Studies on *In Vitro* Regeneration and Genetic Transformation in Peanut (*Arachis hypogaea* L.)

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Studies on *In Vitro* Regeneration and Genetic Transformation in Peanut (*Arachis hypogaea* L.)

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N.C. L. **Dr. Mrs. Sulekha Hazra (Research Guide)**
U.G.A. **Dr. Peggy Ozias-Akins**

In

Memory of

My Beloved Father
Late RASHBIHARI NANDI

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Date: 28th Oct, 2002 (Madhumita V. Joshi)

Place: Pune-8

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Studies on *In Vitro R*egeneration and Genetic Transformation in Peanut (*Arachis hypogaea* L.)"** submitted by Mrs. Madhumita V. Joshi was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Part of the research, mainly the transformation study was carried out at Department of Horticulture, Tifton Campus, University of Georgia, USA, under the supervision of Dr. Peggy Ozias-Akins. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. Mrs. Sulekha Hazra)

Research Guide

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Key to abbreviations

°C	Degree Celsius
2,4,5-T	2,4,5-trichlorophenoxy acetic acid
2,4-D	2,4-di chlorophenoxy acetic acid
2iP	6-γ,γ-dimethylallylaminopurine
ABA	Abscisic acid
ANOVA	Analysis of variance
BAP	6-Benzyl amino purine
cv.	Cultivars
ECFP	Enhanced Cyan Fluorescent Protein
EGFP	Enhanced Green Fluorescent Protein
EYFP	Enhanced Yellow Fluorescent Protein
GA_3	Gibberellic acid
GFP	Green Fluorescent Protein
GUS	□glucuronidase
$HgCl_2$	Mercuric Chloride
IBA	Indole butyric acid
KN	Kinetin (6-furfuryl amino purine)
MS	Murashige and Skoog medium (1962)
NAA	α-Naphthaleneacetic acid
PGR	Plant Growth Regulator
Picloram	4-amino-3,5,6-trichloro picolinic acid
PVP	Polyvinyl pyrrolidone
SAAT	Sonication Assisted Agrobacterium-mediated Transformation
SD	Standard deviation
TBA	Tertiary butyl alcohol (2-methyl propan-2-ol)
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)

ABSTRACT

Groundnut (*Arachis hypogaea* L.), a major oilseed legume, native to South America, is an important commercial crop in Asia and Africa. It is the major oilseed crop of India. The estimated loss in crop yield due to biotic factors that include various diseases caused by fungal pathogens, viruses, bacteria and nematodes is about US $2 billion. (Sharma et al., 2001). Biotechnology offers an impressive option to supplement the ongoing efforts on developing genetically enhanced germplasm for achieving sustainable food production. In addition to classical and molecular breeding approaches, genetic transformation to introduce novel genes into plants for quality production offers an attractive option.

Plant transformation count on a good regeneration protocol. Work was carried out to identify suitable peanut explants for induction of embryonic callus and proliferation of embryos via secondary embryogenesis vis-à-vis appropriate nutrient composition and cultural conditions. Experiments were carried out to standardize an efficient protocol for genetic transformation.

To summarize, the objectives of the present study were:

1. Morphological studies of somatic embryogenesis and embryo germination.
2. Optimization of conditions for morphogenic callus induction and regeneration.
3. Genetic transformation of peanut using two approaches viz. Biolistics/Particle Gun and Agrobacterium Mediated Transformation.

The present studies were carried out at Plant Tissue Culture Division at National Chemical Laboratory (NCL), Pune, India. The major portion of the work on genetic transformation was carried out in the Department of Horticulture, Tifton Campus, University of Georgia, and USA. The thesis has been divided into six chapters along with a summary and list of references.

CHAPTER 1: Introduction

This chapter addresses the importance of peanut as an oil seed crop. The chapter also focuses on present scenario of peanut production as well as constraints in its improvement. Potential of biotechnology in peanut improvement has been discussed. It also presents magnitude and update of research done on *in vitro* culture and genetic transformation of peanut. Finally the objectives of the present study have been discussed.

CHAPTER 2: Materials and Methods

Overall materials used and methods implemented during the course of this work has been described in this chapter. This includes the source of glassware, plastic ware, chemicals, etc.; procedure involved in cleaning and preparation of glassware; preparation of media; surface sterilization of explants; histological methods etc. Materials and methods specific to individual experiment have been dealt separately in respective chapters.

CHAPTER 3: Morphological Studies

In the earlier studies in this lab TDZ was found effective in conversion of somatic embryos by triggering morphogenetic activity in the irregularly developed plumule. To get somatic embryos with well differentiated plumule and having high germination frequency, experiments were conducted to

study the effect of TDZ in early embryo development. It was ineffective in supporting the normal plumule development, but induced morphogenic activity around the equatorial region of the embryos.

Keeping in view the ability of thidiazuron to induce both organogenesis and embryogenesis in peanut tissues, experiments were conducted to define the pathway of morphogenesis in the plumule of rooted somatic embryos. On exposure to TDZ, projections resembling somatic embryos appeared from the plumule. Histological examination revealed that these are caulogenic buds and not somatic embryos.

CHAPTER 4: Induction, Maintenance and Regeneration of Embryogenic Callus

Optimization of a protocol for induction of embryogenic callus and its maintenance and regeneration are described in this chapter. Various explants and combinations of growth regulators were tested for induction of morphogenic callus. Conditions were standardized to maintain embryogenic ability via repetitive embryogenesis. Plants were regenerated from the embryogenic callus.

CHAPTER 5: Studies on Genetic Transformation

First part of this chapter describes the experiments conducted for optimization of particle bombardment parameters using a Green Fluorescence Protein (GFP) gene. We obtained stably transformed lines with GFP gene by visual as well as antibiotic selection and successfully regenerated the transgenic plants with GFP gene. This is the first report on stable transformation of GFP in peanut by visual selection. We have also checked the transient expression of well known reporter gene GUS (ß-glucuronidase) using the optimized bombardment parameters in peanut somatic embryos and obtained the stable transformed cell lines with a selectable marker gene Hygromycin phosphotransferase (*hph*) resistant to hygromycin. Integration and expression of foreign gene has been proved by molecular analysis like PCR, Southern blot and Northern blot analysis.

The particle bombardment approach was further extended to transformation of peanut using a herbicide resistance gene, Phosphinothricine acetyl transferase (*Bar*) as a selectable marker in peanut transformation. The standardization of optimal dose for use of L- Phosphinothricin (PPT) as a selectable marker is reported in this chapter. Stably transformed lines were recovered by liquid culture selection with PPT and presence of *Bar* gene has been proved by PCR analysis. This is the first report in peanut to use of herbicide resistant gene (*Bar*) as a selectable marker.

Second part of this chapter deals with *Agrobacterium* mediated transformation in peanut using somatic embryos as explants. Attempts were made to optimize conditions for high frequency transformation using different chemical and mechanical assistance. In addition to this a new approach identified as Sonication Assisted *Agrobacterium*-mediated Transformation (SAAT) has been used and optimized the parameters with transient expression of reporter gene GUS. The technique deal with the use of *Agrobacterium* as a microprojectile was also evaluated for a high transformation frequency. Both the techniques for *Agrobacterium* mediated transformation were first report in peanut.

SUMMARY

The main findings of the work conducted on peanut morphological and transformation study are summarized in this section.

CHAPTER 1:

INTRODUCTION

Peanuts are grown in tropical and warmer temperate regions throughout the world. The crop provides an excellent source of protein and other nutrients. The global production of peanut averages nearly 24.8 million metric tones from almost 20 million hectare. Average yield varies from approximately 0.50 to nearly 3.0 metric tones per hectare (Knauft and Wynne, 1995). Though India and China together account for over half of the world's production, southeastern Asia, western and southern Africa, Argentina, Brazil and United States are also considered as major peanut producing tracts of the world. About 93.8% of the world's production of groundnut is grown by resource-poor, small farmers in 96.9% of world's groundnut area in developing countries where scanty, unseasonal, and unpredictable rainfall is observed (Nageshwara Rao and Nigam, 2001).

Asia accounts for 66.5% of global groundnut production on 56.8% area while Africa produces 24.7 % on 38 % area subjected to groundnut production. India alone reported 7.36 Million tones of groundnut production in the year 2001- 2002 (CMIE Rep., 2002). Peanut production and quality can be severely impacted under stressful growing conditions such as adverse climate, pests and diseases. However, the last two decades has witnessed considerable progress in peanut improvement via integrated efforts in breeding, genetics and other related disciplines throughout the world. Peanut improvement has received high priority from scientists at the International Crop Research Institute for the Semi-arid Tropics (ICRISAT), which adopted the peanut as a mandate crop in 1976 (Gibbons, 1980).

