Tissue Culture Studies on Peanut
( *Arachis hypogaea*)

COMPUTERISED

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in Chemistry

By

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CONTENTS

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DECLARATION

KEY TO ABBREVIATIONS

ABSTRACT

Chapter I Introduction
Chapter II Materials and Methods
Chapter III Plant Regeneration via Somatic Erabryogenesis from embryo explants.
Chapter IV Erabryogenesis from embryonal leaf explants
Chapter V Plant Regeneration via Organogenesis from embryonal leaf explants
Chapter VI In Vitro Mutagenesis in Peanut
Chapter VII Effect of lysine and threonine stress on the regeneration of embryonal leaflets.

RESUME

BIBLIOGRAPHY

PUBLICATIONS
ACKNOWLEDGMENTS

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11 February, 1993

MRS. S. S. SATHAYE
DECLARATION

This is to certify that the work presented in the thesis entitled "Tissue Culture Studies on Peanut" represents an authentic record of work carried out by Mrs. Sangeeta Sathaye under my guidance.

Dr. A.F. Mascarenhas
Research Guide
ABBREVIATIONS

Growth regulators:
NAA - Napthalene acetic acid
2,4-D - 2,4 dichlorophenoxyacetic acid
BAP - 6-Benzylaminopurine
Kn - Kinetin
Pic - Picloram

Units:
s - seconds
d - days
h - hours
nm - unit for wavelength of light, nanometer ($10^{-9}$ meters)
mm - millimeters
cm - centimeters
m - meters
uM - micromoles
mM - millimoles
°C - degrees Centigrade
mg - milligrams
g - grams
ml - millilitre
l - litre
J - Joules
uE - microEinstein

Others:
MS - Murashige and Skoog basal medium
var - cultivar
ABSTRACT

Peanut belongs to the genus Arachis, a member of the family Leguminosae. Peanut, a protein rich oilseed legume native to South America and cultivated as a major crop in India. It is attacked by a variety of diseases. The Tikka (leaf spot) caused by Cercospora arachidicola inflicts a heavy loss in peanut yield. The seed is attacked by Aspergillus flavus, a fungus which liberates aflatoxin and makes the seed harmful for consumption. Genetic improvement of this crop will thus be beneficial to increase the yield and cultivate resistant varieties.

The conventional methods of crop improvement include introduction of new cultivars via hybridisation. This process is slow due to the fact that peanut is a self pollinated crop. The new in vitro approaches for crop improvement involving genetic transformation, somaclonal variation and other methods offer several opportunities. To fully utilise these approaches however, efficient and effective protocols to facilitate whole plant regeneration are a prerequisite. Reports on regeneration of peanut plants via tissue culture are however very few, suggesting the need for development of a reproducible technique.

With this objective, the present studies were undertaken with a view to developing an efficient in vitro system.

The thesis has been divided into seven chapters. The first two Chapters consist of the Introduction and Materials and Methods respectively. Chapters 3-7 have been further divided into two parts each consisting of an Introduction, Results and Discussion.
Chapter I  Introduction

This chapter deals with general information on the legumes, a family of plants to which the peanut belongs. The status of transformation, somaclonal variation and regeneration in the legumes has also been included with special reference to peanut.

Chapter II  Materials and Methods

The brand and source of the chemicals, glassware and plasticware used have been included in this chapter. The chapter also describes the procedures followed for cleaning of glassware, composition of the media, and the analytical methods used.

Chapter III  Plant Regeneration via Somatic Embryogenesis from embryo explants

Somatic embryogenesis was induced from the immature embryo explants using the auxin 2,4-D, the optimal concentration being 13.75 uM. This chapter deals with optimisation of protocols for induction, maturation and conversion of the somatic embryos. Field data of the plants raised from somatic embryos has also been included. The plants derived were found to be uniform for the morphological characters screened. The protein and lipid contents of the seed was comparable to the controls.

Chapter IV  Embryogenesis from embryonal leaf explants

Plant regeneration via somatic embryogenesis was obtained from embryonal leaf explants on 2,4-D containing medium. 90.50 uM of 2,4-D was found optimum for induction of callus free somatic embryogenesis. Maturation occurred on lowering the 2,4-D concentration to 13.75 uM and in the presence of 6% sucrose. Germination of these embryos took place on MS media containing
activated charcoal. The plants obtained grew to maturity and were fertile. The process of somatic embryogenesis was repetitive and secondary embryogenesis occurred with subculture to fresh media provided with 13.75 uM 2,4-D.

Chapter V Plant Regeneration via Organogenesis from embryonal leaf explants

MS medium supplemented with 5.36 uM NAA as auxin and 4.40 uM BAP as cytokinin induced direct caulogenesis in the embryonal leaf explants. On this media 85% of the explants induced shoot buds.

Multiplication and elongation of the shoot buds occurred in MS medium provided with 13.20 uM and 2.20 uM BAP respectively. Rooting of the shoots obtained was induced on hormone free half strength MS medium. The plants obtained were fertile.

Chapter VI In vitro mutagenesis in peanut

This chapter deals with studies carried out to estimate the effect of UV radiation on whole seeds and the regeneration of shoots from embryonal leaf explants. In one experiment the surface sterilised seeds were irradiated for various time intervals. The plants obtained from these seeds showed distinct variations such as variegation and curling of the leaves on exposure to sunlight.

In the second experiment the embryonal leaflets excised from the seed were irradiated. The regeneration potential of the irradiated leaf explants decreased with increase in exposure time. Multiplication of the regenerated buds was also very slow. The plants obtained showed dwarfism and lack of lateral branches. These plants flowered earlier as compared to the
controls and viable seed was obtained.

Chapter VII Effect of lysine and threonine stress on the regeneration of embryonal leaflets.

This chapter describes experiments carried out to test the effect of lysine and threonine stress on regeneration of leaf explants. The regeneration protocol described in Chapter V was used for all the experiments. Lysine and threonine were supplemented in the induction and multiplication media.

Lysine and threonine severely inhibited regeneration when used beyond all concentrations greater than 1.8 mM. The embryonal leaf explants showed greater tendency towards dedifferentiation in the presence of the stress. These effects were more pronounced if the leaf explants were irradiated with UV light prior to exposing them to the lysine and threonine stress.

Resume

This part of the thesis summarises the main findings from the different studies carried out. Some projections of the work for future application and studies have also been made.
1.1 General Information

Legumes are members of one of the largest families of the angiosperms - the Leguminosae. The Leguminosae consists of approximately 600 genera and 12,000 species. With an annual global production of 160 million metric tons, legumes are the worlds third largest food crop, preceded only by the tuber crops and the cereals (FAO 1983).

The Leguminosae includes annuals, herbs, perennials and tree species and also xerophytes and hydrophytes. Legumes are the most important source of plant proteins and energy and are cultivated throughout the world. The grain legumes are grown in the tropics and the temperate zones. They are among the first food crops to be cultivated by man and constitute a major part of his dietary protein, especially in Asia and Africa. The legumes are also of importance for their food value, as high protein diet for livestock and in fish feed. Duke (1981) described a number of economically important legumes whose uses range from food, fodder, ornaments and wood. Besides being of food value the legumes are endowed with the ability to fix atmospheric nitrogen in symbiosis with bacteria making them an ever increasing source of organic fertilizer.

Legume proteins are very rich in the essential amino acids lysine and threonine and relatively poor in sulphur containing amino acids like methionine and cystine (Bressani 1973). The high level of lysine and threonine in legumes make them an ideal supplement for cereals which lack in these amino acids. Legumes also form a good source of vitamins thiamine and niacin and
minerals such as calcium and iron. In addition they contain 60% of carbohydrates mainly as starch. The oil bearing legumes also contain up to 50% oil.

There has been no significant increase in the production of pulses or oilseeds due to their low static yields and susceptibility to various fungal, viral and bacterial diseases. Soybean alone is affected by several pathogens (Sinclair 1982) while diseases such as leaf spot and root rot cause damage to peanut. Grain legumes need to be improved for their grain yield, methionine and cystine contents, protein digestibility and cooking time caused by seed hardness (Bajaj 1990). Thus for qualitative and quantitative improvements of legumes the breeder has to rely on the extent of genetic variability in the existing base population. The lack of genetic variability has been a major limiting factor in the progress of improvement of these crops; hence the necessity for increasing variability in the existing germplasm.

While many improvements will be brought about by traditional breeding, plant tissue culture - facilitating genetic manipulations at the cellular level - will play an increasing role in plant improvement programmes. Fundamental to the exploitation of tissue culture, is the requirement to regenerate plants routinely and in sufficient numbers from cultured cells.

Several techniques such as anther culture for the production of haploids (Collins and Genovese 1982; Maheswari et al 1982), embryo culture in order to overcome post zygototic incompatibility (Steward 1981, Raghavan and Srivastava 1982), protoplast culture and somatic hybridisation by fusion of protoplasts (Cocking and
Riley 1981) have been used to alter the genetic base and induce variability in many plant species. Though somatic hybridisation was conceived as a promising tool for overcoming the barriers of interspecific and intergeneric incompatibilities in crop species, to date only a few hybrids have been obtained (Wright et al. 1986). Anther culture in legumes has not accomplished the objectives of generating haploids to be used in breeding or in the development of new varieties. Embryo culture to overcome postzygotic barriers to interspecific hybridisation has been employed successfully in some cases (Mroginiski and Kartha 1986).

In the recent past technological advances have opened new vistas for the creation of genetic variability and the selection of desired traits. In this chapter such technological advances in legumes will be discussed with special reference to the following --

1.2 Regeneration in legumes
1.3 Somaclonal Variation
1.4 Genetic Transformation
1.5 Peanut
1.2 Regeneration in legumes

In the last two decades, significant advances have been made in regenerating plants from callus and explants of legumes, particularly in forage crops. Regeneration has been reported to a lesser degree in the large seeded grain legumes and leguminous trees.

Plant regeneration in tissue cultures of legumes has been shown to occur in three principle ways -

1. Shoot bud proliferation: This involves the rapid multiplication of existing apical/axillary shoot buds, in generally a cytokinin containing medium. Such types of cultures have proved to be useful for storage and micropropagation.

2. Organogenesis: Regeneration by de novo formation of shoot meristems in somatic tissue cultures has been routinely applied to regenerate forage as well as large seeded legumes (Bajaj 1990). Plants can be readily obtained by subsequent rooting of the shoots. Histologically, the shoot bud is a unipolar structure and is physically connected to the parent tissue.

3. Somatic embryogenesis: Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into plants through characteristic embryological stages without the fusion of gametes. The embryo formed in this process is a bipolar structure which lacks vascular connections with the mother tissue and resembles a zygotic embryo. Direct as well as indirect somatic embryogenesis has been reported in legumes (Bajaj 1990).
1.2.1 Factors affecting regeneration in legumes

From the numerous reports on regeneration that exist, three major factors that influence legume regeneration are genotype, physiological status of the explant and media regime.

A large number of legumes show genotype specific regeneration as in alfalfa (Bingham *et al* 1975; Phillips 1983; Mitten *et al* 1984; Bianchi *et al* 1988), red clover (Keyes *et al* 1980; Bhojwani *et al* 1984), pea (Malmberg 1979; Rubulo *et al* 1982, 1984; Hussey and Gunn 1984; Kunakh *et al* 1984), pigeon pea (Kumar *et al* 1983, 1984a,b) and *Stylosanthes* (Meijer 1984). The long history of inbreeding and selection for high performance genotypes, leading to reduction in the genetic variability of modern varieties could be the cause of the recalcitrance of the large seeded legumes to *in vitro* regeneration.

In legumes, fast growing, meristematic tissues derived from embryos have been found to be more responsive in culture than those from mature plants. The early stages of embryogenesis in most legumes (specially in grain legumes) can be generally induced by exposing the tissue to the auxin 2,4-D. Induction periods have ranged from 6 months for the variety Mitchell of soybean (Christianson *et al* 1983) to as little as 3-4 days for alfalfa (Brown and Atanassov 1985). Further, removal of auxin has facilitated maturation of the embryos in most cases. Simple hormone regimes have been employed for the regeneration of many legumes via embryogenesis (Hammatt *et al* 1986; Bajaj 1990).
Major reports on *in vitro* morphogenesis in legume cultures with respect to organogenesis and somatic embryogenesis are listed in Table I.

1.2.2 Forage legumes

1.2.2.1 Callus and explant tissues

Regeneration from tissues of *Medicago sativa* are probably the best established systems in the legumes and some of these are already being applied to plant breeding (Hammatt *et al.* 1986). Historically, Saunders and Bingham (1972) regenerated plants from callus tissue initiated from hypocotyl explants through organogenesis and embryogenesis. Since this initial report, the effects of genotype, growth regulators, media composition and ammonia source on the regeneration of this legume have been extensively studied and successful regeneration has been reported for many other species of alfalfa (Table I).

In the genus *Trifolium*, tissue cultures of *T. repens* (white clover) and *T. pratense* (red clover) have been established for a number of years and studied in great detail (Table I). Maheswaran and Williams (1984) regenerated plants via somatic embryogenesis using immature zygotic embryos. *Trifolium* species of lesser economic importance have also been cultured and regeneration obtained in *T. alexandrium*, *T. incarnatum*, *T. repsupinatum*, *T. rubens* and *T. arvense* (Table I).

Many other forage legumes have been regenerated from a variety of explants. The earliest reports are those with *Trigonella foenum–graecum* (Sen and Gupta 1979). Several species of *Stylosanthes* have also been regenerated (Scowcroft and Adamson
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<td>Forage Legumes</td>
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<td>c,s,e</td>
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<td>c,e</td>
<td>Novak and Konecna 1982</td>
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<td>c,e</td>
<td>Chen and Marowitch 1987</td>
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<td>Hypocotyl</td>
<td>s,e</td>
<td>Walker et al. 1979</td>
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<td>McCoy and Bingham 1977</td>
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<td>c,e</td>
<td>Dos Santos et al. 1980</td>
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<td>Arcioni et al. 1982</td>
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<td>Immature embryos</td>
<td>de</td>
<td>Maheswaran and Williams 1985</td>
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<td>2</td>
<td>Clover (Trifolium)</td>
<td>Stolon</td>
<td>de</td>
<td>Bond and Webb 1989</td>
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<td>c,s</td>
<td>Atanassov and Mehandjiev 1979</td>
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<td>Kysley et al. 1987</td>
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<td>Jacobsen and Wilfred 1984</td>
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<td>Tetu et al. 1990</td>
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| 5  | Vigna spp. | Leaf             | s                  | Bhargava and Chandra 1989   |
|    |           | Root            | s                  | Eappen and Gill 1986        |
|    |           | Cell suspension | p                  | Kumar et al 1988            |
|    | V. aconitifolia | Hypocotyl     | p                  | Bowda and Satyan 1988       |
|    | V. unguiculata | Anther         | c                  | Sator et al 1982, 1983      |
|    |             |                | c, p               | Sator 1985a                 |
|    | Lupins      | Immature embryos | s                  | Sator 1985b                 |
|    |             | Seedling explants | s                  | Sator 1985                  |
|    |             | Nodal segments  | p                  | Nadolska-Orczyk 1992        |
|    |             |                  |                   | Sroga 1987                  |
|    |             |                  |                   | Schafar-Meunru 1985, 1986    |

abbreviations: c = callus; s = shoot buds; e = embryogenesis
de = direct embryogenesis; p = plant
1976; Mroginski and Kartha 1981b; Meijer and Broughton 1981; Rey et al 1985). *Lotus corniculatus* (birdsfoot trefoil) is reported to be one of the easiest to manipulate in culture. Embryogenesis has been induced from leaf callus (Arcioni and Mariotti 1982; Mariotti et al 1984b). 2,4-D tolerant lines have been isolated by Swanson and Tomes (1980). Regeneration studies in other forage legumes include *Coronia varia* (Arcioni and Mariotti 1983), *Astragalus sinicus* grown in China (Gao and Xu 1984) and *Melilotus officinalis* (Oelck and Schieder 1983).

1.2.2.2 Suspension and protoplasts

In alfalfa, plant regeneration via somatic embryogenesis was obtained in suspension cultures from shoot tips (Kao and Michayluk 1981), from leaf, petiole, hypocotyl and cotyledons by Novak and Konecna (1982) and from different genotypes by Atanasssov and Brown (1983). *Trifolium* species have also been cultured in liquid medium and plants regenerated from cell types of various origin. Plants via somatic embryogenesis from callus induced from cell suspensions of seedling explants has been reported in *T. rubens* (Parrott and Collins 1982).

Until 1979, there were no reports of plant regeneration via protoplast derived tissues within the Leguminosae, but since then significant advances have been made. Plants were first regenerated from protoplasts of alfalfa via somatic embryogenesis by Kao and Michayluk (1980) using young leaves. Subsequent reports include plant formation from protoplasts derived from mesophyll tissue (Johnson et al. 1981; Teoule 1983a,b), root (Lu et al 1982, 1983b; Pezzotti et al 1984) and cotyledons (Lu et al 1982, 1983b) via embryogenesis.
In *Trifolium rubens*, plants were regenerated from mesophyll protoplasts via somatic embryogenesis (Grosser and Collins 1984) and shoot formation (Gresshoff 1980; Oelck *et al* 1983).

1.2.3 Grain legumes

1.2.3.1 Callus and explant tissue

The grain legumes were considered recalcitrant in the mid-eighties, but since then several reports on regeneration of plants have been published. There was no report of regeneration via somatic embryogenesis of grain legumes until 1980, when Saunders regenerated soybean plants via embryogenesis. Since then reports of regeneration via embryogenesis in grain legumes have steadily increased (Table I). Extensive studies have been carried out in *Glycine* species (soybean) and plants have been regenerated via organogenesis and embryogenesis. *Pisum* (pea), *Vigna*, *Cicer* and *Arachis* spp have also shown regeneration potential with reports on plant recovery via organogenesis and somatic embryogenesis (Table I).

1.2.3.2 Suspension and protoplast culture

Evidence for both early and advanced stages of somatic embryogenesis have been described for suspension cultures of some large seeded legumes, particularly soybean (Beversdorf and Bingham 1977; Phillips and Collins 1981; Gamborg *et al* 1983). Interestingly, early stages of embryogenesis in leaf derived callus were stimulated upon transfer of tissues to liquid medium in pea (Jacobsen and Kysley 1984).

Cell suspension cultures have been a good source of soybean protoplasts. Protoplasts were also isolated from seedling
explants such as roots (Xu et al 1982), cotyledons (Lu et al 1983a; Lin et al 1984) and leaf (Rees et al 1985). Little information is available on pea protoplasts although those isolated from roots (Landgren and Torrey 1973; Crowder et al 1979) and mesophyll (Jia 1982) divided to give callus. Shekhawat and Galston (1983) and Krishnamurthy et al (1984) successfully regenerated plants via embryogenesis and shoot formation through leaf derived protoplasts of Vigna aconitifolia.

1.2.3.3 Status of regeneration in peanut

Although callus can be produced routinely in vitro subsequent plant regeneration is difficult and has been successful only in a few cases (Table II).

Peanut has been regenerated via organogenesis, embryogenesis and meristem culture. Mroginiski et al (1981) regenerated peanut shoots from immature leaflet derived callus. Illingworth (1968) used freeze shattered cotyledons as explants and grew the resultant tissues on moist filter paper to regenerate plants. Plants were also regenerated from epicotyls, ovaries and callus cultures of various explants including leaves, epicotyl, petiole and anthers (Table II). In wild species cultured root discs of A. pusilla and flower buds of A. monticola induced shoots (Shastri et al 1980). After isolation of protoplasts Yung-ru and Yu-hung (1978) obtained callus and roots, but no plants. Schenck and Hildebrandt (1969,1971) isolated protoplasts from cell suspensions of peanut which showed limited divisions, while Oelck et al(1983) reported callus formation from mesophyll protoplasts. Callus containing haploid cells produced a few albino plants (Martin and Rachehault 1976). Mroginiski and Fernandez (1980)
<table>
<thead>
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<th>No</th>
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<th>Response</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1</td>
<td>Pericarp</td>
<td>c</td>
<td>Rangaswamy et al 1965</td>
</tr>
<tr>
<td>2</td>
<td>Anthers</td>
<td>c</td>
<td>Martin and Rabechault 1975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c,p</td>
<td>Mroginiski and Fernandez 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c,p</td>
<td>Bajaj et al 1981</td>
</tr>
<tr>
<td>3</td>
<td>Gynophore</td>
<td>pod</td>
<td>Ziv and Zamski 1975</td>
</tr>
<tr>
<td>4</td>
<td>Cotyledon</td>
<td>r</td>
<td>Guy et al 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>Illingworth 1968</td>
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<tr>
<td></td>
<td></td>
<td>p</td>
<td>Atreya et al 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>Bhatia et al 1985</td>
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<tr>
<td></td>
<td></td>
<td>s,e</td>
<td>Banerjee et al 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s</td>
<td>McKently et al 1989</td>
</tr>
<tr>
<td>5</td>
<td>Apical meristem</td>
<td>p</td>
<td>Shyluk et al 1981</td>
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<td>Different callus</td>
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<td></td>
<td>s,p</td>
<td>Naraisimhulu and Reddy 1983</td>
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<td>7</td>
<td>Hypocotyl</td>
<td>c</td>
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<td>Immature leaf</td>
<td>c,s</td>
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<td>c,s,e</td>
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<td>Ozias-Akins 1989</td>
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<td>Sellars et al 1990</td>
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<td>Ozias-Akins et al 1992</td>
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<td>Durham and Parrott 1992</td>
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</table>

**c** callus  **s** shoot buds  **r** rhizogenesis  **p** plants  **e** embryogenesis  **de** direct embryogenesis
regenerated non haploid plants from *A. lignosa* while Bajaj *et al* (1981) reported a method for production of plants of variable ploidy in *A. hypogaea* and *A. villosa*. Somatic embryogenesis has been reported by Pittman *et al* (1983) and Banerjee *et al* (1988) from young leaflet and cotyledonary node explants. Pittman *et al* (1983) primarily observed organogenesis plus an occasional heart shaped embryo. Banerjee *et al* (1988) did not clearly distinguish between embryogenesis and multiple shoot formation in their cultures. Further development of these embryoids into plants was however not reported. Plant regeneration via somatic embryogenesis was first reported by Hazra *et al* (1989) using immature embryo axes as explants. Subsequently, somatic embryogenesis has also been reported from immature embryos (Ozias-Akins 1989; Sellars *et al* 1990; Durham and Parrott 1992), mature embryo axes (Mckently 1991) and leaf explants (Baker and Wetzstein 1992). Major reports on regeneration in peanut are included in Table II.

1.2.4 Miscellaneous species

Leguminous trees such as *Mimosa pudica*, *Sesbania sesban*, *Dalbergia sisso* and *Leucaena leucocephylla* have been regenerated to plants via callus from various sources (Gharyal and Maheshwari 1982; Khattar and Mohan Ram 1982; Mukhopadhay and Mohan Ram 1981; Nataraja and Sudhadevi 1984). Gharyal and Maheshwari (1981) induced embryoid formation directly on hypocotyl sections of *Albizia lebbeck* cultured on basal medium. *Albizia ricardiana* was regenerated by Tomar and Gupta (1988).
Among the fiber crops, Crotalaria juncea was regenerated from callus by Ramawat et al. (1977) whereas plants were obtained from immature cotyledons and hypocotyl of Indigofera aneaphylla by Bharal and Rashid (1979). Embryogenesis was induced in cell cultures of Cyamopsis tetragonoloba by McHughen and Swartz (1984), whereas in the aquatic legume Neptunia oleracea, clonal propagation was reported by Kakkar and Mohan Ram (1986).

1.2.5 Anther culture in legumes

There are few reports on haploid plant production from anther cultures in forage, grain and woody legumes. Haploid plants were obtained from cultured immature anthers of Trifolium alexandrium (Mokharzaheh and Constantin 1978), from Medicago denticulata in high frequency (Xu 1979), from cultured anthers from 10 lines of Medicago sativa (Zagorska et al. 1984) where haploid, diploid and mixaploid plants were regenerated. Haploid plants have also been reported from soybean (Yin et al. 1980; Jian et al. 1984), from a legume tree Albizia lebbeck (Gharyal et al. 1983) whereas in several other cases either limited morphogenesis or a mixed ploidy of the plants regenerated has been reported (Hammatt et al. 1986).
1.3 Somaclonal variation

Cultured cells are known to display variability (Bayliss 1980; D’Amato 1985; De Klerk 1990). Variability induced by cell cultivation has been termed somaclonal variation. Somaclonal variation commonly occurs in many plant species following regeneration from tissue culture even in the absence of mutagens (Larkin and Scowcroft 1981). The apparent mutagenic effects of cell cultures provide a wealth of variability that can be screened for novel characteristics. Somaclonal variation can be used to introduce non-specific variations into crop plants using standard plant regeneration methods. Such variations may be spontaneous in origin or may be induced in culture with the use of specific mutagens. The latter can be magnified further by application of in vitro selection. While the exact origin of such variations remains elusive, they are believed to be caused by chromosomal rearrangements, deletions and insertions of base pairs, transposable elements etc (De Klerk 1990).

Studies in different legumes has shown that somaclonal variation occurs in tissue culture. Variant phenotypes include chlorophyll deficiency, complete/partial sterility, wrinkled leaves, twin seeds, abnormal leaf morphology, dwarfism and multiple shoots (Bajaj 1990).

The degrees of variation in known genetic traits and morphological features has been studied in regenerants from ethionine-resistant alfalfa (Reisch and Bingham 1981). Groose and Bingham (1984) obtained plants from hypocotyl derived callus. Of these regenerants 21% were variants for one or more characters
whereas at least 11% lost one or more chromosomes. An extensive review on somaclonal variation in *Medicago* has been published by Bingham and McCoy (1982). In *Medicago sativa*, Hartmann et al (1984) selected plants resistant to *Fusarium oxysporum* whereas cell lines of *Medicago coerulae* resistant to NaCl and metholammonium have been reported by Indiojini et al (1986). Recent work in *Medicago media* cultivars revealed that the cultivar and genetic background play an important role in the cytological stability/instability of the regenerants from tissue culture (Nagarajan and Walton 1987). Variations among regenerants has also been reported in *Medicago sativa* (Arcioni et al 1989).

