ANTHER CULTURE, SOMATIC EMBRYOGENESIS AND SYNTHETIC SEED PRODUCTION IN *ARACHIS* Spp.

Thesis submitted in part fulfilment of the requirements for the award of
the degree of Doctor of Philosophy in Biotechnology to the
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CERTIFICATE

This is to certify that the thesis entitled "**ANTHER CULTURE, SOMATIC EMBRYOGENESIS AND SYNTHETIC SEED PRODUCTION IN *ARACHIS* Spp.**" submitted in part fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY (Agriculture) IN BIOTECHNOLOGY**, to the Tamil Nadu Agricultural University, Coimbatore is a record of **bonafide** research work carried out by **Mr.G.NALLATHAMBI** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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Dedicated to my
Wife and Daughters

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ABSTRACT

ANTHER CULTURE, SOMATIC EMBRYOGENESIS AND SYNTHETIC SEED PRODUCTION IN *ARACHIS* Spp.

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The present investigation was carried out in *Arachis* to induce androgenic haploid and somatic embryos from different explants; encapsulation of somatic embryos; regeneration ability and storage potential of encapsulated embryos.

The pretreatment of flower buds at 4°C for 2 d with surface sterilization with 70% ethanol and 0.05% mercuric chloride for 10 min evoked maximum response to anther callusing with least contamination. Anther culture of *A. hypogaea* and *A. pusilla* showed that highest callus induction was achieved with the cultivars of *A. hypogaea* Auxin 2,4-D (4 mg/l) combined with CW (10 ml/l) induced higher callus than 2,4-D (4 mg/l) and pIC (4 mg/l) alone. These calli when transferred to MS medium containing BAP (0.5 mg/l) and NAA (2.0 mg/l) resulted in embryoid formation. Plants were regenerated only in *A. pusilla* and other cultivars did not respond for regeneration. Root tips of the regenerants from anther callus of *A. pusilla* were found to be haploids.

Relatively few plants were regenerated via somatic embryogenesis in cv. Salemkai and VG113 of *A. hypogaea* from immature leaflets culture. The initiation medium contained 2,4-D (20 mg/l) and sucrose (30 g/l) for induction of leaf calli. The subcultured (2,4-D, 3 mg/l) leaf calli in a medium containing BAP (0.5 mg/l) and NAA (0.5 mg/l) resulted in plant regeneration from somatic embryos. Rhizogenesis exhibited in cv. Salemkai in half strength MS medium containing BAP(0.5 mg/l) and NAA (0.5 mg/l).

In de-embryonated cotyledons, MS medium with 2,4-D (4 mg/l) alone and in combination with coconut water (10 ml/l) produced callus, root and shoot formation directly from the explants, while pIC (4 mg/l) developed only callus. Direct embryogenesis and organogenesis were obtained in cv.M13 and organogenesis alone was observed in cv. Salemkai. Indirect embryogenesis was also achieved from the 2,4-D induced callus after subculturing in a medium containing BAP (2mg/l), NAA (1mg/l) and sucrose (30 g/l). The frequency of embryos and plants regeneration was very less compared to mature embryo axis. *In vitro* flowering was achieved in cv. CO2 when 2,4-D induced calli subcultured in a medium containing 2,4-D (0.5 mg/l) and BAP (0.5 to 2.5 mg/l).

Induction of somatic embryogenesis and plant regeneration was achieved higher in mature embryonal axis than immature leaflets and de-embryonated cotyledons. Agar solidified MS medium was found to be superior than liquid medium for embryogenesis. Among the auxins, picloram was found to be superior for induction of somatic embryogenesis, while 2,4-D was also effective but with less frequency. NAA did not favour embryogenesis in mature embryonal axis.

Picloram (4mg/l) in MS medium was found to be optimum for induction of somatic embryogenesis. Somatic embryos developed into plants after subcultured calli transferred to a medium containing TDZ (0.2 mg/l), NAA (0.5 mg/l) and BAP (1.0 mg/l). TDZ was most effective for efficient conversion of somatic embryos into plants in combination with NAA and BAP. Proline (100 mg/l) enhanced somatic embryo formation in induction medium. Globular, heart shaped and cotyledonary stage embryos were obtained from mature embryonal axis of different cultivars. High conversion of plantlets was achieved in cv. M13 (36.4%), TMV12 (32.0%) and VG113 (29.8%).

Somatic embryos induced from mature embryonal axes were encapsulated in sodium alginate (3%) and calcium chloride (0.7%) using MS medium on gel matrix to produce uniform stable beads. Use of sodium alginate at higher concentration reduced the germination percentage. The encapsulated embryos could be stored at 4°C for 30 d without loss of viability. The encapsulated embryos exhibited 4 per cent germination after two months of storage at 4°C, while non-encapsulated somatic embryos failed to germinate.

CONTENTS

ABBREVIATION USED

2,4-D	-	2,4-dichlorophenoxyacetic acid
PIC	-	4-amino-3,5,6-trichloropicolinic acid (picloram)
NAA	-	α-naphthaleneacetic acid
2,4,5-T	-	2,4,5-trichlorophenoxyacetic acid
4-CPA	-	4-chlorophenoxyacetic acid
Dicamba	-	3,6-dichloro-o-anisic acid
IAA	-	Indole-3-acetic acid
MCPA	-	2-methyl-4-chlorophenoxyacetic acid
PcPA	-	p-chlorophenoxyacetic acid
IBA	-	Indole butyric acid
IPA	-	3-indolepropionic acid
2ip	-	6-γ-γ-dimethylallylaminopurine (isopentenyl adenine)
ABA	-	Abscisic acid
GA_3	-	Gibberellic acid
TDZ	-	N-phenyl-N'-1,2,3-thiadiazol-5'-ylurea
BAP	-	6-benzylaminopurine
BA	-	6-benzyladenine
Kn	-	Kinetin
Z	-	Zeatin
CW	-	Coconut water
PM	-	Palmyrah milk
CH	-	Casein hydrolysate
NaOCl	-	Sodium hypochlorite
$HgCl_2$	-	Mercuric chloride
TBA	-	Tertiary butyl alcohol
FAA	-	Formalin-acetic acid-alcohol
MS	-	Murashige and Skoog (1962) medium
M	-	Molar
μ	-	Micron
N	-	Normal solution
h	-	hours
d	-	days
min	-	Minutes
SE	-	Standard error
CD	-	Critical difference
SE/explant	-	Somatic embryos/explant
cv	-	Cultivar or variety
SB	-	Spanish bunch
VB	-	Virginia bunch
VR	-	Virginia runner

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Introduction

1. INTRODUCTION

Groundnut, the "unpredictable legume" is native to South America. The botanical name of *Arachis hypogaea* is derived from two Greek words *Arachis* means a legume and *hypogaea* means below ground, referring to geocarpic nature of pod formation. It is an annual and a tetraploid with 2n=40 chromosome.

It is one of the economically important oil seed crops. It is distributed over a wide range of environments both in tropical and warm temperature regions of the world. It is a protein rich edible oilseed crop accounting for 45 per cent of the oil seed area and 55 per cent of oil seed production in India. The average yield of groundnut in India is low compared to developed countries. Factors responsible for low yield are diseases, pests, salinity and drought.

The main constraints with groundnut breeding are, being a highly self-pollinated crop, difficult in hybridization and limited variations. It is increasingly necessary to produce additional variations artificially. Interspecific hybridization is found to be difficult due to cross compatibility or incompatibility.

Advances in tissue culture and plant molecular biology methods may soon make it possible to transfer genes controlling disease, insect and nematode resistance found in wild species of *Arachis* into cultivated *Arachis hypogaea*. The regeneration of plants from tissue culture is essential to achieve such a goal.

Production of haploids and their subsequent chromosome doubling can reduce the time needed for pedigree or back cross methods to obtain true breeding homozygous line. The diploids obtained from the cultivated tetraploid species may be useful for the study of genomic constitution and can be crossed to a diploid wild species for the *introgression* of desirable genes into the cultivated species. Somaclonal variation, induced by regenerating plants from the callus cultures of different explants will also be of immense use to the groundnut breeder.

The low rate of seed multiplication is another constraint in this crop. In addition to sexual reproduction by seed, scientists have developed yet another means of vegetative propagation in the form of plant tissue culture where plant cells give rise to complete plants. So specialised and mature cells can be manipulated to give rise to multiple copies of parent plant under optimum aseptic conditions and appropriate stimuli.

Regeneration of plants through somatic embryogenesis offers many advantages than organogenesis, especially in crop improvement programmes. Somatic embryos are believed to originate from single cell (Haccius, 1978), whereas organs are regenerated through collective organization of many cells. Therefore, plants derived from somatic embryos tend to be genetically alike, while those regenerated through organogenesis may result in a genetic mosaics. Since somatic embryos were bipolar structures, can easily develop into whole plants. Regeneration *via* somatic embryogenesis is a preferred method for producing stable mutants or cloning genetically engineered plants.

In addition, somatic embryogenesis in *Arachis* offers the following advantages.

i. To overcome post fertilization barriers

ii. *In vitro* selection and screening of genotypes for whole plant characters

iii. Production of artificial seeds and storage

iv. Possibility of cryopreservation

v. Gene manipulation and transformation

Detailed study is necessary to understand somatic embryogenesis and their utilization. In most legumes including groundnut, regeneration is sporadic and transient, due to recalcitrant nature. Different factors are involved in induction of somatic embryogenesis and plant regeneration such as genotypic differences, age of explant, cultural and environmental variabilities. Many of these obstacles have been overcome with implementation of more diverse cultural procedures.

Successful induction of embryogenesis has opened the possibility of using somatic embryos as a potential propagule for seed sown crops (Stuart and Redenbaugh, 1987). Encapsulation of somatic embryos to produce artificial seeds is of great potential value which serves as a novel delivery system (Redenbaugh and Ruzin, 1989). Artificial seeds offer the possibility of a low cost, high volume propagation system that compete with true seeds and transplants. Artificial seeds are also considered as an important material for embryo storage.

The most critical step in artificial seed technology is the ability of encapsulated somatic embryos to germinate directly on soil under a non-sterile environment. To fulfil this requirement, the nutrient matrix of the artificial seed should be provided with suitable additives. Synthetic seed technology, if successful, would provide the benefit of exclusive right of sale and distribution of the synthetic seeds with its producer. This would enable the farmer to introduce such an invention in his field at lower cost.

Considering all these aspects, the present study was undertaken in groundnut with the following objectives.

1. To study the anther culture response of different groups of *Arachis hypogaea* L. and wild diploid species *Arachis pusilla* L.
2. To study the genotypic differences for induction of somatic embryogenesis using immature leaflets, de-embryonated cotyledons and mature embryo axes.
3. To study the effect of different media, growth hormones, amino acids, coconut water and palmyrah milk on somatic embryogenesis.
4. To study the genotypic differences in plant regeneration from somatic embryos
5. To investigate the efficiency of plant regeneration by maturation of somatic embryos
6. To device a feasible method of encapsulation for somatic embryos
7. To study the regeneration ability and storage potential of encapsulated somatic embryos.

Review of Literature

2. REVIEW OF LITERATURE

The *in vitro* culture of cells, tissues and organs of plants is an important area of biotechnology because of its potential to generate improved crops. The concept of regeneration of plants from *in vitro* cultured cells can be traced to the German botanist, Haberlandt (1902) who predicted about totipotency in plants. Later developments provided that single cell can be regenerated into complex multicellular organs and organisms with suitable modification of media and growth hormones.

The wide spread efforts to improve legumes conferring desirable traits through *in vitro* manipulation has been a focal subject of research in recent years. Legumes exhibited a diversity of responses when cultured *in vitro*. Depending on several factors, regeneration occurs *via* organogenesis or embryogenesis, either directly from explanted tissue or indirectly after an intervening callus phase. With few exceptions, legumes were commonly described as recalcitrant with regard to tissue culture of the large-seeded legumes. To-day, there are reports of regeneration from at least 75 species from 25 genera in the leguminosae (Parrott *et al.*, 1992).

Several technique such as anther culture for the production of haploids (Collins and Genovesi, 1982; Maheshwari *et al.*, 1982), embryo culture in order to overcome postzygotic incompatibility (Steward, 1981; Raghavan and Srivastava, 1982), protoplast culture (Shepard, 1981) and somatic hybridization by fusion of plant protoplasts (Cocking and Riley, 1981) have been used to alter the genetic base or induce variability in many plant species.

2.1. Tissue culture in groundnut

Harvey and Schulz (1943) were the first to initiate tissue culture work in groundnut using embryos and embryo segments without success. Two decades later, Rangaswamy *et al.* (1965) reported root formation in groundnut pericarp cultures. Plantlets have been regenerated from explants such as embryonic axis (Braverman, 1975), shoot tips (Russo and Varnell, 1978), epicotyl (Bajaj *et al.*, 1981a), hypocotyl, epicotyl and cotyledonary explant callus cultures (Narasimhulu and Reddy, 1983) and directly from embryo axis (Atreya *et al.*, 1984). Shoots were obtained from cultured root discs of *Arachis pusilla* and flower buds of *A. pusilla* and *A. monticola* among the wild species (Sastri *et al.*, 1981). Shoots, roots and complete plants have been obtained from anther and pollen derived callus of cultivated *Arachis hypogaea* and its wild relatives *Arachis villosa* (Bajaj *et al.*, 1981b). Plants were also regenerated from calli of embryonated and de-embryonated cotyledons (McKently *et al.*, 1990) and from leaf tissue (McKently *et al.*, 1991).

2.1.1. Anther culture

In vitro anther culture technique has enabled the production of large number of haploid plants. This technique has been applied to over 200 species of higher plants (Maheshwari *et al.*, 1982; Keller *et al.*, 1987). Many factors influence androgenesis, including plant genotype, physiological state of the parent plant, pollen stage, pre-treatment of flower buds and culture medium (Maheshwari *et al.*, 1980).

Martin and Rabechault (1976) were the first to report on the anther culture of *A. hypogaea*. They obtained calli with chromosome ranging from haploid to polyploid, the main morphogenetic response on MS medium with NAA (11.0 μM) and 2,4-D (9.0 μM). In addition, they reported the occurrence of a few albino plants.

Mroginski and Fernandez (1979) cultured anthers of *A. hypogaea* (tetraploid), *A. villosa, A. correntina* and an unidentified *Arachis* species (wild diploids) at various stages of microsporogenesis on MS medium with different combinations of NAA, BAP and myo-inositol. The callus cells were found to be differing at ploidy level. In the case of wild species, it was possible to detect early events of androgenesis such as multicellular pollen structures in some calli. In the subsequent year (1980), the same authors observed the plants with 2n=20, when calli from anther cultures of *A. lignosa* and an unidentified *Arachis* species (both haploid) were transferred from MS + NAA (2 mg/l) + BAP (0.5 mg/l) to MS + BAP (0.01 mg/l). The regenerated plants were not haploids.

Bajaj *et al.* (1980a) observed callus, early pollen embryogenesis and multicellular pollen grains from anthers of *A. hypogaea* and *A. glabrata* (both tetraploid) cultured on MS + IAA (22.0 μM) + Kn (9.3 μM). Bajaj *et al.* (1981b) cultured anthers of *A. villosa* and *A. hypogaea* at first pollen mitosis on MS + IAA (22.0 μM) + Kn (9.3 μM) or BAP (8.8 μM) and obtained white compact callus growth in 13% of *A. hypogaea* anthers and 11% of *A. villosa* anthers within five weeks. When callus was subcultured in MS medium with NAA (5.7 μM) and BAP (8.8 μM), shoot differentiation occurred in *A. villosa* and rarely in *A. hypogaea.* If the differentiated shoots were left on the basal medium, they started turning yellow and ultimately dried. However, on transferring to basal MS medium with NAA (2.7 to 5.4 μM), rhizogenesis was noted in some cases. Thus the time taken from the inoculation of anthers to regeneration of plants was about 8-12 weeks. The callus cells of *A. hypogaea* ranged from haploids to octoploids.

Sastri *et al.* (1981) found early embryogenesis from anther calli of *A. hypogaea* and *A. monticola* on MS + NAA (2 mg/l) + BAP (0.5 mg/l) but plantlets were not obtained. However, plants have been observed from anther cultures of two other wild species of *A. pusilla* (diploid) and *Arachis* sp. P.I. 276233 (tetraploid) (Sastri *et al.*, 1982). In another species *A. pintoi*, Pittman (1981) succeeded in inducing limited shoot regeneration by *in vitro* culture of anthers.

Bajaj and Gosal (1983) cultured anthers of *A. hypogaea* cv. M13 and *A. villosa* for 4 to 6 weeks and the resulting calli were stored at -196°C for one year. Anther callus survival was 31% in *A. villosa*.

Seitz and Stalker (1985) obtained a highly morphogenetic callus of *A. paraguariensis* using N6 medium with CH (500 mg/l), picloram (0.20 mg/l), BAP (0.25 mg/l) and proline (3 g/l). In the same medium with reduced concentration of picloram (0.008 mg/l) and BAP (0.25 mg/l), buds appeared after 30-40 d. Shoot development was observed when buds were transferred to MS with 0.01 mg/l of BAP or MS with no hormones. Suspension of callus in N6 medium with reduced picloram levels also produced shoots.

Bajaj (1985) obtained pollen embryogenesis from the anther cultures of *A. villosa* and *A. glabrata*. Anther derived callus and the regenerated plants in *A. villosa* were mixoploids and showed wide genetic variation ranging from haploids to octoploids.

Still *et al.* (1987) noticed highly morphogenetic callus from anthers of *A. paraguariensis* on N6 medium supplemented with picloram (0.2 mg/l) + BAP (0.5 mg/l) and buds and shoots were obtained from the calli.

Sudhakar and Moss (1990) obtained significant differences among sizes of anthers containing pollen at different stages of development in *Arachis hypogaea*. They also suggested that anthers could be collected for culture by standardizing the sizes of flower buds.

Willcox *et al.* (1990) stated that plants grown in a controlled environment were found to have highly synchronized microspore development, both within an anther and among anthers contained in the same bud of *Arachis hypogaea*. In addition, floral bud shape was confirmed as a reliable indicator of anther stage in groundnut.

Willcox *et al.* (1991) observed that early uninucleate microspore was effective for highest anther response rating. They tested anthers of *Arachis hypogaea* in N6 basal medium with NAA (1.0 mg/l), BA (0.1 mg/l), glutamine (3.5 g/l) and sucrose (55 g/l) and observed four nucleate cells.

Tiainen (1992) obtained embryogenesis by pre-treatment of potato anthers at 6°C. He has also stated that sucrose gave the highest embryo (8.1%) and plant yields (3.2%) than maltose, melibiose and mannitol.

Agarwal and Bhojwani (1993) reported that germination of pollen grains was very poor (10%) on B5 or B5 containing GA_3 in anther cultures of *Brassica juncea*. Exposure of the embryos at 4°C for 6 d proved to be the best treatment which induced 66% germination of the embryos.

Kim *et al.* (1994) obtained calli from anther culture of *Cartharanthus roseus* on MS medium supplemented with NAA (1.0 mg/l) and Kn (0.1 mg/l). They reported that 80% of the calli produced somatic embryos and plantlets.

In alfalfa anther culture, genotype, stage of microspore development, phytohormonal composition of the nutrient media and pre-treatment with physical agents alone or in combination affected the efficiency of organogenesis and plant regeneration (Zagorska and Dimitrov, 1995).

Guo and Pulli (1996) found that activated charcoal (150 mg/l) when added to the NLN medium, promoted embryogenesis in microspore culture of

Brassica campestris. The embryo development was faster and the embryo yield was significantly higher than those culture without activated charcoal.

2.1.2. Leaflet culture

In groundnut, young leaflets have been found to be a successful explant for regeneration which was first reported by Mroginski *et al.* (1981). They found maximum number of shoot buds regeneration with NAA (1.0 mg/l) and BAP (1.0 mg/l). Later, several workers have reported shoot bud formation and regeneration of plants from immature leaves (ICRISAT, 1981; Johnson, 1981a; Pittman, 1981; Narasimhulu and Reddy, 1983).

Pittman *et al.* (1983) cultured leaflets from several *Arachis* species, but could get regeneration in the *Arachis* and Extranervosae sections of the genus. Cultures of seedling leaves from all groundnut cultivars tested produced callus, but the frequency of organogenesis was highly cultivar dependent (Johnson, 1981b; Pittman *et al.*, 1983).

Sukumar and Sree Rangasamy (1984) obtained callus and root formation in *A. diogoi, A. duranensis, A. marginata, A. monticola, A. hagenbeckii, A. glabrata* and *A. villosulicarpa* utilising petiole, pinna and stem as explants. They obtained callus in MS + CH (0.196 mg/l) + CW 15% (v/v) + 2,4-D (2 mg/l) and subculturing in MS basal medium resulted in shoot formation.

Shoot bud formation and shoot regeneration was reported in *Arachis villosulicarpa* (Pittman *et al.*, 1984; Johnson and Pittman, 1985). Burtnik *et al.* (1985) obtained regeneration of plants from leaf tissue of *A. pintoi*.

Seitz *et al.* (1987) studied genetic variation for regenerative response in immature leaflet cultures of 47 cultivars in *A. hypogaea*. Significant differences between media for rhizogenesis and callus proliferation were observed. They also observed significant differences among genotypes for rhizogenesis and no differences for shoot formation.

Cheng *et al.* (1992) obtained regenerated plants from *in vitro* cultured petioles with blades attached, leaf segments, epicotyl and petiole sections. Multiple shoots arose in MS medium supplemented with BA (5-25 mg/l) and NAA (0.5 - 3.0 mg/l). Leaflet segments and petiole sections were less responsive in groundnut for normal shoot formation.

Dunbar and Pittman (1992) cultured mature leaf explants of 13 *Arachis* species on MS salts with B5 vitamins, sucrose (30 g/l), NAA (5 μM) and BAP (5 μM). Transfer of these culture in a medium with reduced auxin regenerated shoot buds or shoots from the cultures of *A. burkartii, A. lignosa, A. paraguariensis, A. repens, A. rigonii* and *A. villosulicarpa*. Mature leaf explants of *A. villosulicarpa* produced an average of 50 shoots and buds after 25 d in culture at 25°C with a 16 h photoperiod on a medium with MS salts, B5 vitamins, NAA (5 μM), BAP (5 μM) and solidified with rice starch (120 g/l).

Ozias-Akins *et al.* (1993) obtained transgenic groundnut plants from embryogenic leaf callus via microprojectile bombardment. Eapen and George (1993b) reported shoot regeneration from leaf discs of groundnut and pigeonpea. They also stated that highest frequency of plant regeneration in groundnut was induced by BA in combination with IAA or IAA-L-alanine, while in pigeonpea, BA in combination with IAA or IAA-L-aspartic acid produced best results.

Mansur *et al.* (1993) observed greatest transformation efficiency when leaf or cotyledon explants of *A. hypogaea* were co-cultivated with *Agrobacterium tumefaciens* on solid rather than liquid medium for 48 h. Highest transformation rates were observed on leaves from 7 to 10 d old seedlings with inoculum densities of 1.5 x 10^9 cells/ml.

Chengalrayan *et al.* (1994) cultured immature leaves of *A. hypogaea* on a medium containing 2,4-D (20 mg/l) and somatic embryos developed by further reducing the concentration of 2,4-D (3 mg/l). They obtained shoot elongation from 25% of the embryos in a medium supplemented with 0.5 mg/l each of BAP and Kn. The developed embryos of immature leaflets were germinated either singly or in clusters in a half or full strength MS medium with or without growth regulators.

2.1.3. Cotyledon culture

Normal groundnut plants from de-embryonated cotyledon and cotyledonary fragments were first produced by Illingworth (1968, 1974). Verma and Van Huystee (1970) obtained callus from cotyledons in LS medium with NAA (2.0 mg/l) and Kinetin (0.5 mg/l) along with amino acids. They also obtained homogenous cell lines from cotyledon culture on White's medium supplemented with CW (7%, v/v).

Guy *et al.* (1978, 1980) reported rhizogenesis and vigorous callus growth from cotyledon callus of *A. hypogaea* in MS medium with 2,4-D (2.0 mg/l), NAA (2.0 mg/l) and Kn (0.06 mg/l).

Pittman (1981) cultured the cotyledons of *A. hypogaea* in MS medium with IAA and BAP or NAA, 2,4-D and BAP produced callus. Quian (1981) observed callus from cotyledons of *A. hypogaea* and obtained regeneration from the callus.

Sastri *et al.* (1981) induced shoot buds and shoots on the callus of cotyledons in *Arachis hypogaea*, which were localized at the proximal end of the cotyledons.

Narasimhulu and Reddy (1983, 1985) regenerated plants from cotyledons of *A. hypogaea* in MS medium supplemented with BAP (1.0 mg/l), NAA (0.4 mg/l) and Kinetin (1.0 mg/l). Direct shoot formation was also observed in the medium containing IAA (2.0 mg/l) and Kinetin (2.0 mg/l).

Atreya *et al.* (1984) reported that the shoot induction was predominantly from the cotyledon segments proximal to the embryonic axis in *A. hypogaea.* They also obtained maximum shoot formation on MS medium with BAP (2.0 mg/l), maximum root formation on MS medium with NAA (1.0 mg/l) and complete plants on MS medium with NAA (1.0 mg/l) and BAP (0.5 to 2.0 mg/l).

Rugman and Cocking (1985) obtained multiple shoot buds or callus and shoot buds from the cotyledons culture of *A. hypogaea* in MS medium with Zeatin (2.0 or 4.0 mg/l) or IAA (2.0mg/l) and BAP (1.0 mg/l).

Banerjee *et al.* (1988) induced multiple shoots from cotyledonary nodes of *A. hypogaea* in MS medium with NAA (1.0 mg/l) and BAP (3.0 mg/l). The mean number of shoots observed varied from 1 to 12 per explant and mean length of the shoot varied from 2.0 to 5.6 cm.

Ozias-Akins (1989) reported that an average of 50-60% of the cotyledon segments of *A. hypogaea* produced either nodular outgrowth or somatic embryos in B5 medium containing picloram (0.5 - 2.0 mg/l).

McKently *et al.* (1990) produced highest number of shoots from single embryonated cotyledons of *A. hypogaea* in MS medium with BA (0.5-60.0 mg/l).

Daimon and Mu (1991) obtained highest percentage of multiple shoot from cotyledonary nodes in medium containing BAP (50 mg/l) in *A. hypogaea* .

Durham and Parrott (1992) found repetitive somatic embryogenesis in groundnut by initiation of cultures using individual somatic embryos induced from immature cotyledons in MS medium with 2,4-D (40 mg/l).

Gulati and Jaiwal (1994) obtained highest number of shoots from cotyledons of mungbean in B5 basal medium containing BAP or 2-ip with sucrose (30 g/l).

George and Eapen (1994) induced multiple shoots from the cotyledonary node explants of pigeonpea cultured on BAP enriched medium.

Shivaprakash *et al.* (1994) also noticed multiple shoots from the cotyledonary node region of pigeonpea in MS medium with BAP (2.0 mg/l). The cotyledonary node along with the mass of shoot initials excised from the seedlings, continued to form new shoot initials on MS medium containing BAP (2.0 mg/l) and IAA (0.5 mg/l).

2.1.4. Embryo culture

Earlier studies on *in vitro* embryo culture in groundnut concentrated on regenerating whole plants from embryos of *A. hypogaea*. Martin (1970) recovered plants from small ovules, but Sastri *et al.* (1980) could only produce callus using the same media as used in Martin's experiment.

Sastri *et al.* (1981) obtained hybrid plants from difficult crosses by culturing immature embryos from undeveloped pods of groundnut.

Bajaj *et al.* (1982) and Bajaj and Gosal, (1983) cultured hybrid embryos of *A. hypogaea* x *A. villosa* on MS medium containing IAA (2 μM) and Kinetin (9.3 μM) which produced plantlets.

Bajaj (1985) cultured the hybrid embryos of cultivated *A. hypogaea* x wild diploid *A. villosa*, which generally abort in nature, rescued 30 to 45 d after pollination under *in vitro* and obtained regenerated plants. This hybrid plants showed triploid chromosome numbers (3x = 30).

Moss and Stalker (1987) reported the embryo rescue in wide crosses in *Arachis* for culturing ovules excised from well developed pods or from immature pods derived from flowers treated with gibberellic acid (GA_3). Embryo rescue of CO x *A. glabrata* produced only calli without regeneration (Ramaswamy, 1987).

Hisajima *et al.* (1989) observed multiple shoots from seeds of *A. hypogaea* cultured on MS medium containing BAP. These shoots multiplied further in medium containing BAP (0.5 μM) and IBA (0.025 μM).

McKently *et al.* (1990) obtained multiple shoots formation in cultured embryo axes of groundnut in MS medium containing BA (0.5 -60.0 mg/l).

2.2. Somatic embryogenesis

Somatic embryogenesis was considered to be a distinct development pathway, different from either shoot or root organogenesis in which a single cell gives rise to a structure containing bipolar, meristems and with no direct vascular connections to the maternal tissue (Haccius *et al.*, 1978).

The initiation and development of embryos from somatic tissues derived from the storage taproot was first reported independently by Steward *et al.* (1958) and Reinert (1959) in carrot.

Li *et al.* (1985) obtained globular and heart shaped somatic embryos from immature embryos of soybean in the presence of BA (0.5 mg/l) and IAA (0.01 mg/l).

Direct somatic embryogenesis has been reported from different explants in *Arachis.* Ozias-Akins (1989) noticed embryogenesis from immature embryos of groundnut on B_5 medium containing picloram (0.5-1.0 mg/l).

Hazra *et al.* (1989) obtained direct somatic embryogenesis from immature zygotic embryo axes of *A. hypogaea* in MS medium with 2,4-D (1,3,6 mg/l).

Sellars *et al.* (1990) induced adventitious somatic embryogenesis from cultured zygotic embryos of groundnut and soybean in L2 medium with picloram. They obtained 53% embryogenesis in groundnut and 29% in soybean. *Arachis* produced an average of 29 somatic embryos per explant compared with 16 for soybean. An average of 77% of *A. hypogaea* somatic embryos formed shoots and 61% formed roots compared to 30% shoots and 33% roots for soybean.

McKently (1990) and McKently *et al.* (1990, 1991) observed somatic embryogenesis from mature axes of embryos and leaf tissues in seedling explants of groundnut.

Ozias-Akins *et al.* (1992a) obtained embryogenic callus from immature cotyledon and embryo axis explants of seven genotypes in groundnut on MS medium with picloram (0.5 mg/l) and sucrose (30 g/l). They observed significant differences among genotypes for somatic embryo formation and plant regeneration. Somatic embryo formation from cotyledons showed better correlation with plant regeneration than from the embryo axis.

Baker and Wetzstein (1992) induced somatic embryos from leaflets culture of *A. hypogaea.* Leaflet size influenced per cent somatic embryogenesis; 5-8 mm long cut leaflets were superior to 2-3 mm long uncut leaflets. Maximum embryogenesis of 14.6% obtained after a 15 d incubation on modified MS with B5 vitamins, sucrose (30 g/l), Gel-Gro (4 g/l), 2,4-D (40 mg/l) and Kinetin (0.2 mg/l) followed by transfer to a secondary medium with 2,4-D (5 mg/l) and Kinetin (0.2 mg/l).

Eapen and George (1993a) observed somatic embryos from immature embryonal axes and immature cotyledons of groundnut. They found that 2,4-D was most effective for highest number of somatic embryos per responding culture, while dicamba, picloram, IPA, NAA, 2,4,5.T were also effective for embryogenesis. Among cytokinins, Zeatin slightly enhanced the frequency of somatic embryogenesis, while kinetin, 2-ip and BA were relatively inhibitory. Among the carbon sources, sucrose at 60 g/l produced highest frequency of embryos per explant. Highest plant conversion frequency from somatic embryos was obtained in the presence of dicamba or NAA and using cotyledon explants.

Eapen *et al.* (1993) also obtained somatic embryogenesis from immature cotyledons and immature embryonal axis of *A. hypogaea* in L6 medium with NAA, picloram or 2,4-D at 5-50 mg/l. Immature embryonal axis produced a higher number of somatic embryos in comparison with immature cotyledons. Somatic embryos developed into plants on basal medium supplemented with activated charcoal.

Chengalrayan *et al.* (1994) found embryogenic masses from immature leaves of groundnut on medium with 2,4-D (20 mg/l). These masses developed into somatic embryos following transfer to a medium with 2,4-D (3 mg/l). Shoot elongation was obtained in 25% of the embryos following transfer to a medium with 0.5 mg/l each of BAP and Kn.

Baker *et al.* (1994) initiated somatic embryogenesis from immature zygotic cotyledons of *A. hypogaea*. They observed that percentage of embryogenesis and number of embryos were markedly lower in explants induced on NAA compared to 2,4-D. Embryos produced under 16 h photoperiod were tough, woody and difficult to separate for subsequent germination and those produced under 24 h dark condition were succulent and pliable.

Baker *et al.* (1995) obtained high frequency of somatic embryogenesis in groundnut dry seed embryos using 2,4-D (10-40 mg/l) in the induction medium. Embryos noticed from cultures grown in the dark were easier to remove from the explant than those under 16 h photoperiod. McKently *et al.* (1995) developed transgenic groundnut plants using zygotic embryo axes of mature seeds.

Cheng *et al.* (1996) reported fertile transgenic plants using leaf sections of groundnut with *Agrobacterium tumefaciens* mediated transformation system.