The genus *Arachis* contains a rich diversity of plant types. It is generally believed that peanut, *Arachis hypogaea* L., originated in southern Bolivia or northern Argentina (Gregory *et al.*, 1980) is cultivated for food and oil around the world. 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. Gregory and Gregory (1976) postulated that *A. duranensis* and *A. cardenasii* intercrossed to produce cultivated peanuts. Though *A. hypogaea* has a distinct pair of chromosomes present only in the B genome (usually present in *A. batizocoi*), molecular genetic variability studies has confirmed existence of a large genetic distance between *A. hypogaea* and *A.batizocoi* (Ro *et al.*, 1992; Halward *et al.*, 1992).

Poor yields of peanut in developing countries is mainly due to the biotic and abiotic constraints like erratic rainfall, low residual moisture, lack of high yielding adapted cultivars, damage by pest and diseases, poor agronomic practices, and limited use of inputs (Nageshwara Rao and Nigam, 2001). The loss due to biotic factors that include various diseases caused by fungal pathogens, viruses, bacteria and nematodes is estimated to be US $2 billion. (Sharma *et al.*, 2001). The procedures and objectives for peanut improvement programs largely depend on the use of the crop, whether for oil or food, and on the amount of the inputs used in production. When grown as a subsistence crop by low-resource farmers, measures to manipulate stresses are no longer cost effective. Under these conditions, the crop by itself must be tolerant to range of biotic and abiotic stresses if adequate yields are desired. Breeding for peanut essentially accounts for vegetative, reproductive and quantitative traits that are associated with yield and those with economic value.

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. 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.

Most elaborative area of peanut *in vitro* culture includes studies associated with *in vitro* regeneration via. somatic embryogenesis and organogenesis, genetic transformation, somaclonal variation, and *in vitro* plant regeneration for somatic hybridization and protoplast culture. Successful genetic transformation of plants generally requires a well-established tissue culture system to regenerate whole plants from single cells. Regeneration of plants *in vitro* using somatic embryogenesis has some distinct features such as single cell origin, the consequent number of regeneration (Haccing,

1978; Ammirato 1983; Sato *et al.* 1993). Development of transgenic plants through somatic embryos thus far remains the pathway of choice due to their single-cell origin and conversion ability to genetically stable, nonchimeric plants. Thus the somatic embryogenesis system is being increasingly employed to produce transgenic plants (Ritchie and Hodges 1993; Redenbaugh *et al.* 1986). Successful transformation and development of primary and secondary embryogenesis from single cell was reported by several researchers in different species (Polito *et al.* 1989; McGranahan *et al.* 1988, 1993; Ninkovic *et al.* 1995).

Transformation procedures typically result in transformation of individual cells within the explant tissue (Merkle *et al.* 1990). Whenever it is possible for somatic embryos to arise from single cells, repetitive embryogenesis may be used to first recover a somatic embryo from a transgenic cell, and then to multiply in number of transgenic embryos available for plant recovery. Alternatively, when somatic embryos 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). In essence, 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 somatic embryogenesis and ultimately repetitive embryogenesis.

Somatic embryogenesis can either be direct, developing directly from an organ or indirect, involving a callus phase. In case of indirect embryogenesis it is associated with unorganized and unintegrated cells which initiate new somatic embryos (Williams and Maheswaran, 1986). Repetitively growing morphogenic/ embryogenic cultures are an ideal source of tissue for application of gene transfer techniques. Repetitively growing cultures ensure greater tissue homogeneity than most primary explants. Thus it has a distinct advantage for elevating transformation frequency. Further more, the gradual proliferation of transformed sectors due to the expression of a selectable marker gene and the elimination of nontransformed tissue during prolonged selection reduces the possibility of recovering chimeras.

There is a great need to optimize a protocol for induction of embryogenic callus, somatic embryogenesis and repetitive embryogenesis to maintain the embryogenic capacity for extended period and continue to differentiate large number of plants. Maintenance of embryogenic capacity for more than two years by repeated secondary embryogenesis has been reported in *Asperagus officinalis*

(Delbreil & Marc, 1992), *Camellia japonica* (Vieitez *et al.* 1991), *Clitoria ternatea* (Dhahnalakshmi & Lakshmanan, 1992), *Picea glauca* (Kong & Yeung, 1992), *Manihot esculenta* (Raemakers, 1993) and many more. Preservation of embryogenic lines can be cost effective and selected lines can be multiplied in large quantities by secondary somatic embryogenesis. This indirect embryogenesis protocol is also helpful in overcoming the limitations of post-fertilization barriers which often prevent maturation of the zygotic embryos. Immature embryos of interspecific plants from incompatible crosses (Merkle *et al.* 1990) may be rescued by culturing them for secondary somatic embryogenesis (Raemarkers *et al.* 1995).

Somatic embryogenesis has been obtained in many plant species. However conversion of somatic embryos into plants remains inefficient and limits the application of somatic embryogenesis as regeneration protocols in many systems (Wetzstein *et al.* 1990; Lippmann and Lippmann, 1984; Chee *et al.* 1990). There are several reports of somatic embryogenesis from which whole plants were not obtained, eg. *Vigna radiata* (Eapen and George, 1990), *Glycine max* (Yang *et al.* 1991), *Poinciana regia* (Bajaj, 1989). Earlier reports in soybean (Buchheim *et al.* 1989), peanut (Wetzstein and Baker, 1993), grapevine (Goebel-Tourand et al, 1993), Feijoa (Cruz *et al.* 1990), and carrot (Nickel and Yeung, 1993) have shown an interrelationship between morphological aberrations and lack of conversion of somatic embryos into plants.

Thidiazuron (TDZ), identified as a synthetic growth regulator, induces a wide array of responses with high degree of efficiency (Murthy *et al.* 1998). This growth regulator has been found to substitute for both the auxin and cytokinin requirement for somatic embryogenesis (Saxena *et al.* 1992; Visser *et al.* 1992; Gill *et al.* 1993). Inclusion of TDZ in culture media stimulated *in vitro* somatic embryogenesis in tobacco (Gill and Saxena, 1993), Geranium (Visser *et al.* 1992), Chickpea (Murthy *et al.* 1996), Peanut (Saxena *et al.* 1992; Murthy *et al.* 1995), *Acacia mangium* (Xie and Hong, 2001) and many more. TDZ has proven effective for conversion of abnormal somatic embryos in peanut (Chengalrayan *et al.* 1997). These reports provide evidence of involvement of TDZ in somatic embryogenesis and an efficiency of plant regeneration from embryos. A better understanding of the physiological responses of plant tissues to TDZ will lead to a greater understanding of the process of morphogenesis itself.

Plant transformation is now a core research tool in plant biology and a practical tool for cultivar improvement. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (De Block *et al.* 1984; Horsch *et al.* 1984; Paszkowski *et al.* 1984), has

been extended to over 120 species in at least 35 families (Birch, 1997). There are several reports on development of plant transformation system using *Agrobacterium* (Hooykaas and Schilperoort, 1992; Tepfer, 1990, Zupan and Zambryski, 1995), direct gene transfer into protoplasts (Gad *et al.* 1990; Negrutiu *et al.* 1990; Neuhaus and Spangenberg, 1990), or particle bombardment (Birch and franks, 1991; Christou, 1992; Christou, 1995), and the potential for their practical application (Collins and Dunwel, 1995; Horsch, 1993). The expectation of plant transformation can be met depending on the efficiency and predictability of production of lines with desired phenotype, and without undesired side effects of the transformation process. The fruits of transgenic research are the commercial release of improved varieties in a range of crops, start from "Flavr Savr" in Tomato (Calgene) to "Laurical"in Canola (Calgene), "bollgard" in Cotton; "Newleaf " in Potato; "Yieldgard" in Maize (Monsanto), "Liberty Link" in Corn (Pioneer) and many more.

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), *Agrobacterium tumefaciens* (Lacorte *et al.*, 1991; Mansur *et al.*, 1993, Cheng *et al.*, 1997), and direct DNA delivery using particle bombardment (Ozias-Akins *et al.*, 1993, Brar *et al.*, 1994; Singsit *et al.*, 1997).

There are a few reports on transformation in peanut using various protocols but further improvement is needed to achieve transformation at high frequency. An efficient callus regeneration protocol via somatic embryogenesis and repetitive embryogenesis is a pre-requisite for optimization of a reliable transformation protocol. Present study was formulated to address some basic questions associated with induction, maintenance and regeneration of embryogenic callus; induction, regeneration of somatic embryos from callus; and viable plants from somatic embryos. The work attempts to find suitable medium for induction of embryogenic callus and their proliferation via secondary embryogenesis vis-à-vis appropriate nutrient composition and cultural conditions, in peanut using mature embryo axes.

