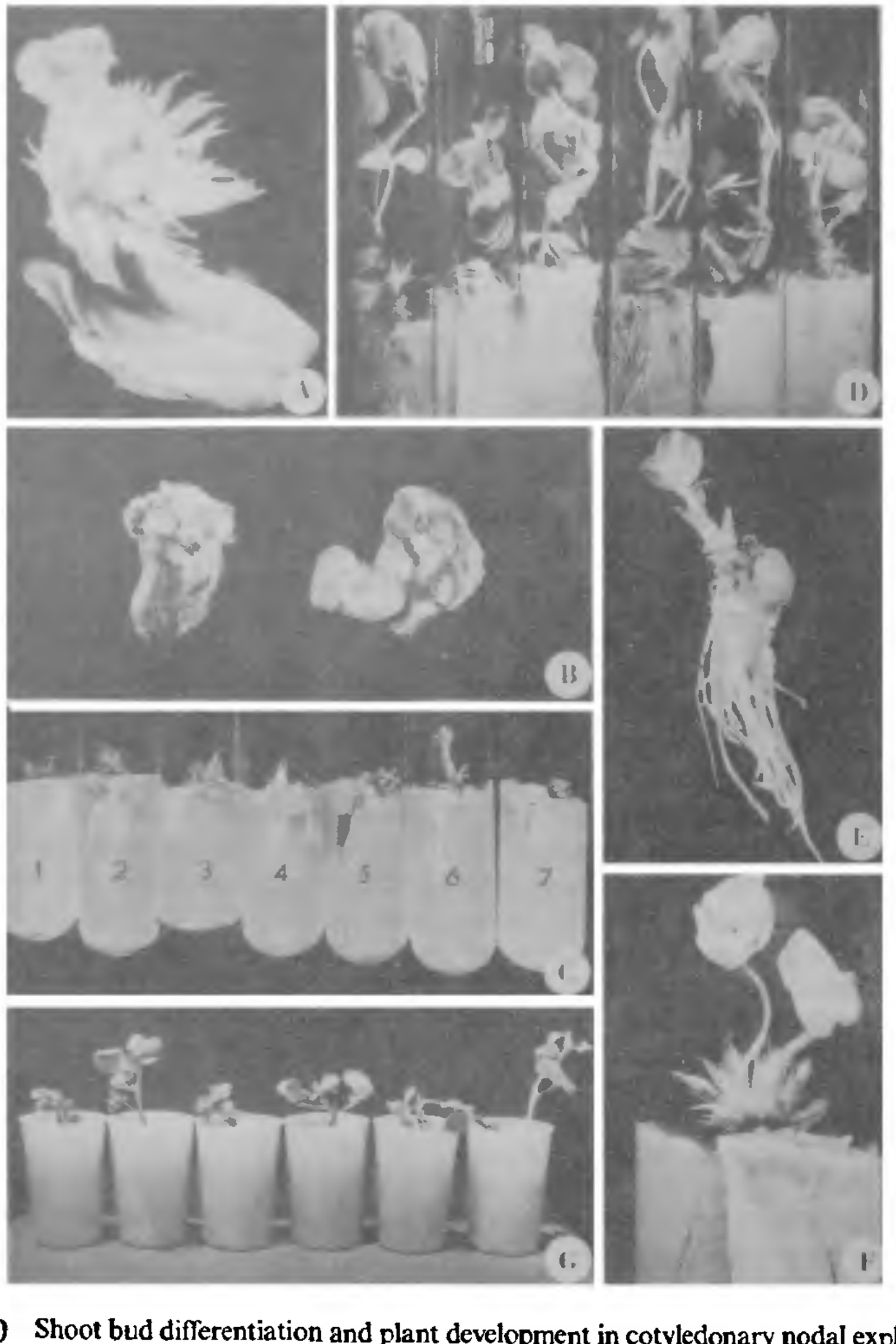
Table 1 *The effect of various concentrations of BAP (mg/l) on shoot bud differentiation in cotyledonary node cultures of six varieties of Arachis hypogaea.*

Variety	0.1	0.5	1	2
TG-3	a	e	e	e
TG-9	a	d	e	c
TG-14	b	c	e	c
TG-17	a	c	e	e
TG-21	b	c	e	e
TG-SI	b	c	d	e

Data scored at the end of 6 weeks, 24 replicates per treatment. a: nil, b: 10–20%, c: 20–40%, d 50%, e 60% or above.

