SOMATIC EMBRYOGENESIS AND GENETIC TRANSFORMATION IN PEANUT

A THESIS

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IN **BIOTECHNOLOGY**

BY

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled, **"Somatic Embryogenesis and Genetic Transformation in Peanut",** being submitted to the **University of Pune** for the award of **Doctor of Philosophy in Biotechnology** by Ms. Shweta Singh, was carried out by her under my supervision in the Plant Tissue Culture Division, National Chemical Laboratory, Pune 411008, India. Materials obtained from other sources have been duly acknowledged in the thesis.

Date: **Dr. Sulekha Hazra**

Place: (Research Guide)

DECLARATION

I hereby declare that the work for the thesis entitled **"Somatic Embryogenesis and Genetic Transformation in Peanut"** has been carried out by me in the Plant Tissue Culture Division, National Chemical Laboratory, Pune under the supervision of Dr. Sulekha Hazra. The work is original and has not been submitted in part or full by me for any degree or diploma to any other University. I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

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Dedicated
To
My Beloved Family

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Acknowledgement

This dream of mine, which at this moment realizes to its full, was once a budding desire that lured me into the thrills of exploring the unknown! It has been a wonderful expedition since then, trailing a spectrum of circumstances, people, and stages of life. Now, reaching the acme of this dream, I gaze back and wonder at all who have helped me to the summit!

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To complete any mission successfully in life, it is not just the roadmap that helps, but also the company of good friends which soars you to the heights greater than those reached alone. I have always been blessed with the best of good hearts, to relieve me from the most tiresome moments of life. I am indebted to all my friends throughout my lifetime for their care and kindness.

While your well-wishers smile when you smile and friends cry when you cry, only your family can 'feel' what you feel! When I think of my family, a special place in my heart is always reserved for my dear father, who shaped my whole career and personality, and made me proud of what I am. At the same time, I find no words to pen down my mother's love and affection, who also gifted me with an additional love, my dear Bhai. I am grateful to all my family members, in-laws, and relatives for their patience and

encouragement. I owe many cherished moments to 'bitiya' who soothed away all my worries with her bright smile and sweet words.

Above all these people, behind all my efforts and aspirations, there was one force that enriched my heart with the hope for tomorrow; that often assured me of the invincible power of care, love, and affection; that never deserted me in desperate times and never ceased to enthuse me when life touched its nadir. It is my beloved husband, who is indeed, the great force behind my perseverance; without whom, nothing would have been possible and nothing is going to be possible in future too!

Shweta Singh

Key to abbreviations

°C	Degree Celsius
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)
ANOVA	Analysis of variance
sd	Standard deviation
MS	Murashige and Skoog medium (1962)
PGR	Plant Growth Regulator
KN	Kinetin (6-furfuryl amino purine)
BA	6-Benzyl amino purine
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
PVP	Polyvinyl pyrrolidone
2,4-D	2,4-dichlorophenoxy acetic acid
Picloram	4-amino-3,5,6-trichloro picolinic acid
IBA	Indole butyric acid
ABA	Abscisic acid
GA_3	Gibberellic acid
SE/ SEs	Somatic Embryo/ Somatic Embryos
SEsis	Somatic Embryogenesis
PM	Primary Medium
SM	Secondary Medium
Taxim	Cefotaxime (antibiotic)
TBA	Tertiary butyl alcohol (2-methyl propan-2-ol)
DPX-4 mountant	189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)
UV	Ultra violet (light)
ISSR	Inter simple sequence repeats
d	Days
MZEDL	Mature zygotic embryo derived leaflet

Abstract

Arachis hypogaea L. (peanut, groundnut), world's most important oilseed crop is a legume, native to South America. This crop grows in a wide range of agro-climatic conditions. Peanut seeds constitute an excellent source of edible oil, protein and other nutrients. The productivity and nutritional value of this crop can be improved by developing cultivars resistant to biotic/abiotic stresses and having better protein and oil quality/quantity. Biotechnology offers the option to supplement the ongoing efforts on developing genetically modified germplasm for desirable characteristics by transformation techniques. Reliable *in vitro* regeneration protocols and suitable transformation strategy are prerequisites for the genetic transformation approach.

Peanut has been studied extensively for regeneration *in vitro* and for genetic transformation. However, none of the existing regeneration protocols is efficient, as the conversion of somatic embryos to plantlet involves several manipulations. Failure of the peanut somatic embryos to convert into plantlets is attributed to developmental abnormalities of the plumule. Keeping in view the limitations of the existing protocols, present program entitled **"Somatic Embryogenesis And Genetic Transformation In Peanut"** was undertaken with the following objectives:

1) Morphological studies to obtain normal fertile peanut plants at high frequency via somatic embryogenesis.
2) Optimization of the protocol for transformation using *Agrobacterium rhizogenes* for genetic improvement.

The data generated from this study is compiled in the five chapter of this thesis. The work is summarized in the last chapter. List of references includes the literature referred.

CHAPTER 1: General Introduction

This chapter covers the existing literature on the importance of peanut as an oil seed crop and on somatic embryogenesis and transformation studies. The objectives and significance of the study are discussed in this chapter.

CHAPTER 2: Materials and Methods

General materials and methods for tissue culture, transformation and molecular studies implemented during the course of the work are described in this chapter. Specific protocols used in individual experiments are incorporated in the respective chapters.

CHAPTER 3: Influence of 2,4-D and TDZ on Somatic Embryogenesis:

Experiments conducted to optimize the concentration and exposure of 2,4-D and TDZ to obtain normal somatic embryos are described in this chapter. Mature zygotic embryo derived leaflets were used as explants. This chapter is divided into three sections A, B and C.

Section A describes the **influence of 2,4-D** concentrations and exposure on embryo development and conversion. Leaflets cultured in embryogenic mass induction medium (MS with 90.5μM 2,4-D) for varying periods (2, 4, 6 and 8 wks) were cultured for 4 wks in 3 secondary media including, SM-1 (MS), SM-2 (MS+13.6μM 2,4-D), SM-3 (MS+90.5μM 2,4-D). In the explants cultured for 6 wks in primary medium, embryogenic mass and subsequently embryo formation initiated. Somatic embryos developed on transfer to all the three secondary media. Optimum conversion frequency of 53% was achieved in embryos developed in SM-2 and cultured for 12 wks in growth regulator free medium. Extended exposure of the leaflet explants for 8 wks in primary medium led to the formation of embryogenic mass and development of embryos but the conversion frequency of these embryos was reduced. **Culturing the leaflets in primary medium for 6 wks and thereafter in SM-2 medium was optimum for conversion of the embryos on transfer to medium devoid of growth regulator.**

Section B describes the **influence of TDZ** at different concentrations and exposures, on plumule differentiation of developing embryos, by incorporating TDZ in three stages of embryo development including (i) embryogenic mass (EM) induction from leaflets; (ii) embryo induction from embryogenic masses; (iii) embryo conversion. Incorporation of TDZ in embryo conversion medium for 24 hrs was the most effective and the conversion frequency attained in the somatic embryos was 43 %.

Section C describes the experiments carried out to assess the uniformity/variability within the regenerants obtained through incorporation of TDZ in the embryo conversion medium by using ISSR primers. ISSR analysis showed more than 99% similarity within the plants.

CHAPTER 4: Meristems as Explants for Somatic Embryogenesis:

This chapter deals with the experiments conducted to assess the potential of mature zygotic embryo axis-derived **(A)** and axenic shoot culture-derived **(B)** meristems as explants for somatic embryogenesis and **(C)** effect of silver nitrate on somatic embryogenesis.

A. *Embryo axis as explant:*

Apical portion of mature zygotic embryo axes having pre-existing meristems were used as explants for somatic embryogenesis. Optimum (8/explant) embryogenesis was noted in explants, cultured in primary medium for 6 wks followed by culturing in SM-2. Optimum conversion frequency of 32% was achieved in these embryos. Origin of embryos from the existing caulogenic buds was confirmed histologically.

B. *Axenic shoot culture derived meristems as explants:*

Protocol for somatic embryogenesis from the existing meristems of the embryo axis derived plumule was extended to the axenic shoot derived caulogenic buds of the cultivar SB-11 and *Arachis duranensis* (a wild species). The difference in potential in the nodes due to their relative position was determined by culturing the nodes in series. The meristem in the first node demonstrated more potential compared to the rest in SB-11. Explants from this cultivar responded consistently in presence of 2,4-D, to give rise to embryos from the axillary meristems. However, the response was sporadic in picloram. On the contrary, in *Arachis duranensis,* shoot culture-derived axillary meristems did not respond in 2,4-D whereas induction of somatic embryos occurred in 2 wks at all concentrations of picloram tested.

C. *Effect of silver nitrate on somatic embryogenesis:*

Incorporation of silver nitrate at low concentrations increased the conversion frequency of the embryos developed from axillary meristems of mature zygotic embryo axis.

CHAPTER 5: Genetic Transformation of Peanut with *Agrobacterium rhizogenes*:

Three agropine-type strains (15834, A4 and 9402) of *A. rhizogenes* were tested for transformation of peanut tissues. Seedling explants including leaf blade, petiole, node and internodes were used for transformation studies. Rhizogenesis was noted in all types of explants at varied frequency with all the strains but regeneration from these transformed roots was lacking. Mature zygotic embryo axis-derived leaflets were also tested for transformation with *A. rhizogenes*. After infection with *A. rhizogenes* leaflets were cultured in embryogenic media that led to the development of somatic embryos. During the whole process of embryogenesis there was no sign of hairy root development from somatic embryos. Hairy roots appeared after transferring these embryos in 2^{nd} passage of MS basal media for conversion. These somatic embryos converted into transformed emblings with hairy roots. Integration of rhizogenes is demonstrated by PCR.

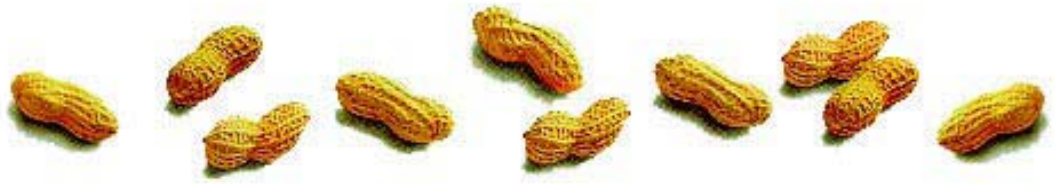
APPENDIX: *Ex Vitro* Performance of Peanut Plants from TDZ-pretreated Seeds:

This section describes the study conducted to test the effect of TDZ on morphology and growth of the seedlings germinated extra vitrum from TDZ treated seeds. Seeds of SB-11 soaked in different concentrations (2.27, 4.54, 9.08, 13.62 and 22.71μM) of TDZ for 12 hrs did not demonstrate variation in germination frequency but the growth of seedlings was retarded with increasing concentration of TDZ. Shoot and root lengths were reduced at higher TDZ concentrations and number of rootlets was less. Initially the leaves of the 22.71μM TDZ treated seed derived plant, were small and wrinkled. On maturity, there was no difference in leaf morphology. Flowering in TDZ treated seed derived seedlings was delayed. In control plants nodule formation was noted in the hypocotyls of the seedlings. However, nodule formation was absent in the hypocotyls of seedlings derived from seeds pre-treated with TDZ. Root nodules were present in control as well as in TDZ treated seed derived seedlings. Optimum pod formation was obtained in plants raised from the seeds pre-treated with 2.27μM of TDZ. The pod yield was enhanced in plants raised from seeds treated with lower concentrations of TDZ. This observation needs to be confirmed with more number of trials and by testing other cultivars prior to consideration of TDZ pretreatment for increased grain yield.

Summary: The main findings of this research work are summarized in this section.

Chapter 1

Introduction



1.1 INTRODUCTION

In vitro applied conditions lead to regeneration of plant tissues through two pathways, namely 'organogenesis' wherein shoot buds are organized by concerted meristematic activity of a number of cells and 'embryogenesis', where usually single cell or a small cluster of cells undergo differentiation to produce somatic embryos (SEs) similar to zygotic embryos (Chandra and Pental 2003).

Somatic embryogenesis (SEsis) is the developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (i.e., bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes (Williams and Maheswaran 1986; Jimenez 2001). Since then SEsis was described for the first time 50 years ago (Steward *et al.* 1958; Reinert 1959), it has been the subject of many studies.

Somatic embryogenesis has many advantages over organogenesis: (a) it permits the culture of large numbers of 'reproductive units' with the presence of both root and shoot meristems in the same element, (b) unlike shoots, SEs frequently originate from single cells and the embryogenic cultures can be synchronized and purified so that one can deal with practically pure cultures of homogeneous material, and, (c) plants derived from SEs are less variable than those derived by way of organogenesis (Ammirato 1987; Terzi and LoSchiavo 1990; Osuga *et al.* 1999; Jimenez 2001).

Various applications of somatic embryogenesis (Jimenez 2001):

- SEsis constitutes is an efficient approach to investigate the initial events of zygotic embryogenesis in plants. Perhaps the primary reason for the limited progress in understanding the developmental events in plant embryos is that zygotic embryos of plants grow within maternal tissues, and it is difficult to collect sufficient embryos for biochemical, physiological and morphological analyses of the events that occur early in the developmental process. SEs provide a good model system in which such problems could be circumvented. Knowledge of many of the events that occur during the early embryogenesis has resulted from experiments on SEsis (Jimenez 2001; Kiyosue *et al.* 1993; Zimmerman 1993).

- Mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of SEsis (Merkle *et al.* 1990).

- Production of plants with different levels of ploidy, i.e., obtaining haploid embryos by cultivating anthers and raising triploids from endosperm have been suggested (Terzi and Lo Schiavo 1990).

- Induction of dormancy and accomplishment of long-term storage, together with the achievement of encapsulation of SEs, has opened up the possibility for their use in the synthetic seed technology (Gray and Purohit 1991; Gray *et al.* 1995; Litz and Gray 1995).

- Gene transfer into embryogenic plant cells has become an indispensable tool for crop improvement. One of the most important prerequisites for genetic manipulation of plants *in vitro* is the ability to regenerate viable plants from somatic cells. Somatic embryogenesis, therefore, is a more efficient pathway for studies involving production of genetically transformed plants (Litz and Gray 1995; Vicient and Martinez 1998).

Moreover, SEsis is a useful tool in basic research on totipontency and on the fundamental processes of plant morphogenesis. Various culture treatments can be manipulated to optimize the frequency and morphological quality of SEs. Understanding the induction, initiation and development of SEs is crucial for better regulation of these processes for various experimental or practical objectives (Griga 2002). Knowledge of regeneration mechanisms including the initiation sites of regeneration and the cells/tissues involved in embryo formation is of high importance for choosing optimum strategy in a particular technology.

Somatic embryogenesis and factors influencing the process

Somatic embryogenesis has been divided into two main phases, namely, the one whereby differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells by means of a complete reorganization of the cellular state, including physiology, metabolism and gene expression (Feher *et al.* 2002), and the phase whereby

the embryogenic cells display their embryogenic competence and differentiate into SEs. A hypothetical model of events underlying SEsis was presented in Fig.1 (Feher 2005).

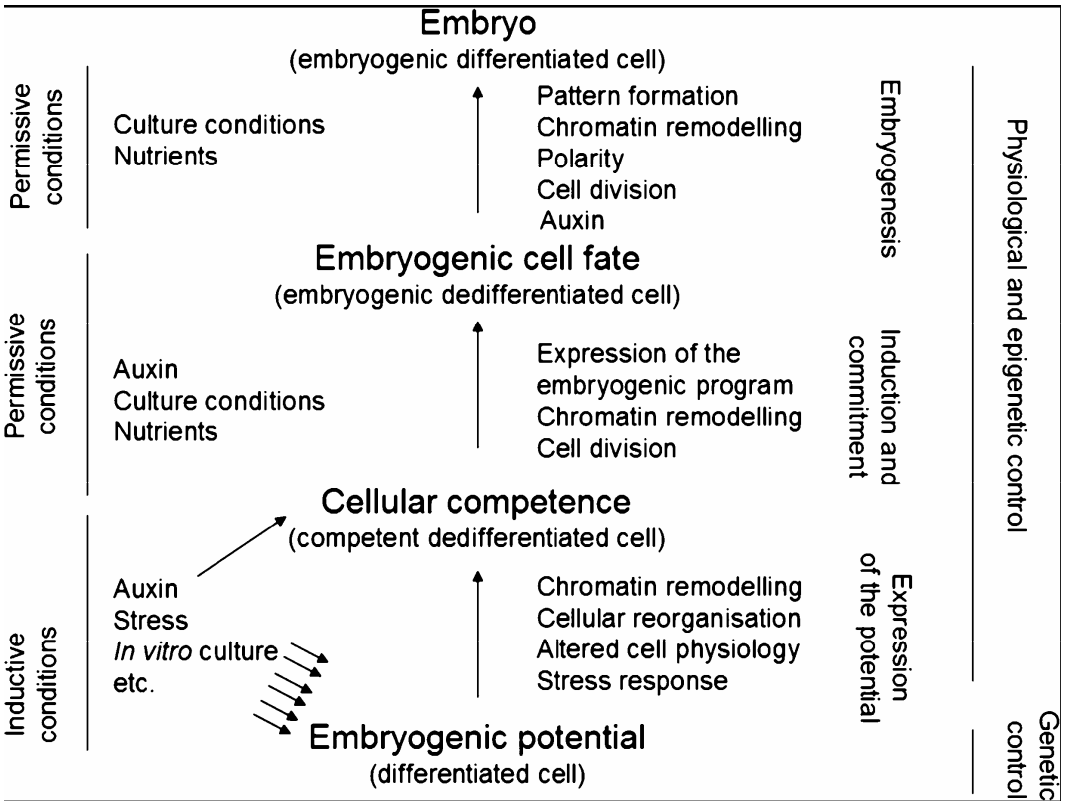


Fig.1 A hypothetical model of events underlying SEsis. A multitude of parallel signals, including auxin (either exogenously supplied or endogenously altered), evoke a wide cellular response including reorganizations at the levels of cell structure, physiology, chromatin and gene expression. As a result, the dedifferentiated cells become competent for embryogenesis. Competent cells will indeed be embryogenic if external and cellular conditions allow the expression of the embryogenic programme that is, in most cases, preceded by or parallel to cell divisions. Further cell divisions together with polarity establishment and pattern formation result in the development of the embryo. The central role of chromatin remodelling can be hypothesized in all phases, including dedifferentiation, embryogenic reprogramming and embryo differentiation. They are all associated with the parallel activation/inactivation of a large number of genes (Feher 2005).

Studies on factors controlling *in vitro* plant morphogenesis are highly desirable not only for the development of improved regeneration systems, but also for the analysis of molecular mechanisms underlying plant embryogenesis. *In vitro* development of cells and tissues depends on different factors, such as, genotype, type of plant, age and developmental stage of an explant, physiological state of an explant-donor plant, and the external environment which includes composition of media and physical culture

conditions (light, temperature etc.). Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development. Such cells, which represent an intermediate state between somatic and embryogenic cells, are called competent (Gaj 2004). Competent cells display sensitivity to physical and chemical stimuli that trigger the embryogenic pathway of their development, while embryogenic cells are already determined in embryogenesis (De Jong *et al.* 1993).

Based on the results from various plant systems, it became obvious that many different factors determine a somatic cell to switch an embryogenic program of development as a result of complicated interaction between a proper physiological state of cultured cells and exogenous signals. Undoubtedly, plant growth regulators used to induce *in vitro* morphogenesis are among key factors in the determination of embryogenic response (Gaj 2004).

Auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation (Francis and Sorrell 2001; Feher *et al.* 2003; Gaj 2004). Influences of exogenously applied auxins, preferentially 2,4-dichloro phenoxy acetic acid (2,4-D), on the induction of SEsis are well documented (Dudits *et al.* 1991; Yeung 1995). In more than 65% of the protocols, 2,4-D was applied singly or in combination with other plant growth regulators (Gaj 2004). The high efficiency of 2,4-D for induction of embryogenic response found in different *in vitro* systems and plant species indicates a specific and unique character of this plant growth regulator (PGR). This synthetic growth regulator and an auxinic herbicide appear to act not only as an exogenous auxin analogue but also as an effective stressor. Examples that 2,4-D brings about different changes in physiology and gene expression of cells implicate its possible role as a stress factor triggering embryogenic pattern of development in cultured plant cells (Feher *et al.* 2003). However, abscisic acid (ABA), ethylene, gibberellic acid (GA_3) and other hormones have regulatory roles, which must not be ignored in culture systems. Moreover, a new generation of PGRs, such as thidiazuron (TDZ), a cytokinin that belongs to the phenylureas, is emerging as a successful alternative for high-frequency direct regeneration of SEs, even from well-differentiated explant tissues (Gairi and Rashid 2004; Panaia *et al.* 2004; Zhang *et al.* 2005).

In many plants like *Zoysia japonica* (Asano *et al.* 1996), *Citrus* (Carimi *et al.* 1999), *Begonia* (Castillo and Smith 1997), *Oncidium* (Chen and Chang 2001), *Acacia* (Nanda and Rout 2003), the addition of cytokinins as the sole source of PGR is sufficient to generate SEs but the cytokinin-induced model of embryogenesis is rare and was less described (Gaj 2004). In pea (Kysley and Jacobsen 1990), soybean (Lazzeri *et al.* 1987) and *Coronilla varia* (Arcioni and Marriotti 1982) addition of cytokinins inhibited the induction of SEsis promoted by auxins. In some legumes e.g. *Cicer arietinum* (Kumar *et al.* 1995), *Vigna aconitifolia* (Kumar *et al.* 1988; Shekhawat and Galston 1983), *Psophocarpus tetragonolobus* (Ahmed *et al.* 1996) and *Dalbergia latifolia* (Rao and Lakshmisita 1996), requirement for cytokinin alone, or in combination with auxin, for SE maturation was reported (Lakshmanan and Taji 2000).

Application of *in vitro* systems based on SEsis for plant regeneration is determined not only by a high efficiency of SE formation, but frequently depends on capacity of the embryos for plant development. In numerous systems, in spite of the high number of SEs produced, problems occurred due to lack or a low frequency of embryo conversion into plants. To stimulate embryo conversion, and to improve the efficiency of plant regeneration, a number of different strategies were tested. To improve conversion rate regeneration media were supplemented with other plant growth regulators such as indole butyric acid (IBA), GA_3, ABA, or cytokinins (Madakadze and Senaratna 2000; Sarasan *et al.* 2001; Yang and Choi 2000).

It should be stressed that in some systems e.g. in asparagus (Levi and Sink 1991), maple (Vlasinova and Havel 1999), *Eschscholzia* (Park and Facchini 2000), abnormal morphology of SEs did not decrease their chances of development into plants. In Arabidopsis system high (above 75%) conversion rate of SEs was achieved even though numerous embryos displayed morphological anomalies (Gaj 2001). Failure of the peanut somatic embryos to convert into plantlets attributed to the morphological anomalies in the plumule (Chengalrayan 2001)

Genetic characterization of regenerants

Plant regeneration systems that limit or avoid genetic chimerism in regenerants are of special value for biotechnology, combining tissue culture with genetic transformation or mutant induction and selection. Genetic modification is a unicellular event, and hence

regeneration from multi-cellular centers frequently results in the formation of genetic chimeras.

Somaclonal variation, first defined and reviewed by Larkin and Scowcroft (1981), is a commonly observed phenomenon in cell and tissue cultures of different species regardless of the regeneration system used. *In vitro* regeneration systems useful for cloning or genetic modifications of plants should eliminate or decrease somaclonal variation. In the light of an increasing number of reports, there is no 'safe' *in vitro* system in which lack of new variation could be guaranteed. Even in micropropagation methods, for which the concept of genetic fidelity of clonally propagated plants has been accepted for many years, there is evidence of somaclonal variation at the morphological, cytological, protein and DNA level (Rani and Raina 2000).

The risk of somaclonal variation is particularly high when SEs are induced in callus tissues, in long-term cultures (Henry *et al.* 1998), or via secondary embryogenesis (Remotti *et al.*1997). On the contrary, direct development of SEs from cultured explants and/or the use of young explant tissue in combination with short term culture usually limit *in vitro* induced variation.

Even though evaluation of the spectrum and frequency of somaclonal variation is an important requirement for every regeneration system proposed, only a limited number of *in vitro* protocols actually analyze variation in regenerants. In less than 30% of the recent protocols somaclonal variation was considered, and in almost 90% of them the lack of the variation was stated when the morphology and chromosome number of regenerants were analyzed. Normal plant phenotype, or even karyotype, do not exclude less distinct genetic changes in the analyzed regenerant, and thus for reliable evaluation of somaclonal variation other approaches should be applied, including molecular markers (Gaj 2004).

DNA markers have the advantage that they do not change in response to environmental factors or the developmental stage of a particular plant tissue. DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analysed objectively. DNA markers have been used for testing the genetic fidelity during micropropagation/ ex situ conservation on the one hand and for characterization of plant genetic resources on the other. Molecular markers have particularly been suggested to be

useful for confirmation of genetic fidelity in micropropagated plants, where life span is long and performance of micropropagated plant could only be ascertained after their long juvenile stage in field conditions (Rani and Raina 2000).

DNA markers are generally classified as hybridization based markers and polymerase chain reaction (PCR) based markers. Restriction Fragment Length Polymorphism (RFLPs), Random amplified polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphism (AFLPs), Simple Sequence Repeats (SSRs), Single Nuleotide Polymorphism (SNPs) etc., are some of the DNA markers in use. Inter simple sequence repeats (ISSR), a promising DNA marker, combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD.

Various types of DNA markers have been used including RFLPs, RAPDs, SSR and AFLPs, to evaluate genetic variation in SE-derived plants. Molecular marker-based analysis revealed either the absence (Heinze and Schmidt 1995; Fouree *et al.* 1997; Gallego *et al.* 1997; Hornero *et al.* 2001), or presence (Hashmi *et al.* 1997; Piccioni *et al.* 1997; Al-Zahim *et al.* 1999; De Verno *et al.* 1999; Vendrame *et al.* 1999; Yang *et al.* 1999) of *in vitro* generated changes in plants DNA.

1.2 *Arachis hypogaea*

Peanut (groundnut), one of the world's most important oilseed crop belonging to the Leguminosae family and the Papilionaceae subfamily, is a native to South America but now grown in diverse environments in six continents between latitudes 40°N and 40°S. It can grow in a wide range of climatic conditions mainly in tropical and warm temperate regions of the world. Asia is the largest producer followed by Africa, North and Central America and South America. Asia accounts for 66.5% of global groundnut production on 56.8% area while Africa produces 24.7 % on 38 % area (CMIE Rep. 2002).

The genus *Arachis* contains a rich diversity of plant types. The cultivated peanut (*Arachis hypogaea*) is a tetraploid (4n= 40). The subspecies *hypogaea* is characterized by alternate branching, a lack of inflorescence on the main stem, and the first branch of the cotyledonary lateral always being vegetative. Plausible progenitor of *A. hypogaea* has been proposed for many years, on the basis of cytological and genetic studies (Joshi

2003). Gregory and Gregory (1976) postulated that *A. duranensis* and *A. cardenasii* intercrossed to produce cultivated peanuts.

In India, peanut is an important oilseed, food and feed crop grown in an area of 6.45 million ha with a total production of 6.57 million tons based on an average of the last five years (FAO 2005). This contributes to 18.5% of world's peanut production and 26.6% of crop area production. Peanut occupies nearly 28.3% of the cultivated area and contributes 31.7% of the production of the total oilseeds in the country. It is widely used as cooking oil, digestible protein, minerals and vitamins in many countries and contributes significantly to food security and alleviating poverty. About 80% of India's peanut production is crushed for oil, 12% for using as seed, 5% for food and 2% for export (Badigannavar *et al.* 2007). The low yields of this crop in India are mainly attributed to unreliable rainfall patterns with frequent droughts, lack of high-yielding adapted cultivars, damage by diseases and pests, poor agronomic practices, and limited use of inputs (Nageshwara Rao and Nigam 2003).

There is a need to increase the productivity and enhance the nutritional value of this pulse crop by developing cultivars resistant to biotic/abiotic stresses and having better protein quality/quantity. Wild species of peanut possess many agronomically desired characteristics. However, some of this variability has not been exploited in the improvement of the cultivated species due to the frequent failures in interspecific crosses. These failures are attributed to post-fertilization barriers leading to abortion of embryos, seed and/or pods, which fail to germinate (Chengalrayan 1997). Thus, there is a need to widen the genetic base and incorporate desired characters. Genetic engineering approaches are comparatively fast, leading to better isolation and cloning of desired traits for combating the various biotic and abiotic stresses. There is an urgent need to use transgenic technologies for the improvement of this crop.

Conventional breeding has led to the improvement of few peanut traits like seed yield and drought tolerance. However, due to its limited applicability, many of the important agronomic traits have yet to be improved. These include resistance to insect pests and diseases, caused by nematodes, viruses like spotted wilt virus and peanut stripe viruses, leaf spot fungi etc. Besides, many economically important traits like tolerance to water stress, uniform fruit maturity, and nutritional quality contribute to quality peanut

production. Although the genetic variability within wild species includes many of these valuable traits, wide hybridization in peanut has limited applicability due to cross incompatibility, low frequency to produce hybrid seeds and linkages with undesired traits. *In vitro* cultures in peanut have been used to introgress germplasm from wild species (Ozias-Akins *et al.* 1992a). Some researchers have been able to rescue embryos by avoiding events partially correlated with quiescence (Moss and Stalker 1987; Halward and Stalker 1987a, b; Pattee and Stalker 1992). Success of these studies has depended on many factors, including the specific genotypic combination (Ozias-Akins *et al.* 1992a) and the growth stage of the culture. Tissue culture can produce haploids followed by doubling of chromosomes to produce plants to bridge introgression of wild germplasm and serve as a source of homozygous breeding lines.

Development of procedures by which plants could be regenerated from single cells and organized tissues and specific genes transferred to plant cells were the prerequisite for practical genetic engineering for grain legume improvement. These techniques have given us the opportunity to create, characterize and select plant cultivars, which could not be obtained by traditional breeding methods (Christou 1997). Last two decades has witnessed considerable progress in peanut improvement via integrated efforts in breeding, genetics and other related disciplines throughout the world. Many traits connected with final plant productivity are the result of multiple gene families, which are complex in their inheritance but difficult to define biochemically (Goodman *et al.* 1987). Further success in plant genetic engineering will rely on a thorough understanding of the molecular, genetic and metabolic characteristics and properties of these traits (Barton and Brill 1983). Peanut improvement has received high priority from scientists at the International Crop Research Institute for the Semi-arid Tropics (ICRISAT), which adopted peanut as a mandate crop in 1976 (Gibbons 1980).

1.2.1 Role of biotechnology in *in vitro* propagation of peanut

In addition to classical and molecular breeding approaches, genetic transformation to introduce novel genes into plants for quality production offers an attractive option. Peanut crop has a narrow germplasm base, without satisfactory resistance to several major pathogenic fungi and viruses. Thus a strong practical incentive exists for the development of an efficient genetic transformation system for peanut (Mansur *et al.* 1995).

One of the conditions for plant transformation is to count on a good regeneration protocol. However the different pathways of regeneration, viz. organogenesis from callus, embryogenesis from callus, organogenesis directly from explants and embryogenesis from explants in a direct mode vary in their amenability to different gene delivery techniques. Efficient regeneration of highly totipotent cells is an essential component of genetic engineering systems, whether the regenerated plant results from a preformed shoot meristem or an undifferentiated callus cell or embryo initial that is competent to express morphogenic program (Ozias-akins and Gill 2001).

Establishment of an efficient regeneration system in peanut has not been trivial and has been built on decades of research beginning with the regeneration of plants from *in vitro*-cultured de-embryonated cotyledons (Illingsworth 1968). Literature reports on regeneration of peanut are tabulated (Table1.1).

1.2.2 *In vitro* regeneration of peanut via embryogenic pathway:

Variety of explants has been employed for initiating SEsis in peanut. These include leaflets (Baker and Wetzestein 1992; Gill and Saxena 1992; Venkatachalam *et al.*1999b), immature cotyledons (Ozias-Akins 1989; Ozias-Akins *et al.*1992; Baker and Wetzstein 1994; George and Eapen 1993), immature embryo axes (Hazra *et al.* 1989; Reddy and Reddy 1993; Roja-Rani *et al.* 2005), mature embryo axis (Mckently 1991; Baker *et al.*1995) mature embryo derived leaflets (Chengalrayan *et al.*1994 and 1997), hypocotyls (Venkatachalam *et al.* 1997) and epicotyls (Little *et al.* 2000).

Peanut Somatic embryogenesis from immature seed derived explants:

Regeneration of plants via direct SEsis in *Arachis hypogaea* was first reported from immature zygotic embryo axis (Hazra *et al.* 1989). Induction and maturation of the SEs was achieved in the medium containing 2,4-D. This PGR was essential for direct SEsis and the size of the immature zygotic embryo axis and the concentration of 2,4-D were important. Reddy and Reddy (1993) and Roja-Rani and Padmaja (2005) reported SEsis from immature zygotic embryo axis in MS medium with 2,4-D. These authors (2005) reported only 31.7% embryogenic response and shoot induction was observed in SEs placed on MS medium supplemented with BAP and NAA.

Table 1.1: Literature on regeneration of peanut.

Type of explant	Type of response	References
Anther	Callus, Organogenesis	Mroginski and Fernandez 1980
	Callus, Organogenesis	Bajaj *et al.* 1981a
Apical Meristem	Organogenesis	Shyluk *et al.*1981
Cotyledon	Organogenesis	Illingworth 1968
	Rhizogenesis	Guy *et al.* 1980
	Organogenesis	Atreya *et al.* 1984
	Organogenesis, Flowering	Narasimhlu and Reddy 1984
	Organogenesis	Bhatia *et al.* 1985
	Organogenesis, Embryogenesis	Banerjee *et al.* 1988
	Organogenesis	Mckently *et al.* 1990
	Embryogenesis	Ozias-Akins *et al.* 1992
	Callus	D`Silva and Podder 1995
	Embryogenesis	Venkatachalam *et al.* 1997
	Organogenesis	Tiwari and tuli 2007
Hypocotyl	Organogenesis	Li *et al.* 1994
Immature cotyledons	Repetitive SEsis	Durham and Parrott 1992
	Embryogenesis	Wetzstein and Baker 1993
	Embryogenesis	Eapen *et al.* 1993
	Embryogenesis	Baker and Wetzstein 1994
	Repetitive embryogenesis	Baker and Wetzstein 1995
	Somatic embryogenesis	Yang *et al.* 2001
Immature embryo axis	Somatic embryogenesis	Hazra *et al.* 1989
	Somatic embryogenesis	Ozias-Akins 1989
	Somatic embryogenesis	Sellars *et al.* 1990
	Somatic embryogenesis	Ozias-Akins *et al.* 1992
	Embryogenesis	Reddy and Reddy 1993
	Somatic embryogenesis	Roja-Rani and Padmaja 2005
Cotyledon, Juvenile leaves	Organogenesis, direct embryogenesis	Gill and Saxena 1992
Immature cotyledons and Young leaves	Organogenesis	Li *et al.* 1994

Table 1.1 contd.

Immature leaf	Callus, organogenesis	Mroginski *et al.* 1981
	Callus, organogenesis, embryogenesis	Pittman *et al.* 1983
	Callus, organogenesis	Seitz *et al.* 1987
	Organogenesis	Mckently *et al.* 1991
	Embryogenesis	Baker and Wetzstein 1992
	Organogenesis	Eapen and George 1993
Mature embryo axis	Somatic embryogenesis	Mckently 1991
	Organogenesis	Brar *et al.* 1994
	Somatic embryogenesis	Mckently 1995
	Organogenesis	Ponsamuel *et al.* 1997
	Somatic embryogenesis	Livingstone and Birch 1999
	Somatic embryogenesis	Magbanua *et al.* 2000
Mature zygotic embryo derived leaflets	Embryogenesis	Chengalrayan *et al.* 1994
	Embryogenesis	Mhaske and Hazra 1994
	Callus, organogenesis, flowering	Chengalrayan *et al.* 1995
	Embryogenesis	Chengalrayan *et al.* 1997
	Embryogenesis, organogenesis	Joshi *et al.* 2003
Mature leaf	Organogenesis	Dunbar and pittman 1992
Ovaries, ovules and pod, peg tips	Callus	Sastri *et al.*1982
Seeds	Embryogenesis, organogenesis	Victor *et al.* 1999
Seedling explants	Callus, organogenesis	Narasimhulu and Reddy 1983
	Organogenesis	Cheng *et al.* 1992
	Organogenesis	Kanyand *et al.* 1994
	Embryogenesis	Murthy *et al.* 1995
	Embryogenesis	Sabitha and Reddy 1996
	Callus, Embryogenesis	Venkatachalam *et al.* 1997
	Callus, Embryogenesis	Venkatachalam *et al.* 1998
	Embryogenesis	Baker and Wetzstein 1998
	Callus, Embryogenesis	Venkatachalam *et al.* 1999

Ozias-Akins (1989) reported SEsis using immature cotyledon, cultured in B5 medium with picloram. Eapen and George (1993) studied the influence of different growth regulators and sugars on peanut SEsis from immature embryonal axis and immature

cotyledon and concluded that 2,4-D was the most effective among the different auxins tested. This PGR produced the highest response frequency and higher number of SEs in each of the responding cultures while 3,6-dichloro-o-anisic acid (Dicamba), 4-amino-3,5,6-trichloro picolinic acid (picloram), indolepropionic acid, NAA, 2, 4, 5-tricholorophenoxypropionic acid and α-Naphthaleneacetic acid were also effective for peanut embryogenesis. Among the various carbon sources tested, sucrose at 6% was the best for embryo induction. But conversion frequency was very less (20%) as recorded at the end of 5 months. Wetzstein and Baker (1993) reported that SEs developed from immature cotyledonary explants in peanut exhibited very divergent morphologies. Six types of SEs were developed based on axis and cotyledon development. The 2,4-D level in the induction medium did not affect embryo rooting or conversion in the cultivar AT127 and had little effect on embryo morphology. Higher auxin concentration during induction decreased precocious germination of embryos (Baker and Wetzstein 1994). However embryo morphology was noticeably affected by photoperiod (Baker *et al.* 1995).

All these protocols were optimized using immature seed-derived explants. To overcome the limitations encountered in using immature seed-derived explants, seedling-derived explants and mature zygotic embryo derived-explants were tested for SEsis. A distinct advantage of this system over the previous SEsis system using immature zygotic explants is a ready year round availability of tissue. Problems of obtaining explants at specific developmental stages are eliminated. Aseptic cultures with little or no contamination are readily obtained.

Peanut Somatic embryogenesis from seedling derived explants:

Baker and Wetzstein (1992) reported SE induction and plant regeneration from seedling derived leaf cultures in modified MS basal medium with B5 vitamins and supplemented with 2,4-D and kinetin. Baker and Wetzstein (1998) further optimized the leaflet development stage, leaflet size, induction medium and time on embryogenic induction medium. Leaflets that were 5-7 mm long had a greater embryogenic response than smaller or larger leaflets. Percent embryogenesis and mean number of embryos were related to the developmental stage of germinating seedlings. A greater response was obtained if leaflets were folded and closely appressed in MS medium with 2,4-D and kinetin. Gill and Saxena (1992) established embryogenic cultures from cotyledon and leaf

explants of 12-day old seedlings and concluded that the SEsis were depending on genotype and growth regulator pretreatment of explants.

Victor *et al.* (1999a) exhibited *de novo* regeneration of SEs at the hypocotyledonary notch region of intact seedling in presence of TDZ. Subsequently analyzing the endogenous level of purine metabolites (Victor *et al.* 1999b), authors demonstrated that the supplementation of the media with TDZ resulted in an overall increase in the endogenous cytokinins, while the purine analog 2,6-diaminopurine inhibited the purine recycling resulting in decreased levels of endogenous adenine and zeatin.

Venkatachalam *et al.* (1997) induced embryogenic calli from hypocotyl of 7-day old seedlings in presence of 2,4-D or NAA in combination with BAP and concluded that the type of auxin, concentrations and genotypes influenced the SEsis in peanut. 2,4-D was the best SE inductor and conversion occurred in BAP and NAA. These authors extended the embryogenic study (Venkatachalam *et al.* 1999) to the immature and mature leaflet and petiole explants excised from axenic seedlings. Venkatachalam *et al.* (1998) initiated the suspension culture from leaflet-derived callus in MS liquid medium supplemented with 2,4-D, NAA and BAP. Large number of SEs differentiated when the suspension was cultured for 7 days in induction medium.

Peanut somatic embryogenesis from mature seed derived explants:

Mckently (1991) reported peanut SEsis from the 2 mm region adjacent to and encircling the epicotyl of mature zygotic embryo axis in presence of picloram and suggested that a 2-mm band of hypocotyledonary tissue surrounding the epicotyl is highly embryogenic in nature. Somatic embryogenesis was evaluated on MS media supplemented with a variety of auxin treatment. Optimum production occurred on medium supplemented with picloram. Baker *et al.* (1995) observed SEsis in cultivar GK-7 on young and expanded leaves of the epicotyl of mature zygotic embryo axis cultured horizontally in MS medium containing 2,4-D. Survey of 14 genotypes indicated that genotype had a large influence on embryogenic capacity, with all genotypes being embryogenic to some extant.

Chengalrayan *et al.* (1994, 1995, 1997, 1998, and 2001) used mature zygotic embryo derived leaflets (MZELs) for induction of SEsis, caulogenesis, callusing and *in vitro* flowering by altering the hormonal composition on the MS basal medium. The protocol

for obtaining a high frequency of plant development via SEsis from MZEDLs of peanut involved multiple stages (Chengalrayan *et al.* 1997). These were induction of embryogenic masses, development of embryos, radicle emergence, conversion of embryos and development of plants from rooted abnormal embryos. Embryogenic masses developed in the base of MEZL in MS basal medium containing 20 ppm of 2,4-D and SEs developed from the embryogenic masses in MS basal medium containing 3ppm of 2,4-D. The SEs were morphologically malformed. These morphologically abnormal SEs germinated and produced roots in medium devoid of growth regulator. Shoot development was triggered from the undeveloped plumule of these rooted SEs in medium containing BAP and kinetin, or in medium with TDZ singly (Chengalrayan *et al.* 1997). Plants grown on TDZ had multiple shoots. Sixteen genotypes were subjected to this protocol and this protocol was effective for all the genotype (Chengalrayan *et al.* 1998) at varying frequencies. The complete process of SEsis depended on the genotype constitution of the original plant. Joshi *et al.* (2003) studied the pathway of morphogenesis of multiple shoot formation in rooted SEs in presence of TDZ. This PGR triggered the proliferation of the plumule meristem and produced multiple caulogenic buds. The buds differentiated into shoots on transfer to medium devoid of growth regulators resulting in formation of plantlets with multiple shoots.

In an attempt to overcome the limitation of SEs conversion in PGR free medium, Mhaske *et al.* (1998) studied the influence of sucrose, mannitol, sorbitol and abscisic acid on maturation accumulation of triglycerides in peanut SEs. An increase in storage lipid in the somatic embryos did not improve conversion of peanut SEs.

Repetitive somatic embryogenesis in peanut

Reports on repetitive embryogenesis in peanut are limited. Durham and Parrott (1992) developed a peanut regeneration system based on repetitive embryogenesis initiated from immature cotyledons cultured in MS liquid medium containing 2,4-D for 30 days. Secondary and tertiary embryos were formed after shifting on MS liquid medium. Approximately 4% embryo conversion frequency was obtained in agar gelled MS basal medium. Ozias-Akins *et al.* (1993) reported repetitive embryogenesis in peanut using immature embryos as explant and picloram as the auxin. Embryogenic callus was maintained in MS medium supplemented with 3mg/l picloram with 3% sucrose and 1g/l filter-sterilized glutamine. Baker and Wetzstein (1995) reported repetitive embryogenesis

from immature cotyledon of peanut in 2,4-D medium. The viability of secondary embryogenesis was maintained for over one year by repeated subculture of isolated SE.

Little *et al.* (2000) had conducted comprehensive study of different auxins on peanut embryogenesis, secondary embryogenesis, genotypic effect, morphology of embryos and their conversion rate by using epicotyls. Picloram and Centrophenoxine were optimum for induction and conversion of repetitive embryos from epicotyls of mature embryo axes.

1.2.3 Genetic transformation:

Although many different techniques (*Agrobacterium*-mediated, electroporation of intact tissues, microprojectile bombardment etc.) have been tested for gene delivery to plant cells, two major methods, namely *Agrobacterium*-mediated and particle bombardment have been extensively employed for genetic transformation of plants (Chandra and Pental 2003). Each method has limitations with regard to the competent target tissues and genotype. *Agrobacterium* infection depends on biochemical factors within plant, and its infection is more genotype dependent. Likewise, electroporation requires the DNA to be in contact with the plasma membrane of competent cells and is most effective with protoplasts, plant cells that have had their cell walls removed (Li *et al.* 1995). Molecular analysis of plants obtained by biolistic transformation generally reveals a complex pattern of transgene integration. Biolistic technique resulted in high frequency of multiple transgene insertions (Fu *et al.* 2000; Ozias-Akins and Gill 2001) which led to infertility and transgene silencing. This is a major drawback of the biolistics technique.

Transformation procedures typically result in transformation of individual cells within the explant tissue (Merkle *et al.* 1990). Whenever it is possible for SEs to arise from single cells, repetitive embryogenesis may be used to first recover a SE from a transgenic cell, and then to multiply in number of transgenic embryos available for plant recovery. Alternatively, when SEs have multicellular origins (Williams and Maheswaran 1986), the original transgenic cell can at best give rise to a transgenic sector within an embryo. An additional cycle of embryogenesis is needed to obtain a fully transgenic embryo from the transgenic sector (Merkle *et al.* 1990). Transformed embryos continue repetitive embryo production under selection, resulting in a reduction in chimeric events and in a greater number of embryos available for conversion into plants (Parrott *et al.* 1991). Hence, any improvement in genetic transformation needs an efficient protocol for SEsis.

Peanut is an important oilseed and protein crop. As such it presents unique targets for genetic engineering in terms of modified composition. A problem to peanut meal is the production of highly toxic mold metabolites during unfavorable storage conditions. One such compound is aflatoxin B1 which is a very potent carcinogen. Additional goals for peanut improvement include development of varieties with higher and more stable yields, early maturity, resistance to various diseases (virus, bacteria) and pests, and drought-tolerance (Bajaj 1984).

The recent development of transformation and regeneration systems has allowed the introduction of useful genes into peanut germplasm (Yang *et al.* 1998; Rohini and Rao; 2001). Genetic transformation of peanut is an alternative for the improvement of the crop, allowing the transfer of individual genes which confer agronomic traits such as pest and nematode resistance or enhancement of protein quality of the seeds (Mansur *et al.* 1995; Lacorte *et al.* 1997). This method overcomes the limitations encountered due to inter-specific, inter-generic or inter-kingdom barriers. Transformation of peanut has been attempted using wild-type strains of *Agrobacterium rhizogenes* (Mugnier 1988; Bolivar et. al. 2007), *Agrobacterium tumefaciens* (Lacorte *et al.* 1991; Mansur *et al.* 1993; Cheng *et al.* 1997a, b), and direct DNA delivery using particle bombardment (Ozias-Akins *et al.* 1993; Brar *et al.* 1994; Singsit *et al.* 1997). The reports on peanut transformation are surmised in Table1.2.