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) influence of this growth regulator was studied in early embryo development. This experiment was conducted in anticipation of obtaining normal somatic embryos. A part of the study was formulated to understand the TDZ induced pathway of high frequency embryo conversion.

For application of genetic transformation approach there is a need to develop reliable protocols for genetic transformation. In this study experiments were conducted to optimize parameters for both particle bombardment and *Agrobacterium*-mediated transformation. Green Fluorescence Protein (GFP) gene; a widely appreciated reporter, GUS (ß-glucoronidase) and Phosphinothricin (*Bar*) a herbicide resistance gene have been used.

To summarize, in this study the experiments were divided in three parts with the following objectives,

1. Studies on somatic embryogenesis for improved plantlet recovery.
2. Optimization of condition for embryogenic callus induction and regeneration via somatic embryogenesis.
3. Genetic transformation of peanut using Particle gun and *Agrobacterium tumefaciens*.

CHAPTER 2:

MATERIALS AND METHODS

The materials used and the plant tissue culture techniques routinely followed during the course of the present investigations have been described in this chapter. It also includes the molecular analysis methods used for detecting the integration of foreign gene. The methods used in genetic transformation study by particle bombardment as well as *Agrobacterium* mediated transformation have been described in chapter V.

2.1. ***Plastic ware***

Pre-sterilized plastic petridishes of 35 mm, 55 mm and 85 mm diameter and filter sterilization units (20 ml) were procured from "Laxbro", India. Eppendorf tubes (1.5 ml and 2 ml capacity), micro-tips (0-200 µl and 200-1000 µl capacity) obtained from "Laxbro" and "Tarsons", India and wide bore micro-tips (0-200 µl) were procured from "Sigma", USA.

2.2. ***Glassware***

The following glassware used in all the tissue culture related experiments was procured from "Borosil", India.

Test tubes (25 mm x 150 mm),
Jam bottles (70 mm x 125 mm) with polypropylene plastic caps,
Plastic petri dishes (85 mm x 15 mm),
Erlenmeyer's flask (100, 250, 500 and 1000 ml),
Pipettes (1, 2, 5, 10 and 25 ml),
Beakers (100, 250, 500 and 1000 ml) and
Measuring cylinders (10 – 1000 ml).

2.2.1. ***Preparation of Glassware, filter paper, instruments, millipore filters***

Glassware used for the experiments were cleaned by boiling in saturated solution of sodium bicarbonate for 1h to ensure absolute removal of dry gelling agents, followed by repeated washing in tap water. These were immersed in 30% nitric acid solution for 30 min followed by repeated washing in tap water. Thereafter they were rinsed with distilled water and dried at room temperature or in an oven at 200 °C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd,

Andhra Pradesh, India). Pipettes, forceps scalpel, millipore filter units etc were wrapped in brown paper and packed in autoclavable polypropylene bag prior to autoclaving. Course quality filter paper pieces were used for aseptic manipulation of tissues. The pieces of Approximately 10 x 20 cm were cut. These pieces were packed in paper bags. The paper bags were packed in polypropylene bag for sterilization by autoclaving. These were used for dissection and for transfer of explants in a laminar air flow cabinet.

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

2.3. *Chemicals*

All major and minor salts used in the tissue culture media were of analytical grade and were obtained from "Qualigens", "S.D fine chemicals" or "Hi-media", India. Growth regulators, vitamins, antibiotics (except Cefotaxime) and Phytagel were obtained from "Sigma Chemical Co.", USA. Cefotaxime was procured from Russel India Ltd., Bombay, India. Sucrose, glucose, fructose, maltose, were obtained from "Qualigens" and agar-agar from "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO®" laboratories, USA. The chemicals used in molecular biology experiments were obtained from "Sigma Chemical Co.," USA.

2.4. *Preparation of culture media*

Glass distilled water was used for the preparation of culture media. After addition of all macro- and micro-nutrients, vitamins, growth regulators and carbohydrates like sucrose or glucose or fructose or maltose, the pH of the media was adjusted to 5.8 using 0.1N NaOH or HCl prior to autoclaving. Agar (0.7%) was used for gelling the plant tissue culture medium and 1.5% Agar-agar (Hi-media) was used for microbial culture. The medium was steamed to melt the gelling agent. Media were dispensed into test tubes and flasks with cotton plugs or in bottles with plastic caps. The media were autoclaved at 121°C under 15 lb psi for 20 min. Specific growth hormones, amino acids, antibiotics etc. were added to autoclaved medium before dispensing. The compositions of Murashige and Skoog's (MS) macro-, micro elements and vitamins used in the present study are given in Table 2.1, 2.2 and 2.3 respectively.

Table 2.1: Composition of macro-element salts of MS (Murashige & Skoog 1962) basal media.

Macro-element	Conc. (mg/l)
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170

Table 2.2: Composition of micro-element salts in MS (Murashige & Skoog 1962) basal media.

Micro-element	Conc. (mg/l)
$MnSO_4.\ 4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025

$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3

Table 2.3: Composition of organics in MS (Murashige & Skoog 1962) basal media.

Organics	**MS (mg/l)**
Thiamine. HCl	0.1
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Myo-inositiol	100
Glycine	2.0

2.5. *Collection of Plant material*

Pods of mature seeds (Fig. 2.1) of a high yielding cultivar of peanut (*Arachis hypogaea* L. cv. JL-24) were collected in the month of April - May from the Agriculture College, Pune, India. The seeds were stored in sealed containers at room temperature and used throughout the year.

2.6. *Preparation of plant material*

2.6.1. *Preparation of petridishes with medium:*

Two sizes of Petri dishes were used i.e. 55 mm and 85 mm. Media were poured in laminar flow aseptically. According to size of dish 10 ml medium were poured for 55 mm dish and 20 ml for 85 mm dish. Dishes were kept open for 5 min and let the medium solidify.

2.6.2. *Surface sterilization of seeds:*

The dehusked seeds (Fig.2.2) of the above mentioned cultivars were washed with 1 % (v/v) liquid detergent (Labolin, S.D.Fine Chem, India) for 5 minutes. The embryo axes were excised and surface sterilized with 0.1 % (w/v) mercuric chloride (Qualigens, India) solution for 4-5 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 dissection of immature leaflets (Fig. 2.3. A, B). Leaflets were excised with sharp, fine forceps and 20-25 leaflets were placed in each 85 mm Petri dish containing 20 ml medium.

Fig. 2.1. "Seeds of peanut cv JL-24 with seed coat"

Fig. 2.2. Dehusked seeds of peanut

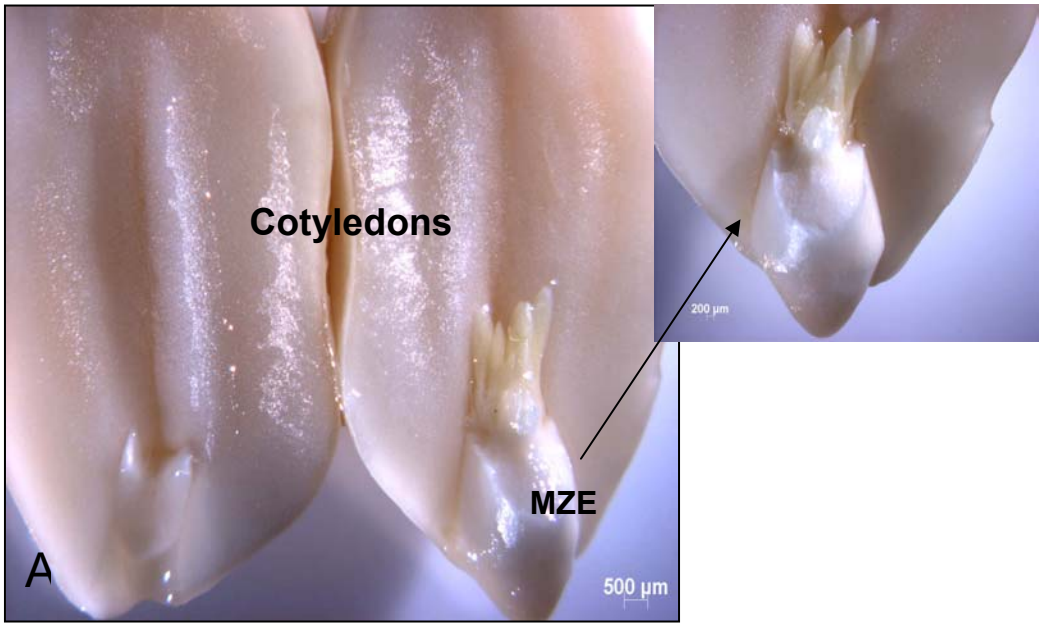


Fig. 2.3.A. Cotyledons of peanut seed with mature zygotic embryo (MZE)