Pelletier and Pelletier (1971) first regenerated plants from cotyledon callus of white clover and reported both chromosomal and morphological variations in the regenerants. On the other hand, genetically uniform plants were reported to be regenerated via direct somatic embryogenesis from immature zygotic embryos by Maheswaran and Williams (1987).

Meijer (1984) observed considerable variation with respect to morphology and vigour among plants regenerated from 2 year old hypocotyl callus of *Stylosanthes guianensis*. Godwin et al (1990) reported plants with aberrant morphology and reduced fertility from callus cultures of *S. guianensis*, *S. lamata* and *S. scabia*.

Mathews et al (1986) showed that 14% of the R1 progeny of the regenerants of mung bean segregated for chlorophyll deficiency and morphological mutations. Quantitative and qualitative variation has also been reported in soybean regenerants (Barwale and Widholm 1987). Complete sterility and wrinkled leaf morphology have been evaluated in 3 or more
generations and both traits are inherited as single recessive nuclear gene mutations. In one family of regenerants 14% of the R3 plants were partially sterile whereas in R4 generation 2 of the plants derived from normal plants in R3 family showed partial sterility. Oil and protein contents were determined in seed of approximately 700 R3 families of A3127, Adams and Capitol. Most somaclones showed oil and protein content similar to that of the controls. However a few showed a 25% increase in oil content.

The use of tissue culture of legumes in studies directed to the induction and selection of mutants is severely constrained mainly because most of the systems available are not conducive to plant regeneration from long term callus cultures, suspension cultures or protoplast-derived cells.
1.4 Genetic Transformation

Genetic transformation may be defined as the uptake and expression of foreign DNA by the cell. The advantage of this approach is that it is possible to manipulate existing species genetically by introducing foreign genes from diverse sources which otherwise would not be possible by classical breeding methods.

Most of the legumes are susceptible to Agrobacterium infection and thus majority of the published material on legume transformation deals with this mode of transformation (Bajaj 1990).

Much of the earlier work on transformation in legumes involved the inoculation of whole plants with wild varieties of Agrobacterium tumefaciens and Agrobacterium rhizogenes. Mariotti et al (1984a) and Pederson et al (1983) reported the formation of galls on Medicago sativa and Glycine max and these galls remained disorganised in vivo as well as in vitro. Webb (1986) reported the formation of teratomous galls on infection of A. tumefaciens to Lotus corniculatus. The simplicity of the oncogenic system helped the spread of transformation to a wide range of legume species, even to tropical tree legumes such as Sesbania rostrata (Vlachova et al 1987) and Stylosanthes species (Manners 1987).

Jensen et al (1986) were able to introduce a chimeric gene leghaemoglobin-chloramphenicol acetyl transferase (leg-cat) into Lotus corniculatus. Shoots regenerated spontaneously and their transformed status was confirmed by expression of cat reporter gene on infection with Rhizobium.
Faciotti *et al* (1985) transformed soybean seedling using an oncogenic plasmid pTICGN609 which had the coding region of nptII linked to the 5 prime portion of the soybean small subunit (ssu) of the ribulose 1,5-ribulose bisphosphate gene. They were able to demonstrate that expression of the chimeric gene Rubisco-npt-II was light inducible in the callus.

An improved protein content in alfalfa (*Medicago sativa*) was obtained via transformation. A genetically engineered alfalfa variety containing a pea albumin gene is presently being tested in Australia (Ford 1988). Since wool growth in sheep is dependant on the availability of sulphur amino acids sheep grazing on this variety show increased productivity. This gene can now be transferred to other forage crops.

Within the legumes, *Agrobacterium* mediated gene transfer has been the most preferred method and very few reports exist on direct gene transfer. Successful transformation of soybean protoplasts was reported by two groups (Hauptmann *et al* 1987 and Christou *et al* 1987). Hauptmanns group reported transient gene expression of chimeric 35S-*cat* construct in soybean cultures but no stable integration. Christou *et al* stably transformed soybean protoplasts with a plasmid containing *nos-npt*-II construct. Transformed cultures were selected on kanamycin containing media.

Peanut was shown to be a suitable recipient plant for transformation using wild *A. tumefaciens* strain (Dong *et al* 1990) as well engineered *A. tumefaciens* (Lacorte *et al* 1990). Transformation of peanut using high velocity microprojectile balistics was recently reported by Clemente *et al* (1990).
1.5 Peanut

1.5.1 General information

Peanut (Arachis) belongs to the large seeded grain legumes. The systematic position of the genus is the Papillonaceae family, tribe Aeschynomeneae, subtribe Stylosantheneae. Its members are perennial or annual herbaceous plants with trifoliate or tetrafoliate leaves, stipulate leaves, papilionate flowers, tubular hypanthium and sessile ovary. The fruits are subterranean, resulting from the presence of a unique structure, the pegs, which are formed by the expansion of the intercalary meristem proximal to the basal ovule and between the remaining ovules.

The genus Arachis has both diploid and tetraploid (2n=40) species. The important diploids include A. diogoi, A. duranensis, A. repens and A. villosa whereas the important tetraploids include A. glabrata, A. hypogaea, A. monticola, A. marginata and A. prostrata. A. pusilla has been reported to be an octaploid form.

Peanut is attacked by a large number of diseases such as leaf spot (caused by Cercospora arachidicola) and rust caused by Puccinia arachidis. Infection with virus TMSV (Tomato spotted wilt virus) causes necrosis. Apart from this, infection of the seed by the fungus Aspergillus flavus produces the toxic metabolite aflatoxin which makes the affected seed dangerous for human consumption.

The wild species of Arachis possess desirable characters which have not been reported in commercial varieties. A. monticola is resistant to Cercospora and A. prostrata is drought
resistant. *A. diogoi* and *A. glabrata* possess better mineral, protein and fat content. *A. villosa* possesses higher oil content, resistance to drought and to pests and foliar diseases.

The conventional methods of crop improvement include introduction, selection hybridisation and recombination followed by pedigree and back cross techniques. Selection procedures do not create new variability, as in peanut which is a self-pollinated crop, it is difficult to accomplish most interspecific crosses. Due to this limitation there is a need to generate additional variability and use the available variability by adopting unconventional methods.

The genetic studies to date suggest that some of the disease resistance genes and genes controlling fatty acid composition can be manipulated *in vitro*. The accumulated literature, especially during the last few years demonstrates the potential and promise of the use of *in vitro* techniques for plant regeneration via plant tissue culture for peanut crop improvement.

### 1.5.3 Objectives

The objectives of the present study were:

1. To regenerate plants via somatic embryogenesis/caulogenesis and screen the regenerated plants for any variation.
2. To apply the regeneration procedures developed for studies on mutagenesis and stress tolerance.
II

MATERIALS & METHODS
2.1 Glassware/Plasticware

Borosil/Corning glassware was used for all experiments. Test tubes (25x125 mm) were plugged with absorbent cotton and autoclaved for one hour prior to use (121°C, 15 lbs pressure). Disposable plastic petri-plates were procured from Laxbro, India. Dissection instruments like forceps, scalpels were sterilised by autoclaving.

2.2 Chemicals and Seed Source

Chemicals used for preparation of the basal media were GR grade and were procured from Loba Chemicals, India or Qualigens India. The auxins, cytokinins, amino acids, vitamins and stains were of Sigma grade. Agar was obtained from Qualigens India, paraffin wax (m.p 58-60°C) from Loba Chemicals, India and solvents used (Analar grade) were procured from SD's Fine Chemicals, India.

Seeds used for all experiments was purchased from the Agriculture College, Pune, India. Immature seeds of all the genotypes (as given in Chapter III; Table VI) were collected in 1987 from the above mentioned college during the two peanut growing seasons. Seeds of cultivars SB-11 and JL-24 were obtained in the growing season from field plots of these cultivars maintained in our laboratory premises.

2.3 Media Preparation

Modified Murashige and Skoog basal medium (Murashige and Skoog 1962) was used in the preparation of the different media formulations in these studies. pH was adjusted to 5.6 with 1N KOH prior to addition of agar. Double distilled water was used in all
the media. Agar, at a concentration of 0.4% was added to all media formulations. Sucrose was used as carbohydrate source and was added at 3% unless specified. Auxins used were 2,4-D (range 4.52 μM to 90.50 μM), NAA (range 5.36 μM to 107.18 μM) Cytokinins used were BAP (range 4.40 μM to 13.30 μM) and Kinetin (4.60 μM to 13.90 μM). The auxins and cytokinins were maintained in the form of stock solutions (10 mg/100ml) and aliquots of these were added during media preparation. When used, activated charcoal (0.25%) was added to the media prior to addition of agar. The media was then melted in a steam steriliser and distributed into pre-autoclaved flasks/tubes. Autoclaving of the media was carried out at 121°C, 15 lbs steam pressure for 20 minutes. Heat labile compounds were filter sterilised through membrane filters of pore size 0.22 um and added to the respective media after autoclaving. The media composition of the most responsive media, among those tested for various experiments has been given in Table III.

2.4 Surface sterilisation of the plant material

Immature pods obtained from the field grown plants were washed thoroughly with detergent followed by 3-4 washes with distilled water. These were then first washed with 70% ethanol for 20 secs followed by surface sterilisation using HgCl₂ at 0.1% as the sterilant. The time of exposure was varied based on the nature of the explant. Pods were exposed for 10 min to the sterilant, followed by a thorough rinsing of the explants with sterile distilled water. When embryo axes were used as explants, they were directly exposed to the sterilant for 6 min followed by thorough washing with sterile distilled water 3-4 times.
### TABLE III

Media composition of the various media standardised

**Basal medium - MS (Murashige and Skoog)**

<table>
<thead>
<tr>
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<th>Composition</th>
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<tr>
<td>1</td>
<td>KM1</td>
<td>MS + 3% sucrose + 13.75 uM 2,4-D</td>
</tr>
<tr>
<td>2</td>
<td>KM2*</td>
<td>MS + 6% sucrose + 90.50 uM 2,4-D</td>
</tr>
<tr>
<td>3</td>
<td>SM1</td>
<td>MS + 3% sucrose + 5.36 uM NAA + 4.40 uM BAP</td>
</tr>
<tr>
<td>4</td>
<td>MK2</td>
<td>MS + 3% sucrose + 2.20 uM BAP</td>
</tr>
<tr>
<td>5</td>
<td>MK3</td>
<td>MS + 3% sucrose + 13.20 uM BAP</td>
</tr>
<tr>
<td>6</td>
<td>GM1</td>
<td>MS1/2 + 2% sucrose + 0.25% activated charcoal</td>
</tr>
<tr>
<td>7</td>
<td>RM3</td>
<td>MS1/2 + 2% sucrose</td>
</tr>
</tbody>
</table>
2.5 Culture of explants

All inoculations into preautoclaved media, were carried out in a sterile laminar air flow cabinet (Microfilt India). Disposable petriplates when used were sealed with Klinwrap (Klinwrap, India). Explants inoculated into stationary liquid media (poured into test tubes) were supported on filter paper bridges. Cultures were incubated at $25^{\circ}C \pm 2^{\circ}C$ temperature under cool white fluorescent light (intensity-50 $\mu$Em$^{-2}$s$^{-1}$) with a 16 hour photoperiod unless otherwise mentioned.

2.6 Evaluation of morphogenesis

All the cultures were periodically examined under a stereomicroscope. For evaluation of the response the explants were examined and the somatic embryos / shoot buds were visually counted to the nearest approximation. The data was represented as percentage response (number of explants showing response to the number inoculated). The frequency of induction was calculated as number of somatic embryos / shoot buds induced per explant.

2.7 Histological studies

Tissues were prepared for histological studies according to the procedure by Johansen (1940). The fixative used was FAA (Formaldehyde : Acetic acid : Alcohol, 5:5:90). The fixed tissues were dehydrated by passing through alcohol series and brought into absolute alcohol. These were then passed through alcohol:xylene series with steadily increasing percentages of xylene. Embedding in paraffin ($58-60^{\circ}C$) was done in a vacuum oven and 10 um thick sections were cut on a rotary microtome (Supercut, Reichert-Jung). The sections were spread on slides and
dried. These were deparaffinised using xylene prior to double staining with haematoxylin and eosin. Haematoxylin was used as the nuclear stain. The sections were finally mounted in DPX and after drying were observed under a microscope. The magnification was calculated by calibration of the microscope with a stage micrometer.

2.8 Photography

Specimen slides were observed under a microscope (Docuval, Zeiss) and photographed with an automatic photography attachment, using ORWO 125ASA film. The morphogenic response was evaluated by observation under a stereomicroscope and photographed in color using a 125 ASA color film (KONICA SRV 100).

2.9 Transfer to soil and hardening

All plants obtained from somatic embryos as well as from shoot buds were transferred to sand: soil mixture (3:1) in small polybags (50 x 120 mm). These bags were arranged in a tray and covered with a clear plastic sheet to maintain humidity. The transplants were watered, misted with a hand sprayer and incubated in racks with light intensity of 13-14 μE m⁻² s⁻¹ from cool fluorescent lights during a 16h photoperiod and 25/18°C day/night temperature. After 5-7 days, the relative humidity was reduced by adjusting the lid to permit air intake and the plantlets fed weekly with 5-7 ml of half strength M6 stock per bag. After 10-12 days, the plastic sheet was removed completely and within the next 5-6 days the tray was placed in a greenhouse (24±2°C; a natural daylength of 12-13 h, relative humidity 50-70% and photon flux of over 550 μE. Next the plants were placed
in pots containing a sand:soil (3:1) mixture one plant/pot and grown to maturity. For controls, seedlings 2-3 week old post germination were used in all experiments.

2.9 Growth of the regenerants and progeny testing

The plants were grown to maturity in the greenhouse. The uniformity / variability of the plants was evaluated by screening for morphological characters such as the height, leaf morphology and phyllotaxy, branching pattern, flowering, color of the flowers and seed morphology and biochemical characters such as the lipid and protein content of the seeds. The regenerants were single threshed on attaining maturity and the seeds were used for progeny testing. The seeds were sown separately and each plant maintained as a separate identity. The F1 and F2 generation plants were also evaluated for the same morphological and biochemical characters. For comparison of the regenerants, seed raised controls of the three generations were maintained simultaneously.

2.10 Protein estimation

The soluble protein content of the seed was estimated using Bradford method of protein estimation (Bradford 1976). The seed, after weighing was crushed and suspended in 0.05 M Tris-HCl buffer of pH 7.8. The homogenate was centrifuged at 5000 rpm and the supernatant was collected. This was used directly to estimate the protein. The standard used was BSA (Bovine Serum Albumin, Sigma). The protein content was evaluated using Coomassie Blue G250 (Sigma) as colour reagent and estimating the resultant
colour at 595 nm. The protein content was expressed as mg of protein per gram of seed.

2.11 Oil estimation

The lipid content of the seed was evaluated using the non destructive NMR (Nuclear Magnetic Resonance) methodology.

2.11.1 Principle

In oilseeds, hydrogen which gives the strongest NMR signal is mainly present in four forms viz oil, moisture (bound water), carbohydrates and proteins. The oil hydrogen is the most mobile among them. As a result of this, the pulsed NMR signal from oil will be present when all the other signals are dead. This makes it possible to measure the oil signal even in the presence of other constituents of the seeds. This oil signal is converted into the quantity of oil with the help of that obtained from weighed oil samples pressed out from the same crop.

2.11.2 Procedure

Oil was extracted mechanically from the stock seed of cultivar SB-11. The hydrogen spectrum of a weighed quantity of this oil sample (a) was obtained. Weighed quantities of the seed which was to be evaluated for oil were cut and used. The NMR spectrum was obtained in the form of peaks localised in specific areas of the spectrum (Plate 1).

2.11.3 Quantitisation of the oil from the spectrum.

The oil content was represented by the integral of the whole area under the peaks (b). This area corresponded to the quantity of the oil from extracted oil sample used. This correlation was used to evaluate the oil content from preweighed
\[ a = \text{wt. of the oil used for the assay.} \]
\[ b = \text{area of the peaks obtained for (a).} \]
\[ c = \text{area under the peaks of the sample seeds.} \]

\[ \text{oil content of the seed} = \frac{c \times a}{b} \]

2.12 Irradiation and stress tolerance experiments

UV (ultraviolet radiation) from a germicidal lamp (principle wavelength of the radiation used was 254 nm) was used as the physical mutagen. All experiments were conducted in petriplates. The seeds and leaf explants were irradiated in special UV chambers for various time intervals. Preliminary experiments showed 48 hours to be lethal for both the explants. The time interval chosen for exposure to UV radiation was from 1 hour to 24 hours.

After irradiation with UV, seeds were transferred to hormone free MS media for germination. The leaf explants were transferred to the shoot bud induction medium (MS media with 5.36 \text{um} \text{ NAA and 4.40 \text{um} \text{ BAP}}). Initially the explants were incubated in dark for 48 hours and then transferred to light. The regenerated plants were evaluated upto the F1 generation for any morphological variation. When used, lysine and threonine was added to the media aseptically from a filter sterilised stock (100 mM) prepared by the addition of equimolar quantities of each amino acid.
III

PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS FROM EMBRYO EXPLANTS
3.1 Introduction

Plants develop from meristems where cells proliferate and get partitioned into layers that eventually differentiate to form the various tissues and organs. A phenomenon unique to plant tissues and organs is their ability to induce de novo a range of developmental patterns. Plant regeneration in vitro occurs via two developmental pathways - embryogenesis and organogenesis. The ability of somatic cells to regenerate in culture implies that they have a complete set of genes that retain the potential to program all differentiated states in the plant (Goldberg et al 1989).

In vivo embryogenesis is concerned with the whole series of post-fertilization events involved in the origin, growth and orderly transformation of the zygote into a full-fledged embryo (Raghavan 1976). In vitro embryogenesis, however, is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without the fusion of gametes. This process has been termed as somatic embryogenesis.

The discovery of somatic embryogenesis is ineluctably tied to the demonstration of totipotency of plant cells. As generally applied to biological systems a totipotent cell is a cell that can regenerate in full multicellularity, sexuality and structure the phenotype of the organism of which it is a part.

The ability of a somatic cell to form a new sporophytic plant is a remarkable developmental feat. Reinert (1958) and Steward et al (1958) for the first time demonstrated the
formation of bipolar embryos originating from the carrot root callus on a synthetic medium. Single cell embryogenesis was reported by Steward (1963) in carrot cell suspension cultures. At about the same time Kato and Takeuchi (1966) described a balanced sequence of development of somatic embryos in single cell cultures of carrot. Since then reports on regeneration via somatic embryogenesis have grown steadily.

Two views prevail regarding the origin of somatic embryos. Haccius (1978) suggested that somatic embryos are new individuals arising from single cells or cell complexes formed from a segmenting single cell. Maheswaran and Williams (1985) however suggested that a multicellular origin of the somatic embryo appears to produce embryos fused with the parent over a broad area while a unicellular origin is more likely to give rise to embryos by a narrow suspensor-like organ.

Somatic embryos pass through typical stages of embryo development: the proembryo, the round globular stage, the torpedo stage with initial cotyledon primordia visible and the cotyledonary stage where well developed cotyledons are visible. Demonstration of somatic embryogenesis in a variety of plants under diverse cultural conditions has proved the fact that somatic embryogenesis in plants is not an aberrant form of development displayed by cultured cells, but is comparable to zygotic embryogenesis. Certain physiological and biochemical evidences also point to a close similarity between zygotic and somatic embryos. Like zygotic embryos, somatic embryos exhibit a form of dormancy that is overcome by cold treatment (Kavalthekar et al 1977; Rajeskaren and Mullins 1979). At the biochemical
level the somatic embryos of diverse plants synthesize qualitatively similar fatty acids (Pence et al 1981a), lipids (Janick et al 1982), storage proteins (Sung and Okimoto 1981; Crouch 1982), anthocyanins (Pence et al 1981b) and alkaloids (Schuchmann and Wellmann 1983) characteristic of maturing zygotic embryos in vivo. Clearly somatic embryos can serve in a limited way as models for the contemporary analysis of embryo development in angiosperms.

Somatic embryos are known to arise directly on the explant or indirectly from callus. According to Sharp et al (1980), direct embryogenesis proceeds from preembryonic determined cells (PEDC), needing only permissive conditions for their expression. Indirect embryogenesis involves the development of induced embryogenically determined cells (IEDC). In the second type a reorganization and redetermination of the differentiated cells is needed prior to their development into embryogenically determined cells.

The evolution of an embryo with its precise regularity of form, from a homogenous mass of cells probably involves several hormone controlled processes, any one of which may be limiting (Rangaswamy 1986). The hormonal control mechanisms combine with environmental factors to foster normal development of the embryo. Thus by supplying the limiting substance exogenously or by compensating for the environmental factors, it is possible to induce normal development of the embryo.

The major events in somatic embryony can be divided into four stages viz. induction, early growth, maturation and
conversion. Induction involves the determination of the cells to undergo embryogenic development. After induction the cells begin their sequential development where the vascular tissue and the polarity is established. The cotyledon primordia are also initiated in early growth. This is followed by the development of the somatic embryo, passing through the typical stages of development evident morphologically in culture. Finally the mature somatic embryo gets converted (germinated) into a plant. All these steps may take place in the same medium (single step) or may require more than one media composition (multiple step).

Abnormal forms of somatic embryos have been reported in *in vitro* cultures (Ammirato 1987). In addition to single embryos, twins, triplets and highly multiple clusters of somatic embryos have been found. The size of the proembryo is one factor that can affect normal development. Developmental events delayed, premature, absent or extended in time result in abnormalities.

The developing somatic embryos respond to different and diverse internal and external stimuli. By suitable alteration of the conditions under which they are grown, the maturation of the embryo can be modulated. Differentiation of the embryo from diverse plant organs or via callus has to be achieved by subtle alterations in the hormonal constituents supplied in the medium.

Among the exogenous factors that have been shown to influence the process of somatic embryogenesis are the explant, culture medium and culture environment. Though somatic embryogenesis can be induced from a range of tissue, immature or young explants have been more responsive with the developmental stage of the explant being of prime importance (Thorpe 1988). The
genotype has also been shown to be of importance in inducing a positive embryogenic response.

Regeneration through somatic embryogenesis has been preferred to organogenesis since both the root and the shoot primordia are simultaneously induced in the somatic embryo and it is not necessary to root the shoots obtained as in organogenesis. Secondly the roots developed from shoot cultures are adventitious roots resulting sometimes in reduced vigor of these plants with time. The probability of getting chimeras in plants regenerated via somatic embryogenesis is less than via organogenesis. Somatic embryos obtained can also be used for encapsulation for the production of artificial seed.

Occurrence of embryoid like structures in cotyledon cultures of peanut was first reported by Banerjee *et al* (1988). Subsequently somatic embryogenesis was reported from immature cotyledons (Ozias Akins 1989), immature embryo axes (Sellars *et al* 1989) and mature embryo axes (McKently 1991). In the present study, attempts were made to regenerate peanut plants via somatic embryogenesis. This chapter describes the induction of somatic embryos using immature embryo explants. The morphology of the somatic embryos combined with histological observations regarding the type of induction and the origin of the somatic embryos has also been included. The plants obtained from the somatic embryos were screened for uniformity/variability and this data has also been incorporated.
3.2 Results and Discussion

Preliminary experiments were carried out using mature and immature embryo explants of cultivar SB-11. Subsequently other genotypes were also tested (Table VI) and the further experiments were carried out with the most responsive of these. Basal medium (MS) was supplemented with the auxins 2,4-D and NAA in various concentrations ranging from 1 mg/l to 10 mg/l (corresponding to 4.52-45.25 uM of 2,4-D and 5.36-53.69 uM of NAA) separately or in combinations. Culture conditions were as described earlier (Chapter II). Fifty explants were inoculated in each media tested.

3.2.1 Mature embryos

Mature embryos were surface sterilized as described earlier (Chapter II). The explants from mature embryos were of 3 types viz. (i) the whole embryo (ii) the embryo axis and (iii) the cotyledons. The cultures were observed every five days and the response scored as a positive or negative embryogenic response. The positive response was evaluated in terms of percentage induction and frequency of induction. Percentage induction was calculated as the number of explants giving positive embryogenic response whereas the frequency of response was the number of somatic embryos formed per explant. The observations recorded 20 days after initiation of cultures are presented in Table IV.

After 20 days mature embryos germinated in all media regimes tried (data not included in table). Thus somatic embryogenesis could not be induced from these explants. However when the cotyledons and embryo axes of the mature embryo were
**TABLE IV**

Responses of mature embryo explants to various media regimes

**Basal medium:** MS + 0.4% agar

**Sucrose:** 3%

**Supplements:** 2,4-D and NAA

**Culture conditions:** 16h photoperiod, 25 ± 2°C

**Period of Incubation:** 20 days

<table>
<thead>
<tr>
<th>Media No.</th>
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<th>NAA uM</th>
<th>Mature axis</th>
<th>Mature cotyledon</th>
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</table>

**Abbreviations used in the table**

- g = germination
- r = rhizogenesis
- c = callusing
- e = embryogenesis
- n = no response
- wc = watery callus
- fc = friable callus
inoculated separately, they responded in a different manner from that of the whole embryo.

Mature cotyledons also failed to show any embryogenic response, although callusing with rhizogenesis under some conditions (in media 12-16) was observed. The callus that developed was white and powdery.