Mary and Jayabalan (1997) induced somatic embryogenesis in hypocotyl derived callus of sesame (*Sesamum indicum*). They stated that 2,4-D was the most effective and resulted highest frequency of responding cultures but 2,4,5-T and NAA were not beneficial for embryogenesis.

Sreenivasu *et al.* (1997) obtained plant regeneration via somatic embryogenesis in cotyledons and leaf explants of pigeonpea (*Cajanus cajan*) in MS medium supplemented with TDZ (10 µM).

Chengalrayan *et al.* (1997) reported that high conversion of abnormal peanut somatic embryos into shoots in a medium with BA, Kn and TDZ (22.7 µM) and obtained plant recovery from 86 to 92%.

Luo and Jia (1998) developed efficient procedure for inducing callus and plant regeneration using hypocotyl segments of forage legume *Astragalus adsurgens* in MS medium containing NAA (0.5 µM) and BA (8.9 µM).

Chengalrayan *et al.* (1998) obtained high frequency of plant development *via* somatic embryogenesis from mature zygotic embryo derived leaflets of groundnut (*A. hypogaea*). They have also observed that the failure of somatic embryos to undergo conversion into plantlets could be a genotype dependent characteristic.

2.3. Histological studies

Histological information on the origin and development of somatic embryos *in vitro* was obtained from studies on callus and suspension cultures of carrot. Embryoids arose directly from small cells of meristematic appearance, scattered among the more typical differentiated epidermal cells.

Tissert *et al.* (1979) reported that bipolar embryoids were formed from aggregated cells. Hazra *et al.* (1989) showed the evidence of somatic embryo origin directly on cultured immature zygotic embryo axis in groundnut. In eastern red bud (*Cercis canadensis* L.), the histological studies indicated that the shoots were formed from actively dividing cells located at the axillary bud (Distabanjong and Geneve, 1997).

2.4. Synthetic seed technology

The historical development and current status of artificial seed production is summarised below.

Somatic embryos was first observed in 1958 (Steward *et al.*, 1958) but only 20 years later that the concept of synthetic seed appeared on paper. The concept of synthetic seed was first mooted by Murashige (1977) during a meeting held in Belgium to discuss the role of plant tissue culture in horticulture. Kitto and Janick (1980) were the first to report synthetic seeds in carrot by forming detachable wafers from a water soluble resin containing polyoxyethylene oxide.

Kitto and Janick (1982) reported 2.5% polyox- a polythelene oxide homopolymer which formed detachable wafers with embedded embryos had the most desirable characteristics for use as encapsulating material.

Redenbaugh *et al.* (1986, 1987b, 1988) demonstrated that calcium alginate gel beads were well suited for encapsulation of somatic embryos of alfalfa, celery, lettuce, carrot and cauliflower.

Datta and Potrykus (1989) reported that calcium alginate coated artificial seeds of barley maintained their germination capacity for 6 months while non-encapsulated embryos did not survive two weeks of storage.

Deng *et al.* (1990) observed that the conversion efficiency of alginate encapsulated somatic embryos of wheat was lower than non-encapsulated embryos. They reported 60% germination for calcium alginate encapsulated embryos and 62% germination for naked embryos.

Ganapathi *et al.* (1992) reported the use of White's medium as substrate for encapsulation which resulted in 100% conversion of encapsulated shoot tips into plantlets in banana.

Lulsdorf *et al.* (1993) demonstrated that the addition of 0.5% (w/v) activated charcoal to the alginate capsule significantly enhanced root development and germination of somatic embryos of interior spruce (*Picea glauca*) but not for zygotic embryos.

Padmaja *et al.* (1995) obtained highest germination percentage (33.3%) and conversion (25.4%) in encapsulated embryos of groundnut on half strength MS medium with 1% sucrose and maltose each. Somatic embryos encapsulated with MS nutrients, germinated 8.2% but failed to convert into plants upon storage for 40 d at 4°C.

Onay *et al.* (1996) obtained a conversion frequency of 14 per cent from encapsulated somatic embryos of pistachio (*Pistacia vera*) after 60 d storage at 4°C.

Piccioni (1997) demonstrated that addition of growth regulators to the artificial endosperm and culture of the single nodes for root primordia initiation for 3,6 or 9 d in darkness before encapsulation allowed production of 58, 60 and 66% of plantlets, respectively.

Janeiro *et al.* (1997) demonstrated that the frequency of *in vitro* germination into plants of artificial seeds was affected by various nutrient additives included in the encapsulating matrix of somatic embryos in camellia (*Camellia japonica*). The addition of Ca-free MS basal medium plus sucrose (30 g) plus gibberellic (14.4 μM) acid and indole-3-acetic acid (28.5 μM) to the alginate capsule resulted in 63% plant recovery rate, which was similar to that of non-encapsulated embryos. Plant recovery of encapsulated embryo was 40% and 30% following storage for 30 and 60 d at 4°C respectively.

Naik and Chikkagouda (1997) produced somatic embryos by reducing the concentration of 2,4-D in young leaves of sugarcane (*Saccharum officinarum*). These embryos encapsulated in sodium alginate (3%) supplemented with activated charcoal (0.1%) to form synthetic seeds. They obtained 94.7% germination on half-strength MS medium with sucrose (30 g/l), NAA (3 mg/l) and BAP (0.5 mg/l).

Materials and Methods

3. MATERIALS AND METHODS

Laboratory experiments were carried out at the tissue culture laboratory, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University (TNAU), Coimbatore.

3.1. Choice of donor

Seven genotypes of cultivated groundnut belonging to three groups of *Arachis hypogaea* L. and one wild species of *Arachis pusilla* L. were collected from the Department of Oilseeds, Centre for Plant Breeding and Genetics, TNAU, Coimbatore. These cultivated and wild species were raised under screen house conditions for collection of explants. The details of these species are presented in Table 1.

3.1.1. Explants

The explants used in *A. hypogaea* and *A. pusilla* are indicated below

Arachis hypogaea	*Arachis pusilla*
Anthers	Anthers
Immature leaflets	
Mature cotyledons	
Mature embryo axes	

3.2. Preparation of media

3.2.1. Preparation of stock solutions

The different nutrient media used were Murashige and Skoog (MS) (1962), Blaydes (1966), B5 (Gamborg *et al.*, 1968), N6 (Chu, 1978) and Nitsch and Nitsch (NN) (1969). The media composition is presented in Tables 2 and 3. The stock solution of macro, micro, minor, KI and vitamins were prepared with double distilled water as per concentrations listed (Tables 2 & 3) and stored in well stoppered bottles inside the refrigerator. While preparing vitamins stock

Table 1. Species / cultivars used for anther culture and somatic embryogenesis

S.No.	Species/ genotypes	Ploidy level	Section/ series code	Major group	Origin/ source
A.	***A. hypogaea* L.**				
1.	CO2	4n	A3	Spanish bunch	Coimbatore
2.	TMV12	4n	A3	Spanish bunch	Tindivanam
3.	TMV10	4n	A3	Virginia bunch	Tindivanam
4.	VG113	4n	A3	Virginia bunch	Vriddhachalam
5.	ICG 92	4n	A3	Virginia runner	ICRISAT
6.	Salemkai	4n	A3	Virginia runner	Tiruchencode
7.	M13	4n	A3	Virginia runner	Junagadh
B.	***A. pusilla* L.**	2n	T	-	Coimbatore

Table 2. Composition of nutrient and preparation of stock solution for MS medium

Ingredients	Composition in the medium (mg/l)	Content in stock solution (mg)	Volume of stock solution (ml)	Volume of stock solution for a litre medium (ml)
a) Macro nutrients			500	50.0
NH_4NO_3	1650.000	16500.0		
KNO_3	1900.000	19000.0		
$CaCl_2.2H_2O$	440.000	4400.0		
$MgSO_4.7H_2O$	370.000	3700.0		
KH_2PO_4	170.000	1700.0		
b) Micronutrients			250	2.5
$MnSO_4.4H_2O$	22.300	2230.0		
$ZnSO_4.7H_2O$	8.600	860.0		
H_3BO_3	6.200	620.0		
c) Minor elements			250	2.5
$Na_2MoO_4 \cdot 2H_2O$	0.250	25.0		
$CuSO_4 \cdot 5H_2O$	0.025	2.5		
$CoCl_2 \cdot 6H_2O$	0.025	2.5		
d) KI	0.830	83.0	250	2.5
e) Iron			100	5.0
Na_2EDTA	37.250	745.0		
$FeSO_4 \cdot 7H_2O$	27.850	557.0		
f) Vitamins			100	5.0
Nicotinic acid	0.500	10.0		
Pyridoxine HCl	0.500	10.0		
Thiamine HCl	1.000	20.0		
Myo-Inositol	100.000	2000.0		
g) Amino acids			100	5.0
Glycine	2.000	40.0		

* Sucrose 30 g/l, agar 8 g/l, pH 5.8

Table 3. Nutrient composition of the media

Ingredients	*Media (mg/l)			
	Blaydes	B5	N6	Nitsch
a) Macro nutrients				
KNO_3	1000.00	2500.000	2830.00	950.000
NH_4NO_3	1000.00	-		720.000
$MgSO_4.7H_2O$	35.00	250.000	370.00	185.000
KH_2PO_4	284.00		400.00	68.000
$CaCl_2.2H_2O$	-	150.000	166.00	166.000
$(NH_4)_2SO_4$	-	134.000	463.00	-
$Ca(NO_3)_2.4H_2O$	-	150.000	-	-
$NaH_2.PO_4 2H_2O$	-	-	-	-
KCl	65.00	-	-	-
K_2HPO_4	20.40	-	-	-
b) Micronutrients				
$MnSO_4.1H_2O$	-	-	-	19.000
$MnSO_4.H_2O$	4.00	10.000	4.40	-
$ZnSO_4.7H_2O$	1.50	2.000	1.50	10.000
H_3BO_3	1.60	3.000	1.60	10.000
$Na_2MoO_4.2H_2O$	-	0.250	-	0.250
$CuSO_4.5H_2O$	-	0.025	-	0.025
$CoCl_2.6H_2O$	-	0.025	-	0.025
c) Iodine source				
KI	0.80	0.750	0.80	-
d) Iron				
Na_2 EDTA	32.00	37.500	32.50	27.800
$FeSO_4.7H_2O$	23.60	27.800	11.20	37.300
e) Vitamins				
Nicotinic acid	0.50	1.000	0.50	5.000
Pyridoxine HCl	0.10	1.000	0.50	0.500
Thiamine HCl	0.10	10.000	1.00	0.500
Myo-Inositol	-	100.000	-	100.000
f) Amino acids				
Glycine	2.00	-	2.00	5.000
pH	5.8	5.5	5.8	5.5

* All media contains, sucrose 30 g/l, agar 8 g/l

solutions myo-inositol was not added to the stock. On the otherhand it was added in the powder form to media as fresh at the time of preparation of media.

3.2.2. Preparation of iron stock solution

A quantity of 557 mg ferrous sulphate and 745 mg of disodium EDTA were taken separately. Disodium EDTA was dissolved in 40 ml of boiling water and then added to 40 ml hot solution of ferrous sulphate with gentle shaking. Then the volume was made upto 100 ml. The stock was stored in brown glass bottle.

3.2.3. Preparation of stock solution of growth hormones

The growth hormone was dissolved in a few drops of the solvent and then the volume made up to the required level. The growth hormones and the solvents are given below.

Growth hormone	Quantity (mg)	Solvent	Volume of stock solution for each hormone (ml)
2,4-D, pIC, NAA	100	2.5 ml of 95% ethanol	100
BAP, Kn	100	2.5 ml of 0.5 N HCl	100
TDZ	100	5 ml of 95% ethanol	100

3.2.4. Carbon source

Sucrose was used as main carbon source for anther culture, somatic embryogenesis and plant regeneration. High purity sucrose (30 g/l) was used. Before adjusting the pH of the medium, sucrose was added.

3.2.5. Organic additives

Coconut water and palmyrah milk were used for different experiments. Six months old tender coconuts were selected for collection of coconut water.

Similarly for collection of palmyrah milk, three months old palmyrah nuts were collected. Coconut water and palmyrah milk collected from the respective tender nuts were filtered and boiled for ten minutes, cooled and the supernatant was filtered. Ten ml of each additive was used per litre of medium. The remaining water was stored in deep freezer. Casein hydrolysate (500 mg/l) was used.

3.2.6. Agar

Either Difco-Bacto agar or other purified agar (Hi-media) was used at a concentration of 8 g L^{-1}. The agar was dissolved slowly in boiling distilled water without any clots.

3.2.7. Preparation of agar medium

From the respective stock solution, required volume of nutrients were pipetted out for 1 litre of medium. The calcium chloride was dissolved separately and added before adjusting the pH of the medium. The growth hormones and sucrose were added and the pH was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH. Eight gram agar was melted separately to which the ingredients of the media were added. The final volume was made up with double distilled sterilized water.

Twenty ml was dispensed into clean and sterilized tubes. The tubes were plugged with non-absorbent cotton and sterilized within 6 h in an autoclave (1.01 kg/cm^2, 15 lb at 121°C) for 20 min. The sterilized tubes were allowed to cool before proceeding with inoculation.

3.3. Culture techniques used for different explants

3.3.1. Preparation of surface sterilants

All tissue culture operations were carried out in laminar air flow chamber. The working bench was cleaned with 70% (v/v) ethanol and UV light was switched on for 15 minutes. Ethyl alcohol and mercuric chloride were used for surface sterilization. The concentration of the chemicals required and time of exposure are presented in Table 4.

The explants were washed with the disinfectant (Table 4). The materials after sterilization were thoroughly washed with sterilized distilled water. Surgical instruments were sterilized in 70% alcohol and flaming to red hot. The different explants *viz.*, anthers, immature leaflets, de-embryonated mature cotyledons and mature embryo axes were inoculated into the medium as per required explants per tube and incubated at 25 ± 2°C in the light / dark for induction of callus, somatic embryogenesis and plant regeneration.

Table 4. Concentration of chemicals for surface sterilization of different explants

S.No.	Disinfectant	Explant	Concentration (%)	Exposure time (min)
1.	Mercuric chloride	Flower buds	0.05	2,5,10,15
2.	Mercuric chloride	Flower buds	0.10	2,5,10,15
3.	Ethyl alcohol + Mercuric chloride	Flower buds	70.00 + 0.05	2,5,10,15
4.	Ethyl alcohol + Mercuric chloride	Flower buds	70.00 + 0.10	2,5,10,15
5.	Mercuric chloride	Immature leaflets	0.50	5
6.	Mercuric chloride	Mature kernels	0.10	15

3.3.2. Anther culture

3.3.2.1. Selection of flower buds

Cultivated *Arachis hypogaea* cultivars CO2, TMV12, TMV10, VG113, ICG92, Salemkai and *Arachis pusilla* were grown in glass house condition.

Flower buds of different sizes were collected in the forenoon (8-10 a.m.) from glass house grown plants at 30 d after sowing. Length of flower bud was measured using eye piece micrometer before the anthers were dissected and stained in 2% aceto carmine. Some fresh, unfixed flower buds were measured, anther dissected and the colour recorded before staining in aceto carmine to observe the stage of microsporogenesis. A minimum of 20 flower buds were studied under each category. The size and stages of flower buds and colour of anthers were recorded.

3.3.2.2. Anther inoculation

Based on flower categorization, approximate size of the flower buds (cv. TMV12) were used to standardising the pretreatment/surface sterilants (Table 4) for anther response. Three anthers were inoculated per treatment. Observation for percentage contamination was recorded for all treatments, 5 d after inoculation.

In all experiments for induction of anther callus, the flower buds were collected and kept for 2 d at 4°C for pretreatment. Then flower buds were surface sterilized with 70% ethanol for one minute followed by 0.05% $HgCl_2$ for 10 min. They were then washed thoroughly with sterile water four or five times. Anthers were excised from flower buds with the help of sterile needles and filaments were removed. Anthers having light yellow in colour were inoculated at the rate of three per tube with a total of 60 anthers per genotype on a solidified agar medium in all experiments.

Out of ten anthers, the large three oblong anthers were used in the present study and other anthers were rejected. After inoculation the cultures were kept under 24 h dark condition for three weeks and then placed in 16 h photoperiod for callus induction and differentiation. Another experiment was set by placing the inoculated tubes continuously under 24 h dark condition and another set of inoculated tubes were placed at 16 h photoperiod (1000 lux) followed by 8 h dark condition.

3.3.2.3. Callus induction

a. Nutrient media

The following nutrient media containing 2,4-D (4 mg/l) were used for callus induction. All media contained sucrose (30 g/l) and agar (8 g/l).

MS, Blaydes, B5, N6, Nitsch

b. Auxins

Experiment No.1

In this experiment twelve hormone combinations each at three levels of 2,4-D alone or in combinations with coconut water and palmyrah milk were used. The per cent of callus induction and fresh weight were recorded. The different treatments used in this experiment are furnished below:

2,4-D (mg/l)	2	3	4	2	3	4	2	3	4	2	3	4
CW (ml/l)	-	-	-	10	10	10	-	-	-	5	5	5
PM(ml/l)	-	-	-	-	-	-	10	10	10	5	5	5

Experiment No.2

The experiment was conducted using MS medium with different levels of picloram for induction of callus and other morphogenetic responses from anthers.

Different level of pIC used in medium were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l.

Experiment No.3

The following experiment was conducted in MS medium with 16 h photoperiod (1000 lux) followed by 8 h dark condition or 24 h dark condition for anther callus induction.

16 h photoperiod and 8 h dark condition						
2,4-D (mg/l)	2	3	4	2	3	4
CW (ml/l)	-	-	-	10	10	10
24 h dark condition						
2,4-D (mg/l)	2	3	4	2	3	4
CW (ml/l)	-	-	-	10	10	10

3.3.2.4. Regeneration

Experiment No.1

The anther calli obtained from different genotypes using 2,4-D (4 mg/l) along with CW (10 ml/l) were subcultured with reduced concentration of 2,4-D (2 mg/l) and CW (10 ml/l) on 30th d. These subcultured 60 d old embryogenic calli were transferred to N6 medium with different concentrations of NAA, pIC, BAP, CH, glutamine and proline.

N6 + pIC (0.2 mg/l) + BAP (0.25 mg/l)+ Proline (0.5 g/l) + CH (500 mg/l)

N6 + NAA (1.0 mg/l) + BAP (0.5 mg/l) + Glutamine (3.5 g/l)

N6 + NAA (1.0 mg/l) + BAP (0.5 mg/l)

N6 + pIC (0.2 mg/l) + BAP (0.25 mg/l) + Proline (0.5 g/l)

Experiment No.2

In another experiment, anther calli obtained from different genotypes using 2,4-D (4 mg/l) were subcultured with lower concentration of

2,4-D (1 mg/l) on 30th d. These subcultured 60 d old embryogenic calli were transferred to regeneration medium (MS) containing various hormones (BAP, NAA, CW, 2,4-D).

2,4-D (mg/l)	0.5	0.5	0.5	0.5	-	-	-	-
NAA (mg/l)	-	-	-	-	1.0	1.0	2.0	2.0
BAP (mg/l)	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0
CW(ml/l)	-	-	10.0	10.0	-	-	-	-

3.3.2.5. Liquid culture

Auxin and cytokinins were used both individually and in combination to study the effect of somatic embryogenesis in suspension culture. Fresh anther calli (25 d old) induced by 2,4-D (4 mg/l) with coconut water (10 ml/l) of various genotypes (CO2, TMV12, TMV10, VG113, ICG-92 and Salemkai) were used in different hormone combinations with MS medium.

BAP (mg/l)	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
NAA (mg/l)	-	-	-	0.25	0.25	0.25	0.5	0.5	0.5	-	-	-
CW(ml/l)	-	-	-	-	-	-	-	-	-	10.0	10.0	10.0

3.3.2.6. Cytological studies

Fresh embryogenic calli of 30, 40 d old were fixed in carnoys solution (6 parts 95% ethanol; 3 parts chloroform and 1 part glacial acetic acid) for 24 h. Then the callus were transferred to 70% ethanol. The fixed materials were washed in distilled water for 5 min with 2 to 3 changes of water. The materials were hydrolysed in 1N HCl at 60°C for 8-10 min. The hydrolysed materials were washed in distilled water for 10 to 15 min. The materials were mordented in 4 per cent iron alum solution for 20 to 30 min.

The materials were washed thoroughly for 15 to 20 min with distilled water. Then the materials were stained with 0.5 per cent haematoxylin solution for 45 to 60 min. The stained materials were squashed on slides using a drop of 45 per cent acetic acid. Then these materials were mounted in DPX mountant and left for observation of haploid cells (Gould, 1984). The root tips of the regenerants were also studied by using acetic acid, ethanol (1:3) fixatives for confirmation of ploidy level.

3.3.3. Immature leaflet culture

Few seeds in each cultivars of CO2, TMV12, TMV10, VG113, M13 and Salemkai were raised under glass house conditions and immature leaflets from 12 d old seedlings were collected. The leaflets were sterilized with 0.5% mercuric chloride solution for 5 min and washed three times with sterile distilled water. The sterilized leaflets were cut into small pieces and inoculated two explants per tube. The culture tubes were kept in darkness (24 h) in culture room for induction of callus and somatic embryogenesis.

3.3.3.1. Callus induction

The immature leaflets of 12 d old seedlings were cultured in MS medium with 2,4-D alone or in combination with coconut water for induction of callus and other responses.

2,4-D (mg/l)	4.0	20.0	20.0
CW (ml/l)	-	-	10.0

3.3.3.2. Regeneration

Experiment No.1

The leaf callus obtained from MS medium containing 2,4-D (20 mg/l) on 25th d were subcultured in MS medium containing 2,4-D (3 mg/l) and sucrose (30 g/l). These subcultured calli (60 d old) of different genotypes were transferred to regeneration medium (MS) with different hormonal combinations.

2,4-D (mg/l)	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0
BAP (mg/l)	-	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0

Experiment No.2

In another experiment the leaf calli induced by 2,4-D (20 mg/l) were transferred to half-strength MS medium with different hormonal combinations.

BAP (mg/l)	0.5	0.5	0.5	0.5	0.5
NAA (mg/l)	-	0.5	0.5	0.5	0.5
CW (ml/l)	-	-	10.0	-	5.0
PM (ml/l)	-	-	-	10.0	5.0

3.3.4. Mature cotyledon culture

The mature seeds of CO2, TMV12, TMV10, VG113, M13 and Salemkai were collected 10 d after harvest of matured pods. The seeds were soaked in distilled water for 1 h to remove the outer testa of the seeds. These seeds were surface sterilized with 0.1% mercuric chloride solution for 15 min and rinsed three times with sterile water. The embryonal axis was carefully excised from the cotyledon and cultured separately. Initially the de-embryonated cotyledons were inoculated two explants per tube and later it was transferred to one per tube with a total of 20 explants per genotype. After inoculation the culture tubes were kept in dark (24 h) for three weeks and then placed in continuous light for 16 h followed by 8 h dark for induction of callus and somatic embryogenesis.

3.3.4.1. Callus induction

The de-embryonated cotyledons of six genotypes were cultured in MS medium supplemented with 2,4-D, 2,4-D combined with coconut water and picloram for somatic embryogenesis.

2,4-D (mg/l)	4.0	4.0	-
CW (ml/l)	-	10.0	-
pIC (mg/l)	-	-	4.0

Thirty d old non responsive de-embryonated cotyledons were cultured again in MS medium with 2,4-D (4 mg/l) for further response.

3.3.4.2. Regeneration

The 2,4-D (4 mg/l) induced de-embryonated cotyledon calli of different genotypes were subcultured on 30th d with reduced concentrations of 2,4-D (1 mg/l). These subcultured calli were transferred to different hormonal combinations of BAP, NAA, CW and pIC in MS medium for induction of somatic embryogenesis and plant regeneration.

BAP (mg/l)	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0	-
NAA (mg/l)	-	-	-	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0	-
CW (ml/l)	-	-	-	-	-	-	-	-	-	10.0	10.0	10.0	-
pIC(mg/l)	-	-	-	-	-	-	-	-	-	-	-	-	0.2

3.3.4.3. *In vitro* flowering

The 2,4-D (4 mg/l) induced de-embryonated calli of CO2, TMV10 and M13 were evaluated with MS medium containing 2,4-D and BAP for somatic embryogenesis and other flowering.

2,4-D (mg/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
BAP (mg/l)	-	0.5	1.0	1.5	2.0	2.5	3.0

3.3.5. Embryo axis culture

The embryo axis excised from the cotyledons were used for mature embryo axis culture. The mature embryo axes of different genotypes (CO2, TMV12, TMV10, VG113, M13 and Salemkai) were inoculated initially three explants per tube and then it was cultured one explant per tube with a total of 30 explants per genotype. The inoculated tubes were kept in the culture room at 24 h dark for three weeks and then placed at 16 h photoperiod and 8 h dark conditions. The temperature and relative humidity were maintained at 24 ± 2°C and 75 per cent respectively. The somatic embryos induced from mature embryo axes were utilized for different experiments.

3.3.5.1. Callus induction

The experiment was conducted with mature embryo axis of six genotypes in MS medium with 2,4-D, CW and pIC for induction of callus and somatic embryogenesis. The callus induction and shoot formation were recorded in each treatment on 15th and 30th d.

2,4-D (mg/l)	4.0	4.0	-
CW (ml/l)	-	10.0	-
pIC (mg/l)	-	-	4.0

3.3.5.2. Regeneration/Somatic embryo formation

The calli induced from mature embryo axis by various treatments of 2,4-D, 2,4-D with CW and pIC of different genotypes were subcultured separately on the respective medium on 30th d. At 45th d the respective calli were transferred to MS medium with NAA, BAP, CW and pIC for induction of somatic embryogenesis. The different responses obtained in each treatment on 60th d were recorded.

NAA (mg/l)	1.0	1.0	-
BAP (mg/l)	2.0	2.0	-
CW (ml/l)	-	10.0	-
pIC (mg/l)	-	-	0.1

3.4. Somatic embryogenesis

3.4.1. Auxins

Experiment No.1

The mature embryo axis excised from cotyledons of CO2, TMV12, VG113 and M13 were cultured in MS medium containing pIC (2, 4, 6 mg/l) for induction of somatic embryogenesis. The embryos produced in each treatment were recorded on 45th d.

Experiment No.2

The effect of two auxins (2,4-D, NAA) in MS medium containing sucrose (30 g/l) and agar (8 g/l) were studied using mature embryo axis of CO2, TMV12, VG113 and M13. The observations were recorded in each treatment on 45th d. The concentration of auxins are 2,4-D (4 mg/l), NAA (4 mg/l).

Experiment No.3

The combined effect of three auxins (2,4-D, NAA and pIC) with MS medium containing sucrose (30 g/l) and agar (8 g/l) were evaluated with mature embryo axis of CO2, TMV12, VG113 and M13 for somatic embryogenesis. The observations were recorded in each treatment on 45th d.

2,4-D (mg/l)	2.0	-	2.0
NAA (mg/l)	2.0	2.0	-
pIC (mg/l)	-	2.0	2.0

3.4.2. Nutrient media

The following nutrient media containing, pIC (4 mg/l) were used with mature embryo axes of CO2, TMV12, VG113 and M13 for somatic embryogenesis. The somatic embryoids developed in each treatment were recorded on 45th d.

MS, B5, Blaydes, N6

3.4.3. Explants

Four different explants *viz.*, anther, immature leaflets, mature de-embryonated cotyledons and mature embryo axis of cv. TMV12 were cultured in MS medium supplemented with pIC (4 mg/l) for induction of somatic embryogenesis. The observations were recorded on 45th d.

3.4.4. **Genotypes**

Six genotypes of three groups i.e., Spanish bunch (CO2, TMV12), Virginia bunch (TMV10, VG113) and Virginia runner (M13, Salemkai) were evaluated for induction of somatic embryogen^sis. The mature embryo axes of different genotypes were cultured in MS medium supplemented with pIC (4 mg/l). The per cent of induction and somatic embroys were recorded on 45th d.

3.4.5. Amino acids

Amino acids proline, glutamine and tryptophan were used at the concentrations of 25, 50, 100, 150 and 200 mg/l in MS medium containing pIC (4 mg/l). The mature embryo axis of cv. TMV12 was used to evaluate the effect of amino acids on somatic embryogenesis. The observations were recorded on 45th d.

3.4.6. Liquid culture

The 30 d calli of mature embryo axis (TMV12) induced by pIC (4 mg/l) were used in MS liquid medium with pIC (0.1 mg/l) and sucrose (30 g/l) for somatic embryogenesis. Subcultures were initiated every two weeks with same medium.

3.4.7. Embryo maturation

Experiment No.1

The developed dicotyledonary somatic embryos (35 to 45 d old culture) of VG113 were evaluated in different media without growth regulators for embryo germination. Sixty somatic embryos per treatment was inoculated.

MS, MS-half, B5, B5-half, Blaydes, Blaydes-half, N6, N6-half.

Experiment No.2

The induced dicotyledonary stage embryos of TMV12 were studied in different concentrations of sucrose (10, 20, 30, 40, 50, 60, 70 g/l) in half-strength MS medium and agar (8 g/l) for embryos germination. In each treatment forty somatic embryos were used. The germination frequencies were recorded.

Experiment No.3

In another experiment the developed dicotyledonary stage embryos of M13 were evaluated in half-strength MS medium with different growth regulators for embryos germination. In each treatment 30 embryos were inoculated.

NAA (mg/l)	0.5	0.5	1.0	1.0	2.0	0.5	0.5	0.5
BAP (mg/l)	0.5	1.0	1.5	3.0	3.0	-	-	-
Kn (mg/l)	-	-	-	-	-	1.0	1.5	2.0

3.4.8. Conversion of whole plantlets

The developed somatic embryos of TMV12, VG113 and M13 were allowed in maturation medium for ten days. After maturation, the embryos of different cultivars were evaluated in half-strength MS medium with different growth hormones for conversion of plantlets. The conversion frequency of whole plants in all treatments were recorded.

BAP (mg/l)	1.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0
NAA (mg/l)	-	-	0.5	0.5	-	-	0.5	0.5
TDZ (mg/l)	-	-	-	-	0.2	0.2	0.2	0.2

3.4.9. Histological studies

Microtome sections were taken following the procedure of Johansen (1940).

3.4.9.1. Materials

The embryogenic calli of cv. TMV12 collected during the culture period (7 d interval) upto the cotyledonary stage were used to confirm the formation of somatic embryos induced by picloram (4 mg/l) from mature embryonal axis.

Composition of FAA solution

Ethyl alcohol (95%)	-	50 ml
Glacial acetic acid	-	5 ml
Formalin	-	10 ml
Distilled water	-	35 ml

3.4.9.2. Fixation

FAA solution was used to fix the materials. The materials were fixed in the fixative for 18 h and then stored in 70% ethanol until dehydration process was started.

3.4.9.3. Dehydration

Fixed materials were passed through different level of tertiary butyl alcohol and ethanol as indicated in Table 5.

Table 5. Ethanol and tertiary butyl alcohol (TBA) for dehydration

S.No.	TBA-Ethanol-Wate mixture			
	TBA (ml)	100% ethanol (ml)	Distilled water (ml)	Duration (h)
1.	20	35	45	12
2.	35	35	30	12
3.	55	30	15	12
4.	75	25	-	12
5.	100	-	-	8
6.	100	-	-	8

After dehydration the embryogenic calli were given two changes with TBA each at eight hours interval. During the final change, a pinch of eosine was added to stain the specimen superficially so as to locate them inside the wax while embedding stage.

3.4.9.4. Infiltration and embedding

The dehydrated materials were transferred to a solution of liquid paraffin and TBA in 1:1 ratio for 1 h. Paraffin wax at 58°C was used for infiltration and embedding. The dehydrated materials along with TBA and liquid paraffin were transferred to a beaker containing wax. The materials were placed in the oven at 60°C for 12 h. Subsequent changes with fresh molten paraffin wax was administrated at 6 h intervals until all traces of TBA and paraffin were removed. The whole process was completed in 24 h. The materials were embedded in paraffin wax contained in a paper boat. After cooling, the edges of the paraffin block was trimmed to form a cube.

3.4.9.5. Microtoming

Uniformly thin ribbons of 10 μm thickness were cut with a rotary microtome and the ribbons were stretched out by placing them in warm water (40°C). These ribbons were cut into appropriate size and placed on the slide coated with a drop of Haupt's adhesive. These were flooded with 4% formalin solution. The slide was slightly warmed until the paraffin stretched to lie flat on the slide. Excess fluid was drained off and the slides were shade dried for 10-12 h in a dust free chamber.

3.4.9.6. Staining

Dried slides with ribbons were passed through xylene-ethanol series for dissolving the wax and hydrating the sections for staining. The sequence of solvent treatment and the period are given below.

Steps	**Min**
Xylene (I)	30
Xylene (II)	30
Xylene : Ethanol (1:1)	30
100% ethanol	10

95% ethanol	10
70% ethanol	10
50% ethanol	10
30% ethanol	10
10% ethanol	10
4% Ferrous ammonium sulphate	30
1% Erlich's hematoxylin (ripened for 30 d)	30
Washing in running tap water	10-15

The sections were stained with hematoxylin stain.

3.4.9.7. Dehydration and mounting

After staining, the sections were dehydrated in ethanol-xylene series.