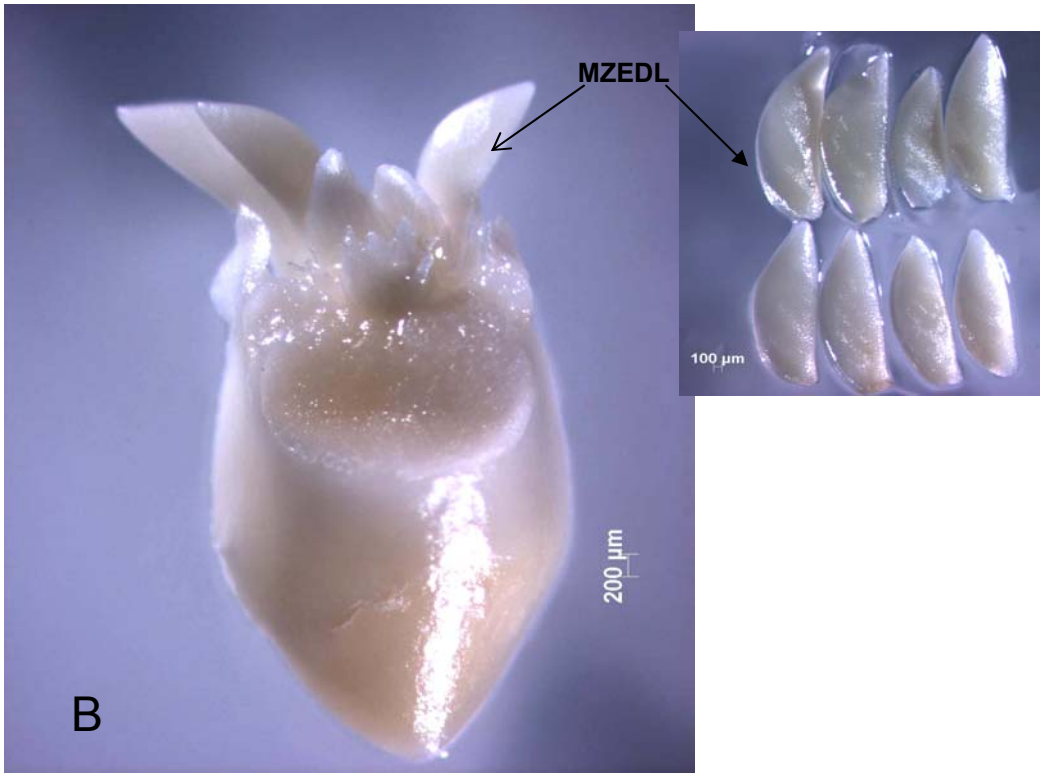


Fig. 2.3.B. Mature zygotic embryo derived leaflets (MZEDL) used as explant

2.7. *Culture conditions*

For the experiments in light, the cultures were incubated at 25 ± 2°C in irradiance of 32 μmol $m^{-2} \cdot s^{-1}$, 16 h photoperiod and for dark, cultures were kept in BOD maintaining the above temperature. The details of the incubation conditions described in respective sections separately. Subcultures were done every 3-4 weeks or according to the need of experiment.

The number of explants used in each replication, number of replication and the number of repeats of each experiment are described in materials and methods of the respective section.

2.8. *Statistical analysis*

Statistical methods were used for comparison of treatment means during optimization of parameters. Means and Standard deviations for the data were calculated and were analyzed using Microsoft Excel package. Analysis of variance was carried out by Randomized Block Design method and significance among the treatments was determined by Student's 'T' test (Panse and Sukhatme, 1967).

2.9. *Hardening of plantlets*

The details of TDZ induced plant recovery via organogenesis and from callus via somatic embryogenesis were described in respective section. *In vitro* raised plantlets were carefully taken out of the test tubes and gently washed under tap water to remove the gelling agent and the medium adhering to the roots. The plantlets were dipped in 1 % aqueous solution of Bavistin®, a systemic fungicide (BASF, India) for 10-15 min prior to transfer to soil. The treated plantlets were transferred to earthen pots of diameter 8 cm containing a mixture of autoclaved soil: sand (1:1). The plants in pots were covered with transparent polypropylene bags to prevent the rapid loss of humidity and were kept in greenhouse 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 3-4 weeks, the polypropylene bags were completely removed.

2.11. *Genetic Transformation*

Details of materials & methods used for particle bombardment and Agrobacterium mediated transformations and maintenance of the transformed cell lines has been described in respective sections.

CHAPTER 3:

MORPHOLOGICAL STUDY

Introduction

Unlike zygotic embryogenesis, where fusion of gametes leads to embryo development, somatic embryogenesis follows a characteristic development pattern, enabling development of differentiated plants from embryos emerging from somatic cells (Tisserat *et al.* 1979; Williams and Maheswaran 1986; Rangaswamy 1986; Zimmerman 1993; Merkle *et al.* 1995). As defined by Williams and Maheswaran (1986), somatic embryogenesis is a pathway of *de novo* regeneration from *in vitro* maintained callus tissue (indirect morphogenesis) or from differentiated cells of leaf, hypocotyls, zygotic embryo etc (direct morphogenesis). But, somatic embryogenesis, as a physiological process designates the ability of individual plant cells to produce embryos in response to appropriate stimuli. However the process has been poorly understood. Since it's very first report in carrot (Steward *et al.*, 1958) remarkable progress has been made in the study of somatic embryogenesis. The potentials of somatic embryogenesis impel from number of factors that involve high regenerative capacity, the ability to regenerate from single cell in both gametophyte and sporophyte tissues, the bipolarity of embryos and compactness and broad metabolic potential of embryogenic tissue (Janick 1993). However the successful application of the somatic embryogenesis protocol largely depend on the ability of the somatic embryos to convert into plantlets.

While reporting somatic embryogenesis in peanut for the first time; Pittman *et al.* (1983) observed occasional heart shaped embryos in the organogenesis pathway from young leaves.

Banerjee *et al.* (1988) observed different developmental stages of embryogenesis from superficial layer of the cotyledonary node explants but failed to report further development.

Several workers using variety of explants and growth regulators (Table 3.1) subsequently reported plant regeneration via somatic embryogenesis in peanut.

Table 3.1: Literature on Somatic embryogenesis in peanut

Explant	**Growth regulators**	**References**
Immature embryo axis	2,4-D	Hazra *et al.* 1989
Immature embryo cotyledons	Picloram	Ozias-Akins 1989
Mature zygotic embryo	Picloram	McKently 1991
Excised immature cotyledon	Picloram	Ozias-Akins *et al.* 1992, b
Sections of cotyledons and juvenile leaves	Thidiazuron (TDZ)	Gill and Saxena 1992
Leaflets from aseptically germinated embryo axes	2,4-D and Kinetin	Baker and Wetzstein 1992
Immature embryonal axes	2,4-D	George and Eapen 1993
Immature embryonal axes and immature cotyledon	2,4-D	Eapen and George 1993
Mature embryo-derived Leaflets	2,4-D	Chengalrayan *et al.* 1994
Immature cotyledons	2,4-D	Baker and Wetzstein 1994
Seedlings	Forchlorfenuron (CPPU)	Murthy and Saxena 1994
Mature dry seed (Whole embryo axis, epicotyl portion of axis, radicle portion of axis	2,4-D	Baker *et al.* 1995
Intact seedling	TDZ	Murthy *et al.* 1995
Seedling leaves	2,4-D and Kinetin	SabithaRani and Reddy 1996
Hypocotyl of germinated seedling	2,4-D with BAP	Venkatachalam *et al.* 1997
Cotyledons from mature dry seeds.	BAP with NAA	Venkatachalam *et al.* 1999a

The major snag in peanut somatic embryogenesis is low conversion frequency of somatic embryos to normal plant. The failure and /or low conversion frequency has often been attributed to the

morphological abnormalities or immaturity of somatic embryos (Ammirato, 1987; Wetzstein and Baker, 1993). Several attempts have been made to obtain normal somatic embryos and improve the frequency of plant recovery (Ozias-Akins *et al.* 1992b; Chengalrayan et al, 1995, 1997). However, information on normal embryo development and conversion of these germinated embryos to plantlets is limited. In most culture systems, somatic embryogenesis is induced by modulation of the ratio of auxin to cytokinin (Dermastia *et al.* 1996; Letham Palni, 1983; Skoog and Miller, 1957). The role of auxin in the induction of somatic embryogenesis has been documented (Michalczuk, 1992). In peanut, the induction of somatic embryogenesis is dependent on the auxin content of the medium (Hazra *et al.* 1989; Sellars *et al.* 1990; Eapen and George, 1993). However, the role of cytokinins in embryo induction and development is poorly understood, due to a lack of cytokinin-dependent experimental systems and effective inhibitors of cytokinin biosynthesis and metabolism (Hutchinson, 1996).