Mature axes germinated in all media containing either 2,4-D or NAA alone (media 1-8). This was also associated with callusing. The nature of the induced callus was watery in NAA containing media whereas it was white and friable in the presence of 2,4-D (Table IV). However, embryogenesis was observed in 5 media containing combinations of the two auxins (media 10,13-16). Among these, the highest percentage (30%) of induction was observed in media 13 (Table IV), followed by a 12% response in media 15. Morphologically, induction was seen in the formation of white protrusions on the mature embryo axes. The induction of protrusions on the mature axes was associated with suppression of its germination and frequency of induction was between 2-5 protrusions per explant. However, these developed into embryos only in a few cases (Fig 1). On longer exposure to the media (beyond 25 days), development of the embryo was restricted and profuse callusing overtook embryo growth. The mature embryo axes thus exhibited a fairly low percentage of somatic embryo formation when combinations of 2,4-D and NAA were used. Moreover conversion of the somatic embryos into plants also could not be achieved because of inhibition of somatic embryo growth. The low percentage of response in mature embryo axes may be due to its higher degree of differentiation as compared to the immature
Fig 1: Somatic embryos induced on mature zygotic embryo axis on MS medium supplemented with 2,4-D (27.12 uM) and NAA (5.36 uM). c = callus, e = somatic embryo X 50x
embryo axes. McKently (1991) could induce somatic embryo formation using mature axes as explants. He obtained greatest embryogenic activity in a 2 mm region adjacent to and encircling the epicotyl. This response was maximum in the presence of 3 mg/l of 2,4,5-T. He observed that the type and concentration of the auxin influenced the embryogenic process. Further the process initiated with the formation of hair instead of protrusions as observed in the present study.

3.2.2 Immature embryos

Immature pods were harvested 15-20 days after the peg penetrated the soil. At this stage the seed coat was still colorless while the cotyledons were approximately 4-9 mm. The nature of these varied from papery white to succulent green. After surface sterilization the pods were opened and the seeds were excised under aseptic conditions. The seed coat was removed and the embryos inoculated whole or dissected into cotyledons and axes which were inoculated separately into the media described earlier.

When inoculated whole, embryogenesis was observed in all media containing 2,4-D (Table VA), whereas in media containing only NAA, no somatic embryo formation was seen. Somatic embryos were induced on the axis but only protrusions were observed on the cotyledons. The percentage response in the responsive media however was low; further the somatic embryos showed poor development. Moreover, it was apparent that among the two auxins tested, presence of 2,4-D in the media was necessary.
In all the media tried, the inoculated cotyledons increased in size and became green in 7-8 days. In low concentrations of NAA (5.36 µM) abnormal rhizogenesis was observed at the embryonal end of the cotyledon (Table VA). The roots formed were thick and fleshy. No other type of morphogenesis was observed in media containing only NAA.

In the presence of 2,4-D the cotyledons first increased in size and became intensely green. Protrusions were seen all over the cotyledons within 10 days in media 1-4 (Table VA; Fig 2). Over 85% of the inoculated cotyledons showed this response. These protrusions gave rise to abnormal elongations which further developed roots at the tip of the projections. The roots formed were thick and fused with each other. In media 1 and 2 there was formation of embryo like structures at the proximal end of the cotyledon. On longer exposure to 2,4-D, the growth regulator failed to support the subsequent development of these structures. If transferred to hormone free media the root end of the embryo developed but shoot formation was not observed. Thus the immature cotyledons showed a positive embryogenic response but the frequency of abnormal development was high and the protrusions did not metamorphose into normal somatic embryos. Similar observations were recorded by Ozias-Akins (1989) in peanut. She obtained somatic embryos on isolated immature cotyledons cultured in Pic. In these studies, NAA and 2,4-D stimulated only nodular outgrowths that rarely developed further.

When immature embryo axes were exposed to 2,4-D the development of the axes was suppressed. Somatic embryogenesis was
**TABLE VA**

Responses of immature embryo explants to various media regimes

**Basal medium:** MS + 0.4% agar

**Sucrose:** 3%

**Supplements:** 2,4-D and NAA

**Culture conditions:** 16h photoperiod, 25 ± 2°C

**Period of Incubation:** 20 days

<table>
<thead>
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<th>Media No.</th>
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<th>NAA uM</th>
<th>Immature embryo</th>
<th>Immature axis</th>
<th>Immature cotyledon</th>
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</table>

**Abbreviations used in the table**

- g = germination  
- r = rhizogenesis  
- c = callusing  
- e = embryogenesis  
- n = no response  
- wc = watery callus  
- fc = friable callus
Fig 2: Protrusions induced on immature cotyledon in medium containing 13.75 uM 2,4-D. c = cotyledon, p = protrusions X 40x
observed in all media with 2,4-D as the only auxin (Table VA). The size of the embryo axes used was from 2 mm to 6 mm in length. Somatic embryos were initiated in the form of small white protrusions which became apparent on the axes within 5 days of inoculation on media with low concentration of 2,4-D (4.52 and 13.75 μM). This induction process was extended to 10 days in the presence of 27.12 μM 2,4-D. At the end of 15 days distinct somatic embryos were observed on embryo axes inoculated in all the 2,4-D concentrations.

The somatic embryos were apparently induced on the embryo axes directly without any intervening callus phase (Figs 3,4). These always appeared at the root shoot junction of the embryo axis. These observations were further confirmed in histological studies. The percentage induction was 100 % in media with low 2,4-D (4.52 and 13.75 μM ). The optimal concentration of 2,4-D was determined subsequently.

When 2,4-D was substituted with NAA the embryo axes growth was not suppressed, instead these germinated in 8-10 days (Table VA) in low NAA (< 16.08 μM) containing media. In media 7 and 8 (those with high NAA concentration) germination was also accompanied by callusing at the shoot end of the axes. Somatic embryo formation was not observed at any NAA concentration. NAA alone was unable to induce embryogenesis in immature embryo axes.

When combinations of these two auxins were tested somatic embryogenesis was induced in four combinations (Table VB). The formation of somatic embryos was accompanied by callusing. As seen in the table, 100 % induction was observed when the 2,4-D and NAA concentration was 1 mg/l each (4.52 μM 2,4-D and 5.36 μM.
Fig 3: Somatic embryos induced directly on immature embryo axis in EM1 medium. s = shoot end of zygotic embryo axis e = somatic embryo c = callus X 40x

Fig 4: Somatic embryos on immature embryo axes 15 days after induction in EM1 medium. e = somatic embryo c = callus X 75x
### TABLE V8

Embryogenic Response of Immature Axes in media containing 2,4-D and NAA Combinations

**Explant:** Immature embryo axes (3-5mm long)

**Media:** MS medium + 3% Sucrose

**Light:** 16h photoperiod

**Supplements:** 2,4-D and NAA (as in table)

**Period of Incubation:** 20 days

<table>
<thead>
<tr>
<th>Media No</th>
<th>2,4-D uM</th>
<th>NAA uM</th>
<th>% Response</th>
<th>Frequency response</th>
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NAA) whereas the frequency of induction was only 3-5. Among the other three responsive combinations the highest percentage induction was only 23% in media no 11 (Table VB).

Thus, preliminary experiments showed that somatic embryogenesis could be induced using mature as well as immature embryo axes as explants. The embryogenic response from the mature embryo axes which had a low percentage response however could not be enhanced. The immature embryo axes were seen to be more responsive. Immature embryos are known to possess a strong morphogenetic potential (Green and Phillips 1975; Vasil 1986,88; Tetu et al 1987a; Barwale et al 1986). Further embryogenesis was induced in these explants in the presence of 2,4-D even at low concentrations. This indicates that 2,4-D was essential for induction of embryogenesis. The role of 2,4-D in the induction of embryogenesis is well documented (Stamp and Henshaw 1982, Ammirato 1983, Brown and Atanassov 1985). A few recent reports have documented the developmental pathway of somatic embryogenesis in peanut from immature zygotic embryos (Ozias-Akins 1989; Sellars et al 1990). In all the cases the somatic embryos were auxin induced and although similar auxins were evaluated variance in response was obtained. Sellars et al (1990) induced embryo formation from the cotyledons and axis of intact immature zygotic embryos of 3 cultivars, with the best response in the presence of 0.001-0.02 mg/l Pic. However 2 mg/l NAA and 5 mg/l 2,4-D were also favorable. In the present study, however, 2,4-D was found to be superior.
The specific effects of auxin on embryogenic induction have been suggested by Henshaw et al. (1982). They proposed that auxins might remove existing developmental constraints from cells and provide the signals which evoke the embryogenic response in the competent cells. The natural growth substances can be regarded as activating agents for cells which are preconditioned to respond in specific ways (Sharp et al. 1980). This is in agreement with Bruinsma and Libbenga (1988) who proposed that the main function of auxin in the organ in which it is synthesized is the stimulation of the cellular processes of division and elongation. Thus it can be presumed here that 2,4-D functioned as an activating agent for the embryogenic cells of the embryo axes and in its presence the cells became meristematic.

3.2.3 Optimization of embryogenesis in immature embryo axes

To obtain a high frequency of normal somatic embryo formation various parameters were tested. These included several physical parameters like size of the axes and light conditions during incubation, chemical parameters like effects of varying 2,4-D and sucrose concentrations, organic supplements and genotypic effects. The explants were inoculated in MS basal medium supplemented with 3% sucrose and 13.75 uM 2,4-D for all these experiments. This medium has been denoted EM1 medium hereafter. The following paragraphs describe the results obtained.
3.2.3.1 Effect of size of explant

In this study embryo axes of 5 different sizes (length of the axis) viz. 2 mm, 3 mm, 4 mm, 5 mm and 6 mm were selected. Ten axes were inoculated per plate containing EM1 medium. These experiments were run in three replicates. The 2 mm embryo axes were translucent soft and watery whereas the 6 mm embryo axes were opaque and hard. Embryo axes in the range of 3 mm to 5 mm were soft and pale green in color.

The response of the immature embryo axes in 2,4-D was size specific (Figs. 5). Normal embryogenesis was initiated only on embryo axes ranging in size from 3-5 mm in length. On embryo axes 5 mm long frequency of induction was however comparatively lower and the embryos formed were abnormal (Fig 6). Beyond a size range of 5 mm the response was delayed and the abortive embryos appeared on the axes after 10 days. This type of response was accompanied by callusing. Thus the most responsive size range for induction of somatic embryogenesis was found to be 3-4 mm. The size of the explant was crucial for induction of embryogenesis as it may represent a particular developmental stage. This is in concurrence with previous reports from other species (Wernicke and Brettell 1980; Litz and Conover 1981, Lu and Vasil 1982; Wang et al. 1984; Maheswaran and Williams 1985,1987 ; Barwale et al 1986; Tetu et al 1987b,1990).

In further experiments this size range was maintained to test the effect of other parameters.
Fig 5: Effect of size of immature embryo axis on induction time and frequency of induction.

X-axis = size of explant in mm.

Y1 axis = Frequency of induction \( \text{se/explant} \)

Y2 axis = Days of response(d)
EFFECT OF SIZE OF EXPLANT ON SOMATIC EMBRYOGENESIS

<table>
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<tr>
<th>RESPONSE TIME (DAYS)</th>
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<td>10</td>
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</tbody>
</table>

SIZE mm -->

- ■ FREQUENCY
- ▬▬▬ RESPONSE TIME (DAYS)
Long axes in EMT medium, as = abnormal embryos

Fig 6: Abnormal somatic embryos (abnormal type) induced on 5 mm
3.2.3.2 Effect of light

To study the effect of light on the induction process two sets of experiments were maintained - one was incubated in dark and another in light with a 16 hour photoperiod.

When incubated in the dark embryo induction was slower than that in light. In the dark, somatic embryos were induced within 8-9 days whereas in light with a 16 h photoperiod they appeared within 5 days. Cultures were incubated in light with a 16 h photoperiod in all further experiments.

3.2.3.3 Effect of 2,4-D concentration

Embryo axes were inoculated in MS basal medium supplemented with 3% sucrose and 2,4-D in various concentrations (4.52 μM, 13.75 μM, 27.12 μM, 36.16 μM and 45.25 μM respectively).

The concentration of 2,4-D affected the percentage and frequency of induction and also the period required for emergence of the first somatic embryos. The quality of the embryos was also affected by the concentration of 2,4-D in the media.

Percentage induction decreased with increase in the 2,4-D concentration in the medium. The embryogenic response was highest (100%) in the presence of 4.52 μM and 13.75 μM 2,4-D. This decreased to 45% when the explants were exposed to 45.25 μM 2,4-D. Frequency of induction and period of response were also affected by concentration of 2,4-D. The frequency of induction was highest in 13.75 μM 2,4-D with an average of 11.42 somatic embryos per embryo axes. Lower frequencies of 5.2 and 3.33 were seen in media with 4.52 μM and 27.12 μM of 2,4-D respectively. Moreover, somatic embryos appeared within 4-5 days in the
Fig 7: Effect of 2,4-D concentration on the percentage and frequency of induction of somatic embryos on immature embryo axes.

X axis = 2,4-D concentration in uM.

Y1 axis = Percentage of induction (no. of explants responding to the number inoculated)

Y2 axis = Frequency of induction (no. of embryos/explant)
EFFECT OF 2,4 D CONCENTRATION ON SOMATIC EMBRYOGENESIS

FREQUENCY / RESPONSE

uM of 2,4-D

FREQUENCY    RESPONSE TIME(DAYS)
Fig 8: Abnormal somatic embryos induced in medium with high 2,4-D content (45.25 µM). as = abnormal embryo × 40x
presence of 13.75 uM 2,4-D whereas they were induced after 10
days when the concentration of 2,4-D in the media was higher than
13.75 uM.

As the concentration of the auxin increased more abnormal
somatic embryos were induced and many abortive types of embryos
were visible in cultures on media with 45.25 uM 2,4-D (Fig 8). In
studies on somatic embryogenesis in peanut using mature embryo
explants, McKently (1991) recorded similar observations. The
logistic analysis performed by him for each auxin, to determine
the effect of concentration on embryo shape was found to be
significant for 2,4-D and Pic, such that the probability an
embryo would be normal in shape decreased with increase in
concentration of the auxin.

Among the various levels of 2,4-D tried the best results
were observed in presence of 13.75 uM of 2,4-D. Thus MS basal
medium supplemented with 13.75 uM 2,4-D and 3% sucrose (EM1) was
used for all further experiments.

3.2.3.5 Effect of sucrose concentration

Sucrose concentrations within a range of 2% to 12% were
tested to evaluate the effect of concentration of sucrose on the
embryogenic response. In this experiment the sucrose content of
the EM1 medium was substituted by that used for the experiment.

Both period and frequency of response varied markedly at
varying sucrose concentrations (Fig 9). The embryogenic response
decreased with increasing sucrose concentration. Clear
morphological differences were also observed under the
stereomicroscope. Normal somatic embryos were induced in media
with 2,3 and 5% sucrose and these were well formed and green (Fig
Fig 9: Effect of sucrose concentration in medium with 13.75 mM 2,4-D on frequency of induction and induction time.

X axis = concentration of sucrose (gm %)

Y1 axis = frequency of induction (se/explant)

Y2 axis = induction time (d)
EFFECT OF SUCROSE CONC.
ON SOMATIC EMBRYOGENESIS

FREQUENCY -->

SUCROSE CONC. -->

<table>
<thead>
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<th>DAYS OF RESPONSE</th>
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</tr>
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<td>2</td>
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<tr>
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</table>
Higher sucrose concentrations caused browning of the explant; further the induced somatic embryos were necrotic (Fig. 11).

Sucrose has been pointed out to be one of the factors influencing the induction of embryogenesis (Thorpe 1988). In peanut induction was optimal in the presence of 3-5% sucrose. Suppression of embryogenesis in higher concentrations of sucrose may be attributed to increased osmolarity in the medium.

3.2.3.6 Effect of various organic supplements

Casein hydrolysate (500 mg/l), yeast extract (500 mg/l), coconut milk (5%) were separately supplemented to EM1 medium prior to autoclaving. Glutamine was filter sterilized and added aseptically at the concentration of 5 mg/l.

All the above organic supplements added to the media were ineffective in enhancing embryogenesis. Callus formation however increased in media supplemented with yeast extract and glutamine, with a slight suppression in the induction of embryos. The stimulation of callus formation and the suppression of the embryogenic response by the presence of organic supplements has been reported by Maheswaran and Williams (1984) and Vasil and Vasil (1981). This effect has been attributed to the presence of auxin like substances in these supplements.

No organic supplements were thus added to the embryo induction medium EM1 in further experiments.
Fig 10: Normal green somatic embryos induced in EM1 medium (3% sucrrose). e = somatic embryo X 40x

Fig 11: Necrosis of explant and low induction of somatic embryos in EM1 medium with 12% sucrose instead of the usual 3%. n = necrotic somatic embryo
3.2.3.7 Effect of genotype

Thirty one different genotypes were selected for this experiment (Table VI). The numbers given in the Table for the various genotypes correspond to the numbers appearing in the graphs (Figs 12, 13, 14). Immature embryo axes were inoculated in EM1 medium to evaluate the genotypic effect on the embryogenic response in terms of percentage and frequency of induction.

Among the genotypes screened many variations were observed in the percentage induction (Fig 12). Genotypes SB-11⁶ and B(4)-A22²⁰ elicited 100% response. Some other responsive genotypes included ICGS-11¹ (86%), ICG(4)FDR⁷ (90%), KADIRI-3⁵, J-11¹⁰, B-32 EC 21127¹⁰, JL-24², B 45 FSB-7-5²¹ (80%). Genotypes such as B(38) EC 24375¹⁹ (28%) B-39 EC24449²⁶ (10%) were the low responding genotypes (Fig 12).

The highest frequency induction was recorded for genotype SB-11 (8-13 se/explant) (Fig 10) whereas genotypes such as ICG(4) FDR⁷ (3 se/explant), NFG-7¹¹ and NFG-9¹² (3-4 se/explant) exhibited low frequency induction (Fig 13).

The appearance of the somatic embryos (days of response) on the explants was also a genotype dependent phenomenon (Fig 14). The somatic embryos appeared the earliest (4-5 days) in genotypes SB-11⁶, S-206¹⁵, B 4 -A22²⁰, B45FSB-7-5²¹, B-43 EC106984²², D-19 DH-3-30 x CHICO²⁵ and B-32 EC 21127¹⁰. The slow responding genotypes included those which responded after 20 days. These were M-13³ (20 days) and B-39 EC 24449²⁶ (22 days).

Response of explants to various media is known to vary with the genotype. Regeneration has been reported to be influenced by
TABLE VI

Data of embryogenic response of peanut genotypes in KML medium

Percentage response = no. of responsive explants / no. inoculated
Frequency response = no. of somatic embryos induced / explant
Days of response = Initiation of embryogenesis after inoculation

<table>
<thead>
<tr>
<th>No.</th>
<th>Genotype</th>
<th>% Response</th>
<th>Frequency Response</th>
<th>Days of Response</th>
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<td>7</td>
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<td>SB-11</td>
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</table>
Fig 12: Effect of genotype on percentage induction of somatic embryos inoculated in EM1.

  X axis = genotypes screened (no. correspond to those of genotype in Table VI).
  Y axis = percentage response.

Fig 13: Effect of genotype on frequency of induction of somatic embryos in EM1 medium.

  X axis = genotypes screened (no. corresponds to those in Table VI)
  Y axis = frequency induction (no. of se/explant)
Fig 14: Effect of genotype on days required for induction of somatic embryos in EM1 medium.

X axis = genotypes screened (no. correspond to those in Table VI).

Y axis = days required for induction (d)
GENOTYPE VS. DAYS OF RESPONSE

DAYS OF RESPONSE

GENOTYPES

DAYS OF RESPONSE
the genotype in many species such as soybean (Barwale *et al* 1986; Parrott *et al* 1989), cotton (Trolinder and Xhixian 1989), *Medicago* (Chen and Marowitch 1987; Brown and Atanassov 1985), *Trifolium* (Campbell and Tomes 1984; Keyes *et al* 1980), *Stylosanthes* (Meijer 1982), *Cajanus* (Kumar *et al* 1983) and *Coronilla* (Mariotti and Arcioni 1983). Variation in the embryogenic response was also observed in peanut. The percentage response and frequency response was maximum in the cultivar SB-11. Genotypes JL-24, S-206 and ICGS-11 also showed high embryogenic response. The readily available and highly responsive genotypes SB-11, JL-24 and ICGS-11 were used for all further experiments.

The results obtained indicated that high percentage (100%) and frequency (8-13 somatic embryos per explant) somatic embryogenesis was obtained when immature embryo axes 3-4 mm in length were exposed to medium with 13.75 μM 2,4-D and 3% sucrose (EM1).

3.2.4 Somatic embryony patterns

3.2.4.1 Light microscopy

As described earlier in the EM1 medium the somatic embryos could be seen as small white protrusions that appeared within 5 days. The induction was associated with the suppression of growth of the main axis. This observation supports earlier findings (Raghavan 1976, Williams and Maheswaran 1986). The suppression of growth of the embryo axes associated with embryogenesis has also been observed in *Ilex* (Hu and Sussex 1971), flax (Pretova and Williams 1986), alfalfa (Maheswaren and Williams 1985, Lupotto 1983) and *Theobroma* (Pence *et al* 1980).
The globular stage embryos were white, glossy with a size of approximately 1 mm. Induction of fresh embryos continued as early somatic embryos matured into further developmental stages. In about 5-6 days the somatic embryos passed from the globular to the torpedo stage. A typical heart stage was not seen frequently as the development was fast at this stage. The torpedo stage embryo was 2-3 mm long. This developed further into the cotyledonary stage in 5-7 days. The somatic embryos formed usually had cotyledons of variable morphology and a relatively long axis (Fig 15).

The system of induction was asynchronous and so all stages were visible in the same clump at a given time. A great variability in morphology was also seen indicating abnormalities in embryo development. Occasionally somatic embryos with one cotyledon (Fig 16) or more than two cotyledons were also seen. In some cases cotyledons developed either as a single tube (Fig 17) or in the form of the neck of a pitcher (Fig 18). Sometimes the embryos ceased to grow at globular stage of development and such embryos showed a pinched shoot pole (Fig 8). This was possibly the result of delayed polarity establishment. In other cases the cotyledons of more than one embryo were fused along the length of the axes. Ammirato (1985), in studies with caraway, has explained the appearance of such variants. According to him, a small proembryo can readily progress through various developmental stages and if the proembryo enlarges to form two growth centers before maturity, twins or multiple embryos develop. If the enlargement is at the shoot end then, the somatic embryos are
Fig 15: Normal somatic embryo induced in EM1 medium with a relatively long axis. a = axis, c = cotyledons X 150x

Fig 16: Somatic embryo with only one cotyledon c = cotyledon X 40x

Fig 17: Somatic embryo with cotyledons in the form of a single tube. t = tube formed by fusion of the cotyledons, r = root end of embryo X 150x

Fig 18: Pitcher shaped somatic embryo with swollen axis and long cotyledon tube. p = cotyledon tube resembling neck of pitcher. X 150x
formed with one root and two shoot primordia. Such types have been observed in peanut cultures.

He further suggested that abnormalities could also arise due to error in the maturation events, that is initiation of the cotyledon primordia and tissue differentiation. These events may be premature, delayed or extended in time. The cotyledons begin to grow from the formation of a broad disk meristem with many divisions at the periphery and center (Ammirato 1987). The cotyledons often appear fused along their margins producing fused, tube like structures. Changes in the cell division, enlargement and differentiation can also affect cotyledon formation (Ammirato 1987). In normal cotyledon formation, the proembryo undergoes a series of cell divisions and a ring of cells forms a cotyledonary collar. Within that ring two growth centers emerge and give rise to two cotyledons with a presumptive shoot apex in the center. If the size of the cotyledonary disc ring is altered by too many cell divisions or enlargement, two or more growth centers will form resulting in development of a somatic embryo with multiple cotyledons. If the divisions are very few, only one cotyledon will form. If the divisions continue after formation of cotyledonary primordia, the cotyledons appear fasciated and fused. Different types of embryo morphology has also been observed in sweet potato (Tsay and Tseng 1979; Chee and Cantcliffe 1988), Atropa (Konar et al 1972) and alfalfa (Stuart et al 1985).
3.2.4.2 Histological observations

Various stages of embryo development were visible in the same clump (Fig 19). The somatic embryos were seen to be formed directly from the root shoot junction of the zygotic embryo axes without an intervening callus phase (Fig 20). They were attached to the parent by their root poles and did not bear any vascular connection with the parent explant. The embryos were formed from the superficial layers of the explant. Direct somatic embryogenesis has been reported in various types of plant species such as clover (Maheswaran and Williams 1984), alfalfa (Maheswaran and Williams 1985), olive (Rugini 1988), soybean (Hepher et al 1988; Finer 1988), cassava (Szabados et al 1987), Cichorium (Dubois et al 1990), flax (Pretova and Williams 1986), sunflower (Freysinnet 1988; Finer 1987), pea (Tetu et al 1990), Ilex (Hu and Sussex 1971), orchard grass (Conger et al 1983), Brassica (Pretova and Williams 1986), Solanum aviculare (Alizbeth and Mantell 1991) and tobacco (Stolarz et al 1991).

According to Sharp (1980) direct somatic embryo formation in culture proceeds from cells which are already determined for embryogenic development prior to explanting i.e. they are pre-embryogenic determined cells (PEDC). The PEDC’s require only growth regulators or favorable conditions to allow release into division and expression of embryogenesis. This theory is an extension of earlier conclusions by Tisserat et al (1979) that the internal state of the explant is of prime importance in the expression of somatic embryogenesis, with other conditions being only permissive for the expression of the predetermined pattern.
Fig 19: Somatic embryos induced directly on embryo axis without an intervening callus phase in EM1 medium. e = embryo, f = fasciated embryo, s = suspensor X 100x

Fig 20: 10µ section of a zygotic embryo axis bearing somatic embryos. z = zygotic axis, r = root pole of the explant. e = somatic embryos. Bar 10 mm = 408 µm
of development. Further with respect to direct somatic embryogenesis the process of development is limited not only to certain cell stages but also to certain cell types. These include embryogenic cells which can be readily accepted as predetermined embryogenic cells (Williams and Maheswaran 1986).

All stages of embryo development from the globular to the cotyledonary stage could be seen in the sections observed (Figs 21, 22, 23). The initiation of embryogenesis was not synchronous. Hence two or more stages of embryogenesis could be observed in the same culture (Fig 22). The cotyledonary stage of the embryo showed a distinct root and shoot pole with the apical meristem (Fig 23).