Agrobacterium-mediated genetic transformation in peanut:

Plant transformation mediated by *Agrobacterium tumefaciens* has become the most used method for the introduction of foreign genes into peanut plant cells and the subsequent regeneration of transgenic peanut plants. Lacorte *et al.* (1991) screened five Brazilian cultivars with four wild type *A tumefaciens* strains and concluded that successful peanut transformation was dependent on specific bacterial strain-plant cultivar interaction. Strain A281 was the most effective for tumor induction. Tumors displayed hormone autonomous growth, were opine positive and contained DNA that was homologous to the T-DNA of the inciting strain. Tumors induced on seed and seedling explants by A281 also expressed the reporter genes *gus* and *npt-11* contained in the binary vector. This study confirmed that peanut is a permissive host for the acceptance of genes from specific gene vectors. Li *et al.* (1997) unequivocally demonstrated foreign gene integration in

multiple transgenic events derived from New Mexico Valencia A. Multiple shoot were induced from cultured leaf sections on BAP and NAA. Egnin *et al.* (1998) investigated different parameters which were enhancing *Agrobacterium*-mediated transfer of foreign genes to peanut cv. New Mexico. An intro-containing *uidA* gene under the transcriptional control of CaMV 35S promoter served as a reporter. The disarmed *Agrobacterium* strain EHA101 was superior in facilitating the transfer of *uidA* gene to peanut cells compared to the disarmed strain C58. Rinsing of explants in half strength MS media prior to infection by *Agrobacterium* significantly increased the transformation efficiency. The polarity of epicotyl during co-cultivation was important, explants incubated in an inverted (vertically) manner followed by vertically upright position was used to obtained transient transformation. Cheng *et al.* (1996) used the same variety and leaf section for *Agrobacterium* mediated transformation. Leaf section explants were co-cultivated with *A tumefaciens* strain EHA105 harboring the binary vector pBI121 containing the genes for *gus* and *nptII*. GUS expression segregated in a 3:1 Mendelian ratio in most T_1 generation plants.

Based on strain A281 harboring a binary vector, Mansur *et al.* (1993) examined the factors that modulate the transformation efficiency of regenerable tissue of peanut and noted that the 7-10day old seedlings derived leaves were co-cultivated on solid medium rather than liquid medium for 48 h had greatest transformation efficiency. Additions of acetosyringone to bacterial culture did not alter the transformation frequency. Eapen and George (1994) co-cultivated the seedling derived leaves with *A tumefaciens* LBA4404 harboring the binary plasmid pBI 121 and confirmed the stable integration and expression of the transgenes by NPT II assay, southern blot hybridization and GUS assay. Rhoini and Rao (2000) developed a tissue culture independent *A. tumefaciens* transformation procedure in peanut. The protocol utilized treatment of *Agrobacterium* with wounded tobacco leaf extract as in Cheng *et al.* (1997) and wounding /co-cultivation of the embryo axis attached to one cotyledon similar to that used in the study of Mckently *et al.* (1995). *Agrobacterium* strain LBA 4404 harboring the binary vector pKIWI105 that carries the gene for *gus* and *nptII* was used for transformation. Molecular characterization of primary transformants and its progeny confirmed stable integration of the foreign genes.

Table 1.2: Literature on peanut transformation

Genotype	Explant	DNA Delivery	Response	References
Tatu, Tatuf, Tatu branco, Tupa, Penapolis	Seedling	*Agrobacterium tumefaciens*	Tumor	Lacorte *et al.* 1991
Tatu (Valencia)	Cotyledons, leaf	*Agrobacterium tumefaciens*	Callus	Mansur *et al.* 1993
Toalson, Florunner	Embryogenic cultures	Particle bombardment	Transgenic plants	Ozias-Akins *et al.* 1993
NC7, Tamnut74, Florunner	Mature zygotic embryos	Particle bombardment	Transgenic plants	Schnall and Weissinger 1993
JL-24	Seeds and Seedling	*Agrobacterium tumefaciens*	Transgenic plants	Sharma *et al.* 1993
TAG 24	Leaf	*Agrobacterium tumefaciens*	Transgenic plants	Eapen and George 1994b
GK 7	Mature zygotic embryos	ACCELL gene delvery method	Transgenic plants	Brar *et al.* 1994
Flrigiant, Florunner, NC7, 435	Cotyledons	*Agrobacterium tumefaciens*	Transgenic plants	Mckently *et al.* 1995
EC5	Cotyledonary protoplasts	Electroporation	Protoplast	Li *et al.* 1995c
Valencia cv New Mexico, Floruuer, GA runner, Sun runner, South runner	Leaf, hypocotyl	*Agrobacterium tumefaciens*	Transgenic plants	Egnin *et al.* 1995
Valencia cv New Mexico	Leaf	*Agrobacterium tumefaciens*	Transgenic plants	Cheng *et al.* 1996
Dhaka-1, DM-1, DG-2 and Acc. No. 12	Leaflet and epicotyl	*Agrobacterium tumefaciens*	Transgenic plants	Sarker *et al.* 1997
Georgia runner, Floruuer and MARC-1	Embryogenic cultures	Particle bombardment	Transgenic plants	Yang *et al.* 1998

Table 1.2 contd.

Georgia runner, Floruuer and MARC-1	Embryogenic cultures	Particle bombardment	Transgenic plants	Wang *et al.* 1998
Java 13	Mature embryo axis	*Agrobacterium rhizogenesis*	Transgenic roots	Akasaka *et al.* 1998
Valencia cv New Mexico, Floruuer, GA runner, Sun runner, South runner	Leaf and epicotyl	*Agrobacterium tumefaciens*	Transgenic plants	Egnin *et al.* 1998
NC-7 and Gajah	Embryogenic cultures	Particle bombardment	Transgenic plants	Livingstone *et al*. 1999
TMV-2	Mature embryo axis with one cotyledon	*Agrobacterium tumefaciens*	Transgenic plants	Rohini and Rao 2002
JL-24	Cotyledon	*Agrobacterium tumefaciens*	Transgenic plants	Sharma and Anjaiha 2000
Georgia Green	Embryogenic cultures	Particle bombardment	Transgenic plants	Ozias-Akins *et al.* 2002
DM-1	Young leaflet	*Agrobacterium tumefaciens*	Transgenic plants	Sarker and Nahar 2003
Georgia Green	Embryogenic cultures	Particle bombardment	Transgenic plants	Yang *et al.* 2003
Luhua14	Epicotyl	*Agrobacterium tumefaciens*	Transgenic plants	Quisheng *et al.* 2005
Georgia Green	Embryogenic cultures	Particle bombardment	Transgenic plants	Joshi *et al.* 2005
JL-24	Cotyledonary node	*Agrobacterium tumefaciens*	Transgenic plants	Anuradha *et al.* 2006
JL-24	Cotyledon	*Agrobacterium tumefaciens*	Transgenic plants	Tiwari *et al.* 2008
Georgia Green	Embryogenic callus	Particle bombardment	Transgenic plants	Chu *et al.* 2008

Venkatachalam *et al.* (1998) reported high frequency of transformants (>20%) by co-culturing of the cotyledonary node, cotyledon, or hypocotyls with *Agrobacterium* in Indian cultivar VRI-2 and TMV-7 based on kanamycin selection. Venkatachalam *et al.* (2000) reported that the cotyledon from germinated seeds of cultivar TMV-2 could be efficiently transformed by co-cultivation with *Agrobacterium* strain LBA 4404 harboring the binary vector pBI121 containing the *uidA* and *nptII* genes. A strong GUS activity was detected in the putatively transformed plants by histochemical assay. Integration of T-DNA into nuclear genome of transgenic plants was further confirmed by southern hybridization with *nptII* gene probe.

Sharma and Anjaiah (2000) produced transgenic plants by using cotyledon explants from mature peanut seeds of two cultivars, one Spanish (JL-24) and one virginia (ICGS-44). Efficient transformation of these cotyledons by using *A. tumefaciens* strain C58 carrying *nptII* and *GUS*, *uidA*, or coat protein gene of the Indian peanut dump virus (IPCVcp) and *nptII* on binary vectors (pBI121, pROKII:IPCVcp) led to the production of transgenic plants. Integration of the transgenes and stable genetic transformants in the progeny were assessed by PCR amplification of 700-bp fragment of *nptII* and 585-bp of IPCVcp genes, and Southern blot hybridizations in the T1 generation of transgenic plants. On an average, 120-150 days were required between the initiation of explant transformation and transfer of rooted plants to the greenhouse. Integration patterns in progeny of transgenic plants were shown for only JL-24. This transformation system appears very straight forward, neither requiring *vir* gene induction treatment nor a lengthy tissue culture phase. This experiment proved the genotypic independency but other researchers using the cotyledon as an explant did not achieve the same frequency or magnitude of regeneration response.

Qiusheng *et al.* (2005) studied the effects of antioxidants (Ascorbic acid (AA), sodium selenite (Se), tocopherol (TOC) and glutathione (GSH)) during the plant regeneration and co-cultivation with *A. tumefaciens,* strain LBA 4404 harboring the binary plasmid PFZY1 was used. This plasmid contained the *GUS* reporter gene and the cowpea trypsin inhibitor (CpTI) gene driven by the CaMV35S promoter, and the *nptII* gene under the control of the nopaline synthase gene promoter. GSH, TOC and Se not only eliminated the formation of H_2O_2 produced in wound tissue during preparation of leaflets and co-cultivation with *A. tumefaciens* and decreased malondialdehyde (MDA) formation, but also enhanced superoxide dismutase (SOD) and catalase (CAT) activities. As a result,

GSH, TOC or Se increased the frequency of plant regeneration and transformation efficiency of peanut explants by *A. tumefaciens*.

Anuradha *et al.* (2006) have generated putative tagged lines in *Arachis hypogaea* L. cv. JL-24 using cotyledon node (CN) as an explant and a promoterless *gus::nptII* bi-functional fusion gene mediated by *Agrobacterium* transformation. Parameters enhancing genetic transformation viz. seedling age, *Agrobacterium* genetic background and co-cultivation periods were studied by using the binary vector p35SGUSINT. Genetic transformation with CN explants from 6-day-old seedlings co-cultivated with *Agrobacterium* GV2260 strain for 3 days resulted in high kanamycin resistant shoot induction. Among the *in vivo* GUS fusions studied with promoterless *gus::nptII* construct, GUS-positive sectors occupied 38% of the total transient GUS percentage. Generated plants showed 3.54% stable integration of the fusion gene as evident from GUS, polymerase chain reaction (PCR) and Southern blot analyses.

Recently *A. rhizogenes* mediated transformation has also been applied for plants improvement. *A. rhizogenes* is well known as a soil-borne bacterium that induces hairy roots in infected plant tissues by transferring T-DNA of the Ri-plasmid into the plant genome. In some species, difficulty in regenerating transgenic plants has been circumvented by development of rapid and efficient transformation protocols using *A. rhizogenes* to produce hairy roots on "composite" plants (an untransformed plantlet with hairy roots). These composite plants have been used in studies focused on root characteristics such as nod-in *L. japonicus* (Stiller *et al.* 1997), soybean (Narayanan *et al.* 1999), and barrel medic (Boisson-Dernier *et al.* 2001). Besides production of composite plants, binary and co-integration vectors introduced into *A. rhizogenes* strains have also been used to transform several plant species, including *Eustoma grandiflorum* (Handa 1996), melon (Toyoda *et al.* 1991), petunia (Kiyokawa *et al.* 1992b), *Tylophora indica* (Chaudhuri *et al.* 2005) and *Gmelina arborea* (Dhakulkar *et al.* 2005).

Reports on transformation of peanuts using *A. rhizogenes* are limited. Akasaka et al (1998), reported induction of transformed hairy roots at the excised site of the epicotyl of dry mature seed of a Spanish type peanut cv. Java 13 after 2 wks of inoculation with wild type strain of *A. rhizogenes*, MAFF-02-10266. Composite plants consist of only transformed root with non-transformed shoots. Forty days after inoculation, composite

plants show a root system with abundant root mass, more lateral branching and high fractal dimension compared to the control.

To deliver a highly defined and enriched resveratrol product, hairy root cultures of peanut were established and tested as a bioproduction system for resveratrol and associated derivatives (Bolivar et. al. 2007). Results demonstrate the capacity of hairy root cultures as an effective bioprocessing system for valued nutraceuticals like resveratrol and resveratrol derivatives. Hairy roots may offer a scalable and continuous product recovery platform for naturally derived, high quality, enriched nutraceuticals, provided effective means to induce and recover high levels of resveratrol and associated derivatives from the media fraction are available.

Particle bombardment mediated genetic transformation in peanut:

Direct DNA transfer methods such as microprojectile bombardment can circumvent the genotype dependence of *Agrobacterium* infection (Ozias-Akins and Gill 2001). Schnall and Weissinger (1993) used zygotic embryos for biolastic-mediated transformation. The apical meristem housing the germ line cells was easily exposed for bombardment without compromising the viability of the plant. Microprojectile bombardment did not substantially impair embryo development. Ozias-Akins *et al.* (1993) transformed embryogenic callus derived from immature embryos by microprojectile bombardment, followed by stepwise selection for resistance to hygromycin. Livingstone and Birch (1999) described production of efficient fertile transgenic plants of diverse peanut cultivars, by particle bombardment of embryogenic callus derived from mature dry seeds, followed by escape free (not stepwise) selection for resistance to hygromycin and brief osmotic desiccation followed by sequential incubation on charcoal and cytokinin-containing media, resulting in efficient conversion of transformed SEs into fertile, non-chimeric, transgenic plants. Reporter gene (*luc*) expression was confirmed in T_1 progeny. Lacorte *et al.* (1997) evaluated the effect of parameters involved in the transformation efficiency of peanut seed tissues by particle bombardment, using the reporter gene *GUS*. They found that transient expression of GUS was affected by both particle and DNA amounts and was positively correlated with gene copy number, but no influence of plasmid size was observed. The gene products of both the Brazil nut methionine-rich 2S albumin and *GUS* genes under the transcriptional control of the 35S promoter were detected by ELISA assays.

Wang et al (1998) demonstrated direct DNA delivery via microprojectile bombardment using embryogenic cultures from three peanut cultivars. They have used two plasmid constructs containing a *uidA* gene controlled by either a soybean vegetative storage protein gene promoter or a cauliflower mosaic virus 35S promoter. Fertile transgenic peanut plants were produced. Yang *et al.* (2001) demonstrated the use of cotyledons of immature peanut zygotic embryos as explants for particle bombardment with a plasmid containing chimeric intron-*GUS* and a chimeric *hph* gene conferring resistance to hygromycin. The presence and integration of foreign DNA in regenerated hygromycin resistant plants was confirmed by PCR and by Southern analysis of the *hph* gene.

Singsit *et al.* (1997) introduced a gene encoding the *Bacillus thuringiensis cryIA(c)* protein into peanut by microprojectile bombardment of SEs initiated from immature peanut cotyledons of two cultivars. DNA from hygromycin-resistant embryogenic cell lines, regenerated plants, and a progeny plant showed the presence and integration of *hph* and *Bt* genes by PCR and or Southern blot analyses. ELISA immunoassay of the CryIA(c) protein from the hygromycin-selected plants showed the expression of CryIA(c) protein up to 0.18% of total soluble protein. Insect feeding bioassay of transformed plants indicated various levels of resistance to the lesser cornstalk borer.

Peanut transgenic for TSWV gene construct was 1st reported by Brar *et al.* (1994). Shoot meristems of mature embryo axis were bombarded with *gus*, *bar* and tomato spotted wilt virus-nucleocapsid protein (*tswv*-np) genes. Screening of GUS activity identified transgenic shoots, and independent transformants were recovered. Molecular analysis of the plants demonstrated the stable integration of the foreign genes into the plant genome. Progeny's had one to multiple copies of integrated genes.

Li *et al.* (1997) mechanically inoculated T1 plants of cv. New Mexico Valencia A, which developed symptoms 10 to15 days later than non-transgenic controls, and were protected from systemic infection.

Yang *et al.* (1998) transformed embryogenic cultures of three commercial peanut cultivars using nucleocapsid protein (N) gene of the lettuce isolate of tomato spotted wilt virus (TSWV). Constructs containing the *hph* gene resistant to the antibiotic hygromycin

and the *tswv* N gene were used for bombardment of peanut SEs. They noted divergent levels of transgene expression in the transgenic plants and their progeny. One transgenic plant, which contained a single copy of the transgene, expressed the N protein in the primary transformant, and the progeny segregated in a 3:1 ratio based upon ELISA determination. Many of the primary transgenic plants became infected with TSWV and were not able to set seed. No resistance data on the remaining plants were presented.

Magbanua *et al.* (2000) transformed embryogenic cultures of two cultivars VC1 and AT120 with TSWV. The selectable marker (hygromycin resistance) and the N gene were on separate plasmids. A total of 207 VC1 and AT120 hygromycin lines were produced. After field trial of transgenic plant resulted that, 76% of the N+ plants were symptomless, while 2% were severely symptomatic and dead. In contrast, only 42% of the plants lacking the N gene were symptom less and 50% were severely symptomatic or dead.

Sclerotinia minor Jagger is the causal agent of Sclerotinia blight, a highly destructive disease of peanut. Livingstone *et al.* (2005) recovered the transgenic peanut plants from embryogenic cultures of Virginia peanut, for increased resistance to *Sclerotinia minor*.

Immature zygotic embryos of peanut was transformed via particle bombardment with a plasmid containing a Bluetongue *VP2* gene (*BTVP2*) comprising neutralizing epitopes (Athmaram *et al.* 2006). Under continuous selection, 12.38% kanamycin resistant embryos were recovered. The expression of the BTVP2 protein was confirmed through RT-PCR (reverse transcription polymerase chain reaction) using the RNA isolated from the transgenic callus employing BTVP2-specific primers.

Yang *et al.* (2003) introduced the bacterial mercuric ion reductase gene, *merA* into embryogenic cultures, in order to test an alternative selectable marker system for the production of transgenic peanut plants. MerA reduces toxic Hg(ll) to the volatile and less toxic metallic mercury molecule, Hg(0), and renders its source Gram-negative bacterium mercury resistant. Stable transgenic plants were recovered through hygromycin-based selection from SE tissues bombarded with the plasmid containing both genes. However, no transgenic SEs were recovered from selection on 50-100 μmol/L $HgCl_2$.

Joshi *et al.* (2005) used green fluorescent protein as a non-destructive marker for peanut genetic transformation and evaluated two fluorescent protein mutants (enhanced GFP (EGFP) and enhanced yellow fluorescent protein (EYFP) for their transient expression efficiency after particle bombardment of embryogenic cultures of the peanut cultivar, Georgia Green. Embryogenic lines selected for GFP expression initially may have been chimeric since quantitative analysis of expression sometimes showed an increase when GFP- expressing lines, that also contained hygromycin-resistance gene, subsequently were cultured on hygromycin. Integration of the *gfp* gene in the genomic DNA of regenerated plants was confirmed by Southern blot hybridization and transmission to progeny.

Complex pattern of transgene integration is the major draw back of direct DNA delivery method technique because of that *Agrobacterium*-mediated transformation still remains to be the most opted method as it is devoid of most of the limitations inherent in the direct gene delivery method (Chandra and Pental 2003). Ease of the protocol with minimal equipment costs makes it most attractive. This technique efficiently introduces variability in transgenic plants due to stable integration and appropriate expression of the limited copy numbers of the transgenes. These advantages were a driving force to adapt this system to many different crops (Finnegan and Flroy 1994; Gould 1997; Kumpatla *et al.* 1998; Hansen and Wright 1999).

OBJECTIVES OF THE PRESENT STUDY

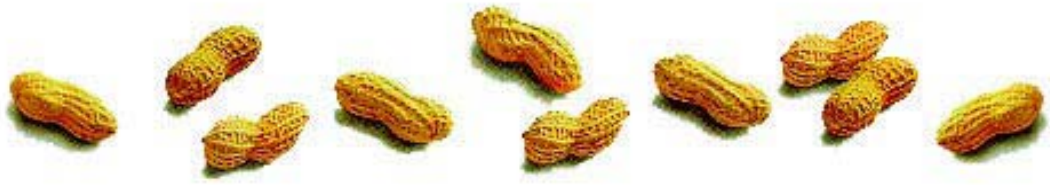
Keeping in view the limitations of the existing protocols, there is increasing demand to develop reliable protocols for *in vitro* regeneration and to study the basic processes involved in peanut. Efficient regeneration protocol via direct SEsis is a pre-requisite for the optimization of a reliable transformation protocol. Present study was formulated to address some basic questions associated with induction and development of SEs and regeneration of these SEs into viable plantlets. The work attempts to find suitable medium and duration for induction of SEs vis-à-vis appropriate nutrient composition and cultural conditions, in peanut using mature zygotic embryo-derived leaflets and mature zygotic embryo axes and axenic culture-derived meristem. Keeping in view the role of TDZ in induction of SEsis, and its role in triggering meristematic activity in the plumule of abnormal SEs of peanut (Chengalrayan *et al.* 1997; Joshi *et al.* 2003) influence of this

growth regulator was studied in early embryo development with respect to pulse treatment. This experiment was conducted in anticipation of obtaining physiologically normal SEs. A part of the study was formulated to understand the TDZ induced pathway of embryo conversion. The resultant emblings were ascertained regarding their uniformity. For the application of genetic transformation approach there is a need to develop reliable protocols for genetic transformation. Hence we have attempted to standardize transformation protocols with *Agrobacterium rhizogenes* as an alternative to the *Agrobacterium* strains used so far, which is expected to result in better efficiencies in terms of numbers of transgenic plants recovered. In this study, various parameters for *Agrobacterium*-mediated transformation were optimized. To summarize, the present study on Peanut was initiated with the following objectives:

1) Morphological studies to obtain normal fertile peanut plants at high frequency via somatic embryogenesis.
2) Optimization of the protocol for transformation using *Agrobacterium rhizogenes* for genetic improvement.

Chapter 2

Materials and methods



INTRODUCTION

This chapter describes the general techniques and materials routinely practiced in plant tissue culture and in isolation and amplification of DNA. The materials and methods, specific to particular experiment, are dealt in details in respective chapters. The method used in genetic transformation study by *Agrobacterium* mediated transformation is described in chapter V.

2.1 PLANT TISSUE CULTURE

2.1.1 MATERIALS

Most of the consumables and chemicals were procured from various local suppliers. Few chemicals including the growth regulators were imported.

Glasswares:

Test tubes (25x150mm), conical flasks (100, 250 and 500ml capacity), pipettes (0.1,0.2,1,2,5,10 ml capacity), Beakers (100, 250, 500 and 1000 ml), jam bottles and measuring cylinders (25ml, 100ml, 1000ml capacity) of Borosil, India, were used for culturing the tissues, and for preparation of media, reagents etc. Autoclavable, screw cap bottles (100, 250 and 500ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60ml capacity) (Tarson, India), slides (Blue Star, India) and cover slips (Micro-Aid, India).

Plastic wares:

Plasticwares including sterile disposable plastic petriplates of 55 and 85 mm diameter were procured from Tarson and Laxbro, Pune, India. Klin wrap, used for sealing the petriplates and bottles were from local market. Micropipette of different precision measurements (1000µl, 200µl, 100µl, 20µl, 10µl and 2µl) were procured from Gilson, USA and microtips (1ml, 200µl, 10µl), Eppendorf tubes (2ml, 1.5ml, 1ml and 0.5ml) were procured from Tarson, Pune, India.

Chemicals:

Chemicals used for surface sterilization procedures were Savlon (Johnson and Johnson Limited, USA) and Mercuric chloride (Qualigens Fine Chemicals, India). An antibiotic, Cefotaxime (Alkem, India) was often used for controlling the growth of the contaminating bacteria in plant tissue culture media.

All major, minor, inorganic salts and vitamins used for preparation of culture media and for other experiments were of analytical grade (BDH, Hi-Media and Qualigens Fine Chemicals, India). Sucrose was procured from Hi-Media (India). Agar agar (bacteriological grade), used as gelling agent in the semisolid culture medium was procured from Hi- Media (India).

Growth regulators including 2,4-dichlorophenoxy acetic acid (2,4-D), 4-amino-3, 5, 6-trichloropicolinic acid (Picloram), Kinetin (KN), 6-benzyl adenine (BA), and N-phenyl-N'-1, 2,3-thidiazol-5-ylurea (Thidiazuron, TDZ) were obtained from Sigma (U.S.A.). The chemicals used for ISSR-PCR were obtained from "Sigma Chemical Co.," USA.

Chemicals used for histological studies including formaldehyde solution, glacial acetic acid and xylene were procured from Qualigens Fine Chemicals, India. Ethanol, 2-methyl propan-2-ol (tertiary butyl alcohol), iron alum was from S.D. fine chemicals, India; paraffin wax (m.p. 58-60°C) from E. Merck, India Ltd., Haematoxylin and Eosin stain from Hi-Media Laboratories Pvt. Ltd., Bombay, and DPX-4 [189-(2-chloro-N- (4-methoxy-1, 3, 5-triazin-2-yl amino carbonyl) benzene sulphanamide)] mountant was from BDH, India.

Equipments:

The major equipments used in the present study include: Electronic Balance (Contech), pH meter (Thermo Orion), Autoclave (Nat Steel Equipment Private Limited Bombay), Laminar airflow ultra clean unit (Klenzoids/ Microfilt, India), Magnetic stirrer (Remi, India), Steamer (Ultradent, India), Temperature controlled oven (Pathak Electricals, India), Light microscope (Carl-Zeiss Jena), Microtome (Reichert Jung), Camera (Nikon/Zeiss), membrane filter sterilizing unit (Laxbro, Pune) and Pipetman (Gilson/Tarson).

2.1.2 METHODS

Preparation of glassware and instruments:

Glassware used in our studies was cleaned by boiling in saturated solution of sodium bicarbonate for 1 h followed by washing in tap water. These were then immersed in 30% nitric acid solution for 30 min and were washed thoroughly with tap water. After rinsing with double distilled water these were allowed to dry on a draining rack.

Except the heat labile materials sterilization of all required materials was carried out by autoclaving at 121°C, 15 lb psi for 1h. The heat labile materials were sterilized aseptically using the 0.22 μm pore size millipore filtration membranes procured from Advanced microdevices (P) Ltd., Ambala, India.

Tubes and flasks were plugged with absorbent cotton (Safe Surgical Industries, Beawar, India). Forceps, scalpels, millipore filter units etc. were wrapped in aluminum foils and then with brown paper. These were packed in autoclavable bag and autoclaved. Ordinary grade filter paper pieces of approximately 10x20cm were kept in stack alternatively with brown paper pieces of similar size. These were packed in autoclavable plastic bags with 20-25 pieces in each bag and autoclaved. Dissection and transfer of explants were carried out on these sterile papers under aseptic conditions and disposed after use. Microtips used for aseptic addition by micropipettes were arranged in cases meant for their size, wrapped with brown paper and autoclaved. Sterilization of the glassware and instruments was carried out by autoclaving at 121°C for 1h in 15 lbs/(inch)^2.

Preparation of media:

Concentrations of the macro and microelements, salts and organic constituents of the MS (Murashige and Skoog, 1962) basal medium are listed in Table 2.1 Stock solutions of the media ingredients were prepared by dissolving weighed amounts of these salts as per Table 2.1 in double distilled water. Appropriate aliquots of these solutions were mixed to prepare the media.

Table 2.1: Composition of Murashige and Skoog basal medium (Murashige and Skoog 1962).

Ingredients	**Amount ($mg.l^{-1}$)**	**Stock solution**
Macronutrients		**(20 X) in 500mL**
KNO_3	1900	19 g
NH_4NO_3	1650	16.5 g
$CaCl_2.2H_2O$	440	4.4 g
$MgSO_4.7H_2O$	370	3.7 g
KH_2PO_4	170	1.7 g
Micro-nutrients		**(100 X) in 100 mL**
$MnSO_4.4H_2O$	22.3	62 mg
$ZnSO_4.7H_2O$	8.6	223 mg
H_3BO_3	6.2	86 mg
KI	0.83	2.5 mg
$CuSO_4.5H_2O$	0.025	0.25 mg
$Na_2MoO_4.2H_2O$	0.25	8.3 mg
$CoCl_2.6H_2O$	0.025	0.25 mg
$FeSO_4.7H_2O$	27.8	278 mg
$Na_2EDTA.2H_2O$	37.3	373 mg
Vitamins		**(100 X) in 100 mL**
Myo-inositol	100	1 g
Thiamine-HCl	0.1	5 mg
Nicotinic acid	0.5	20 mg
Pyridoxine-HCl	0.5	1 mg
Glycine	2	5 mg

Stock solutions of growth regulators (GR) were prepared by adding few drops of solvent (EtOH, 1N NaOH, DMSO etc.) in the weighed amount of growth regulator to dissolve. After dissolution, the volume was made by the addition of sterile double distilled water. The solutions were stored in refrigerator in sterilized bottles. Table 2.2 describes the list of solvent and diluents, stock concentration of growth regulators used.

Table 2.2: Preparation and Concentration of Growth Regulators

Growth Regulator		Molecular weight	Solvent	Diluents	Stock Concentration
Chemical name	Abbreviation				
2, 4-dichloro phenoxy acetic acid	2,4-D	221.0	EtOH	Distilled H_2O	10 mg/100mL
N-phenyl-N'-1, 2, 3-thidiazol-5-ylurea	TDZ	220.2	DMSO/ 1N NaOH	Distilled H_2O	10 mg/100mL
4-amino-3, 5, 6-trichloro picolinic acid	PIC	241.5	1N NaOH	Distilled H_2O	10 mg/100mL
6-benzyl aminopurine	BA	225.3	1N NaOH	Distilled H_2O	10 mg/100mL
Kinetin	KN	215.2	1N NaOH	Distilled H_2O	10 mg/100mL

For media preparation a calculated amount of aliquots were added from stock solutions. Sucrose was weighed and added in required quantity (2%, 3%, 6%) and allowed to dissolve. pH of all the media was adjusted to 5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent. The volume was made up with double distilled water in appropriate flask. Gelling agent (agar agar) was added and were autoclaved for 20 min. at 121°C and 15 lbs/ $(inch)^2$. Autoclaved media was poured aseptically in sterile petridishes and gelled, as and when required for culturing tissues. For 55 mm dish 10 ml medium was poured and 20 ml medium was poured in 85 mm dishes.

For culturing in tubes and bottles, agar was added in the media and heated on water bath for the agar to melt. Molten medium was dispersed into sterile culture tubes (20 ml of media) or bottles (50ml of media) after thorough mixing and was autoclaved for 20 min. at 121°C and 15 lbs/$(inch)^2$. Media additive sterilants like antibiotic taxim (Cefotaxime)

in required concentration were added aseptically to the autoclaved semisolid medium before distributing in culture vessels.

Collection of Plant material:

Seeds (Fig.2.1a) of peanut (cv. SB-11) were procured from the local market in the month of December - January. The seeds were stored at 4^0C and used through out the year for both *in vitro* and *ex vitro* experiments.

Seedling culture:

For seedling cultures, peanut seeds were washed with detergent for 10 minutes followed by repeated washing with distilled water. Thereafter the seeds were treated with 4% savlon for 12 minutes. On removing savlon with repeated washing with sterile water, these seeds were disinfected by 0.1 % $HgCl_2$ treatment for 10 minutes. Repeated washing with double distilled water eliminated the adhering $HgCl_2$. Testa of these seeds (Fig.2.1a) was removed aseptically and the seeds were cultured in agar gelled MS basal medium supplemented with 2 % sucrose. After incubating the cultures for 3 - 4 days in dark for radicle emergence, the seedlings were transferred in 16 hr photoperiod at 25 ± 2°C.

Leaflets culture for somatic embryogenesis:

The mature zygotic embryo axes (Fig.2.1b), excised from the seeds of SB-11 were washed with liquid detergent (Labolin, Qualigens India) for 5 minutes. These were surface sterilized with 0.1 % (w/v) mercuric chloride solution for 2-3 minutes followed by 4-5 rinses with sterile glass-distilled water under aseptic conditions. Embryo axes were soaked in sterile double distilled water for 12-16 hours. Prior to isolation of the explants the soaked embryo axes were surface sterilized once again with 0.1 % (w/v) mercuric chloride and rinsed 4-5 times with sterile glass-distilled water.

Cultures were initiated from the surface sterilized explants. All the dissections were carried out on sterile filter papers. Apical part of embryo axis (Fig.2.1b) and mature zygotic embryo axis derived leaflets (Fig.2.1c) were excised with sharp, fine forceps. During aseptic operations these were heat sterilized intermittently by dipping in rectified spirit and flaming.

After transferring the tissues, the petriplates were sealed with Klin wrap. All cultures were labelled appropriately prior to incubation. The number of explants used in each repeat, and the number of repeats of each experiment are described in materials and methods of the respective section.

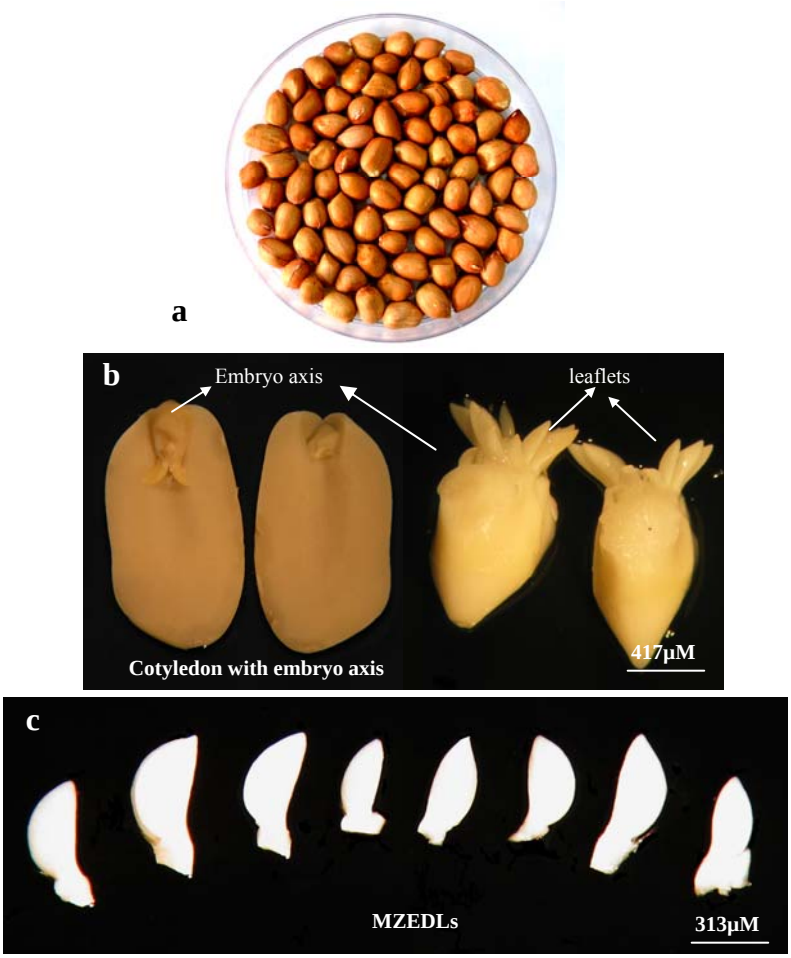


Fig.2.1 (a) Seeds of peanut (var. SB-11) with seed cover/testa.
(b) Mature zygotic embryo axis with and without cotyledon.
(c) Isolated leaflets from mature zygotic embryo axis.

Culture conditions:

Cultures were incubated in 16h photoperiod at 32 µE $m^{-2}s^{-1}$ light intensity at 25±2°C. For dark incubation, cultures were kept in BOD maintained at 25±2°C. During hardening procedures, the cultures were incubated in continuous light (24h), other conditions remaining unchanged.

Transfer of plantlets and Hardening:

In vitro raised plantlets were carefully taken out of the test tubes. The roots were washed gently under tap water to remove the adhering gelling agent and medium. The plantlets were dipped for 10-15 min in 1 % aqueous solution of Bavistin, a systemic fungicide (BASF, India) prior to transfer to soil. The treated plantlets were transferred to autoclaved soil: sand (1:1) mixture in plastic cups and were covered with transparent polypropylene bags to prevent the rapid loss of humidity and were incubated in continuous light (24h) for two weeks. The plants were watered once a week. The top corners of polypropylene bags were cut after two weeks to gradually expose the plants to the outside environment. After 2 weeks pots were shifted in green house. After 3-4 weeks, the polypropylene bags were completely removed and plants were shifted in earthen pots containing a mixture of soil: sand (1:1).

Histological Preparations:

Tissues were prepared for histological studies following the methods described by Sharma and Sharma (1980). Tissues were cut into small pieces (approx 3 x 4 mm) and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48-h at room temperature. These were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 10 μM were cut using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with haematoxylin-eosin and mounted with DPX *(Loba Chemie,* Mumbai, India) for studies under microscope.

Microscopy and Photography:

The morphogenic response in various explants was evaluated under stereo microscope and was photographed. Histological slides were studied under microscope and photographed. Magnifications of the photomicrographs were noted.

Statistical Procedures:

Statistical methods were used for comparison of treatment means during optimization of the parameters for somatic embryogenesis using different explants. Completely Randomized Designs were used. The data was subjected to analysis of variance (ANOVA) and treatment means were compared (Panse and Sukhatme, 1967). The differences among the treatment

means were tested using Duncan multiple range test (DMRT) at a 5% probability level ($P<0.05$), wherever applied. The data was analyzed using Microsoft Excel package.

2.2 MOLECULAR CHARACTERIZATION

2.2.1 MATERIALS

Source of plant material:

Leaves of control seedlings and leaves of somatic embryo derived plantlets were used for DNA isolation and ISSR.

Reagents and Chemicals for ISSR:

- Tris-HCl pH 8.0 (1M); EDTA pH 8.0 (0.5M); NaCl (5M); CTAB (20%); Chloroform:Iso-amylalchol (24:1 v/v); Polyvinyl pyrrolidone; β-mercaptoethanol; cold isopropanol and ethanol (70%)
- Extraction buffer: 100mM Tris-HCl (pH 8.0), 25mM EDTA, 1.5M NaCl, 2.5% CTAB, 0.2% β-mercaptoethanol (v/v) (added immediately before use) and 1% PVP (w/v) (added immediately before use).
- High salt TE buffer: 1M NaCl, 10mM Tris-Cl (pH 8.0) and 1mM EDTA.
- Agarose (Sigma, India)
- Electrophoresis buffer: Tris-acetate-EDTA (0.5x)
- Loading buffer: Bromophenol blue (0.25%) and glycerol (30%)
- Fluorescent dye: Ethidium bromide (10µg/mL)
- Marker: Low range DNA ladder (3 Kb) (Genei, India)
- Enzymes: RNAase A (10mg/mL) and Taq DNA Polymerase (Genei, India)
- Buffers: *Taq* DNA Polymerase buffer with $MgCl_2$ (Genei, India)
- Nucleotides: dNTPs (G, A, T, C) (Genei, India)
- PCR additives: Spermidine (20mM)
- Primers: UBC ISSR Primer synthesized from Sigma was used (Table 2.3).

Table 2.3: ISSR primers used for experiment

ISSR primer	Primer sequence
UBC 808	AGA GAG AGA GAG AGA GC
UBC 811	GAG AGA GAG AGA GAG AC
UBC 818	CAC ACA CAC ACA CAC AG
UBC 819	GTG TGT GTG TGT GTG TA
UBC 823	TCT CTC TCT CTC TCT CC
UBC 825	ACA CAC ACA CAC ACA CT
UBC 826	ACA CAC ACA CAC ACA CC
UBC 827	ACA CAC ACA CAC ACA CG
UBC 834	AGA GAG AGA GAG AGA GYT
UBC 835	AGA GAG AGA GAG AGA GYC
UBC 840	GAG AGA GAG AGA GAG AYT
UBC 845	CTC TCT CTC TCT CTC TRG
UBC 847	CAC ACA CAC ACA CAC ARC
UBC 848	CAC ACA CAC ACA CAC ARG
UBC 851	GTG TGT GTG TGT GTG TYG
UBC 855	ACA CAC ACA CAC ACA CYT
UBC 856	ACA CAC ACA CAC ACA CYA
UBC 857	ACA CAC ACA CAC ACA CYG
UBC 859	TGT GTG TGT GTG TGT GRC
UBC 860	TGT GTG TGT GTG TGT GRA
UBC 878	GGA TGG ATG GAT GGA T
UBC 881	GGG TGG GGT GGG GTG

Equipments:

Milli-RO water system (Millipore, USA) for deionized water, Horizontal electrophoresis unit (Tarson, India), UV Transilluminator (for gel visualization under ultraviolet radiation), Water bath (Julabo), Spin Win (Tarson), Spectrophotometer (Perkin & Elmer), PCR (Eppendorf, Germany) were used for experiment.

2.2.2 METHODS

DNA isolation protocol: The DNA isolation was carried out based on the Khanuja's protocol (Khanuja et al., 1999). The protocol is as follows:

1. About 150 mg of leaf tissue was ground into a fine powder in liquid nitrogen.
2. The powder was transferred to 2ml microfuge tube and added 1ml of freshly prepared extraction buffer, Mixed by inversion to a slurry
3. Incubated at 65 ºC in a waterbath for 30-60mins with occasional mixing.
4. Added 1ml of choloform: isoamyl alchol (24:1) and mixed by inversion for 15 min.
5. Spinned at 8000 rpm for 10 min at 25-30 ºC.
6. Carefully transferred the upper clear aqueous layer to another 2ml microfuge tube.
7. Added 200µl of 5M NaCl and mixed properly (do not vertex).
8. Added 500µl of cold Isopropanol and by careful mixing produced fibrous nucleic acid that can be scooped and transferred to 2 ml microfuge tube and centrifuged.
9. Discarded the supernatant and washed the pellet with 70% ethanol.
10. Dried the pellet in vacuum for 15 min and dissolved it in 300µl high salt TE buffer.
11. Added 5 µl of RNAase A and incubated at 37ºC.
12. Added 300µl of choloroform: isoamyl alchol (24:1) and mixed by inversion.
13. Transferred the aqueous layer to a fresh 1.5ml microfuge tube and added equal volumes of isopropanol.
14. Spinned at 10,000 rpm for 10 min at 25-30ºC.
15. Washed the pellet with 70% ethanol. Dried the pellet in vacuum and dissolved in 200µl of sterile milli Q water and then stored at 4ºC until required.

DNA concentrations were determined by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis and by taking the absorbance at 260 nm. The purity of the DNA was assessed with the ratios of absorptions at 260 nm and 280 nm.

PCR protocol:

- The PCR protocol described by Raina et al. (1995) was followed and carried out in a total volume of 25µl containing following components: 2µl of genomic DNA (20ng); 2.5µl of 10X *Taq* buffer with 1.5mM $MgCl_2$; 2.5µl of dNTPs (1mM/µl); 0.16µl of

Taq Polymerase (3u/µl); 0.5µl of Primer (1.5pm/µl); 1µl of Spermidine (20mM) and 16.34µl of sterile milliQ water.

- PCR amplifications were performed on a Eppendorf under the following program: Initial denaturation at 94°C for 7 min, followed by 44 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, extension at 72°C for 2 min with a final extension at 72°C for 7 min (Fig.2.2).
- Negative controls, containing all PCR components except DNA were also set up and run with each set of reactions.
- A low range DNA ladder (100bp-3000bp) was used for band sizing.
- The ISSR products were loaded on 1.5% agarose gel stained with ethidium bromide for electrophoresis in 0.5X TAE at a constant current 60mA, < 150V for 2 h. Visualization of gel was undertaken in Gel Documentation system.

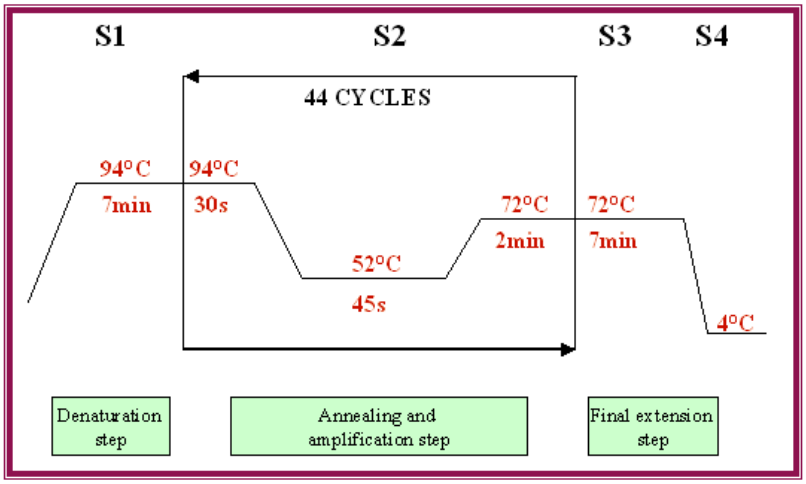


Fig.2.2 Schematic diagram of PCR process.

Data Analysis:

Presence/absence of each scorable fragment was recorded in a binary data matrix and the frequency of each band in sample was determined. Wind Dist software program in NTSYS format was used to produce similarity matrix.

Chapter 3

Influence of 2,4-D and TDZ on somatic embryogenesis

INTRODUCTION

A number of reports exist for regeneration of peanut plants via somatic embryogenesis using a variety of explants and plant growth regulators (PGR). However, plant recovery has been limited (Ozias-Akins 1989; McKently 1991; Baker and Wetzestin 1992; Durham and parrot 1992; Ozias-Akins *et al.* 1993; Wetzestin and Baker 1993; Eapen *et al.* 1993; Reddy and Reddy 1993; Chengalrayan *et al.* 1994). The major snag in peanut somatic embryogenesis is low conversion frequency of somatic embryos into normal plantlet. Failure of the peanut somatic embryos to convert into plantlets is attributed to morphological abnormalities of somatic embryos (Chengalrayan *et al.* 1997, 2001; Wetzestin and Baker 1993). To increase the frequency of plant recovery, attempts have been made to obtain normal somatic embryos by exposing the embryos to the germinating medium (Ozias-Akins 1992a) and subsequently by triggering shoot formation from the plumule of rooted embryos in presence of BAP and KIN (Chengalrayan *et al.* 1995). Somatic embryo conversion was also achieved in presence of BAP and NAA (Venkatachalam *et al.* 1997, 1999a, b). TDZ was effective in inducing differentiation in the plumule of the rooted somatic embryos which were developed in presence of 2,4-D (Chengalrayan *et al.* 1997; Joshi *et al.* 2003). However there is no report on genetic uniformity/variability in the peanut plants generated via somatic embryogenesis.

Our group has been contributing significantly towards regeneration of peanut plants via organogenesis and embryogenesis (Chengalrayan *et al.*1997; Joshi *et al.* 2003). However none of the existing protocols is efficient since the conversion of somatic embryos to plantlet was poor and involves several manipulations for conversion. Keeping in view the influence of 2,4-D and TDZ in triggering morphogenesis in plumule of peanut somatic embryos, the present experiments were conducted to study the effect of 2,4-D and TDZ in early embryo development by incorporating these growth regulators at initial developmental stages for varying durations to trigger plumule differentiation at the right stage of embryo development. In plant propagation, a major concern is to retain genetic integrity of the plants, as *in vitro* culture techniques could induce genetic variability. Thus, genomic stability of these regenerated plants was determined by using molecular markers.

The present investigation was designed and conducted in three parts.

1) ***Influence of 2,4-D exposure on peanut somatic embryogenesis***
2) ***Effect of TDZ at initial stages of somatic embryogenesis in peanut***
3) ***Assessment of plants regenerated via somatic embryogenesis for genetic uniformity/variability***

The objective was to obtain genetically uniform, physiologically mature normal somatic embryos with well-defined plumule and radicle to achieve high conversion frequency, in reduced time, and involving fewer manipulations, using MZEDLs as explant.