The cultured protoplasts underwent division within a week. Repeated divisions (figure 2B) followed and macroscopic colonies were formed which developed into callus masses (figure 2C). Among the tested media B-5[3] was as good as or superior to more complex media such as 8p[4] and V-47[5] in supporting cell divisions and colony formation. A combination of 2, 4-D, NAA and BAP was found suitable for optimum growth of protoplasts in different varieties as represented in table 2. It was noticed that fast growing colonies often produced phenolic substances which led to browning of the cells. Keeping filter paper in the medium improved the growth of colonies. The protoplast derived callus was yellowish green and compact. No regeneration was obtained when protoplast callus was subjected to different hormonal treatments.

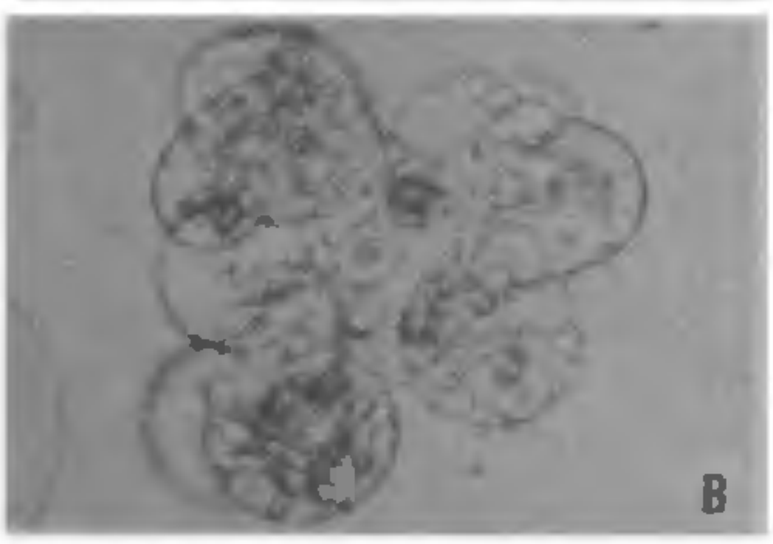
Our observations clearly indicate that induction of multiple shoot buds in *Arachis hypogaea* can be accomplished relatively easily by using cotyledonary node explants isolated from aseptic seedlings. Regeneration of plantlets from cultures of *Arachis* has been reviewed by Sastri *et al*[10]. Atreya *et al*[8] reported shoot bud induction on MS + BAP (2 mg/l) whereas Narasimhulu and Reddy[9] obtained shoots on Blaydes medium containing KIN (3 mg/l) or BAP (4 mg/l). The cotyledonary nodal region of *Arachis* is known to contain several meristematic zones which remain dormant and addition of cytokinin in the germinating medium and subsequent culture of cotyledons from such conditioned seedlings on a fresh medium of the same composition stimulates the meristematic zones to form shoot buds. Thus the successful establishment of *in vitro* technique for plant regeneration from cotyledonary node is significant and can be applied for the rapid propagation of new strains of peanut. In soybean, Cheng *et al*[11] reported that shoot bud regeneration in explants from seedlings already activated by cytokinins was further augmented by