The somatic embryos were attached to the parent over a broad base or by a narrow suspensor (Figs 19, 20). Williams and Maheswaran (1986), suggested that a multicellular origin of the somatic embryo appears to produce embryos fused with the parent over a broad area of the root pole or the axis region, whereas a unicellular origin is more likely to give embryos attached by a narrow suspensor - like organ. In peanut cultures the embryos were induced fused with the parent over a broad area or attached by a narrow suspensor like organ. Haccius (1978) however defined a non-zygotic embryo as a new individual arising from a single cell and having no connection with the mother explant. However the single cell origin of the embryo has been unequivocally demonstrated only in some cases such as walnut (McGranahan et al 1988) and carrot (Nomura and Komamine 1985). On the other hand multicellular origin of somatic embryos has been reported in Borage (Quinn et al 1989) and oil palm (Schwendiman et al 1988).
Fig 21: Globular stage somatic embryo. Bar 10 mm = 133 um

Fig 22: Heart and torpedo stage embryos. h = heart stage embryo, t = torpedo stage embryo, m = meristematic cell layers, p = projection at shoot pole of somatic embryo. 10 mm = 266 um

Fig 23: Cotyledonary stage embryo easily separated from explant. r = root pole, c = cotyledons 10 mm = 165 um

Fig 24: Finger like projections induced in some somatic embryos in EM1 medium. p = projection, e = embryo X 100x
Haccius (1978) attempted to explain the origin of somatic embryos. She explained that somatic embryos were essentially single cell in origin. Proembryonal cell complexes which induce somatic embryos are formed by multiple divisions of a single cell indicating again a single cell origin. This could indirectly suggest that in peanut all somatic embryos originated from single cells.

Some somatic embryos bore finger like projections at the shoot pole of the embryo (Figs 22,24). Secondary embryogenesis was occasionally observed in some cultures (Figs 25,26). The secondary embryos arose directly on the existing primary somatic embryos. The growth of the primary somatic embryos was observed to be suppressed when secondary embryogenesis was initiated.

3.2.5 Conversion and Progeny testing

The induction and maturation of the somatic embryos as manifested by the different developmental stages occurred on the same medium (EM1). There are several reports where induction and maturation of the somatic embryos occurs in two or more steps involving various media compositions (Keysley et al 1987, Nagarajan et al 1986, Li et al 1985). In A. hypogaea, in the present study, the complete process of development from the initial globular to the fully mature embryos occurs in the presence of 2,4-D in 20 days. The process of embryogenesis is thus completed in one step as in mango (Litz et al 1984) and carrot (Nomura and Komamine 1985). The embryos induced in the medium with 13.75 uM 2,4-D matured on the same medium.
Fig 25: Secondary somatic embryos induced on a primary somatic embryo directly. s = secondary embryos, m = primary embryo  X 75x

Fig 26: Secondary somatic embryo formed on the primary embryo without an intervening callus phase. m = primary embryo, s = secondary embryo. 10 mm = 220 um
Germination ensued with root initiation which occurred within 21 days of inoculation (Fig 27). At this stage the somatic embryos were transferred to various hormone free media to achieve optimal embryo germination. These included (i) solid MS medium with 2% sucrose, (ii) solid MS medium with half strength salts and full strength vitamins supplemented with 2% sucrose (MS1/2) and (iii) MS1/2 supplemented with 0.25% activated charcoal.

Germination in hormone free MS basal medium was very poor (2%) and rather than elongation and shoot initiation, the root end of the somatic embryo callused. When the salt concentration was reduced to half, germination percentage improved to 23.5%. Callusing associated with germination was also less. The germination percentage improved remarkably (upto 50%) when MS1/2 medium was supplemented with 0.25% activated charcoal. This medium was used to achieve germination and has been referred to as GM1. Roots in this medium appeared within 4-5 days. Charcoal enhanced germination of the embryos. Activated charcoal has been reported to enhance somatic embryo germination earlier (Ranch et al 1986). This may be because of its ability to absorb inhibitory substances (Fridborg et al 1978; Ziv 1979, Takayama and Misawa 1980) when added to the medium. Activated charcoal is also known to absorb hormones (Fridberg et al 1978; George and Sherrington 1984).

All embryos including the normal and abnormal types developed roots on transfer to GM1 medium. All types of embryos gave rise to normal root development on germination although shoot growth was unpredictable and often abnormal. This
Fig 27: Initiation of germination of somatic embryo by root formation in EMI medium 21 d after induction. r = root, X 80x

Fig 28: Conversion of somatic embryo in GM1 medium.
observation clearly indicated that the abnormality in the embryos was predominantly localized at the shoot end of the embryo and the root always developed normally. This may be because of abnormal organization of the shoot pole during the course of embryo development in peanut.

The normal somatic embryos germinated into plants within 21 days from transfer to GM1 medium (Fig 28).

Fifty percent of the transferred somatic embryos germinated into plants and could be transferred to soil. These were hardened as described earlier (Chapter II). All the plants derived from somatic embryos grew to maturity (Fig 29) and set seed (Fig 30) under greenhouse conditions. A total of 20 plants was used to study their growth behavior and morphological characters by comparison with seed raised controls during the F0 generation (Table VII).

At maturity, these plants were single threshed and the seed obtained was used to screen F1 and F2 generations. Among the seeds obtained from each plant, 10 seeds of every plant were germinated, grown to maturity and compared with plants from seed raised controls (Table VIII). The protein and lipid content of the seeds was evaluated using the methodologies described in Materials and Methods (Chapter II).

The plants screened in the Ro, F1 and F2 generations were fertile. The morphological characters were similar to the control plants in all cases. The results of this study have been included in Table VII and Table VIII.

The data obtained from the somatic embryo derived plants (Ro) is given in Table VII. There was no significant variation in
Fig 29: Seed set in somatic embryo derived plant.
### TABLE VII

**Growth Behaviour of Somatic Embryo derived Plants In Comparison to Seed Raised Controls**

<table>
<thead>
<tr>
<th>No</th>
<th>Morphological character</th>
<th>Controls (seed raised) (average of ten plants)</th>
<th>Somatic embryo derived plants (average of ten plants)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Height of plant (cm)</td>
<td>27 ± 2.32</td>
<td>26.33 ± 1.22</td>
</tr>
<tr>
<td>2</td>
<td>No. of lateral branches</td>
<td>4-5</td>
<td>4-5</td>
</tr>
<tr>
<td>3</td>
<td>Phyllotaxy</td>
<td>Alternate</td>
<td>Alternate</td>
</tr>
<tr>
<td>4</td>
<td>Pubescence on stem</td>
<td>Greenish</td>
<td>Greenish</td>
</tr>
<tr>
<td>5</td>
<td>Texture of leaves</td>
<td>Smooth</td>
<td>Smooth</td>
</tr>
<tr>
<td>6</td>
<td>Flower initiation (d)</td>
<td>46 ± 1.35</td>
<td>48 ± 0.82</td>
</tr>
<tr>
<td>7</td>
<td>Color of flower</td>
<td>Bright yellow</td>
<td>Bright yellow</td>
</tr>
<tr>
<td>8</td>
<td>Pod morphology</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. reticulation</td>
<td>reticulate</td>
<td>reticulate</td>
</tr>
<tr>
<td></td>
<td>b. beak/no beak</td>
<td>no prominent beak 1-2</td>
<td>no prominent beak 1-2</td>
</tr>
<tr>
<td></td>
<td>c. seed/pod</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Seed morphology</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. shape</td>
<td>round</td>
<td>round</td>
</tr>
<tr>
<td></td>
<td>b. color</td>
<td>flesh color</td>
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</tr>
<tr>
<td></td>
<td>c. seed coat</td>
<td>smooth</td>
<td>smooth</td>
</tr>
</tbody>
</table>
TABLE VIII

Growth Behaviour of Somatic Embryo derived Plants as Compared to Seed raised Controls over Two Generations

<table>
<thead>
<tr>
<th>No</th>
<th>Morphological Character</th>
<th>Control Plants (Seed raised)</th>
<th>Somatic Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F2</td>
</tr>
<tr>
<td>1</td>
<td>Height (cm)</td>
<td>31 ± 0.89</td>
<td>32.5 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>No. of lateral branches</td>
<td>4 ± 1.52</td>
<td>4 ± 0.98</td>
</tr>
<tr>
<td>3</td>
<td>Phyllotaxy</td>
<td>alternate</td>
<td>alternate</td>
</tr>
<tr>
<td>4</td>
<td>Pubescence on stem</td>
<td>green</td>
<td>green</td>
</tr>
<tr>
<td>5</td>
<td>Texture of leaves</td>
<td>smooth</td>
<td>smooth</td>
</tr>
<tr>
<td>6</td>
<td>Initiation of flowering (M)</td>
<td>45</td>
<td>44</td>
</tr>
<tr>
<td>7</td>
<td>Flowers in apices</td>
<td>few</td>
<td>few</td>
</tr>
<tr>
<td>8</td>
<td>Colour of flowers</td>
<td>yellow</td>
<td>yellow</td>
</tr>
<tr>
<td>9</td>
<td>Pod Morphology</td>
<td>reticulate rounded 1-2</td>
<td>reticulate rounded 1-2</td>
</tr>
<tr>
<td>10</td>
<td>Seed Morphology</td>
<td>round flesh smooth</td>
<td>round flesh smooth</td>
</tr>
<tr>
<td>11</td>
<td>Seed Protein mg/g</td>
<td>242</td>
<td>224.6</td>
</tr>
<tr>
<td>12</td>
<td>Oil in seed mg/g</td>
<td>323</td>
<td>325.3</td>
</tr>
</tbody>
</table>
growth characteristics when compared among themselves. When they were collectively compared with the seed controls for the same characters also no significant difference could be found (Table VII). The seeds obtained from these regenerants were grown for two more generations and evaluated for the same characters. No morphological difference was found up to F2 generation (Table VIII). The protein and lipid content of the seed estimated was similar to that of the control seeds.

It is now a well established fact that organogenesis from callus, cell suspensions or protoplasts is often associated with genetic and cytological variations (Larkin and Scowcroft 1984, Shepherd 1982, Bayliss 1980, Larkin 1984). It is presumed that this also applies to somatic embryos regenerated from the above mentioned types of cultures (Maheswaran and Williams 1984). However direct somatic embryogenesis appears to be associated with a greater genetic and cytological uniformity (Vasil 1982), since it bypasses a callus phase and offers higher chances of clones of genetically uniform plants. Uniformity of regenerants obtained via direct somatic embryogenesis has also been elucidated in *Trifolium* (Maheswaran and Williams 1988)

3.2.6 Original explant - embryo axes

Somatic embryos were induced from immature embryo axis in the presence of 2,4-D. The induction of the embryos was accompanied by growth suppression of the axis. After maturation of the somatic embryos, the clump of embryos along with the axis was transferred to GM1 medium.
An interesting observation was the germination of the original embryo axes along with germinating somatic embryos (Fig 30). The plants obtained from these embryo axes were fertile and set seed. The morphological characters of these plants were similar to the seed raised and somatic embryo derived plants.

Thus the original explant was capable of renewed growth and germinated to give rise to a fertile plant. These observations point to the fact that the explant after passing through 2,4-D containing medium did not lose its original identity and could revert back to its earlier physiological condition. This resulted in the renewed growth of the axis and formation of a fertile plant. The embryo axis was apparently unaffected except for the formation of somatic embryos directly.

This observation suggests that 2,4-D differentially affected two regions of the embryo axes depending on the differential status of the cells in these regions. It was ineffective in diverting the meristems of the shoot towards dedifferentiation/morphogenesis but only suppressed further growth in the induction medium. On the other hand 2,4-D could induce morphogenesis (somatic embryo formation) in the superficial layers at the root shoot junction of the immature axis. Possibly due to the same reason auxin failed to induce high frequency embryogenesis in the mature axes. This observation also deviates from the theory proposed by Williams and Maheswaran (1986). According to them growth suppression of the main embryo axis is associated with the breakdown in the integrity of the cells as a single group and escape of individual or smaller groups of cells to function autonomously.
Fig 30: Germination of original explant (immature embryo axis) on transfer to GM1 medium (2,4-D free medium).
This result could be interpreted in another way. Except for some cells which gave rise to somatic embryos, the remaining physiological state of the axis remained unchanged and the growth of the axis was suppressed as long as it was under the influence of 2,4-D. Thus the few cells that gave rise to somatic embryos could be the PEDCs (pre determined embryogenic cells) which on exposure to auxin, in this case 2,4-D, expressed their existing potential to grow into somatic embryos. These observations consolidate the theory of the existence of pre-determined cells proposed by Sharp in 1980.

3.2.7 Conclusion

In the present study, the embryogenic potential of the immature embryo was recognised and plant regeneration via somatic embryogenesis could be successfully obtained using immature embryo axes as explants in the presence of 13.75 uM of auxin 2,4-D. The entire process from induction of somatic embryos to their maturation was a single step process i.e induction and maturation occurred in the same medium. The formation of the somatic embryos was essentially from single superficial cells of the embryo axes directly, without an intervening callus phase. The mature somatic embryos converted to plants with 50% frequency. The somatic embryo derived plants exhibited similar morphological characters as well as seed protein and oil content when compared with seed raised controls.

Earlier reports (Banerjee et al 1988; Pittman et al 1983) described embryoid like structures in cotyledon and leaf cultures respectively but no plant regeneration was reported. The present
work represents the first report of plant regeneration via somatic embryogenesis in peanut (Hazra et al 1989). This report was the foundation for further studies on embryogenesis in peanut as is evident from subsequent reports. Ozias-Akins (1989) obtained low frequency conversion of somatic embryos derived from immature cotyledons. Subsequently, successful plant regeneration via somatic embryogenesis was reported, again using immature embryo explants by Sellars et al (1990). These workers also succeeded in inducing repetitive embryogenesis. Suspension cultures have also been established recently by Durham and Parrott (1992) using immature cotyledons.

The procedure described in this chapter can be applied to achieve Agrobacterium tumefaciens mediated gene transfer after further optimisation to help reduce the frequency of abnormal embryo formation.
IV

EMBRYOGENESIS FROM EMBRYONAL LEAF EXPLANTS
4.1 Introduction

Plant tissues normally contain a diversity of cell types. A query that follows is - are all the cells in an explant potentially and equally capable of redetermination in culture? This concept is yet to be fully resolved. It is obvious that in most tissue culture systems only certain cell types get selectively multiplied while others either remain inert or give rise to a recalcitrant callus.

Morphogenesis in vitro is a complex developmental process. Determination is the event which initiates a specific pathway of development by singling it out from the various other possibilities. The end of the induction process is not in emergence of embryos/shoots but the time when the cell or group of cells become committed to follow a particular pathway. Thus according to Waddington (1966) determination to do a piece of morphogenesis necessarily precedes actual morphogenesis.

The excision of tissues from their native environment removes the restrictions on cell behavior which are necessary to integrate behavior of the whole plant. Cells so freed may find themselves in the permissive environment of the culture medium and respond to largely endogenous determinants in following a particular developmental pathway (Halperin 1986).

Somatic embryogenesis occurs naturally in a wide variety of species from both reproductive tissue such as nucellus and synergids and somatic tissues such as leaf margins. Somatic embryogenesis in vitro has been reported from a wide range of cell types, from organised tissues directly or via callus. The
origin and development of somatic embryos in culture have been reviewed by Street (1975), Thomas and Davey (1975), Sharp et al. (1980, 1982) and Williams and Maheswaran (1986).

In all systems the embryogenic cells from which embryos are derived show a number of common features characteristic of rapidly dividing meristematic cells. The cells are small in size, with dense cytoplasmic contents, have large nuclei with a prominent nucleolus and small vacuoles.

Direct somatic embryogenesis is limited not only to developmental stages of the explant, but also to particular cell types. These include embryonic cells, which can be accepted as predetermined embryogenic cells and cells of young seedlings particularly the hypocotyl epidermis which may exhibit minimal differentiation. Embryonic tissue, hypocotyl tissue and young epidermal cells are associated with developmental plasticity and ease of proliferation. These characters are essential for the redetermination process - thus the importance of these explants in inducing morphogenesis. As against this in mature tissues the capacity of cells to revert back towards redetermination is greatly reduced.

The previous chapter dealt with the use of immature embryo axes as initial explants. The main disadvantage with these explants was their collection which is season dependent. Further they are available in the right stage only during a part of the peanut growing season. Somatic embryogenesis induced from immature embryo axes was also not of the repetitive type and was associated with abnormal embryo formation. This chapter describes the successful development of a peanut embryogenic regeneration
system from embryonal leaflets and the regeneration of plants from JL-24, an important Indian cultivar. A distinct advantage of this system over the previous somatic embryogenesis system using immature embryo axes, is a ready year round availability of the explant tissue. Aseptic cultures with little or no contamination could be readily obtained using the following described method. In addition, a leaf derived embryogenic system would be an additional avenue for the development of transformation protocols.
4.2 Results and Discussion

In the previous chapter, the mature and immature embryo axes were seen to exhibit embryogenic potential. Somatic embryogenesis could be induced in high frequencies from the immature embryo axes whereas in the mature axes, this frequency was lowered. However when embryonal leaflets excised from the same mature axes were inoculated independently, they exhibited high morphogenic potential.

4.2.1 Preliminary experiments

Seeds of genotypes JL-24 and SB-11 were used in the following experiments. The seed was split longitudinally and the embryo axis excised gently. These embryo axes were used to obtain the embryonal leaflets. The surface sterilised axes were soaked in sterile distilled water for 24 hours, the leaflets were excised and inoculated into the various media regimes in petriplates.

To draw an estimate of the morphogenic potential, the embryonal leaflets were inoculated in MS basal media supplemented with 3% sucrose and either 2,4-D or NAA as auxins. For each genotype the experiment was initiated in two sets, one set comprising of 2,4-D containing media and the other containing NAA media. The concentration range of the auxins used was from 4.52 - 90.50 μM 2,4-D and 5.36 - 107.18 μM for NAA. The inoculated cultures were maintained under the cultural conditions as described earlier (Chapter II) and observed every 5 days.

Similar responses were observed in both the genotypes used. Low concentrations of NAA (5.36 and 10.72 μM) induced the
Fig 31: Nodular outgrowths on leaf in presence of 5.36 μM NAA

c = callus, n = nodular outgrowths  X 50x
formation of nodular outgrowths on the abaxial surface of 33% of the leaflets (Fig 31). This was also associated with callus. Higher concentrations of NAA (> 32.26 μM) induced callusing and root formation in 14% of the inocula. The nodular outgrowths failed to develop further, even when transferred to lower (0.536 μM) NAA containing medium. Hence this auxin was eliminated for all further experiments.

In media containing low 2,4-D concentrations (4.52 μM and 13.75 μM) the leaflets turned green in 10 days and expanded. Watery callus was observed along the petiolar end of the leaf. Leaflets exposed to 27.12 μM 2,4-D did not show greening as when exposed to low 2,4-D containing media (4.52 μM and 13.75 μM). Instead a pair of bulges were formed on either side of the midvein at the petiolar end on the abaxial side of the leaflets (Fig 32). 72% of the leaves inoculated elicited this response within 21 days of exposure to 2,4-D. Subsequently the developing bulges were outgrown by the growing callus. Similar bulges were formed when the 2,4-D concentration in the medium was higher than 27.12 μM. The percentage of leaflets showing bulges however was found to decrease with an increase in 2,4-D concentration. In the presence of 90.50 μM 2,4-D only 45% of the leaflets showed bulges. The position of the bulges was same in all the explants. Callusing associated with this response decreased with increase in the 2,4-D concentration. Virtually no callus was induced in leaflets on medium with 90.50 μM 2,4-D except at the cut end (Fig 33). The time required for the induction of the bulges however was not affected by the concentration of the 2,4-D in the medium and they appeared on the leaflets in 21 days.
Fig 32: A pair of bulges induced at the petiolar end on abaxial side of leaf accompanied by callusing in 27.12 uM 2,4-D containing medium. c = callus, b = bulges X 50x

Fig 33: No callus formation associated with bulge formation in presence of 90.50 uM 2,4-D. l = explant, b = bulge X 75x
These bulges formed were smooth, glistening and compact. They were equal in dimension on both the sides of the midvein and had a pearly appearance. The formation of the bulges was site specific (Figs 32,33). The other leaf regions as well as leaflets cut into half did not exhibit bulge formation whether intact or injured.

After 4 weeks in the 2,4-D containing medium, the bulges gave rise to globular outgrowths and fasciated somatic embryos (Figs 34,35). Formation of such bulges was considered a positive embryogenic response.

Hormones play an important role in induction of somatic embryogenesis since they may interact directly with factors, like ionic currents which control cell polarity (Brawley et al 1984). A steady ionic current directed along the developmental axis of the somatic embryos may be involved in the determination and maintenance of polarity, at least until the early torpedo stage (Brawley et al 1984). In the present study, as observed with immature embryos, 2,4-D also elicited an embryogenic response in immature leaf explants among the two auxins tried. Somatic embryogenesis has been induced using 2,4-D as auxin in around 57% of the plant species reported so far (Thorpe 1988). Generally low 2,4-D levels are effective in embryo induction, although very high levels are also occasionally reported (Finer 1990, Shoemaker et al 1991, Baker and Wetzstein 1992).

In the present study, the process was initiated with the formation of bulges on the abaxial surface on either side of the midvein at the petiolar end of the leaflet directly without an
Fig 34: Globular outgrowth induced directly on the bulge in presence of 90.50 μM 2,4-D after 25 days in culture. g = globular outgrowth, b = bulge on leaf X 75x

Fig 35: Ill-developed somatic embryos induced from bulges in presence of 27.12 μM 2,4-D after 25 days in culture. Callusing profuse. c = callus, e = somatic embryo X 40x
intervening callus phase. Similar bulge formation prior to initiation of somatic embryos from leaf has been reported in cassava cultures (Szabodas et al 1988). On the other hand, Baker and Wetzstein (1992), reported somatic embryogenesis in peanut on high 2,4-D containing medium (40 mg/l) using leaflets as explants. Somatic embryos developed from intact as well as cut leaflets in a maximum of 14.6 % of their cultures. A clear distinction of the type of embryogenesis (direct or indirect) was not made by these workers.

4.2.2 Parameter studies to optimise induction

Manipulation of physical parameters viz. the position of the leaflets on the axis, the age of the explant and light conditions and chemical parameters such as sucrose concentration, effect of cytokinins, and organic supplements was carried out in attempts to enhance the embryogenic response. For these experiments leaflets were inoculated in MS basal medium supplemented with 3% sucrose and 90.50 μM 2,4-D. This medium has been referred to as EM2 hereafter. The cultures were maintained in the cultural conditions as described earlier (Chapter II).

4.2.2.1 Position of the leaflet on the axis

The axis of the peanut seed bears an older outer pair of leaves and a more juvenile inner pair. These were inoculated in EM2 medium separately and treated as two different explants to study whether they showed a varied embryogenic response.

Both types of leaves gave a positive response. Forty five percent of the outer leaves inoculated and 43% of the inner exhibited bulge formation. It can be concluded that the position of the leaf on the axis did not affect the induction process.
Further experiments were carried out without the separation of the two types of leaflets.

4.2.2.2 Effect of the age of the leaf

For this experiment the mature embryo axes were inoculated in hormone free MS medium after surface sterilisation and incubated in light (16 h photoperiod). The leaflets were excised from these every day for four consecutive days. A set was also initiated using the leaflets obtained from the axes just after surface sterilisation. This was taken as day 0 and the days were counted subsequently as day 1, 2, 3, and day 4. By day 4, greening of the leaflets on the axes commenced. The explants were inoculated in EM2 medium.

0 day old leaflets expanded and greening of the leaves commenced 4 days after inoculation into EM2 medium. Bulge formation was seen after 21 days in 29% of these explants. In one day old leaflets the embryogenic response was induced in 45% of these leaflets in 21 days. This was also preceded by greening of the explants. The older leaves (2-4 days old) turned green and callusing was seen in EM2 medium. Bulge formation did not commence in the 2,4-D containing medium. Only 0-1 day old leaflets exhibited this response whereas older, fully expanded leaves and leaves chosen from the germinating axes did not form somatic embryos under the given conditions. This observation emphasizes the importance of the juvenile tissue towards eliciting the embryogenic response. The developmental stage of the explant is crucial to the expression of somatic embryogenesis and the specificity of the developmental stage has been reported
for many other plant species such as celery (Johri and Sehgal 1966), cacao (Pence et al 1979, 80), sorghum (Wernicke and Brettell 1980), papaya (Litz and Conover 1981), millet (Lu and Vasil 1982), strawberry (Wang et al 1984) and clover (Maheswaran and Williams 1985). Wounding of the explant was also necessary for eliciting of the response. Besides being stage specific the responsive region was also precise and the somatic embryos were formed only on the abaxial surface of the leaflet at the petiolar end (Figs 32, 33). This could be because the petiolar end of the leaf is least differentiated. A similar response has been attributed to less maturity of the cells at the base of the leaf in monocotyledons by Konar and Nataraja (1965) and Conger et al (1983). The petiolar end of the embryonal leaf explants may represent the most responsive stage to induce embryogenesis under the given conditions. In the present study, 2-3 mm size of the leaflets was most responsive, whereas in an earlier report (Baker and Wetzstein 1992) 5-8 mm long leaflets of peanut exhibited a higher embryogenic response.

In the present study, for all further experiments, one day old leaflets were used as explants.

4.2.2.3 Light conditions

Three light conditions were used for this study. One set was maintained in complete darkness, second in continuous light and the third in a 16 h photoperiod. The medium used in this experiment was the EM2 medium.

Leaflets inoculated in continuous light showed a low positive response. Only 11% of the leaflets incubated in continuous light gave rise to the formation of the bulges. The
explants seemed vitrified within 8-10 days and a watery callus was induced on them. The bulges dedifferentiated into callus if the cultures were retained in continuous light. Under 16 h photoperiod, the percentage response was 48% in EM2 medium and bulges appeared on the leaflets within 21 days. In the dark grown cultures, the percentage response remained unchanged. However the days required to elicit this response varied from that of the cultures grown under a 16 h photoperiod and was extended to 35 days. Hence, a 16 h photoperiod was maintained during all further experiments.

4.2.2.4 Sucrose

Higher sucrose concentration (6%) was incorporated in EM2 medium instead of the usual 3%, to study its effect on the induction of embryogenesis.