3.1 INFLUENCE OF 2, 4-D ON PEANUT SOMATIC EMBRYOGENESIS

Among the auxin analogues used for induction of somatic embryogenesis, 2,4-D is by far the most efficient and, hence, used more often for induction of somatic embryogenesis. In more than 65% of the protocols, 2,4-D was applied alone or in combination with other plant growth regulators (Gaj 2004). The high efficiency of 2,4-D for induction of embryogenic response found in different *in vitro* systems and plant species indicates a specific and unique character of this plant growth regulator. This synthetic growth regulator appears to act not only as an auxinic herbicide but also as an effective stressor. It (2,4-D) brings different changes in physiology and gene expression of cells. This implies its possible role as a stress factor triggering embryogenic pattern of development in cultured plant cells (Feher *et al.* 2003). Some attempts have been made to elucidate the genetic and molecular mechanisms of the process. Analyses of the protein products of *LEC1*, *LEC2*, *WUS/PGA6*, *AtSERK1* and *BBM* genes have led to the suggestion that they modulate somatic embryogenesis by promoting embryogenic transition of somatic cells and/or by maintaining their embryogenic identity, but the signaling cascade triggered by 2,4-D in the induction of somatic embryos remains largely unexplored (Raghavan 2005).

Raghavan (2005) reported that, depending on the duration of pre-treatment with 2,4-D, zygotic embryos of *Arabidopsis* display different morphogenic patterns during subsequent growth in the basal medium and found that an optimum period of auxin treatment also promotes the maturation of somatic embryos and programs their shoot apical meristem for the production of leaves. Similarly Filippov *et al.* (2006) suggested

that the period of exposure to auxins had a significant effect on the efficiency of somatic embryogenesis. Thus optimum exposure to auxin is important for the development of somatic embryos and for their maturation.

For peanut somatic embryogenesis, 2,4-D was most effective and widely used. It induced embryogenesis at high frequency and developed highest number of somatic embryos per responding culture. Hazra *et al.* (1989) reported somatic embryogenesis from immature zygotic embryos and achieved induction and maturation of somatic embryos in 2,4-D. Eapen and George (1993) demonstrated that 2,4-D was most effective among the different auxins (NAA, 2,4-D and picloram) tested and resulted in higher number of somatic embryos per culture. Baker *et al.* (1994) used immature cotyledonary explant in the presence of 2,4-D and NAA. The use of 2,4-D compared to NAA in the induction medium resulted in a greater percentage of embryogenesis and mean number of embryos. Venkatachalam *et al.* (1997, 1999b) reported somatic embryogenesis from peanut seedling-derived leaflets and hypocotyl in presence of 2,4-D and NAA. For the induction of embryogenesis as well as for the production of embryos, 2,4-D was more effective. However, critical timing of 2,4-D treatment for acquisition of embryogenic competence of peanut tissue has not been demonstrated. Baker and Wetzstein (1992, 1998) optimized the incubation period of the explants in the induction medium with 2,4-D in combination with cytokinins, but not just with 2,4-D alone. Gross morphological evaluation of somatic embryos have shown that abnormalities are associated with the use of 2,4-D in the induction medium and morphological quality of somatic embryos affects the efficiency of conversion into plantlets (Rodriguez and Wetzstein 1998).

In concurrence with an earlier report from our laboratory in JL-24 (Chengalrayan *et al.*1994), embryos of SB-11 germinated (100%) to form root and development of shoots were restricted. To assess the influence of 2,4-D exposure on embryo development in the present study, leaflets (MZEDL) of SB-11 were cultured for varying periods in embryogenic mass induction medium (Chengalrayan *et al.*1994) or primary medium (PM) containing 90.5 μM 2,4-D and 6% sucrose. Thereafter the embryogenic masses were cultured in three secondary media (SMs) for embryo induction and developed embryos were tested for conversion in MS basal medium without PGR.

3.1.1 MATERIALS AND METHODS

The MZEDLs (Fig.2.1c) were dissected from plumule of surface sterilized mature zygotic embryo axis and cultured in four groups for 2, 4, 6 and 8 weeks (wks) in PM containing 90.5 μM 2,4-D and 6% sucrose. The response in these explants was noted before dividing the explants from each group into three sub-groups to culture in three secondary media (SM) for 4 wks:

(i) MS basal medium with 2% sucrose (SM-1),
(ii) MS basal medium with 13.6 μM of 2,4-D and 6% sucrose (SM-2), and
(iii) MS basal medium with 90.5 μM of 2,4-D and 6% sucrose (SM-3).

Observation of cultures with embryogenic masses (EMs) and embryos developed from EMs were scored twice at intervals of 2 wks in SMs. Subsequently embryos were transferred to conversion medium i.e., MS basal medium with 2% sucrose and without growth regulator. Cultures were maintained in this medium for three passages of 4 wks each.

All 2,4-D containing media were supplemented with 6% sucrose, whereas MS basal medium was supplemented with 2% sucrose. The pH of the medium was adjusted to 5.8 prior to addition of (0.7%) agar. Media were autoclaved at 120°C under 15psi for 20 minutes. The cultures were incubated in 16h photoperiod at 25 ± 2°C under diffuse cool white fluorescent light ($32\mu Em^{-2}sec^{-1}$). Converted plantlets were hardened as described in Chapter 2 (Section 2.1.2). Survival rate of the plants was calculated from number of plants transferred to soil and the number of plants established.

This experiment was repeated thrice with 35-40 replicates for each treatment. Number of explants that responded in culture and demonstrated morphological changes were scored. Data were subjected to ANOVA analysis.

3.1.2 RESULTS AND DISCUSSION

Response in the leaflets after 2, 4, 6 and 8 weeks in PM

Two wks exposure in PM led to swelling (Fig.3.1a) in 78% (Table 3.1) of the MZEDLs. Extended exposure of the leaflets for 4 wks in PM led to the development of EMs (Fig.3.1b) at the base of the leaflets in 63% of the cultures (Table 3.1). Incubation for 6 and 8 wks in PM led to the development of EMs and subsequently embryos (Fig.3.1c, d) in 70% and 62% of the cultures respectively. Reduction in embryogenic response in PM in the cultures incubated for 8 wks was due to dedifferentiation of some of the EMs to calli.

Response of the cultures in SMs after 2 wks in PM

Culturing of the swollen leaflets (Fig.3.1a) in three SMs led to the development of EM with 1%, 2% and 10% of cultures in SM-1, SM-2 and SM-3, respectively (Table 3.1). Average numbers of developed embryos per explant were 0.8, 0.3 and 1.0 in SM-1, SM-2 and SM-3, respectively after 2 wks. This did not change even after 4 wks.

Response of the cultures in SMs after 4 wks in PM

Embryogenic masses (Fig.3.1b) developed in PM in 4 wks were cultured in 3 SMs for 2 wks. In SM-1 somatic embryo formation was infrequent (Table 3.1) and callusing was predominant (Fig.3.1e). In SM-2, frequency of embryo development (Fig.3.1f) was 82% and in SM-3 (Fig.3.1g) it was 79% (Table 3.1). Average number of embryos after 2 wks in SM-1 was 0.5/explant and in both SM-2 and SM-3 it was 1.9/explant. Average number of embryos did not change even after 4 wks. Embryos developed in SM-3 (Fig.3.1g) appeared often abnormal. None of the embryos developed in the 3 SMs converted into plantlets after 4wks in MS medium without PGR (Table 3.1). However root emergence was 100%. Conversion was slow. Conversion of 3% was achieved from embryos developed in SM-2, whereas it was 0.7% from embryos developed in SM-3 medium after 3 passages in PGR free medium (Table 3.1).

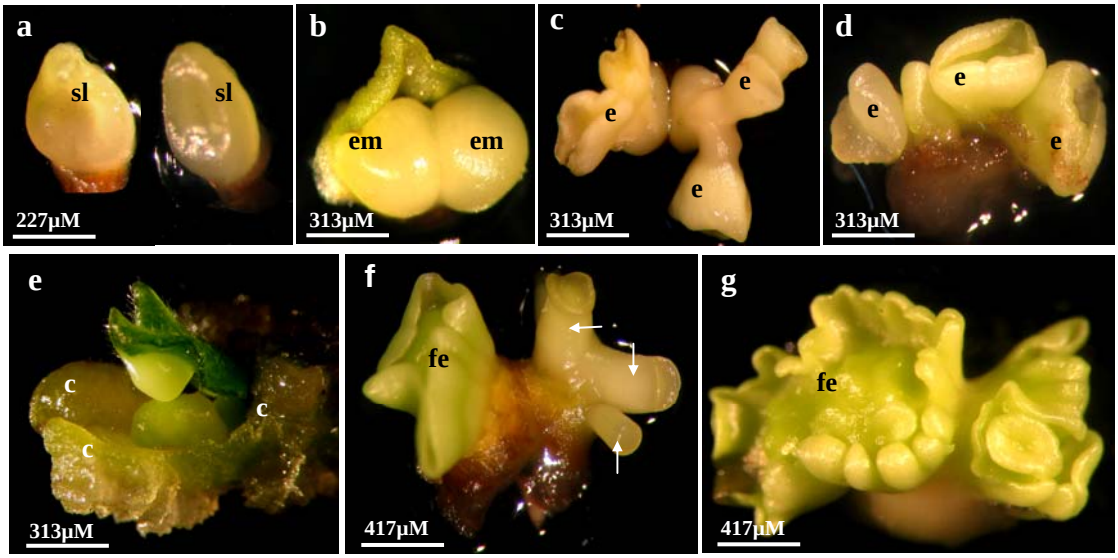


Fig.3.1 a) Swollen leaflet (sl) after 2 wks incubation in PM.
b) EMs developed in the base of MZEDLs after 4 wks in PM.
c) Embryos (e) developed from EM after 6 wks in PM.
d) Embryos (e) developed from EM after 8 wks in PM.
e) EMs developed in PM in 4 wks callused (c) on culturing in SM-1for 4 wks.
f) Single (arrow) and fused embryos (fe) developed from EMs after 4 wks in SM-2 from explants pre-cultured in PM.
g) EMs developed in PM in 4 wks led to the development of fused embryos (fe) after incubating in SM-3 for 4 wks.

Response of the cultures in SMs after 6 wks in PM

Extended incubation of MZEDL in PM for 6wks led to the induction of EMs and subsequently somatic embryos developed in 70% (Table 3.1) of the explants. Transferring the developing embryos to SMs, led to the development of fully formed embryos (Fig.3.2a, b, c) at a frequency of 89, 86 and 91% in SM-1, SM-2 and SM-3, respectively. Average number of embryos ranged between 2-3/explant in all the three SMs (Table 3.1). Embryos developed in all the three SMs were morphologically fused and abnormal (Fig.3.2a, b, c). Embryos developed in SM-1 (Fig.3.2a) were green and less fused as compared to the embryos developed in SM-2 (Fig.3.2b). Somatic embryos developed in SM-3 (Fig.3.2c) were more fused. Shoot and root differentiated (Fig.3.3a) in the embryos developed in SM-1, SM-2 and SM-3 (Table 3.1) on culturing for 4 wks in MS basal medium without PGR. Conversion frequency increased after 2nd and 3rd passage in MS basal medium. It was 27, 40 and 13% after 2nd passage and 33, 53 and 21% after 3rd passage from the somatic embryos developed in SM-1, SM-2 and SM-3 respectively.

Table 3.1 Effect of 2, 4-D exposure on somatic embryogenesis from MZEDLs.

Exposure period in PM	Response in PM (%)	Secondary medium (μM)	Response after 4 wks (EM + embryo formation) mean±sd* (%)	Average no. of embryo (after 2 wks in SM) mean±sd	Average no. of embryo (after 4 wks in SM) mean±sd	Conversion after 1st subculture in MS mean±sd** (%)	Conversion after 2nd subculture in MS mean±sd (%)	Conversion after 3rd subculture in MS mean±sd (%)
2 weeks	(Swelled leaflets) 78	SM-1	1± 1.9 (88)	0.8 ± 1.3	0.8 ± 1.3	-	-	-
		SM-2	2 ± 1.9 (90)	0.3 ± 0.6	0.3 ± 0.6	-	-	-
		SM-3	10± 3.4 (88)	1.0 ± 1.0	1.4 ± 1.7	-	-	-
		ANOVA	S1%	NS	NS			
4 weeks	(EMs) 63							
		SM-1	2 ± 2.9 (70)	0.5 ± 0.9	0.5 ± 0.9	00.00 (3)	00.00	00.00
		SM-2	82 ± 11.5 (101)	1.9 ± 0.2	2.1 ± 0.1	00.00 (166)	1.7 ± 0.7	3 ± 0.9
		SM-3	79 ± 21 (87)	1.9 ± 0.2	2.0 ± 0.3	00.00 (121)	00.00	0.7 ± 1.1
		ANOVA	S1%	S5%	S5%	NS	S1%	S1%
6 weeks	(EMs + embryos) 70							
		SM-1	89 ± 8 (83)	2.1 ± 0.0	2.2 ± 0.1	13.4± 6.7 (138)	27.5 ± 4.8	33 ± 3.2
		SM-2	86 ± 6 (82)	2.7 ± 0.6	3.0 ± 0.7	14.4± 1.3 (189)	40 ± 12	53 ± 10.4
		SM-3	91 ± 2.6 (85)	1.9 ± 0.2	2.0 ± 0.2	4.8± 2.1 (152)	13 ± 6.0	21 ± 4.9
		ANOVA	NS	NS	NS	NS	S5%	S1%
8 weeks	(EMs + embryos) 62							
		SM-1	94 ± 8 (85)	2.0 ± 0.4	2.1 ± 0.3	11.1 ± 4.3 (161)	21.0 ± 3.8	27 ± 4.5
		SM-2	92 ± 7 (86)	2.7 ± 0.4	3.5 ± 0.4	14.0± 3.5 (232)	28.6 ± 6.1	43 ± 4.0
		SM-3	89 ± 2 (86)	2.2 ± 0.2	2.6± 0.1	4.5 ± 4.8 (171)	10.1 ± 3.4	14 ± 3.5
		ANOVA	NS	NS	S1%	NS	S1%	S1%

* Three repeats each with 35-40 replicates; ** Figures in parenthesis indicate total number of embryos tested

Response of the cultures in SMs after 8 wks in PM

Exposure of MZEDL for 8 wks in PM led to the formation of EM and subsequently somatic embryos (Fig.3.1d). Transferring the cultures in SM-1, SM-2 and SM-3 led to the

development of somatic embryos at a frequency of 94, 92 and 89% respectively. After 2 wks in the SMs average number of embryos per explant varied between 2 to 3.

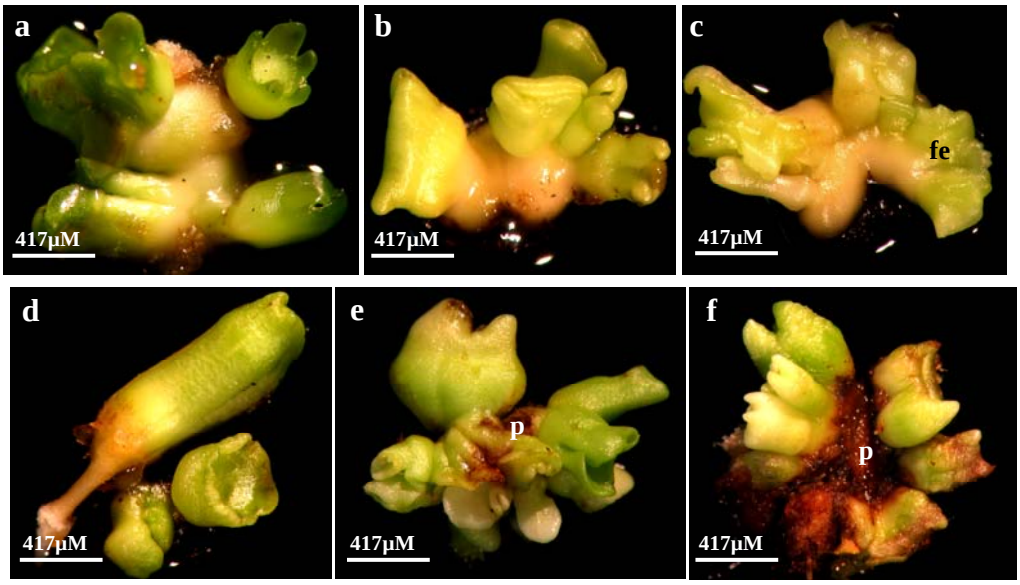


Fig.3.2 a) Somatic embryos (SEs) developed in SM-1 medium after 6 wks pre-culture in PM. Embryos were greener than those developed in SM-2 and SM-3.
b) Fused SEs developed in SM-2 from the EMs that were developed in MZEDLs pre-cultured for 6 wks in PM.
c) More fused SEs (fe) developed in SM-3 from the EMs from MZEDLs pre-cultured for 6 wks in PM.
d) Embryos developed in SM-1 after 8 wks incubation in PM. Embryos were often isolated and greenish than the embryos developed in SM-2 and SM-3.
e) Fused embryos developed in SM-2 after 8 wks pre-culture in PM.
f) Highly fused embryos developed in SM-3 after 8 wks pre-culturing in PM. Production of phenols (p) was noted at the base of developed embryos.

Extended incubation of cultures for 4 wks in SM-2 increased the number of embryos to 3.5 embryos/explant (Table 3.1), whereas it did not increase the number of embryos in SM-1 and SM-3. There was a high degree of abnormality in embryos (Fig.3.2d, e, and f) developed in all the 3 SMs. Presence of phenolics was also observed in embryos developed in SM-3 (Fig.3.2f). Embryos developed in SM-1, SM-2 and SM-3 converted into plantlets in 4 wks in MS basal medium at a frequency of 11, 14 and 4.5% (Table 3.1) respectively. After a second passage in MS basal media the conversion frequencies were doubled in all 3 SMs (Table 3.1). Conversion frequencies in SM-1, SM-2 and SM-3 reached upto 27, 43 and 14% respectively after 3rd passage in MS basal medium.

Converted plantlets appeared normal (Fig.3.3b, c) and survived on transfer in mixture of soil: sand (1:1) in plastic cups. These plants were acclimatized and hardened successfully in greenhouse (Fig.3.3d).

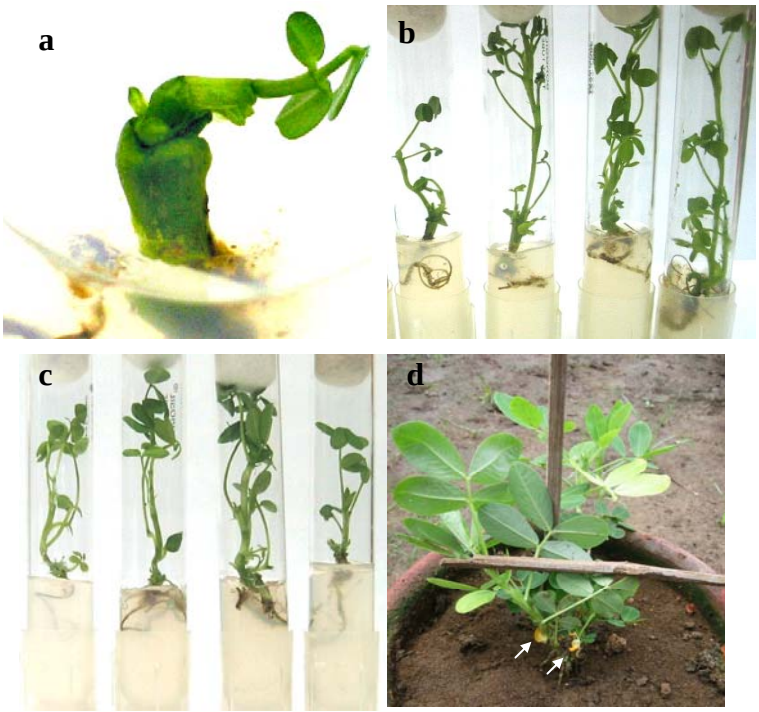


Fig.3.3 a) Conversion of SE in MS medium.
b) SEs developed in SM-2 after 6 wks pre-culturing in PM, converted into plantlets in MS media.
c) SEs developed in SM-3 after 8 wks pre-culturing in PM, converted into plantlets in MS media
d) Converted plant hardened in green house and developed flowers (arrow) successfully.

Earlier report (Chengalrayan *et al.* 1994) from this laboratory described development of abnormal somatic embryos from leaflets in 2,4-D. On transferring these somatic embryos to MS medium without PGR, only the radicle differentiated and conversion occurred in 0.5% of the embryos. To overcome the poor conversion of abnormal somatic embryos, the media composition was manipulated by the addition of TDZ in MS basal medium (Chengalrayan *et al.* 1997) and the rooted somatic embryos were exposed to TDZ. A 92% recovery of plants with stunted root and shoot was reported in 7-8 months with leaves that were not fully opened. However, these reports involved five steps as described below.

Comparison of present experiment with Chengalrayan *et al.* (1997) protocol

Number of steps	Chengalrayan *et al.* 1997	Present system
1	4 wks in EM induction medium	4 wks in EM induction medium
2	4 wks in embryo formation medium	6 wks in embryo formation medium
3	4 wks in germination medium (MS)	12 wks (3 passage of 4 wks each) in germination medium (MS).
4	8 wks in MS+TDZ for plumule differentiation	-
5	8-12 wks in MS for shoot differentiation (2-3 passages of 4wks).	-
Total steps	Five	Three
Total duration	28-32 wks	22 wks

Compared to the earlier report (Chengalrayan *et al.* 1997), in the present experiment plantlets with normal shoot and root could be recovered involving only three steps and without the involvement of any cytokinins in media, in addition to being faster, as described in the above table.

In the present experiment we observed that, optimum incubation period of MZEDL in PM was 6 wks (Table 3.1) to develop physiologically normal embryos. This could be due to gradual decomposition and decrease in 2,4-D concentration in medium with time as the explants were continuously in the same medium. Alternatively it could be due to loss of moisture. In the earlier protocol, (Chengalrayan *et al.* 1994) after 4 wks in PM (90.5 µM 2,4-D) the cultures were transferred in 13.6 µM of 2,4-D for embryo development. Thus there was sudden change in 2,4-D concentration and moisture content in medium. It needs to be tested, whether the sudden change in culture condition led to the development of abnormal embryos. In most of the prevailing protocols on peanut somatic embryogenesis the effect of incubation period in induction medium has not been tested and the explants were cultured, by default, for 4 wks (Eapen and George 1993; Baker *et al.* 1994; Chengalrayan *et al.* 1994, 1997; Venkatachalam *et al.* 1997, 1999a, b). Only Baker and Wetzstein (1992, 1998) optimized the incubation period for somatic embryogenesis from peanut seedling-derived leaflets with 2,4-D in combination with cytokinins. They found that one to two wks exposure in 2,4-D with cytokinins were enough for induction of somatic embryos from seedling-derived leaflets. They also reported that somatic embryogenesis was influenced by developmental stages of explant, leaflet size, induction medium and duration of exposure to induction medium. This report

does not mention the influence of incubation period on conversion of embryos into plantlets. Ammirato (1985) demonstrated that petiolar explants of *Daucus carota* required only 24h pulse treatment with 2,4-D for acquisition of embryogenic competence. In the present experiment, 2 wks incubation in PM led to callusing and infrequent EM formation, whereas 4 wks exposure led to the development of EMs and embryos developed from these EMs in SMs, were with retarded plumule. Optimum incubation period was 6 wks for induction and development of physiologically normal somatic embryos (Table 3.1). The frequency of conversion was 53% in these embryos, which reduced to 43% on incubation of the primary explant for 8 wks in EM induction medium (Table 3.1).

In peanut McKently (1991) reported somatic embryogenesis from mature zygotic embryo axis in 2,4-D and picloram. These authors described that, as 2,4-D and picloram concentrations increased, the probability of obtaining normal-shaped somatic embryos decreased. In soybean, using 90.4 or 180.8 μM 2,4-D for initiation of embryogenic cultures led to the production of somatic embryos which resembled zygotic embryos. These embryos matured and germinated like zygotic embryos (Ranch *et al.* 1986). Baker and Wetzstein (1993) reported that 2,4-D level in the induction medium did not affect embryo rooting or conversion and had little effect on embryo morphology in peanut. Higher auxin concentrations during induction decreased precocious germination of embryo and had no marked effect on somatic embryos in peanut (Baker and Wetzstein 1994).

3.1.3 CONCLUSION

In the present investigation we studied the effect of 2,4-D exposure duration on somatic embryogenesis of peanut keeping the concentrations unaltered. Culturing the leaflets in PM for 6 wks followed by incubation in SM-2 medium was optimum (Table 3.1) for conversion (53%) of the embryos on transfer to medium devoid of growth regulator. Thus extended incubation of MZEDL in PM reduced the number of manipulations and time required for recovery of plants via somatic embryogenesis in peanut. TDZ was used earlier for development of plant from rooted embryos. As the use of TDZ is avoided in the present process, the somatic embryo derived plants were with single shoot and single root like seedlings.

3.2 EFFECT OF TDZ ON SOMATIC EMBRYOGENESIS IN PEANUT

TDZ was first reported to have cytokinin activity in 1982 by Mok *et al.* It is a urea derivative and does not contain the purine ring common to adenine-type cytokinins such as BAP, kinetin, or zeatin. TDZ has been shown to induce a very high intrinsic cytokinin activity in early bioassays (Mok *et a.l* 1982). The nature of TDZ to proliferate and multiply the existing meristematic zone and induce organogenesis was observed in numerous plant species (Huetteman and Preece 1993; Lu 1993). However, little is known about the mechanism by which TDZ induces organogenesis in plants. There is considerable evidence that TDZ may be involved in increasing the biosynthesis or accumulation of endogenous purine cytokinins (Capelle *et al.* 1983; Murthy *et al.* 1995; Hutchinson *et al.*1996). Maxwell *et al.* (2007) reported that, TDZ-induced regeneration is the manifestation of a metabolic cascade that includes an initial signaling event, accumulation, and transport of endogenous plant signals such as auxin and melatonin, a system of secondary messengers, and a concurrent stress response.

By influencing the endogenous levels of both auxin and cytokinins, TDZ induces different morphogenic responses in different tissues. A better understanding of the physiological responses of plant tissue to TDZ will lead to a greater understanding of the process of morphogenesis (Murthy *et al.* 1995).

Peanut regeneration through **organogenesis** and **embryogenesis** in presence of TDZ is reported by several researchers.

- The influence of TDZ on direct **somatic embryogenesis** in peanut was first reported by Gill and Saxena (1992). They demonstrated induction of somatic embryos from seedling explants of peanut eg. cotyledons and juvenile leaves.

- Saxena *et al.* (1992) reported direct **somatic embryogenesis** from morphologically intact seedlings of peanut germinated on a medium supplemented with 10 μM TDZ. They observed that the somatic embryos were induced in the apical region and on the surface of cotyledons and hypocotyls of germinating seedlings, which eventually mature and develop into plants.

- Kanyand *et al.* (1994) reported induction of **multiple shoots** from various parts of the Valencia type peanut seedling in different concentration of TDZ and observed that hypocotyl and cotyledon explants produced higher number of shoots in the medium containing 30 mg/l TDZ. Those shoots rooted on the MS basal medium and gave rise to plantlets.

- Li *et al.* (1994) demonstrated the ability of TDZ in induction of **adventitious shoots** from hypocotyl region of cultured seed explants of peanut. An exposure of one week in 10 μM TDZ was sufficient to stimulate initiation of adventitious shoots that subsequently developed into normal and fertile plants.

- Murthy *et al.* (1995) assessed the regulatory role of thidiazuron and explant factors in imparting **somatic embryogenic** potential in relation to endogenous growth regulator levels in peanut. They demonstrated that by influencing endogenous levels of both auxin and cytokinins, TDZ induced somatic embryogenesis in peanut.

- Murch and Saxena (1997) studied the modulation of mineral and free fatty acid profiles during thidiazuron mediated **somatic embryogenesis** in peanut seedlings. Their observations suggested an alteration of nutrient availability and structural free fatty-acid profiles, affecting both cellular functions and growth patterns. This seems to be part of the mode of action of TDZ and may play an important role in the induction of regeneration.

- Kanyand *et al.* (1997) reported **differentiation of emergences**, a multicellular structure resembling multicellular trichomes, at the cotyledonary node, petiole base, and on the rachis of the peanut leaf where folioles are attached. Greatest number of emergence was found at cotyledonary node grown on medium containing 10 mg/l TDZ for 3 wks and exhibited progressive morphological changes of emergences that differentiated into three types of adventitious structures with one type resembling radially concentric shoots. The greatest extent of shoot differentiation occurred at the cotyledonary node and 11% of those shoot developed roots. The ability of the emergences to form **adventitious shoots** was emphasized because of their superficial origin in epidermal and sub epidermal tissues. For genetic transformation by particle bombardment these tissues are directly exposed.

- Chengalrayan *et al.* (1997) reported the effectiveness of TDZ (22.71 μM) for high-conversion frequency (92%) of abnormal peanut **somatic embryos** by triggering **caulogenic activity in the abnormal plumular meristem of peanut somatic embryos**. Various growth regulators including KIN, BAP, Zeatin, γ,γ - dimethylallylaminopurine (2ip), TDZ, Gibberellic acid and Abscisic acid were tested for triggering morphological activity at the plumule of the abnormal somatic embryos. The study demonstrated TDZ as the most potent growth regulator in triggering differentiation in the meristems of rooted somatic embryos of peanut.

- Gill and Ozias-Akins (1999) reported a highly morphogenic callus induction from immature cotyledons and embryo axes of peanut with various concentration of TDZ. It was optimum in cotyledon with attached embryo axis when cultured in the dark on 10 μM TDZ. Cultures were maintained in the same medium and regenerated via **organogenesis** with a gradual reduction in TDZ concentration and exposure to light.

- Murch *et al.* (1999) reported the role of proline in thidiazuron-induced **somatic embryogenesis** of peanut and indicated that proline plays a key role in directing the route of TDZ-induced somatic embryogenesis. TDZ effectively stimulates a cascade of metabolic events resulting in the production of specific metabolites, including amino acids, required for the regenerative process.

- Victor *et al.* (1999a) reported ***de novo* regeneration of somatic embryos** in peanut at the hypocotyledonary notch region of intact seedling in presence of 10 μM TDZ and suggested that seedlings treated with TDZ undergo a different morphological route of development than that induced by purine based cytokinins.

- Later, they (Victor *et al.* 1999b) demonstrated the role of endogenous purine metabolism in thidiazuron-induced **somatic embryogenesis** of peanut. Analyzing the endogenous level of purine metabolites, they showed that supplementation of the media with TDZ resulted in an overall increase in the endogenous cytokinins, while DAP (2, 6-diaminopurine, purine analogs) inhibited the purine recycling resulting in decreased levels of endogenous adenine and zeatin.

- Akasaka *et al.* (2000) noted that among the cytokinins tested, TDZ was found to be the most efficient for inducing **bud primordia** in peanut. They reported induction of bud primordia from cultured leaf segments in peanut by limited exposure to TDZ. They observed normal conversion of shoot buds to shoots in PGR free medium, when explants cultured for 7 days at 10mg/l or 21 days at 1mg/l TDZ, whereas over exposure on TDZ containing media induced abnormal development of these primordia and they fail to grow into plantlets.

- Joshi *et al.* (2003) suggested that development of multiple shoots in the plumule due to exposure of rooted embryos to TDZ, may either be due to triggering of the existing multiple meristems in the fasciated embryos or due to proliferation of pre-existing shoot meristem.

- Matand and Prakash (2007) reported a protocol that can be applied across a broad spectrum of peanut market types, explant types and geographic regions using TDZ. They used different seedling derived explants and cultured on varying concentration of TDZ for organogenesis. The effect of the timing of TDZ application to the both zygotic embryos and subsequent seedling derived explants on MS medium is also evaluated. Limited application of TDZ (10 d) was sufficient to induce shoot formation in peanut. However, an extended (20 d) use of TDZ and at a higher concentration (30 mg/l) resulted in the highest number of shoots. They found hypocotyl was the best explant that induced highest number of shoots.

- Johsi *et al.* (2008) studied the effect of TDZ and 2,4-D on peanut **somatic embryogenesis and *in vitro* bud development**. They reported appearance of bud-like projections in the embryogenic masses when these were cultured in media containing combinations of 2,4-D and TDZ. These projections developed into buds, which subsequently formed shoots and plantlets. They found that the embryogenic mass was converting into organogenic mass in presence of TDZ.

In the present experiment, we studied the influence of TDZ on different developmental stages of somatic embryos and conversion of the embryos to plantlets. The method standardized in our laboratory to regenerate plants via somatic embryogenesis from

MZEDL, is described in the previous part of this chapter (Section 3.1.2). The method involves five steps (Chengalrayan *et al.*1997). In the fourth stage TDZ was needed for induction of differentiation in the plumule of the rooted embryos, wherein plantlets with stunted shoot and root growth (Chengalrayan *et al.* 1997) was achieved. In present experiments, TDZ was incorporated at three initial embryogenic developmental stages for varying durations:

a) *By incorporating TDZ in EM induction medium (MS medium incorporated with 90.5 µM 2,4-D and 6% sucrose)*
b) *By incorporating TDZ in embryo induction medium (MS medium incorporated with 13.6 µM 2,4-D and 6% sucrose)*
c) *By incorporating TDZ in embryo conversion medium (MS medium with 2% sucrose)*

This experiment was conducted to test, if incorporation of TDZ in the early stages of embryogenesis can support the development of a functionally normal plumule.

3.2.1 MATERIALS AND METHODS

Source of explant and surface sterilization procedure is described in Chapter 2 (Section 2.1.2).

a) Incorporation of TDZ in EM induction medium:

Surface sterilized MZEDLs (described in Chapter 2, Section 2.1.2) were cultured in EM induction media containing MS basal salts, with 90.5 µM 2,4-D, various concentrations (0, 1.36, 4.54, 13.62, 22.71 and 31.78 µM) of TDZ and 6% sucrose. After 24, 48, 72 and 96 h of exposure, TDZ was withdrawn and the explants were transferred in EM induction medium without TDZ. Total culture period was 4 wks including the period of TDZ exposure. Thereafter cultures were transferred in MS medium containing 13.6 µM of 2,4-D (embryo induction medium) for 4 wks. All these manipulations were done in 55mm petridishes containing 10ml medium. Subsequently all cultures were transferred to MS medium devoid of growth regulator in culture tubes containing 10ml of medium. This experiment was conducted thrice with 40-100 replicates for each treatment.

b) Incorporation of TDZ in embryo induction medium:

The MZEDL-derived EMs developed within 4 wks in MS basal medium with 90.5 μM 2,4-D were used as explants in this experiment. These were cultured in MS basal medium composed of 13.6 μM 2,4-D with varying concentrations (0, 1.36, 4.54, 13.62, 22.71 and 31.78 μM) of TDZ. Exposure to these TDZ containing media was for 24, 48, 72 and 96h. Thereafter the cultures were transferred in two different media formulations viz., MS basal medium with 13.6 μM 2,4-D (embryo induction media) and 6% sucrose and MS basal media (without any PGR) and 2% sucrose, for 4 wks. Subsequently all cultures were transferred to culture tubes containing 10ml of MS basal media and 2% sucrose. This experiment was repeated 5 times with 6-9 replicates in each treatment.

c) Incorporation of TDZ in embryo conversion medium:

In this experiment embryos were treated as explants. The embryos were obtained by exposing the MZEDLs in EM induction media for 4 wks, followed by 4 wks in embryo induction media. These were cultured in MS basal medium composed of various concentrations (0, 1.36, 4.54, 13.62, 22.71 and 31.78 μM) of TDZ and 2% sucrose. Exposure to this TDZ containing media was for 24, 48, 72 and 96h. After TDZ treatment, the explants were cultured in MS basal medium (devoid of growth regulator) and 2% sucrose. Total culture period in this step was 4 wks. Experiment was carried out thrice with 10-18 replicates in each treatment.

In all the three experiments, number of explants that responded in culture by showing development of EMs, embryos and then differentiating embryos were scored. All data were subjected to ANOVA analysis. All the 2,4-D containing media were supplemented with 6% sucrose, whereas MS medium was supplemented with 2% sucrose. The pH of the medium was adjusted to 5.8 prior to addition of (0.7%) agar. Media were autoclaved at 120°C under 15psi for 20 minutes. The cultures were incubated in 16h photoperiod at 25 ± 2°C under diffuse cool white fluorescent light ($32\mu Em^{-2}sec^{-1}$).

Converted plantlets were hardened as described in Chapter 2 (Section 2.1.2). Survival rate of the plants was calculated from number of plants transferred to soil and the number of plants established.

3.2.2 RESULTS AND DISCUSSION

a) Influence of TDZ pulse treatment on EM induction:

Exposure of the MZEDLs of SB-11 to 90.5 µM 2,4-D triggered EM formation in 42% (Table 3.2) of the leaflets in the form of a pair of bulges (Fig.3.4a). However, compared to 76% response in JL-24 (Chengalrayan *et al.*1994), the frequency of EM formation in the present experiment was less (42%). This could be due to change in genotype (Chengalrayan *et al.* 1998). Embryogenic mass (Fig.3.4b) formation was optimum (60%) in cultures exposed to 90.5 µM 2,4-D with 4.54 µM TDZ for 24h. This was followed by the response in 90.5 µM 2,4-D with 1.36 µM (56.5%) TDZ exposed for 24h (Table 3.2). Embryogenic masses developed in lower concentration (1.36 µM and 4.54 µM) of TDZ when exposed for short duration (Fig.3.4b) and EMs developed in EM induction medium without TDZ (Fig.3.4a, b) were similar in morphology.

Development of somatic embryos from the EMs pre-exposed in EM induction medium without TDZ was 54% (Table 3.2). On the other hand, development of somatic embryos in cultures pre-exposed in 90.5 µM 2,4-D with 4.54 µM TDZ for 24h was optimum 55% (Table 3.2) on transferring to embryo induction medium. Average number of embryos was similar (2 and 1.9 embryo/explant) in explants pre-cultured for 24h in EM induction medium with 1.36 µM and 4.54 µM TDZ (Table 3.2) and in the explants exposed in EM induction medium without TDZ. Longer exposures (48, 72, and 96h) to TDZ, even at low concentrations inhibited EM induction (Fig.3.4c) and embryo formation. At higher concentrations and longer exposure to TDZ in EM induction medium, often the morphology was completely different. Innumerable dark green undulations (Fig.3.4d) were seen in the entire leaflet. Culturing these explants in embryo induction medium resulted in callus formation.

Embryos induced from EMs developed in 1.36 µM TDZ containing EM induction medium, appeared to be more fused (Fig.3.4e) and abnormal compared to the embryos developed from control (Fig.3.4f). Radicle emergence (Fig.3.4g) was achieved in embryos on transferring to the PGR free medium. Germination was 100% in control (Table 3.2). Germination frequency of embryos was reduced in PGR free medium which were developed from the EMs in media with higher concentration and longer exposure of TDZ.

Table 3.2 Influence of TDZ on embryogenic mass development.

Exposure period (hrs)	EM induction medium without and with TDZ (μM)	Response (EM formation) mean±sd * (%)	Response in 13.6μM 2,4-D (embryo formation) mean±sd (%)	Average no. of embryo per explant mean±sd	Germination in MS medium mean±sd** (%)	Conversion in MS medium mean±sd (%)
24	Control	42.0 ± 18.5 (256)	54.1 ± 4.0	1.9 ± 0.1	100 ± 0.0 (106)	0.0 ± 0.0
	90.5D+1.36T	56.5 ± 19.4 (229)	52.5 ± 25.8	2.0 ± 0.2	100 ± 0.0 (124)	0.4 ± 0.7
	90.5D+4.54T	60.0 ± 19.8 (229)	55.0± 11.6	1.9 ± 0.0	100± 0.0 (136)	0.8 ±1.3
	90.5D+13.62T	4.1 ± 4.6 (95)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+22.71T	2.4 ± 2.1 (102)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+31.78T	0.0 ± 0.0 (98)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	ANOVA	S 1%	S 1%	S1%	S1%	NS
48						
	90.5D+1.36T	46.7 ± 6.7 (237)	47.5 ± 6.8	1.8 ± 0.1	100± 0.0 (95)	6.0 ± 6.3
	90.5D+4.54T	45.7 ± 6.6 (266)	50.0 ± 21.8	1.8 ± 0.1	100± 0.0 (91)	0.6 ± 1.0
	90.5D+13.62T	2.5 ± 2.5 (101)	0.0 ± 0.0	0.0 ± 0.0	0.0± 0.0	0.0 ± 0.0
	90.5D+22.71T	1.0 ± 1.7 (104)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+31.78T	0.0 ± 0.0 (98)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	ANOVA	S 1%	S 1%	S 1%	S1%	NS
72						
	90.5D+1.36T	8.3 ± 14.4(103)	14.3 ± 24.7	0.6 ± 1.0	100± 0.0 (17)	0.0 ± 0.0
	90.5D+4.54T	5.3 ± 9.1(95)	22.2 ± 38.5	0.8± 1.4	55.0 ± 8.5 (18)	0.0 ± 0.0
	90.5D+13.62T	0.0 ± 0.0 (103)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+22.71T	0.0 ± 0.0 (115)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+31.78T	1.3 ± 2.3 (103)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	ANOVA	S 1%	S 5%	S 5%	S 1%	NS
96						
	90.5D+1.36T	6.4 ± 11.0 (99)	16.7± 28.9	0.5 ± 0.9	35.0 ± 4.4 (15)	0.0 ± 0.0
	90.5D+4.54T	6.1 ± 10.5 (103)	16.7 ± 28.9	0.7 ± 1.2	0.0 ± 0.0	0.0 ± 0.0
	90.5D+13.62T	2.4 ± 4.1 (108)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+22.71T	0.0 ± 0.0 (98)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+31.78T	0.0 ± 0.0 (107)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	90.5D+31.78T	0.0 ± 0.0 (107)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	ANOVA	S 1%	S 5%	S 5%	S 1%	NS
*Number of explants tested in three repeats; ** Figures in parenthesis indicate total number of embryos tested						

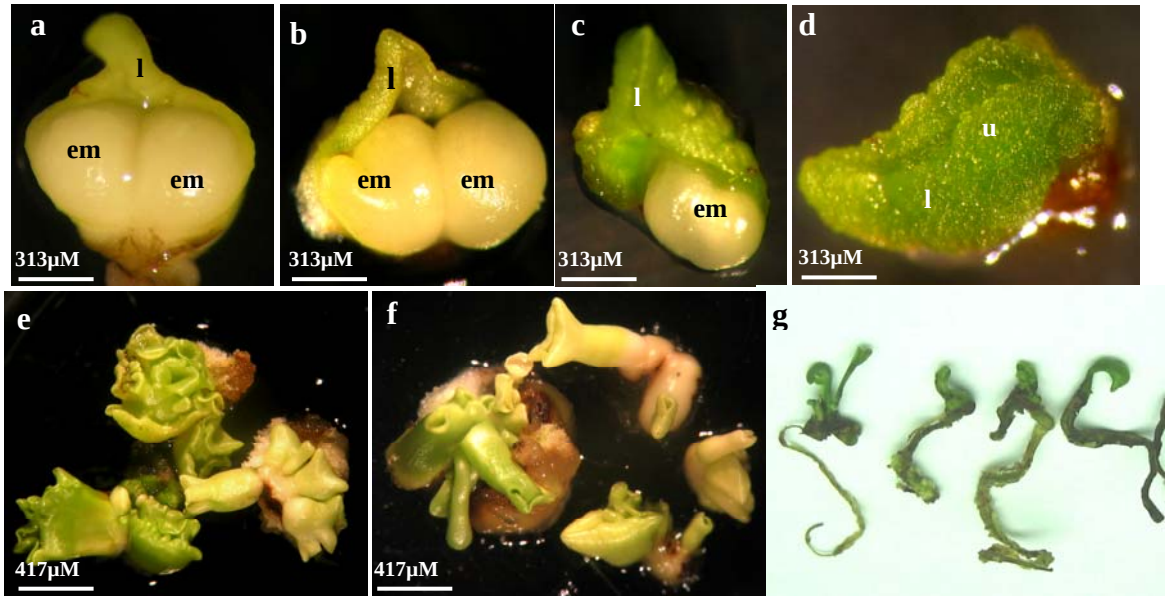


Fig.3.4 a) MZEDL with EM (em) developed in 90.5 µM 2,4-D (control).
b) MZEDL with EM developed in 90.5 µM 2,4-D after 24h pre-culturing in 90.5 µM 2,4-D with 1.36 µM TDZ.
c) MZEDL with less developed EM in 90.5µM 2,4-D after 72h pre-culturing in 90.5 µM 2,4-D with1.36µM TDZ.
d) Dark green undulations (u) developed in the entire leaflet (l). No EM development was noted from the leaflets after 24h pre-culturing in MS medium containing 90.5 µM 2,4-D and 13.62 µM TDZ.
e) Somatic embryos developed in 13.6 µM 2,4-D, from EMs after 24h pre-culturing in MS medium 90.5 µM 2,4-D and 1.36µM TDZ.
f) Somatic embryos developed in 13.6 µM 2,4-D, from EMs developed in 90.5µM 2,4-D without TDZ (control).
g) Somatic embryos germinated in MS basal media.

Incorporation of TDZ at EM induction stage was not effective for development of normal embryos. Thus it did not improve the plantlet recovery. Higher concentrations and longer exposures to TDZ inhibited development of EM and somatic embryo from leaflets. Number of embryos formed per EM and the number of embryos converted to plantlets did not improve effectively as compared to those of control.

b) Influence of TDZ pulse treatment on embryo induction:

Embryogenic masses exposed to embryo induction medium incorporated with different concentrations of TDZ for varying periods, led to the development of diverse morphology after culturing in MS basal medium with 13.6 µM 2,4-D and in MS basal medium without 2,4-D.

Influence of TDZ on embryo induction after culturing the TDZ pre-treated EMs in MS basal medium with 13.6 µM 2,4-D:

Embryos developed from the EMs cultured in embryo induction medium without and with TDZ (Fig.3.5a, b). Average number of embryo formation was similar in all combinations of 13.6 µM 2,4-D with and without TDZ regardless of exposure period (Table 3.3). It ranged from 2.0 to 2.7 embryos/explant. Embryos developed in 13.6 µM 2, 4-D with and without TDZ had no morphological differences. Embryos developed in medium with TDZ (Fig.3.5b) look similar to embryos developed in control (Fig.3.5a).

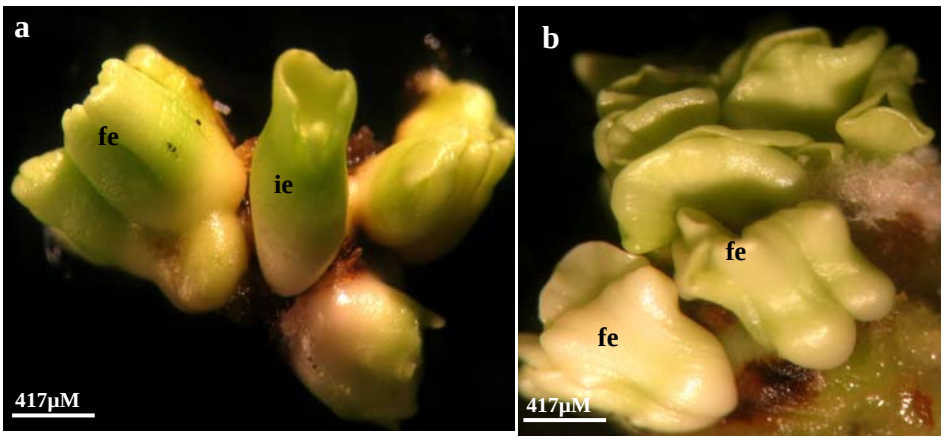


Fig.3.5 a) Both isolated (ie) and fused embryos (fe) developed from EMs in 13.6µM 2,4-D without TDZ.
b) Fused embryos (fe) developed from EMs in 13.6 µM 2,4-D after 72h exposure to 13.6 µM 2,4-D and 1.36 µM TDZ.