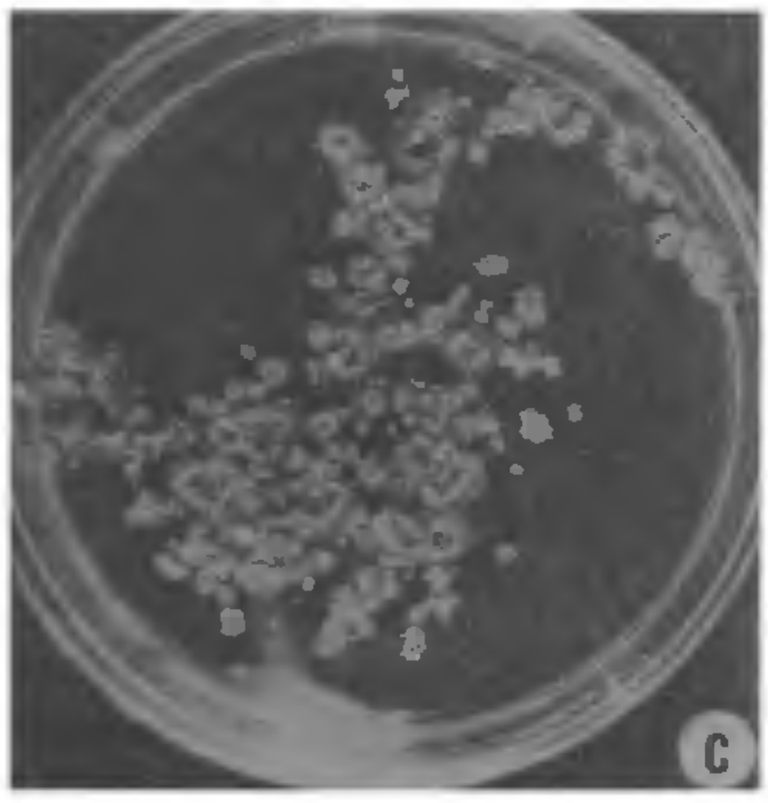


Figures 1(A–G) Shoot bud differentiation and plant development in cotyledonary nodal explants of *Arachis hypogaea*. **A.** Multiple shoot bud formation from cotyledonary node explant on BM + BAP (1 mg/l). **B.** Callus formation in cotyledonary explant devoid of node on MS + BAP (1 mg/l).**C.** Isolated shoot buds on MS with different auxins (1 mg/l). 1, MS; 2, IAA; 3, IBA; 4, NAA; 5, NOA; 6, IAA + IBA; 7, NAA + NOA. **D.** Plantlets with roots [left to right: TG3, TG9, TG14, TG17, TG21, TG SI; medium: $\frac{1}{2}$ MS + IAA (1 mg/l)]. **E.** A developing plantlet with profuse roots [(medium: $\frac{1}{2}$ MS + IAA (1 mg/l)]. **F.** Flower formation directly from shoot bud. **G.** Regenerated plantlets established in soil in paper cups (Left to right: TG3, TG9, TG14, TG17, TG21, TG SI).

growing them on a cytokinin-enriched medium. Regeneration of plantlets from the cotyledons of *Cajanus cajan* has also been reported[12].

The differences in the morphogenetic responses of various varieties to growth substances observed in the present study may be attributed to their genotypes since genetic effects have been reported to contribute to differences observed in tissue cultures of legumes





Figures 2(A-C) Protoplast culture of *Arachis hypogaea*. **A.** Shoot culture of *Arachis* used for the isolation of mesophyll protoplasts. **B.** A multicellular colony derived from protoplasts. **C.** Protoplast-derived calli masses.

such as *Phaseolus*[13], *Medicago*[14] and *Trifolium*[15].

Similarly as far as protoplast culture is concerned the genotype and culture media were the major factors influencing the rate of protoplast division and callus formation. In an earlier report[16] on *Arachis* protoplast

Table 2 *Protoplast isolation, division and callus formation in six varieties of Arachis hypogaea**

Variety	Protoplast isolation	Protoplast division rate	Colony formation	Callus formation
TG-3	c	a	a	a
TG-9	d	c	b	b
TG-14	d	c	c	c
TG-17	b	a	a	a
TG-21	d	c	b	b
TG-SI	d	c	c	c

a: nil, b: low, c: moderate, d good.

only one local variety had been investigated whereas in the present work protoplast isolation and culture has been extended to six more varieties. The failure to get plant regeneration in protoplast callus from six varieties of peanut (present work) as well as previous report on *Arachis* protoplast[16] points out the incapability of peanut callus to undergo differentiation. However, it would be interesting to isolate protoplasts from cotyledons since cotyledons have been successfully used to isolate protoplasts and obtain plants in *Crotalaria juncea*[17].