It was observed that the percentage induction was unaffected at both the sucrose concentrations; however, the days required to elicit the response varied. At higher sucrose concentration (6%) the bulges appeared on the leaflets within 14-16 days as against 21 days in medium containing 3% sucrose.

Sucrose has been reported to affect the embryogenic process in many other plant species. This may be attributed to the fact that in addition to their role as an energy and carbon source, carbohydrates are involved in regulating the osmotic potential of plant cells in vitro (Brown et al 1978; Brown and Thorpe 1980). Somatic embryogenesis in sunflower also was induced only in medium containing 12% sucrose (Finer 1988). Sucrose was reported to promote the embryogenic process in cacao (Kononowicz and

Increased sucrose effected a faster response, thus 6% sucrose was used in all further experiments.

### 4.2.2.5 Cytokinins

BAP and Kinetin were added to EM2 medium separately at varying concentrations (4.40 - 22.20 μM BAP and 4.60 - 23.20 μM Kn). The results of this experiment are included in Table IX.

In all the combinations, the most notable change was greening of the explants within 5-7 days and the presence of a high degree of callusing. This callusing increased with an increase in the cytokinin content and callusing was seen to a greater degree in the 2,4-D and BAP containing media combinations. The nature of the callus was friable in media with BAP up to 13.30 μM. The nature of the callus changed to a nodular type in media with higher content of BAP (22.20 μM) (Table IX). In older cultures the leaflets also gave rise to roots in the BAP containing media. The nature of the callus in Kn containing media remained friable at all concentrations. Rhizogenesis was also exhibited by these cultures. The roots formed were thick and short.

After 28 days in culture embryogenesis was observed in 4% of the explants inoculated in EM2 medium supplemented with 13.30 μM BAP. Two to four somatic embryos were formed per explant which were also accompanied by some shoot buds. The embryos developed to the cotyledonary stage but showed poorly developed cotyledons (Fig 36). If retained in the same medium, the root end of the
**TABLE IX**

Response of immature leaflets to combination of auxin 2,4-D and cytokinins BAP and Kn

**Explant - immature leaflets**

**Medium - MS basal with 3% sucrose**

**Culture conditions - 16h photoperiod at 25±2°C**

**Supplements - 90.50 uM 2,4-D and BAP (4.40 uM to 22.20 uM) or Kn (4.60 uM to 23.20 uM)**

<table>
<thead>
<tr>
<th>No.</th>
<th>2,4-D uM</th>
<th>BAP uM</th>
<th>Kn uM</th>
<th>Response</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>90.50</td>
<td>4.40</td>
<td>0</td>
<td>g,c</td>
</tr>
<tr>
<td>2</td>
<td>90.50</td>
<td>13.30</td>
<td>0</td>
<td>g,fc,e</td>
</tr>
<tr>
<td>3</td>
<td>90.50</td>
<td>22.20</td>
<td>0</td>
<td>g,nc</td>
</tr>
<tr>
<td>4</td>
<td>90.50</td>
<td>0</td>
<td>4.60</td>
<td>g,fc</td>
</tr>
<tr>
<td>5</td>
<td>90.50</td>
<td>0</td>
<td>13.80</td>
<td>g,fc</td>
</tr>
<tr>
<td>6</td>
<td>90.50</td>
<td>0</td>
<td>23.20</td>
<td>g,r</td>
</tr>
</tbody>
</table>

- **g** greening
- **fc** friable callus
- **r** rhizogenesis
- **nc** nodular callus
- **e** embryogenesis
Fig 36: Induction of somatic embryos and some shoot buds on immature leaflets in EM2 medium supplemented with 13.30 μM BAP. e = somatic embryo, s = shoot bud primordia × 50x
somatic embryo callused. These somatic embryos could be detached from the mother explant but failed to germinate on transfer to auxin free medium. Kn did not induce any such response at the concentrations tried.

The embryogenic response was inhibited in EM2 medium supplemented with both the cytokinins tested. No bulges were formed in any combination of BAP and Kn with 2,4-D. Results obtained here are not in agreement with those reported by Baker and Wetzstein (1992) in their studies on somatic embryogenesis from peanut leaflets. They observed an increase in number of somatic embryos induced with increase in the cytokinin content of the 2,4-D medium.

In the present study, BAP in conjunction with 2,4-D induced the formation of shoot buds along with embryogenesis. Shoot bud inducing potential of BAP is well known. The addition of BAP altered the endogenous auxin/cytokinin level which could result in the switching of the otherwise embryogenic cultures towards shoot bud induction.

4.2.2.6 Organic supplements

Yeast extract, casein hydrolysate, coconut milk and glutamine were the supplements used. Glutamine was incorporated in the medium aseptically while the others were autoclaved with the EM2 medium. These were supplemented in the EM2 medium.

All the organic supplements tested were ineffective in altering the response in the leaves. The only change observed was that of increased callusing. Maximum callusing was observed in media supplemented with yeast extract and within 15 days the
callus outgrew the bulges.

Embryogenesis could be initiated without the formation of callus in the presence of 90.50 µM 2,4-D using one day old leaflets. Bulge formation was observed in the EM2 medium after 21 days; however, embryo development from the bulges did not progress beyond the globular stage. Cytokinins or other organic supplements did not enhance the embryogenic response.

For all further experiments MS basal medium supplemented with 6% sucrose and 90.50 µM 2,4-D was used as the induction medium. This has been referred to as EM2* from here on.

4.2.3 Histological studies on induction

At culture initiation, histological preparations of the leaflets showed an immature organisation of cell layers. These consisted of uniform epidermal, parenchymal and vascular traces which were regularly spaced along the sections (Fig 37). There was no differentiation into the typical cell layers as can be seen in the mature leaflets.

The formation of bulges was due to the proliferation of the parenchymal and epidermal layers of the leaf on the abaxial side (Fig 38). Further the bulges were composed of two distinct types of cell layers- the inner layers consisting of loose parenchymal cells and the outer layers with dividing, densely staining compact cells (Fig 39). In their appearance, the dividing cells were typical of meristematic cells (Fig 40). Dividing cells with two nuclei were seen frequently (Fig 40). Under higher magnification, initial periclinal divisions were seen in some of the superficial meristematic layers (Fig 40). Somatic embryos
Fig 37: Immature organisation of the leaf inoculated in EM2 medium. Epidermis (lower and upper), parenchyma and vascular traces seen. red arrows = epidermis, blue = parenchyma, green = vascular traces. Bar 10 mm = 137 um.

Fig 38: Proliferation of the upper epidermis and parenchyma to give rise to bulges. red arrows = epidermis, blue = parenchyma. Bar 10 mm = 330 um

Fig 39: Two proliferating cell types: loose lower parenchymal cells (blue arrow), upper meristematic epidermal cells (red arrow). Bar 10 mm = 72 um

Fig 40: Typical meristematic cells undergoing division. Bar 10 mm = 22 um
originated from these cells as cell division stages similar to the two-celled stage of embryo formation were observed (Fig 41). Sections from older cultures showed early division patterns towards formation of somatic embryos (Fig 42). Subsequently globular somatic embryos developed (Fig 43). The developing somatic embryos did not have any vascular connection with the parent tissue and were formed from the superficial meristematic cells (epidermal in origin) directly without an intervening callus phase. The induction was not only tissue age specific but also exhibited site specificity. Despite the direct contact of the epidermal cells of the adaxial side with the medium, meristematic activity was observed only in the cells of the abaxial side. The meristematic cells were formed due to the proliferation of the superficial cells epidermal in origin. The somatic embryos were induced from these cells subsequently. Thus in the presence of auxin 2,4-D the epidermal cells became induced embryogenic determined cells and these differentiated into somatic embryos. It has been pointed out by several investigators (Steward et al 1958, Esau 1965) that the parenchyma cells of plants are capable of de novo meristematic activity due to their inherent developmental plasticity reflecting their relatively low level of differentiation. Consistent with this are the observations of Conger et al (1983) who observed that parenchymatous cells of Dactylis glomerata show (apparently) predetermination and direct somatic embryogenesis can proceed from individual cells without an intervening callus phase. Mesophyll protoplasts of some plants are also capable of direct somatic embryogenesis (Dijak and Brown 1987). The precise
Fig 41 : Two celled stage in somatic embryo formation
Bar 10 mm = 13 um

Fig 42 : Further early divisions towards somatic embryo formation. Bar 10 mm = 19 um

Fig 43 : Globular stage embryo Bar 10 mm = 72 um

Fig 44 : Cotyledonary stage somatic embryo in 25 day old cultures. Bar 10 mm = 133 um
position of the parenchyma cells within the inductive tissues in which the primary predetermined state is maintained has not been clearly defined in many tissue culture systems (Henshaw et al 1982). In peanut though, the epidermal cells on the abaxial side of the leaf were the cells to become determined and undergo embryogenesis. This again points to the fact that auxins act on different cells of the explant as was observed in immature embryo axes of peanut. In immature embryo axes also the meristematic cells (of the apical meristem) did not give rise to the embryos but the cells at the root shoot junction were activated into embryony. In embryonal leaflets, the highly plastic parenchymal cells did not undergo embryogenesis but the epidermal cells were converted first into IEDCs and they subsequently gave rise to somatic embryos.

The cotyledonary stage was also observed in histological preparations (Fig 44).

4.2.4 Maturation and Conversion

4.2.4.1 Maturation

The above experiments showed that though there was formation of bulges in the 2,4-D medium, this concentration of the auxin did not support any further development of the somatic embryos. If retained in EM2* beyond 25 days, the bulges gave rise to protrusions which occasionally developed into malformed embryos. To achieve normal embryo maturation the leaflets with bulges were transferred to MS basal medium supplemented with 4.52 or 13.75 uM 2,4-D and 6% sucrose.

On transfer to low auxin containing medium, the protrusions
Fig 45: Different stages of somatic embryo formation in medium with 13.75 µM 2,4-D and 6% sucrose. c = cotyledonary stage, t = torpedo stage X 100x

Fig 46: Induction of somatic embryos on the bulges directly without intervening callus phase e = embryo, b = bulge X 75x
Fig 47: Induction of somatic embryogenesis in presence of 90.50 uM 2,4-D. (25 day old culture)

Fig 48: Maturation of the induced somatic embryos in lower 2,4-D (13.75 uM) containing medium.
developed into somatic embryos by passing through the various embryo stages (Fig 45). Callusing did not occur during embryo development when the medium contained 13.75 μM 2,4-D (Fig 46). Hence, MS basal medium supplemented with 6% sucrose and 13.75 μM 2,4-D was used as the maturation medium.

The development of the somatic embryos up to the cotyledonary stage was a two step process. Initiation of embryogenesis occurred in 90.50 μM of 2,4-D (Fig 47) whereas maturation occurred in 13.75 μM 2,4-D (Fig 48). Under higher magnification well developed somatic embryos were visible (Fig 49). Occasionally, the somatic embryo development also showed some abnormalities (Fig 50). This was also observed in soybean cultures when somatic embryogenesis was induced in high 2,4-D. In soybean the somatic embryos were arrested at the globular stage of development (Shoemaker et al 1990).

4.2.4.2 Secondary embryogenesis

Along with maturation of the somatic embryos, secondary embryos were also induced. The secondary embryos arose from the inner sides of the cotyledons as globular structures. On retention in the same medium these developed into funnel shaped embryos. Eight to ten such funnel shaped structures were formed per embryo (Fig 51). These however did not mature into normal somatic embryos nor could they germinate to give rise to plants.

4.2.4.3 Repetitive embryogenesis

Repetitive embryogenesis could be obtained by exposing the culture alternately to high and low 2,4-D containing media (Fig 52). Induction always occurred in the presence of 90.50 μM 2,4-D and 6% sucrose whereas maturation occurred in medium containing
Fig 49: Well developed somatic embryos under higher magnification. e = embryo X 75x

Fig 50: Abnormal somatic embryo a = abnormal embryo X 75x
Fig 51: Funnel shaped secondary somatic embryos induced on the inner side of the cotyledons of the primary embryo. f = funnel shaped embryo, p = primary embryo cotyledon  X 100x

Fig 52: Repetitive embryogenesis e = embryo X 75x
lowered 2,4-D (13.75 μM). The frequency and percentage of repetitive embryogenesis was reduced in each cycle. Repetitive embryogenesis was not observed if the cultures were not passed through a high auxin medium (90.50 μM 2,4-D) between the two low auxin passages.

4.2.4.4 Conversion

The mature somatic embryos initiated germination in the low 2,4-D containing medium by giving rise to a root initial. Such embryos were transferred to MS basal medium with 2% sucrose and supplemented with 0.25% activated charcoal (GM1) where low frequency germination was observed. Only 12% of the transferred embryos converted into plants (Fig 53) within 20 days. These were hardened and transferred to the green house for further growth (Fig 54). They showed similar flowering and other morphological characters when compared to the control plants. The plants were fertile and set seed.

4.2.5 Conclusion

Somatic embryogenesis using leaf explants has been recently reported (Baker and Wetzstein 1992). This report represents the first report of somatic embryogenesis from leaflets of peanut or any large seeded legume. However, the percentage embryogenesis was low (14.6%). The conversion rate of the somatic embryos into plants was also low (2%). Moreover, the formation of somatic embryos was also associated with callus and no clear explanation of the nature of embryogenesis (direct or indirect) was provided. In comparison, the protocol described in the present study is far more superior to the above in more than one ways.
Fig 53: Mature somatic embryos transferred to GM1 medium for conversion.

Fig 54: Plant derived from somatic embryo in pot (30 days after transfer to soil).
Firstly, 45% of the leaf explants inoculated showed direct somatic embryo formation in high 2,4-D (90.50 µM) containing medium. No cytokinin was needed for induction, moreover cytokinin supressed the embryogenic response. Thus embryogenesis was obtained using a simple medium. Secondly, the embryos were single cell in origin. The induced somatic embryos matured in low 2,4-D (13.75 µM) medium. Thirdly repetitive embryogenesis could be obtained by exposing the cultures to high (90.50 µM) and low (13.75 µM) 2,4-D alternately. The conversion rate of the somatic embryos was higher (12%). The plants obtained were fertile and set seed.

Peanut improvement has been limited from integrating resistance to many diseases and pests from wild peanut (Stalker and Moss 1987) because of problems with sterility barriers and genomic incompatibilities associated with traditional breeding. At present, gene transformation methods cannot be successfully applied to peanut improvement in part because most of the organogenic and embryogenic regeneration systems reported lack adequate regeneration rates. Besides, direct somatic embryogenic systems would have some distinct advantages when used for transformation or other applied studies such as isolation of variants etc.

With around 45% embryogenesis (direct) from leaflets and a single cell origin of the somatic embryos, the embryogenic system described in the present study makes it highly adaptable for application to biotechnology and transformation studies.
PLANT REGENERATION VIA ORGANOGENESIS FROM EMBRYONAL LEAF EXPLANTS
5.1 Introduction

In vitro organogenesis is characterised by the formation of a bud primordium with subsequent development of the bud into a shoot, root or a flower. The developing organs induce a procambial strand to establish a connection with the mother tissue. In cultures explants have the capacity to give rise to shoots, roots or floral structures in the presence of exogenously supplied plant growth regulators.

The formation of shoots, roots, or floral structures may arise directly from the explant or via callus. In the first case the immediate precursors of the new organs are cells in the explant itself. The other type of organogenesis involves a dedifferentiation of the explant, callus formation at the cut edges of the explant and induction of new organs from the newly formed callus tissue. Here the exogenously supplied hormone not only controls the process but is required for organogenesis to occur. Caulogenesis leads to the formation of unipolar bud primordia which develop into shoots that must be rooted (Thorpe and Patel 1984).

It is well known that the control of organogenesis in majority of the cultures is largely a function of the exogenous auxin/cytokinlin ratio within a particular range of concentration a principle discovered by Skoog and Miller (1957). This was proved in experiments carried out by Tran Thanh Van et al in 1974. She demonstrated that in superficial cell layers excised from tobacco floral branches, auxin and cytokinin at 10^{-6}M caused the regeneration of floral buds directly without intervening callus. The absolute concentration of hormones is important since
at $10^{-7}$ M cytokinin concentration, floral buds are not formed. If the cytokinin concentration was raised to $10^{-5}$M only vegetative buds are formed.

Regeneration of shoots via organogenesis in vitro is an approach which offers rapid shoot proliferation and has been observed in a large number of plant species. This pathway of plant regeneration is of particular interest when rapid multiplication of a unique trait of any plant is required. It is of use for the propagation of an elite plant species, a valuable somaclone or it can also be used to amplify a transformation of the plant genome. Hybrids which are unique can be propagated via organogenesis to retain the particular characteristic. This pathway of regeneration is of great use where very fast multiplication of few shoots/plants is required.

On the other hand, evidence has accumulated to prove that the genetic integrity of a plant species may get altered in vitro. The term somaclonal variation was introduced to describe the genetic variation in plants from any form of cell culture (Larkin and Scowcroft 1981). The effects of biological (genotype, explant), medium (growth regulators) and physical (duration of culture) factors on somaclonal variation have been noted, but so far the basic knowledge towards the cause of these changes is fragmentary. In the study of somaclonal variation the absence of a straightforward rapid assay to measure its extent is a major obstacle (Orton 1983a,b). Reviews on somaclonal variation focus on its potential for breeding (Larkin and Scowcroft 1981; Evans 1989), cytological aspects (Bayliss 1980; Lee and Phillips
1988; Pijnacker and Sree Ramulu 1990) or on its origin and causes (Orton 1983a,b; Karp and Bright 1985; Gould 1986; Sree Ramulu 1987).

Mericlones, that is, plants originating from the outgrowth of non-adventitious meristems are more true to type: somaclonal variation is observed mostly in plants originating from adventitious meristems (Hussey 1983).

In addition to somaclonal variation epigenetic (non genetic) variation also occurs frequently (Binns 1981; Meins 1983). The main difference between the two is that somaclonal variation is transmitted during meiosis whereas epigenetic variation is not. Further epigenetic variation is reversible during the life of the plant and is primarily a physiological response: therefore is a one direction change (i.e follows a dose response curve).

In peanut, regeneration via organogenesis has been reported from leaves, cotyledons, hypocotyls and epicotyls (Mroginski et al 1981; Narasimhulu and Reddy 1983; Pittman et al 1983), but regeneration frequency was low and plants were rarely obtainable. McKently et al (1989) successfully obtained shoot regeneration from cotyledons in a study evaluating 20 peanut genotypes. Plants were obtained, matured and set seed. Shoot organogenesis and plants were also obtained using immature leaflets (McKently et al 1991). These reports however did give any information on the evaluation of the regenerants. The present chapter describes high frequency regeneration of peanut plants *in vitro* via organogenesis in the cultivar JL-24. The plants obtained were fertile and set seed. These plants were evaluated for any phenotypic variation in comparison with seed raised controls.
5.2 Results and Discussion

5.2.1 Induction of shoot buds

The leaf explants were obtained from mature embryo axes as described earlier (Chapter IV). The leaflets of genotypes JL-24 and SB-11 were inoculated in petri-plates (80 explants/plate) containing shoot bud induction medium SM1 (MS basal + 3% sucrose + 5.36 μM NAA + 4.40 μM BAP). This medium was chosen after a literature survey of regeneration studies reported in peanut. The petri-plates were incubated under cultural conditions as described earlier (Chapter II).

Shoot buds were induced from embryonal leaflets 2-4 mm in length within 10 days. Older leaves did not exhibit morphogenic potential. This was also observed in studies on embryogenesis using leaf explants, when embryogenesis was induced on leaves derived from overnight soaked embryo axes in the presence of 2,4-D. This could reflect the high plasticity of the leaf cells at this age so that they could easily switch from one pathway to another with change in media composition. The size specific response of peanut leaves in the induction of shoot buds has also been reported earlier for various wild varieties of peanut such as A. pusilla, A. monticola, A. pintoi, and A. villosullicarpa. Primary leaves 2-5 mm in length have also been reported to be the best as explants to induce shoot buds directly in pea (Rubluo et al 1982) and Psophocarpus tetragonolobus (Blackmon and Reynolds 1982).
Fig 55: Induction of shoot buds on immature leaflets in SM1 within 10 days. X 50x

Fig 56: Shoot bud primordia in SM1 medium after 20 days. X 70x
In the present study, two approaches were used to follow the process –

5.2.1.1 Light Microscopy

5.2.1.2 Histological observations

5.2.1.1 Light Microscopy

Both the genotypes tested showed similar responses. After 8 days in SM1 the leaflets turned green and enlarged. A small amount of friable callus was observed at the cut end of the leaflets. After 10 days shoot bud primordia were observed at these cut ends. Shoot buds were induced at the petiolar end of the whole leaflets on the abaxial side (Fig 55). This initiation was seen in 85% of the leaflets inoculated. Over the next 10 days the shoot primordia remained essentially unchanged (Fig 56) with only occasional differentiation of some buds into leafy shoot initials (Fig 57). The explants were then transferred to different media to optimise multiplication and elongation of these shoot buds (5.2.2).

5.2.1.2 Histological observations

Leaf explants were fixed in FAA every alternate day for the first 10 days and prepared for histological study as described earlier (Chapter II).

Five day old leaf explants exhibited distinct upper and lower epidermis, inner mesophyll tissue and vascular bundles (Fig 58). Cell division was apparent in the mesophyll cells close to the epidermis (Fig 59) by the next two days. Meristematic activity was observed in localised regions of the leaf within 10 days of exposure of explants to SM1 (Fig 60). The origin of this
Fig 57: Differentiation of some buds into leafy shoot initials s = shoot initial X 75x
Fig 58: Distinct upper and lower epidermis, inner parenchymal tissue and vascular traces in 5 day old leaflets.

Bar 10 mm = 400 u

Fig 59: Cell division apparent in parenchymal cells close to the epidermis. Bar 10 mm = 117 u

Fig 60: Meristematic activity in parenchymal cells of the leaflet 7-8 days after inoculation. Bar 10 mm = 130 u

Fig 61: Establishment of vascular strands in shoot buds from 20 day old cultures. Bar 10 mm = 333 u
meristematic activity was in the mesophyll cell layers. This was evident from the presence of the intact epidermis (Fig 60). Vascular strands were observed in shoot buds from 20 day old cultures (Fig 61). Leafy shoot primordia with leaf initials surrounding the shoot meristem were clearly seen in the older cultures (Fig 62).

5.2.2 Multiplication and elongation

Cytokinins have been used earlier to increase multiplication rate of shoots in many species such as clover (Singh et al 1988), papaya (Rajeevan and Pandey 1986) and banana (Wong 1986). Among the cytokinins, effectiveness of BAP in induction of shoots from meristems has been recognised in peanut (Mhatre et al 1985; Atreya et al 1984) as well as in other plant species such as soybean (Kartha et al 1981) and sunflower (Knopp and Mix 1986; Paterson 1984) and Atractylodes lancea (Hiraoka et al 1984). Both BAP and Kn differ structurally from the natural cytokinins in that both have cyclic structures connected to the amino acid group rather than the carbon atom. Perhaps BAP or Kn bind to a cytokinin binding site but because the fit is imperfect the tissue is stimulated to produce natural cytokinins that enhance regeneration (Wright et al 1986). Thus these compounds may act as promoters of cytokinin synthesis rather than as cytokinins.

In the present studies, after 20 days in SM1 medium, the responsive explants were transferred to either test tubes (one explant/tube) or flasks (3 explants/flask) containing one of the following three media:
Fig 62: Fully differentiated shoot bud in older cultures.

Bar 10 mm = 225 u
Fig 63: New shoot buds induced at the base of the existing ones on transfer to ME2 medium. X 75x

Fig 64: Differentiation of shoot buds into shoots and their elongation in ME2 medium (25 d old cultures).
i) MS + 3% sucrose + 0.536 μM NAA + 4.40 μM BAP (ME1)

ii) MS + 3% sucrose + 2.20 μM BAP (ME2)

iii) MS + 3% sucrose + 13.20 μM BAP (ME3)

Multiplication rate could not be visually assessed due to the close initiation sites of the shoot buds. Multiplication has been described as either rapid or poor.

Multiplication of shoot buds in medium ME1 was very slow and it was also accompanied by the formation of creamy friable callus. Rapid proliferation of shoot buds was not observed at the end of 15 days in ME1 medium. Differentiation of the shoot buds to form leafy shoots occurred after 20 days. The number of shoots formed per tube was 0-2. This medium however did not favor elongation of the shoots and at the end of 28 days these were only 1-2 cm long. Thus it can be concluded that ME1 medium induced neither rapid multiplication nor elongation. This medium was eliminated in all further experiments.

Explants transferred to medium ME2 also showed slow multiplication after 15 days. New shoot buds were induced at the base of the existing ones (Fig 63). These differentiated into leafy shoots and at the end of 25 days 3-4 shoots (5-7 cm long) were formed per explant (Fig 64). Thus in ME2 medium also the rate of multiplication was poor but this medium enhanced shoot elongation. This medium was used as the shoot elongation medium for all further experiments.

Rapid proliferation of shoot buds was observed within 7-8 days of transfer of leaf explants to ME3 (Fig 65). The new shoot buds were induced in the form of green knots. By the end of 15
Fig 65: Rapid proliferation of shoot buds observed 7-8 days of transfer of leaf explants to ME3 medium.

Fig 66: Poor elongation of the shoots in ME3 medium.
Fig 67: Formation of new shoot buds from the pre-existing ones.

p = primary shoot bud, s = new shoot buds induced

Bar 10 mm = 276 u
days the mass of multiplying buds was visibly 4-5 times bigger than when transferred to ME3. The multiplication was associated with the formation of creamy friable callus. The callus could be easily separated from the shoot bud mass. Elongation of the shoot buds was poor in this medium and only 1-2 shoots were formed after 20 days (Fig 66). These shoots were always stunted (2-3 cm long) and possessed very small internodal lengths. Thus high concentrations of BAP suppressed elongation of the shoot buds. In general, high content of BAP in media has been reported to inhibit elongation and rooting (Paterson 1984, Yeoman 1986). In peanut, the resultant shoot buds elongated when transferred to low BAP containing medium. The shoots were healthy and green.

In histological studies, the formation of new shoot buds was seen to originate from the pre-existing shoot buds (Fig 67). The ME3 medium was used as the shoot bud multiplication medium for all further experiments.