Embryos developed in embryo induction medium with or without incorporation of TDZ were transferred to MS basal medium without PGR for conversion. Radicle emergence started and it was 100% in embryos developed in embryo induction medium without TDZ (Table 3.3). Germination frequency of embryos developed in embryo induction medium without TDZ decreased with increasing concentration and longer pre-exposure to TDZ in embryo induction medium (Table 3.3). The conversion frequency was optimum (26.5%) in embryos developed after pre-culturing the explants for 24h in MS basal medium with 13.6 µM 2,4-D and 13.62µM TDZ (Table 3.3). With increasing concentration of TDZ in embryo induction medium, there was increase in conversion of embryos into plantlets till 13.62µM TDZ exposed for 24h, after which it started decreasing. Higher concentration (31.78µM of TDZ) and longer exposure of TDZ inhibited the conversion of embryos into plantlets (Table 3.3). The converted emblings showed normal morphology (Fig.3.6a).

Plants regenerated from the present experiments were transferred to sand: soil (1:1) mixture (Fig.3.6b) and were hardened successfully in greenhouse (Fig.3.6c). There were no morphological differences in plants. Seeds were collected while harvesting (Fig.3.6d).

Table 3.3 Influence of TDZ on embryo development.

Response (in EM formation medium) (%)	Exposure period (h)	Embryo formation medium without and with TDZ (μM)	Average no. of embryo in 13.6μM 2,4-D mean±sd*	Germination in MS medium mean±sd** (%)	Conversion in MS medium mean±sd (%)
51.17	24	Control	2.4 ± 0.4 (203)	100.0 ± 0.0 (238)	2.6 ± 2.8
		13D+1.36T	2.4 ± 0.9 (45)	63.4 ± 37.5 (54)	7.5 ± 14.6
		13.6D+4.54T	2.5 ± 1.2 (42)	61.7 ± 41.6 (54)	10.0 ± 22.4
		13.6D+13.62T	2.0 ± 0.7 (39)	48.2 ± 30.7 (45)	26.5 ± 25.5
		13.6D+22.71T	2.0 ± 0.6 (41)	28.5 ± 26.2 (54)	11.2 ± 12.4
		13.6D+31.78T	2.0 ± 0.6 (38)	04.6 ± 10.2 (61)	1.8 ± 4.1
		ANOVA	NS	S1%	NS
	48				
		13.6D+1.36T	2.7 ± 1.4 (44)	54.0 ± 11.4 (65)	17.2 ± 19.3
		13.6D+4.54T	2.0 ± 0.7 (44)	44.8 ± 15.3 (50)	6.7 ± 9.2
		13.6D+13.62T	2.5 ± 0.9 (40)	42.5 ± 33.5 (53)	13.6 ± 9.1
		13.6D+22.71T	2.7 ± 0.5 (41)	39.2 ± 35.9 (66)	23.6 ± 29.5
		13.6D+31.78T	2.2 ± 0.5 (41)	1.7 ± 3.7 (64)	0.0 ± 0.0
		ANOVA	NS	S1%	NS
	72				
		13.6D+1.36T	2.1 ± 0.3 (43)	53.1 ± 34.2 (61)	7.1 ± 6.1
		13.6D+4.54T	2.2 ± 0.5 (38)	43.1 ± 18.4 (57)	1.3 ± 3.0
		13.6D+13.62T	2.3 ± 0.9 (36)	41.1 ±14.0 (59)	18.3 ± 14.2
		13.6D+22.71T	2.3 ± 0.8 (40)	27.1 ± 19.3 (70)	24.6 ± 21.4
		13.6D+31.78T	2.6 ± 0.5 (38)	8.8 ± 8.3 (71)	2.5 ± 5.6
		ANOVA	NS	S1%	S5%
	96				
		13.6D+1.36T	2.2 ± 0.4 (40)	51.8 ± 30.1 (63)	17.4 ± 21.1
		13.6D+4.54T	2.4 ± 0.3 (32)	35.9 ± 10.9 (64)	9.2 ± 17.4
		13.6D+13.62T	2.2 ± 0.4 (37)	31.0 ± 20.9 (66)	15.9 ± 12.2
		13.6D+22.71T	2.7 ± 0.7 (42)	27.4 ± 27.8 (103)	13.2 ± 9.8
		13.6D+31.78T	2.7 ± 1.0 (42)	6.1± 5.8 (63)	1.7 ± 3.7
		ANOVA	NS	S1%	NS

*Number of explants tested in three repeats; ** Figures in parenthesis indicate total number of embryos tested

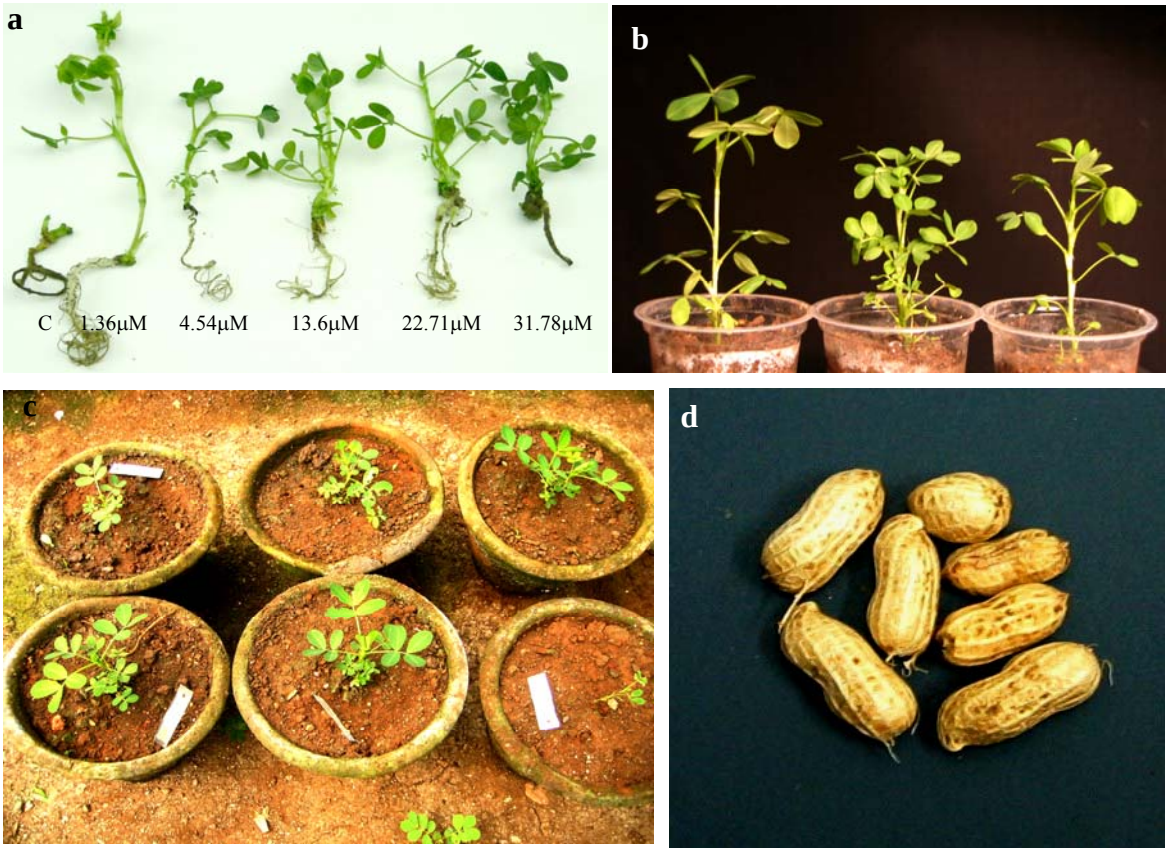


Fig.3.6 a) Somatic embryos developed in 13.6 µM 2,4-D incorporated with different concentrations of (1.36, 4.54, 13.6, 22.71 and 31.78 µM) TDZ converted into plantlets in MS medium.
b) Somatic embryo derived plants were hardened in *in vitro* condition (24h light).
c) *In vitro* hardened plants transferred to earthen pots in green house.
d) Seeds collected during harvesting of hardened pants.

Influence of TDZ on embryo induction after culturing the TDZ pre-treated EMs in MS basal medium without 2,4-D:

Embryogenic masses exposed to embryo induction medium with TDZ and then cultured in MS medium without PGR for 4 wks, did not give rise to embryo or any embryo like structure. Finger-like protrusions (Fig.3.7a) developed on the surface of EMs pre-cultured in embryo induction medium incorporated with lower concentrations (1.36-13.62 µM) of TDZ. Within 2 wks a single row of these protrusions appeared. In next 2 wks these protrusions elongated and appeared as finger like projections (Fig.3.7b) and the number of projections increased. Further culturing in MS medium often led to growth of leafy structures (Fig.3.7c) from these projections. Root emergence was never noted. Rare development of shoot (Fig.3.7e) was observed from the projection developed on EMs pre-cultured in embryo induction medium incorporated with low concentration of TDZ.

Embryogenic masses pre-cultured in embryo induction medium incorporated with higher concentration of TDZ, developed dark green multiple globular lobes (Fig.3.7d). Thus the pre-culturing of EMs in embryo induction medium with TDZ and then transferring in MS medium without any PGR, led to the diversion of embryogenic pathway to caulogenic pathway. Caulogenic responses were optimum (27%) in the EM pre-cultured for 96h in embryo induction medium incorporated with 4.54 μM TDZ (Table 3.4) followed by the EM pre-cultured for 24h in the same medium. This could be compared with a report by Joshi *et al.* (2008) who observed similar response with higher frequency (88%) on 8 wks incubation of EM in 13.6 μM 2,4-D with varying concentrations of TDZ.

Table 3.4 Caulogenic response of EMs in MS medium with different exposure to TDZ (24, 48, 72, and 96h) in embryo induction medium.

Embryo formation Medium (μM)	Caulogenic response mean±sd* (%)			
	24h	48h	72h	96h
13.6D+1.36T	0.0± 0.0 (27)	7.4± 6.4 (27)	18.5± 6.4 (27)	7.4± 6.4 (27)
13.6D+4.54T	18.5 ± 6.4 (27)	3.7± 6.4 (27)	3.7± 6.4 (27)	26.9± 5.8 (27)
13.6D+13.62T	14.8 ± 16.9 (27)	18.5± 12.8 (27)	7.4±12.8 (27)	3.7± 6.4 (27)
13.6D+22.71T	14.8±12.8 (27)	7.4± 6.4 (27)	14.8± 6.4 (27)	11.1± 11.1(27)
13.6D+31.78T	3.7± 6.4 (26)	3.7± 6.4 (26)	7.4±6.4 (26)	4.2± 7.2 (26)
ANOVA	NS	NS	NS	S 5%

Exposure of EM was for 24, 48, 72 and 96h in 13.6 μM 2,4-D with varying concentrations of TDZ. Afterwards EMs were cultured in MS basal medium. In MS basal medium we observed development of two types of morphogenic structures (finger like projection and globular lobes). The present experiment resulted in the development of much-scattered morphogenic structures (that were not differentiated into shoots) unlike that reported by Joshi *et al.* (2008), which resulted in a better organization of similar morphogenic structures that were differentiated into shoots and plantlets. The reason for these differences could be the variation in the durations of exposure with the above report involving an exposure time of 4-8 wks quite longer than that in the present experiments (upto 96h).

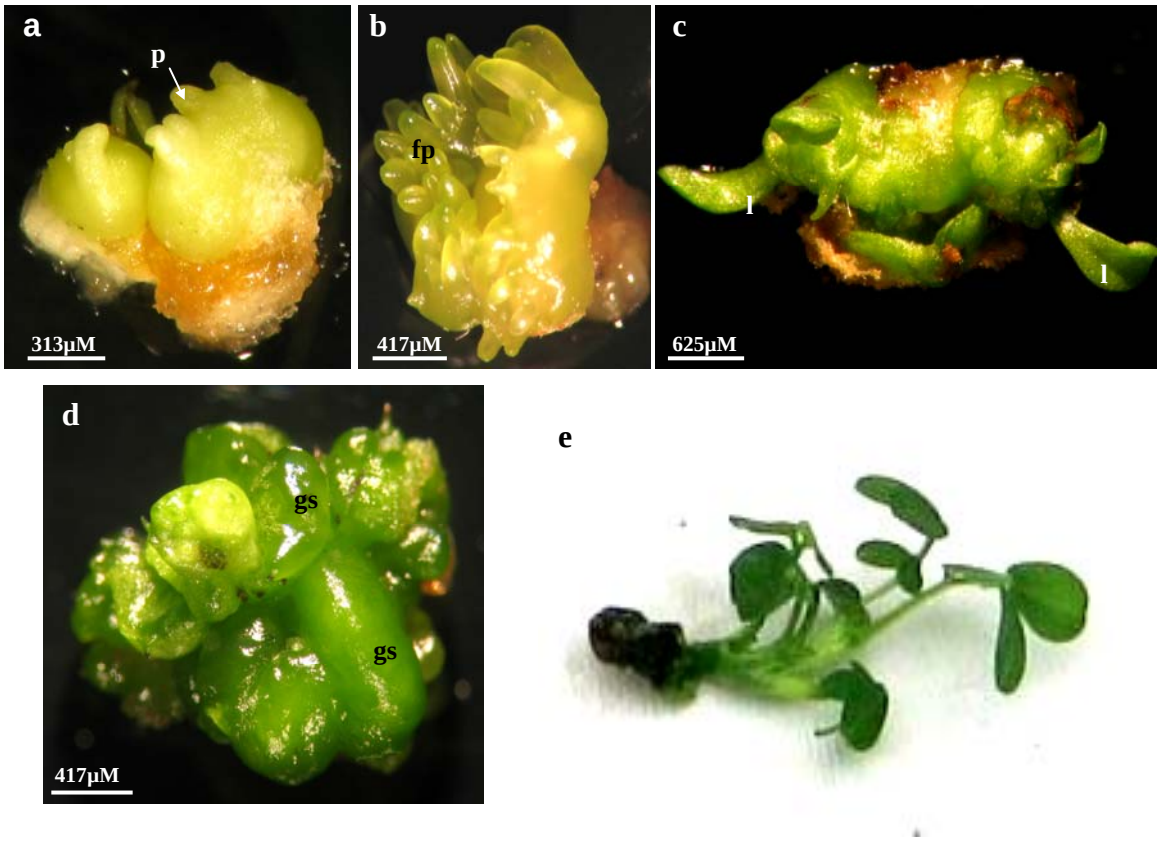


Fig.3.7 a) Induction of protrusions (p) on the surface of EMs in MS media within a wks after 48h exposure to 13.6 µM 2,4-D and TDZ (13.62 µM).
b) Finger like projection (fp) developed in MS medium within 4 wks after 48h exposure in 13.6µM 2,4-D and TDZ (13.62 µM). There was no sign of SE development.
c) Leaves (l) developed from these finger-like structures on further incubation in MS media.
d) Green globular structures (gs) with multiple lobes were developed in MS medium after exposure to 13.6 µM 2,4-D and higher concentration of TDZ (22.71-31.78 µM).
e) Shoot development from the finger-like projection was hardly noticed.

In the present experiment EMs were pre-cultured in embryo induction medium incorporated with various concentrations of TDZ for 24, 48, 72 and 96h and transferred in embryo induction medium, resulting in the development of somatic embryos with differentiation of normal plumule of developing embryos, which were converted into plantlets with normal root and shoot (Fig.3.6a). Optimum conversion (26.5%) was obtained from the somatic embryos developed from the explants exposed in MS basal medium with 13.6 µM 2, 4-D and 13.62 µM TDZ for 24h (Table 3.3), whereas Joshi *et al.* (2008) concluded that the presence of EMs in embryo induction medium containing TDZ

for 4-8 wks, restricted the development of somatic embryos from EMs. This could be due to the longer exposure to TDZ in embryo induction medium.

This suggests that the morphogenic competence of EMs responded in diverse way with varying concentrations and durations of exposure to TDZ. The responses were not only dependent on culture media but also on the duration of exposure to growth regulators. The results of the present experiment also suggest that EMs can be regulated to form embryos or organs by manipulating the growth regulators used.

c) Influence of TDZ pulse treatment on embryo conversion:

After pulse treatment in varying concentrations of TDZ for 24, 48, 72 and 96h the embryos were cultured in MS basal medium to test germination frequency. Germination frequency was 100% in somatic embryos, developed in control (Table 3.5). This is in concurrence with the earlier report in JL-24 (Chengalrayan *et al.* 1994). However, germination frequency was reduced in the embryos treated with TDZ (Table 3.5). Embryos pre-treated in MS medium without (Fig.3.8a) or with (Fig.3.8b) TDZ, appeared morphologically similar in MS medium till 2 wks. After 4 wks in MS medium without any PGR, embryos pre-treated at higher concentrations and/or for longer periods in TDZ resulted in proliferation of the plumular meristem and subsequently led to the development of multiple shoots. Shoot proliferations occurred either from plumule (Fig.3.8c) or from the peripheral region (Fig.3.8d) of the somatic embryos, whereas embryos pre-treated to lower concentrations (1.36μM and 4.54μM) of TDZ for various exposures (24-96h) did not show proliferation in plumule and resulted in normal emblings with single shoot and root development (Fig.3.9a).

Conversion frequency of embryos pre-treated in MS medium with 1.36 μM TDZ increased with exposure duration till 72h and started decreasing later (Table 3.5). The conversion frequency was optimum (43%) in embryos pre-treated in MS medium with 4.54 μM TDZ for 24h (Table 3.5). Increasing exposure duration (Fig.3.9b) and concentration (Fig.3.9c) of TDZ led to the inhibition of radicle differentiation and promotion of organogenesis in plumular part of somatic embryos. This resulted in formation of plantlets with multiple shoots with or without roots (Table 3.5).

Table 3.5 Influence of TDZ on embryo conversion

Response (in EM formation medium) (%)	Response (in embryo formation medium) (%)	Exposure period (h)	Conversion medium (μM)	Germination in MS medium mean±sd* (%)	Conversion in MS medium (Shoot+root) mean±sd (%)	Only shoot formation in MS medium mean±sd (%)
67.94	81.15	24	Control	100.0 ± 00.0 (147)	3.9 ± 0.8	0.0 ± 0.0
			MS+1.36T	92.6 ± 12.8 (30)	20.7 ± 16.7	28.1 ± 10.3
			MS+4.54T	76.4 ± 20.6 (30)	42.6 ± 17.9	46.3 ± 11.6
			MS+13.62T	49.6 ± .3.3 (46)	20.3 ± 9.1	22.7 ±7.6
			MS+22.71T	22.6 ± 6.0 (52)	20.0 ± 7.4	27.3 ± 10.8
			MS+31.78T	09.5 ± 2.6 (42)	9.5 ± 2.6	24.3 ± 9.5
			ANOVA	S 1%	S5%	S 1%
		48				
			MS+1.36T	77.8 ± 38.5 (33)	34.3 ± 16.8	37.1 ± 12.5
			MS+4.54T	65.9 ± 14.3 (30)	28.2 ± 17.3	31.5 ± 12.3
			MS+13.62T	48.4 ± 19.1 (37)	35.2 ± 18.0	47.1± 22.7
			MS+22.71T	21.9 ± 7.58 (38)	21.9 ± 7.5	44.9 ± 10.7
			MS+31.78T	2.8 ± 4.8 (30)	2.9 ± 4.8	36.4 ± 16.7
			ANOVA	S 1%	S5%	S5%
		72				
			MS+1.36T	57.1 ± 2.2 (35)	36.6 ± 16.5	16.9 ± 7.9
			MS+4.54T	40.3 ± 9.6 (35)	20.7 ± 8.1	25.6 ± 15.5
			MS+13.62T	21.4± 8.1 (37)	16.2 ± 0.7	26.9 ± 9.0
			MS+22.71T	13.2 ± 9.1 (50)	13.2 ± 9.1	25.7 ± 2.4
			MS+31.78T	0.0 ± 0.0 (37)	0.0 ± 0.0	28.0 ± 5.0
			ANOVA	S 1%	S1%	S 5%
		96				
			MS+1.36T	54.6 ± 14.8 (35)	32.9 ± 18.9	41.3 ±19.2
			MS+4.54T	38.2 ± 7.4 (30)	17.4± 6.5	45.9 ± 29.3
			MS+13.62T	20.8 ± 17.1 (37)	17.5 ± 11.6	34.9 ± 9.8
			MS+22.71T	4.4 ± 7.7 (51)	2.2 ± 3.9	19.3 ± 12.3
			MS+31.78T	0.0 ± 0.0 (47)	0.0 ± 0.0	24.3 ± 12.9
			ANOVA	S 1%	S1%	S5%

* Figures in parenthesis indicate total number of embryos tested

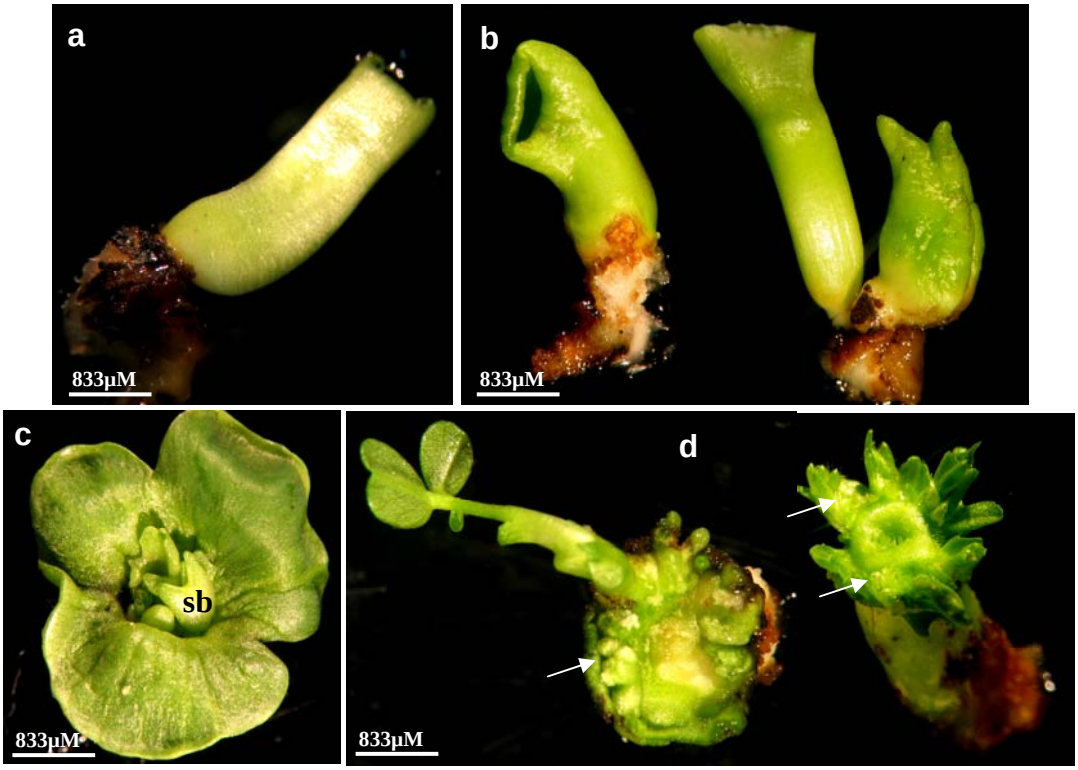


Fig.3.8 a) Germinated SEs in MS medium without TDZ in 2 wks.
b) Germinated SEs in MS media in 2 wks after different exposure in MS media and TDZ.
c) Plumular meristem of SEs proliferated as shoot buds (sb) in the central depression of embryos in MS medium, after exposure to MS media with TDZ.
d) Meristems present at the base and periphery of the SEs proliferated as shoot buds (arrow) and started shoots formation in MS medium after exposure to MS media with TDZ.

To trigger plumule differentiation in abnormal, rooted, somatic embryos of peanut, multiple shoots were induced by extended incubation of 4 wks in TDZ (Chengalrayan *et al.* 1997; Joshi *et al.* 2003). But all those were stunted shoot and root, and shoot buds elongated to a limited extent. On repeated transfer to MS medium, shoot and root were elongated. Nevertheless, in the present experiment we observed conversion of somatic embryos with normal morphology (Fig.3.9a) due to limited exposure to TDZ.

Further, we observed that shoot bud development was restricted to two parts of meristematic zone; it developed either from the plumule or from the periphery of the somatic embryos. However, Joshi *et al.* (2003) demonstrated that in presence of TDZ the meristematic dome of the somatic embryo proliferated to give rise to several caulogenic buds forming peanut plantlets with multiple shoots with single root. TDZ induces

proliferation of the meristematic cells in the plumule of the somatic embryos. This resulted in the formation of a meristematic zone spread between the cotyledons. Several meristematic buds appeared from this zone. The reason behind the spreading of the meristematic zone could be longer exposure (minimum 4 wks) to TDZ. On the other hand, diverse results were noted in the present case could be due to shorter exposure (maximum 96h) of TDZ.

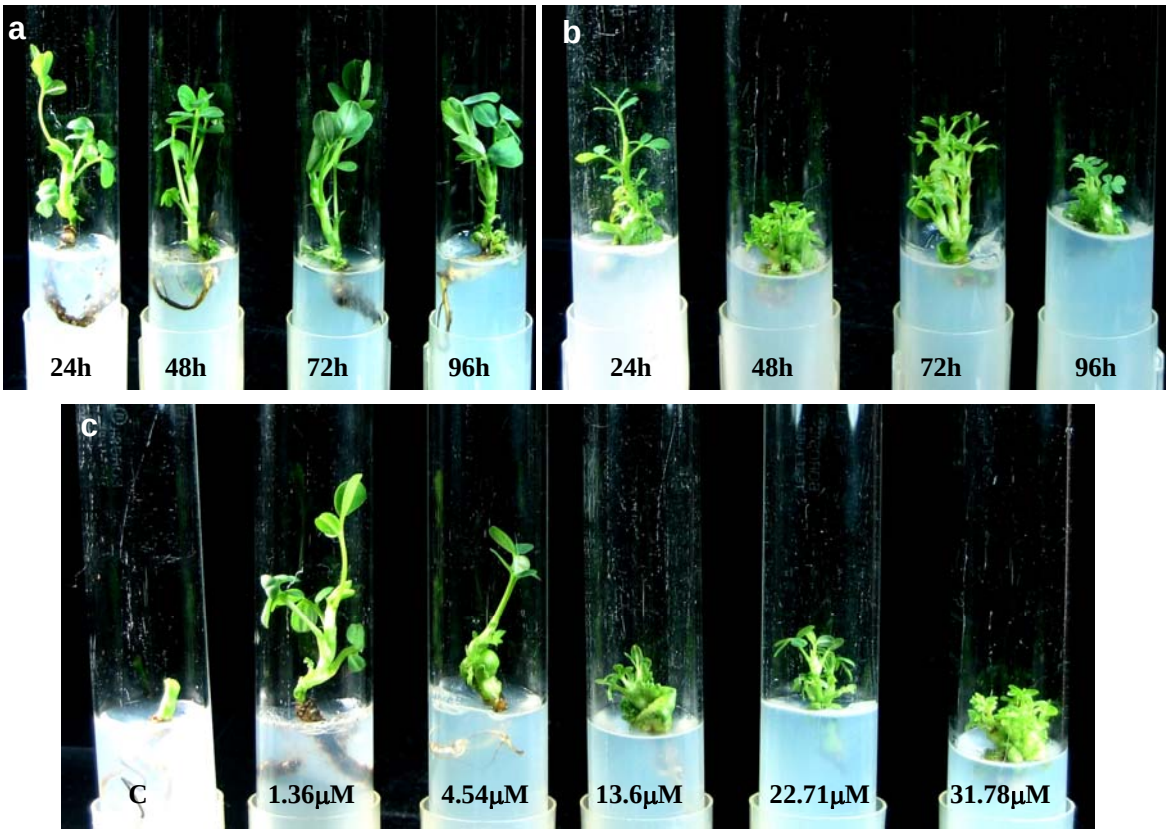


Fig.3.9 a) SEs exposed to MS media incorporated with 1.36 µM of TDZ for 24-96h, converted into normal plantlets in MS medium.
b) SEs exposed to MS media incorporated with 31.78 µM of TDZ for 24-96h, converted into plantlets in MS medium with multiple shoots and less developed roots.
c) SEs exposed to MS media incorporated with 1.36-31.78 µM of TDZ for 24h, converted into plantlets in MS medium. Development of multiple shoots with increasing concentration of TDZ noted.

Kanyand and Prakash (2007) reported the effect of the timing of TDZ application to the culturing of both zygotic embryos and seedling derived explants on MS medium in peanut. Limited application of TDZ (10d) was sufficient to induce shoot formation in peanut. However, an extended use of TDZ and at a higher concentration (30 mg/l)

resulted in the optimum number of shoots. The use of TDZ (0.5 mg/l) for 10 days appeared to be sufficient to promote shoot formation in explants. This experiment is supporting our results that the limited exposures of TDZ led to the development of normal plantlets with single shoot and root.

3.2.3 CONCLUSION

With reference to earlier reports (Chengalrayan *et al.* 1997; Joshi *et al.* 2003) and the data generated in the present experiment, it is apparent that the action of TDZ is always localized to meristematic areas and it varied with the developmental stage of peanut somatic embryos. Its way of action also depends on its exposure duration in combination with other growth regulators.

In present experiments, we find that out of the three stages (EM induction stage, embryo formation stage and embryo conversion stage) of peanut embryogenesis, embryo conversion was the crucial stage to realize the effect of TDZ incorporation and out of the four exposure periods (24, 48, 72 and 96h), 24h exposure to MS basal medium containing 4.54 μM TDZ was the optimum duration for getting normal plantlets with single shoot and single root.

3.3 ASSESSMENT OF PLANTS REGENERATED VIA SOMATIC EMBRYOGENESIS FOR GENETIC UNIFORMITY/VARIABILITY

In plant propagation, a major concern is to retain genetic integrity of the plants, as *in vitro* culture techniques could induce genetic variability, referred as somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation is unpredictable in nature, and can be both heritable (genetic) and non-heritable (epigenetic) (Jain 2001). Tissue culture system itself acts as a mutagenic system because cells are subject to traumatic experiences from isolation, and may reprogramme during plant regeneration, which are different than under natural conditions. Reprogramming or restructuring of events can create a wide range of epigenetic variation in newly regenerated plants (Jain 2000; Kaeppler *et al.* 2000). Many variables influence the amount of somaclonal variation in tissue culture. Beyond the influence of the culture conditions eg. culture media, type of explant, culture duration, the number of subculture, selection pressure, etc., the occurrence and extent of *in vitro* genetic instability may also be highly genotype specific (Meins 1983; Karp and Bright 1985; Peschke and Phillips 1992; Jain 2001; Vazquez 2001; Wilhelm *et al.* 2005). The occurrence of somaclonal variation is associated with point mutations, and chromosomal rearrangements and recombination, DNA methylation, altered sequence copy number and transposable elements (Jain *et al.* 1998; Kaeppler *et al.* 2000). However, gross morphological variations may occur at a much lower frequency than cryptic variations (Evans *et al.* 1984). The frequency of somaclonal variation was found to be dependent on regeneration method and explant source. In general, micropropagated plants obtained from pre-formed meristems have been reported to be uniform (Ostry *et al.* 1994). However, possibility of somaclonal variations cannot be ruled out completely (Rani and Raina 2000). The occurrence of somaclonal and random genetic variations are potential drawbacks when the propagation of an elite plant is intended for the clonal uniformity. On the other hand, stable somaclonal variation of a specific type may be of advantage for the improvement of certain traits. Somaclonal variation can broaden the genetic variation in crop plants, many plant characters can be altered, including plant height, yield, number of flowers per plant, early flowering, grain quality, resistance to diseases, insect and pests, cold and drought, and salt (Jain *et al.* 1998). The value of tissue culture induced variation or somaclonal variation to crop improvement depends on establishing a genetic basis for the variation (Hashmi *et al.* 1997; Jain 2001).

Reliable assays to assess the genetic stability of a genotype throughout tissue culture are highly desirable because the absence of visible variations does not preclude the absence of all variation among micropropagated progeny. Allozyme markers have been used for examining uniformity and cryptic somaclonal variations, but both number and extent of polymorphism and their developmentally regulated expression, limit these markers. DNA markers are a more attractive means for examining genetic similarity/dissimilarity since they are not developmentally regulated (Bindiya and Kanwar 2003). Marker technology based on polymorphism in proteins or DNA has catalyzed research in a variety of disciplines such as phylogeny, taxonomy, ecology, genetics, and plant and animal breeding (Weising *et al.* 2005). Thus molecular tools appear to be more reliable for the assessment of genetic variation (Leroy *et al.* 2001).

In earlier reports on various plant species, variations among somatic embryo derived plants have been observed with different molecular markers. Hashmi *et al.* (1997) detected somaclonal variants by using RAPD markers in peach regenerants initiated from embryo callus culture of leaves. Fourre *et al.* (1997) evaluated somaclonal variation in embryogenic clones initiated from mature zygotic embryos of Norway spruce. Although variation was detected by cytogenetic and morphogenetic analyses, none was observed using RAPD analysis, in spite of using several primers. Vendrame *et al.* (1999) analyzed somaclonal variation by using AFLP in repetitive somatic embryogenesis of pecan initiated from immature zygotic embryos in presence of NAA and BAP. Siragusa *et al.* (2006) reported variation with ISSR and RAPD in regenerant obtained through somatic embryogenesis in style-stigma explants of citrus cultured in synthetic cytokinins (BAP, CPPU, PBU and MDPU). Orbovic *et al.* (2008) detected somaclonal variants by using RAPD in lemon regenerants obtained through somatic embryogenesis from nucellar callus. AFLP analyses of peach palm revealed that 92 % of the regenerated plantlets were true to type produced from shoot meristem through indirect somatic embryogenesis in presence of picloram (Steinmacher *et al.* 2007).

Recently, molecular markers have been successfully used for studying uniformity/variability among micropropagated plants of several crops. Studies on microsatellite markers in trembling aspen (Rahman and Rajora 2001), AFLPs in pecan somatic embryos (Vendrame *et al.* 1999), RAPD and ISSR in peach, almonds, pea and *Codonopsis lanceolata*, (Hashmi *et al.* 1997; Martins *et al.* 2004; Kuznetsova *et al.* 2006;

Guo *et al.* 2006 respectively) and RAPD and cpDNA microsatellites in *Foeniculum vulgare* (Bennici *et al.* 2004) have been reported.

Among the DNA markers, Randomly Amplified Polymorphic DNA (RAPD) has serious limitations with respect to its reliability and reproducibility (Riedy *et al.* 1992). Moreover, RAPDs are dominant diallelic markers; thus, individual parental alleles cannot usually be differentiated in diploid organisms. The sensitivity, reproducibility, co-dominance and strong discriminatory power of microsatellite DNA/SSR (Simple Sequence Repeats) markers or SSR-targeted primers like ISSRs (Inter Simple Sequence Repeats) make them particularly suitable for assessment of uniformity and detection of somaclonal variations (Rehman and Rajora 2001). Moreover, ISSRs offer greater probability than any other PCR marker system in the repeat regions of the genome, which are reported to be the potent regions of genetic variations and this renders ISSRs useful as a supplementary system to any of the random, dominant marker systems (Pandit *et al.* 2007). Molecular markers can be used for the characterization of somaclonal variations with greater precision and less effort than phenotypic and karyologic analyses (Cloutier and Landry 1994).

Morphological variation generated in tissue culture could be visible only at the later stage of maturity (Pietsch and Anderson 2007). Consequently, early detection of variations among the tissue culture raised plants using molecular techniques may be desirable. Clonal uniformity based on morphological and phenological traits is not precise (Rahman and Rajora 2001; Guo *et al.* 2006). However, genomic stability of these regenerated plants can be determined using molecular markers. In the present study, the ISSRs were exploited to generate information on genetic uniformity/variability of *in vitro* grown emblings by using 2,4-D incorporated with TDZ.

3.3.1 MATERIALS AND METHODS

Seeds were surface sterilized as described in Chapter 2 and seedlings grown in MS medium with 2% sucrose, without any growth regulators in 3-4 wks. DNA was isolated from fresh leaf material collected from 18 seedlings derived plants and 62 emblings, using miniprep method reported by Khanuja *et al.* (1999). Emblings were obtained by exposing (24-96h) somatic embryos (developed in 13.6µM 2,4-D from leaflet derived

EM) to various concentration of TDZ (as described in Section of 3.2.1c part of this Chapter and chosen randomly). The details of DNA isolation, checking of purity and PCR protocols are described in Chapter 2.

In preliminary screening with 22 ISSR primers, eight primers (823, 826, 827, 835, 840, 848, 855 & 881), which gave reproducible and unambiguous amplification products, were chosen for genetic uniformity/variability studies. DNA samples of plants were amplified using these eight primers. PCR amplification was done individually for each primer and the experiment was repeated twice. A low range (3Kb) DNA ladder was used for band sizing. Presence/absence of each scorable fragment was recorded in a binary data matrix and the data were analyzed using Win Dist and Win Boot software programs with Dice co-efficiencies.

3.3.2 RESULTS AND DISCUSSION

Visual assessment of the *in vitro* raised emblings (obtained after exposing somatic embryos to various concentrations of TDZ for 24-96h) failed to reveal any morphological differences. In the present study, we tested the somatic embryo raised plants using ISSR markers to assess the uniformity/variability, if any, among the regenerants. Out of 22 ISSR primers, 3 primers did not produce any amplification product, 11 primers gave weak fragments and 8 primers (Table 3.6) produced clear, intense bands. From eight ISSR primers used in the present study, a total of 4240 scorable bands (number of plants analyzed x number of band classes with all the primers) were obtained. The number of bands per primer ranged from 5 to 8 with an average of 6.62 bands per primer. The band sizes ranged from 250 bp to 2380 bp. Among the 53 distinct band classes recorded, 51 (96.2%) were monomorphic for all the emblings and seedling plants and 2 (3.7%) bands were polymorphic for 4 emblings (Table 3.7). Only ISSR primer UBC 835 generated these two polymorphic bands.

PCR profiles of the seedling plants for all the ISSR primers showed no polymorphism. All other primers (Fig.3.10 and Fig.3.11) showed uniformity among the *in vitro* propagated plants except UBC 835. Primer UBC 835 showed minor variations (Fig.3.12). Primer UBC 835 showed different banding patterns among the plants numbered E-23, E-27, E-37, E-48 (Fig.3.12 c-e). The results show that 740 bp size bands were present only

in 3 plants and 660 bp size band was present in single plant, other bands were common to all the plants. From the results, it appears that there are three different groups of plants. Plants numbered E-23, E-27 and E-48 are similar, while E-37 is different from the rest. Remaining 58 emblings have shown identical banding among them and as that of seedling derived 18 plants (Fig.3.12).

Table 3.6: Nucleotide sequence of the primers producing clear amplification products.

ISSR primer	Primer sequence 5'-3'
UBC 823	TCT CTC TCT CTC TCT CC
UBC 826	ACA CAC ACA CAC ACA CC
UBC 827	ACA CAC ACA CAC ACA CG
UBC 835	AGA GAG AGA GAG AGA GYC
UBC 840	GAG AGA GAG AGA GAG AYT
UBC 848	CAC ACA CAC ACA CAC ARG
UBC 855	ACA CAC ACA CAC ACA CYT
UBC 881	GGG TGG GGT GGG GTG
	[Y= (C, T); R=(A, C, G)

Table 3.7: Selected primers with the number of bands and their relative size.

ISSR primer	No. of distinct bands generated	Similarity %	Band sizes (range in bp)
UBC 823	5	100	670-1600
UBC 826	6	100	730-2200
UBC 827	7	100	600-2400
UBC 835	8	75	600-1200
UBC 840	7	100	250-1800
UBC 848	5	100	790-2000
UBC 855	8	100	850-2380
UBC 881	7	100	860-2000

The pair wise matrix of genetic similarities as shown in Fig.3.13 depicts indices of similarities among *in vitro* raised plants of peanut. Estimation of genetic similarity co-efficient based on ISSR band data indicated that out of 80 *in vitro* raised plants selected, 58 plants were 100% identical to each other and to the seedling derived 18 plants and remaining 4 regenerated plants have shown more than 99% similarity to the other plants and seedling derived plants (Fig.3.13). Genetic similarity indices among all the individuals ranged from 0.99 to 1.00. Polymorphic band containing plants had 0.99 similarities to the seedling plants (Fig.3.13), which suggest the reliability of the protocol used for *in vitro* propagation of the peanut cultivar SB-11.

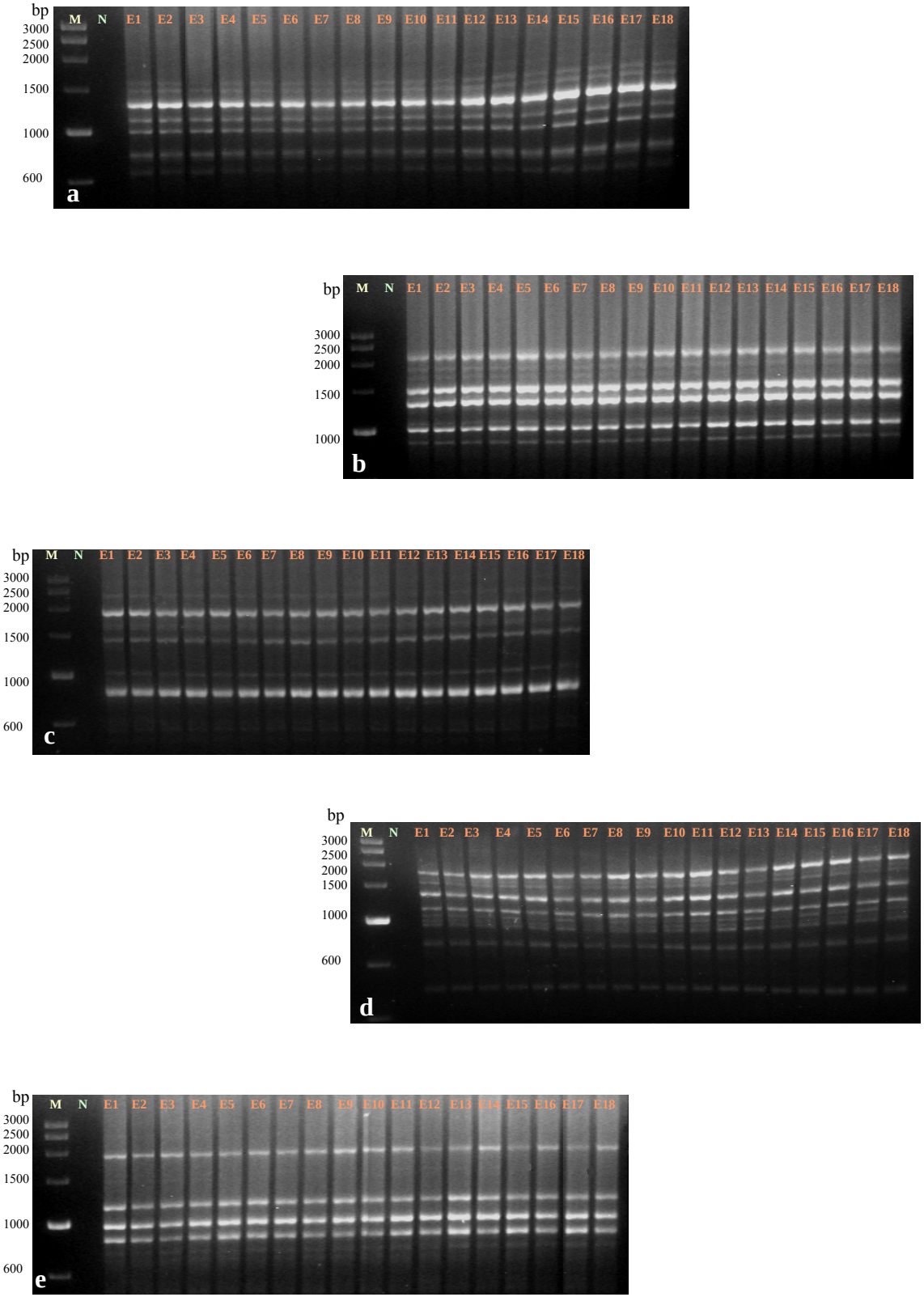


Fig.3.10 Agarose gel electrophoretic pattern of amplification products obtained by using the primer 823 (a), 826 (b), 827 (c), 840 (d) and 848 (e) **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification.

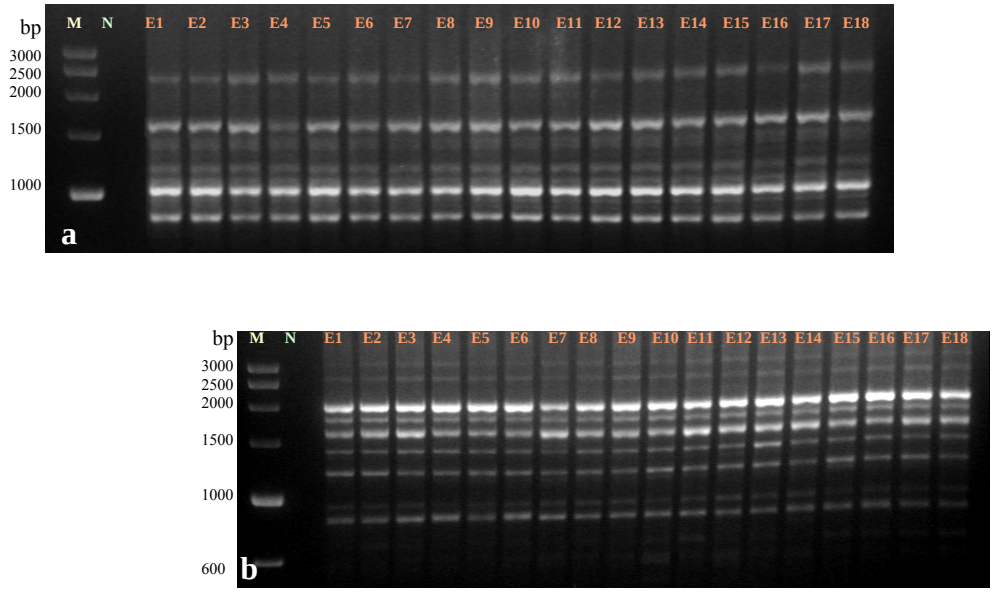


Fig.3.11 Agarose gel electrophoretic pattern of amplification products obtained by using the primer 855 (a) and 881 (b) **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification.

Interestingly, for the same primer (UBC 835), the DNAs of seedling plants grown *in vitro* did not show any polymorphism. Seedlings were only 4 wks old and grown in MS medium with 2% sucrose, without any growth regulators whereas emblings culture were 20-24 wks old and developed in MS medium incorporated with various concentrations of growth regulators (2,4-D and TDZ) and with 2-6% sucrose as described in Sections 3.2.1c of this chapter. Tissue culture, being comprised of a de-differentiation process and a highly stressful condition whereby normal control on cellular level is disrupted and consequently, both genetic and epigenetic alterations are often induced (Phillips *et al.* 1994; Kaeppler *et al.* 2000).

In contrast to our results of uniformity (more than 99%) of peanut somatic embryo derived plantlets, Thomas *et al.* (2006) reported 33-35% of genetic variability of somatic embryo derived tea plants obtained from single line cotyledonary culture with ISSR. Siragusa *et al.* (2006) also reported somaclonal variation among the Citrus plants developed from somatic embryos by using ISSR and RAPD markers and observed that, diphenylurea derivatives induce higher levels of somaclonal variability as compared to BAP. In our present experiment we exposed somatic embryos to TDZ but for short duration and got uniformity in emblings.