Steps	Min
10% ethanol	5
50% ethanol	5
70% ethanol	5
100% ethanol	5
Ethanol : Xylene (1:1)	15
Xylene (I)	15
Xylene (II)	15

Then the sections were mounted with DPX mountant. Histological observations were made, in a day or two after the slides got dried.

3.5. Synthetic seeds

3.5.1. Production of synthetic seeds

3.5.1.1. Standardisation of sodium alginate and calcium chloride for beads formation

Different concentrations of sodium alginate (2.0, 2.5, 3.0, 3.5, 4.0%) solution was prepared by dissolving sodium alginate in boiling MS medium or double distilled water with constant stirring. Calcium chloride solution

(0.5, 0.7%) was prepared in double distilled sterilized water. The solutions were autoclaved at 15 psi for 20 minutes. Various combinations of sodium alginate (2.0, 2.5, 3.0, 3.5, 4.0%) and calcium chloride (0.5 and 0.7%) were tried to get firm beads following the procedure of Bapat and Rao (1989). The embryos (M13) with distinct cotyledonary stage were used for encapsulation. The embryos along with sodium alginate solution was added drop by drop by means of a dropper into a flask containing calcium chloride solution with continuous shaking. In this way the external layer of sodium alginate was complexed. The beads containing the embryos was kept in the solution for 30 minutes. Optimum concentration of sodium alginate which formed stable beads was selected. Later the beads were removed and washed in sterile distilled water 3-4 times, to remove all traces of calcium chloride.

3.5.2. Effect of sodium alginate concentration on germination

The encapsulated embryos by different concentrations of sodium alginate (2.5, 3.0, 3.5, 4.0%) with 0.7% calcium chloride were compared with fresh embryos for germination in half-strength MS medium containing BAP(1.0 mg/l), TDZ (0.2 mg/l), sucrose (30 g/l) and agar (8 g/l). Forty seeds per treatment were inoculated. The germination percentage of synthetic seeds in each treatments of the following were recorded.

3.5.3. Effect of media on conversion of whole plants

The following nutrient media were used to evaluate the rate of conversion of encapsulated and non-encapsulated embryos into whole plantlets. Thirty seeds each of encapsulated and non-encapsulated embryos were inoculated. The encapsulated embryos developing into plantlets were recorded in the following media.

MS, MS-half strength, N6, N6-half strength, B5, B5-half strength.

3.5.4. Effect of support media for conversion

The encapsulated embryos of M13 were tested in different media for conversion to whole plantlets. The number of d for root and shoot emergence and percentage of germination out of 20 seeds per treatment were recorded.

Liquid half strength MS medium

Agarised half strength MS medium

Agarised half strength MS + growth hormones

Filter paper wetted with water

Filter paper wetted with MS medium containing growth hormones

Agar only

Distilled water

3.5.5. Effect of storage of synthetic seeds

The somatic embryos encapsulated using MS medium and distilled water in their gel matrix were stored at 4°C for 7, 15, 30, 45 and 60 d. The viability test of these stored encapsulated embryos was done in germination medium (half strength MS with BAP, 1.0 mg/l and TDZ, 0.2 mg/l). Forty seeds per treatment were inoculated and kept under continuous light 16 h and dark 8 h for germination. The percentage of germinating seeds to form root and shoots was noted. The encapsulated (MS medium in gel matrix) and non-encapsulated embryos were stored aseptically at 4°C for 60 d and the germination test was conducted.

3.6. Cytological analysis of regenerants

The root tips of some of the regenerants were fixed with acetic acid : ethanol (1:3) for 24 h and the fixed root tips were transferred to 70% ethanol and cytological studies were carried out to confirm the regenerants from the somatic embryogenesis.

3.7. Statistical analysis

The frequency of induction of callus and somatic embryos on various treatment combinations of different cultivars were calculated as the ratio of the number of explants responding to total number of explants inoculated and was expressed in percentage as follows.

$$\text{Percentage of response} \quad \frac{\text{No. of explants responded}}{\text{No. of explants inoculated}} = \times 100$$

Number of somatic embryos and different developmental and morphological types of somatic embryos per individual explant was calculated. Conversion frequency of somatic embryos, synthetic seeds and fresh embryos on different media and growth hormones were calculated. All data collected were analysed for significance following the methods of Panse and Sukhatme (1967). The values recorded as percentage were transformed for analysis. Chi-square test was used to test the differences (Onay *et al.*, 1996) on escapsulated and non-encapsulated embryos stored at 4°C for 60 d.

Results

4. RESULTS

In vitro response of different explants *viz.*, anthers, immature leaflets, mature cotyledons and mature embryo axes of *Arachis hypogaea* L. (2n=40) and anthers of the wild species *Arachis pusilla* L. (2n=20) were evaluated with different levels of auxins and cytokinins individually and in combination with different media for callus induction, androgenic haploid, somatic embryogenesis. In addition, cytological and histological studies were made for confirmation. Statistical analysis with arcsine transformation were carried out.

4.1. Anther culture

4.1.1. Selection of flower buds

Results indicated that there exists significant variation for flower bud sizes in different groups of *Arachis* species (Table 6). The flower bud size (Plate I-1g) was smaller in *A. pusilla* (2.68 mm) at uninucleate stage when compared with spanish bunch (3.98 mm), virginia bunch (4.99 mm) and virginia runner (5.27 mm) of *A. hypogaea* (Plate I-1a to f). The colour of the anthers at uninucleate stage of microspores was always the same in different genotypes. Anthers bearing uninucleate pollen grains were light yellow, but flowers at earlier or later stages showed transparent, white and deep yellow anthers.

4.1.2. Effect of surface sterilants

Results showed a significant difference in the per cent contamination (Table 7). The lowest contamination (2.2%) was obtained by pre treatment of flower buds of cv. TMV12 at 4°C for 2 d followed by surface sterilization with 70 per cent ethanol for 1 min and then sterilization with 0.05 per cent $HgCl_2$ for 10 min. The control without pre treatment had 100 per cent contamination.

Table 6. Size of flower buds and colour of anthers in glass house grown groundnut

Stage of development	Colour of anthers	Size of flower buds (mm)*									
		Arachis hypogaea									*Arachis pusilla* (t_4)
		Spanish bunch			Virginia bunch			Virginia runner			
		CO2	TMV12	Mean (t_1)	TMV10	VG113	Mean (t_2)	ICG92	Salemkai	Mean (t_3)	
MEIOSIS											
Early stages	Transparent	2.25	2.26	2.26	2.85	2.87	2.86	3.26	3.28	3.27	1.80
Tetrad	White	2.56	2.58	2.57	3.44	3.48	3.46	3.62	3.62	3.62	2.20
POLLEN GRAINS											
Uninucleate	Light yellow	3.97	3.98	3.98	4.99	5.00	4.99	5.26	5.28	5.27	2.68
Binucleate	Yellow	4.88	4.90	4.89	5.96	5.98	5.97	6.88	6.90	6.89	2.92
Mature	Deep yellow	5.62	5.64	5.63	6.24	6.26	6.26	7.28	7.30	7.29	3.20
SE mean				0.23			0.23			0.30	0.19

t (1,2) = 2.770*
t (1,3) = 2.827*
t (1,4) = 2.243@

t (2,4) = 2.612*
t (3,4) = 2.562*
t (2,3) = 2.210@

* Mean of ten flower bud size
* Significant at 5% level
@ Not significant at 5% level

PLATE I. Flower bud size and induction of anther callus in different cultivars of *Arachis*

1. Appropriate flower bud size for anther culture

   a. CO 2 b. TMV12 c. TMV10 d. VG113
   e. ICG92 f. Salemkai g. *A. pusilla*

2. Inoculated anthers for callus induction

3. 2,4-D (2 mg/l) induced anther callus

   a. CO 2 b. TMV12 c. ICG92 d. TMV10

4. 2,4-D (4 mg/l) induced anther callus

   a. TMV10 b. TMV12 c. ICG92

5. 2,4-D (2 mg/l) + CW (10 ml/l) induced anther callus

   a. ICG92 b. Salemkai c. *A. pusilla*

6. 2,4-D (4 mg/l) + CW (10 ml/l) induceo anther callus

   a. CO 2 b. TMV12 c. ICG92 d. *A. pusilla*

7. 2,4-D (4 mg/l) + CW (5 ml/l) + PM (5 ml/l) induced anther callus

   a. TMV12 b. VG113 c. ICG92

8. 2,4-D (2 mg/l) + CW (5 ml/l) + PM (5ml/l) induced anther callus

   a. TMV10

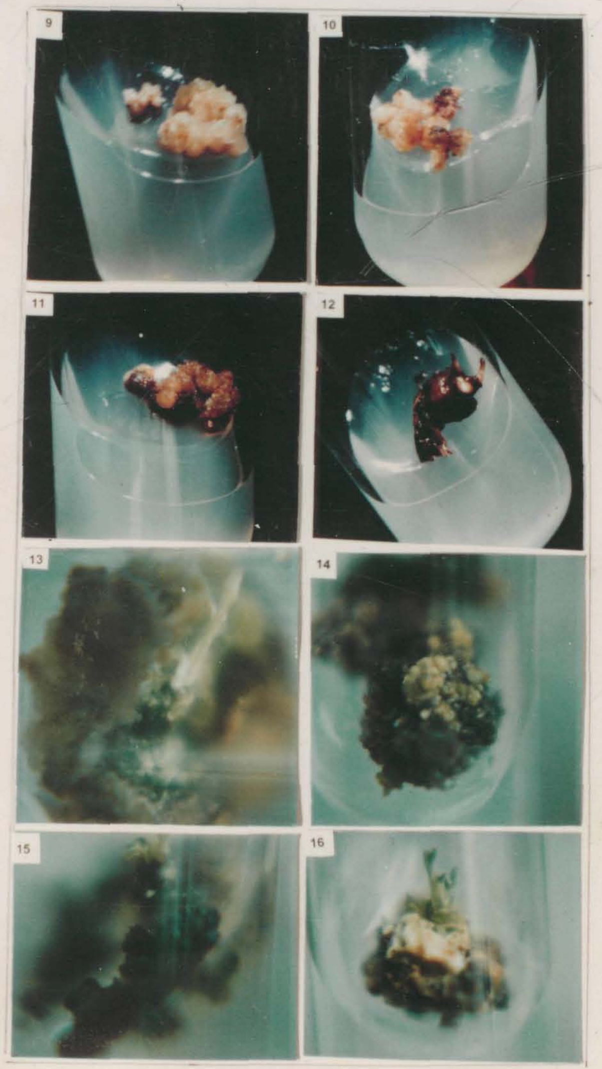
PLATE II. Development of embryoids in anther callus and plant regeneration

Callus induction medium	:	MS + 2,4-D (4 mg/l) + CW (10 ml/l)
Regeneration medium	:	N6 + BAP (0.5 mg/l) + NAA (1 mg/l) + Glutamine (3.5 g/l)

9. TMV12 - Proliferation of callus with differentiation
10. *A. pusilla* - Globular stage of embryoids
11. *A. pusilla* - Advance stage of globular embryoids
12. Salemkai - Shoot initiation from embryoids

Callus induction medium	:	MS + 2,4-D (4 mg/l)
Regeneration medium	:	MS + BAP (0.5 mg/l) + NAA (2 mg/l)

13. Salemkai - Shoot initiation from embryoids
14. *A.pusilla* - Clusters of somatic embryoids
15. *A.pusilla* - Germination of embryoids
16. *A.pusilla* - Regeneration of plantlets from embryoids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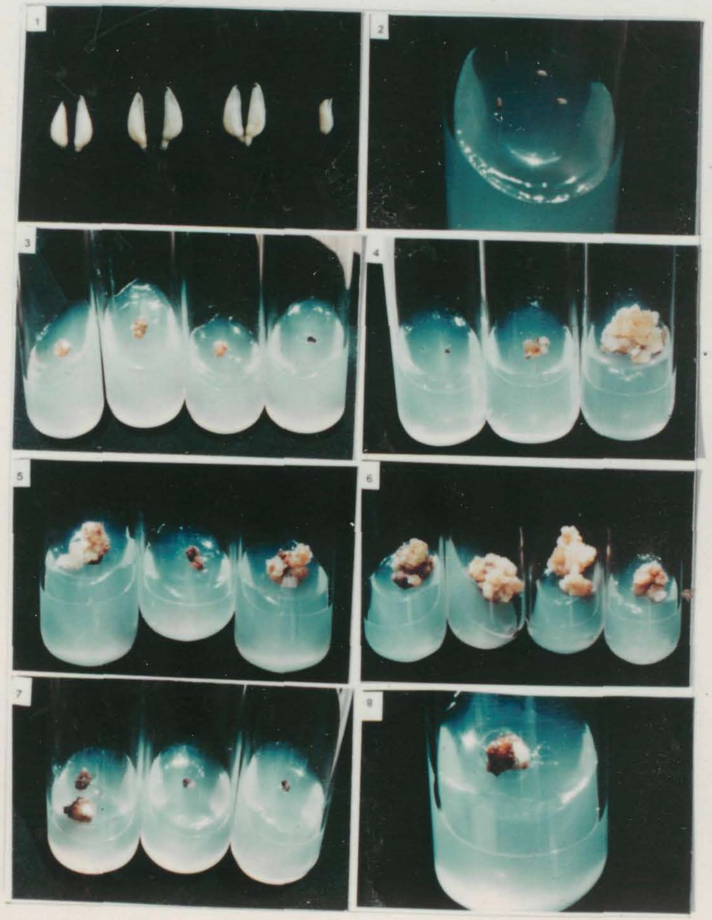
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Table 7. Effect of surface sterilants on anther culture of cv. TMV12

Treatments			Mercuric chloride treatment time				
Pre-treatment at 4°C (d)	Ethanol (%)	$HgCl_2$ (%)	2 min	5 min	10 min	15 min	Mean
			Contamination (%)				
-	-	0.05	98.2 (82.3)	97.8 (81.5)	95.2 (77.3)	98.2 (82.3)	97.3 (80.5)
-	-	0.10	97.2 (80.4)	96.8 (79.7)	90.6 (72.1)	93.2 (74.9)	94.4 (76.3)
-	70	0.05	92.2 (73.8)	90.2 (71.8)	88.2 (69.9)	89.2 (70.8)	89.9 (71.5)
-	70	0.10	90.8 (72.3)	87.2 (69.0)	80.6 63.9)	82.6 (65.3)	85.3 (67.5)
2	70	0.05	32.2 (34.6)	28.6 (32.3)	2.2 (8.5)	26.2 (30.8)	22.3 (28.2)
2	70	0.10	48.4 (44.1)	45.2 (42.2)	41.8 (40.3)	46.2 (42.8)	45.4 (42.4)
Control	-	-	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)
F-test					*		
SEd					0.682		
CD					1.668		

* Significant at 5% level ; Figures in parentheses indicate arcsine values

4.1.3. Callus induction

4.1.3.1. Effect of different media

Six genotypes of *A. hypogaea* were evaluated for callusing in five media *viz.*, MS, Blaydes, B5, N6 and Nitsch supplemented with 2,4-D (4 mg/l).

The mean values for anther callusing frequency revealed that MS medium recorded the higher callus induction (40.1%) than the other media tested (Table 8). Blaydes medium recorded the least callus induction (13.1%), B5, N6 and Nitsch media recorded 16.0, 16.9 and 22.4 per cent callus induction. Among the varieties, TMV12 showed comparatively higher callus induction (26.7%) followed by CO2 (23.2%). The local cv. Salemkai gave the least callus induction (17.4%).

The TMV12 anthers in MS medium gave the maximum callus induction (51.6%) followed by CO2 (48.6%). The local cv. Salemkai on Blayde's medium gave as low as 10 per cent callus induction. The callus induction of CO2, TMV12, TMV10 and VG113 in MS medium were significantly different from other media.

4.1.3.2. Effect of 2,4-D, coconut water and palmyrah milk

The experiment was conducted with twelve hormone combinations each at three levels of 2,4-D alone and in combinations with coconut water and palmyrah milk for callus induction by anthers of *A. hypogaea* (six genotypes) and wild species. The per cent of callus induction and fresh weight of callus are presented in Tables 9 and 10.

Significant differences observed between hormonal combinations, genotypes and interaction effects for induction of callus from anthers (Table 9). The combination of 2,4-D (4 mg/l) and coconut water (10 ml/l) recorded significantly higher per cent of callus induction (61.1) followed by 2,4-D (3 mg/l)

Table 8. Effect of different media and varieties on callus induction in anther culture (2,4-D, 4 mg/l)

Media	Callusing (%)						
	CO2	TMV12	TMV10	VG113	ICG92	Salemkai	Mean
MS	48.6	51.6	37.6	39.7	32.6	30.3	40.1
	(44.2)	(45.9)	(37.8)	(39.1)	(34.8)	(33.4)	(39.3)
Blaydes	12.6	16.8	11.2	13.2	14.8	10.0	13.1
	(20.8)	(24.2)	(19.5)	(21.3)	(22.6)	(18.4)	(21.2)
B5	15.2	18.7	16.2	16.2	17.3	12.3	16.0
	(22.9)	(25.6)	(23.7)	(23.7)	(24.6)	(20.5)	(23.6)
N6	16.6	20.8	15.2	16.8	18.2	13.9	16.9
	(24.0)	(27.1)	(22.9)	(24.2)	(25.2)	(21.9)	(24.3)
Nitsch	22.8	25.8	20.0	21.8	23.6	20.6	22.4
	(28.5)	(30.5)	(26.6)	(27.8)	(29.1)	(26.9)	(28.2)
Mean	23.2	26.7	20.6	21.5	21.3	17.4	21.7
	(28.8)	(31.1)	(26.9)	(27.6)	(27.5)	(24.6)	(27.8)

Source	SEd	CD at 5%
Genotype	0.880	2.262
Media	2.201	6.109
Genotype x Media	6.912	14.135

Figures in parentheses indicate arcsine values

Table 9. Effect of 2,4-D, CW and PM in MS medium on anther callus induction (%)

Treatment			*Arachis hypogaea*									*Arachis pusilla*	Overall mean
			Spanish bunch			Virginia bunch			Virginia runner				
2,4-D (mg/l)	CW (ml/l)	PM (ml/l)	CO2	TMV12	Mean	TMV10	VG113	Mean	ICG92	Salemkai	Mean		
2	-	-	20.4	25.2	22.8	18.2	22.2	29.2	22.3	15.8	19.0	16.2	20.0
			(26.8)	(30.1)	(28.5)	(25.2)	(28.1)	(26.7)	(28.2)	(23.4)	(25.8)	(23.7)	(26.6)
3	-	-	31.2	35.8	33.5	29.2	22.3	25.7	32.2	26.2	29.2	25.2	28.9
			(34.0)	(36.7)	(35.4)	(32.7)	(28.2)	(30.5)	(34.6)	(30.8)	(32.7)	(30.1)	(32.5)
4	-	-	36.4	43.3	39.8	33.5	38.2	35.8	38.2	30.3	34.2	30.2	35.7
			(37.1)	(41.1)	(39.1)	(35.4)	(38.2)	(36.7)	(38.2)	(33.4)	(35.8)	(33.3)	(36.7)
2	10	-	38.2	44.6	41.4	36.2	40.2	38.2	39.2	32.6	35.9	33.2	37.7
			(38.2)	(41.9)	(40.0)	(37.0)	(39.3)	(38.2)	(38.8)	(34.8)	(36.8)	(35.2)	(37.9)
3	10	-	41.2	49.2	45.2	42.0	43.2	42.6	39.2	34.6	36.9	35.4	40.7
			(39.9)	(44.5)	(42.2)	(40.4)	(41.1)	(40.7)	(38.8)	(36.0)	(37.4)	(36.5)	(39.6)
4	10	-	75.3	84.8	80.0	54.2	59.2	56.7	58.3	50.4	54.3	45.3	61.1
			(60.2)	(67.0)	(63.4)	(47.4)	(50.3)	(48.8)	(49.8)	(45.2)	(47.5)	(42.3)	(51.4)
2	-	10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
			(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)
3	-	10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
			(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)
4	-	10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
			(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)	(4.5)
2	5	5	22.2	26.2	24.2	13.6	15.8	14.7	14.2	12.4	13.3	10.8	16.4
			(28.1)	(30.8)	(29.5)	(21.6)	(23.4)	(22.5)	(22.1)	(20.6)	(21.4)	(19.2)	(23.9)
3	5	5	27.8	31.8	29.8	14.2	16.8	15.5	15.8	13.2	14.5	12.2	18.8
			(31.8)	(34.3)	(33.1)	(22.1)	(24.2)	(23.2)	(23.4)	(21.3)	(22.4)	(20.4)	(25.7)
4	5	5	33.3	35.2	34.2	16.2	19.2	17.7	17.2	15.2	16.2	14.2	21.5
			(35.2)	(36.4)	(35.6)	(23.7)	(26.0)	(24.9)	(24.9)	(22.9)	(23.7)	(22.1)	(27.8)
	Mean		27.2	31.3	29.2	21.4	23.1	22.2	23.0	19.2	21.1	18.5	23.4
			(31.4)	(34.0)	(32.7)	(27.6)	(28.7)	(28.1)	(28.7)	(26.0)	(27.3)	(25.5)	(28.9)

Source	SEd	CD (5%)
Genotype	0.770	1.884
Treatment	0.952	2.095
Genotype x Treatment	1.321	2.615

Figures in parentheses indicate arcsine values

with coconut water (10 ml/l) which recorded 40.7 per cent callus induction (Fig.1). The callus induction by 2,4-D alone (4 mg/l) and 2,4-D (2 mg/l) with coconut water (10 ml/l) were statistically on par. The lowest per cent (16.4) of callus induction occurred in media containing 2,4-D (2 mg/l), coconut water (5 ml/l) and palmyrah milk (5 ml/l). The 2,4-D combined with palmyrah milk did not have any impact.

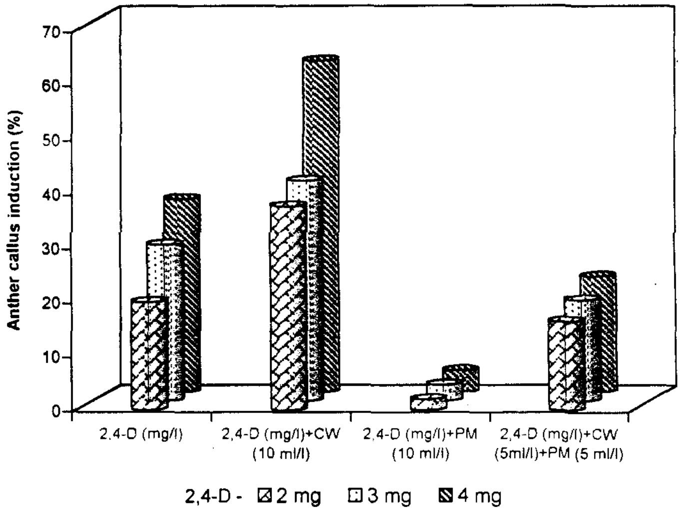
The cultivated genotypes of *A. hypogaea* responded significantly higher callus induction (24.2%) than the wild species (18.5%). Among the three groups of groundnut cultivars tested, Spanish bunch group (TMV12, CO2) responded significantly higher callus induction (29.2%) than virginia bunch (22.2%) and virginia runner (21.1%) and the latter two groups were statistically on par.

The anthers from spanish bunch cultivars in medium containing 2,4-D (4 mg/l) and coconut water (10 ml/l) showed significantly higher callus induction in TMV12 (84.8%) followed by CO2 (75.3%). The order of callus induction in different genotypes were TMV12 > CO2 > VG113 > ICG92 > TMV10 > Salemkai > *A. pusilla.* The time taken for initiation of callus varied from 16 to 24 d in different treatments.

There were genotypic differences in the nature of calli and proliferation. The light brown embryogenic calli were observed in spanish bunch (CO2, TMV12), virginia runner (ICG92, Salemkai) and wild species, whereas non embryogenic compact, dark brown and pale green callus observed in virginia bunch (TMV10 and VG113) when 2,4-D was used alone at 2 to 4 mg/l. The growth of callus was slow in lower concentration (Plate I-3) compared with higher concentration of 2,4-D (4 mg/l) (Plate I-4).

When coconut water at 10 ml/l was used along with three levels of 2,4-D, friable embryogenic callus mass was obtained (Plate I-5,6) in all

Fig.1. Effect of 2,4-D, CW and PM on anther callus induction



genotypes except cv. TMV10. Highly embryogenic proliferating of calli (Plate I-6) were obtained in medium containing 2,4-D (4mg/l) and CW (10 ml/l) than 2,4-D alone (Plate I-3,4) or 2,4-D combined with CW and PM (Plate I-7,8). The 2,4-D with coconut water and palmyrah milk produced dark brown, non embryogenic calli with very slow growth (Plate I-7,8).

Significant differences were observed for fresh weight of callus (Table 10). Medium containing 2,4-D (4 mg/l) and CW (10 ml/l) was found to be significantly superior in respect of callus production. Among the genotypes, TMV12 gave as high as 0.76g fresh weight of callus (mean weight) followed by CO2 (0.64 g). These genotypes significantly differed from virginia bunch (0.56g) and virginia runner cultivars (0.42g) on callus weight. The lowest callus weight (0.31g) was recorded in wild species. Highest fresh callus weight (1.45g) was observed in TMV12.

4.1.3.3. Effect of picloram

The experiment was conducted with different level of picloram (0.5 to 4.0 mg/l) for induction of callus. The differences in induction of calli were significant in various concentrations of picloram (Table 11). After three weeks of culturing, 7.2 to 22.5 per cent of the cultured anthers responded to produce white and light brown calli. Increase in callusing per cent was observed as the doses of picloram increased upto 2.0 mg/l (22.5%) and thereafter no proportionate increase was evident. The per cent of callus induction on overall mean ranged from 8.4 to 16.3 in different genotypes. Higher per cent of callus induction was observed in spanish bunch cultivars (15.8%) compared to *A. pusilla* (8.7%). In general, picloram treatment was not effective for callus induction when compared to 2,4-D (Fig.2). Moreover, when subcultures were done two to three times, no embryos formed from the callus.

Table 10. Effect of 2,4-D, CW and PM in MS medium on callus fresh weight

Treatment			Callus fresh weight (g) (30 d after inoculation)										
			Arachis hypogaea									*Arachis pusilla*	Overall mean
			Spanish bunch			Virginia bunch			Virginia runner				
2,4-D (mg/l)	CW (ml/l)	PM (ml/l)	CO2	TMV12	Mean	TMV10	VG113	Mean	ICG92	Salemkai	Mean		
2	-	-	0.41	0.56	0.48	0.36	0.40	0.38	0.27	0.22	0.24	0.16	0.34
3	-	-	0.52	0.67	0.59	0.44	0.48	0.46	0.31	0.26	0.28	0.20	0.41
4	-	-	0.62	0.76	0.69	0.53	0.59	0.56	0.40	0.31	0.35	0.24	0.49
2	10	-	0.74	0.90	0.82	0.65	0.70	0.67	0.52	0.43	0.47	0.32	0.61
3	10	-	0.90	1.07	0.98	0.74	0.88	0.81	0.63	0.52	0.57	0.40	0.73
4	10	-	1.30	1.45	1.37	0.94	1.10	1.02	0.84	0.73	0.78	0.61	0.99
2	5	5	0.40	0.45	0.42	0.34	0.37	0.35	0.35	0.30	0.32	0.26	0.35
3	5	5	0.44	0.49	0.46	0.38	0.40	0.39	0.39	0.35	0.37	0.30	0.39
4	5	5	0.48	0.51	0.49	0.42	0.44	0.43	0.43	0.39	0.41	0.32	0.43
	Mean		0.64	0.76	0.70	0.53	0.59	0.56	0.46	0.39	0.42	0.31	0.52

Source	SEd	CD (5%)
Genotype	0.021	0.051
Treatment	0.032	0.073
Genotype x Treatment	0.042	0.083

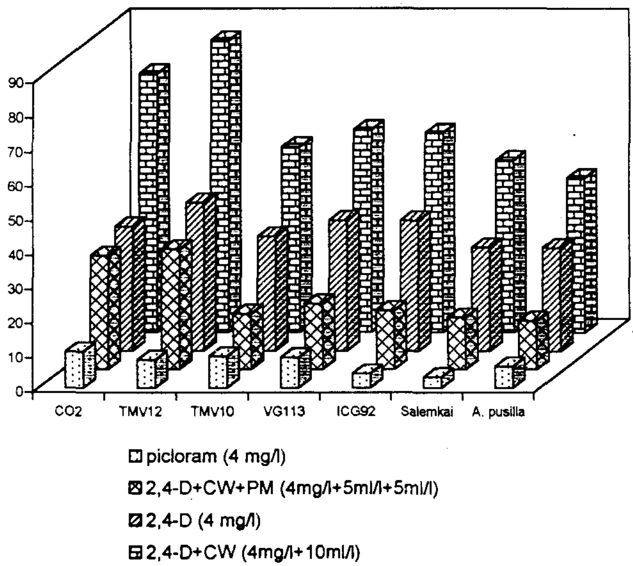
Table 11. Effect of picloram in MS medium on callus induction from anthers

Treatment	Callusing (%)										
	Arachis hypogaea									*Arachis pusilla*	Overall mean
	Spanish bunch			Virginia bunch			Virginia runner				
pIC (mg/l)	CO2	TMV12	Mean	TMV10	VG113	Mean	ICG92	Salemkai	Mean		
0.5	8.4 (16.8)	10.2 (18.6)	9.3 (17.8)	6.4 (14.6)	7.2 (15.6)	6.8 (15.1)	5.8 (13.9)	4.2 (11.8)	5.0 (12.9)	2.2 (8.5)	6.3 (14.5)
1.0	10.3 (18.7)	11.4 (19.7)	10.8 (19.2)	9.3 (17.8)	10.4 (18.8)	9.8 (18.2)	8.2 (16.6)	9.6 (18.0)	8.9 (17.4)	6.2 (14.4)	9.3 (17.8)
1.5	15.2 (22.9)	16.3 (23.8)	15.7 (23.3)	13.2 (21.3)	15.6 (23.3)	14.4 (22.3)	12.4 (20.6)	11.2 (19.5)	11.8 (20.0)	8.2 (16.6)	13.1 (21.2)
2.0	28.4 (32.2)	30.4 (33.5)	29.4 (32.8)	26.3 (30.8)	29.3 (32.8)	27.8 (31.8)	15.6 (23.3)	13.2 (21.3)	14.4 (22.3)	14.2 (22.1)	22.5 (28.3)
2.5	16.4 (25.4)	20.6 (26.9)	19.5 (26.2)	17.2 (24.5)	17.8) 24.9)	17.5 (24.7)	12.2 (20.4)	11.0 (19.4)	11.6 (19.9)	13.2 (21.3)	15.8 (23.4)
3.0	16.8 (24.2)	18.8 (25.7)	17.8 (24.9)	15.6 (23.6)	14.8 (22.6)	15.2 (22.9)	10.8 (19.2)	8.2 (16.6)	9.5 (17.9)	10.8 (19.2)	13.7 (21.7)
3.5	15.4 (23.1)	14.6 (22.5)	15.0 (22.8)	12.0 (20.3)	13.2 (21.3)	12.6 (20.8)	8.2 (16.6)	6.8 (15.1)	7.5 (15.9)	8.2 (16.6)	11.2 (19.5)
4.0	10.6 (19.0)	8.2 (16.6)	9.4 (17.8)	9.2 (17.7)	8.9 (17.4)	9.0 (17.5)	4.2 (11.8)	3.2 (10.3)	3.7 (11.1)	6.3 (14.5)	7.2 (15.6)
Mean	15.4 (23.1)	16.3 (23.8)	15.8 (23.4)	13.6 (21.6)	14.6 (22.5)	14.1 (22.1)	9.7 (18.1)	8.4 (16.8)	9.0 (17.5)	8.7 (17.1)	12.4 (20.6)

Source	SEd	CD (5%)
Genotype	0.423	1.035
Treatment	1.062	2.511
Genotype x Treatment	0.985	1.973

Figures in parentheses indicate arcsine values

Fig.2. Effect of 2,4-D, coconut water, palmyrah milk and picloram on callus induction from anthers



4.1.3.4. Effect of 2,4-D, coconut water, light and dark condition

This experiment was conducted with 16 h photoperiod and 24 h dark conditions. There was significant difference in the induction of callus from anthers incubated at 16 h photoperiod (1000 lux) and 24 h dark conditions. The mean per cent of callus induction was higher in 16 h photoperiod (37.1%) compared to 24 h dark conditions (24.1%). Highest response for callus induction was observed in 2,4-D (4 mg/l) combined with coconut water (10 ml/l) both at 16 h photoperiod (60.6%) and 24 h dark conditions (32.8%). Genotypic differences were significant for callusing at both the conditions.

In general callus induction per cent was high in all the genotypes incubated at 16 h photoperiod compared to 24 h dark conditions (Fig.3). Among the genotypes tested, spanish bunch cultivars TMV12 (46.5%, 31.6%) and CO2 (40.2% 28.3%) recorded the highest callus induction at both the conditions, whereas lowest per cent of callus induction was observed in *A. pusilla* (32.2%; 18.0%). However, most of the genotypes produced embryogenic calli mass from anthers incubated at 24 h dark condition compared to 16 h photoperiod.