5.2.3 De novo flower bud formation

In ME2 medium, it was observed that among the slow multiplying shoot buds, occasionally there was formation of flower buds which differed in their appearance from the others (Fig 68). The induction of such buds was a sporadic and an unpredictable event. These arose in groups of 3-4 buds and metamorphosed into flower buds within 10-12 days of their appearance.

In plants, the transition to flowering marks the beginning of reproductive development, during which meristems produce flowers rather than leaves, stem and associated vegetative
structures. Internal signals related to developmental age or external signals such as daylength or temperature can induce the onset of flowering and this may involve both promotive and inhibitory substances (Evans 1960; Lang et al. 1977; Bernier 1988). It is well established that some of these substances are generated in leaf tissues (Lang 1965; Zeevaart 1976), but other plant organs may also influence the induction and development of flowers (Bernier 1986). The involvement of leaf and roots in floral evocation in vitro in tobacco was shown by Wagner et al. (1989).

In studies on in vitro floral morphogenesis in tomato Rastogi and Sawhney (1986) showed that the presence of a cytokinin was important in the initiation of carpel primordia of the flower, as well as in the growth and maturation of all the floral parts. The requirement of a cytokinin for the in vitro growth and development of floral parts has also been reported for Nicotiana tabacum (Hicks and Sussex 1970), Cyperus rotundus (Mohan Ram and Batra 1970), Begonia francois (Berghoef and Bruinsma 1979), Zea mays (Polowick and Greyson 1982), Lycopersicon esculentum (Rastogi and Sawhney 1987) and Arachis hypogaea (Narasimhulu and Reddy 1984, 1987). In the present study also, flowering was induced in the presence of cytokinin (BAP). As early as 1970 it was suggested by Skoog that the influence of cytokinins was possibly mediated through their action as modulators of protein biosynthesis and their influence on the synthesis of several growth factors including thiamine.

In earlier reports of in vitro flower bud induction in peanut (Narasimhulu and Reddy 1984, 1987), exclusive flowering
Fig 68 : Induction of flower buds among shoot bud in ME2 medium  

f = flower bud  X 50x

Fig 69 : Development of the flower buds upto the pre-blooming stage in ME2 medium.
could be induced on de-embryonated cotyledons directly in the presence of 2.20 μM BAP. The use of embryonated cotyledons required higher cytokinin (13.20 μM BAP) to induce flowering. Moreover this was always associated with formation of shoots. The flowering was site specific and was influenced by the concentration and type of cytokinin in the medium. Only BAP could induce flowering and flower buds were induced only at the embryonal end of the cotyledon. This observation lends support to the early evidence forwarded by Fortainer (1957) that flower bud initials in peanut are present very early in development and are located in the embryonic region of the seed.

In the present study the immature leaflets also were derived from the same region. One reason for the formation of flower buds could possibly be the precocious expression of pre-existing determined cells. The induction of floral meristems can also be interpreted in another way. It is known that flowers develop from groups of undifferentiated cells that grow from the flanks of shoot apical meristems. The cells in these floral primordia divide and then differentiate into appropriate number of floral organs, in appropriate places. This could mean that in peanut cultures some of the cells differentiated into shoot meristems while some others simultaneously gave rise to floral meristems. The milieu of the cells possibly played an important role in the shift of the morphogenic pathway to either of the two types. Besides, although anatomical differences between vegetative and floral organs are substantial, several types of evidence suggest that floral organs are likely to be modified vegetative
Fig 70: Flower buds and shoot buds in the same explant. s = shoot bud, f = flower bud Bar 10 mm = 333 μ

Fig 71: The four whorls clearly visible. Bar 10 mm = 400 μ
structures (Arber 1937; Wilson and Just 1939; Bailey and Swamy 1951; Kamalay and Goldberg 1980).

The flower buds developed further but failed to open in this medium (Fig 69). Flower opening could be achieved by mere substitution of cytokinin BAP with an equimolar concentration of another cytokinin Kn. In peanut, under in vitro conditions, induction, development and maturation of the flower buds and subsequently, their blooming were thus two separate events each requiring a specific cytokinin type. This emphasises the development related hormonal specificity for this process. In histological preparations it was observed that the shoot buds originated from the superficial mesophyll cells whereas the flower buds had their origin in the vascular tissue (Fig 70). Premature versions of the four whorls of a flower bud (calyx, corolla, anthers and ovary) were clearly visible (Fig 71).

5.2.4 Indirect caulogenesis

Callus induced in ME3 could be separated and maintained in the same medium. Localised green spots were observed on this callus within 12-14 days of transfer to fresh ME3 medium (Fig 72). Within the next 4-5 days these spots metamorphosed into shoot buds. The further development of these shoot buds was similar to those induced directly from leaf explants. The callus origin of the shoot buds was also evident from histological studies (Fig 73). This regenerating callus showed shoot bud induction for 5 subcultures after which the regeneration potential of this callus was seen to decrease.
**Fig 72**: Localised green spots on the callus induced in ME3 medium in 12-14 d.

**Fig 73**: Shoot buds originating in the callus induced in ME3. c = callus, s = shoot bud primordium. Bar 10 mm = 167 u
Thus besides inducing shoot bud proliferation directly 13.20 μM BAP also supported formation of caulogenic callus. The first event in the process was the formation of localised green spots. These could correspond to induction of meristematic activity in the dedifferentiated callus. It has been suggested that BAP or cytokinins in general are primarily responsible for the formation of meristematic zones during shoot morphogenesis (Vasil 1986).

It must be mentioned here that this explant showed the potential for three types of morphogenesis (direct caulogenesis, indirect caulogenesis and floral morphogenesis) in the presence of BAP and fine manipulation of the media could direct a specific pathway of development.

5.2.5 Rooting

Elongated shoots (4-5 cm long) from ME2 medium were transferred to four types of media to achieve rooting (Fig 74). Twenty five shoots were inoculated in each medium composition. The media compositions were as follows:

1) MS basal + 2% sucrose + 0.4% agar (RM1)
2) MS basal with half strength inorganic salts and full strength vitamins + 2% sucrose (liquid medium RM2).
3) RM2 + 0.4% agar (RM3)
4) RM2 + 0.4% agar + 0.25% activated charcoal (RM4)

Rooting was observed in all the four media tested but percentage of shoots inducing roots differed (Table X). Further time required for induction of rooting also varied with each
Fig 74: Transfer of elongated shoot in RM3 medium for rooting.
TABLE X

Effect of various hormone free media on rooting of shoots obtained via caulogenesis

Basal medium - MS

Culture conditions - 16h photoperiod, 25 ± 2°C

<table>
<thead>
<tr>
<th>No</th>
<th>Medium</th>
<th>% rooting</th>
<th>Callusing</th>
<th>Secondary rooting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RM1</td>
<td>29</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td>2</td>
<td>RM2</td>
<td>52</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td>3</td>
<td>RM3</td>
<td>89</td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>4</td>
<td>RM4</td>
<td>62</td>
<td>present</td>
<td>absent</td>
</tr>
</tbody>
</table>
media composition. The best response was seen in RM3 medium with 89% of the shoots showing root formation within 15 days. In this medium, 2-3 adventitious roots were formed per shoot and secondary rooting was also observed (Fig 75). Very sparse callusing was associated with root formation in RM3 medium. In the other three media tested (RM1, RM2 and RM4) only a single root was initiated, which was accompanied by callusing (Fig 76). Thus medium RM3 was used as the rooting medium hereafter.

Shoots of some species multiplied in vitro lack a good root system (Yeoman 1986). Rooting is usually induced by subculture to medium devoid of cytokinins with or without rooting hormones. Factors such as shoot quality and the use of cytokinins at the multiplication stage affect rooting efficiency. Cytokinins are known to inhibit rooting and BAP widely used for shoot multiplication does so strongly (Hussey 1986). Moreover it is well known that de novo initiation of roots depends on low cytokinin to high auxin ratio and the hormonal requirement for shoot multiplication is exactly the opposite (Skoog and Miller 1957). The residual cytokinin from the multiplication stage may be high enough to suppress root formation. The rooting in these shoots can be achieved optimally in hormone free medium because carry over of the inhibitory influence of cytokinin would be less. This was also found to be true in the present study and rooting was achieved in hormone free medium. Another cause for suppression of rooting is the presence of high salt in the medium. This can be overcome with the use of a rooting medium with reduced salt content (Kartha et al 1974, 1981; Lane 1979; Skirvin and Chu 1979). Successful rooting using lower salt
Fig 75: 2-3 adventitious roots as well as secondary roots induced in RM3 medium. Almost no callusing observed.

Fig 76: Single root induced in RM4.
concentration has been achieved in several plant species (Kartha et al 1974; Weng 1978; AnCora et al 1981; Ben-Jaakov and Dax 1981; Werner and Boe 1980; Snir and Erez 1980). In peanut cultures also the reduction in salt content of the medium favored rooting.

5.2.6 Hardening and Soil Transfer

The rooted shoots were hardened as described earlier (Chapter II). Ninety six percent of the transferred plants survived in soil. After attaining an height of 10-12 cm these plants were transferred to larger pots, one plant /pot and grown to maturity in the green house (Figs 77,78).

5.2.7 Growth Behaviour of the Regenerants

To obtain a large number of plants, the shoot buds were initially multiplied in ME3 medium for five subcultures. These were subsequently elongated in ME2 medium and then rooted. The growth profile of 53 plants (Ro generation) so obtained and ascribed numbers P1, P2, ...P53 was studied in comparison to seed raised controls (Fo generation).

The overall growth rate, flowering time and seed set of the regenerants was comparable to the seed raised controls. Among these, some however exhibited deviations from the normal growth pattern.

Abnormal leaflet number was observed in leaves of one plant P15, with 5 leaflets instead of the usual 4. The fifth leaflet was always present at the base of the inner leaflets on the abaxial side of the leaf (Fig 79). This type of leaf appeared after every 3 normal leaves in this plant. This sequence was
Fig 77: Transfer of rooted shoots to small pot containing a sand:soil mixture (3:1).

Fig 78: Plants transferred to the greenhouse.
followed only on the main stem of the plant.

Abnormality in leaf morphology was observed in another plant P \(_8\). Here the leaflet number /leaf was unaltered, but the upper (inner) pair of leaves were very small in size and curled. The lamina of these abnormal leaflets remained folded at the midvein (Fig 80).

Flowering was initiated late in one regenerant P \(_{32}\). The first flush of flowering appeared 15 days later than in the other regenerants.

All the regenerants were fertile and set seed. The seed yield of the regenerants and the controls was similar. Eight to twelve pods were obtained per plant (Fig 81). Ninety two percent of the pods of each plant were two seeded.

Variation in the Ro generation was observed in 6% of the regenerant population. A query that naturally followed was - what could the presence of such deviations in morphology and development be attributed to and secondly, were these heritable changes?

The possible causes of the variations could have been:

a) Prolonged culture: Prolonged culture of the adventitious buds before their differentiation into shoots could be one reason for deviation from normal behaviour. It is known that genetic variation increases with prolonged culture (De Klerk 1990). In agreement with this are studies reported in celery (Orton 1985), pea (Natali and Cavallini 1987), maize (Armstrong and Phillips 1988), wheat (Hartmann et al 1989) and pine (Franklin et al 1989).

b) Mutagenic substances: It has been assumed that medium
Fig 79: Abnormal leaf in plant $P_{15}$ with five leaflets instead of the usual four.

Fig 80: Inner pair of leaflets curled in leaves of plant $P_8$.

Fig 81: Pods obtained from one of the regenerants $P_{12}$. 
components, especially certain growth regulators are mutagenic (Vajrabhaya 1977; George and Sherrington 1984). The reason for this is the triggering of chromosomal aberrations by these regulators at high concentrations (Bayliss 1980). However, NAA, 2,4-D and BAP at a concentration of 1 mg/l were reported to be ineffective in causing an increase in the frequency of mutation in tissue cultures (Dolezel and Novak 1984).

c) Response to stress: Conventionally, the genome is considered to be a stable entity. More recently, however, evidence has accumulated to suggest that the genome is actually in a continuous state of flux with changes occurring both in the mitotic as well as meiotic cycles (Walbot and Cullis 1985). McClintock (1984) advocated the view that when plants are exposed to an environmental stress beyond their capacity to adapt by epigenetic changes, they may enter a state of "genome shock" that activates transposons and thereby brings about rapid revolutionary changes. Tissue culture is envisaged as a form of stress and could therefore result in similar changes that form a part of an adaptive strategy.

d) The changes may be due to change in the chromosome number. However it has been observed by many workers that plants with correct chromosome numbers may have an aberrant phenotype as reported in sugarcane (Liu and Chen 1976), potato (Creissen and Karp 1985; Fish and Karp 1986; Gill et al 1986; Sree Ramulu et al 1986; Osifo et al 1989), wheat
(Maddock and Semple 1986; Chen et al. 1987) and *Lolium multiflorum* (Jackson and Dale 1989).

The variations observed in the regenerants persisted throughout their life cycle. However in the F1 generation the variations were not observed. These results indicate that the variations observed were possibly epigenetic in nature. Screening of a higher number of plants or an alternative method for measuring somaclonal variation suggested by De Klerk (1990) may give a more clear picture of the status of heritable variation in peanut regenerants.

5.2.8 Conclusion

The protocols described in this chapter provide efficient regeneration of peanut plants from immature leaflets via organogenesis. Besides induction of shoots (direct caulogenesis), the same explant was also capable of inducing a regenerating callus (indirect caulogenesis) and floral meristems. This regeneration system is thus of great value for basic and applied studies.

Gene transfer systems depend on an ability to regenerate whole plants from transformed cells. For many dicot species, cells in the margin of the excised leaf pieces can be induced to undergo rapid division *in vitro* to form regenerable callus or shoots. These cells are thus attractive targets for transformation, whether mediated by *Agrobacterium* (Gasser and Fraley 1989) or by microbead bombardment (Tomes et al. 1990). The present protocol can also be used for isolation of variants and to study exactly which conditions govern the ability of the leaf explant to undergo a particular type of morphogenesis.

96
VI

IN VITRO MUTAGENESIS
IN PEANUT
6.1 Introduction

Mutations are the ultimate source of all variability in organisms. Variability caused by induced mutations is not essentially different from variability caused by spontaneous mutations during evolution.

In plants, use of mutations is a very valuable supplementary approach to plant breeding specially when one or two identifiable characters present in an otherwise well adapted variety are to be altered. The main advantage of this approach is that the main genotype is usually only slightly altered (as compared with the hybridisation of two different varieties) while the improved character is added. Early maturity, resistance to diseases and certain improvements in the grain quality can be introduced in well adapted species without altering their other attributes. Early studies on mutagenesis were carried out on whole plants and seeds *ex vitro*. Chemical mutagens and radiations were used to treat whole seeds and parts of plants such as shoots, tubers, rhizomes etc commonly used for vegetative propagation. Release of the varieties of wheat (Sharbatli Sonora), rice (Reimei) and barley (Luther) have resulted from initial mutagen treatments (Manual on Mutation Breeding 1970). Radiation has also been used to achieve mutation in crop species in the form of translocations and duplications (Hadberg 1966), haploidization (Bender 1963) and for induction of sexuality (Julen 1961). In legumes, mutant varieties released include those of soybean, various kinds of beans, pea and peanut. A review on the various aspects of mutation research with respect to application for breeding,
studies on mutagenic mechanisms and on DNA repair in particular has been reported by Negrutiu (1990).

6.1.1 Mutagenesis in vitro

The constant development and sophistication of cell and tissue culture techniques in plants has made it possible to extrapolate the strategies of selection used *ex vitro* to *in vitro* cultured cells and tissues. This has resulted in a wide spectrum of biochemical variants (Flick *et al* 1983; Negrutiu *et al* 1984). These studies have also furnished information on the effects of irradiation and chemical mutagens on cell and tissue survival and mutation rates (King 1984; Negrutiu 1984).

The living cell has evolved a series of enzymes which act to maintain the integrity of the genetic material by repairing the DNA that has been damaged spontaneously or by mutagens. Rescuing the cell from potentially lethal damage to the genome is often at the expense of making errors from which various mutations arise (Howland and Hart 1977; Negrutiu 1990).

Mutation damage to the genome can be classified as macromutations involving deletions, duplications and rearrangements (inversions and translocations) and micromutations such as base pair substitutions and frame shift mutations (Drake 1969).
6.1.2 Types of mutagens

6.1.2.1 Chemical mutagens

These include the base analogues, alkylating agents and other compounds such as sodium azide.

a) Base Analogues

These are compounds closely related to the DNA bases. They get incorporated into the DNA during replication which results in pairing errors (Heslot 1977). 5-bromouracil and 5-bromodeoxyuridine are the base analogues commonly used. Similarly maleic hydrazide, a structural isomer of uracil has been shown to be able to induce sister chromatid exchanges in *Vicia faba* and *Allium cepa* (Cortes et al 1987).

b) Alkylating agents

The alkylating agents (alkyl sulfates and sulfonates) have one or more reactive alkyl groups which can be transferred to phosphate groups such as those of the purines and pyrimidines (Heslot 1977). It is unclear whether the alkylating agents directly alkylate the DNA or cause mutations indirectly.

c) Sodium azide

This compound was established as an effective mutagen under defined conditions with a high number of advantages namely a high frequency of point mutations, safe handling, non-persistent and inexpensive (Heslot 1977). This compound is a rare example of a proximal mutagen acting indirectly through a compound synthesised from it by metabolic processing. This was shown to occur in some plant species such as barley and the metabolite synthesised *in vitro* was identified as azidoalanine (Nilan 1981; Owais et al

Chemical mutagens are generally very toxic to handle and have very small shelf lives. Their administration has to be carried out under critical conditions and the removal of the compound is necessary after the treatment. Some of these limitations can be overcome with the use of physical mutagens.

6.1.2.2 Physical mutagens

This type is characterised by radiations-ionising and non-ionising. These radiations include X-rays, gamma rays and UV radiations. These are safer to use and do not possess the drawback of removal of the mutagen after the treatment. The effect of these radiations is however not targeted.

The characteristic features of each of these radiations are as given below:

a) X-rays

These are electromagnetic radiations produced by electrically accelerated electrons in high vacuum. Their wavelength is between 10 and 0.001 nm and hard X-rays (with shorter wavelengths) have higher penetration values (Briggs and Constantin 1977).

b) Gamma radiations

These are ionising radiations. These have wavelengths shorter than X-rays and thus have more penetrating power. These are obtained from radioisotopes such as $^{60}\text{Co}$ and $^{137}\text{Cs}$.

c) Neutrons

These are produced in atomic reactors and accelerators ($^{235}\text{U}$ fuel) and have been reported to be highly effective in inducing mutations in plants (Briggs and Constantin 1977).
The above types of radiations are ionising radiations and these need a very elaborate experimental set up for their use.

**UV radiation**

These are non-ionising radiations and are a part of the non-visible range of light. UV radiations produce maximal biological effects in the range of 250-290 nm, a window of maximal light absorption by the nucleic acids. The most commonly used source of UV light is the germicidal lamp. The wavelength of this light is around 254 nm and this radiation is strongly absorbed by DNA. This radiation has been shown to be very effective in inducing single mutations (Negrutiu 1990).

UV light induces both frame shift mutations and base pair substitutions as in *Neurospora* (Drake 1969). Pyrimidine dimers are caused due to UV radiations. The dimers can get repaired by photoactivation. Pyrimidine dimers lead to the formation of single strand breaks in the DNA both during excision repair and replication. The aberrant repair of these gaps then produces mutations. Howland (1975) in his studies with wild carrot cells demonstrated that dimer excision after low doses of UV was nearly 100%, but after higher doses excision was drastically reduced. He also showed that the rate of dimer excision was initially very rapid but was essentially zero after 24 hours.

In plants, certain aspects of mutagenesis biochemistry and repair have been studied in seeds and cultured cells (Veleminsky and Gichner 1978). Recent contributions to this field are those related to the repair of UV light induced DNA damage (McLennan 1987) and the DNA repair in pollen grains (Jackson 1987).
Work on mutagenesis with \textit{in vivo} systems has shown that goals can be reached when little is known about the genes that control the character in question. The coupling of mutagenic treatment with cell culture methods is likely to be most effective when the desired character is controlled by a single gene and when this is expressed in the homozygous recessive condition as most mutations are from the dominant to recessive form of the allele.

Among the various types of radiation mutagenesis, UV mutagenesis offers wide applications due to its specificity, availability, convenience and relative safety. The most commonly used source of the UV lamp is the germicidal lamp. These radiations are very effective in inducing mutations or cell killing because the energy emitted (primarily 250-300 nm) is strongly absorbed by the DNA. Irradiation of plant tissues with UV results in somewhat well defined damage of DNA. This damage is in the form of intrastrand dimerisations of the adjacent pyrimidines resulting in a lesion shown to be important in cell killing. Pyrimidine dimers are reported to increase with increasing UV fluence, until very high concentrations accumulate (Howland 1975). Eriksson (1967) isolated a stable variant exhibiting an altered karyotype and a high propensity for anthocyanin production from UV irradiated \textit{Haplopappus} cell cultures.

Peanut has been shown to be susceptible to ionising radiations and phenotypically altered plants have been isolated post X-ray irradiation (Patil and Mouli 1977). However to date information on the susceptibility of peanut cultures to UV
radiation is fragmentary (Verma and van Huystee 1971).

Over the years plants have developed effective means for limiting the genetically destructive effects of UV. A higher incidence of UV radiations across the stratosphere represents further reasons to develop a systematic study on the UV mutagenic mechanisms. A beginning has been made in this direction and interesting recent contributions to this field are those related to the damage of UV light-induced DNA damage in plants (McLennan 1987) and DNA repair in pollen grains in *Petunia* (Jackson 1987).

In the present study, the effect of UV radiation on whole seeds of peanut and regeneration potential of leaf explants was estimated under *in vitro* conditions.
6.2 Results and Discussion

6.2.1 Whole seeds

Seeds have been the most widespread material used for irradiation. Their obvious advantage is that they can be dried to become biologically inert. This makes it possible to handle them under harsh controlled environmental conditions used for radiation monitoring, without causing significant biological damage.

In the present study, the following experiments were carried out, to determine response of whole seeds to increasing doses of UV radiation (intensity 120 J/m²). Irradiation of the seeds was performed under aseptic conditions. Seeds of cultivars JL-24 and ICGS-11 were surface sterilised as described in Chapter II and divided into two parts.

6.2.1.1 Set I: In vitro control plants

This set consisted of plants obtained by in vitro germination of seeds of the two cultivars JL-24 and ICGS-11. Twenty seeds of each cultivar were inoculated into tubes containing RM3 medium and incubated under standard conditions of light (16 h photoperiod) to achieve germination.

6.2.1.2 Set II: Experimental plants

The second set consisted of plants obtained from surface sterilised seeds of both cultivars which were first irradiated with UV radiation (intensity 120 J/m²) followed by their in vitro germination. In this experiment, surface sterilised seeds were divided into 8 parts (20 seeds/part) and each part was irradiated separately with UV radiation for various time intervals ranging
from 1 h to 24 h. In each case the seeds were placed in petriplates at a distance of 30 cms from the UV source. After irradiation, the seeds were inoculated into tubes containing RM3 medium (1 seed/tube), labelled and incubated first in dark (at 25+2°C) for 5 days and subsequently in light under standard light conditions. Germination of these irradiated seeds was found to be 100%.

Both the sets were initiated simultaneously.

The plants from both sets after attaining a height of 8-9 cms (15 day old seedlings), were transferred to polybags containing a sand : soil (3:1) mixture and hardened following the procedure described in Chapter II.

6.2.1.3 Set III Ex vitro control plants

Twenty seeds of both the cultivars were sown directly in polybags containing a sand : soil (3:1) mixture to obtain plants. These were used as the ex vitro control plants. The seeds in this case were not exposed to radiation.

6.2.1.4 Field study

Plants obtained from all the three sets were transferred to the field 25 days after germination. The two controls viz in vitro and ex vitro germinated plants were grown in separate rows. The experimental plants were also planted in rows with each row corresponding to plants obtained from seeds exposed to a particular radiation dose. The growth behaviour of the experimental plants was compared to the two controls. Observations were taken every week and the results obtained after 60 days in field have been recorded in Table XI.

After transfer to field and exposure to daylight, distinct
TABLE XI

Growth Characteristics of Plants derived from Seeds of cultivars JL-24 and ICGS-11 subjected to UV radiation for various time intervals

Controls: In vitro (C1)
Ex vitro (C2)

Data obtained from plants derived from seeds of both cultivars irradiated for 16-24h

<table>
<thead>
<tr>
<th>No</th>
<th>UV exposure (h)</th>
<th>0 (C1)</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Curling</td>
<td>C2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>JL-24</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ICGS-11</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Variegation</td>
<td>C2</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>JL-24</td>
<td></td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>ICGS-11</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Flowering</td>
<td>C2</td>
<td>45-49</td>
<td>57-61</td>
<td>57-61</td>
<td>55-58</td>
<td>55-58</td>
</tr>
<tr>
<td></td>
<td>after transfer</td>
<td>JL-24</td>
<td>45-50</td>
<td>57-61</td>
<td>57-61</td>
<td>55-58</td>
<td>55-58</td>
</tr>
<tr>
<td></td>
<td>to field (d)</td>
<td>ICGS-11</td>
<td>45-47</td>
<td>45-48</td>
<td>45-48</td>
<td>45-48</td>
<td>45-48</td>
</tr>
</tbody>
</table>

| 4  | Internodal     | C2     | 5-7 | 5-7 | 4-6 | 3.5-4.5| 3-4 | 3-4 |
|    | Distance (cm)  | JL-24  | 5-7 | 5-7 | 4-5 | 3-5   | 3-5 | 3-4 |
|    |                | ICGS-11| 5-7 | 5-7 | 4-5 | 3-5   | 3-5 | 3-4 |

Degree of variegation
++ slight (only apical meristem affected)
+++ apical as well as in lateral branches
++++ extensive in all shoot and leaves

Signs used
- absent
+ present
variations were apparent within 20 days. Of the two genotypes, JL-24 was affected by the radiation to a higher degree as compared to ICGS-11. All the differences observed between the controls and the experimental plants were more pronounced in JL-24. The effect of radiation has been reported to be genotype dependent, with some genotypes being more sensitive to radiation (Manual on Mutation Breeding 1970).