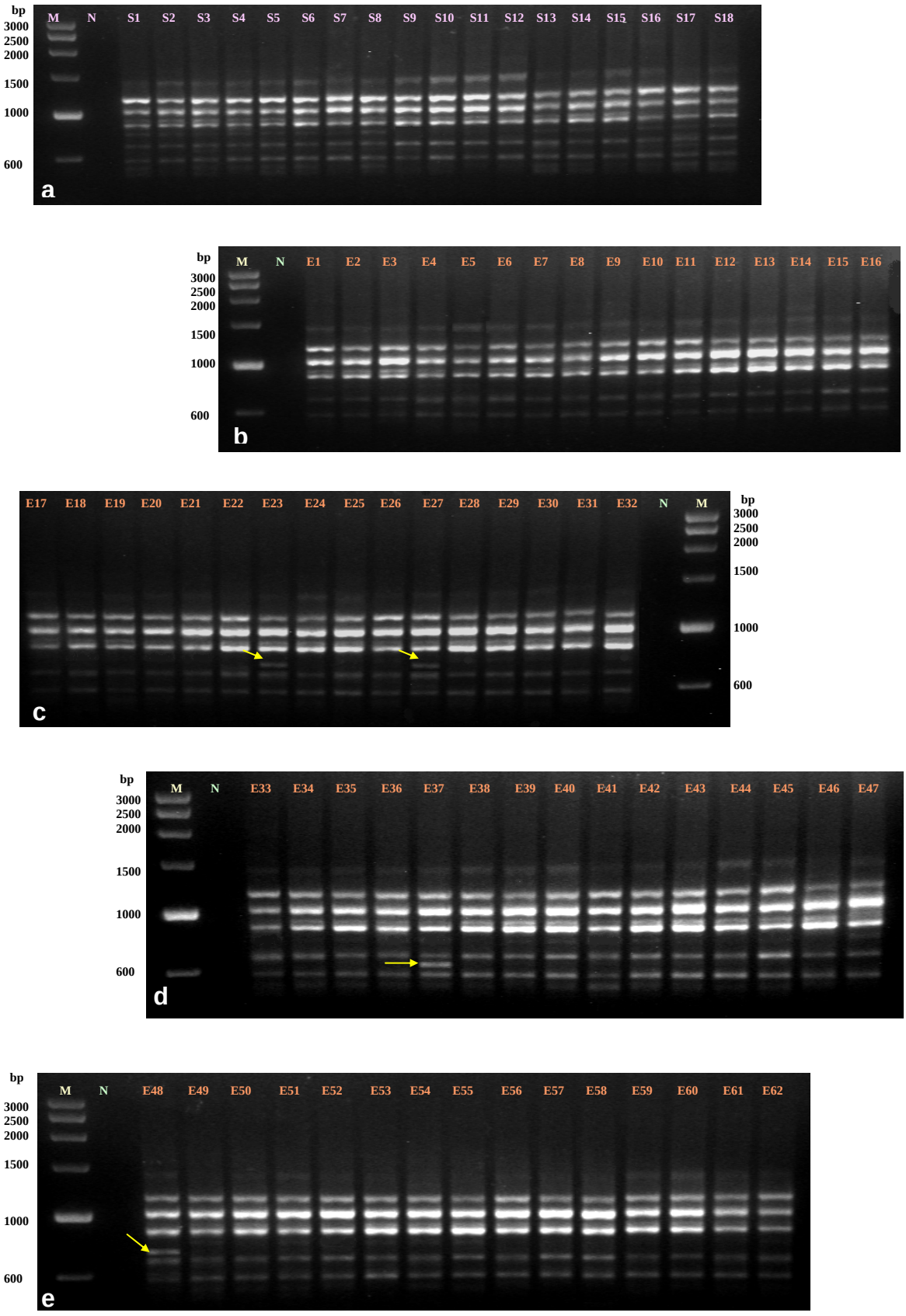


Fig.3.12 Agarose gel electrophoretic pattern of amplification products obtained with eighty plants (a-e) using the primer 835. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification. Lane S1-S18 represents seedling plants and lane E1-E62 represents SE derived plants.

Fig. 3.13 Matrix of similarities for ISSR markers for *in vitro* raised emblings and seedling of peanut

In the present study with ISSR markers, only one type of polymorphism appeared i.e. presence of novel band with fragment size differences and there is no null phenotype (absence of a fragment). Difference in the band intensity was also observed. The presence of variations (as revealed by ISSR-PCR) among the morphologically indistinct plants indicates that visible evaluation may not be sensitive enough to detect minor variations at DNA level (Rahman and Rajora 2001). Our study demonstrated that the uniformity of emblings could not be assured always, and that somaclonal variation cannot always be detected at the gross morphological level.

3.3.3 CONCLUSION

The present study on the molecular characterization of *in vitro* raised plants revealed the uniformity among the plants as assessed with ISSR markers and showed reliability of our protocol for somatic embryogenesis.

Chapter 4

Meristems as explants for somatic embryogenesis

INTRODUCTION

A deeper understanding of induction, initiation and development of somatic embryos is crucial for better regulation of these processes towards various experimental or practical objectives. Knowledge of regeneration mechanisms, including the initiation sites of regeneration and the cells/tissues involved in embryo formation is of high importance for choosing optimum strategy in a particular technology (Griga 2002). The location of initiation sites within the explant as well as the mode of embryo initiation and the cells/tissues involved may be affected by a number of factors, the most important of which include, explant type, its physiological state and position on agar medium, type and concentration of auxin used, and duration of auxin treatment (Hartweck *et al.* 1988; Yeung 1995).

Protocols for somatic embryogenesis in peanut have been developed using explants including immature embryo axes (Hazra *et al.* 1989; Ozias-Akins *et al.* 1989; Roja Rani and Padmaja 2005) and immature cotyledons (Eapen and George 1993; Baker *et al.* 1994). Keeping in view the limitations (i.e., obtaining plant material at the correct developmental stage and contamination) in using immature zygotic embryo derived initial explants, alternative methods for *in vitro* regeneration of peanuts via somatic embryogenesis have been developed using various explants including seedling-derived leaflets (Baker and Wetzstein 1992; Venkatachalam *et al.* 1999b), mature zygotic embryo axis (Mckently 1991; Baker *et al.* 1995), mature zygotic embryo derived leaflets (Chengalrayan *et al.* 1994) and hypocotyl (Venktachalam *et al.*1997). The majority of these protocols are inefficient possibly due to morphological abnormalities in the apical meristem of the somatic embryos as demonstrated in the cultivar JL-24 (Chengalrayan *et al.* 1997, 2001). The abnormalities in somatic embryos lead to low frequency of embryo conversion and plant recovery in medium devoid of growth regulator. This limitation is overcome by several *in vitro* manipulations, which involves time and labor to recover the plants while maintaining sterility. In an attempt to regenerate peanut transgenics an elaborate method involving repeated culturing of the embryos in various media formulations was adopted by Joshi *et al.* (2005). Such protocols are not only time consuming but also pose the risk of microbial contamination during *in vitro* manipulations. Thus, for transformation of peanut, reducing the time to transgenic plant recovery remains an important goal for both biological and direct DNA delivery methods

(Joshi *et al.* 2005). Therefore continuous efforts are being made to develop efficient protocols for *in vitro* regeneration of this important oil seed crop. In a recent study, attempt was made to induce normal embryo differentiation by culturing the EMs in embryo development medium containing 2,4-D and various concentrations of TDZ (Joshi *et al.* 2008). However, somatic embryo development was restricted in the presence of TDZ. In an earlier study, TDZ was used for conversion of abnormal somatic embryos developed from mature zygotic embryo-derived leaflets (Chengalrayan *et al.* 1997).

Somatic embryogenesis system is developed in peanut using embryo axis-derived explants from harvested, dry, stored seeds (Baker *et al.*1995). The explants were cultured horizontally in the medium. In this study, embryos developed on the epicotyl portion of the embryo axes, primarily on the young, expanding leaves. Somatic embryogenesis from the leaf explants is reported (Chengalrayan *et al.* 1994). Various explants have been tested for somatic embryogenesis in peanut except the meristematic cells, which have the potential to differentiate into shoot.

In the present investigation, our objective was to test the embryogenic potential of the determined meristematic cells as explant, presuming that somatic embryos developing from the cells programmed and determined to form shoot may give rise to embryos with potent plumule. Until date, there is no report on development of somatic embryos from defined caulogenic meristems in peanut. Development of somatic embryos from the existing meristems was confirmed histologically. Moreover, regeneration of plants via somatic embryogenesis from the axillary buds may reduce the possibility of appearance of somaclonal variation. Culturing the embryo axis-derived explants horizontally lead to induction of embryos from the leaves (Baker *et al.*1995) differentiated from the meristems. Leaf differentiation from the meristems was restricted by culturing the explants vertically. To test the effect of varied 2,4-D exposure on the process of embryogenesis and embryo conversion, the primary explants were cultured in 2,4-D for varying periods. In addition to the above experiments, the effect of silver nitrate on peanut somatic embryogenesis and embryo conversion was also investigated.

To explore the possibilities of obtaining normal embryos with well-defined plumule and to understand the phenomenon of the origin of somatic embryos the present investigation was designed and conducted in three parts:

1) ***Mature zygotic embryo axis-derived meristems as explants for somatic embryogenesis***
2) ***Axenic shoot culture-derived meristems as explants for somatic embryogenesis***
3) ***Effect of silver nitrate on somatic embryogenesis***

4.1 MATURE ZYGOTIC EMBRYO AXIS DERIVED MERISTEMS AS EXPLANTS FOR SOMATIC EMBRYOGENESIS

Regeneration of plant via somatic embryogenesis from the axillary buds has additional advantages. For instance, developing somatic embryos directly from this explant reduces the possibility of appearance of somaclonal variation. A reliable protocol for regeneration of peanut plants via somatic embryogenesis from the existing meristems will be useful for genetic transformation using direct DNA delivery approach. This approach has been used for genetic transformation of *Tylophora indica* by injection of agrobacterial suspension in the nodal part (Chaudhuri *et al.* 2005).

4.1.1 MATERIALS AND METHODS

Plumule end of embryo axis having three meristems including the shoot tip and a pair of nodal meristems was dissected and used as explant (Fig.4.1a, b). Dissection was done carefully from below the pair of nodal meristems. These were cultured vertically in agar-gelled MS basal medium (Murashige and Skoog 1962) following the same method as mentioned in Chapter 3 (Section 3.1.2) for the standardization of 2,4-D exposure in EM induction medium. In each petridish 10-12 explants were cultured in 10 ml medium in 55 mm petridishes. The experiment was repeated thrice with 30-40 replicates in each repeat. All data were subjected to ANOVA analysis.

Histology:

Histological studies were carried out with the seed derived embryo axis soaked over night in distilled water and, with the explants with developing somatic embryos in the axillary nodes. These were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48h at room temperature and were dehydrated using graded concentrations of tertiary butyl alcohol. The tissues were embedded in paraffin wax using the procedure described (Sharma and Sharma 1980). Serial sections of 10 μm were cut using a rotary microtome. Sections were double stained with haematoxylin-eosin and mounted with DPX *(Loba Chemie,* Mumbai, India) prior to examination under microscope.

4.1.2 RESULTS AND DISCUSSION

All three meristems could be identified in the explant on isolation from the de-embryonated embryo axes (Fig.4.1a, b). On culturing these explants in medium devoid of growth regulator, the meristems turned green (Fig.4.1c) within a few days. Shoot differentiated from the shoot apex confirming the viability of the explants.

After 2 wks in the PM (MS + 90.5 μM of 2,4-D) EM like structures appeared in the axillary meristems (Fig.4.1d) of 89% of the cultures (Table 4.1). These masses (75%) grew in size with days of culture in PM and were obvious in 20-25 days. This reduction in response frequency from 89% to 75% on extended incubation for 2 wks in the PM was due to dedifferentiation of some of the EMs to calli. Apical meristem of the plumule in these explants was swollen and remained white in all the explants tested (Fig.4.1d). Absence of shoot differentiation in the apical meristem indicates 2,4-D induced suppression of shoot differentiation in the tip. On transfer to SM-1, after 2 wks of culture in the PM, 22.8% (Table 4.1) of the explants responded and somatic embryos developed from the mass in the cotyledon node. At the end of the incubation period of 4 wks in this medium the embryos were well developed and both the shoot and root poles could be distinguished (Fig.4.1e). These well-differentiated, bipolar embryos were loosely attached to the explants. Occasionally the shoot tip meristem of the explant that was suppressed under the influence of 2,4-D, differentiated on withdrawal of the growth regulator to form the main shoot whereas somatic embryos developed from the axillary meristems (Fig.4.1e). It is assumed that 2 wks exposure in PM was not enough for total suppression of differentiation of the determined meristematic cells of the apical meristem. Thus, the

cells still carrying the signal to form the plant organs differentiated to form the shoot or leaves on withdrawal of 2,4-D. This presumption was further strengthened on parallel culturing of the similar explants in SM-2 (35.4%) and SM-3 (35.5%) that contains 2,4-D.

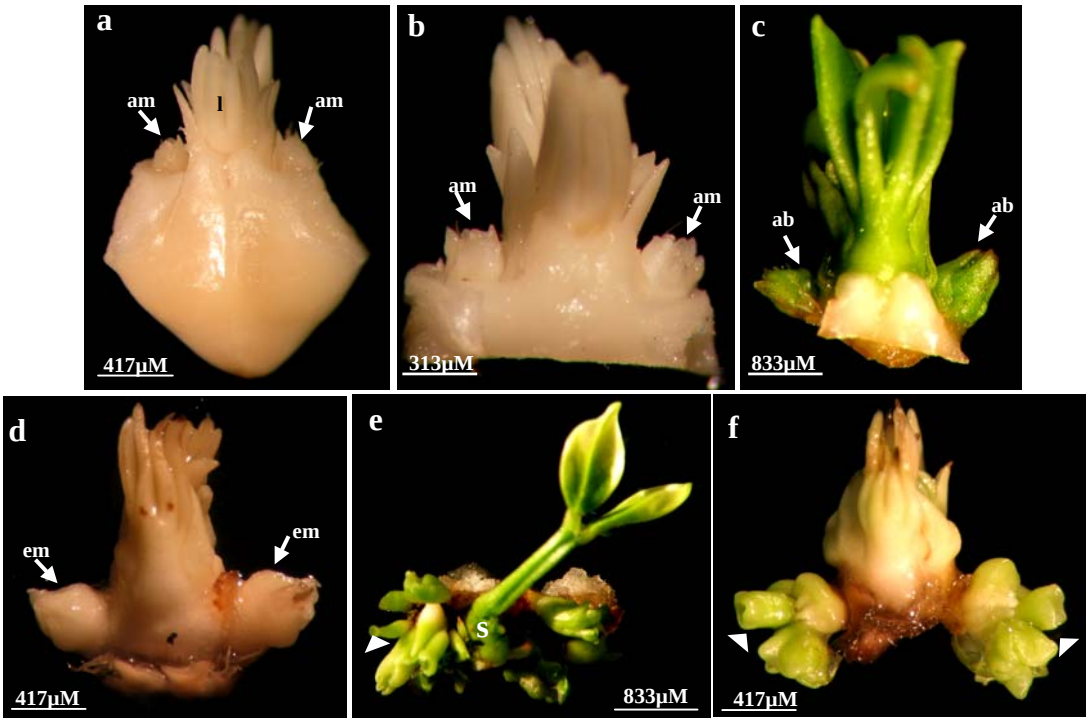


Fig.4.1.a) Seed derived mature zygotic embryo axis (MZEA) with three meristems- apical meristem (covered with leaflets-l) and two axillary meristems (am).
b) One third apical part of MZEA derived explant with all three meristems.
c) Shoot differentiation from the apical meristem and developed shoot primordia in the axillary buds (ab) after 10d in MS medium.
d) Embryogenic mass (em) developed from the axillary meristems after 2 wks in PM.
e) Isolated embryos (arrowhead) developed from the EMs at the axillary meristem in SM-1, after pre-culture for 2 wks in PM. Shoot (s) differentiated from apical meristem.
f) Embryos (arrowhead) developed from the EM at axillary meristems in SM-2 after pre-culture for 2 wks in PM. No differentiation in shoot apex.

There was no shoot or leaf formation from the shoot tips in any of these cultures (Fig.4.1f). A similar observation was reported in *Pisum sativum* (Griga 2002). Using shoot apical meristem of *Pisum sativum* as an explant it was demonstrated that when auxin induced stimulus for somatic embryogenesis was not optimum (early subculture from auxin medium to hormone free medium), the pathway could be converted back to organogenesis (leaf and shoot bud differentiation dominated over embryogenesis). In peanut, in contrast to the bipolar embryos (Fig.4.1e) developed in SM-1, the embryos developed in SM-2 and SM-3 were bipolar but fasciated and appeared abnormal with fused cotyledons (Fig.4.1f). Extended incubation for 4 wks in SM-1, SM-2 and SM-3,

number of embryos developed from each explant increased marginally (4.0 and 5.0%). On transferring the embryos from these cultures to medium devoid of growth regulator, 5.8-13.9% of the embryos germinated and converted into plantlets.

Table 4.1 Effect of 2,4-D exposure on somatic embryogenesis in embryo axis derived axillary meristems.

Exposure period in PM	Response (EM + embryos) (%)	Secondary Medium (MS + Different conc. of 2,4-D) (μM)	Response (EM + embryo) mean±sd* (%)	Average no. of embryo after 2wks in SM mean±sd	Average no. of embryo after 4wks in SM mean±sd	Conversion after 1st subculture in MS mean±sd** (%)	Conversion after 2nd subculture in MS mean±sd** (%)	Conversion after 3rd subculture in MS mean±sd (%)
2 weeks	89	SM-1	22.8 ± 5.1 (75)	2.8 ± 1.0	4.0 ± 2.0	00 ± 00 (56)	00 ± 00	5.8 ± 5.6
		SM-2	35.4 ± 10.7 (83)	3.0 ± 0.9	5.0 ± 2.2	00 ± 00 (75)	00 ± 00	6.6 ± 3.6
		SM-3	35.5 ± 13.2 (83)	1.5 ± 1.3	4.1 ± 2.0	00 ± 00 (46)	2.4 ± 4.1	13.9 ± 9.1
		ANOVA	NS	NS	NS	NS	NS	NS
4 weeks	75							
		SM-1	37.3 ± 17.9 (74)	3.9 ± 0.6	4.4 ± 0.2	00 ± 00 (54)	0.9 ± 1.6	4.0 ± 4.5
		SM-2	39.7 ± 13.7 (74)	4.9 ± 0.5	5.5 ± 0.3	00 ± 00 (62)	7.9 ± 1.0	11.0 ± 2.5
		SM-3	42.1 ± 14.5 (54)	2.6 ± 1.1	3.6 ± 2.8	00 ± 00 (60)	00 ± 00	7.3 ± 4.9
		ANOVA	NS	S 5%	NS	NS	S1%	NS
6 weeks	79							
		SM-1	56.6 ± 3.7 (99)	3.9 ± 0.7	4.3 ± 0.6	3.4 ± 2.1 (223)	10.0 ± 3.0	15.4 ± 3.2
		SM-2	59.9 ± 8.6 (92)	6.5 ± 0.7	7.5 ± 1.4	12.2 ± 6.0 (347)	20.2 ± 7.6	32.3 ± 3.9
		SM-3	47.2 ± 10.6 (92)	3.9 ± 1.2	3.8 ± 1.2	1.4 ± 1.6 (193)	8.2 ± 3.8	14.2 ± 6.0
		ANOVA	NS	S 5%	S5%	S5%	NS	S1%
8 weeks	79							
		SM-1	60.8 ± 4.13 (97)	4.1 ± 0.9	4.2 ± 0.9	6.3 ± 2.4 (227)	6.2 ± 0.9	17.4 ± 3.6
		SM-2	61.5 ± 2.3 (98)	5.7 ± 0.4	6.6 ± 0.3	7.3 ± 5.8 (305)	10.7 ± 9.5	26.0 ± 4.7
		SM-3	54.3 ± 2.6 (91)	4.0 ± 0.4	4.6 ± 0.8	0.3 ± 0.5 (233)	4.3 ± 0.4	12.9 ± 2.8
		ANOVA	NS	S 5%	S5%	NS	NS	S5%

* Three repeats-each with 30-40 replicates, ** Figures in parenthesis indicate total number of embryos tested

On extending the culture period to 4 wks in PM containing 90.5 µM 2,4-D, the masses in the axils were more prominent (Fig.4.2a). Occasionally embryo development was noted in some cultures in PM before transferring to secondary media. Appearance of somatic embryos never occurred in the shoot tip of the plumule. However, the bases of the leaf primordia surrounding the apex were swollen. On transferring these cultures to SM-1 shoot differentiation never occurred from the apex. This was in contrast to the observation (Fig.4.1e) in the cultures in SM-1 after 2 wks pre-culture in PM. This suggests silencing of the apical meristem due to longer exposure in PM. In SM-1, SM-2 and SM-3 the frequencies of embryogenic responses were 37.3, 39.4 and 42% respectively, after 4wks pre-culture in PM. In these cultures somatic embryos appeared from the EMs of the axillary meristems in multiples (Fig.4.2b, c). On culturing the 4 wks exposed primary explants in SM-1, somatic embryos appeared from the EM of the axillary meristems in multiples. Some of these embryos developed into singular, bipolar structures (Fig.4.2b) and the rest were fasciated. In SM-2 and SM-3 somatic embryos developed from EMs of the axillary meristems appeared in clusters. Embryos (Fig.4.2c) were fasciated and appeared morphologically abnormal with fused cotyledons. No single embryos were identified in these clusters.

From these results, it appears that 2,4-D at high concentration, induced transition of the organogenic cells of the axillary meristems to embryogenic cells. Longer exposure (4 wks) in this growth regulator suppressed the caulogenic ability of the shoot apex irreversibly and supported proliferation of the embryogenic cells to develop large masses (Fig.4.1d) in the axils. It affected the process of embryogenesis adversely resulting in the formation of fasciated embryos (Fig.4.1f and Fig.4.2c). When the plumule explants were cultured vertically, the axillary meristems differentiated predominantly over the apical meristems, resulting in the formation of embryos. The degree of suppression of the shoot apex varied with the exposure in 2,4-D. The results of this experiment suggest that by monitoring the exposure in 2,4-D and the orientation of the plumule explant it is possible to obtain both types of morphogenetic activity in two parts of the same explant (Fig.4.1e). Embryos developed in SM-1 (Fig.4.2b) were greener compared to the embryos developed in SM-2 and SM-3 (Fig.4.2c). The average number of embryos developed after 2 wks in SM-1, SM-2 and SM-3 were 3.9, 4.9 and 2.6, which marginally increased to 4.4, 5.5 and 3.6 respectively, on incubation for further 2 wks. Conversion of somatic embryos (4%-

11%) developed after 4 wks in all the three SMs was achieved in MS medium without PGR (Table 4.1).

When explants were incubated for more than 4 wks (6 and 8 wks) in PM, somatic embryos started developing in EMs itself before getting transferred to SMs. This could be due to gradual dehydration of the medium and less availability of 2,4-D to the explants. But embryos developed in any of the SMs after 6 (Fig.4.2d, e) and 8 wks (Fig.4.2f) of incubation in PM were morphologically abnormal and highly fused.

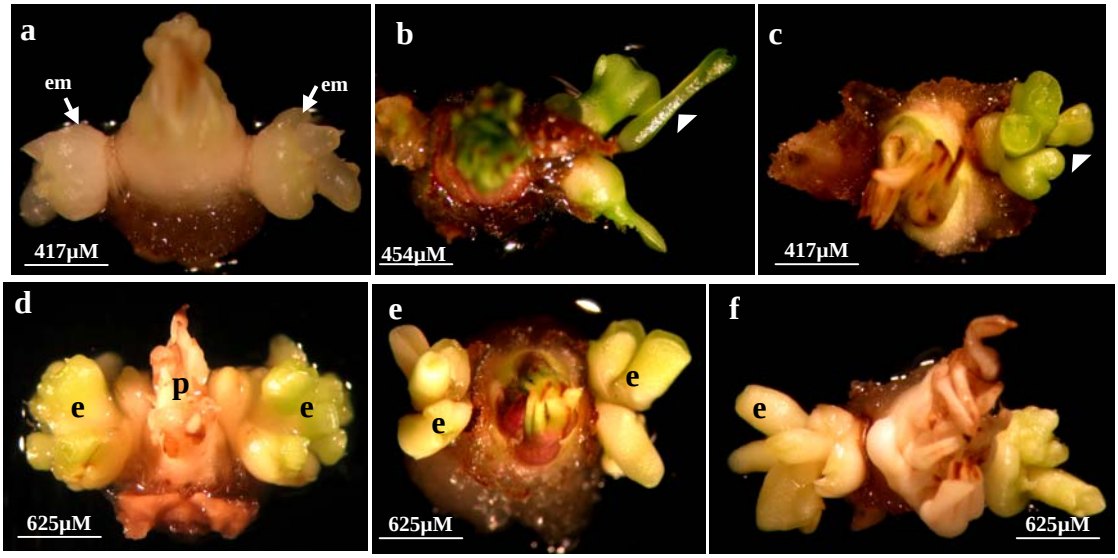


Fig.4.2a) Embryogenic masses (em) in axillary meristems grew larger on incubation for 4 wks in PM.

b) Both single (arrowhead) and fused types of embryos developed from the axillary meristem after 4 wks in SM-1, in explants pre-treated in PM for 4 wks.

c) Bipolar embryos (arrowhead) with fused cotyledons developed from axillary meristem after 4 wks in SM-2. Explants were in PM for 4 wks.

d) Embryos (e) with fused cotyledons developed from axillary meristem after 4 wks in SM-1. Explants were in PM for 6 wks. (P-plumular end)

e) Embryos (e) with fused cotyledons developed from axillary meristem after 4 wks in SM-2. Explants were in PM for 6 wks.

f) Embryos (e) with fused cotyledons developed from axillary meristem after 4 wks in SM-2. Explants were in PM for 8 wks.

Repetitive embryogenesis (Fig.4.3a) was noted occasionally in explants developed in SM-2 and SM-3 after pre-culture for 6 and 8 wks in PM. Isolating apical part of the embryo axis from close to the axillary buds led to the development of embryos from the apical

meristem (Fig.4.3b) but these embryos were very small in size in contrast to embryos obtained from axillary buds and their growth was very slow. Culturing in MS medium for conversion, embryos get detached very easily from the explants (Fig.4.3c) developed in any of the SMs.

In PM after 6 wks and 8 wks embryogenic response varied from 47-59.9% and 54.3-61.5%, respectively (Table 4.1). Extended incubation (6 wks and 8 wks) in PM altered the number of embryos/explant significantly. The number of embryos/explant increased after incubation for 2 wks in all the SMs after 6 (3.9-6.5%) and 8 (4-5.7%) wks pre-treatment in PM (Table 4.1). Optimum average number of embryos developed after 2 wks in SM-2 following 6 wks pre-culture in PM was 6.5, which increased to 7.5 on extended incubation in SM-2 for further 2 wks (Table 4.1). On the other hand, the optimum average number of embryos was 6.6 after 4 wks in SM-2 following 8 wks pre-culturing in PM (Table 4.1).

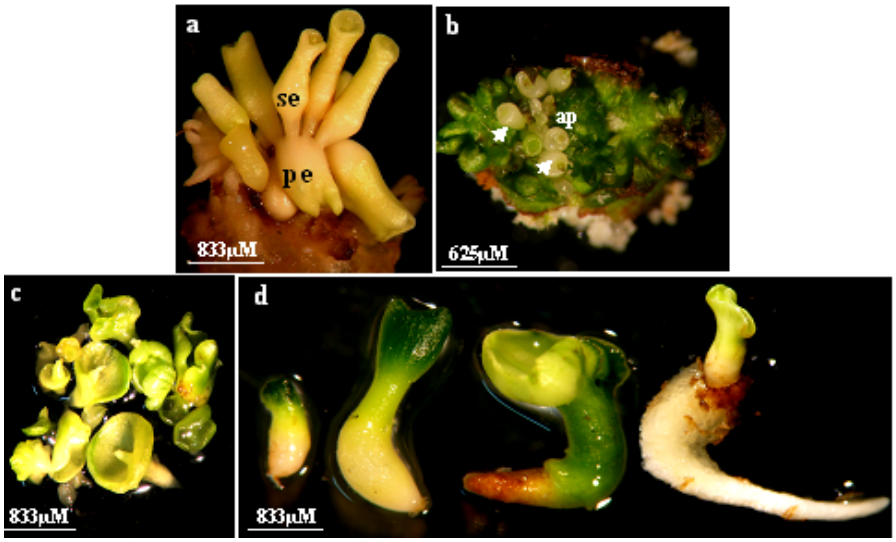


Fig.4.3 a) Secondary embryos (se) developed in SM-2 after 4wks. The explants were pre-cultured in PM for 6 wks.(pe-primary embryo).
b) Bipolar embryos (arrow) with fused cotyledons developed from apical meristem (ap) after 4wks in SM-1. Explants were pre-cultured in PM for 6 wks.
c) Isolated embryos.
d) Radicle emergence from the embryos in MS basal medium.

From the results it is apparent that the frequency of embryogenic response and the number of embryos developed/explant in secondary medium (SM1, SM2 and SM-3), varied with exposure (2, 4, 6 and 8 wks) in the PM (Table 4.1). Frequency of embryogenic response increased with increasing incubation period in PM. It was optimum (61.5%) in the explants pre-cultured in PM for 8 wks (Table 4.1) and then transferred to SM-2 and minimum (22%) in SM-1 in the explants pre-cultured for 2 wks in PM (Table 4.1). The average number of embryos increased with increasing incubation period in PM till 6 wks and afterwards it started decreasing. It was optimum (7.5/explant) in explants cultured in SM-2, after 6 wks pre-culturing in PM.

Embryos germinated (Fig.4.3d) and shoot formation (Fig.4.4a) started in MS basal medium. The optimum conversion frequency was 32% in the embryos developed in SM-2 after 6 wks pre-culturing in PM (Table 4.1) and 25% in the embryos developed in SM-2 after 8 wks pre-culturing in PM (Table 4.1). From the results it is apparent that the conversion frequency was higher for the embryos developed in SM-2 than that for the embryos developed in SM-1 and SM-3 after 4, 6, and 8 wks pre-treatment in PM (Fig.4.5). Thus among all the three SMs, SM-2 is more efficient in facilitating the development of embryos, irrespective of the duration (4, 6 and 8 wks) of pre-culturing in PM, which results in optimum conversion. While the results of the present experiment demonstrate the effect of 2,4-D concentration on somatic embryogenesis, a report by Baker *et al.* (1995) indicates that the concentration of 2,4-D in the medium does not affect embryogenic responses when the apical portion of mature zygotic embryo axis was cultured horizontally, in which case the embryos developed from young expanded leaves of the epicotyl. Chengalrayan *et al.* (1998) evaluated the embryogenic responses and conversion frequency in 16 genotypes of peanut by applying a single protocol (Chengalrayan *et al.* 1994, 1997) initially optimized for peanut cv. JL-24 with MZEDLs. They reported only 4% conversion frequency in this genotype (SB-11) compared to the high conversion frequency (32%) obtained in the present experiment by using meristems as explants. The converted plants acclimatized in plastic cups and hardened (Fig.4.4b) in green house successfully. Pods (Fig.4.4c) were collected during harvesting.

Fig.4.4 a) Converted plantlets.
b) Plants hardened and flowered successfully in greenhouse.
c) Seeds collected from harvested plants.

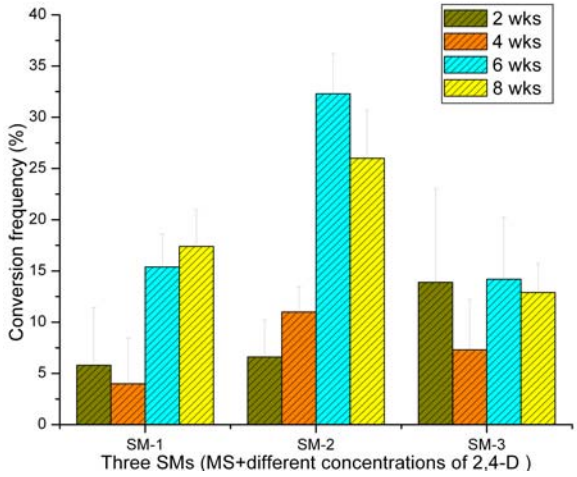


Fig.4.5 Conversion frequency of embryos developed in SM-1, SM-2, and SM-3 after pre-treatment in PM for 2, 4, 6, and 8 wks.

In the present study, development of somatic embryos occurred specifically from the axillary meristems while the growth of shoot apex seized. McKently (1991) cultured mature zygotic embryo axes of peanut and noted embryo development from a 2-mm band of hypocotyledonary tissue surrounding epicotyls.

The role of 2,4-D in plant tissue culture has been reviewed extensively by Feher *et al.* (2003). It has been suggested that 2,4-D has a dual effect in culture above a certain concentration: it acts as an auxin directly through endogenous IAA metabolism or as a stressor. It has also been suggested that 2,4-D affects electrical patterns, membrane permeability, IAA binding to the auxin-binding protein (Deshpande and Hall 2000) and photosynthesis of algae (Fargasova 1994). The protocol for direct somatic

embryogenesis from the existing meristems of peanut provides a suitable system to study some of these phenomena described in algae under the influence of 2,4-D.

Wetzstein and Baker (1993) observed that the concentration of 2,4-D in the induction medium had little effect on embryo morphology and no effect on conversion of somatic embryos in peanut. However, the influence of 2,4-D exposure was not tested. Filippov *et al.* (2006) showed that with extended exposure to 2,4-D, there was an increase in the rate of somatic embryogenesis and number of regenerated wheat plants. Appropriate time duration for mature embryo formation in *Arabidopsis* was determined (Raghavan 2005). Exposure to 2,4-D for more than 10 days showed no indication of increase in the number of mature stage somatic embryos formed during subsequent growth in the basal medium.

Histological analysis of development of embryos from axillary meristems

Two axillary meristems (Fig.4.6a) were obvious in isolated mature zygotic embryo axis. Histological investigation of these initial explants (Fig.4.6b) confirmed the presence of three meristems (two axillary meristems and one apical meristem). Leaf initials and leaf primordia surrounded each bud (Fig.4.6b). Culturing the 1/3rd apical portion of embryo axis in MS basal medium without PGR led to the differentiation of plumular meristem into shoot and development of axillary meristems to axillary buds (Fig.4.6c, d). Vasculatures of axillary buds connected with main vascular strand of the explant were noted (Fig.4.6d).

Five days old explants in PM, showed only swelling at the axillary meristems (Fig.4.6e). Histological studies of these explants showed a dense tissue at the axillary meristems (Fig.4.6f). Culturing in PM for 10 days led to the development of more prominent EM like structures from the axillary meristems of the explants (Fig.4.6g). Histological studies demonstrated the absence of vascular connection (Fig.4.6h) of EMs to the main vascular system of the 10 days old explants. These EMs appeared meristematic and preliminary leaves were present at the base of these EMs (Fig.4.6f, h).

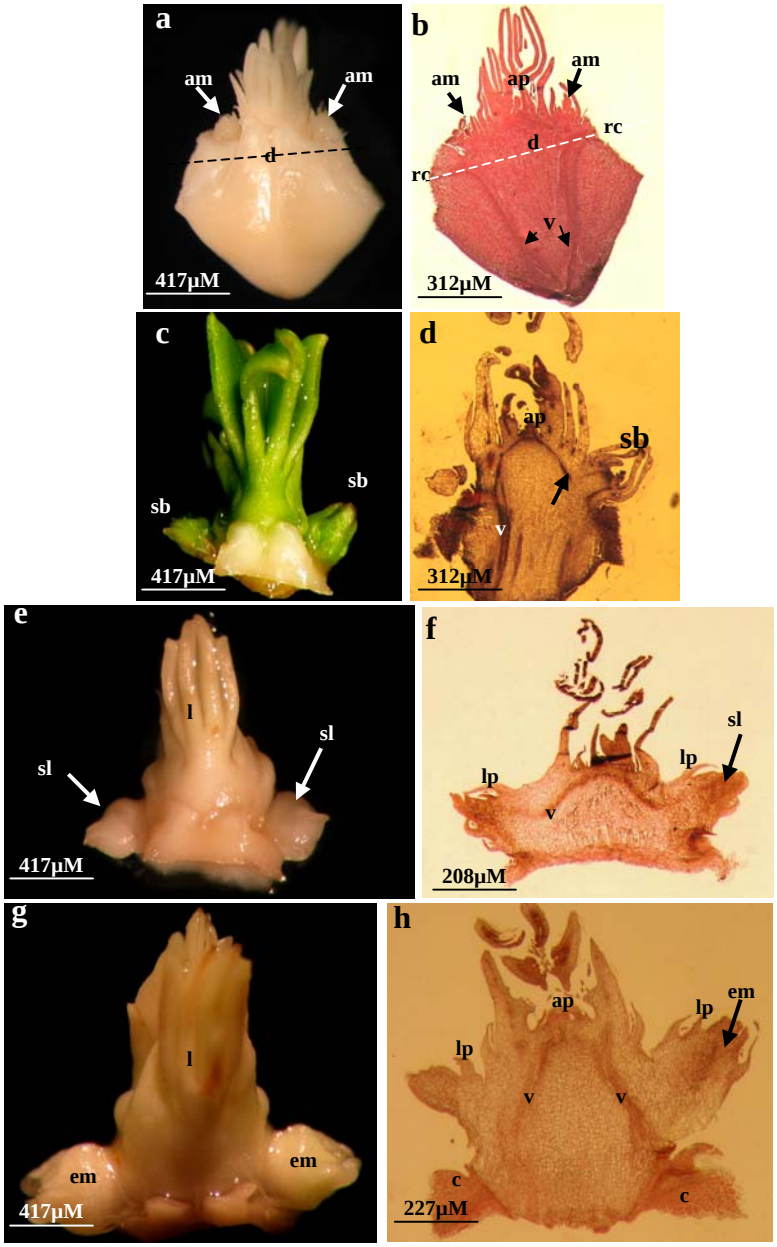


Fig.4.6a) Mature zygotic embryo axis with three meristems. Apical meristem covered with leaflets and two axillary meristems (am). Dotted line (d) is the line of incision to isolate the plumule explant with 3 meristems.

b) Cellular morphology of mature zygotic embryo axis with apical shoot meristem (ap) and two axillary meristems (am). Well-developed vasculature (v) present till cut ends of the removed cotyledons (rc).

c) Shoot differentiation from the apical meristem (ap) and development of shoot primordia in the axillary buds (sb) after 10 d in MS basal medium.

d) Cellular morphology of the explant after 10 d in MS basal medium. Vasculature (arrow) of differentiating apical meristem and axillary bud were connected.

e) Swelling (sl) observed at the axillary meristem after 5d in PM.

f) Histology of the 5d old explant in PM, showing slight connectivity of axillary meristem with apical meristem via vasculature (v).

g) More prominent EM (em) observed at the axillary meristem after 10d in PM.

h) Absence of vasculature strands in axillary buds of the10d old explant in PM. Vasculature (v) present in the mother explant.

Origin of EMs from the meristematic cells of axils could be seen in 2 wks old explants in PM (Fig.4.7a, b). Morphology of the leaflets adjacent to the meristematic buds remained unaffected confirming that the EMs developed specifically from the meristematic cells. Transition of the meristem to mass of cells was obvious and some partially dedifferentiated leaflets and cells at the tips of the masses could be seen. Size of EMs increased with increased incubation in PM.

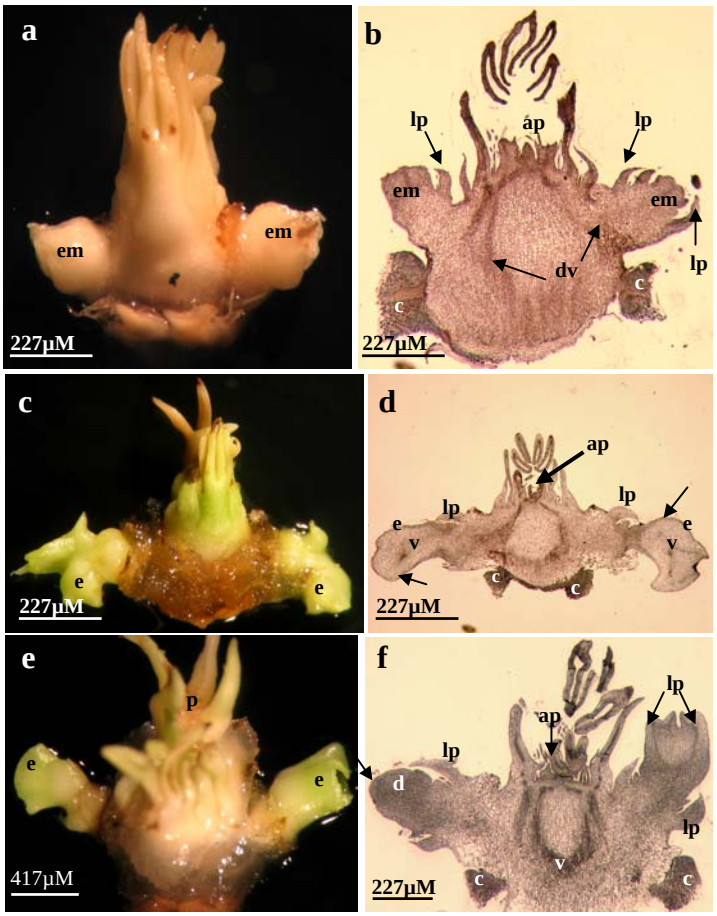


Fig.4.7 a) Developed EM (em) like structure from axillary meristems in PM from 2wks old explant.

b) Morphology after 2 wks in PM shows disintegration of the vasculature (dv), restriction of differentiation of apical meristem (ap), transition of axillary meristem to embryogenic mass (em). Leaf primordia (lp) maintained the integrity. Remaining part of cotyledon (c) is seen at the base of the section.

c) Developed somatic embryos (e) from the EM in the axils. Explants were in SM-1 for 2 wks after pre-culturing in PM for 2 wks.

d) Vasculature (v) and well-developed epidermis (arrow) observed in somatic embryos (e) with broad suspensor after 2 wks in SM-1. Explant pre-cultured in PM for 2 wks. (Leaf primordia -lp).

e) Developed somatic embryos (e) from the EM in the axils. Explants were in SM-2 for 2 wks following 2 wks pre-culture in PM.

f) Cellular morphology, showing dome (d) shaped structure with well-developed epidermis (arrow) from the axils of 2 wks old explants in SM-2 and pre-cultured in PM for 2 wks. Apical meristem (ap) and leaf primordia (lp) maintained the integrity.

Histological studies of the cultures in SMs after 2 wks in PM

Observation of two wks old cultures in SM-1 (Fig.4.7c, d) and in SM-2 (Fig.4.7e, f), pre-cultured in PM for 2 wks confirmed direct somatic embryogenesis in the axillary meristems. It showed that EMs originated from the meristematic cells (Fig.4.7e, f) of the axils, which further led to the development of somatic embryos from these EMs. Epidermis of the somatic embryos developed in SM-1 was much clearer and these embryos attached with mother explant with broad suspensor (Fig.4.7d). Development of vascular strands could be seen in these embryos. Preliminary leaves were present at the base of somatic embryos (Fig.4.7d). Meristematic cells were present at the base of the plumular preliminary leaves. Near the cut of the embryo axis where the cotyledon attached, cells were loosely arranged. In explants, which were in SM-2, embryos were not much developed as compared to SM-1 explants. Leaf primordia, surrounding the meristems demonstrated meristematic activity. Distinct epidermis was observed around the meristematic domes (Fig.4.7f) of the explants, which were in SM-2.

Culturing for further 2 wks (4 wks in total) in SMs (SM-1 and SM-2) resulted in well-developed embryos with distinct vascular strands and epidermis (Fig.4.8a-d). Both fused and single types of embryos were present in the same explant (Fig.4.8d). Many vascular strands were visible in the fused embryos. Fused embryos were attached to the parent explant with broad suspensor (Fig.4.8b, d). Isolated embryos (bipolar embryo) were noted to have only single vascular strands and the embryos were connected to the parent plant with a well-developed narrow suspensor (Fig.4.8d). This result confirms multicellular origin of somatic embryos. At the base of the embryos, callus was also visible (Fig.4.8b, d). At the base of the last preliminary leaf of the caulogenic bud, meristematic swelling was observed (Fig.4.8d). Presence of meristematic mass like structure (Fig.4.8b) at the base of the preliminary leaf of plumule was also noted.

Histological studies of the cultures in PM for 4 wks

Extended exposure of explants to PM, till 4 wks led to the formation of embryos from the caulogenic buds (Fig.4.8e, f). Meristematic structure was also observed in the plumule. Preliminary leaves still maintained their integrity. Development of embryos was noted in PM itself (Fig.4.8f).

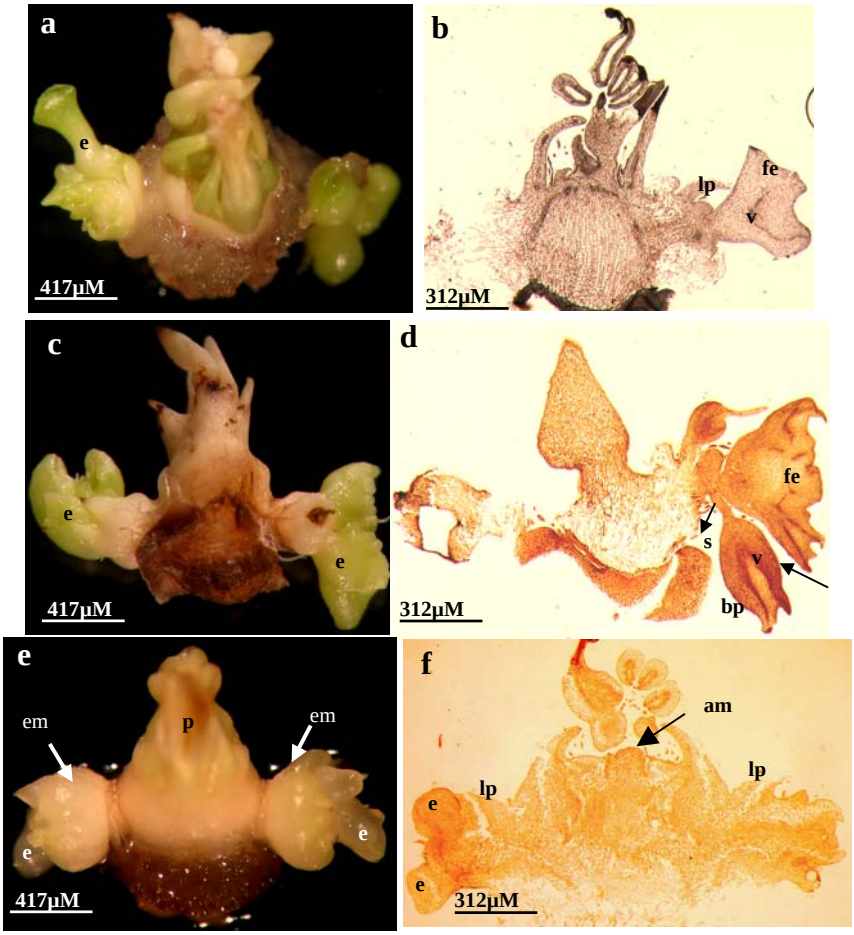


Fig.4.8 a) Somatic embryos (e) developed from the EM in the axils. Explants were in SM-1 for 4 wks following 2 wks pre-culture in PM.
b) Fused embryos (fe) with vasculature (v) developed from the axil after 4 wks in SM-1 following 2 wks pre-culture in PM.
c) Somatic embryos (e) developed from the EM in the axils after 4wks in SM-2 following 2 wks pre-culture in PM.
d) Bipolar (bp) and fused (fe), both types of embryos with well-formed suspensor (s), epidermis (arrow) and vasculature (v) developed from axillary meristem after 4 wks in SM-2. Explants pre-treated in PM for 2 wks.
e) Somatic embryos (e) started developing from EM (em) after 4 wks in PM.
f) Embryos (e) developed from axillary meristem after 4 wks in PM. Apical meristem (am) was clearly visible. Leaf primordia (lp) maintained the integrity.