As suggested by Mok et al, (1987) cytokinin can be classified broadly into two groups, according to their chemical structure; the adenine and the phenylurea derivatives. Cytokinins from adenine group are N6-substituted derivatives of the nitrogenous purine base adenine characterized by the ability to stimulate cell division in tissue culture. On the other hand TDZ is a substituted phenylurea compound which was developed for mechanized harvesting of cotton bolls and has now emerged as a highly efficacious bioregulant of morphogenesis in the tissue culture of many plant species. Application of TDZ induces a diverse array of culture response ranging from induction of callus to formation of somatic embryos (Murthy *et al.* 1998). These authors suggested that TDZ exhibits a unique property of modulation of both auxin and cytokinin effects on growth and differentiation of cultured explants, although structurally it is different from either auxin or purine- based cytokinins. TDZ molecule has two functional groups; (I) phenyl and (II) thidiazol group and the replacement of either of these groups with other ring structures results in the reduction in the activity (Mok et al, 1982). The activity of TDZ varies widely depending on its concentration, exposure time, type of explants and tissue. A number of physiological and biochemical events in cells are likely to be influenced by TDZ which are responsible for the ultimate result of morphogenic responses. A survey of published reports revealed the intense research efforts directed toward the development of TDZ mediated regeneration system in different tissues.

Murthy et al, (1995) assessed the regulatory role of TDZ and explant factors in imparting somatic embryogenic potential in relation to endogenous growth regulator levels. They suggested that the site of action of TDZ might influence the precursors of the auxin, cytokinin and ABA. Therefore, it is possible that TDZ has an influence on the mitochondrial electron transport chain or the oxidative

pentose phosphate pathway, which produces precursors for these growth regulators. So 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.

Role of TDZ in Peanut Tissue Culture

Several researchers have reported the use of TDZ for peanut regeneration via both organogenesis and embryogenesis. 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 thidiazuron-induced 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, surface of cotyledons and hypocotyls of germinating seedlings, which eventually mature and develop into plants.

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 plants.

Kanyand et al (1994) reported induction of multiple shoots from hypocotyl and cotyledon explants of Valencia type peanut in the medium containing 30 mg/l TDZ. Those shoots rooted on the MS basal medium and gave rise to plantlets.

The regulatory role of thidiazuron and explant factors in imparting somatic embryogenic potential was assessed by Murthy *et al.* (1995), 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. 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 leaf where folioles are attached. Explants grown on a medium containing 10mg/l TDZ of 3 weeks exhibited progressive morphological changes of emergences which differentiated into three types of adventitious structures with one type resembling radially concentric shoots. Shoots differentiated from these structures and greatest extent of shoot differentiation occurred at the cotyledonary node. The ability of the emergences to form adventitious shoots was emphasized because of their superficial origin in epidermal and subepidermal tissues. For genetic transformation by particle bombardment these tissues are directly exposed.

Chengalrayan *et al.* (1997) reported the effectiveness of TDZ for high-frequency conversion of abnormal peanut somatic embryos. Various growth regulators including Kinetin, Benzylaminopurine, 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. Eventually 92% of the embryos could be converted to plantlets in presence of 22.7 10 μM TDZ.

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.

Victor *et al.* (1999b) exhibited *de novo* regeneration of somatic embryos 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 demonstrated the role of endogenous purine metabolism in thidiazuron-induced somatic embryogenesis of peanut (Victor *et al.*, 1999a). 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 (purine analogs, 2,6-diaminopurine) inhibited the purine recycling resulting in decreased levels of endogenous adenine and zeatin.

Gill and Ozias-Akins (1999) reported a highly morphogenic callus induction from immature cotyledons and embryo axes of peanut 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.

Akasaka *et al.* (2000) reported a plant regeneration protocol by induction of bud primordial from cultured leaf segments in peanut by limited exposure to TDZ. They observed a 34.7% conversion from shoot buds to shoots when cultured for 7 d at 10 mg/l TDZ or 21 d at 1 mg/l TDZ and an over exposure on TDZ medium induced abnormal development of these primordia.

A protocol for high frequency somatic embryogenesis from mature embryo-derived immature leaflets of peanut has been reported from this laboratory (Chengalrayan et al, 1994). On transferring these somatic embryos to growth regulator free medium, only the radicle differentiated, while the plumule turned green but remained stunted. The failure of the embryos to convert into plantlet was attributed to the abnormal plumule development. To trigger differentiation in the plumule and overcome the failure in conversion, the rooted embryos were cultured in media with various growth regulators including GA_3, ABA, BAP, Kin, 2ip and TDZ (Chengalrayan et al, 1997). Various conversion rates were observed in presence of different growth regulators. Highest conversion of 92 % was achieved with TDZ (22.7 μM) followed by BAP (22.2 μM). TDZ was found effective in conversion of somatic embryos by triggering morphogenetic activity in the irregularly developed plumule. It was suggested that development of multiple shoots in the plumule may either be due to triggering of the existing multiple meristems in the fasciated embryos or due to proliferation of pre-existing shoot meristem (Chengalrayan et al, 1995). Despite these reports, the mechanism of conversion influenced by TDZ at physiological / cytological level is ill-understood. To explore the possibilities of obtaining normal embryo with well defined plumule and to understand the phenomenon of multiple shoot formation in the plumule of the abnormal embryos the present investigation was designed and conducted in two parts:

1) Effect of TDZ in early embryo development was studied to get somatic embryos with well-differentiated plumule having a high germination frequency.
2) Morphogenesis in the plumule of rooted embryos were studied at an optimum concentration of TDZ.

3.1. Effect of TDZ in Initial Stage of Somatic Embryogenesis in Peanut.

The objective of the present investigation was to obtain somatic embryos with well-differentiated plumule and having high germination frequency. Keeping in view the influence of TDZ in triggering morphogenesis in plumule of peanut somatic embryos, experiments were conducted to study the effect of TDZ in early embryo development.

Materials and Methods

A high yielding cultivar of peanut (JL-24) was used in this study. Source of explant and surface sterilization procedure is described in the materials and method chapter (section 2.6).

Experimental media and culture conditions

Embryogenic masses were induced in the mature embryo derived immature leaflets of peanut following the method described by Chengalrayan *et al.* (1994). In each petri dish (85 mm) containing 20 ml of Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) with 0.18 M sucrose and 90.5 µM 2,4- dichlorophenoxyacetic acid (2,4-D), 20 - 25 explants were cultured. After incubation for 4 weeks in light the embryogenic masses were transferred to embryo development medium containing MS basal salts with 13.6 µM 2,4-D and varying concentration of TDZ (2.27 – 45.41µM). Medium with 13.6 µM 2,4-D and without TDZ was used as control. In each petri dish (85 mm) containing 20 ml of medium, 8-10 explants were cultured and 4-5 petri dishes were used for each medium in each replication. Observations were noted after 6 weeks. Numbers of explants (embryogenic masses) that responded in culture and demonstrated morphological changes were scored.

After incubation for 6 weeks, embryos were subcultured in three different media: (1) Fresh medium of similar composition, (2) MS with TDZ 9.08 µM, and (3) MS basal medium. All the media were supplemented with 0.18 M sucrose and solidified with 0.6% agar. The pH of the media was adjusted to 5.8. The cultures were incubated in light.

After 4 weeks cluster of shoot buds (with opened leaves) from the above three media were isolated and transferred to MS basal medium without growth regulator and incubated in light for 2-3 weeks.

All experiments were repeated three times with 5 replications each. Observations were noted from 4 weeks onward for 8-10 weeks. The mean data were subjected to statistical analysis.

Rooting, transfer to soil and hardening

Elongated shoots (3-5 cm) from MS basal medium were transferred to rooting medium containing MS and 0.54 µM NAA with 0.09 M sucrose. Cultures were incubated for 4-6 weeks for growth of both shoots and roots.

Plantlets with healthy roots and shoots (6-8 cm) were transferred to pots containing sand: soil (2:1) mixture and hardened (Materials and methods 2.9). Survival rate of the plants was calculated from number of plants transferred to soil and the number of plants established. The appearance of new leaves after 3 – 4 weeks indicated establishment of the plants in soil.