Ten days after transfer to field, curling and variegation was observed in leaves of 95% of the UV exposed JL-24 plants. The curling and variegation was more prominent in the apical regions and leaf apices of the plants (Figs 82, 83). Variegation increased with increasing UV dosage (Table XI). Both the controls showed no changes.

Embryos in mature seeds consist of leaf primordia, axillary buds and apical meristematic cells. Any induced mutation will thus appear in only a sector of the mature plant, because an irradiated meristem will have mutated as well as non-mutated cells. This could explain why the irradiated seeds in the initial experiments showed only localised alterations in morphology but no overall loss of the growth characteristics. The resultant leaves and the meristems appeared variegated and curled (Figs 82, 83).

Appearance of the variegation and curling indicated that the UV radiation caused genotypic changes which were reflected in the phenotype and further the manifestation of these genotypic changes was a light dependent phenomenon.

The variegation did not persist permanently and within the
Fig 82: Variegation and curling of leaves seen in plants obtained from seeds (var JL-24) irradiated with UV light.

Fig 83: Variegation apparent towards the apex and margins of the leaf as yellowish white patches. c = patches
next 30 days the leaves turned green. At the time of flowering the chlorotic patches were scattered and by the time of seed set the patches disappeared giving an uniform green appearance (Fig 84). This was possibly because of the photorepair and excision repair mechanisms operating in the plant. Such mechanisms of repair have been reported in plants. Early reports indicated that visible light could partially reverse the lethal (Bawden and Kleczkowski 1952; Klein 1963; Trosko and Mansour 1969a) and mutational (Ikenaga and Mabuchi 1966; Fuji 1969) effects of UV irradiation. These were followed by demonstrations of a light-dependent increase in the rate of loss of cyclobutane-type pyrimidine dimers from the DNA of UV-irradiated cells of Nicotiana tabacum (Trosko and Mansour 1968), Ginkgo biloba (Trosko and Mansour 1969b) and Daucus carota (Howland 1975; Howland and Hart 1977), of Lathyrus sativus seedlings (Soifer and Tsieminis 1974, 1977a,b) and intact plants of Wolffia microscopica and Spirodela polyrhiza (Degani et al 1980). Such results have been interpreted as evidence for the enzyme-catalysed monomerisation of the dimers. A photoreactivating enzyme has been detected and partially purified from maize pollen and from many types of bean such as lima bean (Phaseolus lunatus) and pinto bean (Phaseolus vulgaris) (Saito and Werbin 1969). The excision repair of pyrimidine dimers from a UV-irradiated plant was first shown in the grass pea, Lathyrus sativus (Soifer and Tsieminis 1974, 1977). Excision repair has also been demonstrated in protoplasts of carrot, Haploappus, Nicotiana and Petunia (Howland and Hart 1977) and whole irradiated duckweed (Degani et al 1980) but was reported to be absent from cultured soybean cells (Reilly and
Fig 84: Disappearance of white patches on the leaf 50 days after transfer to soil.
Besides variegation and curling of the leaves, the experimental plants subjected to UV light for 18 h and over, of both cultivars possessed shorter internodal distances as compared to the two controls (Table XI).

Flowering was initiated earlier in experimental plants exposed to UV for 20 h of var. ICGS-11 as compared to the controls. It occurred later than controls in var. JL-24 (Table XI).

Seed set in all the plants was normal and plants were single threshed to obtain seed.

6.2.2 Leaf explants

From experiments using whole seeds of peanut it was evident that high doses of UV radiation could effect genomic changes which were repaired eventually by inherent repair systems. Secondly, cultivar JL-24 was generally more susceptible to the radiation than ICGS-11. Following the results with whole seeds, in the next experiment leaf explants of cultivar JL-24 were used to study the effect of UV radiation on their regeneration in vitro. Regeneration via organogenesis was attempted using the procedure standardised in Chapter V (as in Flow sheet 1).

6.2.2.1 Preliminary experiments

In order to determine whether radiation effected changes in the medium components could affect regeneration a preliminary experiment was conducted. The leaf explants were obtained as described in Chapter V. These were inoculated in petri-plates containing SM1 medium (MS + 5.36 µM NAA + 4.40 µM BAP; 80
Flow Sheet - 1

Embryonal leaflets

SM1
(5.36 μM NAA + 4.40 μM BAP)

ME2
(2.20 μM BAP)
FLOWERING
ELONGATION
OF SHOOTS

ME3
(13.20 μM BAP)
MULTIPLICATION
OF SHOOT BUDS
REGENERATING
CALLUS

RM3
(MS 1/2 + 2% SUCROSE)

PLANTS
explants/plate) in two sets a) C1 - petri-plates which were not subjected to UV irradiation and b) C2 - plates which were exposed to UV radiation for 12 h prior inoculation. (In both cases the explants were not exposed to radiation). All the petri-plates were first incubated in the dark for 48 hours and then in light (16 h photoperiod, 50 µEm⁻²s⁻¹).

In all C1 petri-plates, shoot buds were induced on 87% of the leaf explants. This induction was associated with mild callusing. However, where the SM1 medium had been exposed to the radiation (C2 plates), extensive callusing occurred in the leaf explants. All the explants gave rise to a watery and non regenerable callus. Shoot bud induction was also greatly suppressed (16%). From the results it was evident that pre irradiation of the medium affected components of the medium thereby inhibiting regeneration.

It has been reported that radiation can produce chemical changes in the medium in addition to the direct changes produced in the irradiated cells. The indirect effects on the growth and differentiation of explants due to changes in media components have been found to increase with increased exposure of the medium to radiations (Ammirato and Steward 1969; Bajaj 1971). Zeevaart and Lee (1968) reported that Haplopappus callus tissue failed to grow on medium exposed to near UV, but these workers did not identify the components in the medium responsible for this inhibition. On the other hand, some enhanced morphogenetic effects were also ascribed to the radiolysis. Embryo formation in Citrus ovular callus was stimulated by gamma irradiation to the cells plus medium or to the medium alone, while irradiation of
the callus alone was ineffective (Spiegel-Roy and Kochba 1973).

It can be concluded from the above results that irradiation of the regeneration medium caused changes in its composition and all further experiments were carried out using SM1 medium which was not irradiated. Thus any variations in the regeneration potential that would be observed would be due to irradiation of the explants alone.

6.2.2.2 Exposure of only the explants to the UV radiation

Since it was evident from the earlier experiment that composition of the regeneration medium was affected by radiation, the following experiments were carried out with unexposed SM1 medium. To achieve this, the leaf explants were first inoculated in petri-plates containing hormone free MS half strength medium supplemented with 2% sucrose and irradiated for various time intervals ranging from 4-48 h with UV light (5 plates with 80 explants/plate were irradiated for each time interval). Immediately after irradiation, the leaf explants were transferred to fresh petri-plates containing SM1 medium and labelled (as per the UV exposure) and then incubated in the dark (25°C) for the following 48 h. Thus, the leaf explants were exposed to radiation but the regeneration medium (SM1) was not. The petri-plates were subsequently incubated under standard light conditions (16 h photoperiod). Weekly observations were taken and after 20 days the percentage regeneration was calculated. The results obtained were compared with the in vitro control set (SM1 medium and leaf explants unexposed) initiated and maintained simultaneously.
TABLE XII

Effect of length of exposure of explants to UV radiation on percentage shoot bud induction

Explant used: embryonal leaflets

Media used: SM1 (MS + 5.36 μM NAA + 4.40 μM BAP)

Incubation conditions: 23 ± 2°C; 16h photoperiod

Observations recorded after 20 days

<table>
<thead>
<tr>
<th>No.</th>
<th>Exposure time (h)</th>
<th>Percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig 85: Bleaching of the leaves exposed to 48 h of UV light.
After 20 days, shoot bud initiation in the irradiated explants was slower (occurring within 18-22 days) as compared to that in the controls (12-15 days) and was more obvious in the leaf explants irradiated for longer intervals (16 to 24 h). There was decrease in the percentage response with increase in the hours of exposure (Table XII). Further callusing also increased with increase in the exposure time. An exposure period of 48 hours proved lethal for the explants and all of them were bleached within 7 days (Fig 85). Low doses (4 h) of the radiation however did not affect the regeneration percentage.

The exact effect of radiation on the regeneration potential in vitro is not yet fully understood. In Anthurium callus cultures the regeneration potential was reported to strongly decrease with increasing radiation dose (Pierik and Steegmans 1976). Similar observations were recorded in maize (Wang et al 1988; Moustafa et al 1989).

6.2.2.3 Effect of pre-irradiation of explants on plant regeneration

From the above experiments it was observed that longer exposures of the leaf explants to UV radiation adversely affected shoot bud induction. To test the effect of pre-irradiation of the explants on plant regeneration, in this set of experiments, the leaf explants were exposed to 24 h (sub lethal dose, as 48 h was lethal) of UV radiation and inoculated in petri-plates containing SM1 medium as described in 6.2.2.2. Shoot buds were induced on 29% of the leaf explants within 22 days. These shoot bud induced leaf explants were then transferred to tubes (one explant/tube) containing ME3 medium (MS + 13.20 uM BAP) to achieve
**Fig 86**: Control (unirradiated) leaf explants in ME3 medium.

Good growth of callus as well as shoot buds (indicated by arrows)

**Fig 87**: Experimental leaf explants (irradiated). Slow growth and formation of brown callus after 21 in ME3 medium.
**Fig 88**: Control (unirradiated) shoot in ME2 medium showing good elongation.

**Fig 89**: Slow growth and elongation of shoots from experimental (irradiated) explants in ME2 medium.
multiplication of the shoot buds. After 25 days the shoot buds were transferred to fresh ME3 medium and this was repeated for subsequent five passages. A control set (non irradiated) was also initiated simultaneously. In this set the explants were inoculated into SM1 medium directly after surface sterilisation.

The multiplication of shoot buds induced on irradiated explants was slower as compared to the controls (Figs 86,87). This slow multiplication continued over 5 subcultures. This was also accompanied by callusing. The shoot buds obtained from the control as well as the irradiated explants after 5 subcultures were transferred to tubes containing ME2 medium (MS + 2.20 μM BAP) to achieve elongation. Even after repeated transfer to fresh ME2 medium only few shoot buds induced on irradiated explants elongated (Figs 88,89). As a result, only 13 shoots were obtained from the irradiated explants.

The shoots were transferred to RM3 medium (MS1/2 + 2% sucrose) to achieve rooting (Fig 90). Shoots obtained from the controls were also inoculated in RM3 simultaneously. All the shoots produced adventitious roots within 15 days of transfer to RM3. These were subsequently removed and planted in polybags containing a sand : soil mixture (3:1) and hardened as described in Chapter II. Of the 13 plants regenerated from irradiated explants, only 6 survived. The hardened plants were transferred to pots (one plant/pot) and maintained in the greenhouse along with the in vitro derived control plants for further growth.

It was evident from these experiments that irradiation of the explants affected not only the induction of shoot buds but
Fig 90: Rooting of the shoots obtained from irradiated explants 12 d after transfer to RM3.
also their multiplication and elongation. Callusing occurred to a greater degree. Rooting however was unaffected, at a frequency of over 90%.

6.2.2.4 Growth behaviour of the regenerants (Ro)

In this field study, plants raised directly from seeds were maintained as controls together with plants raised through tissue culture without irradiation. Plants obtained from irradiated explants were also grown and referred to hereafter as experimental plants. Observations of the growth behaviour of control and experimental plants were recorded at different time intervals (Table XIII).

6.2.2.4 a) Vegetative Growth

Growth of the experimental plants at 20 and 50 days showed very marked differences in height being over 50% less than the controls (Table XIII). A similar observation was seen in the development of lateral branches which were totally absent on experimental plants (Table XIII; Fig 91). In peanut, plant height and branching pattern are each single gene dependent characters with dwarfism being the recessive expression of the gene and branched being dominant to unbranched (Wynne 1975). Both these characters have been shown to be affected by radiation in peanut by Mouli and Patil (1976) and Patil and Mouli (1977).

The inter nodal distance and the leaf sizes in the experimental plants were also smaller as compared to the controls (Table XIII). Glandular hair were very small and the leaves had an overall smooth appearance in the experimental plants unlike in the controls where glandular hair were present on the entire plant surface. The experimental plants also did not exhibit
TABLE XIII

Comparison of Growth Behaviour of Experimental and Control Plants

Data presented corresponds to observations of all six experimental plants compared to that of fifteen control plants of each type

<table>
<thead>
<tr>
<th>No.</th>
<th>Character</th>
<th>Control (seed raised)</th>
<th>Control (in vitro)</th>
<th>Experimental plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Height of plants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20d</td>
<td>12.5 ± 1.32</td>
<td>13.8 ± 0.28</td>
<td>6.5 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>50d</td>
<td>28.5 ± 0.73</td>
<td>27.3 ± 0.89</td>
<td>10.2 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>Lateral Branching</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20d</td>
<td>1-2</td>
<td>1-2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50d</td>
<td>3-4</td>
<td>3-4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Internodal Distance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-6</td>
<td>5-6</td>
<td>5-6</td>
<td>1.8-2</td>
</tr>
<tr>
<td>4</td>
<td>Pubescence on stem</td>
<td>present</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td>5</td>
<td>Appearance of first flower (d)</td>
<td>25-32</td>
<td>27-34</td>
<td>16-18</td>
</tr>
<tr>
<td>6</td>
<td>Continuation of flowering (d)</td>
<td>60-64</td>
<td>60-68</td>
<td>22-23</td>
</tr>
</tbody>
</table>
pubescence on the stem.

The experimental plants thus showed an overall suppressed vegetative growth as compared to the controls.

6.2.2.4 b) Flowering

Flowering in the experimental plants was initiated earlier than in both the controls (Fig 91). Flowers were formed at the lower most node in 18 days of transfer to soil with peg initiation 15 days later. Only one flush of flowering was seen in the experimental plants. No flower buds were induced on any other node except the lower most. About 6-7 flowers were formed at this node. The flowers were pale yellow in color and small. Flowering in the control plants was initiated in 30 days after transfer to soil and was present on the lateral branches as well as on the main stem. It continued for 3 weeks and peg formation was initiated in 15 days. The flowers were bright yellow in color.

Early flowering was observed only in the experimental plants and is possibly one more effect, besides dwarfism, of irradiation of the explant prior to regeneration.

Both the experimental and control plants grew to maturity and set seed. The number of pods obtained from the experimental plants was 1-3 as against 4-6 pods in the controls.

An interesting observation was that all the six experimental plants exhibited an identical growth behaviour.

6.2.2.5 Progeny Testing

Seed collected from the experimental and control plants was used to screen plants of the F1 generation. The seeds were planted directly into pots (1 seed/pot) and maintained under
Fig 91: Absence of lateral branching as well as early flowering in experimental plant (derived from irradiated explants) 18 days after transfer to soil.
green house conditions for germination.

Growth behaviour of the experimental plants was comparable to that of the controls. Seed set in the experimental plants was also normal.

These observations imply that the changes observed in the experimental plants in the Ro generation were not permanent, heritable changes since they were not exhibited in the first generation.

Dwarfism observed in the experimental plants was possibly because of preferential expression of the recessive allele (dwarfism is recessive to tallness in peanut) in response to radiation stress.

6.2.3 Conclusion

UV radiation affected growth behaviour of the plants obtained both from seeds as well as leaf explants. The effect of radiation on whole seeds was genotype dependent. Genomic changes occurred leading to curling and variegation of the leaves when seeds were exposed for 24 h to UV radiation. These changes were however reversible, possibly due to the presence of inherent repair systems.

When leaf explants were irradiated, regeneration potential of the explants was adversely affected. Percentage induction of shoot buds decreased with increase in the UV dose administered. Low induction was observed in explants irradiated for 24 h. Multiplication and elongation of the shoot buds was also adversely affected. Plant regeneration frequency was extremely low. The regenerants showed suppressed growth and early flowering.
The peanut genome was sensitive to radiation. Though UV radiation was effective in inducing genomic changes in peanut and also affected regeneration potential, no permanent and heritable changes were observed in the plants obtained. This could be attributed to the presence of an efficient inherent DNA repair system.

These observations are of great significance for the study of UV mutagenic mechanisms.

Approximately 7% of the total solar output of electromagnetic radiation lies within the ultraviolet region (100-400 nm). However, absorption of the shorter wavelengths by atmospheric ozone results in a considerable attenuation at the earth’s surface such that the incident UV wavelengths are predominantly in the UVA (320-390 nm) and UVB (280-320 nm) regions with virtually no UVC (200-280 nm) present (McLennan 1987). Nevertheless, the highly actinic nature of UV light makes it a biologically significant form of radiation. Studies have shown that plants possess both photoreactivation and excision-repair systems for the repair of UV induced pyrimidine dimers, but little is known about the lethal or mutagenic effects or the repair of other UV induced lesions (which may be major products of the UVA and UVB wavelengths), or of damage tolerance mechanisms. Many of the practical difficulties inherent to plant systems have now been overcome through development of cell culture techniques. The coupling of tissue culture and UV mutagenesis could thus provide the necessary stimulus for further research in this potentially rewarding area.
VII

EFFECT OF LYSINE AND THREONINE STRESS ON THE REGENERATION OF EMBRYONAL LEAFLETS
7.1 Introduction

Cultured cells are known to display extensive genetic variability (Bayliss 1980; De Klerk 1990). Such changes have been designated as somaclonal variation (Larkin and Scowcroft 1981). This random variability present in cultured cells could be exploited in plant breeding and improvement.

A possible application of somaclonal variation would be in the combination of in vitro culture with in vitro selection. In vitro selection using callus, suspension or protoplast cultures is advantageous because this approach makes handling of a large inoculum possible at a given time. Tissue cultures have been used to isolate plants resistant to a variety of compounds including specific drugs, antimetabolites, metabolite analogues, antibiotics or pathotoxins (Maliga 1984; Yeoman 1986).

Besides the recent developments including somatic hybridization and transformation, direct selection for agronomic traits in cell cultures may seem a little primitive. However, the attraction lies in the relative simplicity of this approach. While the techniques are still not routine, and success still constitutes an intellectual challenge, the methodology is accessible to small teams and individuals working in only moderately endowed institutions. Besides the unequivocal demonstration of sexual inheritance of diseases, herbicide and salt resistance, selected in cell cultures, confirms the potential of this approach (Yeoman 1986). Further, this approach is valuable for the investigation of the genetic and physiological basis of developmental sequences in plants and
basic problems of primary and secondary metabolism. Thus variant cell lines and procedures to obtain them have a great deal to offer in a number of pure and applied areas of plant biology.

Among the various approaches used, knowledge of the metabolic pathways and their control may suggest means of selecting lines which can accumulate specific compounds, e.g. production of amino acid overproducing lines by selection for resistance to amino acid analogues/amino acids. The amino acid analogue, as the name suggests are compounds similar in structure to an amino acid and can get incorporated into the polypeptide during translation. These are generally toxic to the plant because they are incorporated into the protein in place of the amino acid or may cause false feedback inhibition leading to starvation for the amino acid. A mutation leading to decreased sensitivity of the enzyme will overcome the feedback inhibition and cause the accumulation of the amino acid beyond the normal level to successfully compete with the analogue. Similarly, selection for resistance to growth inhibition by lysine plus threonine has been proposed as a means of obtaining feedback resistant mutants in the lysine-threonine-methionine-isoleucine biosynthetic pathway (Green and Phillips 1974).

Lysine, threonine, methionine and isoleucine are synthesised from aspartate via a highly branched pathway in which regulatory controls have been identified (Bryan 1980). The major form of regulation of synthesis is a feedback inhibition expressed at the level of aspartate kinase, the first enzyme of the pathway and at the level of dihydrodipicolinate synthase the first enzyme of the lysine pathway (Shewry and Miflin 1977; Gengenbach et al 1978).
Fig 92: A simplified representation of the biosynthetic pathway by which the amino acids methionine, threonine, lysine and isoleucine are synthesised via aspartate. (Taken from Yeoman 1986)
Enzymes probably subject to feedback control:

A = Aspartokinase
B = Homoserine dehydrogenase
C = Threonine deaminase
The threonine synthesis is also regulated at the level of homoserine dehydrogenase, the first enzyme of the branch leading to threonine and methionine (Fig 92).

Selection methods to identify potential mutants characterised by increased production of lysine and/or threonine have been developed for various species such as maize (Hibberd and Green 1982; Miao et al 1988; Diedrick et al 1990), barley, carrot, Arabidopsis (Cattoir-Reynaerts et al 1981) and tobacco (Heimer and Filner 1970; Mathews et al 1986). These are based on the growth inhibition caused by a combination of lysine and threonine present in the culture medium, or by addition of lysine analogue S-2 aminoethylcysteine. The resistance to lysine and threonine in these cases has been attributed to a single dominant gene mutation or alteration in the regulation of aspartate kinase (Hibberd et al 1980; Bright et al 1982; Rognes et al 1983; Cattoir-Reynaerts et al 1983).

This approach will be of great importance if applied to grain legumes (which have been neglected so far) with an aim to help overcome their inherent deficiencies in certain essential sulphur containing amino acids such as methionine (Salunke et al 1985) and specially to peanut which in addition, is also deficient in lysine and threonine (Oke et al 1975).

With the above background, attempts were made to regenerate peanut plants via organogenesis in the presence of lysine and threonine (lys + thr). The induction of shoot buds was carried out in the presence of these amino acids using the protocol described in Chapter V.
7.2 Results and Discussion

Equimolar quantities of lysine and threonine were used to prepare the stock solution (100 mM stock). Aliquots of this were added to SM1 medium to achieve the desired concentration of lys + thr in the medium.

Regeneration protocol used for all the experiments was as described in Chapter V. Composition of the media used in this experiment were as mentioned in Table III (Chapter II). Leaf explants were obtained from mature axes as described earlier and inoculated in SM1 medium containing various concentrations of lys + thr (mentioned below). All cultures were incubated in light under standard conditions (Chapter II).

7.2.1 Effect of lysine + threonine concentration on regeneration in vitro - Optimisation of dose for positive selection in culture

This experiment was carried out to study the growth inhibitory effect of lys + thr on embryonal leaf explants in vitro and optimise the concentration of the two amino acids to be used for selection of overproducing clones.

The leaf explants were inoculated into petri-plates (80 explants/plate) containing SM1 medium supplemented with various concentrations of lys + thr in the range of 0.1 to 3 mM (Table XIV). Four petri-plates were maintained for each chosen concentration of the amino acids. The cultures were incubated at 25±2°C and a 16 h photoperiod. A control set with petri-plates consisting of leaf explants inoculated in SM1 without lys + thr was initiated simultaneously. The observations recorded after 15 days are presented in Table XIV.
Table XIV

Effect on regeneration of leaf explants in presence of lysine and threonine

Explants: embryonal leaflets

Medium: SM1 (MS + 5.36 μM NAA + 4.40 μM BAP)

Supplements: Lysine and threonine in varying concentrations (0.1mM - 3mM)

Incubation conditions: 16h photoperiod (50 μE m⁻² s⁻¹), 23 ± 2°C

<table>
<thead>
<tr>
<th>No</th>
<th>Conc of lys+thr mM</th>
<th>Greening of leaves</th>
<th>Bleaching of leaves</th>
<th>Callusing</th>
<th>% Shoot bud Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>53</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>2.0</td>
<td>–</td>
<td>+</td>
<td>++</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>2.5</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3.0</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations used:
- absent
+ present
Degree of callusing:
++ moderate
++++ extensive
In control petri-plates (containing lys + thr free SM1), leaflets turned green within 5-7 days and shoot buds were induced on 87% of the explants. This was associated with mild callusing (Fig 93).

In all petri-plates supplemented with lys + thr upto 0.4 mM, the leaflets showed greening, mild callusing and a shoot bud induction frequency of 85-87% (Table XIV). No growth inhibition of the leaf explants was observed in the presence of 0.4 mM of lys + thr as the regeneration frequency also was comparable to the controls.

Leaflets exposed to lys + thr concentrations in the range of 0.4 - 1.8 mM also turned green within 6-8 days. Callusing however increased with increase in the lys + thr content in the medium. Further shoot bud induction frequency was severely affected at higher concentrations of lys + thr, dropping to a low 8% and 3% in presence of 1.4 mM and 1.8 mM (Table XIV). Growth and regeneration of the leaf explants was inhibited in the presence of 1.4 and 1.8 mM of lys + thr.

When the lys + thr content of the medium was greater than 1.8 mM (i.e. between 2.0-3.0 mM), the leaflets were completely bleached within 10 days and shoot bud induction was severely inhibited (< 3%) (Table XIV). There was however formation of mucilagenous callus on 80 % of the leaf explants (Fig 94). This callus turned brown gradually in the next 20 days and growth ceased. Bleaching (loss of normal pigmentation) of the leaves was an indication of growth inhibition.

This severe growth inhibition due to lysine and threonine
Fig 93: Induction of shoot buds within 15d in SM1 medium on leaflets not exposed to lys + thr stress.

Fig 94: Bleaching and callusing in leaflets on SM1 medium with high concentrations (2.5 mM) of lys + thr.
can be attributed to a methionine deficiency arising from the feedback inhibition of aspartate kinase by lysine and threonine. This, in turn, would lead to inhibition of protein synthesis because by inhibiting an enzymatic step required for methionine synthesis, the level of this amino acid would become too low to fulfill the requirement for further protein synthesis, causing growth inhibition and eventually death of the tissue. This has been shown to occur in wheat (Bright et al 1978), corn (Green and Phillips 1974; Bryan 1980; Green and Donovan 1980), barley and Arabidopsis (Cattoir-Reynaerts et al 1981) and sorghum (Piryns et al 1988). Proof that methionine starvation occurs has been forwarded by many workers (Dunham and Bryan 1971; Green and Donovan 1980; Piryns et al 1988) who showed that reversal of growth inhibition caused by lysine and threonine occurs on addition of methionine or its precursor homoserine.