Histological studies of the cultures in SMs after 4 wks in PM

Transferring the cultures to SMs (SM-1 and SM-2) after 4 wks pre-treatment in PM led to the development of well-developed somatic embryos with well-developed cotyledons and vascular strands and these embryos were attached to the mother explants with broad suspensors (Fig.4.9a-d). These structures had a continuous epidermis with the parental

tissue, which could be due to sub-epidermal cell division of the explants, demonstrating the multicellular origin of the somatic embryos. In SM-2, repetitive embryos were noted from the base of the somatic embryos as well as from the meristematic cells present in the plumule (Fig.4.9e, f), which were also multicellular in origin. On the contrary, repetitive embryogenesis was not observed in SM-1, which implies that the presence of 2,4-D in the previous case (SM-2) could be responsible for the development of secondary embryos.

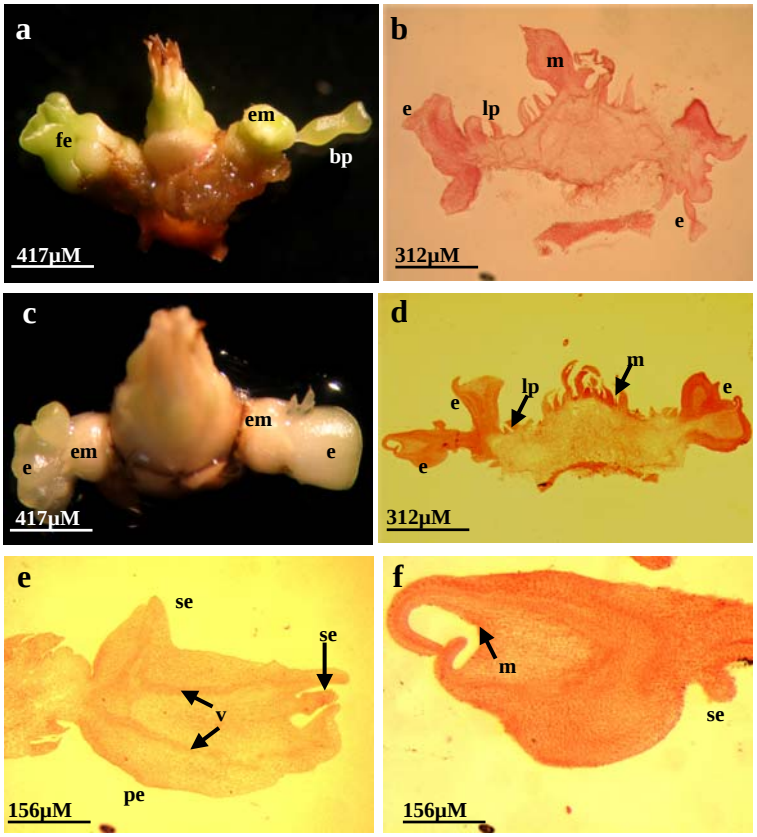


Fig.4.9 a) Fused (fe) and bipolar (bp), both types of somatic embryos developed from the EM in the axils after 2 wks in SM-1 following 4 wks pre-culture in PM.
b) Well-developed somatic embryos (e) attached to the mother explant with broad base. Meristematic (m) structure was present at plumular end. Leaf primordia (lp) maintained the integrity.
c) A cluster of somatic embryos (e) developed from the EM (em) in the axils after 2 wks in SM-2 following 4 wks pre-culture in PM.
d) Somatic embryos (e) developed from the EM in the axils after 2 wks in SM-2. Meristematic (m) swelling was present at the base of plumular leaves.
e) Secondary embryo (se) developed from the plumular part of the primary embryo (pe). Obvious vasculature (v) in primary embryo.
f) Secondary somatic embryo (se) developed from the base of the primary embryo (pe). Meristem (m) is clearly visible in primary embryo.

We observed that the meristem at the shoot tip did not demonstrate any morphological change after 2 wks exposure in PM (90.5 µM 2,4-D). This explained our observation (Fig.4.1e) of shoot apex differentiation into shoots and the development of somatic

embryos from the axils. However, 4 wks exposure (Fig.4.8f) to PM followed by exposure to SM-1 (Fig.4.9b) and SM-2 (Fig.4.9d), were effective in inducing the meristematic activity in the bases of the leaf primordia surrounding the apical meristem (Fig.4.1f). This resulted in the swelling of the shoot apex with a broad base. Nevertheless, the development of somatic embryos was noted only from the EMs present at the axils.

Maheswaran and Williams (1985) suggested that the origin of somatic embryos from one to number of adjacent cells is possible depending on the synchrony of their internal pre-embryogenic determined states and their ability to interact as a group as opposed to individual cells. In the present system, it appears that direct somatic embryogenesis could be of multicellular origin. The suspensors connecting the somatic embryos to the parent tissue were noted to be of both, broad (Fig.4.8d and Fig.4.9b, d) and narrow (Fig.4.8d) types. A similar result was obtained by Hu and Sussex (1971) and Fernando *et al.* (2001) in papaya. According to Maheswaran and Williams (1985), the embryos attached by a narrow suspensor like organ appeared to have arisen superficially, whereas those attached by a broader suspensor might have arisen from meristematic regions within the buds. In the cultures used in the present experiment, direct somatic embryogenesis is associated with suppression of the main zygotic embryo axis. The relationship appears analogous to that between apical and lateral buds. According to Maheswaran and Williams (1985), the growth suppression of the main embryo axis is presumably associated with the breakdown of integration of the cells as a single embryonic group, and escape of individual cells or smaller groups to function autonomously.

4.1.3 CONCLUSION

Our results demonstrate:

(i) The effect of 2,4-D exposure on the frequency and morphology of the peanut embryos.

(ii) It also demonstrates that by altering the orientation of the explant on an appropriate medium, the determined organogenic cells of an explant can be made embryogenic.

(iii) By culturing the determined meristems vertically and by altering the auxin exposure both pathways of morphogenesis can be demonstrated in the same explant.

(iv) Histological studies confirmed that the development of somatic embryo occurred specifically from the axillary meristems.

To the best of our knowledge this is the first systematic study on the influence of 2,4-D on the determined meristems of peanut explants at a particular orientation and on control of two morphogenetic pathways simultaneously in the same explant. This protocol will not only be useful for *in situ* studies on understanding the pathways of morphogenesis and signal transduction but also for genetic transformation using either direct DNA delivery approach or by infection with *Agrobacterium* mediated transformation. The possibility of extending this approach for genetic transformation in *in vivo* system through direct DNA delivery or *Agrobacterium* injection in meristems can also be explored.

A part of this work has been communicated for publication
Somatic embryogenesis from the axillary meristems of peanut (*Arachis hypogaea* L.)

4.2 AXENIC SHOOT CULTURE-DERIVED MERISTEMS AS EXPLANTS FOR SOMATIC EMBRYOGENESIS

In the above experiment we used determined meristematic cells of mature zygotic embryo axis with three meristems as explants for somatic embryogenesis and observed that embryogenesis occurred from the axillary meristems. To test the embryogenic potential of the other meristems of the same species and of a wild species, we extended the protocol to the axenic shoot cultures derived meristems of *Arachis hypogaea* (cv. SB-11) and in *Arachis duranensis* (wild species).

The wild genotypes of peanut are a valuable source of resistant genes against several pests and pathogens besides having high oil and protein contents. The growing concern over the collection, rescue, conservation, multiplication and characterization of the wild species of *Arachis* germplasm relies on the fact that they contain useful genes for the genetic improvement of peanut (Gagliardi *et al.* 2000). However, the multiplication and maintenance of wild *Arachis* germplasm is very labour-intensive and involves specific protocols because many accessions are grown mostly under green house conditions. Even with optimum storage practices, seed germinability and germplasm losses are inevitable (Dunbar *et al.* 1993), since the seeds display a sub-orthodox behavior due to their high lipid contents and thin seed coat, which result in short viability (Vasquez-Yanes and Arechiga 1996). Therefore, there is a limited supply of wild germplasm from the gene bank and it becomes difficult to maintain wild species of *Arachis* for its use in breeding programme. Consequently, *in vitro* germplasm conservation constitutes a viable option for their preservation. *In vitro* protocols for plant regeneration have been reported in different *Arachis* species via indirect way of organogenesis (Gagliardi *et al.* 2000; Rey *et al.* 2000 and 2006; Vijaya Laxmi and Giri 2003). Indirect way of somatic embryogenesis was reported in *A. paraguariensis* (Sellars *et al.* 1990), *A. pintoi* (Rey *et al.* 2000 and 2006) and *A. glabrata* (Vidoz *et al.* 2004). Both direct and indirect ways of somatic embryogenesis were reported in *A. pintoi* (Rey *et al.* 2006*)* by using leaflets. Several wild groundnut species have been successfully regenerated from seed explants including cotyledon, mature and immature leaflets and embryo axes (Rani and Reddy 1996; Vidoz *et al.* 2004; Rey *et al.* 2000; Rey and Mroginski 2006; Gagliardi *et al.* 2000), but studies on the morphogenetic potential of nodal buds from *in vitro* plants are not explored. The methods based on the activation of pre-formed meristems (shoot tips and axillary buds),

which retain the potential to recover true-to-type plants, is desirable for *in vitro* conservation programs.

Micropropagation through direct somatic embryogenesis would help in the mass scale propagation of the wild species and also facilitate germplasm conservation *in vitro*. In addition, the protocol can be exploited for generating new genetic variability in peanut by somatic hybridization through protoplast fusion. This is an attempt to study the effect of growth regulators on morphogenic response of axenic shoot culture derived shoot apices and axillary buds.

4.2.1 MATERIALS AND METHODS

Seeds of cultivated species, *Arachis hypogaea* (cv. SB-11) were procured from local market and wild species, *Arachis duranensis* (ICG No. 8200) were obtained from ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), Hyderabad. Axenic shoot cultures of both the species were maintained in *in vitro* condition for 5 years to preserve the germplasm. Shoot cultures of *A. hypogaea* were maintained in MS basal medium containing 2.22 μM BAP and 2.32 μM KN and 2% sucrose. Shoot cultures of *A. duranensis* were maintained in MS basal medium containing 4.44 μM BAP and 2% sucrose. The cultures were incubated in light.

Cluster of axenic shoots (*A. hypogaea* and *A. duranensis*) was removed from the culture bottles and the shoots were separated aseptically. Leaves were removed (Fig.4.11a1, a2). Explants (shoot tip and nodes) of 3-4 mm (Fig.4.11a3) were dissected from the axenic shoots of both the species (*A. hypogaea* and *A. duranensis*). The length of the shoots varied. Therefore the number of explants obtained from each shoot varied from 2-5 with the length of the axenic shoot. From the shorter shoot only 2 nodal explants could be obtained in addition to the shoot tip. Explants carrying the apical meristem with axillary meristem (shoot tip with 1st node) and explants carrying only a single axillary meristem (node) were cultured vertically in the primary medium (PM) composed of MS (Murashige and Skoog 1962) basal salt with 90.5 μM 2,4-D and 6% sucrose (Chengalrayan *et al.* 1994) in 85 mm petridishes. To determine the difference in potential if any, in the axillary buds due to their relative position from the shoot apex, the explants were arranged in sequence as in the axenic shoots. The cultures of both the species (*A. hypogaea* and *A.*

duranensis) were divided into two groups. One group was incubated in light and the other group was incubated in dark for 4 wks. Embryogenic response was recorded. Afterwards the cultures were transferred to secondary medium (SM) composed of MS basal salt supplemented with 13.6 μM 2,4-D and 6% sucrose. The explants were cultured in the same order as it was in the PM and incubated for 4 wks in their respective culture conditions. Number of embryos was noted. Thereafter all the cultures (including those incubated in light as well as in dark) were transferred to MS medium without PGR and incubated in light. Experiments were repeated twice with 72 and 74 explants in SB-11 and 70 and 75 explants in *A. duranensis*.

The same set of experiments was carried out for both the species, by extending the incubation period of cultures in PM to 8 wks without sub-culturing. Embryogenic response was recorded after incubating the cultures for 8 wks in PM and after 4 wks in SM. The experiment was repeated 5 times with 40-50 replicates in each repeat.

Both the experiments were repeated with medium in which 2,4-D was substituted by picloram and the concentration of sucrose was reduced to 3%. Picloram has been chosen for this study because it has been reported earlier for embryogenesis in immature zygotic embryos (Ozias-Akins *et al.* 1993) and MZEDLs (Joshi 2003) of peanut. Various concentrations (2.07, 4.14, 8.28, 12.42, 16.56 and 20.71 μM) of picloram were tested for induction of somatic embryos from the meristematic explants of both the species (*A. hypogaea* and *A. duranensis*). Similar to the experiment with 2,4-D, the cultures were divided into two groups and one group was incubated in light and the other one was incubated in dark. Cultures of *A. hypogaea* were incubated for 4 and 8 wks and the observations were noted. The explants of *A. duranensis* were incubated initially for 4 wks in both light and dark conditions and the responses were noted. Incubation period was extended for next 4 wks (total 8 wks) in case of explants incubated in light, as they did not show embryogenic response in the first 4 wks. After incubation in different concentrations of picloram, all the cultures (embryos) were transferred to 2.07 μM picloram for the next 4 wks after which they were transferred to MS medium devoid of PGR in light for conversion. These embryos were further transferred twice to MS medium at intervals of 4 wks. The experiments were repeated thrice with 12 replicates each.

The pH of all the media was adjusted to 5.8 prior to the addition of (0.7%) agar. The media were autoclaved at 120°C under 15psi for 20 minutes and distributed in 85mm petridishes. Cultures were incubated in 16h photoperiod at 25 ± 2°C under diffuse cool white fluorescent lights (32μEm^{-2}sec^{-1}) and in dark condition at 25 ± 2°C. All data were subjected to ANOVA analysis.

4.2.2 RESULTS AND DISCUSSION

We did not observe any EM like structures in meristem explants in PM. Development of somatic embryos were observed only in some explants of 1st axillary node (near to shoot tip) of SB-11 cultured in SM for 4 wks after pre-treatment with PM for 4 wks and incubation in light, whereas the shoot tip and other nodes turned brown. Embryogenic response was only 4.5% and average number of embryos per explant was 1.5 (Fig.4.10). These embryos germinated successfully in PGR free media but shoot development was restricted.

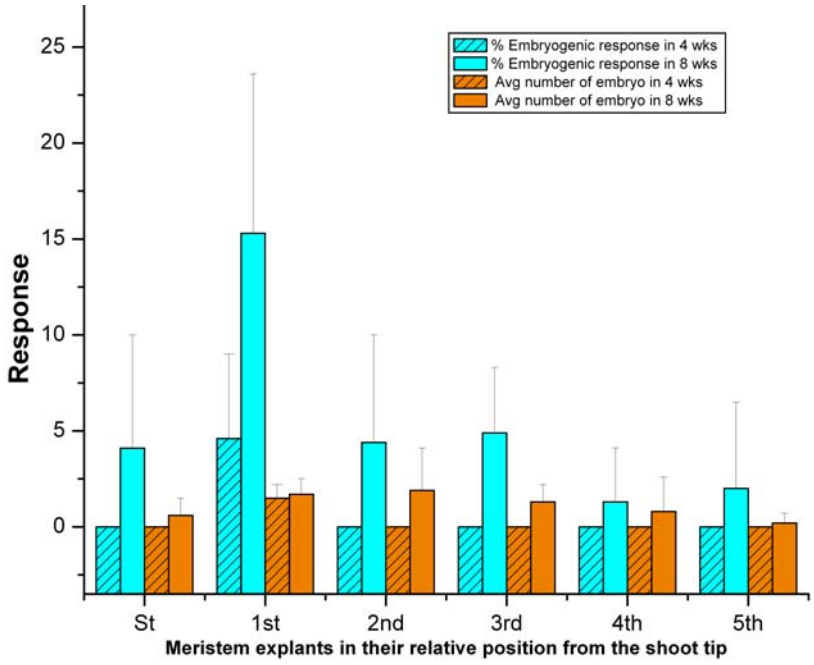


Fig.4.10 Embryogenic response from axenic shoot culture derived meristem explants of *A. hypogaea*, incubated for 4 and 8 wks in PM.

Extended incubation in PM for 8 wks in light and then 4 wks in SM, led to embryogenic response in all the axillary buds tested with varying frequency (Fig.4.10) except in 6th axillary node. Embryogenic response was 4% in shoot tip and it was optimum (15%) in

1st axillary node (Fig.4.10). In 2nd (4.4%) and 3rd (4.9%) axillary node, embryogenic response was almost similar. In 4th and 5th axillary node, embryogenic response was reduced. The average number of embryos per explant was 1.9 in 2nd axillary node (Fig.4.10).

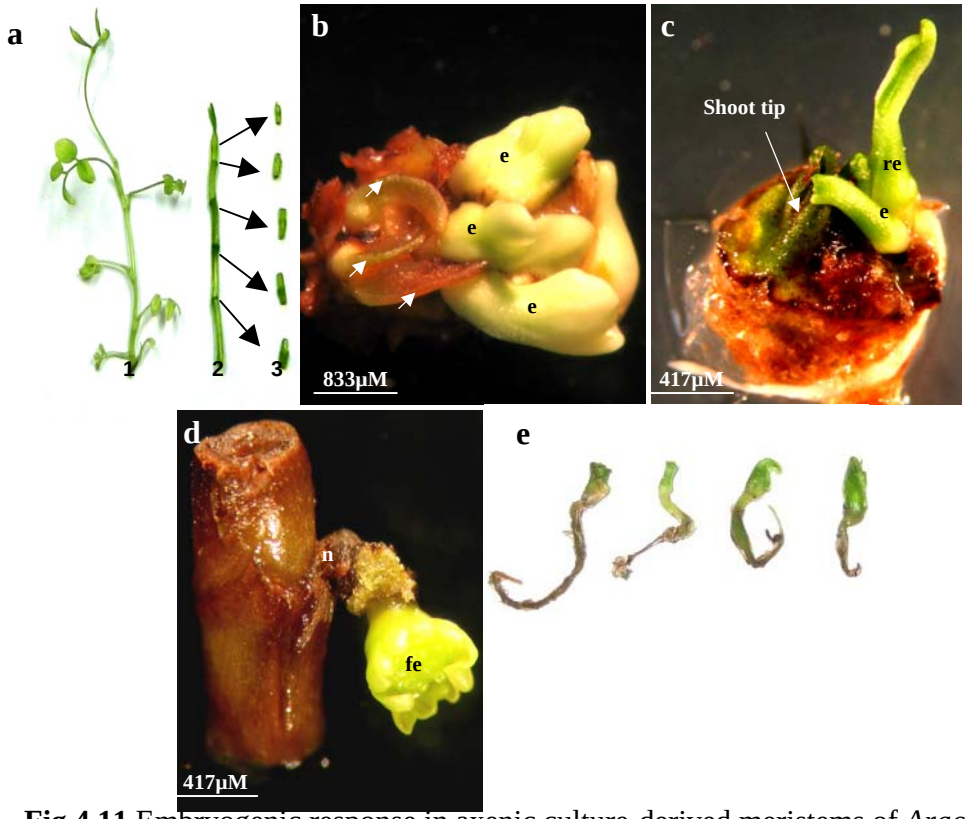


Fig.4.11 Embryogenic response in axenic culture-derived meristems of *Arachis hypogaea*.

a) Axenic shoot culture (1), shoot without leaves (2), isolated nodal parts (3) with nodal bud from different nodes of axenic shoot culture.

b) Embryos (e) developed from shoot apical meristems in SM, pre-treated in PM for 8 wks. Primary leaves (arrow head) turned brown.

c) Embryos (e) developed from 1st nodal meristems in SM, pre-treated in PM for 8 wks started rooting (r). Shoot tips were covered with primary leaves.

d) Single fused embryo (fe) developed from 2nd nodal meristems (n) in SM, pre-treated in PM for 8 wks.

e) Embryos rooted in MS media without PGR.

Shoot tip and 1st axillary node rarely responded together. Mostly either shoot tip (Fig.4.11b) or 1st nodal bud (Fig.4.11c, d) was showing embryogenic potential. Embryos were both of bipolar (Fig.4.11c) and fused types (Fig.4.11b, d). Embryos developed from different axillary nodes and from shoot tip were easily separated from the explants. Somatic embryos germinated (Fig.4.11e) successfully in PGR free media. Germination frequency of embryos was 100%, whereas conversion frequency varied with origin of the

embryo with respect to the position of the nodal buds. Somatic embryo obtained from the 1st and 2nd axillary nodal buds converted into plantlet (Fig.4.12a). Conversion frequency was optimum (9%) in embryos derived from 1st nodal buds (Table 4.2). The converted plantlets hardened successfully (Fig.4.12b). Embryos developed from other nodal buds and from shoot tips did not convert into plantlets in PGR free media.

Table 4.2 Conversion frequency of embryos in MS medium, developed in SM after 8 wks exposure in PM.

Explant	Shoot tip	Node 1	Node 2	Node 3	Node 4	Node 5
Conversion mean±sd* (%)	0±0 (40)	8.8±8.4 (47)	1.7±3.7 (22)	0±0 (10)	0±0 (4)	0±0 (1)
* Figures in parenthesis indicate total number of embryos tested						

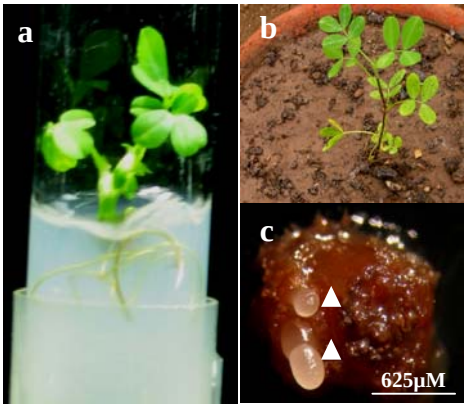


Fig.4.12 a) Conversion of somatic embryo (developed from axillary nodal bud) into plantlet.
b) Hardened plant in green house.
c) Induction of embryos (arrowhead) from 2nd nodal bud in 12.42 µM picloram in dark. Rest of the explant became dark brown.

Increasing exposure duration of PM led to an increase in embryogenic response in the present experiment. Four wks exposure rarely led to the development of somatic embryos that too only in the 1st node (Fig.4.10), whereas 8 wks exposure to PM resulted in embryogenesis from shoot tip to 5th nodal buds with varying frequency (Fig.4.10). This confirms our earlier observation (Section 4.1) that culturing the meristem explants vertically led to the formation of somatic embryos from the axillary buds.

Incubation of explants in dark with PM (irrespective of incubation period) was ineffective in *A. hypogaea* (cv. SB-11) for embryogenesis and only callus was noted. On the other hand, in the case of picloram containing media, all cultures became dark brown after 4 wks exposure in all concentrations of picloram (2.07, 4.14, 8.28, 12.42, 16.56 and

20.71μM), both in light and dark conditions. Embryogenic response was hardly visible (2 out of 36 explants) in explants incubated in dark condition in 12.42 μM picloram for 8 wks but they never grew further (Fig. 4.12c).

Experiments on induction of somatic embryogenesis using axenic culture-derived meristem explants for SB-11 were extended to *A. duranensis* -a wild species, to test the applicability of the protocol for other species.

In contrast to the effect noted in *A. hypogaea* (cv. SB-11), in *A. duranensis*, culturing the explants in the media containing various concentrations of picloram and incubation in dark was highly effective and led to the induction of somatic embryos (Fig.4.13a, b) from axillary buds within 2 wks. Embryogenic response frequency (Table 4.3) was varying in all the concentrations of picloram tested. Within 4 wks repetitive embryogenesis (Fig.4.13c, d) was also apparent. After 4 wks all the cultures were transferred to MS basal media composed of 2.07 μM picloram for maturation of developing embryos. With respect to the distance from the shoot tip, in 8.28, 12.4, 16.56 and 20.71 μM picloram, embryogenic response frequency increased till 2^{nd} node and then started decreasing (Table 4.3). Embryogenic response in the 2^{nd} nodal explants cultured in 12.4 μM and 16.56 μM picloram was 44% and 39 %, respectively (Table 4.3). Next to 2^{nd} nodal axillary buds, the 3^{rd} nodal axillary buds showed higher embryogenic response in 8.28, 12.4, 16.56 μM of picloram (Table 4.3).

The average number of embryos with respect to the explants position on stem, in various concentration of picloram reveals that, in all nodal buds (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th}), average number of embryos was optimum in explants cultured in 16.56 μM picloram (Table 4.3). Within 16.56 μM picloram, the average number of embryos was optimum (13.4) in 4^{th} node followed by 3^{rd}, 2^{nd} and 1^{st} node (Table 4.3). In 12.42 μM of picloram, it was optimum in 2^{nd} node, whereas in the remaining concentrations of picloram, the average number of embryos was not significantly different.

After culturing all the embryos in MS medium without PGR for conversion and incubation in light, the plumular part of embryos turned green (Fig.4.13e) within 4 wks. Embryos could be easily isolated. They showed different types of morphology though all of them appeared morphologically abnormal (Fig.4.14a).

Table 4.3 Embryogenic responses in different concentration of picloram in different nodes of axenic shoots culture of *A. duranensis*.

Explants	2.07 µM PIC mean±sd	4.14 µM PIC mean±sd	8.28 µM PIC mean±sd	12.42 µM PIC Mean±sd	16.56 µM PIC mean±sd	20.71 µM PIC mean±sd
Embryogenic response frequency (%)*						
Shoot tip	0.0 ± 0.0 (36)	5.6 ± 9.6 (36)	5.6 ± 4.8 (36)	0.0 ± 0.0 (36)	2.8 ± 4.8 (36)	13.9 ± 12.7 (36)
Node 1	2.8 ± 4.8 (36)	30.6 ± 45.9 (36)	11.1 ± 12.7 (36)	22.2 ± 9.6 (36)	27.8 ± 9.6 (36)	19.4 ± 17.3 (36)
Node 2	22.2± 19.2 (36)	11.1 ± 19.2 (36)	30.6 ± 25.6 (36)	44.4 ± 17.4 (36)	38.9 ± 4.8 (36)	30.6 ± 24.1 (36)
Node 3	14.7± 13.8 (27)	9.5 ± 16.5 (25)	30.1 ± 16.2 (26)	30.2 ± 2.8 (23)	37.8 ± 20.9 (27)	12.6 ± 14.6 (26)
Node 4	15.3± 16.8 (16)	23.3 ± 25.2 (13)	6.7 ± 11.6 (5)	8.3 ± 14.4 (10)	37.5 ± 12.5 (16)	16.7 ± 28.9 (15)
Node 5	16.7 ± 28.9 (2)	0.0 ± 0.0 (9)	0.0 ± 0.0 (4)	0.0 ± 0.0 (5)	19.1 ± 32.9 (8)	0.0 ± 0.0 (5)
ANOVA	NS	NS	NS	S1%	NS	NS
Average number of embryos per explant						
Shoot tip	0.0 ± 0.0	3.8 ± 5.8	8.0 ± 7.0	0.0 ± 0.0	0.3 ± 0.6	3.5 ± 3.3
Node 1	1.3 ± 2.3	3.0 ± 5.3	1.4 ± 1.7	3.7 ± 1.3	7.3 ± 1.9	2.1 ± 2.0
Node 2	1.3 ± 1.4	4.8 ± 8.4	3.8 ± 2.2	5.0 ± 2.0	9.3 ± 3.0	6.4 ± 3.6
Node 3	3.7 ± 3.2	1.2 ± 2.0	4.0 ± 3.5	3.3 ± 1.9	12.5 ± 6.5	7.5 ± 7.5
Node 4	1.7 ± 2.1	1.0 ± 1.7	0.7 ± 1.2	1.7 ± 2.9	13.4 ± 5.7	1.7 ± 2.9
Node 5	1.3 ± 2.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 3.3	0.0 ± 0.0
ANOVA	NS	NS	NS	S5%	S1%	NS
Germination frequency (%)**						
Shoot tip	0.0 ± 0.0 (0)	4.8 ± 8.3 (22)	5.6 ± 4.9 (24)	0.0 ± 0.0 (0)	0.0 ± 0.0 (1)	5.3 ± 4.6 (25)
Node 1	8.3 ± 14.4 (4)	9.2 ± 15.9 (91)	10.0 ± 17.3 (11)	11.4 ± 10.3 (27)	8.9 ± 5.0 (77)	8.9 ± 7.8 (23)
Node 2	3.0 ± 5.3 (16)	12.1± 20.9 (58)	14.4 ± 13.7 (43)	12.8 ± 2.5 (88)	13.8 ± 3.1 (132)	12.3 ± 13.8 (91)
Node 3	12.2± 10.7 (21)	4.8 ± 8.3 (7)	40.3 ± 8.7 (34)	3.0 ± 5.3 (22)	11.2 ± 1.9 (106)	11.1 ± 10.2 (30)
Node 4	8.3 ± 14.4 (9)	6.7 ± 11.6 (8)	0.0 ± 0.0 (2)	0.0 ± 0.0 (5)	10.5 ± 4.3 (82)	0.0 ± 0.0 (5)
Node 5	0.0 ± 0.0 (4)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	2.9 ± 5.02 (23)	0.0 ± 0.0 (0)
ANOVA	NS	NS	NS	S5%	S1%	NS
Conversion frequency (%)**						
Shoot tip	00 ± 00 (0)	0.0 ± 0.0 (22)	0.0 ± 0.0 (24)	0.0 ± 0.0 (0)	0.0 ± 0.0 (1)	0.0 ± 0.0 (25)
Node 1	0.0 ± 0.0 (4)	0.7 ± 1.3 (91)	3.3 ± 5.8 (11)	0.0 ± 0.0 (27)	3.9 ± 4.1 (77)	0.0 ± 0.0 (23)
Node 2	0.0 ± 0.0 (16)	0.6 ± 0.9 (58)	4.8 ± 4.6 (43)	5.7 ± 5.1 (88)	7.7 ± 2.4 (132)	7.4 ± 9.5 (91)
Node 3	0.0 ± 0.0 (21)	0.0 ± 0.0 (7)	35 ± 16.8 (34)	0.0 ± 0.0 (22)	5.5 ± 0.6 (106)	0.0 ± 0.0 (30)
Node 4	0.0 ± 0.0 (9)	0.0 ± 0.0 (8)	0.0 ± 0.0 (2)	0.0 ± 0.0 (5)	2.7 ± 2.5 (82)	0.0 ± 0.0 (5)
Node 5	0.0 ± 0.0 (4)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (23)	0.0 ± 0.0 (0)
ANOVA	NS	NS	S1%	S5%	S1%	NS

* Three repeats with 12 replicates ** Figures in parenthesis indicate total number of embryos tested

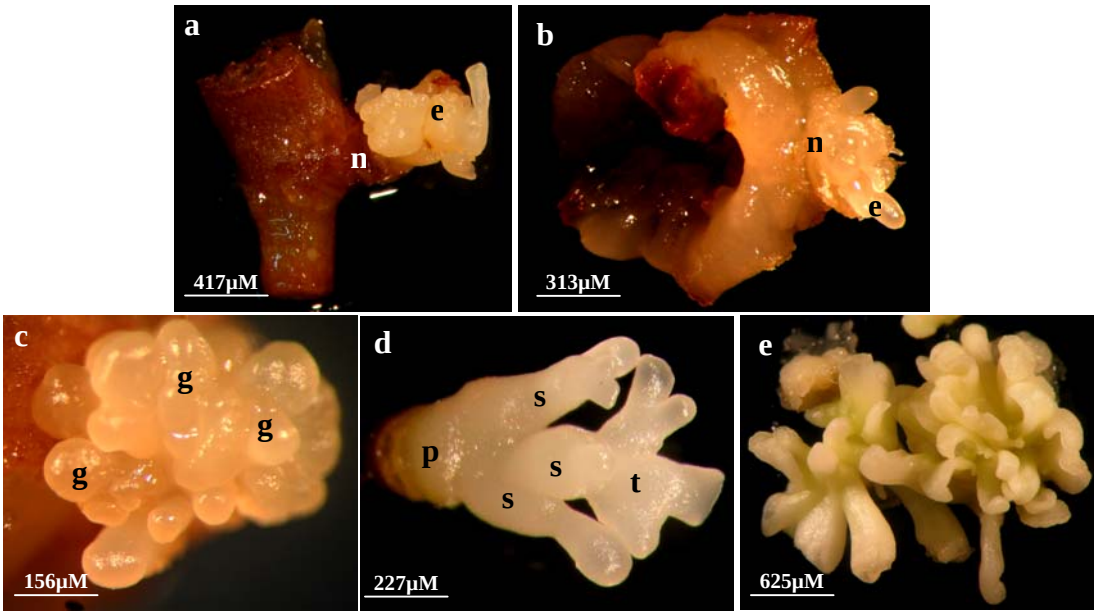


Fig.4.13 Development of somatic embryos (e) in the nodal axillary meristems of *Arachis duranensis.*

a) Induction of direct somatic embryos (e) from 2nd nodal bud after 2wks in 12.42 µM of picloram in dark. (n-position of nodal bud).

b) Induction of direct somatic embryos from 3rd nodal bud after 2wks in 16.56 µM of picloram in dark. (g-globular embryos).

c) Induction of repetitive embryos from 3rd nodal bud after 4wks in16.56 µM of picloram in dark.

d) Repetitive embryogenesis showing primary (p), secondary (s) and tertiary (t) embryos in the same culture.

e) Embryos started differentiating and became partially green in MS medium in light.

After 2nd transfer in MS medium, the embryos started germinating and converted into plantlets (Fig.4.14b). Optimum germination (40%) and conversion frequency (35%) was obtained in 3rd nodal axillary bud- derived embryos, developed in 8.28 µM picloram. There was no pattern in germination and conversion frequency (Table 4.3) with respect to the position of nodal buds on the axenic shoot. In 16.56 µM picloram 1st to 4th nodal bud-derived embryos converted into plantlets with varying frequencies and it was optimum (8%) in 2nd axillary bud-derived embryos. The conversion frequency of somatic embryos, developed in the remaining concentrations of picloram, was quite low (Table 4.3) in MS basal medium without PGR. In 2.07 µM picloram, none of the embryos converted into plants developed from any of the nodal buds. The roots of converted embryos were very thin and short (Fig.4.14b) till 2nd passage in MS medium. Root growth of emblings became better in 3rd passage of PGR free medium (Fig.4.14c).

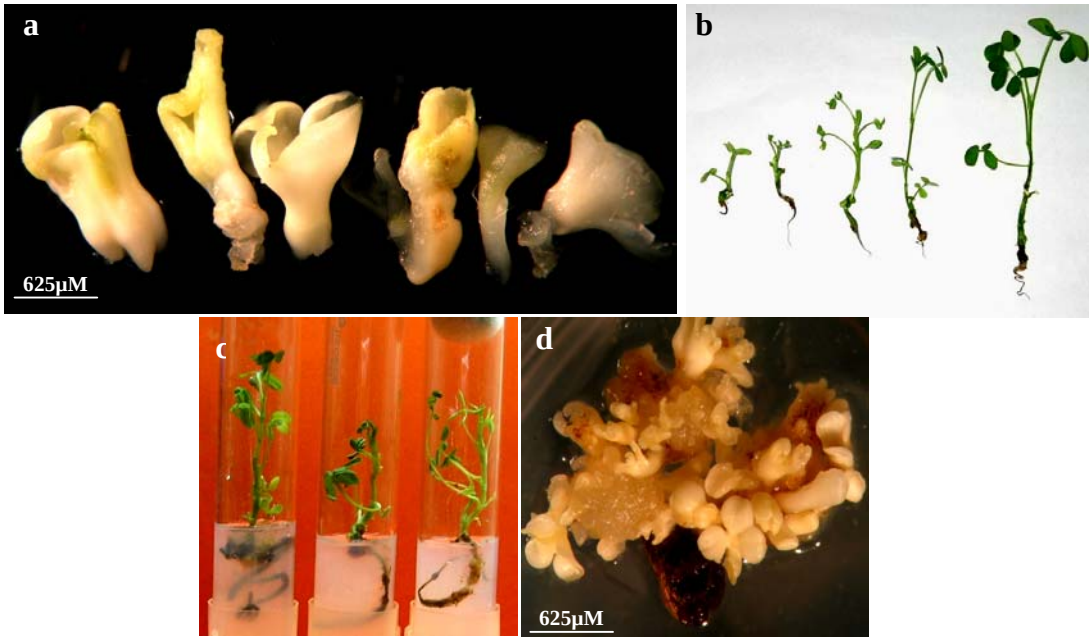


Fig.4.14 a) Somatic embryos of *A. duranensis* developed with different morphologies in picloram.
b) Converted plantlets with asynchronous shoot growth and less-differentiated root.
c) One more passage in MS media led to normal root growth in plantlets.
d) Induction of embryos in SM after pretreatment of explant in PM and incubation in dark.

The present experiments demonstrate that the embryogenic response (44%) and average number of embryos (13.4) were optimum in 12.42 µM and 16.56 µM of picloram respectively (Table 4.3), whereas, the frequency of conversion was optimum (35%) in embryos developed in lower concentration (8.28 µM) of picloram (Table 4.3). Incubation of the cultures in light did not showed any embryogenic response and only callus induction was noted.

In *A. duranensis* 2,4-D was ineffective in light irrespective of exposure period to PM, whereas out of 36 axenic explants only one explant responded in SM pre-treated in PM for 8 wks and incubated in dark (Fig.4.14d).

Thus the present investigation has demonstrated that nodal explants of two species, responds differently, and each species responds differently with different growth regulators. In *A. hypogaea* (cv. SB-11), embryogenic response of meristem explants was noted in 2,4-D, whereas in *A. duranensis* embryogenic response was ensued in picloram. Culture condition also played an important role because *A. hypogaea* responded in light and *A. duranensis* responded in dark. However, it is important to note the difference in

MS media composition used to propagate and maintain (for 5 years) the axenic shoot cultures *in vitro* viz., MS media containing 4.44 μM BAP for *A. duranensis* and MS media containing 22.2 μM BAP and 2.32 μM KIN for *A. hypogaea,* which could be responsible for the differences in embryogenic response between the two species.

In *A. pintoi* (Rey *et al.*2000) and *A. correntina (*Vidoz *et al.* 2004*),* culturing the leaf explants in 2,4-D produced only shoots, whereas in all the accessions of *A. glabrata* (Vidoz *et al.* 2006) tested neither somatic embryogenesis nor organogenesis were observed when leaf were cultured in 2,4-D instead of picloram. In contrast, somatic embryos were obtained in cultivated varieties of peanut in 2,4-D (Baker and Wetsztein 1995; Chengalrayan *et al.* 1994; Little *et al.* 2000). These reports support the observations of the present investigation that 2,4–D is effective only in cultivated variety and hardly responded to wild variety.

Dudits *et al.* (1995) reported the involvement of an early stage dedifferentiation of the induced cells in *in vitro* plant regeneration pathways, which leads to a total reprogramming of differentiated cells at the molecular level. One of the most extreme examples of this plasticity in plant development is the capability of several cell types to initiate embryogenic development. Our result is one of the examples of extreme plasticity because caulogenic nodal buds, which were pre-determined for shoot formation converted into somatic embryos by changing the culture condition and orientation of the explants.

4.2.3 CONCLUSION

The present experiment confirmed that the axillary meristem explants could be worked out for embryogenesis in different species of peanut. It is also confirmed that embryogenic response in peanut tissues relies on genotype, auxin type, concentration, exposure, culture conditions and orientation of the explants. This is the first report on somatic embryogenesis from caulogenic buds of *A. hypogaea* (cv. SB-11) and *A. duranensis*. This protocol will not only be useful for *in situ* studies on understanding the pathways of morphogenesis but also for genetic transformation. These protocols aid in preserving the wild and cultivated varieties *in vitro.* At the same time, it is not required to depend on the availability of seeds from the market (**Shweta *et al.*** Manuscript under preparation).

4.3 EFFECT OF SILVER NITRATE ON SOMATIC EMBRYOGENESIS

Regeneration through somatic embryogenesis is one of the most widely employed methods in transformation techniques. Induction and regeneration of somatic embryos are very sensitive to culture conditions such as the composition of the medium, the physical environment of the culture, the genotype and explant sources. An important *in vitro* factor, unexplored in peanut, is the role of silver nitrate in modifying the effect of ethylene accumulation in the gaseous phase of culture vessels. The involvement of ethylene in plant tissue growth and differentiation has been widely investigated. Application of ethylene precursors and/or inhibitors has shown that ethylene may often have diverse effects in similar tissue culture systems (Fuentes *et al.* 2000). As ethylene production appears to be as universal in cell and tissue culture as it is in intact plants, the ethylene that accumulates in the vessel atmosphere can influence explant growth and morphogenesis (Santos *et al.* 1997). Several researchers have attempted to elucidate the possible influence of ethylene on plant tissue culture. For many plant regeneration systems, ethylene may act as an inhibitor. However, it remains difficult to make generalizations regarding its effects because ethylene may also have promotive effects in certain species. For example, the addition of silver nitrate ($AgNO_3$), a potent inhibitor of ethylene action (Beyer 1976) was shown to promote regeneration in *Brassica campestris* (Palmer 1992) and *Helianthus annuus* (Chraibi *et al.* 1991). Similarly, $AgNO_3$ improved somatic embryogenesis in *Hevea brasiliensis* (Auboiron *et al.*1990), *Solanum tuberosum* (Tiainen 1992), *Hordeum vulgare* (Evans and Batty 1994) *and Picea glauca* (Kong and Yeung 1994). On the other hand, somatic embryogenesis has been shown to be stimulated by ethylene in *Coffea canephora* (Hatanaka *et al.* 1995). In *Daucus carota* somatic embryogenesis, ethylene may act either as an inhibitor (Roustan *et al.* 1992) or a stimulator (Nissen 1994).

In this connection and in continuation with the demonstration of meristems as explants for somatic embryogenesis in the previous sections of this chapter, here we proceed further by investigating the effect of silver nitrate on embryogenic responses and conversion frequency of somatic embryos developed from axillary meristems ($1/3^{rd}$ apical portion of mature zygotic embryo axis). More specifically, the present experiment involves the addition of $AgNO_3$ to the PM and the resulting embryos were monitored for their ability to develop into normal plants.

4.3.1 MATERIALS AND METHODS

To study the effect of silver nitrate on somatic embryogenesis, filter-sterilized $AgNO_3$ was added to the autoclaved PM aseptically at concentrations of 0, 25, 50, 75 and 100 μM. Embryo axis-derived plumule explants were cultured in these media for 6 wks and incubated in light. The embryogenic responses were scored before transferring these cultures to SM-2 for 4 wks and incubated in light. Number of embryos were scored in both PM (with/without $AgNO_3$) and SM-2 medium and mean number of embryos per explant was determined. Obtained embryos were cultured in MS basal medium for conversion into plantlets. The experiment was repeated thrice with 60 replicates in each repeat. All data were subjected to ANOVA analysis.

The pH of all the media was adjusted to 5.8 prior to addition of agar. Media were autoclaved at 120°C under 15 psi for 20 minutes and distributed in 55mm petridishes. In each petridish 10-12 explants were cultured. Cultures were incubated in 16h photoperiod at 25 ± 2°C under diffuse cool white fluorescent lights (32 $\mu Em^{-2}sec^{-1}$).

4.3.2 RESULTS AND DISCUSSION

The media containing $AgNO_3$ turned brown due to photo-oxidation of the latter. While the presence of $AgNO_3$ markedly enhanced the production of somatic embryos, there was no effect on the embryogenic response frequency in PM. The average number of embryos in SM-2 from the explants pre-cultured in PM containing 50 μM $AgNO_3$ for 6 wks (Table 4.4) was higher (6.7) than that obtained from the control cultures. However, at other concentrations (25, 75, 100 μM) of $AgNO_3$, the average number of embryos was lesser than that from control cultures. Embryos induced in SM-2 from the explants pre-cultured in PM incorporated with 25 μM $AgNO_3$ were morphologically bigger (Fig.4.15b) and more isolated than the embryos developed in other concentrations (Fig.4.15a-e). Embryos were not well developed (Fig.4.15e) and mostly EM like structure appeared at the axillary meristem in SM-2 from the explants pre-cultured in PM with 100 μM $AgNO_3$. Transferring all the embryos (developed from explants pre-cultured in PMs with various concentrations of $AgNO_3$) from SM-2 to MS basal medium for germination and conversion to plantlets. All the embryos germinated (Fig.4.15f) successfully. Optimum

(32%) conversion frequency was obtained from embryos developed from cultures pre-treated in PM with 25 μM of $AgNO_3$ (Fig.4.15g). On the other hand, $AgNO_3$ at higher levels inhibited the development of normal embryos (Fig.4.15e) and further conversion of these embryos into plantlets (Table 4.4). Thus the present results indicate a stimulatory effect at low concentration and inhibitory effect at higher concentrations of $AgNO_3$.

Table 4.4 Effect of $AgNO_3$ on peanut somatic embryogenesis

Induction Medium (PM+ different conc. Of $AgNO_3$) (μM)	**Response after 6 wks in PM (EM + embryo) mean±sd* (%)**	**Average no. of embryo (after 6 wks in PM) mean±sd**	**Average no. of embryo (after 4wks in SM) mean±sd**	**Conversion mean±sd** (%)**
PM (control)	45.4 ± 9.4 (272)	4.7 ± 1.0	5.3 ± 1.2	27.4 ± 6.5(280)
PM + 25 Ag	38.2 ± 16.1(279)	4.3 ± 0.0	4.8 ± 1.1	32.3 ± 12.7(289)
PM + 50 Ag	45.0 ± 7.7(284)	4.9 ± 0.9	6.7 ± 1.4	11.9 ± 3.1(204)
PM + 75 Ag	36.6 ± 14.5(291)	3.9 ± 0.5	4.7 ± 1.1	8.4 ± 1.5(218)
PM + 100 Ag	42.3 ± 16.9(292)	4.1 ± 0.8	4.5 ± 0.9	8.9 ± 2.2(225)
ANOVA	NS	NS	S5%	S1%
*Five repeats-each with 60 explants, ** Figures in parenthesis indicate number of embryos tested				

These results could be compared with the literature reports on the stimulatory effect of low concentrations (30-60 μM) of silver nitrate on somatic embryo formation in *Coffea canephora* and the inhibitory effect at higher concentrations on the regenerative capacity (Fuentes *et al.* 2000). In another report, increase in frequency of secondary embryo formation in *C. arabica* was observed in presence of 40μM silver nitrate (Giridhar *et al.* 2004a). Incorporation of $AgNO_3$ at 10–70 μM concentration in the culture medium enhanced the direct somatic embryogenesis of both *C. arabica* and *C. canephora* coffee (Giridhar *et al.* 2004b). Pullman *et al.* (2003) improved the embryogenic response with 20 μM of $AgNO_3$ in pine.