Results and Discussion

Effect of TDZ on the on developing somatic embryos

Morphogenesis in plants via embryogenesis has certain advantages over organogenesis since somatic embryos, have both root and shoot meristems. However, often plant recovery remains a difficult task in embryogenesis protocol because of its low frequency conversion to plantlets. Role of TDZ as an effective stimulant in conversion of peanut somatic embryos by triggering the morphogenic activity in the irregularly developed plumule of rooted embryos is demonstrated (Chengalrayan *et al.*, 1997; Joshi *et al.* 2003). In contrast to that, in the present investigation TDZ was incorporated in the early embryo development medium with an objective to induce normal differentiation of the plumule during embryogenesis to obtain somatic embryos with high conversion frequency.

Embryogenic masses in form of a pair of bulge were induced in the mature zygotic embryo derived leaflets (Fig. 3.1.1) in the medium supplemented with 90.5 µM 2,4-D. On transfer to medium with lower 2,4-D (13.6 µM) with or without TDZ, somatic embryos appeared within 3-4 weeks of incubation in light. Development of bipolar embryos in more than 92 % of the explants in medium with lower (13.6 µM) 2,4-D was reported earlier (Chengalrayan *et al.* 1994). In contrast to the somatic embryos in medium devoid of TDZ (Fig.3.1.2), elongation of the embryos to form bipolar structures was restricted in presence of TDZ in majority of the cultures. Instead of the bipolar structures it often gave the appearance of fused cellular structures with meristematic protrusions on the surface in 37.6-87.65 % of the explants (Table 3.1.1). The response in embryogenic mass was optimum (87.65%) in medium with 9.08 µM TDZ and reduced with increasing concentration. In medium with 45.41 µM TDZ the response was only 37.6%. The data was subjected to statistical analysis and ANOVA was significant.

Among the responding explants two types of morphological structures were noted. Often both types of structures were seen in the same explant. In some of the structures the poles of the embryos could not be distinguished and gave the appearance of single/multiple lobe/s. In the rest of the cultures though the elongation was restricted but the abnormal somatic embryos with fused cotyledons could be distinguished. Both these types of structures were with or without morphogenic structures on the surface.

Fig. 3.1.1. Mature zygotic embryo derived leaflet of peanut with Embryogenic Mass developed in 90.5 µM 2,4-D (Bar = 500 µm).

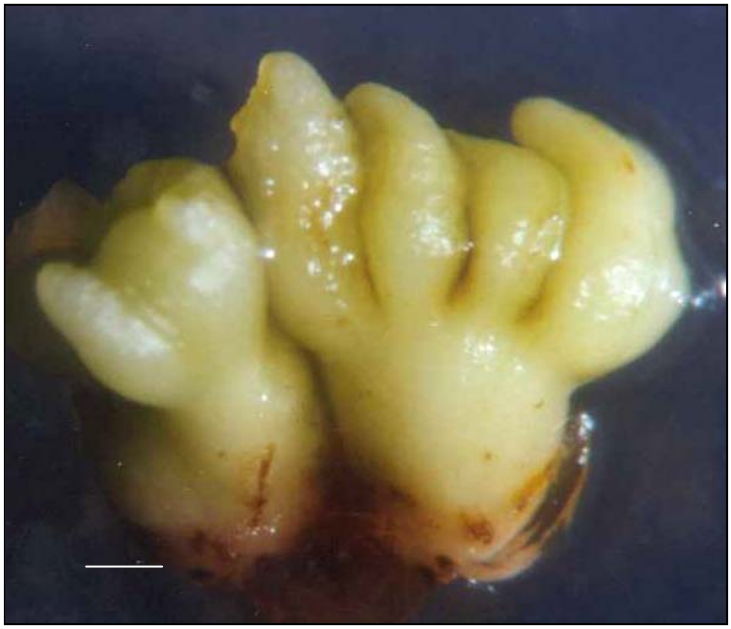


Fig. 3.1.2. embryogenic mass with developing somatic embryos in 13.6 µM 2,4-D (Bar = 500 µm).

Formation of multiple globular structures was noted at varying frequencies in all concentrations of TDZ. It ranged between 60.86 to 79.29 % (Table 3.1.1). In the higher concentration of TDZ (45.41 µM) often the number of lobes formed were more. However the variation in response with increase in TDZ was not significant statistically. The newly developed lobes were greenish in color and distinct (Fig.3.1.3). Formation of morphogenic protrusions was noted on the surface of most of these lobes. In some of the structures the morphogenetic protrusions were around the middle/ equatorial region. In others they were scattered in the plumule.

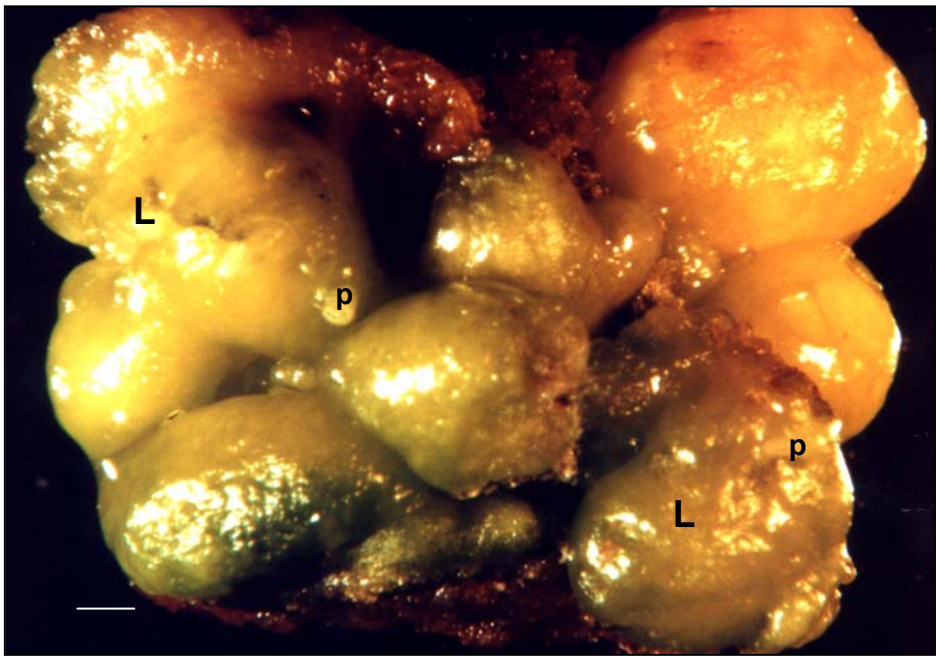


Fig. 3.1.3. Globular structures developed in medium containing 2, 4-D (13. 57 µM) and TDZ (45.41µM) giving appearance of multiple lobes (L). Morphogenic protrusions (p) appeared from lobes (Bar = 250 µm).

On incubation for 3-4 weeks, in lower concentrations (2.27 µM) of TDZ, single row of small protrusions appeared around the equatorial region (Fig 3.1.4) of the 90% cultures (Table 3.1.1). In higher concentration of TDZ the site specific origin could be noted briefly in 14 % of the cultures after 3 weeks of incubation. On further incubation followed upto 8 weeks more rows of structures appeared below the initial row of structures (Fig 3.1.5). These events were relatively slow and could be followed stepwise in the cultures grown in 2.27 µM TDZ. In the rest of the cultures the protrusions appeared simultaneously all over the plumule part of the embryos. With the increase in concentration of TDZ this site specific response was less obvious as these protrusions were dense and scattered in the plumular half of the embryos (Fig. 3.1.6). At higher concentration the response was rapid and the rows could not be distinguished as the projections were diversely distributed in the apical half of the

embryos. The growth and differentiation of the meristematic buds were asynchronous. Some of the structures resembled cup shaped and heart shaped somatic embryos (Fig 3.1.7).

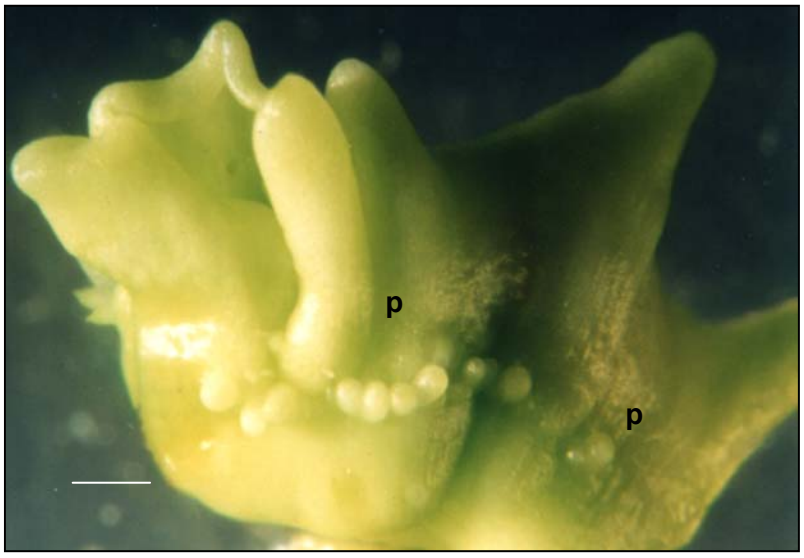


Fig 3.1.4. Row of protrusions (p) in equatorial region of developing somatic Embryos appeared in TDZ (2.27 µM) after 3-4 weeks (Bar = 500 µm).