10 June 1985

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ANNOUNCEMENT

51ST ANNUAL MEETING OF INDIAN ACADEMY OF SCIENCES, BANGALORE 7–10 NOVEMBER 1985 AT MADURAI

The 51st Annual Meeting of the Indian Academy of Sciences will be held at Madurai from 7–10 November 1985. The programme is as follows.

7 November 1985 (Thursday) – Inaugural function including Presidential address.

8 November 1985 (Friday) – Symposium on 'The Genome in Flux'. Convener K. Dharmalingam, Madurai Kamaraj University, Madurai – Introduction. (1) H. Sharat Chandra, Indian Institute of Science, Bangalore – '*Some genetic and evolutionary problems posed by sexuality*' (2) M. S. Kanungo, Banaras Hindu University, Varanasi – '*Change in conformation and expression in the genome of a mammal during its life span*'. (3) D. S. Pradhan, Bhabha Atomic Research Centre, Bombay – '*Mitochondrial DNA repair and replication during anaerobic–aerobic transition of S. cerevisiae*'. **Lecture presentations by Fellows and Young Associates:** (1) M. R. Das, Centre for Cellular and Molecular Biology, Hyderabad – '*Molecular biology of cancer*'. (2) P. Babu, Tata Institute of Fundamental Research, Bombay – '*Gene expression in the fruitfly bithorax as a model*'. (3) K. N. Ganesh, Centre for Cellular and Molecular Biology, Hyderabad – '*Gene fragments: Synthesis, chemistry and biological importance*'. (4) T. J. Pandian, Madurai Kamaraj University, Madurai – '*Food nitrogen as a predictor of digestive efficiency*'. (5) A. S. Kolaskar, Centre for Cellular and Molecular Biology, Hyderabad – '*Nucleic acids sequence analysis—Structural and biological implications*'. **Evening lecture** by R. Cowsik, Tata Institute of Fundamental Research, Bombay – '*Architecture of the universe*'

9 November 1985 (Saturday) – Symposium on 'Fifth Generation Computers' Convener: Prof. V. Rajaraman. Speakers (1) V. Rajaram, Indian Institute of Science, Bangalore – *Introduction.* (2) H. N. Mahabala, Indian Institute of Technology, Madras – '*Nature of computer applications in the fifth generation*'. (3) P. V. S. Rao, Tata Institute of Fundamental Research, Bombay – '*Speech and visual input/output to computers in the fifth generation*'. (4) K. V. Ramanathan, Tata Institute of Fundamental Research, Bombay – '*Integrated circuits – past, present and future*'. **Special lecture** by G. Neuweiler, University of Munich, F.R.G. – '*Echolocation in South Indian bats*'. **Lecture presentation by Fellows and Young Associates** (1) K. S. Yajnik, National Aeronautical Laboratory, Bangalore – '*Control of turbulence*'. (2) T. N. Shorey, Tata Institute of Fundamental Research, Bombay – '*Recursive sequences*'. (3) N. Mohan Kumar, Tata Institute of Fundamental Research, Bombay – '*Curves in space*'. (4) B. R. Iyer, Raman Research Institute, Bangalore – '*Black holes are not for ever*'. (5) V. Balakrishnan, Indian Institute of Technology, Madras – '*Random walks*'. **Evening lecture** by I. Mahadevan, Madras – '*A computer study of the Indus script*'.

10 November 1985 (Sunday) – Lecture presentations by Fellows and Young Associates – (1) E. D. Jemmis, University of Hyderabad, Hyderabad – '*Theoretical study of an organometallic reaction*'. (2) M. G. Kulkarni, National Chemical Laboratory, Pune – '*Applications of super absorbent polymers in the separation process*'. (3) R. A. Rajadhyaksha, University of Bombay – '*Zeolite catalysts: promise and performance*'. (4) V. K. Gaur, National Geophysical Research Institute, Hyderabad – '*Earthquake prediction research*'. (5) P. Ramachandrarao, Banaras Hindu University, Varanasi – '*Synthesis of phase diagrams*'. (6) J. S. Singh, Banaras Hindu University, Varanasi – '*Certain emerging properties of central Himalayan forest ecosystems*'.