Fig.4.15 a) Embryos (e) developed in SM-2 from the axillary meristems in pre-cultured in PM (90.5 µM 2, 4-D) (control). (p-plumule of the embryo axis)
b) Embryos (e) developed in SM-2 from the cultures pre-treated in PM with 25 µM $AgNO_3$. The embryos were morphologically more isolated than control.
c) Embryos (e) developed in SM-2 from the cultures pre-treated in PM with 50 µM $AgNO_3$. The embryos were highly fused and smaller in size.
d) Embryos (e) developed in SM-2 from the cultures pre-treated in PM with 75 µM $AgNO_3$. The embryos were highly fused.
e) Prominent swelling (sl) noted at the axillary meristems in SM-2 from the cultures pre-treated in PM with 100 µM $AgNO_3$. At this concentration embryos were not well developed.
f) Embryos (e) rooted successfully in MS basal media.
g) Converted plantlets.

Zhang *et al.* (2001) reported that, the addition of $AgNO_3$ to the regeneration medium improved the regeneration frequency and reduced callus formation in all tested cultivars of cassava. They also observed that the optimum concentration of $AgNO_3$ were cultivar-dependent. Addition of the ethylene antagonist, $AgNO_3$, into callus induction medium significantly enhanced embryogenic callus production in buffalograss (Fei *et al.* 2000). Study in white spruce has demonstrated that even higher concentration (100 µM) of $AgNO_3$ stimulates embryo formation by influencing endogenous ABA levels (Kong and

Yeung 1995). In black spruce, the addition of $AgNO_3$ to the maturation medium did not affect either ethylene concentration or somatic embryo production. It was concluded that ethylene accumulation during maturation has no effect on somatic embryo production in black spruce (Meskaoui and Tremblay 1999) and no differences were observed in the germination of embryos obtained from different treatments compared to the control.

Cho and Kasha (1992) hypothesized that ethylene may be beneficial during the induction phase of somatic embryogenesis in barley but detrimental in subsequent stages. Also, Roustan *et al.* (1992) reported that $AgNO_3$ did not affect the induction of somatic embryogenesis in *Daucus carota*, but enhanced the differentiation of somatic embryos, as we had observed in the present study.

4.3.3 CONCLUSION

In the present investigation, we demonstrated the stimulatory effect of $AgNO_3$ at low concentration (25 μM) added to the PM, on direct somatic embryogenesis and conversion of the embryos into plantlets. The results were discussed with respect to the hypothesis that $AgNO_3$ acts as a direct inhibitor of ethylene action, thereby regulating the availability of ethylene in the culture vessel during specific stages of peanut embryogenesis. Nevertheless, it would be necessary to measure ethylene concentration in different stages during embryogenesis to confirm this assumption. Although the concentration of ethylene was not determined in this study, the results emphasize the importance of studying the effect of $AgNO_3$ concentrations on induction stages of embryogenesis and its further development of embryo and its conversion into plantlets.

Chapter 5

Genetic transformation of peanut with Agrobacterium rhizogenes

INTRODUCTION

Genetic engineering has opened new avenues to modify crops, and provided new solutions to satisfy specific needs for creation of genetic variability and selection of desired traits. It offers an impressive option to supplement the ongoing efforts on developing genetically enhanced germplasm for achieving sustainable food production. Genetic transformation techniques should provide a potentially faster alternative to conventional breeding, once appropriate methodologies have been developed (Sticklen *et al.* 1994). In addition to classical and molecular breeding approaches, genetic engineering is being extensively used to introduce desirable agronomic traits through genes encoding high-value recombinant proteins into a variety of crops for quality production (Mansur *et al.* 1995).

Many factors influence transformation efficiency, among which the key factor is the choice of a suitable explant resulting in a high frequency of regeneration. As the optimization of transformation procedure is a lengthy process, monitoring transient expression enables early results of experiments and optimization of procedures to target specific cells that are easily regenerated for assaying gene transfer efficiencies. Apart from explant, efficiency of plant transformation also depends on the genotype, technique employed and regeneration system (Gelvin 2000).

The successful production of transgenic plants relies on several factors: i) means of stably introducing foreign DNA into the genome of a plant cell, ii) method of plant regeneration directly or indirectly from transformed cell, iii) and method by which transgenics can be identified among population of untransformed individuals (Somers *et al.* 2003).

Several techniques (*Agrobacterium*-mediated, electroporation of intact tissues, microprojectile bombardment etc.) have been tested for gene delivery to plant cells. Two major methods, namely *Agrobacterium*-mediated and particle bombardment have been extensively employed for genetic transformation of plants (Chandra and Pental 2003). Each method has limitations with regard to the competent target tissues and genotype. *Agrobacterium* infection depends on biochemical factors within plant, and its infection is more genotype dependent. Likewise, electroporation requires the DNA to be in contact with the plasma membrane of competent cells and is most effective with protoplasts, plant

cells that have had their cell walls removed (Li *et al.* 1995). Molecular analysis of plants obtained by biolistic transformation generally reveals a complex pattern of transgene integration and resulted in high frequency of multiple transgene insertions (Fu *et al.* 2000; Ozias-Akins and Gill 2001). This led to infertility and transgene silencing and was found to be one of the major drawbacks of the biolistic technique. Transgene silencing is often observed when multiple copies of a transgene are integrated, or when the sequence of the inserted genes is homologous to that of an endogenous gene and triggered transgene methylation (Muller *et al.* 1996; Chandra and Pental 2003). In addition, delivery of longer DNA fragments is challenging because the sequence can break while delivering. Although the fate of introduced DNA is not clear, ligation of the transgenic DNA fragments before integration is proposed to account for the observation of arrays of transgenic DNA integrated at the same site into the plant genome. This can result in reduction of transgene expression by co-suppression (Pawlowski 1998). *Agrobacterium*-mediated transformation remains to be the most opted method as it is devoid of most of the above limitations inherent in the direct gene delivery method. In addition, the integration of the transgene are more well-defined in *Agrobacterium*-mediated transformation (Chandra and Pental 2003) and this is the most attractive method because of the ease of the protocol coupled with minimal equipment costs. This technique efficiently introduces variability in transgenic plants due to stable integration and appropriate expression of the limited copy numbers of the transgenes. These advantages make the system adaptable to many different crops (Finnegan and Elroy 1994; Gould 1997; Kumpatla *et al.* 1998; Hansen and Wright 1999).

To date *Agrobacterium* appears to be the only known organism capable of performing permanent gene transfer to a number of plants. During the process of transformation, a specific segment of the Ti-plasmid, T-DNA, might be engineered by initial disarming (removal of the bacterial tumorigenic genes contained in the T-DNA) and a suitable marker and genes of interest, are transferred from the bacterium to the host plant cells and inserted into the nuclear genome (Deblaere *et al.* 1985; Hamilton 1997; Torisky *et al.* 1997). These functions are mediated by a set of virulence genes with optimal expression occurring at acidic pH and in the presence of phenolic inducers, such as acetosyringone, which are released by wounded plant cells (Hooykaas *et al.* 1984; Horsch *et al.* 1986; Hansen and Chilton 2000). To date, a number of economically important crops and

ornamental plants have used these established systems to introduce foreign genes into their plant genomes.

Experience with other legumes (*Vigna species*, *Lens culinaris*, *Phaseolus vulgaris* etc.) suggests that the introduction of foreign genes into peanut using *Agrobacterium* will not be a straightforward task. Problems associated with *Agrobacterium* host specificity and low frequencies need to be addressed. It is possible that utilization of alternative *Agrobacterium* strains will result in better efficiencies in terms of number of transgenic plants recovered and also additional varieties which can be engineered directly. Therefore, an alternative technique to transform the peanut is required (Christou 1997).

A. rhizogenes causes hairy root disease of plants via genetic transformation in a manner similar to the crown gall disease caused by *A. tumefaciens* (Grant *et al.* 1991). *A. rhizogenes* is well known as a soil-borne gram-negative bacterium that induces hairy roots in infected plant tissues by the integration of one or both of two transferred DNAs (T_L and T_R) of the Ri-plasmid into the plant genome (Tepfer 1984). *A. rhizogenes*-mediated transformation has been used to obtain transgenic plants in 89 different taxa, representing 79 species from 55 genera and 27 families (Christey 2001). A diverse range of dicotyledonous plant families is represented, including one Gymnosperm family. In addition to the Ri plasmid, over half of these plants have been transformed with foreign genes, including agronomically useful traits (Christey 2001). The natural host range of *A. rhizogenes* appears to be limited to a small number of plant species: apple, cucumber, tomato, or melon (Weller *et al.* 2004). However, under laboratory conditions, more than 450 different species of plants are found to be susceptible to infection by *A. rhizogenes* (Porter 1991; Yibrah *et al.* 1996). Most plant tissues and organs, including the hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, and tuber, susceptible to be infected and genetically transformed *by A. rhizogenes*, with the resulting production of hairy roots (Giri *et al.* 2000). However, the response varies with the *A. rhizogenes* strain and its interaction with the plant species and tissue type.

Selection of transgenics is an important part of the transformation process. In general, the gene of interest is co-delivered with a suitable marker to identify and encourage the growth of recipient cells. Selectable markers usually confer resistance to chemical agents, such as antibiotics or herbicides that inhibit various cellular functions (Wilmink 1993). *A.*

rhizogenes harboring Ri plasmid has an advantage, because plant cell transformed by *A. rhizogenes* is easily distinguishable by the emergence of hairy roots from the transformed tissue and the explants grow well on hormone free medium (Tepfer 1984; Rao and Ravishankar 2002). Thus it enables the development of transgenic plants via a marker-free selection through the use of hairy root morphology as the primary indicator of transformation (Christensen *et al.* 2008). Furthermore, targeted genes are easily inserted between T_L and T_R regions (Deblaere *et al.* 1987). On the contrary, when *A. tumefaciens* is used as a vector, it is often difficult to distinguish non-transformed cells from transformed tumor cells. Hence, this method necessitates the addition of a selectable marker gene. Antibiotic resistance markers are commonly used in the creation of genetically modified organisms (GMOs), but releasing GMO containing antibiotic resistance markers will be banned in the near future by the European Union (European Union 2001).

The use of *A. rhizogenes*-mediated transformation enables the development of transgenic plants via marker-free selection through use of hairy root morphology as the primary indicator of transformation. Due to high rates of co-transformation the genes of interest on the binary vector are also likely to be present (Christey 2001). *A. rhizogenes* mediated transformation combined with a visual selection for green fluorescent protein (GFP) has been applied effectively in carrot transformation. It was demonstrated that the selection of transformants can be performed visually with a high accuracy and the use of commonly applied antibiotic or herbicide genes for screening can be omitted (Baranski *et al.* 2006). Alternatively, having the genes of interest incorporated in the Ri T-DNA also allows for use of hairy root morphology as the primary indicator of transformation eliminating the need for additional marker genes. Additionally, *A. rhizogenes* mediated transformation requires no selection agents that enables a reduction of labour and time necessary for tissue culture (Baranski *et al.* 2006). *A. rhizogenes* mediated transformation can be selected in a wide range of plant species with desirable genes many of which can be regenerated into plants, often spontaneously (Christey 2001). *A. rhizogenes* is confined to specific uses with potential applications for plant breeding and plant improvement including: root system alteration, use of *A. rhizogenes* and *rol* genes for altered phenotype, and the introduction of desirable foreign genes (Christey 2001). In carrot, tobacco and morning glory, transgenic plants were produced from genetic transformations using wild-type *A. rhizogenes* exhibited the distinct phenotypes of curled leaves,

shortened internodes, reduced apical dominance, reduced fertility, and plagiotropic roots, and were characterized as "hairy-root phenotype" or "T phenotype" (Tepfer 1984).

Transformation of peanut has been accomplished via biolistics (Ozias-Akins *et al.* 1993; Brar *et al.* 1994; Singsit *et al.* 1997; Joshi *et al.* 2006) and *A. tumefaciens* (Lacorte *et al.* 1991; Mansur *et al.* 1993; Cheng *et al.* 1997), and a number of potentially useful genes have been introduced into peanut plants (Chapter 1). Literature on *A. rhizogenes* mediated transformation in peanut is limited (Akasaka *et al.* 1998; Karthikeyan *et al.* 2007; Bolivar *et al.* 2007). Akasaka *et al.* (1998) reported composite plants by induction of transformed hairy roots at the excised site of the epicotyl of dry mature seed of a Spanish type peanut. Karthikeyan *et al.* (2007) reported induction of hairy roots from hypocotyl explants excised from seven-day-old aseptically grown seedlings of peanut. Bolivar *et al.* (2007) established hairy root cultures from peanut seedling derived explants for synthesis of resveratrol and associated derivatives. Till now *A. rhizogenes* mediated transformation is not explored for development of transgenic peanut plants.

This chapter describes the results of the experiments conducted to optimize parameters for *Agrobacterium rhizogenes* mediated transformation of peanut. This experiment includes:

1. *Optimization of A. rhizogenes infectivity for seedling derived explants of peanut.*
2. *Regeneration of transgenic plants from transformed hairy roots and from A. rhizogenes infected mature zygotic embryo axis-derived leaflets of peanut.*

5.1 MATERIALS AND METHODS

5.1.1 Plant Material

Seeds of *Arachis hypogaea* L. (SB-11) were obtained from the local market. Surface sterilization of seeds and mature zygotic embryo axis and establishment of seedling culture is described in Chapter 2 (Section 2.1.2).

5.1.2 Bacterial Strains and culture conditions:

Bacterial strains

Three wild-type agropine strains of *A. rhizogenes*, ATCC15834 (harboring pRi15834), LBA9402 (pRi1855) and A4 (pRiA4), were used for transformation. *A. rhizogenes*, ATCC15834 was obtained from Dr. Sujata Bhargava (Dept of Botany, University of Pune) while LBA9402 and A4were obtained from Dr. Sumita Jha (Center of Advanced Studies in Cell & Chromosome Research, Dept. of Botany, University of Calcutta, Kolkata).

Bacterial medium

Composition of YEB (Yeast extract medium) medium (g/l)

Beef extract ---------- 5.0

Bacto-peptone---------- 5.0

Sucrose ---------- 5.0

Yeast Extract Powder-- 1.0

MgSO4 ---------- 0.49

pH adjusted to 7.2 before autoclaving.

Bacterial culture conditions

The bacteria were maintained on nutrient YEB agar (15 g/l) gelled media. A single bacterial colony of the *Agrobacterium* was picked up and inoculated in 10 ml of YEB nutrient broth medium in culture tube and the culture was placed on rotary shaker at 28°C at 180–200 rpm in dark for 12-16h till the OD_{600} was about 1(approximately 10^8 cells/ml). The OD of the culture was tested using 'Spectrophotometer' with absorption at 600 nm.

5.1.3 Culture conditions for infection and Co-cultivation for seedling derived explants

Explants including leaves, petioles, axillary nodes, internodes and hypocotyls were excised aseptically from *in vitro* grown 3-4 wks old sterile seedlings. Bacterial suspension of 20ml (ATCC15834, LBA 9402 and A4) was poured in empty sterile petridish of 85mm. The excised explants were transferred in the bacterial suspension culture and were wounded with a sterile hypodermic needle. Leaves were wounded at the mid-rib, while petiole, internodal segment, hypocotyls were wounded along their length and axillary

nodes at the axillary bud. Number of explants obtained from each seedling, varied. The explants were left in the bacterial suspension for 5-6 minutes after wounding and were then transferred on a sterile filter paper to eliminate the adhering bacterial suspension. These were cultured in 85mm petridishes containing 20ml of semisolid MS basal medium. The cultures were incubated in dark for 2-3 days until bacterial growth was visible around the explants. Subsequently, the explants were transferred in culture bottles containing 100 ml antibiotic solution (cefotaxime-600 mg/l) to wash off the adhering bacteria. The culture bottles containing the explants suspended in the antibiotic solution was shaken gently and the antibiotic solution was discarded. This process was repeated twice. Explants washed with antibiotic were transferred on a sterile filter paper to eliminate the adhering bacterial suspension. Explants wounded with sterile hypodermic needle in liquid YMB medium were treated as control. Explants were then cultured in semisolid MS basal medium containing 600 mg/l cefotaxime. Every week the explants were transferred to MS basal media with half the concentration of cefotaxime used in the earlier week and finally cultures free of bacterial growth were transferred to MS basal medium without antibiotic. All cultures were incubated in light. Ten to fifteen explants were cultured in 20 ml MS basal medium in 85mm petridish. The experiments were repeated thrice. After co-cultivation with bacteria, few explants became vitrified and subsequently turned brown. These brown cultures were discarded. Survival rate of the explants were calculated by scoring the live tissue out of total explants used for the experiments. Induction of roots from each explant was recorded after 4 wks of culture in MS media and the number of explants with hairy root development was scored.

In order to test further development of these hairy roots, each of the transformed root segments (2-3 cm long) were cultured individually in 30ml of liquid MS basal media with 2 % sucrose in 250ml flask and placed on a rotary shaker at 90 rpm and incubated in light. Observation of hairy root growth was noted at intervals of 2 wks.

MS basal medium was supplemented with 2% sucrose and the pH of the medium was adjusted to 5.8 prior to addition of (0.7%) agar. Media were autoclaved at 120°C under 15psi for 20 minutes and before gelling the molten autoclaved media (20ml) was distributed in 85mm petridishes. Cultures were incubated in 16h photoperiod at 25±2°C under diffuse cool white fluorescent lights ($32\mu Em^{-2}sec^{-1}$) and in dark condition at 25±2°C. All data were subjected to ANOVA analysis.

5.1.4 Regeneration of transgenic plants

5.1.4.1 *Culture condition and media for induction of morphogenic callus from transformed roots*

Transformed hairy roots developed from seedling-derived explants after infection with *A. rhizogenes* were cut in small segments (approximately 1 cm each). These root segments were cultured for induction of morphogenic callus in MS basal medium supplemented with various concentrations of picloram including 2.07, 4.14, 8.28, 12.42, 16.56 and 20.71 μM with 3% sucrose and in MS basal medium supplemented with various concentrations of TDZ including 0.045, 0.23, 0.45, 2.25, 4.54, 6.75, 9.08 μM with 2% sucrose. Cultures were transferred thrice in fresh media with the same compositions once in every 4 wks. Picloram has been used earlier for induction of embryogenic callus from immature zygotic embryos (Ozias-Akins *et al.* 1993) and MZEDLs (Joshi 2003) of peanut whereas TDZ has been used for multiple shoot induction from various parts of peanut seedling (Kanyand *et al.* 1994).

The pH of the medium was adjusted to 5.8 prior to addition of (0.7%) agar. Media were autoclaved at 120°C under 15psi for 20 minutes. Root segments were cultured in each petridish (55mm) containing 10 ml of the medium. The experiments were repeated thrice each with 10 replicates.

Root segments cultured in picloram were incubated at 25±2ºC in dark, whereas root segments cultured in TDZ were incubated in 16h photoperiod at 25±2°C under diffuse cool white fluorescent light (32 $\mu Em^{-2}sec^{-1}$).

5.1.4.2 *Culture condition and medium for infection of MZEDL with* ***A. rhizogenes and regeneration through somatic embryogenesis***

Leaflets from surface-sterilized mature zygotic embryo axis were excised aseptically. Bacterial suspension (500 μl) (ATCC15834, LBA 9402 and A4) was poured in empty sterile eppendorf tubes of 1ml capacity. Leaflets (50-60) were transferred in each eppendorf tube containing the bacterial suspension. These were co-cultivated at room temperature for 5-6 minutes. The leaflets were transferred on a sterile filter paper to eliminate the excess suspension. For control, the leaflets were co-cultured in 1ml of the liquid YMB medium, in eppendorf tubes.

The MZEDL were infected with different strains (ATCC15834, LBA 9402 and A4) of *A. rhizogenes* and cultured on MS basal medium to test the susceptibility of the leaflets to infection with *A. rhizogenes*. The infected leaflets were cultured in 10 ml of MS basal medium without any PGR in 55 mm petridishes. Leaflets were co-cultivated in dark for 2-3 days until the bacterial growth zone appeared around the leaflets. Subsequently, they were washed in antibiotic solution of cefotaxime (600 mg/l), blotted on a sterile filter paper and transferred to agar gelled MS basal medium containing 600 mg/l cefotaxime. Explants were incubated in light for 4 wks. Appearance of hairy roots from the explants was noted to detect the susceptibility of the agrobacterial strains to infect peanut explants. The experiment were repeated thrice each with 50 replicates.

The above experiments revealed that two of the *Agrobacterium* strains including ATCC15834 and LBA 9402 were effective in infecting MZEDLs and produced hairy roots. These two strains were used for further experiments. After co-cultivation with these bacterial strains, the leaflets were cultured in 10 ml of MS basal medium containing 90.5 µM 2,4-D and 6% sucrose in 55mm petridishes. This medium was used for induction of embryogenesis in MZEDLs (Chengalrayan *et al.* 1994). Explants were co-cultivated in dark for 2-3 days until the bacterial growth appeared around the leaflets. Subsequently, they were washed in solution of cefotaxime (600 mg/l), blotted on a sterile filter paper and then transferred to semisolid MS basal medium containing 90.5 µM 2,4-D and 600 mg/l cefotaxime. The cultures were incubated in 16h photoperiod. Development of EMs was scored after 4 wks. For embryo development explants were cultured in MS basal medium with 13.6 µM 2,4-D for 4 wks. The clusters of embryos developed from the EM were transferred to MS basal medium for radicle emergence for 1-2 wks. The embryos with emerged radicle were then transferred to MS basal medium containing 22.71 µM TDZ and 2% sucrose for induction of shoots for one week. This medium is slight modified (duration of exposure of TDZ) of earlier optimized medium for the peanut cultivar JL-24 (Chengalrayan *et al.* 1997), where they exposed rooted embryos to TDZ for 4 wks. After TDZ exposure, all cultures were transferred to MS basal medium for shoot differentiation for 3-4 passages (each with 4wks). The experiment was repeated thrice each with 80-100 replicates. To check the presence of *rol* genes in the aerial part of the transformed emblings, leaves of converted plants both with and without hairy roots were isolated and cultured in 20 ml of MS basal medium with 2% sucrose in 85 mm petridishes.

All media containing 2,4-D was supplemented with 6% sucrose whereas medium without PGR was supplemented with 2% sucrose. The pH of the media was adjusted to 5.8 prior to addition of (0.7%) agar. The media were autoclaved at 120°C under 15psi for 20 minutes. In 85mm petridishes 20ml of autoclaved media was distributed whereas in 55 mm petridishes 10 ml of medium was poured. Cultures were incubated in 16h photoperiod at 25±2°C under diffuse cool white fluorescent lights ($32\mu Em^{-2}sec^{-1}$). For co-cultivation cultures were incubated in dark at 25±2°C. All data were subjected to ANOVA analysis.

5.1.5 Polymerase Chain Reaction (PCR)

Genomic DNA isolation from the putatively transformed and untransformed roots, EM and leaves was carried out as mentioned in the Chapter II (Section-2.2).

5.1.5.1 *Stock solutions*

Solution A: 50 mM Glucose, 10 mM EDTA, 0.1% Triton X-100, 25 mM Tris-HCl pH 8.0 containing 2 mg/ml lysozyme freshly added.

Solution B (lysis buffer): 0.2 M NaOH, 1% SDS (w/v).

Solution C (5 M Potassium acetate pH 4.8): Prepared by adding glacial acetic acid to 5 M Potassium acetate until pH 4.8.

3 M Sodium acetate pH 5.2: Prepared by dissolving 40.8 g sodium acetate in 100 ml water and the pH was adjusted with 3 M Acetic Acid.

RNAse A: stock 10.0 mg/ml.

5. 1.5.2 *Plasmid DNA isolation*

Plasmid DNA from the *Agrobacterium* strains was isolated using the alkaline lysis method (Sambrook *et al.* 1989) with minor modifications.

1. About 1.5 ml of overnight grown culture (16 h) was taken in 1.5 ml eppendorf tube and pelleted out at 10,000 rpm for 1 min.
2. Supernatant was discarded and the pellet was re-suspended in 100 µl of solution A by vortexing for 5s.
3. The mixture was incubated in ice for 10 min.
4. To the mixture, 200 µl of freshly prepared solution B was added and the contents were mixed by gentle inversion. Again the mixture was incubated on ice for 5 min.

5. To this, 150 µl of 5 M Potassium acetate pH 4.8 was added and the contents were mixed by vortexing.
6. The mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to another eppendorf.
7. To the above mixture 3 µl of RNAase A (10mg/ml) was added and the mixture was incubated at 37^{o}C for 15 min.
8. To this, 500 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1) was added, the contents were mixed by inversion and centrifuged at 10,000 rpm for 10 min.
9. The supernatant was collected in another eppendorf and 40 µl of 3 M Sodium acetate pH 5.2 and 1.0 ml of cold (-20^{o}C) absolute ethanol were added. The contents were mixed and incubated at -20^{o}C for 1 h.
10. The mixture was centrifuged at 12000 rpm for 15 min and the pellet was washed with 70% cold (-20^{o}C) ethanol and air dried at room temp.
11. The pellet was re-suspended in 40 µl of sterile deionized water.

5. 1.5.3 ***Estimation of DNA concentrations***

DNA concentrations were determined by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis with known size of λ-DNA marker and also by taking the absorbance at 260 nm.

5.1.5.4 ***PCR reaction for confirmation of transgenics***

Primers used

Transformants were screened for presence of *rol A, B* and *C* (synthesized by MWG-Biotech, Bangalore, India) genes using the sequence specific primers.

For *rol A* gene: For- 5′-CAGAATGGAATTAGCCGGACTAA-3′
Rev- 5′-CGTATTAATCCCGTAGGTTTGTTT-3′

For *rol B* gene: For- 5′-ATGGATCCCAAATTGCTATTCCCCCACGA-3′
Rev- 5′-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3′

For *rol C* gene: For- 5′- CATTAGCCGATTGCAAACTTG -3′
Rev- 5`- ATGGCTGAAGACGACCTG -3`

PCR condition

The PCR reactions were carried out in a total 50 µl volume and consisted of 200 ng of DNA, 10 pm/ µl primer, 200 mM dNTP, 1U of Taq DNA polymerase, 1X PCR buffer and 1.5 mM $MgCl_2$. DNA amplifications were performed in a thermal cycler (Mastercycler personal, Eppendorf, Germany) using the programme: initial denaturation at 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min, 52.5 ºC (for *rolB* gene)/62 ºC (for *rolA* and *C* gene) for 1.5 min and 72 ºC for 2 min and a final extension at 72 ºC for 10 min. The amplification products were visualized on 1.5% w/v agarose gel stained with ethidium bromide (0.5 µg/ml).

5.2 RESULTS AND DISCUSSION

5.2.1 Assessment of *A. rhizogenes* infectivity for seedling derived explants of peanut

Development of watery callus (Fig.5.1) was noted in control explants after 4 wks in MS basal media and there was no sign of root induction. However, in the explants co-cultivated with *A. rhizogenes* hard white callus (Fig.5.2 and Fig.5.3a, b, d) appeared from the injured part of the explants in 2 wks of culture. Afterwards root development started (Fig.5.2b and Fig.5.3a) from these calli. Root induction without the intervention of callus (Fig.5.3c) was noted from the cut ends of the hypocotyl explants. Few explants turned brown or vitrified after co-cultivation and never responded. This was the reason for reduction in the survival rate of explants (Fig.5.4).

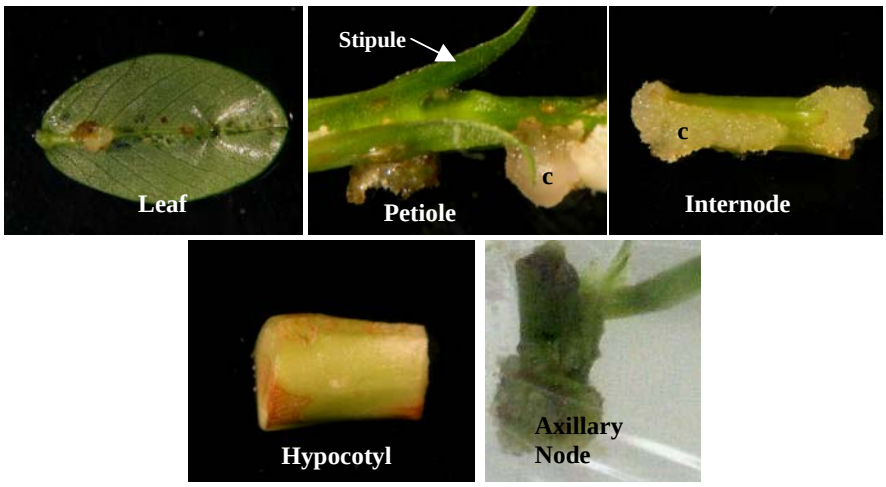


Fig.5.1 Control explants (infected with YMB medium) after 4 wks in MS medium.

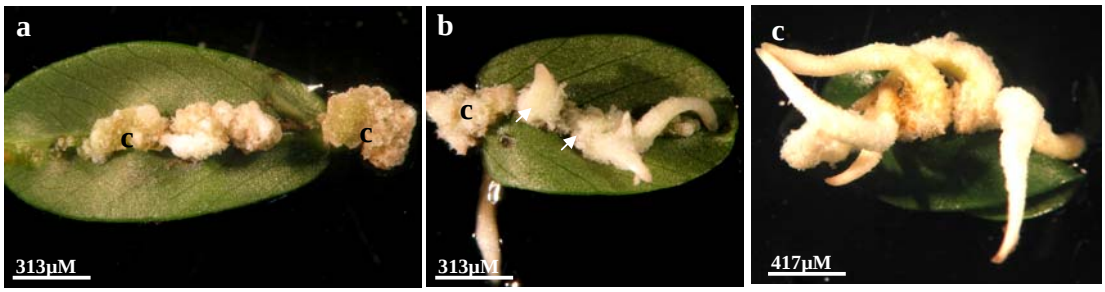


Fig.5.2 a) Initiation of white hard callus (c) from midrib of the leaf.
b) Induction (white arrow) of root from callus (c) developed from midrib of the leaf.
c) Development of root from midrib of the leaf without intervening of callus phase.

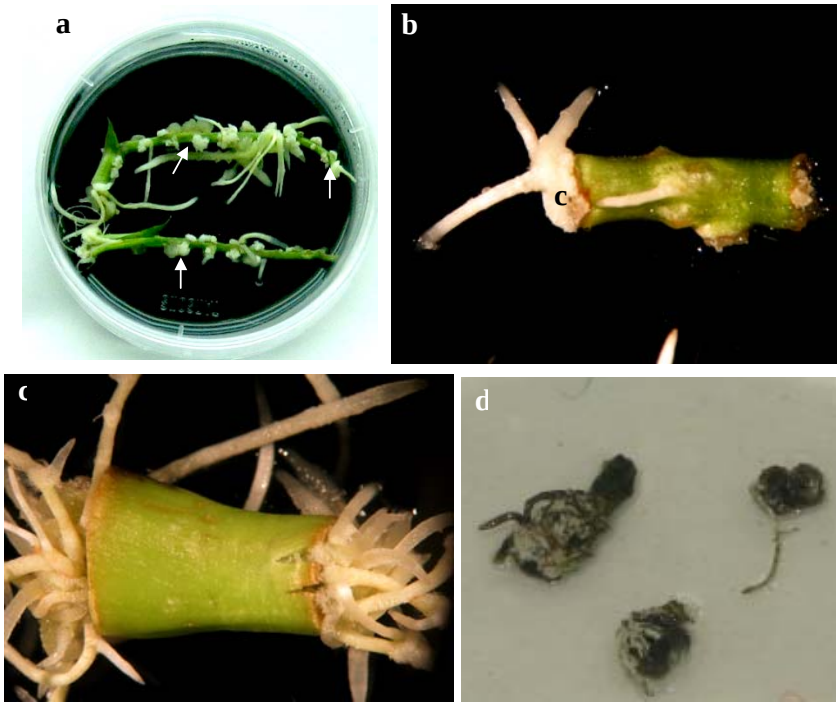


Fig.5.3 Induction of hairy root from (a) petiole (via callus-arrow), (b) internode (via callus (c)), (c) hypocotyl (direct) and (d) axillary node explants.

The induction of hairy roots was correlated with bacterial strain and explant type. Infection with strain ATCC15834 led to the optimum (73%) root induction in the leaves followed by the hypocotyl (69%) (Fig.5.5). All cultures, which showed induction of roots from the injured parts of the explants, did not develop further into hairy root. Hairy root formation was optimum (58.5%) in the leaves followed by the nodal explant (44%)

(Fig.5.6). In case of infection with strain LBA9402, induction of root was almost similar in all the explants but hairy root formation was optimum (94.5%) in the leaves (Fig.5.6) as in the case of strain ATCC15834. However in strain A4, induction (Fig.5.5) and hairy root development from different explants were not significantly different (Fig.5.6).

The frequency of hairy root formation was optimum (94.5%) with *Agrobacterium* strain LBA9402 in the case of the leaves (Fig.5.6). Peanut leaves were more susceptible to infection with the *Agrobacterium* strain LBA9402 (94.5%) than ATCC15834 (58.5%) and A4 (56.8%) for the development hairy roots (Fig.5.6). On the other hand, petiole was more prone to infection and hairy root formation with A4 strain (52%) than to ATCC15834 (43.7%) and LBA9402 (35.6%) strain (Fig.5.6). Internodes and nodal explants were slightly more vulnerable (44.4 and 42.6% respectively) to infection with ATCC15834 than A4 (31.1%) and LBA9402 strain (41.7%). In the case of hypocotyls, A4 was slightly more virulent (50.8%) as compared to ATCC15834 (45.8%) and LBA9402 (36.1%).

The results demonstrated that leaves of *Arachis hypogaea* were more susceptible to strain LBA9402 than to strain ATCC15834 and A4. Virulence or infectivity of *Agrobacterium* strains varies among plant hosts and explant type (Hobbs *et al.* 1989; Bush and Pueppke 1991, Baranski *et al.* 2006) and the transformation efficiency of host plant species can vary between different bacterial strains (Godwin *et al.* 1991, Hu and Alfermann 1993).

Leaves of peanut seedlings injured along the midrib were the best target explants for hairy root formation (Fig.5.6). The higher transformation rates noted in the wounded sites was possibly due to the actively dividing cells at these sites (midrib of the leaves). Cell division in the host target explants tissue is required for successful *Agrobacterium* transformation (Binns and Thomashow 1988). The T_L-DNA (*rolB* in particular) is responsible for the ability of the transformed plant cells to respond to auxin (Cardarelli *et al.* 1987; Shen *et al.* 1988). Therefore, explants capable of auxin synthesis are anticipated to show higher transformation rates.

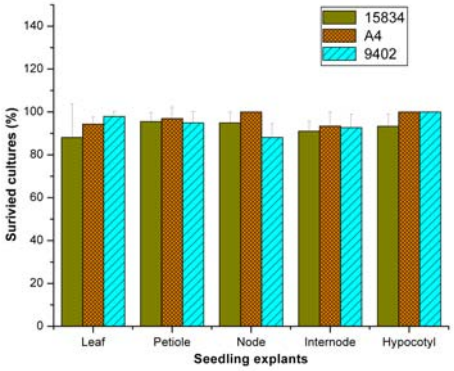


Fig.5.4 Survival rate of the different explants after co-cultivation with different *Agrobacterium* strains.

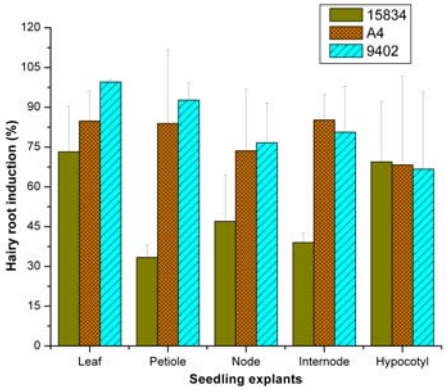


Fig.5.5 Induction of hairy root from the different explants after infection with different *Agrobacterium* strains.

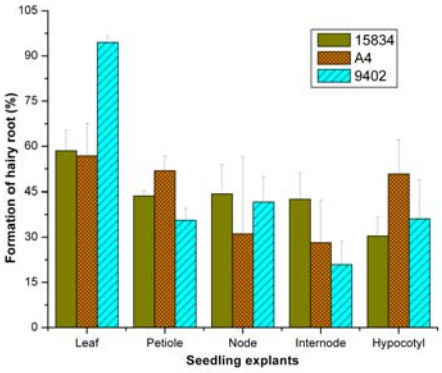


Fig.5.6 Development of hairy roots from the different explants after infection with different *Agrobacterium* strains.

Peanut hairy roots showed a phenotype that had no root hairs along the main root but with higher lateral branching (Fig.5.7a). Earlier report in peanut transformation with *A. rhizogenes* also reported the absence of root hair on transformed root (Bolivar *et al.* 2007). The absence of root hairs has been reported in *A. rhizogenes*-derived transformed roots from *Trichosanthes kirilowii* (Savary and Flores 1994). Growth of hairy root was rapid on sub-culturing the roots in liquid medium, and within 2 wks it formed root bunch (Fig.5.7b). Rapid growth of the roots confirmed the transgenic nature of the culture. Development of hairy root from the transformed tissue is an indication of transgenics, developed after *A. rhizogenes* mediated transformation (Tepfer 1984). Thus we established the hairy root culture of peanut successfully. Hairy root cultures of peanut have been used for production of resveratrol (Bolivar *et al.* 2007). This compound is having anti-cancer properties.

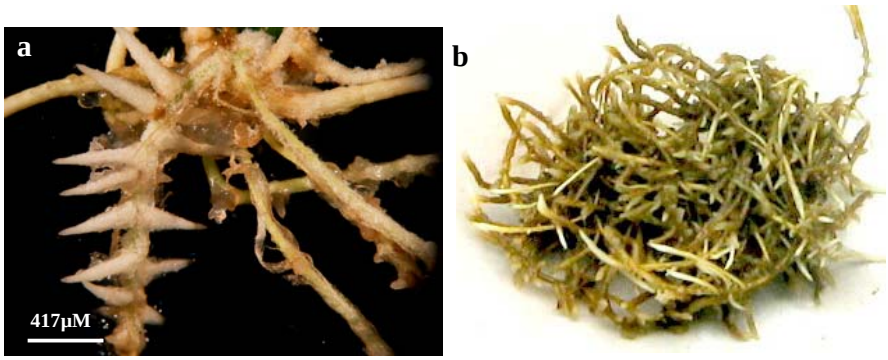


Fig.5.7 (a) Hairy roots with lateral branches, **(b)** bunch of hairy root developed from single explant in liquid culture.

Confirmation of transgenic status

Further confirmation of transgenic status of the tissue was done by the PCR amplification of the DNA isolated from the hairy roots using forward and reverse primers of *rol* genes (*rolA*, *rolB* and *rolC*). *A. rhizogenes* (colony PCR) served as the positive control and DNA from the non- transformed seedlings roots served as the negative control. All transformants (infected with different strains) showed presence of the *rolA* (300bp), *rolB* (780bp) and *rolC* (590bp) in DNA amplified product (Fig.5.8). Presence of the amplified products of the expected size in the positive control and hairy root lines, confirmed the identity of this amplification product and the transgenic nature of the hairy root lines.

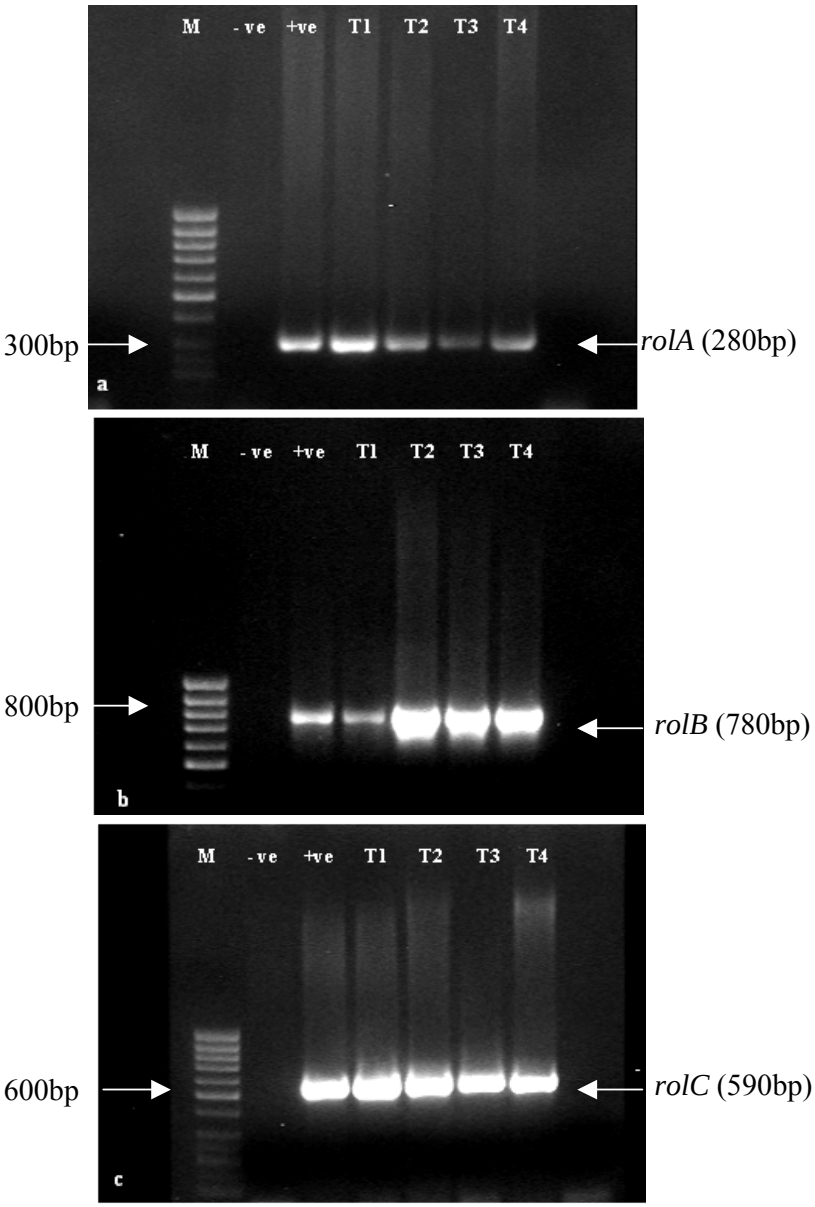


Fig.5.8 PCR amplification of (a) 280 bp fragment of the *rolA* gene, (b) 780 bp fragment of the *rolB* gene, (c) 590 bp fragment of the *rolC* gene. Lane 1 = molecular weight marker (100 bp ladder); lane 2 = DNA from non-transformed roots (negative control); lane 3 = *A. rhizogenes* DNA (positive control); lane 4 = sample 1; lane 5 = sample 2; lane 6= sample 3; lane 7= sample 4. Samples 1, 2 and 3 were DNA of transformed root obtained after infection with strain ATCC15834, LBA9402 and A4 respectively and sample 4 was DNA of transformed EM obtained after infection with strain ATCC15834.

5.2.2 Optimization of protocol for regeneration from hairy roots

A. rhizogenes mediated transformation was selected in a wide range of plant species with desirable genes many of which could be regenerated into plants, often spontaneously (Christey 2001). In peanut, *A. rhizogenes* method has been used either for the production of composite plants (Akasaka *et al.* 1998) or the development of hairy roots for resveratrol production (Bolivar *et al.* 2007). These transformed roots were never tested for development of transgenic peanut. However, regeneration from transformed roots via somatic embryogenesis has been reported in many other species- *Carica papaya* (Cabrera-Ponce *et al.* 1996), *Panax ginseng* (Yang and Choi 2000) and *Cucurbita pepo* (Leljak-Levani *et al.* 2004) by using different composition of auxins and cytokinins.

In different cultivars of peanut, picloram was noted to be efficient in the induction of embryogenic callus. For eg., Ozias-Akins *et al.* (1993) reported embryogenic callus from immature embryos of peanut cultivars; Joshi (2003) obtained embryogenic callus from MZEDLs, of an Indian cultivar JL-24. Little *et al.* (2000) suggested picloram as an effective auxin for induction of embryogenic callus in mature embryo axis of peanut cultivars. On the other hand TDZ has been used in peanut for organogenic (Kanyand *et al.* 1994; Matand and Prakash 2007) response as well as for embryogenesis (Li *et al.* 1994; Murthy *et al.* 1995; Murch and Saxena 1997). We tested transformed hairy roots of peanut cultivar SB-11 for induction of embryogenic response in MS medium containing picloram and for organogenic response, root explants were cultured in MS basal medium containing TDZ.

Callus was induced (Fig.5.9a) from the root segments of the hairy roots, incubated in MS medium containing 12.42, 16.56 and 20.71 μM picloram (Table 5.1). Optimum callus induction (46.7%) was noted in explants infected with strain ATCC15834 and cultured in 16.56 μM picloram followed by the response in 20.71 μM (30%) picloram. Callusing frequency was 27.8% in explants infected with strain LBA9402, and cultured in 16.56 μM picloram. In the case of strain A4, callus induction was 10% in 20.71 μM picloram. But these calli never differentiated further. In lower concentration (2.07, 4.14, and 8.28 μM), there was no dedifferentiation and callus induction in roots and explants turned dark brown (Fig.5.9b).

Table 5.1: Induction of callus in different concentrations of picloram tested from hairy roots obtained after infection with different *Agrobacterium* strains.

Conc. of Picloram (µM)	ATCC15834 mean± sd* (%)	LBA9402 mean± sd* (%)	A4 mean± sd* (%)
2.07 PIC	0.0±0.0 (30)	0.0±0.0 (30)	0.0±0.0 (30)
4.14 PIC	0.0± 0.0 (30)	0.0±0.0 (30)	0.0±0.0 (30)
8.28 PIC	0.0± 0.0 (30)	1.1± 1.9 (30)	0.0±0.0 (30)
12.42 PIC	24.4± 6.9 (30)	13.3± 3.3 (30)	3.2±3.3 (30)
16.56 PIC	46.7±12.0 (30)	27.8± 5.1 (30)	8.9± 1.9 (30)
20.71 PIC	30± 8.8 (30)	16.7±3.3 (30)	10±3.3 (30)
ANOVA	S%1	S%1	S%1

*Three repeats-each with 10 explants.

Fig.5.9 (a) Callus developed from transformed root in 16.56 µM picloram.

(b) Transformed root turned brown and there was no callus induction in 4.14 µM picloram.

(c) Hairy root derived green callus (arrow) in 0.045 µM TDZ.

Initially callus obtained in medium containing 16.56 µM picloram was compact, translucent and appeared embryogenic but after next subculture in the same composition of medium, the callus turned brown in colour. Reports by Little *et al.* (2000) and Ozias-Akins *et al.* (1993) suggested picloram as an effective auxin for the induction of embryogenic callus in mature and immature embryo axis of peanut, respectively. In the present experiment callus obtained from transformed roots did not differentiate into embryos.

In TDZ, callus formation was observed only in the lowest (0.045 µM) of all the concentrations of TDZ tested. Callus formation occurred in only two out of the thirty explants tested in this particular concentration. All other explants turned brown as in picloram, irrespective of the bacterial strain. The callus (Fig.5.9c) did not differentiate either into somatic embryos or into organogenic buds. Hoshino and Mii (1998) reported

similar results in snapdragon. These authors cultured hairy roots in TDZ containing media and obtained only green calli. Shoots regeneration was not achieved from the green calli. Conversely in cabbage only the use of TDZ promoted shoot regeneration (Christey *et al.* 1997).

In contrast to the above observations, a report by Tepfer (1990) demonstrates frequent regeneration of plants from the transgenic roots when *A. rhizogenes* is used. However, it has been suggested that leguminous species are mostly recalcitrant for inducing shoot regeneration in *in vitro* culture, and highly species-specific methods are required to overcome the difficulty (Atkins and Smith 1997) except for *Lotus corniculatus*, in which hairy root cultures regenerated adventitious shoots spontaneously without phytohormones within 30 days of culture (Petit *et al.* 1987). Various open reading frames, such as an auxin synthesis gene and *rol* genes, which regulate growth and differentiation are present in T-DNA. These affect the hormonal status of explants (Hoshino and Mii 1998), demanding various media manipulations to get transgenic regenerants from hairy roots.