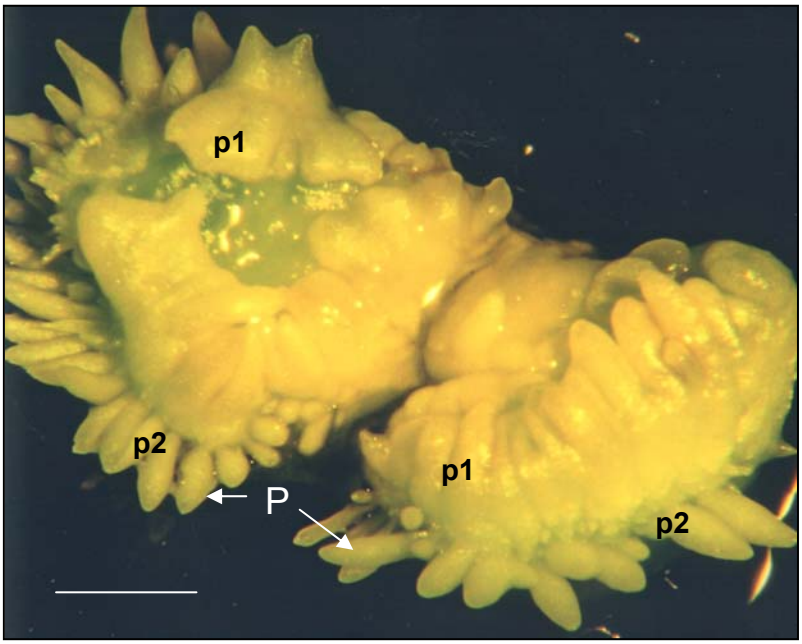


Fig. 3.1.5. Rows of protrusions (P) after 8 weeks in 2,4-D and TDZ. p1: Row of structures appeared first; p2: Row of structures appeared on longer incubation (Bar = 500 µm).

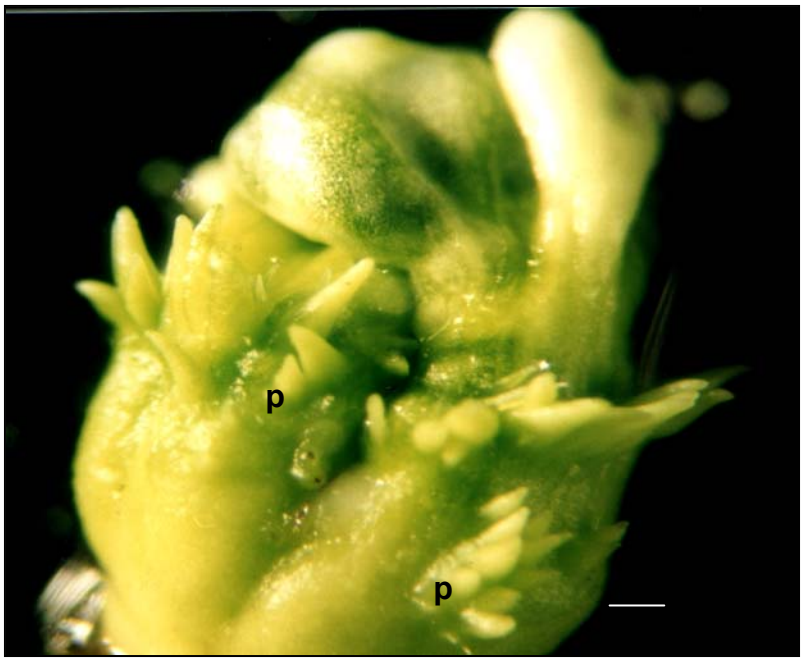


Fig. 3.1.6. Morphogenic protrusions (p) scattered in the plumular half of the developing somatic embryos seen predominantly in higher conc. of TDZ (13.62 – 45.41 µM) (Bar = 500 µm).

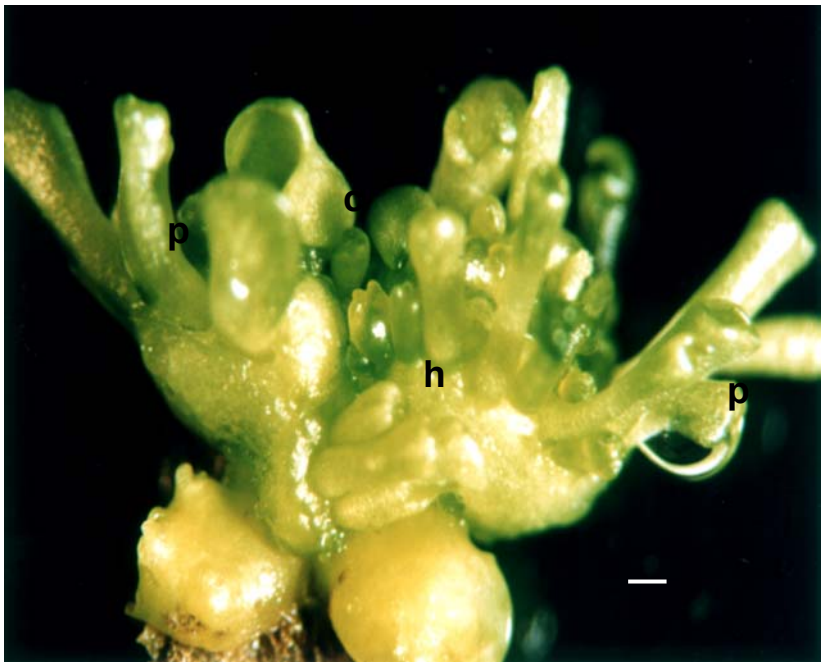


Fig. 3.1.7. Asynchronous growth and differentiation of meristematic buds (p) after 8 weeks of incubation in medium containing 2,4-D and TDZ. Projections resembling cup (c) and heart (h) shaped somatic embryos (Bar = 500 µm).

TDZ was found ineffective in supporting the normal plumule development in the primary embryo, but induced morphogenic activity around the equatorial region of the embryos. Induction of

bud primordias in the specific site indicates the site-specific activity of TDZ. Induction of shoot primirdia in peanut seedling was noted by Kanyand *et al.* (1994). It was suggested that this initiation was close to the cotyledonary nodal region. They also found that nodal region in peanut is the most regenerative part of the cotyledon and possibly contains pre-existing meristems. Similar observation was also made by McKently *et al.* (1991). Even for induction of somatic embryogenesis, Murthy *et al.* (1995) suggested that the primary site of TDZ action is in the cotyledons and the growth regulating compounds produced in the cotyledons may be transported to the other tissues. Kanyand *et al.* (1997) observed the initiation of adventitious shoots called as emergence, were restricted to specific locations on the plant and the number of emergences is significantly different across sites. In contrast to these earlier observations, in the present study TDZ induced meristematic bud formation was observed on the surface of the somatic embryos which forms the outer surface of the cotyledon, on development of the embryos of the cotyledonary stage. Unlike the restricted response in the cotyledonary nodal region, the meristematic activity is noted all round the equatorial region.

The nature of TDZ to proliferate and multiply the existing meristematic zone and perform 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. In some reports, TDZ was suspected to promote regulated morphogenesis in plants through the modulation of endogenous cytokinin and auxin (Gill and Saxena, 1992; Capelle *et al.* 1983; Thomas and Katterman, 1986).

The embryos with bud primordia were transferred to media (1) with corresponding similar combinations of 2,4-D (13.6 µM) and TDZ (2.27- 45.41 µM), (2) in media with TDZ (9.08 µM) singly and (3) in medium devoid of growth regulators. These three media were formulated keeping in view the influence of 13.6 µM 2,4-D in differentiation of peanut somatic embryos (Chengalrayan *et al.* 1994) and ability of TDZ in induction and proliferation of meristematic buds in the plemule of abnormal peanut somatic embryos (Joshi *et al.* 2003). MS basal medium supported differentiation and elongation of caulogenic buds of peanut (Chengalrayan *et al.* 1995).

The embryos with meristematic buds did not differentiate into shoots on transfer to the respective medium with 2,4-D (13.6 µM) and TDZ (corresponding conc.) supplement. In the media with lower concentrations of TDZ (2.27 to 9.08 µM) initially some new buds appeared and a few earlier buds rolled out into leaves. Subsequently dedifferentiation was noted at the origin of the bud (Fig 3.1.8). In the medium with higher TDZ meristematic buds gradually turned brown and necrotic.