The time taken for initiation of callus from anthers varied from 15 (CO2) to 21 d (Salemkai) in 16 h photoperiod and 20 (CO2) to 26 d (Salemkai) at 24 h dark condition in different treatments of 2,4-D and coconut water (Fig.4). The cultured anthers incubated at 24 h dark required more days for initiation of callus (24 d) than the 16 h photoperiod (19 d).

4.1.4. Regeneration of callus

4.1.4.1. Effect of NAA, pIC, BAP, CH, glutamine and proline

The subcultured 60 d old embryogenic calli were transferred to N6 medium with different concentrations of NAA, pIC, BAP, CH, glutamine and proline for redifferentiation and regeneration ability. After two weeks in the

Fig.3. Effect of light and dark conditions on callus induction in anthers

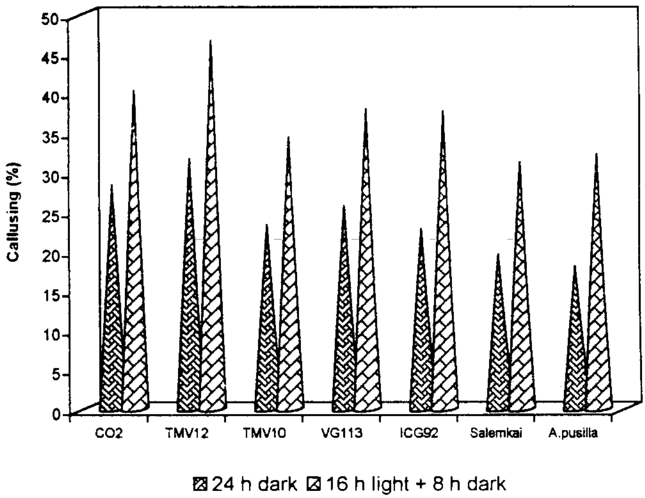
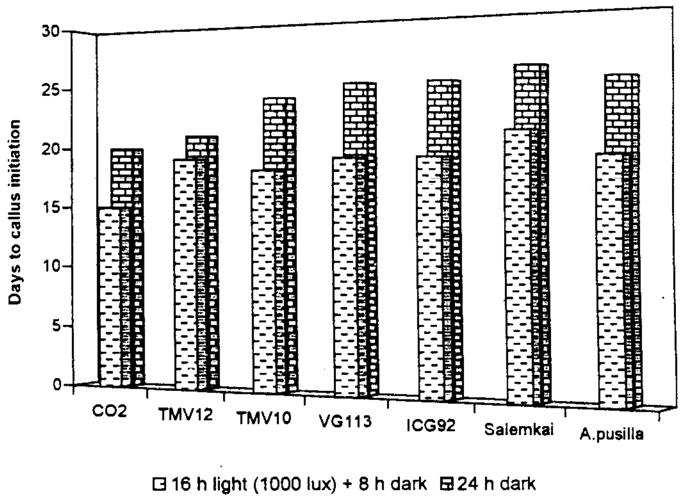


Fig.4. Effect of light and dark conditions on days to callus initiation in anthers



regeneration medium, proliferation of callus was observed in most of the genotypes. However differentiation in TMV12 (Plate II-9), embryoids formation in *A. pusilla* (Plate II-10,11) and shoot initiation in cv. Salemkai (Plate II-12) were observed in the N6 medium supplemented with BAP (0.5 mg/l), NAA (1 mg/l) and glutamine (3.5 g/l). The plant regeneration did not occur from TMV12, Salemkai and *A. pusilla* .

4.1.4.2. Effect of BAP, NAA and their combinations

Transfer of 60 d old embryogenic calli to MS medium supplemented with BAP (0.5 mg/l), NAA (2.0 mg/l) and sucrose (30 g/l) resulted in differentiation and production of embryoids in the callus of cv. Salemkai and wild diploid species.

Embryoids developed from the callus of wild diploid species were found in clusters (Plate II-14) than the cultivated cv. Salemkai. It was difficult to estimate the per cent of embryo induction and maturation in wild species because the embryoids were formed in clusters. The local cv. Salemkai produced only 2 to 3 embryoids. Embryoids at developmental stages (globular, heart shaped) were noticed in the medium because the pathway of embryogenesis was asynchronous. The mature embryoids regenerated into whole plants (Plate II-15,16) and three plants regenerated in the *A. pusilla*. The cv. Salemkai produced only shoot from embryoids and did not develop into whole plant (Plate II-13). The anther calli of spanish bunch (CO2, TMV12), virginia bunch (TMV10, VG113) and virginia runner (ICG92) do not show any differentiation and formation of embryoids in the regeneration medium.

4.1.4.3. Use of liquid medium

The calli (25 d old) induced by MS medium with 2,4-D (4 mg/l) alone or 2,4-D in combination with coconut water (10 ml/l) of various genotypes were transferred to liquid MS medium with BAP and NAA supplements for induction of embryogenesis. After subculturing every two weeks in the same medium, the compact cell aggregates became smaller and finely dispersed in the medium.

After four months of continuous culture, a fine suspension of white callus masses were observed when NAA was present at the concentration of 0.25 mg/l along with 0.5 to 1.5 mg/l of BAP and coconut water (10 ml/l). Embryoid like structures when transferred to germination medium, did not germinate.

4.2. Immature leaflet culture

4.2.1. Effect of 2,4-D and coconut water on callus induction

The immature leaflets of 12 d old seedlings of six genotypes were cultured with 2,4-D alone (4, 20 mg/l) and in combination with coconut water (10 ml/l) for induction of callus and other morphogenetic responses.

Significant differences were observed in per cent callus induction in different genotypes (Table 12). In the lower concentration of 2,4-D (4 mg/l) induction of callus from spanish bunch (Plate III-17b) was highest (66.5%), whereas lowest response on callus induction was observed in virginia bunch (16.7%) and virginia runner (16.6%) and these two groups were statistically on par. The genotypes TMV10 (Plate III-17a) and M13 do not produce callus at 4 mg/l of 2,4-D, but produced callus at higher concentration of 2,4-D (20mg/l) (Plate III-18).

In higher concentration of 2,4-D (20 mg/l) alone or 2,4-D combined with coconut water (10 ml/l), higher per cent on callus induction was noticed (Fig.5) in virginia bunch (92.8; 93.1) and virginia runner (88.7; 91.6) and these cultivars were statistically on par but significantly superior to spanish bunch

Table 12. Effect of 2,4-D and CW on callus induction in immature leaflets

Genotype	Callusing (%)			Mean	Response (20[th] d after inoculation)					
					Nature of the callus			Proliferation of the callus		
	2,4-D (4 mg/l)	2,4-D (20 mg/l)	2,4-D+CW (20 mg/l+10 ml/l)		2,4-D (4 mg/l)	2,4-D (20 mg/l)	2,4-D+CW (20 mg/l+10 ml/l)	2,4-D (4 mg/l)	2,4-D (20 mg/l)	2,4-D+CW (20 mg/l+10 ml/l))
CO2	66.7 (54.1)	28.6 (31.0)	40.0 (39.2)	45.1 (42.2)	CBP	FLB	LBP	High	Slow	Slow
TMV12	65.6 (54.1)	57.1 (49.1)	54.5 (47.6)	59.1 (50.2)	CLB	FW	LBP	High	Slow	Slow
TMV10	0.0 (4.5)	100.0 (90.0)	100.0 (90.0)	66.7 (54.8)	CLB	FW	LBP	Slow	High	High
VG113	33.4 (33.3)	85.7 (67.8)	86.2 (68.2)	68.4 (55.8)	CLB	FW	LBP	Slow	Moderate	Moderate
M13	0.0 (4.5)	93.3 (75.0)	100.0 (90.0)	64.4 (53.4)	CW	FLB	LBP	Slow	High	High
Salemkai	30.2 (33.3)	84.2 (66.6)	83.2 (65.8)	65.8 (54.2)	CW	FW	LBP	Slow	High	High
Mean	32.6 (34.8)	64.8 (60.0)	77.3 (61.5)	61.8 (51.8)	CBW					
Mean (SB)	66.5	42.8	47.2	52.1						
Mean (VB)	16.7	92.8	93.1	67.5						
Mean (VR)	16.6	88.7	91.6	65.6						

Source	SEd	CD (5%)
Genotype	5.584	13.664
Treatment	5.651	24.316
Genotype x Treatment	2.953	6.230

CBP : Compact Brown Powdery
CLB : Compact Light Brown
CW : Compact White
FLB : Friable Light Brown
FW : Friable White
LBP : Light Brown Powdery
SB Spanish Bunch
VB : Virginia Bunch
VR : Virginia Runner

Figures in parentheses indicate arcsine values

PLATE III. Induction of callus and plant regeneration in immature leaflets

17.	a. TMV10	-	No response on callus induction in 2,4-D at 4 mg/l
	b. CO2	-	Callus induction in 2,4-D at 4 mg/l
18.	TMVI0	-	Callus induction in high dose of 2,4-D at 20 mg/l
19.	VG113	-	Embryogenic leaf callus in MS + 2,4-D (20 mg/l)
20.	VG113	-	Globular stage of somatic embryos in MS + 2,4-D (0.5 mg/l) + BAP (1.5 mg/l)
21.	Salemkai	-	Globular stage of somatic embryos in MS + 2,4-D (0.5 mg/l) + BAP (1.5 mg/l)
22.	Salemkai	-	Regeneration of plantlets from somatic embryos (MS + NAA, 0.5 mg/l + BAP, 0.5 mg/l)
23.	Salemkai	-	Rhizogenesis in ½ MS + NAA (0.5 mg/l) + BAP (0.5 mg/l) + CW (10 ml/l)
24.	Salemkai	-	Compact, white powdery mass in ½ MS + NAA (0.5 mg/l) + BAP (0.5 mg/l) + PM (10 ml/l)

PLATE IV. Induction of callus and direct shoot and root formation in de-embryonated cotyledons

25.	a. Salemkai	-	Root formation	MS + 2,4-D (4 mg/l)
	b. TMV12	-	Brown callus formation	
26.	a. CO2	-	No response for callus induction in MS alone	
	b. CO2	-	Shoot and root formation in MS + 2,4-D (4 mg/l)	
27.	M13	-	Direct plantlets formation with roots in cotyledons (MS + 2,4-D, 4 mg/l)	
28.	M13	-	Direct embryogenesis from subcultured de-embryonated cotyledons in MS + 2,4-D (4 mg/l)	
29.	M13	-	Direct plantlets formation with roots in subcultured de-embryonated cotyledons in MS + 2,4-D (4 mg/l)	
30.	Salemkai	-	Direct plantlets formation with roots in subcultured de-embryonated cotyledons in MS + 2,4-D (4 mg/l)	

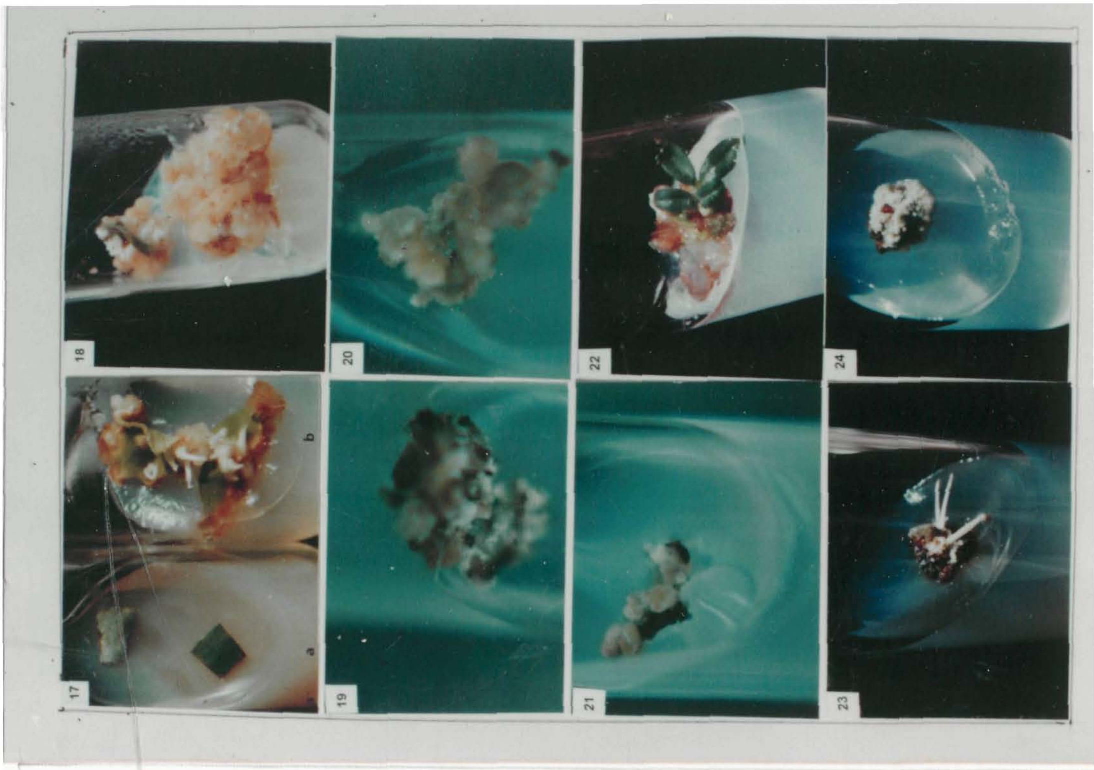

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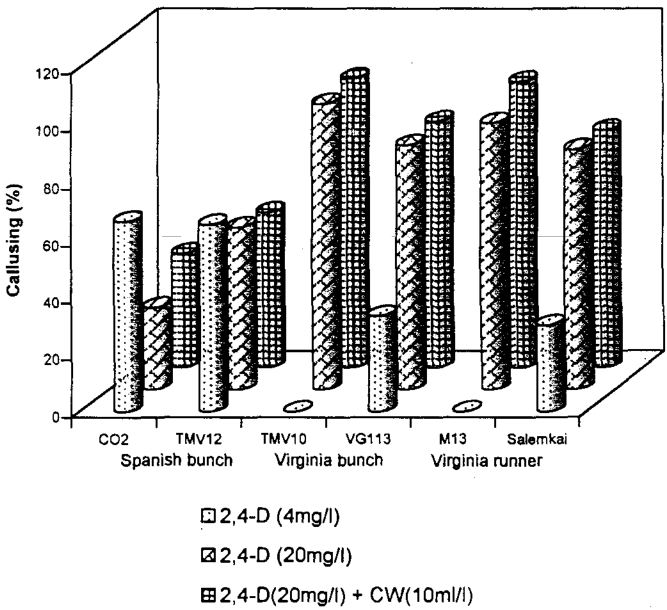
Fig.5. Effect of 2,4-D and CW on callus induction in immature leaflets



(42.8%; 47.2%). At lower concentration of 2,4-D (4mg/l), compact, brown, light brown, white and powdery callus masses were observed. But at higher concentration of 2,4-D, friable, light brown, white and embryogenic callus were obtained. Most of the genotypes produced light brown and powdery callus masses at 20 mg/l, 2,4-D with coconut water (10 ml/l). The proliferation of callus growth was high in spanish bunch cultivars at lower concentration of 2,4-D, whereas in virginia bunch and virginia runner, the callus growth was higher with increased dose of 2,4-D alone and in combination with coconut water.

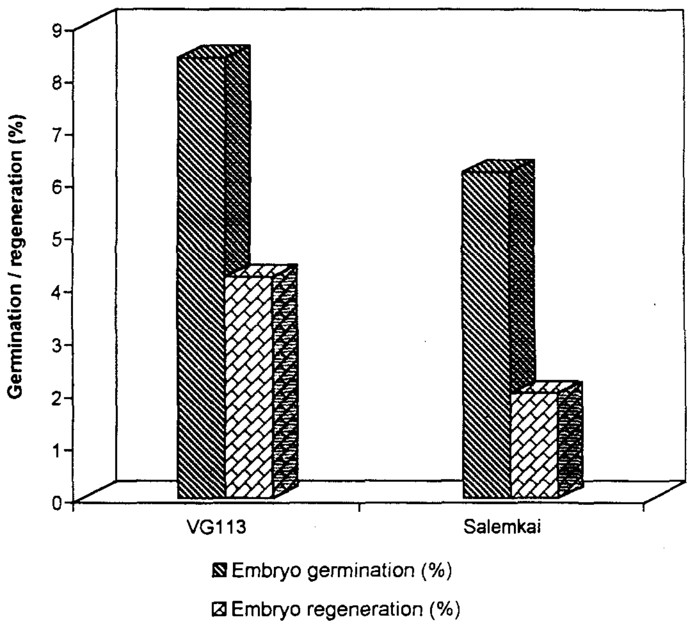
4.2.2. Response of leaf callus in regeneration medium

4.2.2.1. Effect of 2,4-D, NAA, BAP, CW and PM

Leaf callus obtained in MS medium with 2,4-D were subcultured in MS medium supplemented with 2,4-D (3 mg/l) and sucrose (30 g/l) for further differentiation. After four weeks of first subculture, the embryogenic leaf callus (Plate III-19) of each genotype was transferred to full strength and half strength MS medium with different doses of hormone combinations. Among six genotypes tested, two genotypes i.e., one from virginia bunch (Plate III-20) and another from virginia runner (Plate III-21) responded for induction of somatic embryos in MS medium supplemented with 2,4-D (0.5 mg/l) along with BAP (1.5 mg/l) and sucrose (30 g/l). In this treatment two to three embryos per explant were produced. They were then transferred to MS medium supplemented with 0.5 mg/l each of BAP and NAA for conversion into plantlets (Plate III-22).

The conversion of plantlet from embryo was poor (Fig.6) in cv. Salemkai (2.0%) and higher in cv. VG113 (4.2%). The plants developed by this method were grown in sand : soil mixture and survived to maturity. The other genotypes viz., CO2, TMV12, TMV10 and M13 did not responded for somatic

Fig.6. Response of 2,4-D induced leaf calli in regeneration medium



embryogenesis in spite of several hormonal manipulations. The half strength MS medium supplemented with 0.5 mg/l each of NAA and BAP along with CW (10 ml/l) induced rhizogenesis in cv. Salemkai (Plate III-23), whereas the same treatment of NAA and BAP combined with PM (10 ml/l) produced compact, white powdery callus mass (Plate III-24) in most of the genotypes tested. There was no significant response in half strength MS medium combined with NAA, BAP, CW and PM on leaf callus.

4.3. Cotyledon culture

4.3.1. Callus induction

4.3.1.1. Effect of 2,4-D, coconut water and pIC in MS medium

The experiment was conducted with de-embryonated cotyledons from six genotypes employing different combinations of 2,4-D, 2,4-D with CW and pIC at 24 h dark condition for three weeks and then 16 h light and 8 h dark for induction of callus and somatic embryogenesis.

The mean values (Table 13) for response to callusing revealed that MS medium supplemented with pIC (4 mg/l) gave the highest callus induction (91.7%) followed by 2,4-D (4 mg/l) with 68.4% and 2,4-D (4mg/l) + CW (10ml/l) with 49.5% (Fig.7). Significant differences were observed between hormonal combinations but the effect of pIC and 2,4-D were on par statistically. In pIC treatment, inhibition of shoot and root formation were observed. Whereas shoot and root formation in 2,4-D (4 mg/l) and root formation only in 2,4-D (4mg/l) combined with coconut water (10 ml/l) were recorded. The callus growth was high in pIC treatment than other treatments.

Twenty five days after inoculation, induction of callus from the proximal region of the de-embryonated cotyledons was observed (Plate IV-25b) in all cultivars but the frequency of callus induction, shoot formation and root formation (Plate IV-25a) varied between cultivars. Induction on callus was

Table 13. Effect of 2,4-D, coconut water and picloram in MS medium on callus induction in cotyledons culture

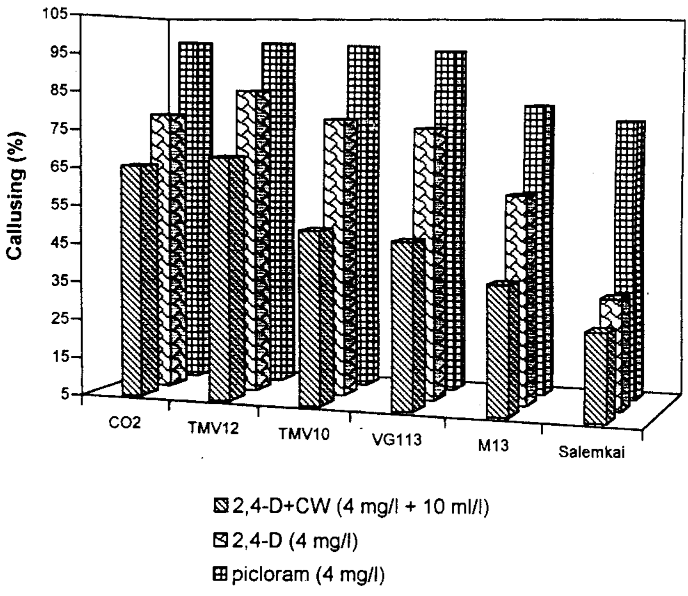
Genotype	Callusing (%)			Mean	Response (30th d after inoculation)					
					Nature of the callus			Proliferation of the callus		
	2,4-D (4 mg/l)	2,4-D+CW (4 mg/l+10 ml/l)	pIC (4 mg/l)		2,4-D (4 mg/l)	2,4-D+CW (4 mg/l+10 ml/l)	PIC (4 mg/l)	2,4-D (4 mg/l)	2,4-D+CW (4 mg/l+10 ml/l))	pIC (4 mg/l)
CO2	78.5 (62.4)	65.5 (54.0)	98.2 (82.3)	80.7 (63.9)	FLB+Shoots+ Roots	FLB+Roots	DB	S	VS	H
TMV2	85.2 (67.4)	68.2 (55.7)	98.2 (82.3)	83.9 (66.3)	FLB+Roots	FLB	LB	S	VS	H
TMV10	78.0 (62.0)	50.0 (45.0)	97.5 80.9)	75.2 (60.1)	DBP+Roots	DB+Roots	DB	M	S	H
VG113	76.0 (60.7)	48.0 (43.8)	96.3 (78.9)	73.4 (58.9)	DBP+Roots	DBP	LB	M	S	H
M13	59.3 (50.4)	38.2 (38.2)	82.0 (64.9)	59.8 (50.6)	DB+Roots	DB	LBP	S	VS	H
Salemkai	33.6 (35.4)	27.3 (31.5)	78.2 (62.2)	46.4 (42.9)	DBP+Roots	DBP	LBP	S	VS	H
Mean	68.4 (55.8)	49.5 (44.7)	91.7 (73.3)	69.9 (56.8)						
Mean (SB)	81.8	66.8	98.2	82.3						
Mean (VB)	77.0	49.0	96.9	74.3						
Mean (VR)	46.4	32.7	80.1	53.0						

Source	SEd	CD (5%)
Genotype	1.410	3.625
Treatment	6.350	27.324
Genotype x Treatment	5.620	11.858

FLB : Friable Light Brown
LBP : Light Brown Powdery
DBP : Dark Brown Powdery
LB : Light Brown
DB : Dark Brown
VR : Virginia Runner
SB : Spanish Bunch
VB : Virginia Bunch
VS : Very slow
S : Slow
M : Moderate
H : High

Figures in parentheses indicate arcsine values

Fig.7. Effect of 2,4-D, CW and pIC on callus induction in cotyledons



higher in spanish cultivars (82.3%) whereas lowest induction of callus was observed in virginia runner (53.0%). In 2,4-D (4mg/l) treatment, cv. CO2 produced directly shoot and root formation (Plate IV-26b) and all other genotypes were responded both for callus initiation and root formation. The percentage of rhizogenesis was higher in virginia runner (62.2% in Salemkai; 33.7% in M13) followed by virginia bunch (19.2% in VG113; 16.7% in TMV10) and spanish bunch (10.2% in CO2; 9.0% in TMV12) (Fig.8). In coconut water (10 ml/l) combined with 2,4-D (4 mg/l), genotypes CO2 and TMV10 responded for callus and root formation and all other genotypes produced only callus.

Thirty days old non responsive de-embryonated cotyledons were cultured again in MS medium supplemented with 2,4-D (4mg/l) for morphogenetic response. After 25 d of first subculture, direct plantlet formation with roots (Fig.8) were observed more frequently from M13 (Plate IV-27,29) and Salemkai (Plate IV-30).

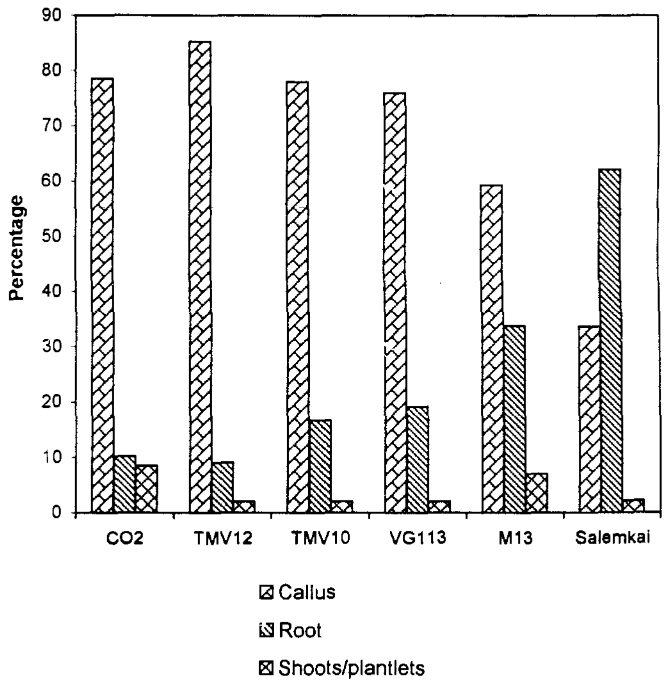
Among the different groups of groundnut tested virginia runner was highly responsive for direct plantlet formation. Cultivar M13 responded both for direct embryogensis (Plate IV-28) and organogenesis (Plate IV-27,29) and cv. Salemkai responded only for organogenesis (Plate IV-30). The frequency of organogenesis (Fig.8) obtained in cv.M13 was higher (7.0%) than the cv. Salemkai (2.2%).

4.3.2. Regeneration

4.3.2.1. Response of 2,4-D induced callus

The 2,4-D induced cotyledon callus of different genotypes were subcultured in the same medium combination. After third subculture, friable embryogenic callus of different genotypes were transferred to different hormone combinations of BAP, NAA, CW and PIC for induction of somatic embryos and plant regeneration.

Fig.8. Effect of 2.4-D (4 mg/l) on cotyledon culture in various genotypes



Treatment BAP alone (1.0 to 2.0 mg/l) or in combination with lower levels of NAA (0.5 mg/l) resulted dark brown to light brown callus only. Whereas culturing with BAP (2 mg/l) and NAA (1 mg/l) in MS medium resulted in the formation of somatic embryos in spanish bunch (TMV12), virginia bunch (TMV10, VG113) and virginia runner (M13) (Fig.9). The frequency of embryo production varied from 3.20 (M13) to 8.20 (VG113) per explant and number of shoots obtained also varied from 1.12 (M13) to 6.20 (VG113) per explant from somatic embryos.

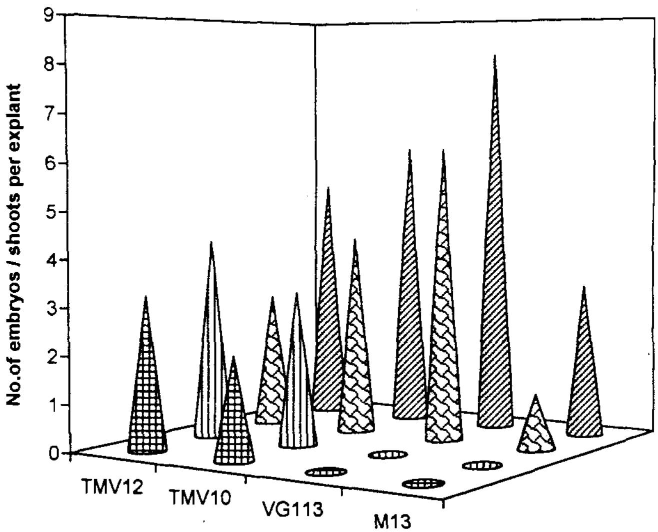
The shoots arose with or without leaves (Plate V-31,32) from the proximal portion of the cotyledonary stage of embryos. Multiple shoots with three plantlets were observed in TMV10 (Plate V-32). Higher frequency of embryos (8.20 per explant) and shoot formation (6.20 per explant) were observed in VG113. Cotyledonary type of embryos with shoot initiation (Plate V-33), multiple shoots without leaves (Plate V-34) and multiple shoots with leaflets (Plate V-35) formation occurred in BAP (2 mg/l) and NAA (1 mg/l) containing MS medium (120 d after inoculation).

Lower frequency of embryos with shoots and plant regeneration were (Fig.9) observed in coconut water (10 ml/l) combined with BAP (2 mg/l) and NAA (1 mg/l). In this treatment, multiple shoots without leaves (Plate V-36), cotyledonary stage of embryos (Plate V-37) and plant regeneration from somatic embryos (Plate V-38) were observed in different cultivars. Induction of indirect somatic embryogenesis and plant regeneration from de-embryonated cotyledon callus occurred most frequently in virginia bunch cultivars (TMV10, VG113) in MS medium supplemented with BAP (2mg/l), NAA (1mg/l) and sucrose (30 g/l).

4.3.3. *In vitro* flowering

The callus from de-embryonated cotyledons of three genotypes (CO2,

Fig.9. Response of 2,4-D induced cotyledon calli on regeneration medium -120 d



No.of shoots per explant } MS + NAA (1.0 mg/l) + BAP (2.0 mg/l) + CW (10 ml/l)
No.of embryos per explant

No.of shoots per explant } MS + NAA (1.0 mg/l) + B AP (2.0 mg/l)
No.of embryos per explant

PLATE V. Somatic embryos and multiple shoots formation in cotyledon callus of different cultivars

Callus induction medium : MS + 2,4-D (4 mg/l)

Regeneration medium : MS + BAP (2 mg/l) + NAA (1 mg/l)

31. TMV12 - Multiple shoot formation without leaves in embryos of cotyledon callus
32. TMV10 - Multiple shoot formation with leaves in embryos of cotyledon callus
33. VG113 - Cotyledonary type of somatic embryos with shoot initiation
34. VG113 - Multiple shoot formation without leaves in embryos of cotyledon callus
35. VG113 - Multiple shoot with leaves, cotyledon stage of embryos

Callus induction medium : MS + 2,4-D (4 mg/l) + CW (10 ml/l)

Regeneration medium : MS + BAP (2 mg/l) + NAA (1 mg/l) + CW (10 ml/l)

36. TMV12 - Multiple shoots without leaves
37. M13 - Cotyledonary stage of embryos
38. TMV10 - Plantlets formation from embryos of cotyledon callus

PLATE VI. ***In vitro* flowering of regenerants from cotyledons and induction of callus and shoots in mature embryo axis**

39. CO2 - *In vitro* flowering of regenerants obtained in cotyledon callus (MS + 2,4-D, 0.5 mg/l + BAP, 0.5 mg/l)

40. CO2 - Regenerated plantlet did not response for *in vitro* flowering (MS + 2,4-D, 0.5 mg/l + BAP, 2.5 mg/l)

Mature embryo axis culture in MS + 2,4-D (4 mg/l)

41. CO2 -
a. Shoot initiation with root
b. Slow development of shoot
c. Early regenerants

42. TMV12 -
a. Shoot formation
b. shoot formation with root
c. Early regenerants

43. VG113 -
a. Shoot initiation
b. Shoot initiation with thin stem
c. Early regenerants

44. TMV10 -
a. Shoot formation with slow growth
b. Shoot initiation
c. Early regenerants

45. M13 -
a. Root initiation
b. Brown callus
c. Early regenerants
d. Shoot formation with root
e&f shoot formation

46. *Salemkai* -
a. Globular type callus
b&c. Plantlets formation with slow growth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45a
45c
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TMV10 and M13) induced by 2,4-D (4 mg/l) were subcultured with MS medium in different combinations of BAP and 2,4-D. The regenerated plants after 120 d of inoculation produced *in vitro* flowering (Plate VI-39) when BAP (0.5 to 2.0 mg/l) combined with 2,4-D (0.5 mg/l). If the BAP concentration exceeds 2.0 mg/l, flowering did not occur (Plate VI-40). Among the cultivars tested, only spanish bunch cultivar (CO2 only) responded for *in vitro* flowering.

4.4. Embryo axis culture

4.4.1. Callus induction

4.4.1.1. Effect of 2,4-D, coconut water and picloram

The experiment was conducted with mature embryo axis of six genotypes in MS medium supplemented with 2,4-D alone (4 mg/l), 2,4-D (4 mg/l) with CW (10 ml/l) and pIC (4 mg/l) (Table 14).

Fifteen days after culture, compact globular type callus masses were observed in all genotypes. Significant differences were observed among different treatments. The effect of pIC and 2,4-D were on par statistically. The mean values for response to callusing revealed that MS medium supplemented with pIC (4mg/l) gave the higher frequency of callus induction (94.7%) followed MS medium with 2,4-D (90.3%) or 2,4-D and CW (77.0%).

On 30th d after inoculation (Fig.10), shoots were formed with 33.3 per cent in 2,4-D (4 mg/l) and 32.1 per cent in 2,4-D (4 mg/l) combined with coconut water (10 ml/l). Whereas callus induced by pIC treatment did not response for regeneration but the callus masses from all the genotypes were embryogenic nature.