5.2.3 Regeneration from MZEDL of peanut through somatic embryogenesis after infection with *A. rhizogenes*

Morphogenesis in plants via embryogenesis has certain advantages over organogenesis, since somatic embryos have both root and shoot meristems (Chapter I). Procedure for induction of somatic embryogenesis was followed as reported by Chengalrayan *et al*. (1994, 1997) with slight modification in exposure period of conversion medium.

On testing the susceptibility of MZEDL to infection with *A. rhizogenes*, no sign of root development was observed (Fig.5.10a) in control (uninfected explants). Out of the 3 bacterial strains (ATCC15834, LBA9402 and A4) tested, 2 strains (LBA9402 and ATCC15834) were found to infect MZEDL resulting in the induction of hairy roots (Fig.5.10b) after 45-60 days in MS basal medium. However, the frequency of hairy root formation was low. In explants infected with strain ATCC15834 and LBA9402 the frequency of infections were 4.5% and 3.7% respectively (Table 5.2). Strain A4 was totally ineffective for leaflet infection and there was no sign of hairy root induction. Gilbert *et al.* (1996) used a different bacterial strain for the transformation of carrot and reported that bacterial strains differ in their virulence and the choice of the appropriate

strain is an important factor for successful transformation. This was in concurrence with our results obtained in MZEDL of peanut.

Table 5.2 Induction of hairy roots in MZEDL cultured in MS medium without PGR after infection with different strains of *A. rhizogenes*

Strain	Survival of MZEDLs (explant) mean±sd (%)	Development of leaf from MZEDL mean±sd (%)	Induction of hairy roots mean±sd (%)
ATCC15834	78.0±11.8 (150)	63.4±21.0	4.5±0.5
LBA9402	86.3±6.0 (150)	60.6±21.2	3.7±0.4
A4	87.4±3.4 (150)	66.4±4.3	0.0±0.0
ANOVA	NS	NS	S1%
* Figures in parenthesis indicate 50 replicates in three repeats.			

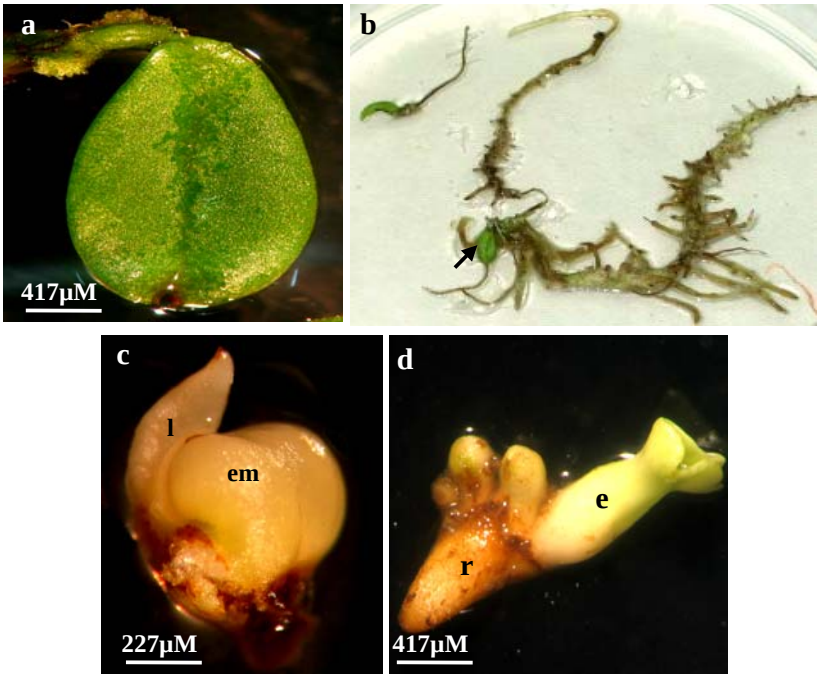


Fig. 5.10 (a) MZEDL cultured in MS basal medium without infection, did not show root development.
(b) Induction of hairy roots in MZEDL (arrow) grown in MS without auxin after infection with *A. rhizogenes*.
(c) Embryogenic mass developed at the base of MZEDL in 90.5µM 2, 4-D after co-cultivation with *A. rhizogenes*.
(d) Radicle emergence from embryo (developed from EM after infection with *A. rhizogenes*) in MS basal medium.

Further, we carried out infection of MZEDL by the LBA9402 and ATCC15834 strains for regeneration through embryogenesis. Leaflets cultured in MS basal medium with high

concentration of 2,4-D (90.5 μM) after infection with *A. rhizogenes* (LBA9402 and ATCC15834), did not show development of hairy roots whereas presence of *rol genes* in EMs was confirmed by PCR (Fig.5.8, lane 7= sample 4). This suggested that the presence of 2,4-D restricted the development of roots possibly by competing with the auxin synthesized within the tissue. Development of EM in the presence of *rol* genes indicates that the genes responsible for embryogenesis are not affected by the auxins synthesized internally. Thus 2,4-D is still effective in induction of EM. Embryogenic masses in the form of a pair of bulges were developed at the base of MZEDL (Fig.5.10c) within 4 wks. Response of EM was similar (Table 5.3) in the explants infected with Strain ATCC15834 (68 %) and LBA9402 (50%). On transfer to MS basal medium with lower concentration (13.6 μM) of 2,4-D, somatic embryos developed within 4 wks. This again supports the observation that the process of embryogenesis is not affected by the presence of the *rol* genes transferred from *A. rhizogenes* to peanut tissue. Radicle emergence (Fig.5.10d) of somatic embryos occurred in MS medium. These somatic embryos did not show any hairy root phenotype even after the removal of PGR. The differences in frequencies of somatic embryo development were not significant between the two (LBA9402 and ATCC15834) strains tested (Table 5.3). Plumule differentiation of somatic embryos was triggered in 22.71 μM of TDZ (Chengalrayan *et al.* 1997). Transfer of these cultures to MS basal medium for 4 wks led to the shoot bud differentiation (Fig.5.11). Till this stage we did not observe any hairy root development from the somatic embryos. The conversion frequency was similar (58 and 63%) in the somatic embryos obtained from MZEDLs infected with either strain (Table 5.3). The emblings exhibited stunted shoot and slow growth rate possibly due to exposure of TDZ.

Table 5.3 Embryogenic and transgenic responses in MZEDLs after infection with two strains of *A. rhizogenes*.

Strain	**Survival of MZEDL (explant) mean±sd* (%)**	**Development of EM mean±sd (%)**	**Development of somatic embryo mean±sd (%)**	**Conversion mean±sd** (%)**	**Converted plants with hairy roots mean±sd (%)**
ATCC15834	75.0 ± 5.9 (293)	68 ± 11.6	34.8 ± 12.7	58.2 ±7.1(86)	31.3 ± 3.9
LBA9402	78.9 ±12.5 (252)	50 ± 11.1	44.5 ± 2.8	62.7 ± 5.5(44)	37.9 ± 11.2
ANOVA	NS	NS	NS	NS	NS

* Figures in parenthesis indicate total number of explant tested in three repeats;
****** Figures in parenthesis indicate total number of embryo tested

Throughout the embryogenic process, we never observed development of hairy root like structure or 'T-phenotype'. But PCR analysis of DNA isolated from EMs developed at the base of leaflets after infection with *A. rhizogenes* have shown integration of T-DNA by amplifying the *rol* genes (Fig.5.8, lane 7= sample 4). It is presumed that EMs developed after infection with bacteria have integrated T-DNA but they were unable to express the genes at initial stages of embryogenesis due to the presence of high concentration of 2,4-D. We never observed any hairy root development from any part of the embryos developed from EMs till 2nd culturing in MS basal medium for conversion. In these cultures we noted hard white callus from different parts of the embryos (Fig.5.11a). Development of callus was mostly observed from edges (Fig.5.11a) of the fused cotyledon, outer edge (Fig.5.11b, c) and also from the inside of the fused cotyledon of the embryo. Hairy root like structure started developing from these calli within a week (Fig.5.11a). Nature of development of hard callus and then induction of root from these calli was the same as in the case of seedling-derived explants of peanut (Fig.5.2b and Fig.5.3a). The number of converted plants with hairy root was similar (31 and 38%) in both of the strain (Table 5.3).

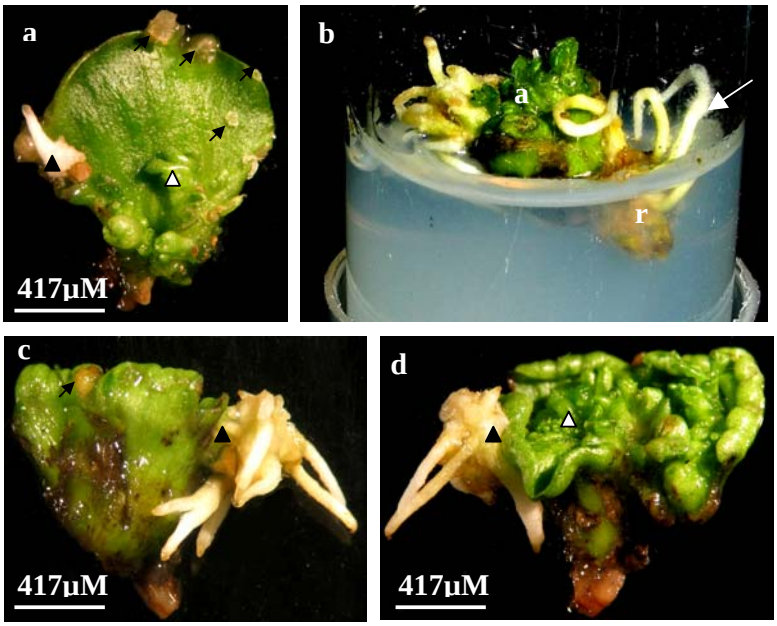


Fig.5.11 Induction of hairy roots from different part of somatic embryos (SE).

(a) Induction of callus (black arrow) and root (black arrow head) from inside and edge of the fused cotyledon of SEs.
(b) Roots developed from apical (a) and radicle end (r) of the SE after callus came in contact with media (embryo cultured horizontally on MS media). Few hairy structures (white arrow) were present at the root. Shoot bud (a) differentiated.
(c) Callus (arrow) and roots (black arrow head) developed from the outer edge of SE.
(d) Shoot buds (white arrowhead) differentiated from the plumular part and roots (black arrowhead) developed from the outer edge of the SE in MS medium.

We observed that part of the differentiating embryos that were away from the medium developed callus. The callus development was scattered on the somatic embryos and development of root from these calli were very slow. Whenever these parts of embryo (with callus) came in contact with medium, root induction (Fig.5.11b, c, d) occurred within 2-3 days. Root hair (Fig.5.11b) from the developing root was seldom observed. Induced hairy roots in contact with the medium (Fig.5.12a) showed vigorous growth (Fig.5.12b) within 4 days. The growth of transformed roots was more vigorous than the non-transformed roots. This made it possible to visually select transformed plants (Christensen *et al.* 2008). Shoot differentiation (Fig.5.12b) became slow when the development of hairy root from differentiating embryos was fast. In control explant, shoot (Fig.5.12c) differentiation was faster than the embryos developed from leaflet after infection with *A. rhizogenes*.

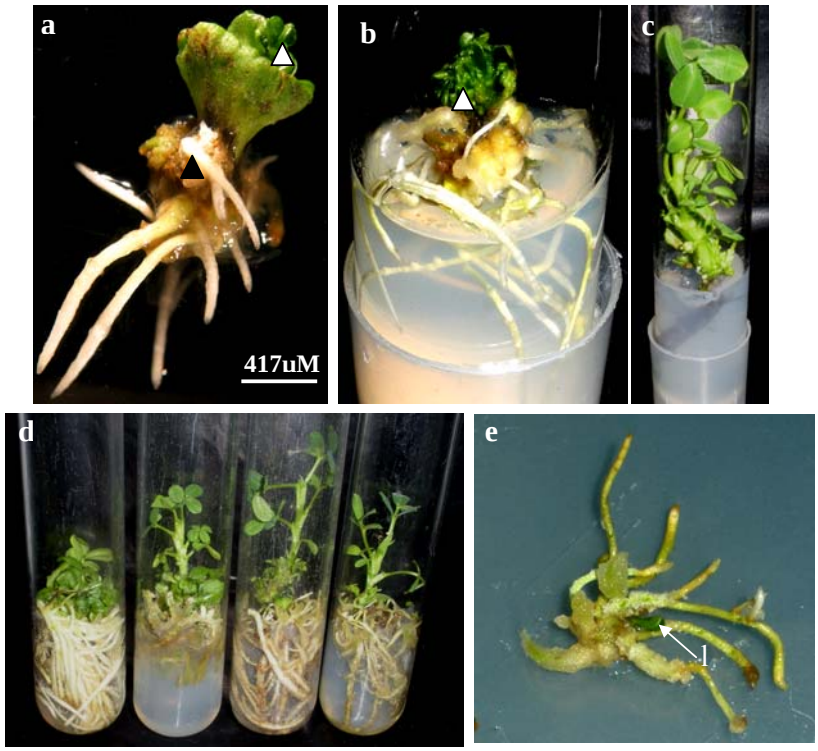


Fig.5.12 (a) Induction of hairy root from the radicle (black arrowhead) part of the embryo and shoot bud (white arrow head) differentiation from the apical portion.
(b) Above explant (Fig.5.12a) after 4 days. Embryo with hairy root and shoot buds (white arrowhead).
(c) Embryo differentiated into root and shoot in control explant.
(d) Transgenic plants with developed shoot and profuse hairy roots.
(e) Induction of root from the isolated leaf (l) of the transgenic plants in MS basal medium.

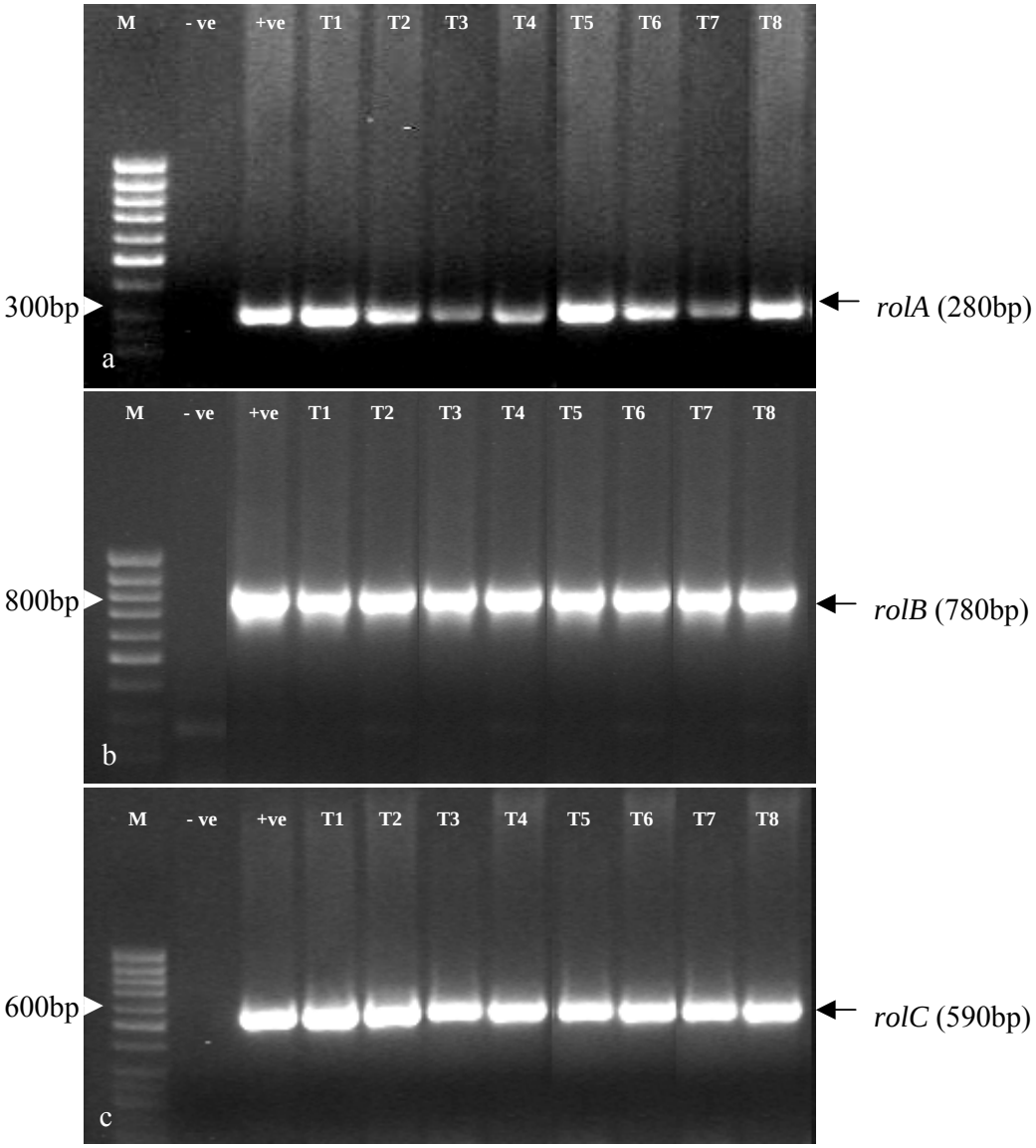


Fig.5.13 PCR amplification of (a) 280 bp fragment of the *ro*lA gene, (b) 780 bp fragment of the *rolB* gene, (c) 590 bp fragment of the *rolC* gene using DNA derived from leaves of emblings with hairy root (obtained after infection with strain ATCC15834 and LBA9402). Lane 1 = molecular weight marker (100 bp ladder); lane 2 = DNA from non-transformed plants (negative control); lane 3 = *A. rhizogenes* DNA (positive control); lane 4-11 = sample 1-8 (T1 to T8). Samples 1-8 were leaf DNA of transformed plants.

Development of hairy root differentiation occurred in the radicle part of the embryos developed from the leaflets co-cultivated with *A.rhizogenes* (Fig.5.12d) whereas rhizogenesis was absent in the aerial parts. To check the presence of *rol* genes in the aerial part of the emblings, leaf of converted plants both with and without hairy roots were isolated and cultured in MS basal medium. Vigorous rhizogenesis in these leaves (Fig.5.12e) indicate presence of *rol* genes in the aerial parts of the plants away from medium. Presence of these genes and their stable integration was additionally confirmed by PCR analysis of DNA isolated from the leaves of transformed plants. PCR analysis also indicated the integration of T-DNA by amplifying the *rol* genes (Fig.5.13a-c).

Direct regeneration of transgenic shoots from the explants, with no intervening hairy root phase, has been reported after *A. rhizogenes*-mediated transformation. In Mexican lime, internodal stem culture on medium with growth regulators yielded adventitious shoots regenerated directly from the 76% of co-cultured explants in 55–60 days with no hairy root phase. The collected transgenic, shoots rooted easily after 21 days in media without PGR and presence of gene were confirmed by PCR (Balch and Alejo 1998). We recovered transgenic embryos without the intervention of any hairy root phase and the differentiation of embryos to shoot followed by simultaneous development of hairy root after two subcultures in MS basal medium. Yamakawa and Chen (1996) also achieved *A. rhizogenes*-mediated transformation by direct formation of adventitious buds on infected petioles of kiwifruit. In rose, transgenic shoots were obtained after co-cultivation of friable embryogenic tissue with *A. rhizogenes* (Firoozabady *et al.* 1994) without any hairy root phase. The transgenic nature of the plants was confirmed by enzyme assays, PCR and Southern hybridization.

Similar results were also reported in the case of chrysanthemum (Tsuro *et al.* 2005). In the *A. rhizogenes*-infected cut leaves, hairy roots were not observed on any tested medium or culture condition. When explants were cultured on shoot induction medium, calli were formed at the cut edge within 4–6 wks of culture, and shoot primordia were observed on the callus surface after 2 wks of callus formation. Consequently, with *gus* introduction, a significantly higher transformation rate was observed for *A. rhizogenes* (6.0%) compared with *A. tumefaciens* (3.3%). The transgenic nature of the plants confirmed by PCR and Southern hybridization. These results indicate that *A. rhizogenes* effectively introduces T-DNA of the binary plasmid into the chrysanthemum genome by

introducing Ri T-DNA even though (presence of Ri T-DNA) they did not show any T phenotype character.

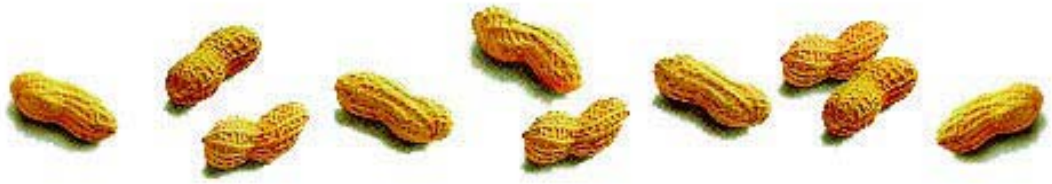
5.3 CONCLUSION

The present study reports the optimization of a system for *A. rhizogenes*-mediated transformation of peanut seedling derived explants. Our results indicate that infection of different explants with 3 different strains of *A. rhizogenes* have response with varying frequencies. Among the seedling-derived explants, leaves have the best potential for infection with strain LBALBA9402. But regeneration could not be achieved from transformed hairy roots in different concentrations of Picloram and TDZ tested. Only green calli were obtained in TDZ. To get transgenic regenerants from hairy roots, more growth regulators either alone or in combination should be tested.

Infection of MZEDLs with different strains (ATCC15834, LBA9402) of *A. rhizogen*es and culturing in embryogenic medium (2,4-D) led to the development of transgenic EMs and then somatic embryos which converted into plantlets. This is the first report to efficiently obtain transgenic peanut plants via *A. rhizogenes* transformation. Thus *A. rhizogenes* can be used as a valuable tool for the production of transgenic peanut plants. The obtained results show that the selection of transformants could be performed visually with a high accuracy and the use of commonly applied antibiotic or herbicide genes for screening can be omitted. The method is time-saving as it requires only 6-8 months to obtain the transgenic plants. In essence, results obtained in the present study suggest that *A. rhizogenes* mediated gene transfer might be a useful alternative for development of peanut transgenics.

Appendix

Ex vitro performance of peanut plants from TDZ-pretreated seeds



INTRODUCTION

Plant growth regulators (PGRs) are known to influence plant growth and development at very low concentrations (Jules *et. al.* 1981). Thidiazuron (TDZ), a substituted phenyl urea (Mok *et al.* 1982) has been established as a potent regulator of morphogenetic responses in a large number of species as well as diverse experimental systems. It induces as many or more adventitious shoots than adenine-type cytokinins in most species in which it has been tested (Lu 1993). It has a diversity of physiological effects on treated plants, which depends on concentration, time of application, plant species and cultivar. This in association with environmental conditions might have substantial impacts on the plant physiological response to the product (Amarante *et al.* 2002). In an experiment with apple trees two cvs., 'Gala' (characterized by having low fruit set) and 'Fuji' (characterized by having high fruit set), when sprayed with TDZ at different doses, increased shoot growth. Fruit set increased with TDZ dose in 'Gala' but not in 'Fuji'. It was concluded that TDZ sprayed at full bloom might improve fruit set in cultivars with deficient pollination (Amarante *et al.* 2002). In soybean (cvs. 'Pungsan' and 'Manlee'), spraying 2-(2,4-dichlorophenoxy) propanoic acid (2,4-DP) and benzyl amino purine (BAP) at early reproductive stages demonstrated that exogenous PGRs significantly influenced reproductive and growth characteristics, and consequently seed yield. With the exception of a low (0.5 mM) BAP treatment in cv. 'Pungsan', all treatments increased the number of pods with varying numbers of seeds per pod. Low 2,4-DP (0.04 mM) and BAP (0.5 mM) significantly reduced flower abortion (Cho *et al.* 2002) and delayed abscission of pods in both genotypes, resulting in increased pod setting. Foliar spray of some PGRs (GA3, IAA (indole-3-acetic acid), BAP) on *Vicia faba* in a field trial led to significant changes in plant height, average number of leaves, leaf area per plant and dry weight of the shoot (Ibrahim *et al.* 2007). Application of BAP and IAA at 100 ppm caused a reduction in the percentage flower abscission and produced the highest number of pod setting. Spraying the foliage of lentil plants with kinetin (10, 20 and 40 mg/l) resulted in reduced stem height, an increase in the number of leaves, branches, shoot dry weight, number of produced flowers per plant, number and weight of pods and seeds per plant (Khalil *et al.* 2006). In peanut, foliar application of a low concentration (0.25 mg/l) of 28-homobrassinolide during flowering and pegging increased pod yield (Ramraj *et al.* 1997). The above studies were conducted on fully-grown plants and the PGRs were sprayed either

before, during or after the plant reached the reproductive phase. However, there is no literature on growth and yield of a plant developed from seeds pretreated with PGRs.

The effects of TDZ on *in vitro* morphogenesis in peanut have been studied widely (Saxena *et al.* 1992, Murthy *et al.* 1995, Chengalrayan *et al.* 1997, Joshi *et al.* 2003). The activity of TDZ varied with concentration, exposure, explant and species (Murthy *et al.* 1998). In *in vitro* studies it was demonstrated (Joshi *et al.* 2003) that incorporation of TDZ into medium influences the meristem of somatic embryos resulting in the formation of plants with multiple shoots. Elongation of the shoots could be achieved on dilution of TDZ in the plantlets by repeated transfer to PGR-free medium. However the productivity of these plants with multiple shoots was never assessed. As somatic embryos are similar to the zygotic embryos, the present study was conducted to test the influence of TDZ on germination, branching pattern, flowering and pod yield in plants raised from TDZ-pretreated seeds having zygotic embryos.

MATERIALS AND METHODS

Peanut (*Arachis hypogaea*) seeds of cv. 'SB-11' were procured from local market. Seeds were selected visually for uniformity. These were soaked for 12 h in aqueous solution of TDZ of concentrations 2.27, 4.54, 9.08 and 22.71 μM. Seeds soaked in distilled water without any PGRs were treated as control. Soaked seeds with adhering TDZ were planted in sand soil mixture (1:1) in earthen pots 22 cm in diameter and 18 cm in height. Plants were grown in green house at 25/18°C (day/night temperature) under natural day length.

The normal crop duration (from germination to harvesting) of 'SB-11' is 110-115 days. Till flowering the plants were identified as seedlings. The morphological characters like shoot, root and hypocotyl length, number of branches per plant and pod yield were noted when plants were harvested after 17 weeks (119 days). The main shoot was measured for shoot length. The length of the hypocotyl was determined by measuring the length between the remainder of the cotyledon and the point from where the root started. Root length was scored by measuring the distance from the end of the hypocotyl to the tip of the main root. Plant

yield was scored by counting the pods, number of seeds from each plant and by weighing the seeds/plant.

The experiment was repeated five times with 10 seeds for each treatment. However, all seeds did not germinate and a few were lost before harvesting. Therefore the data was collected from the surviving plants (Table 1) after 119 days. All data were subjected to analysis of variance (ANOVA). Data were analyzed by one-way ANOVA and the means were compared by Fisher's LSD test at $P \leq 0.05$. Values are the means of five independent experiments. The differences among the treatment means were tested using Duncan's multiple range test (DMRT) at the 5% probability level ($P<0.05$).

RESULTS AND DISCUSSION

This is the first report on effect of TDZ on peanut seed germination and plant growth in soil. Plant growth regulators including BAP (Ibrahim *et al.* 2007), kinetin (Khalil *et al.* 2006), homobrassinolide (Ramraj *et al.* 1997), TDZ (Amarante *et al.* 2002), among others, have been tested to increase growth/yield in crops. However TDZ have never been tested to increase the growth/yield of peanut. Secondly, the majority of PGRs are used as a foliar spray in mature plants to test the effect on crop yield. Keeping in view the persistence of TDZ in peanut tissues even after removal of the PGR in *in vitro* studies (Joshi *et al.* 2003), peanut seeds were soaked in TDZ solution and the coating of TDZ on the seed surface was retained during planting of the seeds.

Germination started in the seeds without TDZ treatment in 5-8 days and displayed normal seedling development. The germination period of seeds was similar to the control at 2.27, 4.54, and 9.08 μM TDZ and was delayed by 7-10 days in seeds treated with 22.7 μM TDZ. However, the frequency of germination (Table 1) was not significantly affected. In seeds of large-seeded grain legumes (*Phaseolus acutifolius*, *P. aureus*), cytokinins did not affect seed germination (Malik and Saxena 1992).

The periods taken by the seedling to develop into mature plant to flower differed in TDZ-treated seed derived seedlings and in the control. Flowering started in control plants in 4-6

weeks from date of planting. After 8 weeks, the plant length reached their optimum (Fig.1a) and thereafter, the vegetative growth in control was retarded and the reproductive phase (flowering and pegging) was enhanced. Flowering was delayed in plants grown from seeds treated with TDZ (Table 2). In 2.27 µM TDZ treated seed derived plants, flowering was delayed by only 2-3 days but in 4.54 µM and 9.08 µM of TDZ it was delayed by 7-10 days and in 22.71 µM of TDZ it was delayed by 14-20 days. After 17 weeks in pots (Fig.1b), plants were harvested. The optimum shoot length in control plants was 12.4 cm (Table 1). As the concentration of TDZ increased, the shoot length decreased (Fig. 1a, 1b); it was minimum (6.2 cm) in plants obtained from 22.71 µM TDZ-treated seeds (Fig.1d). In seedlings raised from 22.71 µM TDZ-treated seed, initially the leaves were wrinkled and small (Fig.1c) after germination but on maturity, there was no difference in leaf morphology. On measuring the hypocotyl and roots in the harvested plants it was observed that TDZ not only retarded shoot length but also influenced root length and growth of rootlets (Fig.1d). Root length decreased as the concentration of TDZ increased and shortest roots (5.2 cm) were noted in plants derived from seeds treated with 22.71 µM of TDZ. Roots were longest in the control at 15.7 cm (Table 1). In contrast to shoots and roots, TDZ did not have any effect on the elongation of hypocotyls. An *in vitro* experiment with lotus (Nikolic *et al.* 2006) reported that TDZ retarded shoot length up to 1/3rd of the control even at lower concentrations of TDZ whereas at higher concentrations, the inhibition was stronger. In the present experiment, we observed that TDZ retarded both shoot and root elongation as the concentration of TDZ increased (Table 1).

Table 1. Germination and growth characteristics (all values mean ± SD) of peanut plants raised from TDZ-pretreated seeds.

TDZ (µM)	Germination (%)	Shoot length* (cm)	Hypocotyl length (cm)	Root length (cm)	№ of branches	Total № of pods	№ of seeds per plant	Seed wt per plant (g)
Control	38 ± 8.4	12.4 ± 1.6 a (18)	3.4 ± 1.3	15.7 ± 2.0 a	5.7 ± 0.7	13.1 ± 3.8 ab	20.4 ± 9.5 a	3.3 ± 1.5 a
2.27	38 ± 16.4	9.3 ± 0.4 b (18)	4.1 ± 0.9	9.9 ± 1.5 b	5.5 ± 0.6	15.7 ± 4.4 a	17.1 ± 3.7 ab	2.4 ± 0.3 ab
4.54	30± 14.1	8.9 ± 2.0 bc (15)	3.8 ± 0.9	8.8 ± 1.8 bc	5.3 ± 1.1	10.4 ± 3.3 ab	16.7 ± 4.5 abc	2.4 ± 0.5 abc
9.08	36± 13.4	8.1 ± 0.9 bcd (16)	4.4 ± 0.9	8.1 ± 0.9 bc	4.9 ± 1.0	11.7 ± 3.5 ab	13.2 ± 2.9 abc	2.1 ± 1.1 abc
22.71	24± 21.9	6.2 ± 1.6 d (12)	4.0 ± 1.5	5.2 ± 1.9 d	4.3 ± 0.9	6.0 ± 5.8 b	6.7 ± 7.1 c	0.2 ± 0.3 d
ANOVA	NS	*P*<0.01	NS	*P*<0.01	NS	*P*<0.05	*P*<0.05	*P*<0.05

[a-d] Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at P≤0.05.
*Figures in parenthesis indicate number of plants

Table 2. Delayed flowering in TDZ pre-treated seed derived peanut plants in comparison to control plants.

TDZ (µM)	2.27	4.54	9.08	22.71
Delayed flowering (Days)	2-3	7-10	7-10	14-20

The effects of cytokinins on seed germination, elongation of seedling shoots and roots, the frequency of regeneration, and the number of regenerants (somatic embryos or shoot bud) per seedling were determined in *Lotus cornicelatus* in an *in vitro* experiment (Nikolic *et al.* 2006). They reported that not all parts of shoots were equally affected. The hypocotyls were not inhibited by cytokinins. In the present experiment we noted that there was no significant difference in hypocotyl length (Table 1) between control and plants raised from TDZ-treated seed. Root nodules were present in control as well as in plants raised from TDZ treated seed; interestingly there were no sign of nodules on the hypocotyls of plants raised from TDZ-treated seed whereas in controls, the hypocotyl was full of nodules (Fig.1e). In leguminous plants, nodules are formed on roots in response to infection with *Rhizobium leguminosarum*. The absence of nodules in hypocotyls of plants raised from TDZ treated seed indicates the lack of infection in these plants.

There were no significant difference in the number of branches per plant between control and plants raised from TDZ-treated seed (Table 1). Multiplication of shoots of pongamia (Sujatha and Hazra 2006), and pigeonpea (Singh *et al.* 2003) in the presence of TDZ *in vitro* was demonstrated earlier. However, in these studies the tissues were continuously exposed to the PGR.

In contrast to somatic embryo-derived plants developed in the presence of TDZ, there was no multiple shoot development in plants grown from seeds treated with TDZ (Table 1). As flowering was delayed in plants raised from seeds pretreated with 22.71 µM of TDZ, flower and flower buds were present (Fig.1f) at the cotyledonary node of these plants at the time of harvest.

Fig. 1 Effect of TDZ on peanut plants raised from TDZ-pretreated seeds. (**a**) Plants after 8 weeks of planting. (**b**) Plants after 17 weeks of planting (before harvesting). (**c**) Seedling with wrinkled leaf raised from seed treated with 22.71 µM of TDZ. (**d**) Plants after harvesting. Plants raised from seeds treated with TDZ (2.27-22.71 µM of TDZ) were shorter in length). (**e**) Roots on harvesting. Nodule formation was restricted to the hypocotyls of the plants raised form TDZ-treated seeds. (**f**) Plant grown from seeds pre-treated with 22.71 µM of TDZ, showing flowering from the cotyledon nodes (black arrow) at the time of harvesting. The pods were removed earlier. (**g**) Pods obtained from the plants raised from seeds pretreated with TDZ and control seeds. Total number of pods was higher in plants raised from seeds treated with 2.27 µM of TDZ. Many of the pods were immature and had a soft shell.

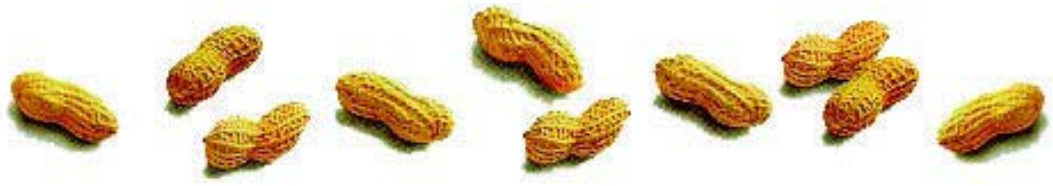
Among all TDZ treatments, the number of pods per plant was maximum when seeds were treated with 2.27 μM of TDZ. This was followed by the control plants. It was lower than the control in other concentrations of TDZ, and was minimum at 22.71 μM of TDZ (Table 1). During harvesting, almost all pods obtained from control plants had a hard shell. However, some of the pods on plants derived from TDZ-treated seed were still soft (Fig.1g) indicating the immaturity of the pods. As the concentration of TDZ increased, so too did the number of immature pods. There was no obvious difference in pod shape compared to control (Fig.1g). The soft-shelled pods were smaller in size and had immature seeds at various stages of development. The number of seeds and seed weight per plant were optimum in the control (Table 1). The number of seeds decreased as the concentration of TDZ increased. Late flowering (Table 2), in plants raised from TDZ-treated seed, resulted in delayed seed set. As all plants were harvested at the same time, the number of small pods with soft shell and undeveloped seeds were more in plants which had delayed flowering. Some of the seeds in the pods were too minute and were not scored as seed. This resulted in reduction in seed number and seed weight (Table 1). Although the number of pods was higher in plants developed from seeds treated with 2.27 μM TDZ, the total number of seeds per plant was less. This was due to the fact that some of the pods collected from these plants were immature and did not bear seed while others were soft-shelled and with immature seeds. It needs to be tested if on extended incubation the immature pods would mature to produce more seeds in pots in the greenhouse giving a higher yield by weight. However, that would increase the crop duration. TDZ has never been tested for increase in peanut crop yield. In the *in vitro* studies (Chengalrayan *et al.* 1997, Joshi *et al.* 2003), exposure of somatic embryos to medium containing TDZ gave rise to plants with multiple shoot primordia, a typical reaction of many plant species *in vitro* to TDZ. Differentiation of these shoot primordia to shoots could be achieved by repeated transfers to PGR-free medium. It was hypothesized that TDZ influenced the meristematic cells (Joshi *et al.* 2003) to proliferate and produce multiple buds and that it persisted in the plants causing retarded elongation of the shoots. Retarded elongation in TDZ-induced shoots has been reported in peanut (Chengalrayan *et al.* 1997, Joshi *et al.* 2003) and in tamarind (Mehta *et al*. 2004). In the present experiment, seeds were exposed in TDZ for only 12 h prior to sowing. The amount of TDZ absorbed and adsorbed by the seeds influenced the plant through out the life cycle of 119 days.

CONCLUSION

Shoot and root elongation was reduced in the plants raised from TDZ-treated seeds whereas the number of pods was optimum in plants developed from seeds treated with the lowest concentration (2.27 μM). The effect of TDZ pretreatment has never been tested on peanut seed germination and plant growth. Retarded growth and delayed flowering indicated that the amount of TDZ absorbed by the seeds affected the plant through its entire life cycle.

This work has been published
***Ex vitro* Performance of Peanut Plants from TDZ-pretreated Seeds.**
Singh *et al.* Seed Science and Biotechnology 2(2): 70-73, 2008.

Summary



Groundnut (*Arachis hypogaea* L.) is a major oilseed legume native to South America and cultivated globally. The crop is susceptible to various pests and diseases from sowing till harvesting and also during storage. Enormous loss in crop yield is due to unreliable rainfall patterns with frequent droughts, lack of high yielding adapted cultivars, damage by diseases and pests, poor agronomic practices, and limited use of inputs (Nageshwara Rao and Nigam 2001). Biotechnology offers an impressive option to supplement the ongoing efforts on developing genetically modified germplasm for achieving sustainable food production. Genetic engineering is a tool for crop improvement that extends our access to beneficial traits beyond sexually compatible crosses. Genes from virtually any organism can be cloned and introduced into plants. In addition to classical and molecular breeding approaches, genetic transformation offers an attractive option to introduce novel genes into plants for quality production. One of the conditions for plant transformation is to count on a good regeneration protocol. The major snag in peanut somatic embryogenesis is, very low conversion frequency of somatic embryos to a normal plant. Keeping in view the limitations of the existing protocols, there is a demand to develop reliable protocols for *in vitro* regeneration and to study the basic processes involved in peanut. Efficient regeneration protocol via direct somatic embryogenesis is a pre-requisite for the optimization of transformation protocol. With this objective, attempts were made to standardize protocols for in vitro regeneration and genetic transformation of peanut tissues.

The present study was formulated to address some basic questions associated with peanut embryogenesis and transformation of peanut. The work was carried out with two major objectives:

1) Morphological studies to obtain normal fertile peanut plants at high frequency via somatic embryogenesis.
2) Optimization of the protocol for transformation using *Agrobacterium rhizogenes* for genetic improvement.

The present study was planned as a means to address some basic questions associated with induction and development of somatic embryos and regeneration of these somatic embryos into viable plantlets. The work attempted to find suitable medium and duration for induction

of somatic embryos vis-à-vis appropriate nutrient composition and cultural conditions, in peanut using mature zygotic embryo axes and axenic culture derived explants.

We optimized the effect of concentration and exposure of 2,4-D on embryo development and conversion by using MZEDLs of peanut as explants. Leaflets cultured in EM induction medium (MS with 90.5µM 2,4-D) for varying periods (2, 4, 6 and 8 wks) were cultured for 4 wks in 3 secondary media viz., SM-1 (MS), SM-2 (MS+13.6µM 2,4-D), SM-3 (MS+90.5µM 2,4-D). In the explants cultured for 6wks in PM, EM and subsequently embryo formation initiated. Somatic embryos developed on transfer to all the three secondary media. Optimum conversion frequency of 53% was achieved in embryos developed in SM-2 and cultured for 12 wks in growth regulator free medium. Extended exposure of the leaflet explants for 8 wks in primary medium lead to formation of embryogenic mass and development of embryos but the conversion frequency of these embryos was reduced. Culturing the leaflets in primary medium for 6 wks and later in SM-2 medium was optimum for the conversion of the embryos on transfer to medium devoid of growth regulator.

Keeping in view the role of TDZ in induction of somatic embryogenesis, and its role in triggering meristematic activity in the plumule of abnormal somatic embryos of peanut (Chengalrayan *et al.* 1997; Joshi *et al.* 2003) the influence of this growth regulator was studied in early embryo development with respect to pulse treatment. Here, the influence of TDZ at different concentrations and exposures, on plumule differentiation of developing embryos was studied by incorporating TDZ, in three stages of embryo development including (i) embryogenic mass (EM) induction from leaflets; (ii) embryo induction from embryogenic masses; (iii) embryo conversion from embryos. Incorporation of TDZ in embryo conversion medium for 24hrs was the most effective and the conversion frequency attained in the somatic embryos was 43 %.

A part of the study was formulated to understand the TDZ-induced pathway of embryo conversion. The resultant emblings were ascertained regarding their uniformity. The experiments to assess the uniformity/variability within the regenerants obtained through incorporation of TDZ in embryo conversion medium were carried out using ISSR primers. ISSR analysis showed more than 99% similarity within the plants.

Experiments were conducted to assess the potential of mature zygotic embryo axes derived meristems as an alternative explants for somatic embryogenesis. Apical portions ($1/3^{rd}$) of mature zygotic embryo axes having pre-exiting meristems were cultured in PM (**Shweta and Hazra,** *communicated*). Optimum (8/explant) embryos were noted in explants, cultured in PM for 6wks followed by culturing in SM-2. An optimum conversion frequency of 32% was achieved in these embryos. Origin of embryos from the existing caulogenic buds was confirmed histologically. Incorporation of silver nitrate at low concentrations increased the conversion frequency of the embryos.

Protocol for somatic embryogenesis from the existing meristems of the embryo axis derived plumule was extended to the axenic shoot derived caulogenic buds of the cultivar SB-11 and *Arachis duranensis* (a wild species). The difference in potential in the nodes due to their relative positions was determined by culturing the nodes in series. The meristem in the first node demonstrated higher potential compared to the rest in SB-11. The explants from this cultivar responded consistently in presence of 2,4-D, to give rise to embryos from the axillary meristems. However, the response was sporadic in picloram. On the contrary, in *Arachis duranensis,* shoot culture derived axillary meristems did not respond in 2,4-D, whereas induction of somatic embryos occurred in 2 wks at all the concentrations of picloram tested.

The experiments were conducted to optimize parameters for *Agrobacterium*-mediated transformation. Three agropine-type strains (ATCC15834, LBA9402 and A4) of *A. rhizogenes* were tested for transformation of peanut tissues. Seedling derived explants including leaf blade, petiole, axillary node, internodes were used for transformation studies. Rhizogenesis was noted in all types of explants at varied frequency with all the strains but regeneration from these transformed roots was lacking. Mature zygotic embryo axis derived leaflet were also tested for transformation with *A. rhizogenes*. Only two out of the three strains (ATCC15834 and LBA9402) were capable of infecting the leaflets. Thus after infection with above two strains of *A. rhizogenes,* the leaflets was cultured in embryogenic media that led to the development of transformed somatic embryos. During the whole process of embryogenesis there was no sign of hairy root development from somatic embryos. Hairy roots appeared after transferring these embryos in 2^{nd} passage of MS basal

media for conversion. These somatic embryos were converted into transformed emblings. Integration of rhizogenes is demonstrated by PCR and by culturing isolated leaves in MS basal media from the transformed emblings.

Experiments were conducted to test the effect of TDZ on morphology and growth of the seedlings germinated *extra vitrum* from TDZ treated seeds (**Shweta *et al*., Seed Science and Biotechnology, 2(2): 70-73, 2008**). Seeds of SB-11 soaked in different concentrations of TDZ for 12 hrs did not demonstrate variation in germination frequency but the growth of seedlings was retarded with increasing concentration of TDZ. Shoot and root lengths were reduced at higher concentrations and the number of rootlets was less. Initially the leaves of the 22.71μM TDZ treated seed derived plant, were small and wrinkled. On maturity, there was no difference in leaf morphology. Flowering in TDZ treated seed derived seedlings was delayed. In control plants nodule formation was noted in the hypocotyls of the seedlings. Whereas nodule formation was absent in the hypocotyls of seedlings derived from seeds pre-treated with TDZ. Root nodules were present in control as well as in TDZ treated seed derived seedlings. Optimum pod formation was obtained in plants raised from the seeds pre-treated with 2.27μM of TDZ. The growth of peanut plants was retarded due to TDZ treatment but the pod yield was enhanced in plants raised from seeds treated with lower concentrations of TDZ. This observation needs to be confirmed with more trials and other cultivars prior to consideration of TDZ pretreatment for increased grain yield.

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1. **S. Shweta, MM** Jana and Sulekha Hazra (2008) Ex vitro performance of peanut plants from TDZ- pretreated seeds. *Seed Science and Biotechnology*. 2(2):70-73.

2. Meera Parthasarathy, S**hweta Singh**, Sulekha Hazra, Vijaymohanan K. Pillai, (2008) Imaging Stomatal Physiology of Somatic Embryo-derived Peanut Leaves by Scanning Electrochemical Microscopy. *Analytical and Bioanalytical Chemistry.*

3. **Shweta Singh** and Sulekha Hazra (2005) Modulation of peanut embryogenesis protocol for fast recovery of morphologically normal plants. Plant Biotechnology: New Frontiers. Proceeding of National Symposium pp. 321-327,CIMAP Lucknow.

4. **Shweta Singh** and Sulekha Hazra. Somatic embryogenesis from the axillary meristems of peanut (*Arachis hypogaea* L.). (*Communicated*).

5. **Shweta Singh**, UJ Mehta and Sulekha Hazra. Somatic embryogenesis from nodal bud of axenic shoot culture of cultivated and wild species of peanut (under preparation).

PRESENTATIONS AT NATIONAL SEMINARS / SYMPOSIA

1. **Shweta Singh** and Sulekha Hazra (2006) "Modulation of peanut embryogenesis protocol for fast recovery of morphologically normal plants". Poster presented on National Science Day at NCL, Pune.

2. **Shweta Singh** and Sulekha Hazra (2005) Modulation of peanut embryogenesis protocol for fast recovery of morphologically normal plants. Poster presented and Abstract published during 'National Symposium on Plant Biotechnology: New Frontiers' (November 18-20, 2005) in the 27th Annual Meeting of Plant Tissue Culture Association (India) held at CIMAP, Lucknow.