Secondly the feedback inhibition of aspartate kinase would also result in unusually high amounts of aspartic acid—a precursor of other biosynthetic pathways such as the pyrimidines. Thus presence of stress of the amino acids in the medium could also result in alteration in the phenotypes of the plant. This is because where a particular amino acid acts as a biosynthetic precursor of some other metabolite, an abundance of the amino acid will cause an enhanced production of that metabolite. A typical example of this type of alteration was the 5-methyltryptophan resistant line in tobacco (Wildholm 1972a,b; Sung et al 1979). These cells grew without the addition of auxin apparently because of the elevated levels of IAA.

In the present study, it can be concluded that 2.0 mM
concentration of lysine and threonine was lethal to the explants. For all further experiments, leaf explants were inoculated in SM1 medium supplemented with 2.0 mM lys + thr.

7.2.2 Experiment II Selection of putative resistant explants in vitro

From the previous experiment it was evident that 2 mM lys + thr inhibited growth totally. This concentration of the amino acids was incorporated into SM1 medium in this experiment in an attempt to recover lys + thr resistant variants.

The leaf explants were inoculated in petri-plates containing SM1 medium supplemented with 2 mM each of lys + thr. Forty plates each containing 80 explants were initiated and incubated under light conditions described earlier (Chapter II). Observations were taken after every 10 days.

After 10 days mucilagenous callus was induced on 80% of leaf explants, but no shoot bud induction was observed. In the next 10 days the callus turned brown and necrotic. Low frequency shoot bud induction (5-7 shoot buds/leaflet) was observed in 3% of the inoculated explants. Growth of these buds was very slow. The responsive leaf explants represented putative variants. The next step was thus to multiply the shoot buds induced and obtain shoots before confirming their resistance to lysine and threonine stress.

For multiplication, the leaf explants with shoot buds were transferred to ME3 medium supplemented with 2 mM lys + thr. After 20 days multiplication was very slow. However callusing persisted in the form of a brown, slow growing callus. To improve
the multiplication rate, these explants were transferred to fresh ME3 medium supplemented with 1 mM of lys + thr instead of the usual 2 mM. After 20 days it was observed that multiplication as well as callus growth improved (Fig 95). Shoot buds which were situated farthest from the medium proliferated the most. The shoot buds were multiplied for a further two subcultures after which they were transferred to ME2 medium supplemented with 1 mM lys + thr for elongation. A total of 16 shoots (2-4 cm in length) were obtained at the end of 30 days. These shoots represented putative variant shoots. The resistance to lys + thr stress exhibited by these could be attributed to a) mutation - alteration of the biosynthetic pathway regulation b) some mechanism by which entry of these amino acids into tissues was prevented thereby effecting growth c) position of the shoot bud proliferating centers with respect to media surface.

To confirm the resistance of the shoots to the presence of lys + thr, the shoots were equally distributed among test tubes (1 shoot/tube) containing either ME2 medium which was lys + thr free or supplemented with 2 mM lys + thr. The length of the shoots transferred was 3-4 cm.

Shoots transferred to lys + thr free ME2 medium survived and attained a height of 5-6 cm in 20 days. On the other hand, shoots failed to survive in medium containing 2 mM of lys + thr and died within 22 days of their transfer. Next, the elongated shoots from lys + thr free ME2 medium were exposed to 2 mM lys + thr. These shoots also did not survive.

The results obtained clearly indicated that the putative variants were not true variants as in both cases the shoots died.
Fig 95: Poor rate of multiplication of shoot buds accompanied by brown callus in ME3 medium supplemented with 2.0 mM lys + thr.
Thus no mutation rendering resistance was evident. A query that followed was how did the shoot buds multiply and elongate in the presence of \textit{lys} + \textit{thr} stress? A possible explanation for this would be that because the proliferation occurred in regions farthest to the medium surface, the underlying callus and other shoot buds acted as filters thus exposing the proliferating masses to tolerable levels of \textit{lys} + \textit{thr}.

\textbf{7.2.3 Conclusion}

The results of these experiments suggest that a number of metabolic changes, including probable decrease in protein synthesis could accompany growth inhibition. No true mutants could be isolated by subjecting the leaf explants to amino acid stress. The probability of isolating variants could possibly be increased by use of peanut callus cultures or by coupling the selection process with mutagenesis. On the other hand, selecting characters such as resistance to amino acid stress means tampering a single component of a multicomponent and highly interactive system. This would make it difficult to anticipate the consequences for other aspects of metabolism.

However, the present studies could provide some insight to the mechanisms whereby specific exogenous amino acids influence the overall metabolism of a cell, could contribute to a better understanding of amino acid synthesis and regulation in this plant and may represent a first step towards manipulation of protein quality. These studies also demonstrate the potential power of \textit{in vitro} selection in plants, especially when based on a sound biochemical principle.
The conventional methods of crop improvement include introduction of new cultivars via hybridisation. This process is slow due to the fact that peanut is a self pollinated crop. The new in vitro approaches for crop improvement involving genetic transformation, somaclonal variation and other methods offer several opportunities. To fully utilise these approaches however, efficient and effective protocols to facilitate whole plant regeneration are a prerequisite. Reports on regeneration of peanut plants via tissue culture are however very few, suggesting the need for development of a reproducible technique.

With the above objective in mind, regeneration protocols were worked out for embryogenesis and organogenesis using embryo explants. Embryogenesis could be induced in immature embryos as well as mature embryo axes. Embryonal leaves (leaves present in the mature embryo axes) proved to be very responsive in culture and both embryogenesis and organogenesis could be induced using these explants.

A high frequency (100% response; 8-13 se/explant) direct embryogenesis was obtained from superficial cell layers of immature embryo axis (3-4 mm in size) in the presence of 13.75 um 2,4-D. The induction process was influenced by the type of auxin, concentration of auxin, size of embryo axis and genotype. Somatic embryos were induced and matured on the same medium (MS basal medium supplemented with 3% sucrose and 13.75 um 2,4-D). Plants could be obtained in 45-50 days. The obtained plants were fertile and uniform in morphological characters and seed storage content among themselves and with the seed derived controls over two
generations. The regeneration protocol worked out represents the first report on plant regeneration via somatic embryogenesis in peanut. However, embryogenesis was non repetitive and only low frequency secondary embryogenesis could be induced.

An interesting observation in this study was the germination of the explant itself into a complete fertile plant on transfer to hormone free medium. Under the influence of 2,4-D, growth of the axes was restricted, but it gave rise to somatic embryos. However, the axes germinated in auxin free medium. This clearly indicated that the auxin affected the explant at two different levels, 1) the growth of the apical meristem was merely suppressed and 2) somatic embryos were induced from some of the cells at the root shoot junction around the apical meristem. This observation could mean that either the cells giving rise to the somatic embryos were predetermined (thus supporting the existence of pre-determined embryogenic cells (PEDCs) as suggested by Sharp, 1980) or were exclusively triggered to induce somatic embryos. The intriguing aspect of this observation is the differential action of the auxin on the two cell types.

In preliminary studies it became evident that mature embryo axes also possessed morphogenic potential. This potential could be enhanced several fold by using embryonal leaves from these axes. Direct somatic embryogenesis was induced from the embryonal leaves in the presence of relatively high 2,4-D (90.50 μM) containing media. Here also, only 2,4-D, among the two auxins tested evoked an embryogenic response in the leaves. Sucrose and auxin content of the medium influenced the process. Somatic embryos originated from epidermal cells which became meristematic.
in the presence of 2,4-D. These cells could represent the induced embryogenic determined cells described by Sharp (1981). Repetitive as well as secondary embryogenesis could be obtained by exposing the explant to high (90.50 μM) and low (13.75 μM) 2,4-D alternately. The entire process of somatic embryogenesis was a two step process, with induction in presence of 90.50 μM 2,4-D and maturation in 13.75 μM 2,4-D containing medium. As compared with the earlier protocol using immature embryo axis, the conversion rate was low. The plants obtained were fertile.

High frequency direct caulogenesis was induced from the same explant (embryonal leaf explants) using NAA in combination with BAP. The induced shoot buds multiplied in presence of high BAP (13.20 μM) and elongated in low BAP (2.20 μM) containing medium. The shoots rooted in hormone free medium and plants so obtained were screened under green house conditions. Development deviated from normal in 6% of the total plants screened in the Ro generation but no differences were observed in the F1 generation. The changes observed in the Ro generation were possibly epigenetic in nature. High BAP (13.20 μM) also induced the formation of regenerating callus which subsequently gave rise to shoot buds (indirect caulogenesis). Low BAP containing medium (2.20 μM BAP) also supported occasional formation of floral meristems (floral morphogenesis). The flower buds matured in the same medium but opening of the same could be achieved only by substituting BAP with equimolar concentrations of another cytokinin Kn.
From the above regeneration studies it was apparent that the embryo, mature or immature, possessed high morphogenic potential and this potential increased with dissection of the embryo explants. Moreover 2,4-D almost exclusively induced embryogenesis whereas NAA favored organogenesis. Morphogenesis in embryonal leaves could be diverted from embryogenesis to organogenesis by change in the auxin type and concentration. The explants also gave rise to floral meristems and regenerating callus under the influence of cytokinin BAP. This reflects the high degree of plasticity of these explants and could be used for protoplast isolation and subsequent regeneration as well as for genetic transformation using the leaf disc approach.

Following the working out of regeneration protocols, the effect of UV light and lysine and threonine stress on the regeneration of leaf explants via organogenesis was studied.

Whole seeds were used initially to estimate the effectiveness of UV. The radiation caused variegation of the leaves of plants derived from these treated seeds indicating genomic changes taking place. These changes were possibly eliminated by photorepair as manifested by the disappearance of the variegation within 35-40 days. When leaf explants were used, induction of shoot buds in leaf explants gradually decreased with increase in the time of exposure of the explants to the radiation. Multiplication of the shoot buds was also inhibited and the cultures exhibited increased tendency towards dedifferentiation. The regenerants showed dwarfism, lack of lateral branching and early flowering. These changes were however
reversed in the F1 generation indicating the absence of stable changes. The above experiments proved that peanut genome was susceptible to radiation and though no mutants could be isolated it should be possible to study the effect of UV light at the cellular level using this approach. On the other hand use of more penetrating radiations such as gamma rays or X rays may be more useful in the isolation of variants.

In another experiment, embryonal leaves were exposed to a lysine threonine stress directly. It was observed that in this case also, regeneration was severely affected and the cultures exhibited a high degree of growth inhibition. These studies suggest that a number of metabolic changes take place on subjecting the explants to the amino acid stress. Further selecting characters such as resistance to amino acid stress means tampering a single component of a multicomponent and highly interactive system. This would make it difficult to anticipate the consequences for other aspects of metabolism.

**Salient features of the regeneration protocols devised**

a) Simple recipe of the media.

b) Regeneration obtained under standard culture conditions.

c) All the three processes for plant production are complete.

Somatic embryos have been shown to contain embryogenic and storage proteins, a characteristic that they only share with zygotic embryos (Crouch 1982). The somatic embryo complete and similar to the zygotic embryo has been shown to accumulate storage products (similar to the zygotic embryo) in the course of its development.
Two plant products that have been synthesised by somatic embryos *in vitro* are anthocyanins in *Theobroma cacao* (Pence *et al* 1981) and storage lipids in *Theobroma cacao* (Pence *et al* 1981) and *Simmondsia chinensis* (Wang and Janick 1984). The lipids synthesised had similar fatty acid and triglyceride composition to those maturing *in vitro*. Plant regeneration via embryogenesis could thus be used to follow storage lipid biosynthesis in the developing somatic embryos.

Somatic embryos could also be used in the production of artificial seeds. Artificial seeds offer the possibility of a low cost, high volume propagation system that will compete with true seeds and transplants. The potential uses of artificial seeds are two fold: as delivery system and as an analytical tool (Redenbaugh 1987). The potential uses of artificial seed as an analytical tool are for the production of large numbers of identical embryos, study of seed coat formation and study of endosperm function. Artificial seeds in peanut could be used for such analytical purposes.

Gene transfer methods allow for the introduction of genetic material not normally accessible by conventional means, in contrast to traditional breeding methods which are limited by sexual incompatibility. Recombinant DNA technologies have tremendous potential in crop improvement applications. However, before biotechnology methods can be utilised, a reliable regeneration system adaptable to transformation methods is needed.
Peanut improvement has been limited from integrating resistance to many diseases and pests from wild peanut (Stalker and Moss 1987) because of problems with sterility barriers and genomic incompatibilities associated with traditional breeding. At present, transformation method cannot be successfully applied to peanut improvement in part because of low frequency regeneration rates.

Somatic embryogenic systems exhibit some distinct advantages when used for transformation. High number of regenerates can be obtained originating from few or single cells which increases the likelihood of achieving transformed plants. Chimeric transformants cycled in repetitive systems can lead to obtaining wholly transformed plants. The present study describes successful development of peanut regeneration via embryogenesis as well as organogenesis from leaf explants and subsequent plant regeneration from S8-11 and JL-24 two important commercial cultivars. The ability of most genotypes of peanut screened to produce somatic embryos at a reasonable frequency and magnitude and to regenerate plants using a single protocol is encouraging for transformation of elite genotypes. Repetitive embryogenic cultures have been especially useful for the transformation of cotton (Finer and McMullen 1990), soybean (McMullen and Finer 1991) and citrus (Hidaka et al 1990) and could similarly be useful in peanut.

The single cell origin of somatic embryos in tissue culture makes it eminently suited for mutant selection and other genetic manipulations of the plant. Selection of favorable mutants at the
cellular level is one of the most important possibilities offered by tissue culture (Scowcroft 1977; Thomas et al 1979). On the other hand the possibility of genetic variability and/or somaclonal variation has also been emphasised in the clones derived in culture (Larkin and Scowcroft 1981). Variation in the regenerants obtained via organogenesis in the present study would be useful to select important and improved peanut cell lines. So far, peanut crop improvement has been achieved mainly by the use of conventional breeding methods. In the recent past, however, the importance of the use of plant tissue culture and biotechnology in establishing high yielding and better adapted cultivars has been realised. The use of efficient in vitro regeneration coupled with various biotechnological approaches such as gene transfer, mutant/variant selection and storage lipid manipulation would certainly prove beneficial for peanut crop improvement.


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138


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150


PUBLICATIONS
We have regenerated plants of *Arachis hypogea* cultivar SB-11 from immature zygotic embryo axis via direct somatic embryogenesis without an intermediate callus stage. Induction and maturation of the somatic embryos was achieved on the same medium, 2,4-dichlorophenoxy acetic acid (2,4-D) was essential for this response. Both the size of the immature zygotic embryo axis and the concentration of 2,4-D were important for direct somatic embryogenesis. Embryos germinated in hormone free medium, and plants survived in a sand:soil mixture.

The successful exploitation of *in vitro* techniques in plant biotechnology depends on the establishment of efficient regeneration systems. Among the modes of plant development in cultured plant tissues, organogenesis has been observed in many plant species and offers rapid shoot proliferation. This method has proven valuable in the propagation of various forest trees and bulbous monocots and dicots. In embryogenesis, somatic embryos, like naturally occurring zygotic embryos, develop as bipolar structures bearing both a root and shoot apex. Thus, both meristems necessary for complete plant development are initiated simultaneously. Since the first observation that somatic embryos could be grown directly from somatic cells, repeated attempts have been made to regenerate plants via this pathway, and somatic embryogenesis has now been observed in many plant taxa.

Somatic embryos can develop either from callus or directly from organs without involving any intermediate callus stage. Since regeneration of plants from callus cultures often show genetic variability, direct somatic embryogenesis from organs can be more efficiently used for application of biotechnology in the improvement of crops. Direct somatic embryogenesis has now been achieved in several legumes.

There are several reports on plant regeneration via organogenesis in *Arachis hypogea*. Regeneration of plants from callus or tissues via somatic embryogenesis has, to our knowledge, not been reported. Here we describe a method for regeneration of plants of *A. hypogea* via direct somatic embryogenesis.

**RESULTS AND DISCUSSION**

Immature zygotic embryo axes of various sizes (from 1 to 6 mm) were used as primary explants. Growth and development of these embryo axes were restricted in the presence of auxin irrespective of the size of embryo axis or concentration of the auxin. This observation supports earlier findings. Maheswaran and Williams in studies with *Trifolium repens* proposed that growth suppression of the main embryo axis is associated with the breakdown in the integrity of the cells as a single embryogenic group, and escape of individual or smaller groups of cells to function autonomously. In *A. hypogea* suppression of growth in the immature zygotic embryo axis is followed by appearance of white protrusions. These white protrusions subsequently develop into embryos.

Plant regeneration via organogenesis from callus, suspension, or protoplast cultures is often associated with genetic and cytological variation. It is presumed that this applies to regeneration by induced somatic embryogenesis from the same type of cultures. On the other hand, embryogenic cell lines maintain their competence for long periods of time and give rise to genetically uniform and normal plant populations. Since the embryonic cells are accepted as predetermined embryogenic cells, direct somatic embryogenesis from immature zygotic embryos offers the possibility for avoiding passage through callus and obtaining a clone of genetically uniform plantlets.

Direct somatic embryogenesis in *A. hypogea* was observed on the axis of immature zygotic embryos of sizes ranging from 3–6 mm in length. This result indicates that the size of the zygotic embryo axis (3–6 mm) represents a particularly responsive developmental stage. Specificity of explant developmental stage for induction of somatic embryogenesis has also been reported in other systems, such as *Sorghum bicolor*, *Panicum maximum*, orchard grass, and strawberry. Development of the embryo was visible in media with 1, 3 and 6 mg/l 2,4-D (Fig. 1). Substitution of 2,4-D with similar concentrations of naphthalene acetic acid (NAA) alone failed to elicit embryogenesis from the embryo axis. At low levels of 1 and 3 mg/l, swelling and callusing of sexual embryos was observed whereas at 6 mg/l only swelling was noted. When the NAA containing media was supplemented with 2,4-D (1 mg/l each), initiation of embryogenesis with callusing occurred. This result demonstrates that the...
TABLE 1 Response of immature embryos of A. hypogea to various concentrations of 2,4-D and NAA.

<table>
<thead>
<tr>
<th>Auxin</th>
<th>Conc. mg/l</th>
<th>Explants forming embryos (N=100)</th>
<th>Response</th>
<th>Days for response</th>
<th>Embryos per zygotic embryo axis (20 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Embryogenesis</td>
<td>Callus swelling</td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>1</td>
<td>100</td>
<td>+</td>
<td>−</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100</td>
<td>+</td>
<td>−</td>
<td>5</td>
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<tr>
<td></td>
<td>6</td>
<td>70</td>
<td>+</td>
<td>−</td>
<td>10</td>
</tr>
<tr>
<td>NAA</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td></td>
<td>3</td>
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<td></td>
<td>6</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>2,4-D + NAA</td>
<td>1+1</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

FIGURE 2 A. Longitudinal section of somatic embryos developing in a row on the immature zygotic embryo axis after 19 days. Immature zygotic embryo (ze), torpedo (t) cotyledonary (c) stages. Bar represents 220 μm. B. Longitudinal section of a somatic embryo at cotyledonary stage Cotyledons (c) radicle and (r) and meristem (m). Bar represents 100 μm. Sections were stained with hematoxylin and eosin.

FIGURE 3 A group of embryos exhibiting fasciations and distortion. Developing embryo (e) with suspensor (s) fertilized embryo (f). Mag. ×200.

FIGURE 4 Germination of somatic embryo with defined cotyledons (c) and root (r) in medium containing 0.25% charcoal.

FIGURE 5 Plantlet with well established shoot and root system, developed from somatic embryo.

presence of 2,4-D is essential for induction of embryogenesis. In the presence of 2,4-D alone (1 and 3 mg/l) or combined with NAA (1 mg/l each) small, white globular, protrusions appeared from the embryonic axis after 5 days. To our surprise, this response was delayed by increasing the concentration of 2,4-D, becoming 10 days at 6 mg/l 2,4-D as against 5 days at 1 or 3 mg/l (Table 1). Further development gave rise to stages similar to zygotic embryogenesis (Figs. 2A, B). Various stages of embryogenesis could be seen in the same clump of embryos as the development was asynchronous. The close proximity of the embryo initiation sites produced frequent fasciations and distortions of cotyledonary lobes (Fig. 3). Embryos were normally attached to the parent tissue at the root pole and could be detached easily. All immature zygotic embryo axes inoculated produced somatic embryos in presence of 2,4-D (1 and 3 mg/l) alone or combined with NAA (Table 1). This frequency was reduced when the 2,4-D concentration was increased to 6 ppm. The role of 2,4-D in the induction of somatic embryogenesis is well.
documented. In the present investigation, the number of embryos produced from each zygotic embryo axis varied with the concentration of 2.4-D (Table 1). 2.4-D at 3 mg/l was found to be optimum, with 8 to 15 embryos developing from each zygotic embryo axis.

There are several reports in which initiation and maturation of the embryos occurs in two or more steps involving various media compositions. In A. hypogea, the complete process of development from the initial globular stage to the fully mature embryo occurs in the presence of 2.4-D within 20 days. This method thus provides a one step process for embryogenesis as in mango and carrot. Fifty percent of the mature embryos transferred to germination medium developed shoots and roots and formed a plantlet within 7 days (Figs. 4, 5). When the embryos were subcultured or allowed to remain in the 2.4-D containing media, the appearance of a second generation of embryos was noted from the embryo axis. Plants from the second crop of embryos have not yet been obtained.

Embryos that germinated were transferred to pots containing a sand-soil (3:1) mixture after they had attained a height of 5 cm.

Direct somatic embryogenesis provides a model system for studying the basic developmental morphology of non-zygotic embryos and the influence of hormones and other factors on embryo development. In addition, this method could be applied for generating in vitro variability in peanuts and for obtaining plants after Agrobacterium-mediated gene transfer.

EXPERIMENTAL PROTOCOL

Expant source. Immature pods of A. hypogea (cultivar SB-11) were collected from the field. These were surface sterilized by a rinsing for 1 minute with 70% ethanol followed by a 0.1% HgCl2 treatment for 10 minutes. The pods were washed thoroughly with sterile water. Pods were opened aseptically and the immature embryo axes, ranging from 1-6 mm in length, were excised.

Culture conditions and experimental media. Two media were used. The induction medium, in which the excised embryo axes were inoculated, contained Murashige and Skoog (MS) salts, MS vitamins, 2.5% sucrose and 2.4-D or NAA. Media were solidified with 0.4% agar. The auxins tested were used in various combinations singly and in combination (Table 1). Clumps of embryos at various stages of development were transferred to germination medium containing 1% half-strength, 2% sucrose and 0.25% activated charcoal. All cultures were incubated at 25 ± 2°C under white fluorescent light intensity of 50 mE m⁻² s⁻¹ with a 16 h photoperiod.

Induction and Maturation. Embryos matured on the same medium used for induction. The frequency response was calculated from the number of zygotic embryo axes inoculated relative to the number of embryo axes that developed embryos. Somatic embryos were counted under a stereomicroscope. After 20 days in culture, clumps of embryos at various stages of development were transferred to germination media.

Germination and plant formation. Embryos on germination medium grew to a height of 5 cm within 3 weeks. At this stage the plants were transferred to pots, containing a sand-soil mixture (3:1) for further growth.

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Direct Somatic Embryogenesis

Somatic embryos, unlike zygotic embryos, develop from callus or directly from plant organs. However, like zygotic embryos, the somatic embryos develop bipolar structures bearing a root and shoot apex containing both meristems necessary for complete plant development. Since regeneration of plants from callus cultures often exhibit genetic variability, Hazra, Sathaye, and Mascarenhas (32) regenerated plants of peanut (Arachis hypogea) via direct somatic embryogenesis.

Immature pods of cv. SB-11 were collected from the field, surface sterilized, opened aseptically, the immature embryo axes (1-6 mm in length) were excised, and placed on induction MS medium containing salts, vitamins, 2.5% sucrose, and 2,4-D or NAA. The medium was solidified with 0.4% agar. About one week later, clumps of embryos at various stages of development were transferred to a germination medium containing half-strength MS salts, 2% sucrose, and 0.25% activated charcoal. All cultures were grown under white fluorescent lights with a 16 hr photoperiod. After 3 weeks, the plants were 5 cm high and were transferred to pots for further growth.

Embryos developed from each zygotic embryo axis. This method provided a one step process for embryogenesis and may be a useful model system for studying developmental aspects of, and the influence of various compounds on, non-zygotic embryos.

Comments of A.F. Mascarenhas: Peanut is a protein rich oil seed crop cultivated universally. A major problem affecting this crop is its susceptibility to several diseases and insect pests, for which efforts are being made by conventional and the more recent methods to evolve resistant lines. One of the requirements for application of recombinant DNA technique is the development of an efficient regeneration system through tissue culture. Our paper describes a procedure for regenerating plants in vitro via direct somatic embryogenesis. This opens up the area for future application to obtain resistance in groundnut. Direct somatic embryogenesis from predetermined embryonic tissue without involving a callus phase gives rise to clones with greater genetic and cytological uniformity.

The complete process of development of somatic embryos from the initial globular stage to the fully mature embryo occurs in one step within 20 days thereby providing a model system for studies on the basic developmentnal morphology of non-zygotic embryos. The various stages of somatic embryo development in peanut are depicted in a subsequent paper.

Plants regenerated by somatic embryogenesis from immature zygotic embryos have grown to maturity in the greenhouse. First generation seeds will be tested to determine the uniformity/variability of the regenerants. The main thrust of our future research will be directed towards utilisation of our procedure for isolation of disease and insect resistant lines using standard recombinant DNA methods.

Endosperm Protoplasts

Hevea brasiliensis is also known as Para Rubber or Caoutchouc Tree and is planted as an ornamental or used as an important source of Para rubber in Brazil. Little is known about the subcellular organization of cyanogenesis in this species. Recently, Selmar, Frehner, and Conn (33) have reported on the development of a method for obtaining protoplasts from the highly cyanogenic endosperm tissue of this plant in order to establish the location of l-nammarase activity at the cellular level. Cyanide inhibits the cytochrome molecule in the electron transport system and is poisonous to most plants.