Variation occurred in the regeneration efficiency of induced callus from 2,4-D (4 mg/l) and in combination with CW (10 ml/l). Higher responses for regeneration of whole plants were observed in TMV12 (45.8%), followed by CO2

Table 14. Effect of 2,4-D, coconut water and picloram in MS medium on callus induction in mature embryo axis (24 h dark - 3 weeks and then 16 h light - 1000 lux)

Genotype	Callusing (%) – 15th d			Mean	Nature of response – 30th d					
					Regeneration efficiency (%)			Nature of callus		
	2,4-D (4 mg/l)	2,4-D+CW (4 mg/l+10 ml/l)	pIC (4 mg/l)		2,4-D (4 mg/l)	2,4-D+CW (4 mg/l+10 ml/l)	pIC (4 mg/l)	2,4-D (4 mg/l)	2,4-D+CW (4 mg/l+10 ml/l))	pIC (4 mg/l)
CO2	95.2 (77.3)	80.2 (63.6)	99.2 (84.9)	91.5 (73.0)	35.2 (36.4)	34.3 (35.8)	-	B+RG	LB+RG	Embryo genesis
TMV12	97.3 (80.5)	86.2 (68.2)	99.2 (84.9)	94.2 (76.1)	45.8 (42.6	44.6 (41.9)	-	B+RG	LB+RG	Embryo genesis
TMV10	91.2 (72.7)	78.2 (62.2)	96.3 (78.9)	88.6 (70.3)	31.6 (34.2)	30.2 (33.3)	-	B+RG	LB+RG	Embryo genesis
VG113	93.3 (75.0)	82.2 (65.0)	94.2 (76.1)	89.9 (71.5)	35.0 (36.3)	33.2 (35.2)	-	B+RG	LB+RG	Embryo genesis
M13	88.3 (70.0)	70.2 (56.9)	90.3 (71.3)	82.9 (65.6)	28.2 (3[illegible].1)	27.5 (31.6)	-	B+RG	LB+RG	Embryo genesis
Salemkai	76.4 (60.9)	65.2 (53.8)	89.2 (70.8)	76.9 (61.3)	24.3 (29.5)	23.1 (28.7)	-	B+RG	LB+RG	Embryo genesis
Mean	90.3 (71.8)	77.0 (61.3)	94.7 (76.7)	87.3 (69.1)	33.3 (35.5)	32.1 (34.5)	-	-	-	-
Mean (SB)	96.2 (78.8)	83.2 (65.8)	99.2 (84.9)	92.9 (74.5)	40.5 (39.5)	39.4 (38.9)	-	-	-	-
Mean (VB)	92.2 (73.8)	80.2 (63.5)	95.2 (77.3)	89.2 (70.8)	33.3 (35.2)	31.7 (34.3)	-	-	-	-
Mean (VR)	82.3 (65.1)	67.7 (55.4)	89.7 (71.3)	79.9 (63.4)	26.2 (30.8)	25.3 (30.2)	-	-	-	-

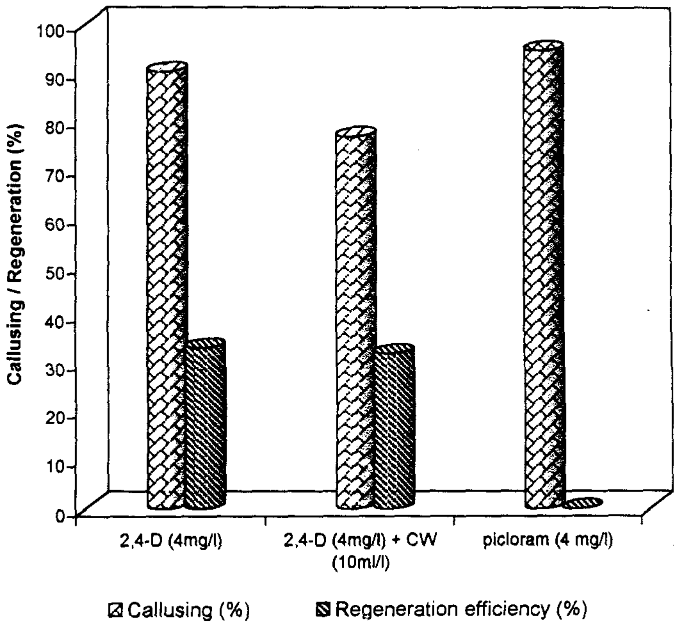
Source	SEd	CD (5%)
Genotype	2.162	NS
Treatment	4.542	14.542
Genotype x Treatment	2.137	4.508

B : Brown callus
LB : Light Brown callus
RG : Regeneration

SB : Spanish Bunch
VB : Virginia Bunch
VR : Virginia Runner

Figures in parentheses indicate arcsine values

Fig.10. Effect of 2,4-D, CW and pIC on organogenesis in mature embryo axis (30^{th} d)



(35.2%), VG113 (35.0%), TMV10 (31.6%), M13 (28.2%) and Salemkai (24.3%) in 2,4-D induced callus. The response for shoot formation and growth pattern of regenerants varied between explants of same genotype in the cultured medium and also differed among genotypes during organogenesis.

In general fast growth of shoots and very slow growth of roots were observed in mature embryo axis culture during organogenesis. Both shoots with or without roots were observed in CO2 (Plate VI-41), TMV12 (Plate VI-42), M13 (Plate VI-45) and Salemkai (Plate VI-46), whereas shoot formation alone was observed in VG113 (Plate VI-43) and TMV10 (Plate VI-44). Among the genotypes, poor growth pattern of regenerants was observed only in virginia runner cv. Salemkai (Plate VI-46).

4.4.2 Regeneration

The calli from mature embryo axis of different genotypes were subcultured separately on the respective medium. At 45th d, the respective calli were transferred to MS + NAA (1mg/l) + BAP (2mg/l), MS + NAA (1mg/l) + BAP (2mg/l) + CW (10ml/l) and MS + plC (0.1mg/l). The results obtained on 60th d are presented in Table 15.

The shoot formation in the regeneration MS medium supplemented with NAA (1 mg/l) and BAP (2 mg/l) alone and combined with coconut water (10 ml/l) were 67.1% and 65.6% respectively. Higher percentage of regeneration of shoots was observed in spanish bunch than virginia bunch and virginia runner during organogenesis. However, the callus induced by picloram did not produce shoot, but developed somatic embryos.

The somatic embryos produced per explant ranged from 3.0 to 15.2. The genotype M13 produced the maximum of 15.2 somatic embryos (Plate VII-47) followed by VG113 (14.2) (Plate VII-49). The embryos of these two genotypes

Table 15. Response of 2,4-D, 2,4-D with CW and pIC induced embryo axis callus in regeneration medium (MS) - 60th d

Genotype	Regeneration frequency (%)		SE/explant
	NAA+BAP (1 mg/l)+(2 mg/l)	NAA+BAP+CW (1 mg/l)+(2 mg/l)+(10 ml/l)	Picloram (0.1 mg/l)
CO2	78.2	77.2	10.8 ± 0.73
TMV12	85.2	86.2	12.0 ± 1.22
TMV10	78.1	76.2	7.3 ± 0.40
VG113	66.6	64.2	14.2 ± 0.35
M13	52.2	48.4	15.2 ± 1.40
Salemkai	42.6	41.6	3.0 ± 0.20
Mean	67.1	65.6	10.4 ± 0.72
Mean (SB)	81.7	81.7	11.4 ± 0.97
Mean (VB)	77.1	70.2	10.7 ± 0.37
Mean (VR)	47.4	45.0	9.1 ± 0.80

PLATE VII. Somatic embryogenesis and genotypic differences of regenerants in mature embryo axis

Callus induction	:	MS + pIC (4 mg/l)
Embryo formation	:	MS + pIC (0.1 mg/l)
Regeneration	:	MS + NAA (1 mg/l) + BAP (2 mg/l)

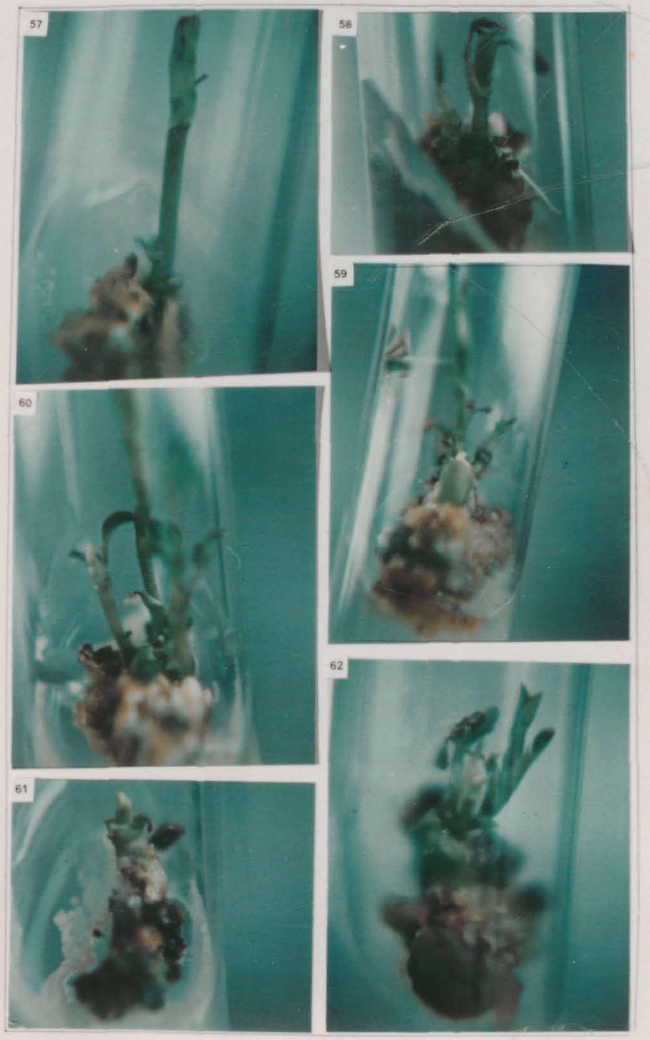
47.	M13	-	Somatic embryos formation
48.	M13	-	Regeneration of plantlet from somatic embryo
49.	VG113	-	Somatic embryos formation
50.	VG113	-	Regeneration of shoot from somatic embryo

Genotypic differences of regenerants

51.	TMV12	-	Somatic embryo formation in mature embryo axis callus
52.	TMV12	-	Germination of somatic embryos
53.	TMV12	} -	Multiple plantlets formation in somatic embryos of mature embryo axis callus
54.	TMV12		
55.	CO2	} -	Regeneration of plantlets from somatic embryos
56.	CO2		

PLATE VIII. Genotypic differences of regenerants from somatic embryos in mature embryo axis

57.	VG113	-	Shoot formation
58.	VG113	-	Multiple shoot formation (2 Nos.)
59.	VG113		
60.	VG113	-	Multiple shoot formation (3 Nos.)
61.	M13	-	Shoot initiation
62.	M13	-	Multiple shoot formation (2 Nos.)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when tested further in the regeneration medium (MS + NAA + BAP) developed into plantlets (Plate VII-48,50). Among the genotypes tested, TMV12, VG113 and M13 were found to be highly responsive for somatic embryogenesis in pIC treatment.

4.5. Somatic embryogenesis

4.5.1. Effect of different auxins

The different concentrations of picloram (2,4,6 mg/l) with four cultivars were evaluated for embryogenesis. The tubes containing cultured mature embryo axis were placed for three weeks at 24 h dark for induction of callus and further differentiation. Later it was placed at 16 h photoperiod (1000 lux) after subculturing with reduced concentration of auxin. The embryos developed at 45th d were counted and the results are presented in Table 16.

When the concentration of pIC was increased from 2 to 4 mg/l, somatic embryos per explant increased. Further increase of pIC concentration to 6 mg/l, reduced the production of somatic embryos per explant in all genotypes. The optimum concentration of picloram for the induction of somatic embryogenesis and production of somatic embryos was found to be 4 mg/l.

The effect of 2,4-D and NAA (4mg/l each) on somatic embryogenesis was studied using four cultivars (TMV12, CO2, VG113 and M13). The results are presented in Table 17.

Higher number of somatic embryos were produced in TMV 12 (17.3) on MS medium containing picloram (4 mg/l) which was followed by M13 (16.2), VG113 (15.2) and CO2 (8.6) (Table 16). Among the auxins tested, picloram induced high embryogenesis ranging from 51.2 to 99.3% and produced 8.6 to 17.3 SE/explant. The 2,4-D induced embryogenesis with low frequency (15.2 to 35.2%) and produced 1.8 to 5.9 SE/explant, whereas NAA did not induce

Table 16. Effect of *picloram* on *somatic embryogenesis* in different genotypes

Genotype	pIC (mg/l)	Somatic embryo*		Nature of embryo
		Induction (%)	SE / explant	
TMV12	2	56.2	9.6 ± 0.86	Mostly globular
CO2	2	22.4	3.2 ± 0.24	Poor development
VG113	2	50.3	7.4 ± 0.64	Normal development
M13	2	51.2	6.5 ± 0.54	Mostly globular
TMV12	4	99.3	17.3 ± 1.24	Mostly globular
CO2	4	51.4	8.6 ± 0.78	Poor development
VG113	4	91.2	15.2 ± 1.18	Mostly globular
M13	4	92.3	16.2 ± 1.20	Mostly globular
TMV12	6	58.3	9.7 ± 0.87	Normal development
CO2	6	24.4	4.4 ± 0.39	Poor development
VG113	6	53.5	9.0 ± 0.79	Poor development
M13	6	49.2	8.4 ± 0.73	Normal development

* 45th d after inoculation

Table 17. Effect of auxins on somatic embryogenesis in embryo culture

Genotype	Auxin (4 mg/l)	Somatic embryo*		Response
		Induction (%)	SE / explant	
TMV12	2,4-D	35.2	5.9 ± 0.49	Embryogenic callus and embryos formation
CO2	2,4-D	15.2	1.8± 0.11	Embryogenic callus and embryos formation
VG113	2,4-D	31.4	5.2 ± 0.46	Embryogenic callus and embryos formation
M13	2,4-D	28.8	4.9± 0.38	Embryogenic callus and embryos formation
TMV12	NAA	0.0	0.0± 0.0	No callus. Rhizogenesis and organogenesis
CO2	NAA	0.0	0.0± 0.0	No callus, rhizogenesis only
VG113	NAA	0.0	0.0 ± 0.0	No callus, rhizogenesis only
M13	NAA	0.0	0.0± 0.0	No callus. Rhizogenesis and organogenesis

* 45^{th} d after inoculation

somatic embryogenesis (Table 17). In NAA treatment, TMV12 and M13 produced both rhizogenesis and organogenesis whereas rhizogenesis alone was observed in CO2 and VGI13.

The combined effect of three auxins were also evaluated for the induction of somatic embryogenesis (Fig.11). Most of the genotypes produced brown with powdery callus masses on 15th d after inoculation and the growth was faster with picloram combined with 2,4-D or NAA. When 2,4-D was combined with NAA, there was no production of powdery callus masses and the growth of the callus was very slow than other treatments.

The embryo production was observed on 45th d in the subcultured medium with reduced concentration of auxins. Among the treatments, picloram with 2,4-D induced high embryogenesis (38.2 to 73.2%) and produced 7.2 to 14.2 SE/explant, whereas 2,4-D combined with NAA, produced low embryogenesis (10.4 to 30.2%) and produced 1.0 to 3.2 SE/explant.

4.5.2. Effect of different media

Induction of somatic embryogenesis was observed in different cultivars using various media (Table 18). The mean induction frequency was higher in MS medium (86.4%) followed by N6 (39.7%), B5 (19.1%) and Blaydes (14.2%). Higher number of SE/explant was observed in MS medium (11.8) followed by N6 (5.3), B5 (4.6) and Blaydes (3.1) with respect to overall genotypes. In general irrespective of genotypes, MS medium was significantly superior over other media.

4.5.3. Role of explants

Four different explants *viz.*, anther, immature leaflets, mature cotyledons and mature embryo axis of TMV12 were cultured in MS medium supplemented with of pIC (4 mg/l). Among the four explants tested, higher percentage of

Fig.11. Effect of auxins on the development of somatic embryos per explant

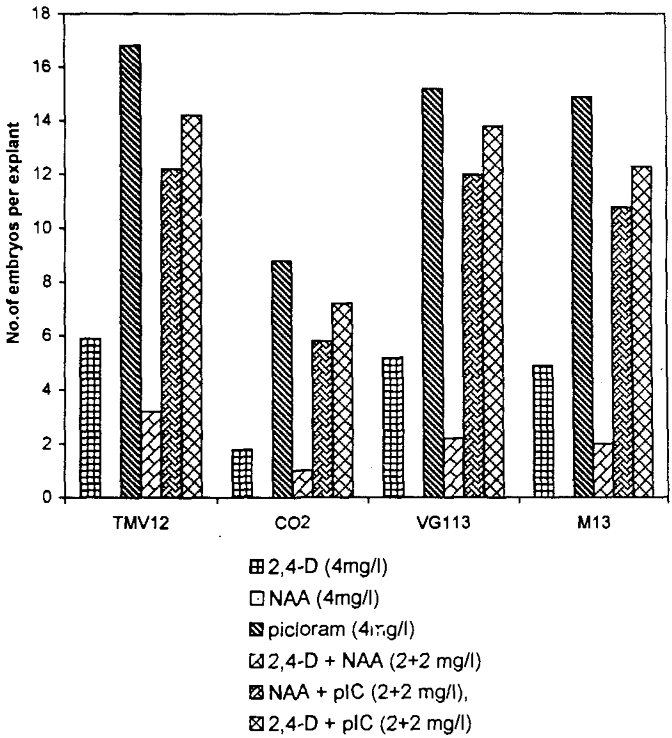


Table 18. Effect of picloram (4 mg/l) in different media on the development of somatic embryos

Media	Induction (%)					SE/explant				
	TMV12	CO2	VG113	M13	Mean	TMV12	CO2	VG113	M13	Mean
MS	98.2	62.2	95.2	90.2	86.4	14.2±1.24	7.4±0.82	13.2±1.10	12.3±1.02	11.8±1.04
B5	25.2	10.8	22.4	18.2	19.1	6.2±0.52	2.2±0.28	5.2±0.38	4.8±0.42	4.6±0.40
Blaydes	20.2	8.2	20.2	8.2	14.2	4.2±0.36	1.2±0.20	3.80±0.18	3.3±0.20	3.1±0.23
N6	46.2	28.2	44.2	40.2	39.7	7.2±0.62	2.4±0.38	6.4±0.58	5.3±0.48	5.3±5.32
Mean	47.4	27.3	45.5	39.2	40.0	7.9±0.68	3.3±0.42	7.1±0.56	6.4±0.53	6.2±0.55

callus was observed (Fig.12) in mature embryo axis (99.2%), followed by cotyledons (98.2%), immature leaflets (42.2%) and anthers (8.2%). This treatment produced compact and light brown callus in anthers; compact, white, powdery callus in immature leaflets and compact, brown callus in mature cotyledons, whereas embryo formation was observed in callus of mature embryo axis of TMV12.

The days to callusing varied from 15 to 25 d. The mature cotyledons required longer duration (25 d) and shorter duration for mature embryo axis (15 d).

The embryos formed in TMV12 (Plate VII-51), CO2, VG113 and M13 during somatic embryogenesis were separated by dissection forceps under aseptic conditions. The separated embryos were transferred to maturation medium without adding any growth regulators. After maturation, the embryos of CO2, TMV12, VG113 and M13 were transferred to regeneration medium (MS) supplemented with NAA (1 mg/l) and BAP (2 mg/l) to know the effect of conversion of embryos into whole plantlets.

The embryo germination was observed in TMV12 (Plate VII-52) after a week in the regeneration medium. The regeneration of whole plants from somatic embryos were observed in TMV12 (Plate VII-53,54), CO2 (Plate VII-55,56), VG113 (Plate VIII-57 to 60) and M13 (Plate VIII-61,62).

4.5.4. Effect of amino acids

Three amino acids *viz.*, proline, glutamine and tryptophan were evaluated by supplementing 25-200 mg/l in MS medium for their role on somatic embryogenesis in TMV12 (Fig.13).

Proline at 25 mg/l had no effect on somatic embryogenesis, whereas 100 mg/l gave the maximum number of SE/explant (18.2). Higher concen-

Fig.12. Type of explant on callus induction in MS medium (pIC 4 mg/l)

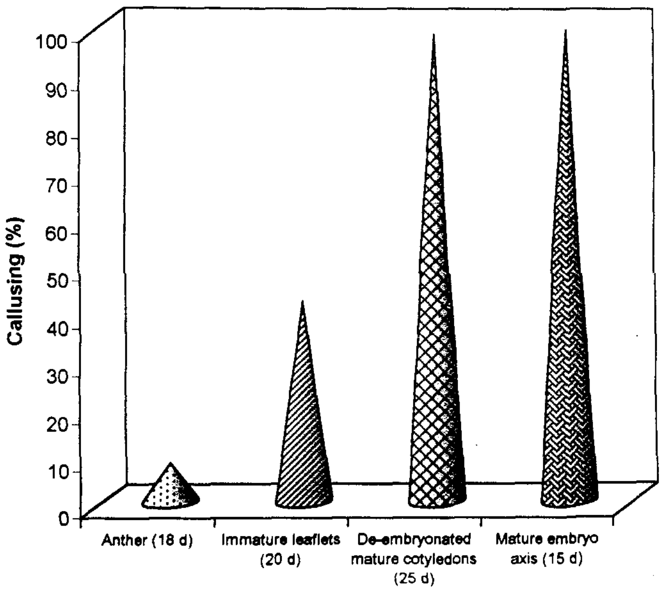
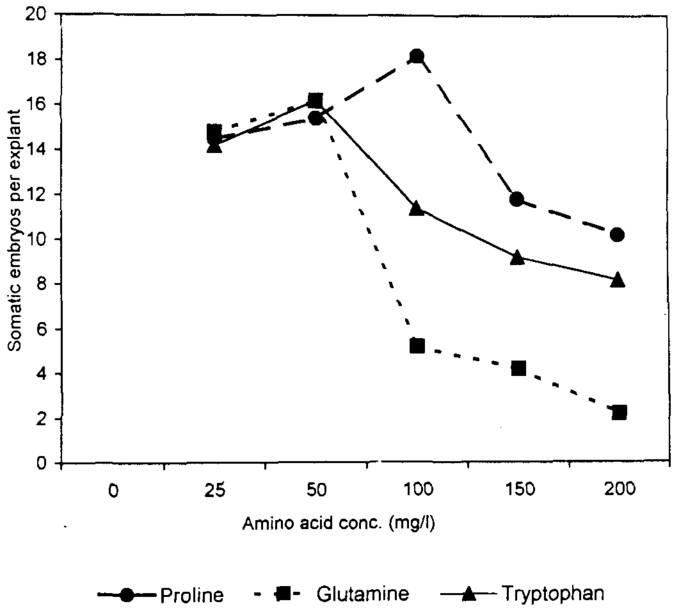


Fig.13. Effect of amino acids on the development of somatic embryos per explant (TMV12)



tration of proline decreased the SE/explant. Glutamine 25 mg/l had no significant role on embryogenesis, while 50 mg/l produced higher number of somatic embryos (16.2%). Further increase in the concentration suppressed the embryogenesis and production of somatic embryos per explant. Embryo abnormality was observed at higher concentrations of glutamine (150 and 200 mg/l). Tryptophan at 50 mg/l supported the embryogenesis and produced higher somatic embryos per explant (16.2) when compared to other concentrations.

4.5.5. Effect of liquid culture

The thirty days old calli of mature embryo axis (TMV12) induced by. picloram (4 mg/l) in MS medium, was transferred to liquid MS medium containing pIC (0.1 mg/l). Subculturing was done every two weeks in the same medium by decanting the old medium. Among the inoculated, 62% of embryo axis exihibited embryogenesis. Induction of embryogenesis and subsequent development were delayed by 10 and 45 d respectively. In liquid medium, less SE/explant (2.4) were produced compared to solid cultures (17.3).

4.5.6.Development of somatic embryos during embryogenesis

Asynchronous development of somatic embryos were observed in 30-45 d old cultures of TMV12, VG113 and M13. In general four types of somatic embryos were identified from the embryogenic clump. The above three cultivars exhibited varying frequencies of globular type (24.4%; 20.2%; 27.4% respectively). Heart shaped embryos were observed in TMV12 (12.4%), VG113 (8.2%) and M13 (11.8%). In these cultivars cotyledonary stage embryos were also observed and the frequency was higher in TMV12 (18.4%) followed by VG113 (10.2%) and M13 (9.2%). Various stages of embryo development and plant regeneration were presented for cv. TMV12 (Plate IX-63 to 66), VG113 (Plate IX-67 to 71) and M 13 (Plate X-72 to 81).

PLATE IX. *Developmental stages of somatic embryos* and plant regeneration in mature embryo axis of cv. TMV12 and VG113

Callus induction	:	MS + pIC (4 mg/l)
Embryo formation	:	MS + pIC (0.1 mg/l)
Regeneration	:	MS + NAA (1 mg/l) + BAP (2 mg/l)

63.	TMV12	-	Globular stage of embryos
64.	TMV12	-	Advance stage of globular and initial stage of heart shaped
65.	TMV12	-	a. Mature stage of globular b. Advance stage of heart shaped
66	TMV12	-	Regeneration of plantlets from somatic embryos
67.	VG113	-	a. Heart shaped embryos b. Globular embryos
68.	VG113	-	a. Globular embryos b. Heart shaped embryos
69.	VG113	-	a. Globular embryos b. Development of shoot meristem
70.	VG113	-	Cotyledonary stage of embryos
71.	VG113	-	Regeneration of plantlets from somatic embryos

PLATE X. Developmental stages of somatic embryos and plant regeneration in mature embryo axis of cv. M13

72.	M13	-	Early proembryo formation
73.	M13	-	Advance stage of proembryo
74.	M13	-	a. Early stage of globular b. Heart shaped embryos
75.	M13	-	a. Mature stage of globular embryos b. Early stage of heart shaped embryos
76.	M13	-	Advance stage of heart shaped embryos
77.	M13	-	Shoot meristem formation
78.	M13	-	Multiple shoot formation from somatic embryos
79.	M13	-	Multiple plantlets and shoot formation from somatic embryos
80.	M13	-	Plantlets formation
81.	M13	-	Multiple plantlets formation from somatic embryos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4.5.7. Histological studies

Histological observations were carried out on embryogenic callus of TMV12 collected during the culture period (7 d interval) upto the cotyledonary stage to confirm the formation of somatic embryos.

The microtome sections of differentiating callus confirmed the various stages of development of somatic embryos. In the sectioning, a group of dividing embryogenic cells with dense cytoplasm were observed (Plate XI-82). At the same time as the cells acquired embryogenic characteristics, a fragmentation of these cellular masses could frequently observed (Plate XI-83). Because of their subsequent evolution, these cellular groups can be considered as young globular somatic embryos (Plate XI-84). They acquired a protoderm and procambial bundle, then shoot and root meristems leading to the globular (Plate XI-85), heart shaped (Plate XI-86) and cotyledonary stage (Plate XI-87). Somatic embryos showed different degrees of morphological conformity. Simultaneously, as soon as two somatic embryos were formed (Plate XI-88,89) many of them became the site of active adventitious embryogenesis.

4.5.8. Effect of media, sucrose and hormones on somatic embryo maturation

The developed dicotyledonary somatic embryos (35 to 45 d old culture) of VG113 were transferred to different media to select a suitable medium for germination (Table 19). Half strength MS medium showed maximum germination of somatic embryos (50.0%) than full strength MS medium (26.7%) and it was also significantly superior to other media tested. Other half strength media evaluated (N6, B5 and Blayde's) showed germination per cent 20 to 30. The half strength MS medium promoted vigorous embryos germination (Plate XII-90), whereas slow germination was observed with their full strength MS medium (Plate XII-91). Significant differences were observed for embryo

PLATE XI. *Histological studies on somatic embryogenesis*

82. Group of dividing embryogenic cells with dense cytoplasm and densely stained nucleolus (100x)

83. Fragmentation of a group of embryogenic cells (100x)

84. a. Early globular embryo (40x)
    b. Heart shaped embryo (40x)

85. Globular embryo (100x)

86. Early stage of heart shaped embryo (100x)

87. Late stage embryo showing the cotyledons with shoot meristem (100x)

88. Simultaneous development of twin embryos showing cotyledons stage (40x)

89. Simultaneous development of twin embryos (100x)

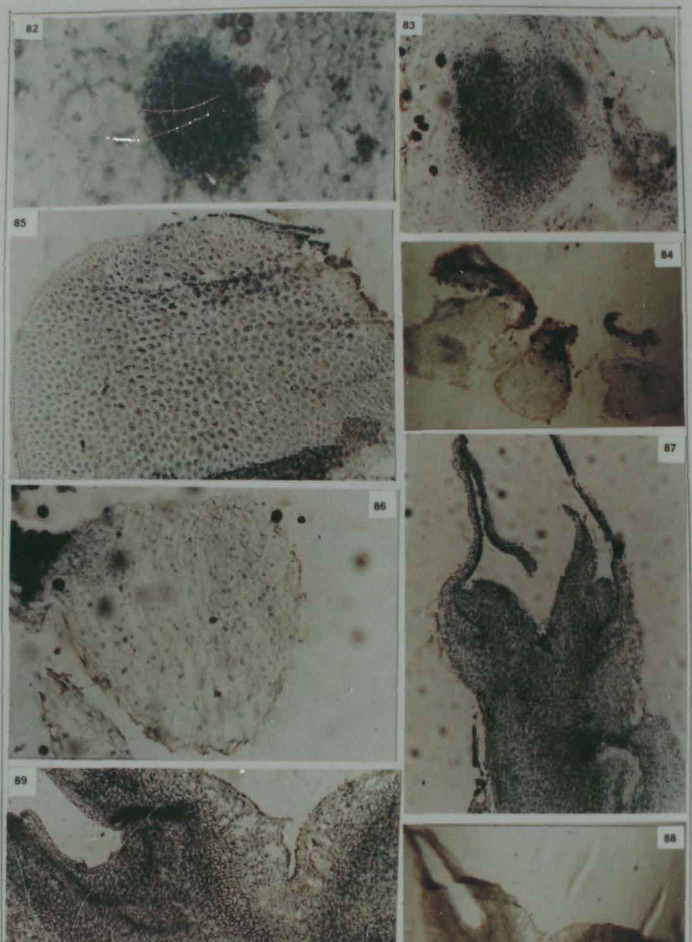
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Table 19. Effect of media on somatic embryo germination (cv. VG113)

*Media	No. of embryos inoculated	No. of embryos germinated	Germination (%)	Response
MS	60	16	26.7 (31.1)	Slow growth
MS half	60	30	50.0 (45.0)	Vigorous growth
B5	60	9	13.3 (21.4)	Poor germination
B5 half	60	16	26.7 (31.1)	Very slow growth
Blaydes	60	6	10.0 (18.4)	Poor germination
Blaydes half	60	12	20.0 (26.6)	Very slow growth
N6	60	8	15.0 (22.8)	Poor germination
N6 half	60	18	30.0 (33.2)	Slow growth
F test			**	
Sed			2.365	
CD			8.858	

* Sucrose, 30 g./l; ** Significant at 5% level Figures in parentheses indicate arcsine values

PLATE XII. Effect of media and sucrose concentration on somatic embryo germination

90. VG113 - Vigorous growth of germination in ½ MS medium

91. VG113 - Slow growth of germination in MS medium

92. TMV12 }
93. TMV12 } - Vigorous growth of germination at 30 g/l sucrose in ½ MS medium

94. TMV12 - Normal growth of germination at 20 g/l sucrose in ½ MS medium

95. TMV12 - Normal growth of germination at 40 g/l sucrose in ½ MS medium

PLATE XIII. Pathway of different morphological changes and plant regeneration during somatic embryogenesis

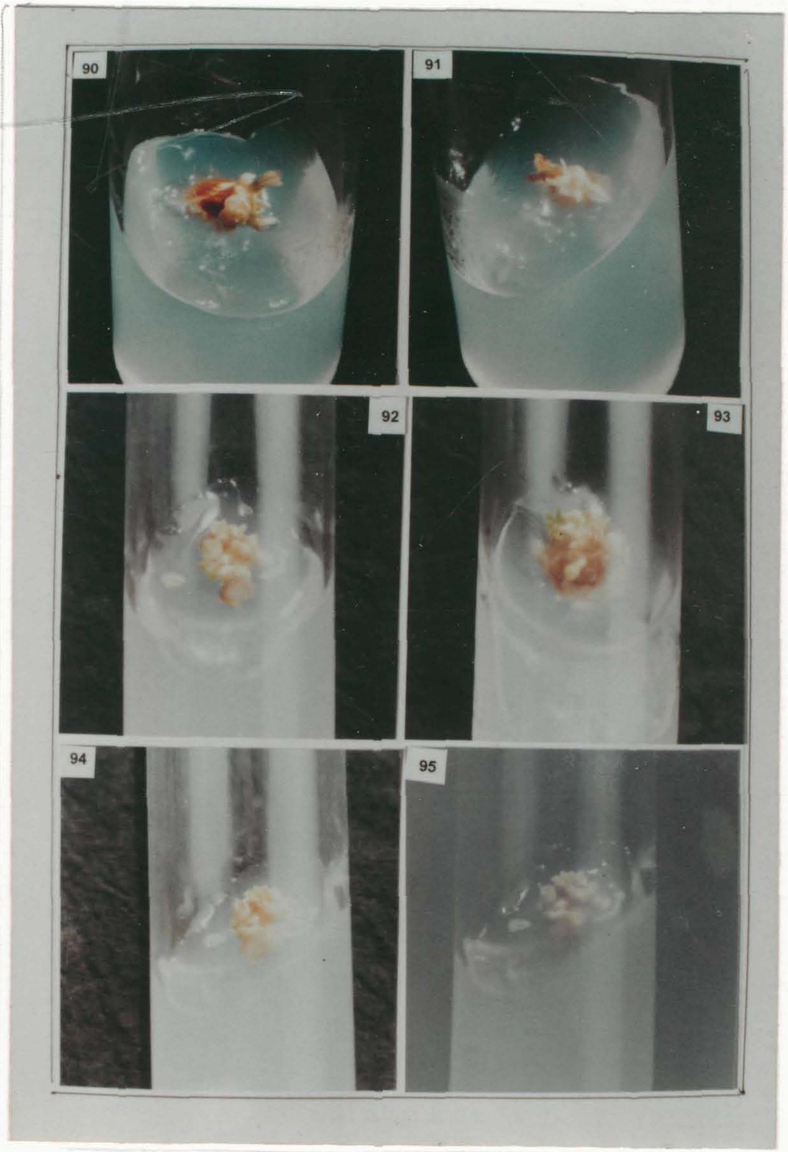
I. MS + pIC (4 mg/l) + Sucrose (30 g/l)

II. MS + pIC (0.1 mg/l) + Sucrose (30 g/l)

III. ½ MS + Sucrose (30 g/l)

IV. ½ MS + NAA (0.5 mg/l) + BAP (1.0 mg/l) + TDZ (0.2 mg/l) + Sucrose (30 g/l)

96.	TMV12	- Globular embryos formation in MS + pIC (0.1 mg/l) in mature embryo axis callus
97.	TMV12	- Globular and heart shaped embryos in maturation medium (½ MS + Sucrose 30 g/l)
98.	TMV12	
99.	TMV12	- Different stages of cotyledonary embryos
100.	TMV12	
101.	TMV12	
102.	TMV12	
103.	TMV12	- Regeneration of plantlets from somatic embryos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germination in different sucrose concentrations. Maximum somatic embryos' germination (50.0%) was observed at 30 g/l sucrose (Table 20; Plate XII-92,93). Further increase or decrease of sucrose concentration reduced the germination frequency as well as suppressed the shoot development. Normal germination was observed in 20 g (Plate XII-94) and 40 g (Plate XII-95) sucrose per litre.

There exists significant differences in embryos germination between different concentrations of auxin/cytokinins tested (Table 21). The half strength MS medium without growth hormones and combined with BAP (0.5 mg/l) and NAA (0.5 mg/l) were found to promote embryo germination (50.%) and these two treatments were on par statistically. But increased concentrations of hormones suppressed the embryo germination (10 to 36%). Half strength MS medium containing kinetin suppressed the embryos germination considerably (5 to 12%).

4.5.9. Effect of growth hormones on conversion of somatic embryos into plantlets

The developed somatic embryos of three cultivars (TMV12, VG113 and M13) were matured (germinated) for 10 d in half strength MS basal medium containing sucrose (30 g/l). Ten d old germinated embryos (Plate XIII-96 to 98) were tested in half strength MS medium containing eight hormonal combinations of NAA, BAP and TDZ to know the conversion of germinated embryos into whole plants (Table 22).

The pathway of different morphological changes occurred in regeneration medium for conversion to whole plants from somatic embryos (Plate XIII- 99 to 103). The matured globular embryos (Plate XIII-97) undergoes various stages like heart shape (Plate XIII-98), cotyledonary stage (Plate XIII-99 to 102) and finally, a whole plant regeneration (Plate XIII-103).

Table 20. Effect of sucrose concentrations on somatic embryo germination in half strength MS medium (cv. TMV12)

Sucrose (g/l)	No. of embryos inoculated	No. of embryos germinated	Frequency (%)	Response
10	40	6	15.0 (22.8)	Poor germination
20	40	15	37.5 (37.8)	Normal growth
30	40	20	50.0 (45.0)	Vigorous growth
40	40	14	35.0 (36.3)	Normal growth
50	40	12	30.0 (33.2)	Slow growth
60	40	8	20.0 (26.6)	Suppression of shoot
70	40	3	7.5 (16.1)	Poor germination
F test			*	
SEd			3.728	
CD			9.125	

* Significant at 5% level; Figures in parentheses indicate arcsine values

Table 21. **Effect of hormones on somatic embryo germination in half strength MS medium (cv. M13)**

Treatments		No. of embryos inoculated	Germination (%)	Response
NAA (mg/l)	BAP (mg/l)			
Control	-	30	50.0 (45.0)	Good germination
0.5	0.5	30	50.0 (45.0)	Good germination
0.5	1.0	30	36.0 (36.9)	Callusing and germination
1.0	1.5	30	14.0 (22.0)	Callusing and germination
1.0	3.0	30	10.0 (18.4)	Callusing and germination
2.0	3.0	30	22.0 (28.0)	Poor development
	Kinetin			
0.5	1.0	30	5.0 (12.9)	Callusing
0.5	1.5	30	8.0 (16.4)	Poor germination
0.5	2.0	30	12.0 (20.3)	Poor germination
F test			*	
SEd			4.185	
CD			9.650	

* Significant at 5% level ;

Figures in parentheses indicate arcsine values

Table 22. Effect of hormones on regeneration of whole plants from somatic embryos (½ MS + sucrose 30 g/l)

Treatment			No. of germinated embryos inoculated			No. of plants regenerated			Regeneration frequency (%)			
NAA	BAP	TDZ	TMV12	VG113	M13	TMV12	VG113	M13	TMV12	VG113	M13	Mean
-	1.0	-	25	18	22	4	2	4	16.0	11.1	18.2	15.1 (22.9)
-	2.0	-	25	18	22	2	1	2	8.0	5.5	9.1	7 5 (15 9)
0.5	1.0	-	25	18	22	7	4	6	28.0	22.2	27 3	25 8 (30 5)
0.5	2.0	-	25	18	22	6	3	5	24.0	16.7	22 7	21 1 (27.3)
-	1.0	0.2	25	18	22	9	6	9	36.0	33.3	40 9	36.7 (37 3)
-	2.0	0 2	25	18	22	11	8	11	44.0	44 4	50 0	34.6 (36.0)
0.5	1.0	0.2	25	18	22	15	12	16	60.0	66.7	72.7	66.5 (54.6)
0.5	2.0	0.2	25	18	22	10	7	11	40 0	38.9	50 0	42 9 (40 9)
Mean			25	18	22	8	5	8	32 0	29.8	36.4	32 7 (34 9)
F test												*
SEd												4 204
CD												9 941

* Significant at 5% level; Figures in parentheses indicate arcsine values

Higher conversion of somatic embryos into whole plants (66.5%) was obtained in half strength MS medium supplemented with NAA (0.5 mg/l), BAP (1.0 mg/l) and TDZ (0.2 mg/l). This combination was found to be significantly superior over other combinations tested.

Variable frequencies of germination and plantlets recovery occurred in three cultivars *viz.*, TMV12 (SB), VG113 (VB) and M13 (VR). Cultivars TMV12 exhibited (Fig.14) maximum germination (62.5%) of somatic embryos followed by M 13 (55.0%) and VG 113 (45.0%). Among the genotypes, M13 responded for high frequency of regenerants (36.4%) followed by TMV12 (32.0%) and VG113 (29.8%). The root tips studies were carried out with somatic embryos derived plants. The regenerants of M13, TMV12 and VG113 showed tetraploidy, as observed in plants. The regenerated plants flowered and set seed in the field. The progeny exhibited general growth characteristics of the cultivars.

4.6. Synthetic seeds

4.6.1. Effect of sodium alginate and calcium chloride on beads formation

Somatic embryos (M13) during distinct cotyledonary stage (Plate XIV-105 to 107) was the most suitable among the various stages (Plate XIV-104) for encapsulation in sodium alginate. During encapsulation, calcium chloride at 0.5% concentration with 2.0 and 2.5% of sodium alginate did not produce any beads (Table 23). The increased concentration of sodium alginate (3.0 and 3.5%) with 0.5% calcium chloride produced beads. The beads so formed immediately collapsed.

At lower concentration (2.5%) of sodium alginate with calcium chloride (0.7%), beads were light with varied size and shape (Plate XIV-108, 109). Beads of uniform size and shape were obtained by using 3% sodium alginate (Plate XIV-110,111). At a higher percentage of sodium alginate (3.5, 4.0%), beads were harder which may suppress the ability of shoots and roots emergence.

Fig.14. Genotypic effect of embryo germination and conversion frequency of whole plant regenerants

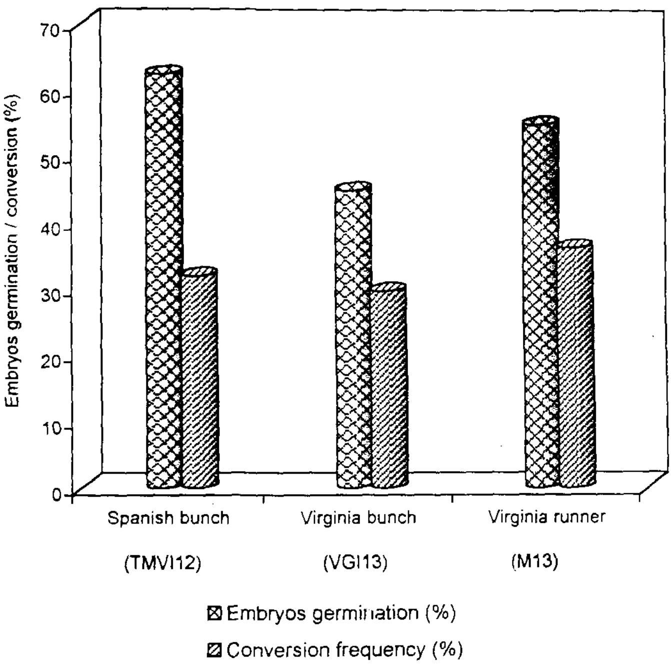


Table 23. Effect of sodium alginate and calcium chloride on beads formation for encapsulation of somatic embryos (cv.M13)

S.No.	Sodium alginate (%)	Calcium chloride (%)	Consistency of beads
1.	2.0	0.5	Not produced
2.	2.5	0.5	Not produced
3.	3.0	0.5	Collapsable beads
4.	3.5	0.5	Collapsable beads
5.	4.0	0.5	Collapsable beads
6.	2.0	0.7	Not produced
7.	2.5	0.7	Fragile beads
8.	3.0	0.7	Stable beads
9.	3.5	0.7	Hard beads
10.	4.0	0.7	Very hard beads

4.6.2. Effect of sodium alginate concentration on germination

The synthetic seeds produced with different concentrations of sodium alginate (2.5 to 4.0%) were compared with fresh embryos for germination (Table 24) in half strength MS medium containing BAP (1.0 mg/l) and TDZ (0.2 mg/l). Maximum germination percentage (60%) was observed in fresh embryos and it was significantly different from encapsulated embryos. The encapsulated somatic embryos at 3% sodium alginate ($CaCl_2$ 0.7%) showed moderate germination (32.5%) in 20 d with better development of shoots and roots. The encapsulated embryos at 2.5% sodium alginate resulted in 27.5% germination in 20 d and it turned brown after germination. When the concentration of sodium alginate increased to 3.5% a reduction of germination was noticed (5%) and took longer days (35) for emergence of shoots and roots. Further increase of sodium alginate concentration to 4% did not induce germination. Sodium alginate (3.0%) with 0.7% calcium chloride was found to be optimum for encapsulation of somatic embryos (32.5% germination).

4.6.3. Effect of media on plantlet formation

Different basal media were evaluated to increase the conversion rate of encapsulated embryos (Table 25). The conversion frequency of encapsulated and fresh embryos on different media were significantly different. Fresh embryos showed a high frequency (44%) of conversion in half strength MS basal medium. The lowest conversion for fresh embryos (21.5%) was obtained using B5 full strength medium. In comparison to fresh embryos, the conversion frequency for encapsulated embryos was low; the maximum response of 33.2% on half strength MS medium and lower response in B5 (11.5%) and N6 (11.5%) full strength media. Although the percentage of conversion was low (17.5%) on half strength N6 medium, the time required for emergence of shoots and roots was reduced (15 d).

Table 24. Effect of sodium alginate concentration on germination (cv.M13)

Treatment		Seeds tested (No.)	Germination period (d)	Seeds germinated (No.)	Germination (%)	Response
Sodium alginate (%)	Calcium chloride (%)					
2.5	0.7	40	20	11	27.5 (31.6)	Germinate and turn brown
3.0	0.7	40	20	13	32.5 (34.8)	Fast germination and development of shoots and roots
3.5	0.7	40	35	2	5.0 (12.9)	Poor germination
4.0	0.7	40	-	-	0.0 (4.5)	Not germinated
Non-encapsulated embryos (Control)	-	40	15	24	60.0 (50.8)	Fast germination and development of shoots and roots
F test					*	
SEd					2.776	
CD					22.837	

* Significant at 5% level; Figures in parentheses indicate arcsine values

Table 25. Effect of media on conversion of non-encapsulated and encapsulated embryos (cv.M13)

Medium	Conversion frequency (%)		Response
	Fresh embryo	Encapsulated embryo	
MS	37.2 ± 0.8	21.0 ± 1.5	Fast growth and development of shoots and roots
MS half strength	44.0 ± 0.7	33.2 ± 1.1	Shoot and root emergence in 15 d
N6	25.5 ± 1.1	11.5 ± 0.8	Shoot and root emergence in 20 d
N6 half strength	35.5 ± 0.8	17.5 ± 1.7	Shoot and root emergence in 15 d
B5	21.5 ± 1.1	11.5 ± 0.8	Slow growth and germination was delayed
B5 half strength	30.2 ± 0.3	15.5 ± 1.7	Slow growth and germination was delayed

LSD at 5% level;	Media type (A)	:	2.01
	State of embryo (B)	:	7.18
	Interaction (A x B)	:	3.31

Significant differences were observed in different support media tested for germination of encapsulated embryos (Table 26). Higher germination (50%) was observed in agarised half strength MS medium with growth hormones BAP (1.0 mg/l) and TDZ (0.2 mg/l) followed by agarised half strength MS medium (35.0%). These two treatments were found to be superior than other support media for germination of encapsulated embryos. The synthetic seeds placed in the filter paper with wetting of water or agar or distilled water did not germinate.

4.6.4. Effect of storage on germination of synthetic seeds

The encapsulated somatic embryos in MS nutrient medium and distilled water were stored at 4°C and their viability was evaluated at different intervals in the half strength MS medium with BAP (1.0 mg/l) and TDZ (0.2 mg/l).

The synthetic seeds could be stored for a period of 30 d without any change in the viability, if the encapsulated embryos were supplemented by MS nutrients to the gel matrix (Table 27). Without MS nutrients in the gel matrix, the stored (7 d) synthetic seeds germinated with 2% efficiency only. The encapsulated embryos with MS nutrients was found to be significantly superior than the embryos encapsulated with water for different periods of storage.

However, the different periods of storage of encapsulated embryos in MS nutrients for germination differed significantly. The encapsulated embryos stored at 4°C for 7, 15 and 30 d did not differ much in germination percentage and conversion to whole plants (52.5, 52.5 and 47.5% respectively). There was significant reduction in germination and conversion frequency at 45^{thd} (70%) and 60th d (87.5%) of storage. The encapsulated embryos stored at 4°C for 30 d (Plate XV-112) developed into whole plants with shoots and roots (Plate XV-117) in 25 d after inoculation in the regeneration medium. The

Table 26. Effect of physical state of support media on synthetic seed germination (cv.M13)

Treatment	Seeds tested (No.)	Germination (d)	Seeds germinated (No.)	Germination (%)
Liquid ½ MS medium	20	25	4	20.0 (26.6)
Agarised ½ MS medium	20	20	7	35.0 (36.3)
Agarised ½ MS + growth hormones	20	20	10	50.0 (45.0)
Filter paper wetted with water	20	-	-	0.0 (4.5)
Filter paper wetted with MS medium containing hormones	20	30	2	10.0 (18.4)
Agar only	20	-	-	0.0 (4.5)
Distilled water	20	-	-	0.0 (4.5)
F test				*
SEd				6.012
CD				14.229

* Significant at 5% level; Figures in parentheses indicate arcsine values

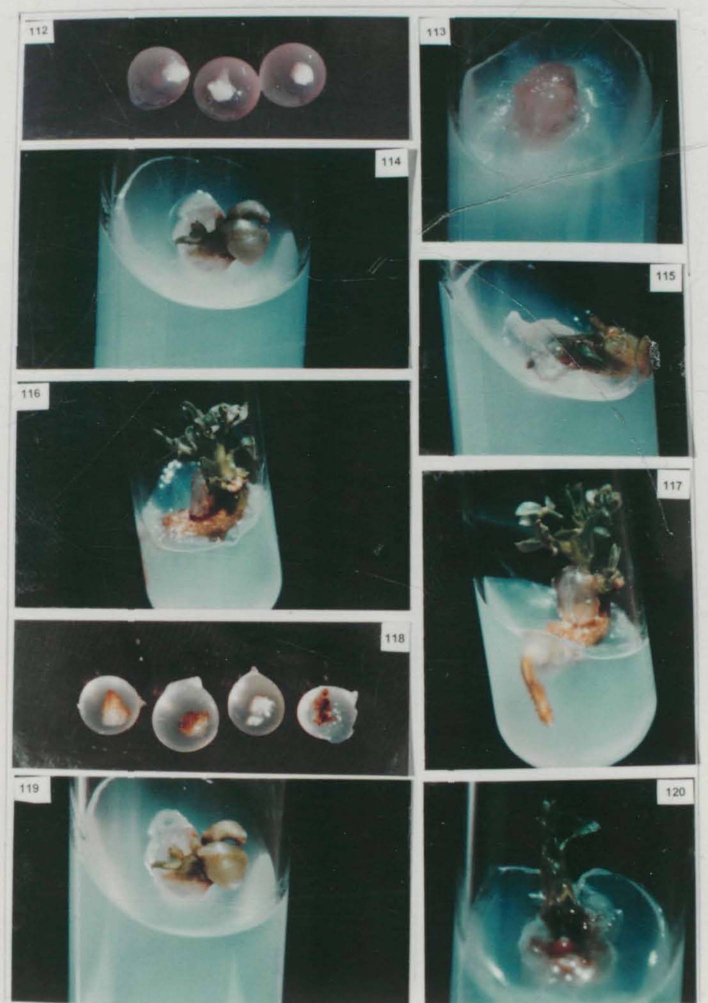
Table 27. Effect of storage on germination of synthetic seeds of groundnut (½MS + BAP (1.0 mg/l) + TDZ (0.2 mg/l)

Storage period (d)	Gelmatrix with MS nutrients		Gelmatrix with water		Response of encapsulated seeds with MS nutrients
	Seeds tested (No.)	Germination (%)	Seeds tested (No.)	Germination (%)	
7	40	52.5 (46.4)	40	2.0 (8.1)	Rapid emergence and development of shoot and root in 25 d
15	40	52.5 (46.4)	40	0.0 (4.5)	Rapid emergence and development of shoot and root in 25 d
30	40	47.5 (43.6)	40	0.0 (4.5)	Rapid emergence and development of shoot and root in 25 d
45	40	30.0 (33.2)	40	0.0 (4.5)	Rapid emergence and development of shoot and root in 25 d
60	40	12.5 (20.7)	40	0.0 (4.5)	Poor germination and slow development of shoots in 35 d
F test		*		-	
SEd		4.973		0.719	
CD at 5%		13.805		NS	
t (1,2) at 5%		6.965*			

* Significant at 5% level; Figures in parentheses indicate arcsine values NS Not significant

PLATE XV. Stages of whole plant regeneration from stored (30 d and 60 d) encapsulated embryos at 4°C

112. Stored synthetic seeds (30 d at 4°C)
113. Inoculated synthetic seed for germination
114. Germination of 30 d stored synthetic seed
115. Development of shoot from stored synthetic seed (30 d)
116. Regeneration of whole plant from stored synthetic seed (30 d)
117. Regeneration of whole plant with root from 30 d stored synthetic seed
118. Stored synthetic seeds (60 d at 4°C)
119. Germination of 60 d stored synthetic seed
120. Regeneration of whole plant from 60 d stored synthetic seed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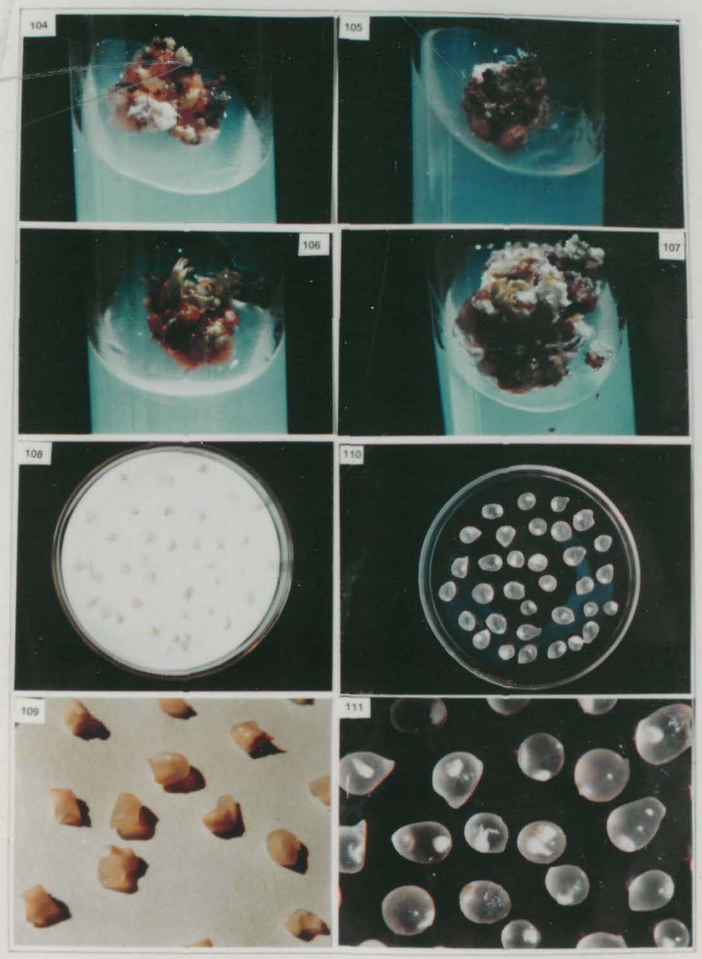
104
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111

Table 28. Conversion frequency in encapsulated and non-encapsulated fresh and stored somatic embryos (cv.M13)

Nature of embryos	No. of embryos cultured	Embryos with roots (%)	Embryos with shoots (%)	Conversion frequency (%)
Non-encapsulated fresh	40	80	70	75
Non-encapsulated stored at 4°C for 60 d	40	40	-	-
Encapsulated fresh	40	62	40	50
Encapsulated stored at 4°C for 60 d	40	12	8	4
χ^2 (3 df)		$P > 0.001$	$P > 0.001$	$P > 0.001$

different stages of conversion of whole plants from encapsulated embryos are presented in Plate XV-113 to 117. Sixty days stored encapsulated embryos (Plate XV-118) took longer days (35 d) for regeneration into whole plants (Plate XV-119,120).

Chi-square analysis of the data showed highly significant differences ($P>0.001$) between the frequencies of conversion to plantlets of non-encapsulated stored, non-encapsulated fresh, encapsulated stored and encapsulated fresh somatic embryos (Table 28). The fresh unstored somatic embryos registered maximum frequency of 75% to form plantlets. Whereas conversion was nil when fresh embryos were stored for two months at 4°C. Encapsulated fresh somatic embryos exhibited 50% of conversion directly after encapsulation. After two months of storage at 4°C, 4 per cent conversion was recorded in encapsulated embryos.

Discussion

5. DISCUSSION

Recent advances in the application of plant cell and tissue culture and plant molecular biology have created unprecedented opportunities for genetic manipulation of plants. The potential impact of these novel and powerful technologies on the genetic improvement of crop plants has generated considerable interest, enthusiasm and optimism in the scientific community. However, serious problems exist in the transfer of this technology to groundnut breeding programmes. Most of the current strategies for the application of agro-biotechnology to crop improvement envisage the regeneration of whole plants from plant cell and tissue culture.

Systematic approach for regeneration of whole plants from the plant cell and tissue culture is necessary for implementation of this technology to groundnut improvement programmes. In the present study systematic approaches on various aspects of groundnut tissue culture comprising anthers, immature leaflets, de-embryonated cotyledons and mature embryo axes of *Arachis hypogaea* and anthers in wild diploid species of *Arachis pusilla* were carried out for haploid / somatic embryogenesis and production of synthetic seeds. The results obtained on various experiments are discussed here under.

5.1. Anther culture

Induction of androgenic haploid is the one of the most important problems of plant cell cultures. Production of haploids through anther culture has been standardized in many plant species since the first report of Guha and Maheshwari (1964). The simplicity of the anther culture method developed by Guha and Maheshwari (1966) and the higher number of haploids obtainable are of great advantage.

Considerable success have been obtained with the anther cultures of alfalfa, tobacco, rice, maize and potatoes (Zagorska *et al.*, 1984; Coumans *et al.*, 1989; Uhrig, 1985). But in most species the success rates were disappointingly low or some species were totally recalcitrant. Research on leguminous crops, especially groundnut anther culture is yet insufficient and still a challenging task because of recalcitrant nature. No reliable tissue culture method is available to produce doubled haploid plantlets in large numbers in order to assist breeding programmes.

A few scientists (Martin *et al.*, 1974; Martin and Rabechault, 1976; Bajaj *et al.*, 1981b; Mroginiski and Fernandez, 1979, 1980) have tried anther culture in *Arachis* and secured very low frequency of haploid cells in the callus. Anther cultures of *A. hypogaea* (Bajaj *et al.*, 1980b; Sastri *et al.*, 1982) and the wild species *A. correntina* (Mroginski and Fernandez, 1979), *A. glabrata* (Bajaj *et al.*, 1980a) and *A. monticola* (Sastri and Moss, 1982) exhibited early stage of embryogenesis. In *Arachis* though plant regeneration has been obtained from anthers, occurrence of haploids has not been reported (Mroginski and Kartha, 1984; Bajaj, 1984). However, in the present study plant regeneration was obtained only from anthers of wild diploid species, *Arachis pusilla* and plant regeneration could not be obtained from anthers of cultivated *Arachis hypogaea*.

5.1.1. Selection of flower buds

In most species, uninucleate pollen has been reported as the most successful to use for anther culture. The stage of development of pollen microspores at the time of culture has been shown to be an important factor in the production of haploids *via.* anther culture (Maheshwari *et al.*, 1980).

In anther cultures of *Arachis* species, selection of flower buds at "uninucleate" stage is difficult and time consuming. In the present study, flower buds of different cultivars of *A. hypogaea* and *A. pusilla* were standardised (Plate I-1) for selection of uninucleate stage based on the size and colour of anthers (Table 6). Difference in sizes between the stages of each genotype were significant in all groups of *Arachis* species. The colour of the anthers at a particular stage of microspores was always the same. Anthers bearing the uninucleate pollen grains were shades of yellow but earlier or later stages were eliminated by rejecting transparent, white and deep yellow anthers. The results were in agreement with the findings of Martin *et al.* (1974) and Sudhakar and Moss (1990). They stressed the use of anther colour in selecting the anthers for culture.

A number of factors affect the pollen plant production in anther cultures (Maheshwari *et al.*, 1980; Heberle-Bors, 1985). Among these, the developmental stage of the pollen at the time of inoculation and the physiological state of the parent plant play important roles. In the present experiment anthers containing uninucleate stage of androgenesis in the cultured anthers of *Arachis hypogaea* and *Arachis pusilla*. This is in agreement with the findings of Mroginski and Fernandez (1980) and Bajaj *et al.* (1980a, 1981b). Willcox *et al.* (1991) stated that early uninucleate stage was the best for anther response in groundnut.

5.1.2. Surface sterilants

Maintenance of complete aseptic condition in the flower buds is the most important pre-requisite for successful culturing without contamination. The results of present investigation demonstrated that pre-treatment of flower buds at 4°C for 2 d followed by treatments using ethanol (70%) for one minute and then $HgCl_2$ (0.05%) for ten minutes resulted in the least contamination (Table 7) and good response for induction of callus.

The large difference in contamination between the control and treated flower buds suggested that the surface was the main source of contamination. Use of mercuric chloride for a longer time and with higher concentration impaired the growth and reduced the response. The result was consistent with the report of Bhojwani and Razdan (1983). Hu and Wang (1985) have shown that the use of one per cent NaOCl might cause damage or cell death in plants. However, Willcox *et al.* (1991) surface sterilized the flower buds in 70% ethanol for 30 seconds followed by one per cent sodium hypochlorite for 2.5 min.

Pre-treatment of inflorescence can have a positive effect on the development of the microspore (Wenzel, 1980) and organ induction from the anther calli of alfalfa (Zagorska and Dimitrov, 1995). Several investigations revealed a high frequency of callus or embryo formation in barley without any pre-treatment (Powell, 1988). A negative effect of low temperature on anther response in wheat has been reported (Marsolais *et al.*, 1984).

5.1.3. Anther callus induction

5.1.3.1. Nutrient medium

Medium composition is widely known to be one of the most important factors affecting callus formation and induction of *in vitro* organogenesis or somatic embryogenesis. Different species and varieties have specific requirements for nutrients and hormones. Since no reference data on induction media for androgenesis are available in groundnut, different nutrient media were examined.

The nutrient media such as MS (Murashige and Skoog, 1962), Blaydes (Blayde's, 1966), B5 (Gamborg *et al.*, 1968), NN (Nitsch and Nitsch, 1969) and

N6 (Chu, 1978) supplemented with 2,4-D (4 mg/l) were tested. The results showed that the best callus induction and development was observed in almost all genotypes of *Arachis hypogaea* using Murashige and Skoog medium (1962). The other media such as Nitsch, N6 and B5 were equally important on callus induction, while Blaydes medium was least effective (Table 8).

The best medium identified in this study was in agreement with the results obtained by Bajaj *et al.* (1980a, 1981a). However the available literature does not allow recommendation of any anther culture medium for general applicability. The basal media commonly used have been mainly N6 medium (Chu, 1978) or that of Murashige and Skoog (1962) medium in groundnut anther culture.

5.1.3.2. Auxins

IAA or NAA were the auxins extensively tested so far for the induction of callus from anthers of *Arachis hypogaea* and its related species and the use of 2,4-D has been to a limited extent (Mroginski and Kartha, 1984; Bajaj, 1984). Anther callus induced by pIC has been found to be morphogenetic for plant regeneration in several species of *Arachis* (Still *et al.*, 1984; Seitz and Stalker, 1985). In all these cases cytokinin, BAP or Kn have been used along with auxin for callus induction. The most effective auxin for callus induction and proliferation was 2,4-D, which is a powerful suppressant of organogenesis and often induces callus even in the absence of any endogenous cytokinin (Dodds and Roberts, 1985).

In the present study, 2,4-D, pIC, 2,4-D with CW, 2,4-D with CW and PM were used for induction of callus and other morphogenetic responses. The results showed that the callus formation was higher by using 2,4-D (4mg/l) along with CW (10 ml/l) (Fig.2) followed by 2,4-D alone, 2,4-D with CW and PM, and pIC (Tables 9 and 11). 2,4-D combined with PM did not have any effect on induction of callus (Fig.1).

Significant genotypic effects have been reported for oat androgenic and somatic tissue culture (Rines, 1983; Gana *et al.*, 1995). The present results showed differences in callus induction capacity and embryogenic response related to genotypes or species. Callus induction rates of cultivated genotypes *A. hypogaea* were better compared to *A. pusilla* (Tables 9 and 11). Spanish bunch cultivars (CO2, TMV12) responded with high frequency for callus induction when compared to virginia bunch (TMV10, VG113) and virginia runner (ICG92, Salemkai) cultivars (Fig.2). Such differential response of genotypes for callus induction from anthers and other explants of *Arachis* have been reported by Bajaj *et al.* (1980a), Murashige (1982) and Mroginski and Kartha (1984).

In the present investigation, callus induction in TMV12 was 84.8% using 2,4-D and CW and in CO2, 75.3% (Table 9). Bajaj *et al.* (1981b) obtained a maximum of 13% callus induction from anthers of TMV2 using IAA (4 mg/l) and kinetin (1 mg/l). The increase rate of callus induction might be due to the action of 2,4-D, which belongs to the phenoxy group of auxin (Leopold and Kriedmann, 1975) and growth promoters present in CW. Coconut water showed an influence on high embryogenic callus with increased fresh weight (Table 10). Narasimhulu and Reddy (1985) observed that addition of coconut water to hormone supplemented media yielded larger amounts of callus compared to control media with CW. Cytokinin like properties of coconut water have been demonstrated in tissue cultures by many workers (Klein and Klein, 1970; Lethan, 1974).

When 2,4-D was used alone, light brown embryogenic calli were observed in spanish bunch (CO2, TMV12), virginia runner (ICG92, Salemkai) and wild diploid species, whereas non-embryogenic, compact, dark brown calli masses were observed in virginia bunch (TMV10, VG113). The growth of callus was decreased in lower concentration (Plate I-3) compared to higher concentration of 2,4-D (Plate I-4). But the use of coconut water along with 2,4-D resulted, a friable embryogenic and proliferated calli (Plate I-5,6) in most of the genotypes. Cultivar TMV10 showed compact with chlorophyllous type of callus most probably induced by the cytokinin like compounds present in CW. Such cytokinin stimulated chlorophyllous callus had been observed by number of workers in the callus cultures of many species (Israel and Steward, 1967; Woznv and Szwevkowska, 1975). 2,4-D combined with CW and PM resulted in the formation of compact dark brown, non-embryogenic callus with very slow growth (Plate I-7,8). The inhibition of the growth of callus may be due to the effect of PM present in the medium.

In another experiment, picloram was found to be less effective for induction of callus from anthers (Table 11). However the callus growth rate was higher when picloram was supplemented in the MS medium. This result was consistent with report of Furmanowa *et al.* (1997). They have observed rapid induction of callus (*Taxas* sp.) in modified White's medium with picloram. No differentiation and formation of embryos were observed in the pIC induced anther calli when subcultured two to three times with intervals of four weeks. But Eapen and George (1993a) stated that pIC was effective for induction of somatic embryogenesis.

5.1.3.3. Light and Dark

Many factors including photoperiod can influence both callus induction and embryo formation. Varying light and dark conditions were used for groundnut somatic embryogenesis. Light has been reported to have both promoting and inhibiting effects on somatic embryogenesis. In *Podophyllum hexandrum* somatic embryogenesis was completely suppressed by light (Arumugan and Bhojwani, 1990). Michler and Bauer (1991) found that no embryos differentiated in Populus embryogenic cultures in light. Continuous darkness was needed for differentiation (Baker and Wetzstein, 1992). In contrast, Gleddie *et al.* (1983) cultured egg plant (*Solanum melongena*) leaf explants in either darkness or continuous light and found that induction was light dependent; although some callus formed, no embryos were developed under dark conditions. Groundnut somatic embryogenesis has been reported under both dark (Ozias-Akins, 1989; Baker and Wetzstein, 1992; Wetzstein and Baker, 1993 and a 16 h photoperiod (Hazra *et al.*, 1989; Sellars *et al.*, 1990; McKently *et al.*, 1991).

In the present investigation, the effect of photoperiod was compared under similar culture conditions. Embryogenic callus occurred under both light and dark conditions. The induction of callus at 16 h photoperiod (1000 lux) was higher (37.1%) compared to 24 h dark condition (24.1%) in most of the genotypes tested (Fig.3). This observation is in accordance with the report by Furmanowa *et al.* (1997). They have reported 11.4 fold increase in callus under light compared to 9.3 fold in dark conditions (*Taxas* sp.) in WR and B5 media containing pIC.

Genotypic differences were also observed at both the conditions (Fig.3). Spanish bunch, cv. TMV12 responded 46.5% and CO2 with 40.2% callus induction under 16 h photoperiod, whereas lower percentage of callus induction was observed under 24 h dark condition (TMV12 - 31.6%; CO2 - 28.3%). But the nature of callus, proliferation and differentiation differed between two conditions. Cultures placed under continuous dark has the ability to form embryogenic calli with differentiation in most of the genotypes tested, whereas light has influence on the production of embryogenic and non-embryogenic calli lacking differentiation at 16 h photoperiod. These results was consistent with the report of Baker and Wetzstein (1992). They found that dark was the optimum cultural environment to obtain embryos of high quality.

Others have reported aberrant embryo associated with light. Lazzeri *et al.* (1987) compared the light intensity on somatic embryogenesis in soybean. Embryogenesis was noticed both in light and darkness, however embryo development was impaired under high light conditions. Michler and Lineberger (1987), described abnormal leaf cotyledons in Carrot (*Daucus carota*) under light treatments whereas dark grown cultures had predominantly fleshy cotyledons which were associated with normal embryogenesis. In the present experiment, cultured anthers incubated at 24 h dark condition took more days for initiation of callus than anthers placed at 16 h photoperiod (Fig.4).

5.1.4. Regeneration

Regeneration from tissue and cell cultures can be accomplished *via* organogenesis or somatic embryogenesis. In the callus cultures of dicots, a low ratio of auxin and cytokinin has been found to be essential (Murashige, 1982). Auxin to cytokinin ratio and their individual concentrations required for the regeneration of plants have been found to vary with respect to species, genotypes and explants. The levels of auxins and cytokinin used in the callus induction medium have also been found to influence the regeneration of plants (Maheswaran and Williams, 1985).

In the present investigation, different types of anther calli induced from different hormonal combinations were used in the regeneration medium. The transfer of 2,4-D with CW induced anther calli to N6 medium containing BAP, NAA and glutamine, resulted proliferation of callus masses in most of the genotypes. When the white friable callus was transferred to a medium containing BAP, proliferation of the callus was more even though no auxin was present in the medium. This must be due to the production of endogenous auxin stimulated by the presence of the exogenous cytokinin (Syono and Furuya, 1972). When BAP (0.5 mg/l), NAA (1.0 mg/l) and glutamine (3.5 g/l) were present in N6 medium, calli differentiation in TMV12 (Plate II-9) and shoot initiation in Salemkai (Plate II-12) of *A. hypogaea* and embryoids formation in *A. pusilla* (Plate II-10,11) were observed. Such type of morphogenetic response was observed by Willcox *et al.* (1991) in the same medium combination as *A. hypogaea*. The regenerated plants were not haploids.

The amino acid, glutamine has been shown to stimulate embryogenesis in both barley (Olsen, 1987) and wheat anther cultures (Henry and De Buyser, 1981). Willcox *et al.* (1991) reported that glutamine concentration of 3.5 g/l resulted in the highest anther response but it was not significantly different from other concentrations. In case of tobacco, culturing immature microspores in medium with glutamine resulted in gametophytic development. If microspores were initially cultured in medium free of glutamine then transferred to medium with sucrose and glutamine, cell division and embryogenesis were observed (Kyo and Harada 1985, 1986). In the present investigation, plant regeneration could not be obtained from responsive cultivars of TMV12 and Salemkai of *A. hypogaea* and wild diploid species *A. pusilla* in N6 medium supplemented with BAP (0.5 mg/l), NAA (1.0 mg/l) and glutamine (3.5 g/l).

Anther callus induced by pIC was found to be morphogenetic for plant regeneration in several species of *Arachis* (Still *et al.*, 1984; Seitz and Stalker, 1985). In the present experiment, N6 medium having pIC, BAP, proline and CH produced friable light brown callus only and not induced any embryogenesis.

Growth regulators and composition of medium can influence the rate of haploid embryogenesis in many species. Cytokinins have been shown to stimulate microspore embryogenesis in *Brassica napus* (Charne and Beversdorf, 1988) but most often a combination of cytokinin and auxin appears to be beneficial (Lichter, 1981). In the present experiment, anther calli induced by 2,4-D were tested in various concentrations of BAP and NAA in MS medium. The MS medium containing BAP alone, did not induce any differentiation or haploid embryogenesis. When BAP (0.5 mg/l), NAA (2.0 mg/l) and sucrose (30 g/l) were used in MS medium, differentiation and embryogenesis in the callus of *A. hypogaea* (cv. Salemkai) and wild species *A. pusilla* were observed. This type of morphogenetic response in anther culture was observed in *A. hypogaea* (Martin and Rabechault, 1976; Mroginski and Fernandez, 1979; Bajaj *et al.*, 1980a, 1981b; Sastri *et al.*, 1981; Bajaj and Gosal, 1983), *A. pusilla* (Sastri and Moss, 1982) and *A. villosa* and *A. glabrata* (Bajaj, 1985).

According to the present study, ability to produce embryogenic structures was not related to the callus initiation capacity among the tested genotypes. Embryos formed from the anther calli of wild diploid species (*A. pusilla*) were developed in clusters (Plate II-14). It was difficult to estimate the frequency of induction and maturation in wild species because of embryoids formed in clusters. The cv. Salamkai produced 2 to 3 embryos per explant and a maximum of eight somatic embryos were observed. Embroys at different developmental stages (globular, heart shaped) were noticed in the embryo induction medium because the path way of embryogenesis was asynchronous. The mature embryoids of *A. pusilla* germinated into whole plants (Plate II-15,16). This observation was in agreement with that of Sastri and Moss, (1982).

They have obtained regenerated plants from anther calli of *A. pusilla* in MS medium supplemented with BAP (0.5 mg/l) and NAA (2.0 mg/l).

The anther derived callus and regenerated plants obtained from *A. villosa* were mixoploids and showed wide genetic variation ranging from haploids to octoploids (Bajaj, 1985). Such type of regeneration in other wild diploid species have been reported by Bajaj (1985) in *A. glabrata* and in *A. paraguariensis* (Seitz and Stalker, 1985). In the present investigation, the frequency of regenerated plants from *A. pusilla* was very low. The cv. Salemkai produced only shoot (Plate II-13) from embryoids of anther callus and no regeneration into whole plant. This stimulation of shoot morphogenesis elicited by NAA (0.5 mg/l) and BAP (2.0 mg/l) was in agreement with the reports in *A. hypogaea* (Bajaj and Gosal, 1981; Bajaj *et al.*, 1981a) and *A. pintoi* (Pittman 1981). The regenerated plants from anther callus in *A. hypogaea* reported by Bajaj (1981b) and Sastri and Moss (1982) were not haploids.

In the present investigation, the only species which produced regenerants was *A. pusilla* In contrast, other genotypes (CO2, TMV12, TMV10, VG113 and ICG92) with higher callus induction rates than wild diploid species and local cv. Salemkai, did not produce any differentiated structures. This was in agreement with the reports of Logue *et al.* (1993) in barley and Kiviharju *et al.* (1997) in oat anther cultures.

5.1.5. Suspension culture

In legumes, use of liquid medium for callus induction and regeneration was only a few (Bajaj and Gosal, 1981; Mroginski and Kartha, 1984). However, reliable protocols have been developed for establishing callus and suspension cultures in groundnut (Sastri *et al.*, 1981; Roffs *et al.*, 1987; Venkateswaralu *et al.*, 1993).

In the present experiment, anther calli induced by 2,4-D alone and combined with CW were tried in liquid MS medium supplemented with BAP and NAA. In none of the above combinations, morphogenetic response was observed. Callus masses were often seen on anther wall and connective tissues but the origin of the developing embryos was not obvious in the suspension culture. Still *et al.* (1987) got regeneration from the suspension culture of *A. paraguariensis* using N6 medium with pIC and BAP.

5.2. Immature leaflet culture

5.2.1. Callus induction

The successful regeneration of grain legumes from leaf explants have been accomplished with only a few species such as *Arachis hypogaea* (McKently *et al.*, 1991; Eapen and George, 1993a), *Vigna radiata* L. (Mendoza and Futsuhara, 1990) and *Glycine max* L. (Wright *et al.*, 1987). In groundnut, immature leaves have been found to be the highly responding organs for plant regeneration (Mroginski and Kartha, 1984). Regeneration of plants have also been obtained from immature leaves of several wild relatives of *Arachis hypogaea* (Mroginski *et al.*, 1981; Pittman, 1981; Pittman *et al.*, 1983; Johnson, 1981a; Sukumar and Sree Rangasamy, 1984; Narasimhulu and Reddy, 1983, 1985; McKently *et al.*, 1991; Chengalrayan *et al.*, 1994) and mature leaves in *Arachis hypogaea* (Pittman *et al.*, 1982, 1984; Johnson and Pittman, 1985, 1986).

In the present study, callus induction occurred from immature leaflets of young seedlings of *A. hypogaea* (Six cultivars) using 2,4-D (4,20 mg/l) alone or combined with CW (10 ml/l). The callus induction was achieved in 20 d after incubation at 24 h dark condition but the frequency of embryogenic mass formation varied among genotypes and concentrations of auxin used.

Chengalrayan *et al.* (1994) have cultured the immature leaflets of groundnut on medium containing different concentrations of 2,4-D.

In general, hormone concentrations successfully used for callus initiation in seed legumes were usually specific (Evans *et al.*, 1981; Flick *et al.*, 1983). It was in accordance with the present results that 2,4-D at 4 mg/l was effective for spanish bunch (Plate III-17b) and 20 mg/l for virginia bunch (Plate III-18) and virginia runner cultivars (Table 12; Fig.5). Narasimhulu and Reddy (1983) obtained maximum frequency of callus from MS medium with 2,4-D (2.0 mg/l) and kinetin (0.5 mg/l).

In the present experiment somatic embryos were not obtained in the induction medium with 2,4-D (20 mg/l) alone and combined with CW (10 ml/l). But Baker and Wetzstein (1992) have got somatic embryos mostly induced from leaflets placed on induction medium with 30 or 40 mg/l of 2,4-D. The number of alfalfa (*Medicago sativa*) somatic embryos was dependent on the 2,4-D and Kinetin concentrations in the induction medium (Meijer and Brown, 1987a). Baker and Wetzstein (1992) have used high auxin levels to enhance embryo induction from immature leaflets in groundnut.

The embryogenic mass was highest in 2,4-D (20 mg/l). This was in accordance with the results of Chengalrayan *et al.* (1994). However, the proliferation of callus growth was high in spanish bunch group at lower concentration of 2,4-D. Virginia bunch and virginia runner exhibited high callus growth in MS medium containing 2,4-D (20 mg/l) alone or combined with CW (10 ml/l).

5.2.2. Regeneration on leaf calli

Generally, plant regeneration is difficult for seed legumes (Evans *et al.*, 1981). In groundnut, it has been proved that regeneration was possible with immature leaflets (Mroginski *et al.*, 1981; Narasimhulu and Reddy, 1985). The concentration of growth regulators is critical for control of morphogenesis and growth of plant. Generally low auxin and high cytokinin concentrations in the medium resulted in shoot formation. The critical factor for successful plant regeneration using immature leaflets is the age of the leaflets cultured. Mroginski *et al.* (1981) found that immature leaflets from 3-5 d old seedlings were highly responsive than the matured leaflets obtained from 52 d old plants. Somatic embryogenesis and plant regeneration from leaflets of groundnut (*A. hypogaea*) was first reported by Baker and Wetzstein (1992). Recent studies with *A. hypogaea* have indicated that young leaf tissues or zygotic embryos can be induced to regenerate plants via direct organogenesis or somatic embryogenesis (McKently *et al.*, 1991; Cheng *et al.*, 1992; Chengalrayan *et al.*, 1994).

In the present study, leaf calli induced from immature leaflets (12 d old seedlings) using 2,4-D (20 mg/l) was subcultured with reduced concentration of 2,4-D (3 mg/l) for further differentiation and induction of somatic embryogenesis. The transfer of subcultured calli (six genotypes of *A. hypogaea*) to MS medium with different concentrations of 2,4-D and BAP resulted in the formation of somatic embryos in VG113 (Plate III-20) and Salemkai (Plate III-21). Such type of somatic embryogenic masses were observed from immature leaves of groundnut by others (Baker and Wetzstein 1992; Chengalrayan *et al.*, 1994).

In the present investigation, embryos of various shapes and sizes were visible in the same clusters indicating that the process of embryogenesis was asynchronous. Somatic embryos were observed within 16 d. The frequency of embryo germination (Fig.6) to plantlets formation was low (2.0%) in local cv. Salemkai and high in virginia bunch cv. VG113 (4.2%). This was in accordance with the results of Baker and Wetzstein (1992).

Leaflets which induced somatic embryos usually had less callus than leaflets without somatic embryos. This was similarly reported in leaflet and petiole explants of alfalfa (Meijer and Brown, 1987a,b) and leaflets of groundnut (Baker and Wetzstein, 1992).

Genotype has been a large factor in determining regeneration potential in legumes (Pederson, 1986; Meijer and Brown, 1987a,b; Chen *et al.*, 1987; McKently *et al.*, 1990, 1991; Sellars *et al.*, 1990). Regeneration was successful with the genotypes, VG113 and Salemkai (Plate III-22) while unsuccessful in the other four cultivars (CO2, TMV12, TMV10 and M13). Such genotypic differences were observed by Narasimhulu and Reddy (1985). They have obtained regeneration with two genotypes, ICG436 and TMV2, while the other two cultivars were not responded (TG198 and US48).

In the present investigation, virginia bunch and virginia runner group of cultivars were more responsive for production of somatic embryos and plant regeneration from immature leaflets culture than spanish bunch cultivars. This is in accordance with the results of Seitz *et al.* (1987). They have cultured immature leaflets from 47 genotypes and observed that virginia type had high potential for regeneration. On the contrast, Daimon and Mu (1989) reported that multiple shoot formation in leaflets culture was higher in spanish bunch than virginia type.

In another experiment leaf calli induced by 2,4-D (20 mg/l) was also tested in half strength MS medium with different hormonal combinations of NAA, BAP, CW and PM. Medium supplemented with 0.5 mg/l each of NAA and BAP along with 10 ml/l of coconut water induced rhizogenesis in local cv. Salemkai (Plate III-23) whereas PM (10 ml/l) combined with NAA and BAP (0.5 mg/l each) produced compact white powdery calli in cv. Salemkai (Plate III-24).

Mroginski *et al.* (1981) and Pittman *et al.* (1983) have reported shoot morphogenesis in groundnut leaflets explant but they have used BAP and NAA in the basal medium. In the present study only rhizogenesis was observed from leaflet cultures by using half strength MS medium with BAP, NAA and CW. Incorporation of ABA, GA, Z, BAP and Kn in the embryo induction medium was ineffective in improving the frequency of somatic embryo (Chengalrayan *et al.*, 1994).

Conversion of somatic embryos into regenerated plants were obtained with medium supplemented with 0.5 mg/l each of NAA and BAP. Based on these reports it is clear that a variety of growth regulators and basal media will support the regeneration of whole plants in cv. Salemkai and VG113 of *A. hypogaea*. The regeneration of plantlets from leaf derived somatic embryogenic system open an additional avenue for the development of transformation protocols in groundnut.

5.3. Cotyledon culture

5.3.1. Callus induction

Cotyledons from mature seeds had a greater propensity to form shoot buds and shoots at the nodal either directly or through a callus phase. Cotyledons were the most frequently used explants for establishment of callus (Rugman and Cocking, 1985; Banerjee *et al.* (1988) and cell suspension cultures (Verma and Van Huystee, 1971; Van Huystee, 1976; Maldonado and Van Huystee, 1980).

In the present experiment, mature seeds of de-embryonated cotyledons of six genotypes (CO2, TMV12, TMV10, VG113, M13 and Salemkai) of *A. hypogaea* were cultured in MS medium with 2,4-D or 2,4-D with CW and plC which produced callus, shoot and root formation directly from cotyledons culture

(Table 13; Fig.7). The callus formation was observed only at the proximal region of cultured cotyledons (Plate IV-25b). This is in agreement with the results of many workers (Guy *et al.*, 1978, 1980; Pittman, 1981; Sastri *et al.*, 1981; Narasimhulu and Reddy, 1983; Atreya *et al.*, 1984; Bhatia *et al.*, 1985; Nataraja and Sumangala Patil, 1987). The formation of callus at the proximal region of the de-embryonated cotyledons might be due to the wound caused by the excision of embryos (Rao, 1969).

Auxins, pIC and 2,4-D have similar effect for induction of callus compared with 2,4-D combined with CW. But the response of these auxins varied significantly. Picloram (4 mg/l) in MS medium produced only callus, whereas 2,4-D (4 mg/l) with MS medium induced callus, shoot and root formation (Fig.8) and 2,4-D (4 mg/l) combined with CW (10 ml/l) produced callus and root formation. Addition of BAP or Kinetin to the medium, increased the production of friable callus (Ozias-Akins, 1989). Nalini *et al.* (1992) observed callus formation in cotyledons of mature seeds using the hormone combination of NAA (0.01-10.0 mg/l) and BAP (0.1-25.0 mg/l). In cotyledon culture Atreya *et al.* (1984) observed induction of callus and roots by using NAA alone. Nataraja and Sumangala Patil (1987) have obtained callus as well as roots in MS medium with 2,4-D (1.0-5.0 mg/l). A similar response was also noted in the present study involving 2,4-D or 2,4-D combined with CW. These studies have shown that spanish and virginia bunch cultivars were more amenable for induction of callus from cotyledon culture.

5.3.2. Direct shoot formation and rhizogenesis

Cotyledons are a good source for *in vitro* manipulation and that their own nutrient storage is sufficient for initiation of shoots in large numbers. Cotyledons were the best source for clonal propagation (Sastri *et al.*, 1981).

Hypocotyl and cotyledon explants were the most responsive as they exhibited higher frequency of shoot development than leaf, leaf lamina, petiole and internode explant (Kanyand *et al.*, 1994).

In the present study, incorporation of picloram (4 mg/l) in MS medium, did not produce any shoot or nodular out growth or somatic embryos from the cotyledon. Whereas, Ozias-Akins (1989) reported either nodular out growth or somatic embryos by incorporation of pIC (0.5 to 2.0 mg/l) in B5 medium from 50-60% of the cotyledon segments.

Direct shoot and root formation were observed in MS medium containing 2,4-D (4 mg/l) (Plate IV-26b,27,29,30). Such types of regenerated plants were obtained directly from cotyledon segments in MS medium supplemented with NAA and BA (Atreya *et al.*, 1984). Rugman and Cocking (1985) have got shoot buds regenerated from cotyledons cultured on media with Zeatin (2.0 or 4.0 mg/l) or IAA (2.0 mg/l) and BAP (1.0 mg/l) . Nalini *et al.* (1992) have observed maximum number of shoot buds and shoots at nodal end directly by combining NAA (2 mg/l) and BAP (25 mg/l).

In the present study cultivar differences in cotyledon cultures were observed in callus induction and direct shoot formation This was similar to the findings of Mhatre *et al.* (1985) and Bhatia *et al.* (1985). Reddy (1986) also observed intervarietal differences in cotyledon culture among four varieties of groundnut studied. Direct plantlet with root formation were observed more frequently from de-embryonated cotyledons of M13 (Plate IV-27,29) and Salemkai (Plate IV-30) by using 2,4-D (4 mg/l). All other genotypes did not respond for direct plant regeneration (Fig.8). These findings are in agreement with earlier reports of Sastri *et al.* (1981); Atreya *et al.* (1984); Bhatia *et al.*, 1985, 1986). Direct embryogenesis was observed in the cultivar M13 (Plate IV-28) when MS medium was supplemented with 2,4-D (4 mg/l).

Rhizogenesis from the de-embryonated cotyledons were observed at the proximal region in MS medium supplemented with 2,4-D alone (Plate IV-26b) or when combined with CW. The intensity of rhizogenesis and rate of root growth was high in virginia runner followed by virginia and spanish bunch cultivars. This is in accordance with report of Guy *et al.* (1980). They observed rhizogenesis in the de-embryonated cotyledons cultured in MS medium containing 2,4-D, NAA and Kn (each at 2.0 mg/l). Atreya *et al.* (1984) have got maximum rhizogenesis in NAA (1.0 mg/l).

5.3.3. Regeneration

Groundnut cotyledon was found to be a difficult explant for regeneration from callus (Guy *et al.*, 1980; Pittman, 1981). Quian (1981) obtained callus from cotyledons and a single plantlet regenerated from the callus. Shoot bud regeneration from callus of cotyledons culture were obtained by Sastri *et al.* (1981), Atreya *et al.* (1984) and Narasimhulu and Reddy (1983, 1985).

In groundnut, auxin stimulated somatic embryogenesis have been reported (Ozias-Akins, 1989; Hazra *et al.*, 1989; McKently *et a.*, 1991; Eapen and George, 1993a; Eapen *et al.*, 1993). In the present study, 2,4-D (4 mg/l) induced embryogenic calli from cotyledons were used for somatic embryogenesis. 2,4-D has been widely used for somatic embryogenesis in groundnut (Hazra *et al.*, 1989) and soybean (Lazzeri *et al.*, 1987). The combination of BAP (2 mg/l) and NAA (1 mg/l) produced somatic embryos in spanish bunch (TMV12), virginia bunch (TMV10, VG113) and virginia runner (M13). The response of different cultivars during embryogenesis were presented in Plate V-31 to 35. Coconut water (10 ml/l) combined with BAP (2 mg/l) and NAA (1 mg/l) resulted in lower frequency of embryos and plant regeneration (Plate V- 36 to 38). Such type of somatic embryos produced from immature zyotic cotyledons were reported (Baker *et al.*, 1994).

The somatic embryos production varied from 3.20 (M13) to 8.20 (VG113) per explant and number of shoots obtained also varied (Fig.9) from 1.12 (M13) to 6.20 (VG113) per explant (Plate V-31 to 35). These conversion of somatic embryos into plants was dependent on the type and concentration of auxin used.

In the present experiment highest plant conversion of somatic embryos were obtained in NAA (1 mg/l) with BAP (2 mg/l). This was in accordance with the results of Eapen and George (1993a,b). Sellars *et al.* (1990) found that an average of 80% of somatic embryos of groundnut produced shoots, while an average of 61% produced roots. In earlier studies, McKently *et al.* (1990) recovered a maximum of 12 shoots per cotyledon explant cultured on BA (25 mg/l). Cheng *et al.* (1992) obtained an average of 1.6 shoots per explant on a medium containing BA (25 mg/l). Among the cultivars tested, virginia bunch cultivars TMV10 and VG113 responded more for induction of somatic embryogenesis. Therefore, the de-embryonated cotyledons can be used for *in vitro* regeneration because of their easy availability and handling. A large number of shoots can be regenerated from a single cotyledon within a shorter time thus making them a good candidate for use in genetic engineering in future.

5.3.4. *In vitro* flowering

The phenomenon of flowering which is a consequence of a change in the activity of apical meristem from young vegetative structures to sex organs, has been a subject of interest (Zeevart, 1976). Flowering which ensures the sexual method of reproduction allows recombination of gene controlled characters and permits rare favourable mutations to spread in the gene population (Narasimhulu and Reddy, 1984).

In vitro flowering was achieved in the present study, using de-embryonated cotyledon calli induced by 2,4-D (4 mg/l) and further culturing with BAP (0.5 to 2.0 mg/l) and 2,4-D (0.5 mg/l). The production of flower buds from de-embryonated cotyledons was completely regulated by exogenous cytokinin (Narasimhulu and Reddy, 1984). Cytokinin induced floral morphogenesis were observed in tobacco (Wardell and Skoog, 1969) and groundnut (Narasimhulu and Reddy, 1985; Rugman and Cocking, 1985; Reddy, 1994).

Efficiency of *in vitro* flower in three cultivars (CO2, VG113 and M13) belonging to three major groups of *Arachis* were evaluated. Only spanish bunch cultivar CO2 (Plate VI-39) showed positive response. This was in accordance with the results of Reddy (1994). The size of cotyledons was found to influence *in vitro* flowering response (Reddy, 1994). With a decreased cotyledon segment inoculated, there was a reduction in the frequency of flowering from 16.6% to 9.09% (Shoba Anand, 1990). However, biochemical and molecular changes that take place at the site of flower primordia initiation are important in understanding the phenomenon of flowering. The quantitative enzyme levels of peroxidase and acid phosphatase was studied by Reddy (1994) and concluded that the presence of peroxidase and acid phosphatase in high levels may be influencing the induction of *in vitro* flowers in *Arachis hypogaea*. The successful induction of *in vitro* flowering in the present investigation offers an unique system in groundnut to study the molecular and physiological basis of flowering. The findings also have an implication for possible improvement in the reproductive efficiency of groundnut by hormonal manipulation.

5.4. Embryo axis culture

5.4.1. Callus induction

Plant regeneration from embryo axis culture is an important step to overcome post fertilization problems during incompatibility crosses and transfer of gene of interest from wild to cultivated species. Most of the earlier reports described regeneration *via*. organogenesis from a wide variety of sources including anthers, cotyledons, epicotyl, hypocotyl, leaves and embryos (Durham and Parrott, 1992).

This study was performed with the aim to induce somatic embryogenesis from mature embryo axis of groundnut. Depending upon the hormones used and their concentrations, different responses were observed. Callus and shoot formation were obtained in 2,4-D (4 mg/l) and 2,4-D (4 mg/l) combined with CW (10 ml/l) in MS medium (Fig.10). This result was in accordance with the reports of other workers (Nalini and Sastri, 1983a,b; Bajaj and Gosal, 1983; Atreya *et al.*, 1984, Stalker and Eweda, 1988).

Callus induction from mature and immature embryos of *Arachis* at high concentrations of auxins have been reported by Bajaj *et al.* (1982) and Nalini and Sastri, 1983b). However, when pIC (4 mg/l) was used with MS medium, maximum induction of callus (94.7%) with embryogenic state was observed compared to 2,4-D or 2,4-D combined with CW (Table 14). This observation is similar to that of Ozias-Akins (1989). He has obtained embryogenic callus from immature embryo axis cultured using different concentrations of pIC (0.5 to 3 mg/l).

The response for shoot formation and growth pattern of regenerants varied between explants of same genotype and between genotypes. Some of the genotypes responded rapid growth of shoots and slow growth of roots (Plate VI-41 to 46) in 2,4-D. Bajaj *et al.* (1982) observed the same pattern of response in MS medium containing a high concentration of IAA and low concentration of Kn. During organogenesis, shoots with or without roots were formed in bunch cv. CO2 and TMV12 (Plate VI-41,42) and virginia runner cv. M13 and Salemkai

(Plate VI-45,46) Whereas shoots formation alone was seen in virginia bunch, cv. VG113 and TMV10 (Plate VI-43,44).

5.4.2. Regeneration

The subcultured calli resulted 67.1% and 65.6% shoots formation respectively in MS medium containing NAA (1 mg/l) and BAP (2 mg/l) and in combination with CW(10 ml/l) (Table 15). This type of organogenesis induced by manipulations of exogenous phytohormone levels occurs directly from callus phase. Atreya *et al.* (1984) got normal plants from embryo axis of groundnut. Even in callus mediated organogenesis, organ forming capacity limited to primary callus indicates that the potential existence of meristems embedded in the explant (Parrott *et al.*, 1992).

Mature embryonal axis induced calli from pIC resulted somatic embryos formation (Table 15) in MS medium containing pIC (0.1 mg/l). Genotype M13 produced the maximum of 15.2 somatic embryos per explant (Plate VII-47) and 14.2 embryos per explant was recorded in cv. VG113 (Plate VII-49). In the preliminary attempts, plants and shoots were obtained in cv. M13 (Plate VII-48), and VG113 (Plate VII-50) after transfer of somatic embryos (Clumps) in MS medium containing NAA (1 mg/l) and BAP (2 mg/l). Similar type of results were noticed by Ozias-Akins (1989) and Ozias-Akins *et al.* (1992a,b) when immature embryo axis was cultured with pIC (0.5 to 1.0 mg/l) in B5 medium.

5.5. Somatic embryogenesis

Somatic embryogenesis is a process whereby a cell or group of cells from somatic tissues form an embryo. These embryos develop into plants through characteristic stages like globular, heart, torpedo and cotyledonary stages. In general, development of somatic embryos parallels that of zygotic embryos. As with organogenesis, somatic embryogenesis in legumes is not inherently different from other plants and the same fundamental principles appear to apply in all cases (Parrott *et al.*, 1992; de Jong *et al.*, 1993). Depending on the species, somatic embryogenesis can occur indirectly from an intervening callus phase or directly from explanted tissues. Indirect embryogenesis was observed in alfalfa by Meijer and Brown (1987a,b), while direct embryogenesis from explants was noticed by Parrott *et al.* (1988) and Buchheim *et al.* (1989) in soybean.

5.5.1. Effect of auxins

Auxin type, concentrations and exposure time have also proven important for initiation of somatic embryogenesis. Groundnut appears to be responsive to a variety of synthetic auxins *viz.*, 2,4-D (0.5-40.0 mg/l), NAA (1.0-3.0 mg/l) and picloram (0.001-0.02 mg/l) for induction of somatic embryogenesis (Durham and Parrott, 1992). However in groundnut immature embryo culture, pIC was used for induction of somatic embryogenesis (Ozias-Akins 1989, Ozias-Akins *et al.*, 1992a,b; Eapen and George, 1993a).

In the present experiment, MS medium supplemented with picloram (2-6 mg/l) on mature embryo axis showed that increased concentration of pIC (2-4 mg/l), increased the number of somatic embryos/explant (Table 16). Further increase of pIC levels (6 mg/l) decreased the number of somatic embryos production per explant. The concentration, 4 mg/l was found to be optimum for induction of somatic embryogenesis. Similar observations were noticed by Ozias-Akins (1989), Ozias-Akins *et al.* (1992a,b) and McKently (1995) in B5 medium with pIC (0.5 to 1.0 mg/l), and MS basal salts supplemented with B5 vitamins and pIC (12.42 μM).

Substitution of pIC with similar concentration of 2,4-D and NAA on mature embryo axis resulted in the induction of somatic embryogenesis

(Tables 16,17). It was high in pIC (4 mg/l) (51.4 to 99.3%). The induction of embryogenesis by 2,4-D was less (15.2 to 35.2%) compared to pIC (Table 16), whereas NAA did not induce embryogenesis (Table 17). Sellars *et al.* (1990) observed highest frequency of somatic embryos when pIC was used in the induction medium, and lower frequencies were observed with either NAA or 2,4-D. Similarly Ozias-Akins (1989) observed somatic embryogenesis using pIC, but little success when NAA was used. Hazra *et al.* (1989) reported that cv. SB11 did not respond to NAA for embryogenesis, as have been observed in the present studies. NAA was not able to induce somatic embryogenesis from shoot apices of pea (Loiseau *et al.*, 1995) though NAA seems to be efficient on immature zygotic embryos of pea (Tetu *et al.*, 1990) and soybean (Barwale *et al.*, 1986; Lazzeri *et al.*, 1987). It appears that different legumes responded differently to 2,4-D, NAA and pIC.

Attempts were also made to see the combined effect of 2,4-D, NAA and pIC on mature embryo axis culture. Picloram combined with 2,4-D induced high embryogenesis (73.2%) in cv. TMV12 with 14.2 SE/explant, whereas 2,4-D combined with NAA produced less (30.2%) with 3.2 SE/explant (Fig.11).

5.5.2. Effect of media

Composition of the nutrient medium is an important factor in the successful development of somatic embryogenesis. Different media with specific modifications have been used for induction of somatic embryogenesis and plant regeneration in legumes.

Among the four types of media *viz.*, MS, N6, B5 and Blaydes on induction of somatic embryogenesis, MS medium was found to be suitable for mature embryo axis culture (Table 18). The four media MS (1962), N6 (1978), B5 (1968) and Blaydes (1966) differed mainly in their inorganic salts and vitamins.

However, Walker and Sato (1981) suggested that the requirement of reduced nitrogen in the form of ammonium was responsible for the dedifferentiation of somatic embryos. In the present experiment, MS medium (1.65 g of NH_4NO_3/l) induced more SE/explant (14.2) compared to other media.

5.5.3. Effect of explants

The choice of explant can have a greater influence on the success of an embryogenesis. The present experiments have shown that selection of suitable explant was important for obtaining high frequency of somatic embryogenesis and plant development. Mature embryonal axis was found to be the best source in comparison with anthers, immature leaflets and mature cotyledons for induction of somatic embryogenesis *via.* intervening callus (Fig.12). McKently (1990, 1995), McKently *et al.* (1990), George and Eapen (1993) and Baker *et al.* (1995) have obtained somatic embryogenesis from mature embryonal axes. Previous workers have used immature embryonal axes (Hazra *et al.,* 1989; Ozias-Akins *et al.,* 1992a,b; Eapen and George, 1993a; Eapen *et al.,* 1993; Ramdev and Reddy, 1993a,b), immature cotyledons (Ozias-Akins, 1989; Durham and Parrott, 1992; Eapen and George, 1993a; Eapen *et al.,* 1993; Wetzstein and Baker, 1993; Baker *et al.,* 1994, 1995), mature cotyledons (Gill and Saxena, 1992), leaves (McKently *et al.,* 1991; Baker and Wetzstein, 1992; Gill and Saxena, 1992; Chengalrayan *et al.,* 1998) and whole immature embryos (Sellars *et al.,* 1990). The present results showed that mature embryo axes were highly responsive in culture. Similar results were obtained by Baker *et al.* (1995) in dry seeds of cultivar GK7 of *Arachis hypogaea.* In general mature vegetative tissues are more convenient and readily available than immature or floral tissues (Brown *et al.,* 1995).

5.5.4. Effect of genotypes

Genotype is well established as an important factor influencing the embryogenic response *in vitro*. In dicots, variability in both the occurrence and frequency of somatic embryogenesis have been observed among species or individuals of a cultivar (Mitten *et al.*, 1984; Chen *et al.*, 1987; Matheson *et al.*, 1990).

Genotypic variation for embryogenesis existed in the present study and responsiveness to embryogenesis differed significantly between individuals of the same cultivar. Similar result was obtained in cotton (Trolinder and Xhixian, 1989). Among six genotypes of three major groups, spanish bunch cv. TMV12 produced maximum number of SE/explant (17.3) compared to virginia bunch cv. VG113 (15.2) and virginia runner cv. M13 (16.2). The frequency of somatic embryogenesis on mature embryo axis varied between different groups of *Arachis*. However cv. TMV12 responded higher induction of somatic embryogenesis (99.3%) than other cultivars in MS medium containing pIC (4 mg/l) (Table 16). Genotype remains the factor with the greatest influence on frequency of somatic embryogenesis from mature axes. Genotypic effects had previously been reported for embryogenesis from other explants of groundnut (Sellars *et al.*, 1990; Ozias-Akins *et al.*, 1992a,b; George and Eapen, 1993; Eapen *et al.*, 1993). However genotypes from varieties *hypogaea* and *vulgaris* had a higher embryogenic frequency and produced significantly more somatic embryos than those from variety, *fastigiata* (McKently, 1995).

The embryos formed in cvs. TMV12 (Plate VII-51,52), CO2, VG113 and M13 were placed in the hormone free medium for germination. The embryos developed into plantlets when transferred into growth regulators containing medium (Plate VII-53 to 56 and VIII-57 to 62). Similar type of responses have been reported in mature embryos axes (McKently, 1990, 1995; McKently *et al.*, 1990; George and Eapen, 1993; Baker *et al.*, 1995) and immature embryonal axes of groundnut (Ozias-Akins, 1989; Ozias-Akins *et al.*, 1992a,b, Eapen and George, 1993a; Eapen *et al.*, 1993).

The quantity of somatic embryos produced was much greater in spanish bunch cv. TMV12 (Plate IX-63 to 65) and virginia runner cv. M13 (Plate X-72 to 76). Genotypes with the highest competence for somatic embryogenesis (TMV12) do not necessarily gave the highest rate of conversion to plants. This behaviour was also observed in *Glycine max* by Komatsuda and Ohyama, (1988). Parrott *et al.* (1989) noticed the same relationship between percentage and number of somatic embryos formed on cotyledons of soybean.

5.5.5. Effect of amino acid

This study was performed with three amino acids (Proline, glutamine and tryptophan) at different concentrations (Fig.13). Proline at 25 mg/l had no significant effect on embryogenesis, however, addition of 100 mg/l to the nutrient medium increased the number of embryos per explant (18.2).

Somatic embryogenesis was stimulated by addition of Proline in alfalfa (Stuart and Strickland, 1984b; Shetty and McKersie, 1993) barley (Rengel and Jelaska, 1986) carrot (Ronchi *et al.*, 1984) and Pepper (Buyukalaca and Mavituna, 1996). In alfalfa, proline has only a minimal effect in stimulating somatic embryogenesis by itself but produced higher effect when combined with an optimal concentration of ammonium. An increase in mitotic divisions has a significant effect when proline was added during the growth of the cultures in the presence of hormones (Ronchi *et al.*, 1984). The increase of mitotic index may be connected with the formation of cells competent for embryogenesis.

The higher concentration of proline (150 mg/l) showed negative result on embryogenesis in groundnut. According to Meijer and Brown (1987b), high concentrations proline, did not result an increase of embryo yield or embryo quality in alfalfa. The high external osmotic potential is normally considered inhibitory to the growth of plants. According to Stuart and Strikland (1984b) proline and other amino acids are taken up by cells to create an osmoticum within the cell cultures.

Glutamine has positive effect on somatic embryogenesis. In mature embryo axis culture, glutamine (50 mg/l) supported the maximum somatic embryos production in cv. TMV12 (16.2). Further increase in the concentrations of glutamine suppressed the embryogenesis. In alfalfa, addition of amino acids to the medium increased the number of somatic embryos (Skokut *et al.*, 1985; Stuart and Strickland, 1984b) and yielded the best embryo conversion into plantlets (Stuart and Strickland, 1984a).

Addition to tryptophan (50 mg/l) to the nutrient medium helped to form maximum somatic embryos per explant (16.2). Further increase in the concentrations of tryptophan suppressed the embryogenesis. Enhancement of somatic embryogenesis by tryptophan was reported in rice by Siriwardana and Nabors (1983).

5.5.6. Liquid culture

The transfer of embryogenic calli of mature embryo axis in liquid medium resulted lower number of somatic embryos production per explant (2.4) compared to solid medium (17.3). In groundnut, Gill and Saxena (1992) reported that agar solidified medium was found to be superior to the liquid medium for the development of embryos and shoot buds. However, repetitive embryogenic cultures in liquid medium were reported in soybean by Finer and Nagasawa (1988) and in *Arachis* by Durham and Parrott (1992).

5.5.7. Development of somatic embryos

The transfer of embryogenic callus of mature embryo axis into a medium with reduced concentration of pIC (0.1 mg/l) resulted in the production of somatic embryos in different cultivars. During the first three days after transfer into a low auxin medium, the rate of cell division within the embryogenic cluster was low. After that rapid cell division occurred in the cell clusters leading to the formation of embryos.

In alfalfa, different patterns of somatic proembryo formation was observed depending on the region of the explant in which the embryo was initiated (Santos *et al.*, 1983). A similar pattern was observed in the present study in cv. M13 (Plate X-72,73). In corn, transitional structure analogous to the proembryo stage was observed (Fransz and Schel, 1991). The globular stage of somatic embryogenesis clearly marks the beginning of structural differentiation. Various stages like globular, heart and cotyledonary were observed during embryogenesis in different cultivars *viz.*, TMV12 (Plate IX-63-66), VG113 (Plate IX-67, 71) and M13 (Plate X-72 to 81). Although little is known about the specific interactions at the surface of plants (de Vries *et al.*, 1988), the cell surface components are the most likely candidates for the generation of various shapes of the somatic embryo.

Schiavone and Cooke (1985) observed that prior to the formation of the heart shaped embryo, axil elongation of the inner isodiametric cells of the globular embryo leads to the formation of a longitudinal extension near the lower end of the embryo to form oblong shaped embryo. Such oblong shaped embryo was observed in cv. TMV12 (Plate IX-64) at the time of embryogenesis. Schiavone and Cooke (1985) further suggested that the formation of the oblong-shaped embryo is the first sign of incipient procambium formation.

Differentiation of organelles was also observed at the globular stage (Plate IX-69, X-77). Plastids begin to turn green in the terminal portion of the developing somatic embryos (Street and Withers, 1974).

The next important morphogenetic events observed in the different cultivars were heart shaped and cotyledonary stage. In dicots, cotyledons arise as small protrusions from the peripheral region of the terminal end of the somatic embryos. In carrot somatic embryos, cotyledon primordia are composed of dense isodiametric cells. Variable number and a variety of cotyledonary forms can be found in the same culture (Plate IX-70, X-76). In soybean somatic embryos, a range of cotyledon morphologies have been observed *i.e.*, mono, di, poly and fused forms (Buchheim *et al.*, 1989). The cotyledons highly influenced the germination process. Buchheim *et al.* (1989) demonstrated that there was direct relationship between the amount of cotyledonary tissue and conversion time required for recovery of plantlets or plants from somatic embryos.

5.5.8. Histological studies

The histological studies of the somatic embryos initiated from the callus of mature embryo axis confirmed the various stages of development of somatic embryos (Plate XI-82 to 89). During mature embryo axis culture, some groups of meristematic cells progressively became embryonic and among those groups of embryonic cells, fragmentation occurred, leading to the formation of somatic embryos. Globular stage embryos were originated from the superficial embryogenic cells leading to heart shape and cotyledonary stage. The longitudinal section of cotyledonary embryo showed the presence of shoot and root meristems which was typical of embryo development. Mitotic stimulus in superficial cells might be the probable reason for embryo development. The formation and development of somatic embryos originated directly as superficial cells of cotyledon surface in pea (Tetu *et al.*, 1990) and groundnut (Gill and Saxena, 1992) and on mature embryo axis culture (Hazra *et al.*, 1989).

In Trifolium, somatic embryos were produced directly from the hypocotyl epidermis (de Jong *et al.*, 1993). According to Alemanno *et al.* (1996), one somatic embryo was derived from one group of embryonic cells, which was itself derived from the division of a single meristematic cell. When comparing the early stages of somatic and zyygotic embryo formation, similar pattern of development have been observed in rice (Jones and Rost, 1989) and Vitis (Altamura *et al.*, 1992). In the present study somatic embryos also exhibited morphological abnormalities (Ammirato, 1983).

5.5.9. Embryo maturation

Testing different maturation media showed that, half strength MS basal medium was found to suitable for maturation of somatic embryos (Plate XII-90; Table 19). This type of observation was also made previously in soybean (Lazzeri *et al.*, 1987), groundnut (Hazra *et al.*, 1989) and pepper (Buyukalaca and Mavituna, 1996).

Embryo maturation is often associated with a reduction or omission of auxin from the medium. In some species, no additional culture steps were needed for maturation of embryos. However, in many other species the development is blocked at the globular stage (Roberts, 1991). In Glycine max (Christou and Yang, 1989) maturation was improved by increasing the sucrose concentration in the medium. Attempts made in the present study indicated that sucrose 30 g/l was found to be optimum for embryo germination (Table 20; Plate XII-92,93) and conversion into plantlets. In groundnut somatic embryogenesis, sucrose, 60 g/l produced the best results (Eapen and George, 1993a), while in soybean lower concentration were more beneficial (Lazzeri *et* *al.*, 1988; Komatsuda *et al.*, 1992). Sucrose (30 g/l) in the maturation medium ensured the full development of embryos and from these embryos high conversion rates to normal plantlets were obtained.

Addition of BAP (0.5 mg/l) and NAA (0.5 mg/l) to half strength MS medium containing sucrose (30 g/l) did not have any impact for germination of embryos (Table 21). In the case of Nerine, substitution of 2-ip (10 µM) for BA and elevation of the sucrose concentration for 3 to 6% enhanced embryo maturation (Lilien-Kipnis *et al.*, 1994). In carrot, 50% of somatic embryos induced with cadmium were reported to germinate, while only 15% of somatic embryos induced with 2,4-D (Kamada *et al.*, 1989). Endogenous cytokinin levels have been observed to decrease the development of both zyogotic embryos of soybean (Aung *et al.*, 1982) and somatic embryos of celery (Danin *et al.*, 1993). Further more, exogenous cytokinins can lead to abnormal development or suppression of the main embryo axis (Maheswaran *et al.*, 1985).

5.5.10. Conversion of somatic embryos into plantlets

The matured somatic embryos of TMV12, VG113 and M13 placed in a medium containing NAA, BAP and TDZ resulted higher conversion of somatic embryos into plantlets (66.5%) (Table 22). Although somatic embryos were induced and developed on the same auxin containing medium, approximately 40% of them could be converted into plants in shoot apices of pea when they were transferred into a medium devoid of growth regulators (Loiseau *et al.*, 1995). In the present study, NAA and BAP in combination with TDZ have induced multiple shoots formation. Such combination of BAP and TDZ in Eastern red bud induced multiple shoot formation (Distabanjong and Geneve, 1997). However, TDZ has been reported to have cytokinin like properties and has been demonstrated to stimulate *in vitro* meristem and shoot formation at low concentration (Huettman and Preece, 1993).

In the present experiments an additive effect was noticed using both compounds. The highest number of shoots formation from somatic embryos were obtained in half strength MS medium supplemented with NAA (0.5 mg/l), BAP (1.0 mg/l) and TDZ (0.2 mg/l). Further increase in plant growth regulator concentrations did not increase the number of plantlets formation. Malik and Saxena (1992) demonstrated the usefulness of TDZ in stimulating shoot regeneration in seed cultures of dry bean, a regeneration recalcitrant legume. On the basis of high frequency regeneration of several cultivars obtained with TDZ, it was suggested that this compound may serve as a general inductive stimulus for *de novo* differentiation of shoots in large-seeded grain legumes (Malik and Saxena, 1992). Similarly, germination of groundnut seeds and prolonged culture of seedlings in the presence of TDZ produced somatic embryos (Saxena *et al.*, 1992).

The best plant conversion frequency was obtained (66.5%) in the present study when picloram was used in induction medium and subsequent plantlets conversion with NAA, BAP and TDZ (Table 22). Similarly when dicamba and picloram was used for somatic embryo induction from cotyledons, 25% of plant conversion was obtained (Eapen and George, 1993a). Sellars *et al.* (1990) found that an average of 80% of somatic embryos of groundnut produced shoots, while an average of 61% produced roots. In the experiment of Ozias-Akins (1989), the conversion frequency ranged from 0-18%. However, embryos initiated from cotyledons in the presence of NAA, 2,4-D or picloram, only 10% of the somatic embryos developed into complete plantlets (Eapen *et al.*, 1993). In soybean, although larger number of somatic embryos were produced, NAA favoured for subsequent conversion in to plants (Barwale *et al.*, 1986; Lazzeri et *al.*, 1987).

Cultivar TMV12 showed maximum germination of somatic embryos (62.5%), followed by cvs. M13 (55%) and VG113 (45%) (Fig.14). The pathway of different morphological changes occurred from germination to regeneration are presented in (Plate XIII-96 to 103). Similar genotypic differences in soybean was reported (Komatsuda and Ohyama, 1988). Similarly, in groundnut the percentage of somatic embryos forming shoots ranged from 3% for genotype 803 to 45% for 487 B (McKently, 1995) in mature embryo axis culture.

The analysis of 65 randomly selected regenerants of cv. M13, TMV12 and VG113 revealed that all the regenerants were tetraploids as were the source plants. The results of the present investigation indicated that spontaneous variation in ploidy level did not occur The relative ease with which groundnut can be regenerated from somatic embryos of mature embryo axis makes the system particularly attractive for further study on Agrobacterium mediated genetic transformation for improvement of groundnut programmes in future.

5.6. Synthetic seeds

The concept of synthetic seeds was developed as a result of increased knowledge of somatic embryogenesis and biotechnology. It has been recently defined as artificially encapsulated somatic embryos, shoots or other tissues that can be used for sowing under *in vitro* or *ex vitro* conditions (Aitken-Christie *et al.*, 1995). The potential uses for artificial seeds are numerous, including delivery of elite germplasm, hand pollinated hybrids with reduced seed fertility and genetically engineered plants with sterile or unstable genotypes. The size of the synthetic seed and the coating around the somatic embryo potentially are advantageous for storage, handling, transportation and planting (Redenbaugh *et al.*, 1988).

Groundnut is a seed propagated plant but multiplication ratio is low compared to cereals. Since somatic embryogenesis has been successful, propagation through use of synthetic seeds may be desirable. The somatic embryos (M13, TMV12, VG113) with distinct cotyledonary stage (Plate XIV-105 to 107) was used for encapsulation in the present study.

5.6.1. Effect of sodium alginate for beads

Sodium alginate was the most beneficial encapsulating agent due to its low cost, easy use and low toxicity (Redenbaugh *et al.*, 1987a). The term hydrogel was used for hydrated encapsulation that can provide protection and convenient handling (Redenbaugh *et al.*, 1986). Such a capsule can serve as a reservoir of nutrients (an artificial endosperm) at the time of germination (Liu *et al.*, 1992). Encapsulation can provide a physical protection to the somatic embryos as well as a mean to carry nutrients, growth regulators, antibiotics and fungicides to assist in germination and plant survival (Kitto and Janick, 1985; Janick *et al.*, 1989). Fujii *et al.*, (1987) suggested that the main use of encapsulation was for mass propagation of field crops which required a protective covering.

Synthetic seeds were made by mixing the embryos in sodium alginate solution and dropping the embryos into a calcium salt solution or by inserting an embryo into a drop of sodium alginate as it was falling into the calcium chloride solution (Redenbaugh *et al.*, 1987a). In the present study, somatic embryo along with sodium alginate solution was dropped into a calcium chloride solution by means of a dropper. Concentration of sodium alginate plays a crucial role during encapsulation. Sodium alginate (3%) with calcium chloride (0.7%) was found to be effective (Table 23) for encapsulation of somatic embryos (Plate XIV-110 to 111). Gel capsules formed at 2.5% sodium alginate were too soft to handle (Plate XIV-108,109) while those formed with 4% alginate were too hard and hindered the emergence of root and shoot. Sodium alginate 3% was used by Janeiro *et al.* (1997) for encapsulation of somatic embryos in camellia (*Camellia japonica*).

Bapat and Rao (1989) used 2.5 per cent sodium alginate in mulberry and sandalwood and Datta and Potrykus (1989) employed 3.2 per cent in barley. In ginger, buds were successfully encapsulated using 4 per cent sodium alginate gel (Sharma *et al.*, 1994). Ruffoni *et al.* (1994) recommended 2 per cent for lisianthus. However, in woody species sodium alginate from 2.0 to 3.0% w/v can efficiently reduce the problem of capsule hardness and shape (Piccioni and Standardi, 1995). The capsule hardness was a function of guluronic acid and mannuronic acid ratio in sodium alginate (Redenbaugh *et al.*, 1993). The beads formed using 4% sodium alginate might have higher content of guluronic acid to form the formation of hard beads.

5.6.2. Effect of sodium alginate concentration on germination

The germination and conversion of encapsulated somatic embryos is governed by supramolecular structure of the alginate gel beads which is affected by alginate concentration, time of gelation, mannuronic-guluronic acid ratio, temperature and pH of the solution (Saucedo *et al.*, 1989). The concentration of sodium alginate needed for encapsulation and optimal conversion, varied among different beads. Embryos that were encapsulated at 3.0% sodium alginate showed the highest conversion of plantlets (32.5%) with better development of shoots and roots compared to 2.5% and 3.5% concentrations (Table 24). Similar reports of a maximum plantlet regeneration frequency with an optimum concentrations of 3.2% sodium alginate was reported in alfalfa and Celery (Redenbaugh *et al.*, 1986) and 3% for *Solanum melongena* (Rao and Singh, 1991). The concentration of complexing agent

($CaCl_2.2H_2O$) also affected the frequency conversion of encapsulated embryos (Rao and Sing, 1991; Ghosh and Sen, 1994).

When non-encapsulated embryos were subjected to germination, a conversion of 60% was obtained in the present experiment. As discussed earlier, encapsulated somatic embryos registered a maximum of 32.5 per cent germination only. Similar depressing effect of encapsulation on conversion was reported by Redenbaugh *et al.* (1986) and Ghosh and Sen (1994). Redenbaugh (1990) suggested that lower conversion due to alginate encapsulation was due to leaking of nutrients and sucrose. Harada *et al.* (1990) reported a 30 to 80 per cent conversion in carrot encapsulated embryos. In *Eucalyptus citriodora*, the encapsulated somatic embryos had an *in vitro* germination rates of about 40% (Muralidharan and Mascarenhas, 1995) but only 10% germination was achieved in sandal wood embryos encapsulated using 3% sodium alginate solution (Bapat and Rao, 1988).

5.6.3. Effect of media on conversion frequency

For commercialization of artificial seeds, efficient conversion into complete plantlets should be achieved. The present study with different media showed that half strength MS medium was found to be suitable (Table 25) for maximum conversion of plantlets (33.2%). This might be due to the optimal requirements of the nutrients for germination of the artificial seeds. However the use of full strength MS basal medium for conversion also gave the highest conversion frequency of artificial seeds in *Solanum melongena* (Rao and Singh, 1991), sandal wood (Bapat and Rao, 1988) and in *Asparagus cooperi* (Ghosh and Sen, 1994). Ganapathi *et al.* (1992) reported highest rate (100%) of plantlets conversion in banana using white's medium.

Attempts were also made on the effect of different support media. Highest germination (50%) was achieved in agarised half strength MS medium containing the growth hormones (Table 26). Ghosh and Sen (1994) and Rao and Singh (1991) have reported maximum response of asparagus synthetic seed as full strength MS medium compared to half strength MS medium. The germination of encapsulated somatic embryos was influenced by interaction of media and stage of embryo (Kitto and Janick, 1985). Plantlet formation was also noticed on substrate of moist cotton and filter paper but the frequency was low compared to plantlet development on MS medium (Ganapathi *et al.*, 1992).

In the present experiment, the synthetic seeds placed on the filter paper with wetting of water or agar or distilled water did not helped germination. Liu *et al.* (1992) stated that carrot synthetic seed had a higher germination when planted on agar or vermiculite containing nutrient medium. Li (1993) observed that carrot synthetic seeds were capable of germinating in non-sterile vermiculite containing medium (87%), when a "preservative" (fungicide) was included in the alginate capsule.

5.6.4. Effect of storage on germination

In the present experiment the conversion frequency of encapsulated embryos containing MS nutrients and distilled water was variable in different storage periods. The encapsulated embryos with MS medium in their gel matrix germinated and produced complete plants (52.5% after 7 and 15 d storage and 47.5% in 30 d storage) (Plate XV-112 to 117). When embryos encapsulated with water in the gel matrix produced only 2% conversion after 7 d and none after 15 d storage (Table 27). Sixty day storage of encapsulated embryos took longer days for regeneration into whole plants (Plate XV-118 to 120). The same trend was observed by Ghosh and Sen (1994) in *Asparagus cooperi* encapsulated embryos stored at 4°C. Bapat and Rao (1988) examined the effect of 45 d at 4°C cold storage on germination of encapsulated embryos of *Santalum album*. They have reported a reduction in germination frequency. The same trend was observed in the present study when encapsulated embryos of groundnut stored at 45 d (4°C) reduced the germination percentage from 52.5 to 30.0%. Datta and Potrykus (1989) also reported a reduction in germination frequency from 65 to 37.5% for encapsulated microspore derived barley embryos after 6 months storage at 4°C. Padmaja *et al.* (1995) observed a low frequency of germination (8.2%) from synthetic seeds of groundnut stored for 40 d at 4°C.

In contrast, encapsulated embryos of *Santalum album* retained their germinability (18%) after storage at 4°C for 45 d (Rao and Bapat, 1993). Lulsdorf *et al.* (1993) claimed that both encapsulated and non-encapsulated somatic embryos of interior and black spruce, survived one months storage at 4°C with no loss of germination capacity but their criterion for germination was simply radicles elongation greater than 2 mm. The decline in germination into plants of stored encapsulated embryos may be related to both oxygen deficiency in the gel bead and its rapid drying (Redenbaugh *et al.*, 1991).

Attempts were made in the present investigation to store the encapsulated somatic embryos and non-encapsulated somatic embryos at 4°C for 60 d (Table 28). The results revealed that encapsulated embryos exhibited 4% conversion after two months of storage while non-encapsulated somatic embryos failed to germinate. This was in agreement with the report of Onay *et al.* (1996) clearly indicating the possibility to optimise the technique for production of plantlets after storage. However a 100% germination of sodium alginate encapsulated carrot somatic embryos after two month storage at 4°C was reported by Liu *et al.* (1992). So a successful encapsulation procedure must ensure that embryos retain their viability for longer period and are able to germinate. Future experiments could also test if oxygen supply might be a limiting factor and further investigation with capsule nutrient supplementation could be attempted.

Summary

6. SUMMARY

Anther, immature leaflets, de-embryonated cotyledons and mature embryo axes of *A. hypogaea* L. and anthers in *A. pusilla* L. were used for induction of androgenic haploid, somatic embryogenesis and synthetic seed development. Plant regeneration was attempted by using different media, growth hormones and amino acids. Histological and cytological observations were carried out to confirm the regenerants obtained *via* somatic embryogenesis. The salient findings are presented below.

6.1. Anther culture

Light yellow colour of the anthers indicate the uninucleate stage of microspores. Pretreatment of flower buds at 4°C for 2 d, sterilization with 70% ethanol for 1 min followed by treatment with 0.05% mercuric chloride for 10 min. were the best suitable conditions for anther callus induction (97.8%).

MS medium was found to be suitable for induction of higher callus (40.1%) than the other media.

Spanish bunch cultivars responded better (29.2%) for induction of anther callus than virginia bunch (22.2%) and virginia runner (21.1%) of *A. hypogaea* and *A. pusilla* (18.5%).

Order of anther callus induction for different genotypes were TMV12 > CO2 > TMV10 > VG113 > ICG92 > Salemkai > *A. pusilla*.

Combination of coconut water (10 ml/l) with 2,4-D (4 mg/l) was found to be suitable for anther callus induction.

Callus induction was higher in 16 h light exposure (37.1%) than 24 h dark condition (24.1%).

Embryogenic nature of calli were higher in 24 h dark condition compared to 16 h light and 8 h dark condition.

N6 medium with BAP (0.5 mg/l), NAA (1.0mg/l) and glutamine (3.5 g/l) induced differentiation (TMV12), embryoids formation (*A. pusilla*) and shoot initiation (Salemkai) but no regenerants was noticed .

Anther calli in MS medium with BAP (0.5 mg/l) and NAA (2.0 mg/l) responded for embryoid formation in cv. Salemkai and *A. pusilla*. Regeneration of haploid plants was obtained only in *A. pusilla*.

6.2. Immature leaflet culture

Spanish bunch cultivars induced leaf calli at lower dose of 2,4-D (4mg/l), whereas virginia bunch and virginia runner responded at higher concentration of 2,4-D (20 mg/l).

Somatic embryos were obtained in cv. VG113 and Salemkai in MS medium containing 2,4-D (0.5 mg/l) and BAP (1.5 mg/l) from leaf calli.

Regeneration of plantlet was obtained from somatic embryos of cv. VG113 and Salemkai in MS medium containing 0.5 mg/l each of BAP and NAA.

Leaf calli of cv. Salemkai produced rhizogenesis in half-strength MS medium containing 0.5 mg/l each of BAP and NAA.

6.3. De-embryonated cotyledon culture

Picloram (4 mg/l) induced higher callus (91.7%) from cotyledons when compared to 2,4-D (4 mg/l) or 2,4-D (4mg/l) combined with CW (10 ml/l).

The induction of rhizogenesis was higher in virginia runner cultivars compared to virginia bunch and spanish bunch.

The non-responsive cotyledons subcultured in the same medium (MS + 2,4-D, 4mg/l) induced both direct embryogenesis and organogenesis in cv. M13 and organogenesis only in cv. Salemkai.

Cotyledon calli produced somatic embryos in cv. TMV12, VG113, TMV10 and M13 in MS medium containing BAP (2 mg/l) and NAA (1mg/l).

In vitro flowering was achieved when the 2,4-D induced calli was placed on to MS medium containing BAP (0.5 to 2.5 mg/l) with 2,4-D (0.5 mg/l).

6.4. Embryo axis culture

MS medium with pIC (4mg/l) exhibited maximum callus induction (94.7%) compared to 2,4-D (4mg/l) or 2,4-D (4mg/l) combined with CW (10 ml/l).

Organogenesis was induced from undifferentiated callus mass of embryo axis in MS medium containing 2,4-D alone (4mg/l) or in combination with CW (10 ml/l).

Spanish and virginia bunch cultivars were highly responsive to organogenesis compared to virginia runner cultivars.

Somatic embryos formation was observed in MS medium with pIC (0.1 mg/l).

6.5. Somatic embryogenesis

Picloram 4mg/l was found to be optimum for the induction of somatic embryogenesis (17.3 SE/explant). Picloram induced high embryogenesis ranging from 51.4 to 99.3% compared to 2,4-D (15.2 to 35.2%), whereas NAA did not induce embryogenesis.

NAA at 4 mg/l produced both rhizogenesis and organogenesis in cv. TMV12 and M13, whereas rhizogenesis alone was induced in cv. TMV12 and M13.

Among the four media tested, MS medium exhibited higher embryogenesis (86.4%).

Among the four explants tested, mature embryo axis was found to be suitable for embryogenesis induction.

Cultivar TMV12 (SB), showed 99.3% embryogenesis with 17.3 SE/explant, M13 (VR) 92.3% embryogenesis with 16.2 SE/explant and VG113 (VB) 91.2% embryogenesis with 15.2 SE/explant.

Among the three amino acids tested, proline, 100 mg/l, increased the production of somatic embryos (per explant). Tryptophan and glutamine each at 50 mg/l also favoured somatic embryogenesis.

Globular, heart shaped and cotyledonary stage of embryos were observed in cv. TMV12, VG113 and M13 during embryogenesis.

Liquid MS medium with pIC (0.1 mg/l) produced lesser SE/explant (2.4) compared to solid culture (17.3).

Histological studies showed that the somatic embryos developing from embryogenic calli were passing through globular, heart shaped and cotyledonary stages.

Half-strength MS medium was found to be suitable for embryo maturation (50%).

Among different sucrose concentrations evaluated, maximum germination of somatic embryos was observed at 30 g/l sucrose in half strength MS medium.

Half-strength MS medium with TDZ (0.2 mg/l), NAA (0.5 mg/l) and BAP (1.0 mg/l) produced higher conversion of somatic embryos into whole plantlets.

Conversion of somatic embryos into whole plants were observed in cv. M13 (36.4%) followed by TMV12 (32.0%) and VG113 (29.8%).

6.6. Synthetic seeds

Encapsulation of somatic embryos using 3 per cent sodium alginate and 0.7% calcium chloride resulted in the formation of beads with uniform size and shape.

Encapsulated embryos at 3% sodium alginate showed moderate germination (32.5%) with better development of shoots and roots.

Half-strength MS medium was found to be suitable for conversion of encapsulated embryos into whole plants.

Among different support media tried agarised half strength MS medium containing growth regulators improved the germination of encapsulated embryos into whole plants (50.0%).

The encapsulated fresh embryos produced 50% conversion into whole plantlets, whereas after two months storage at 4°C produced only 4 per cent conversion. The non-encapsulated embryos failed to recover plants after two months storage at 4°C.

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* Originals not seen.