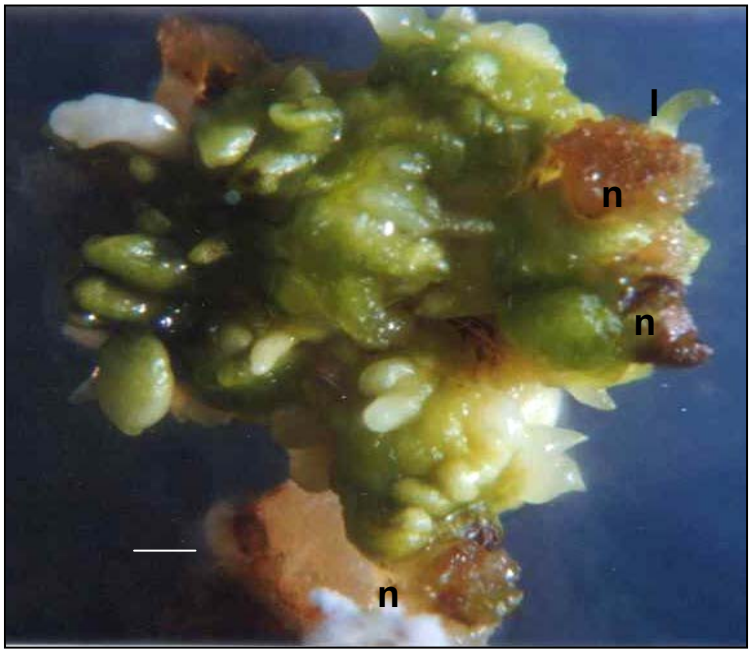


Fig. 3.1.8 Meristematic buds on incubation for 4 weeks in corresponding similar combinations of 2,4-D (13.6 µM) and TDZ (2.27- 45.41 µM). Few opened leaves (l) and necrotic (n) buds noted. Differentiation of buds restricted (Bar=1mm)

Fig. 3.1.9. Elongated tubular projections (ep) in TDZ (9.08 µM) medium after 3 weeks of incubation. Branch (b) with thin and pointed tip (Bar=1mm).

In the medium with TDZ (9.08 µM), singly the meristematic projections elongated in 3 weeks to form tubular structures (Fig 3.1.9) which subsequently differentiated into leaves. Some times

branching was noted from these structures. These branches often had thin and pointed tip. After incubation for 4 weeks these structures elongated further and tips become cup shaped. Some of those look like abnormal buds with abnormal plumule (Fig. 3.1.10) as describe earlier by Akasaka *et al.* (2000). No further development of those buds was observed. Additional structures initiated from the long projections. Some of the structures differentiated and leaves start opening after 2-3 weeks.

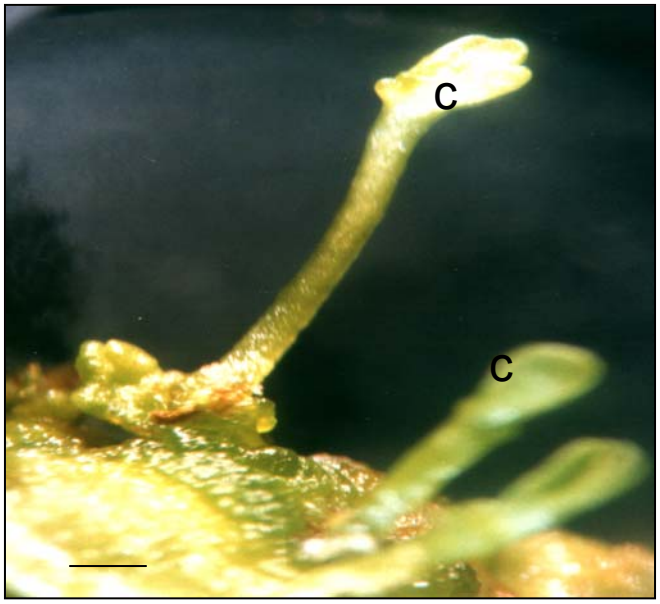


Fig. 3.1.10. Elongated tubular structures with cup (c) shaped tip in medium containing TDZ after 4 weeks (Bar = 500 µm).

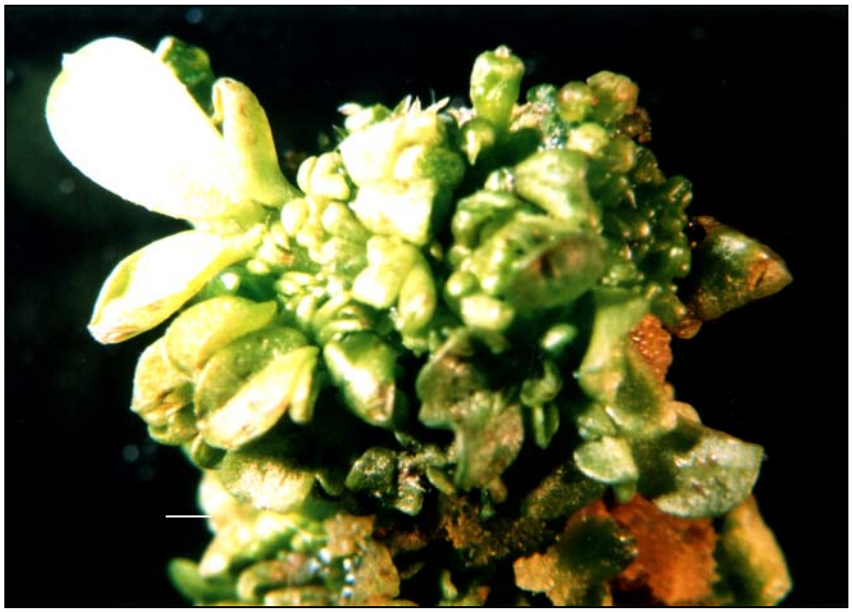


Fig. 3.1.11. Differentiation of leaves from the somatic embryo derived meristematic buds in MS basal medium devoid of growth hormones (Bar=1mm).

In MS medium differentiation of leaves occurred first (Fig 3.1.11). Subsequently in 2-3 weeks shoots differentiated. Elimination of growth regulators supported differentiation of the buds. However, development of caulogenic buds in these cultures was asynchronous. Therefore, prolific shoot differentiation was observed when the elongated shoots were removed and rest of the cluster of buds was subcultured in fresh medium in tube (Fig 3.1.12).

All the buds in the above three media differentiated to form shoots on transfer to MS basal media. After 2-3 weeks shoots elongated to 3-5 cm. were transferred to rooting medium.

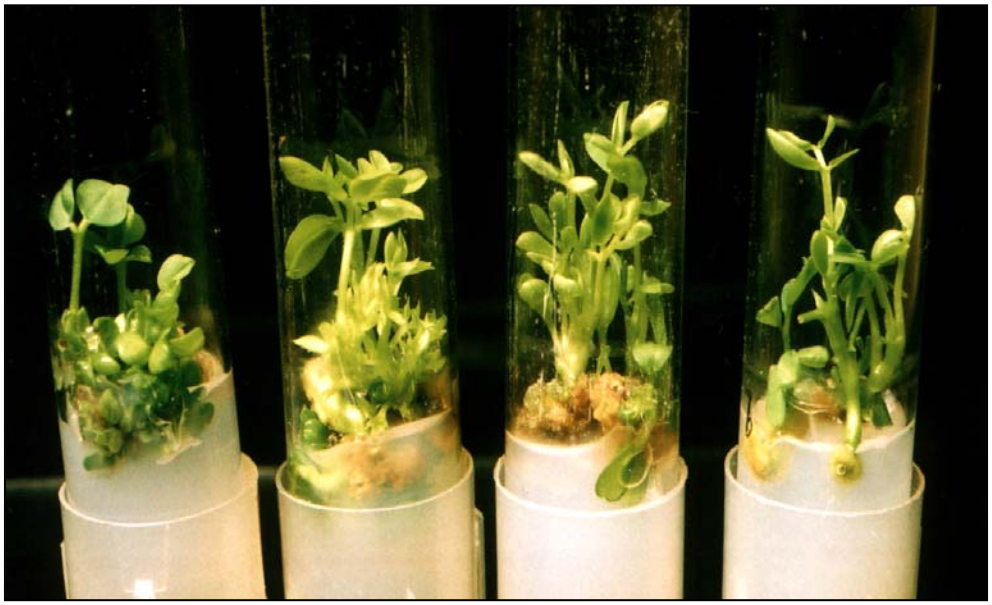


Fig. 3.1.12. Elongation and proliferation of somatic embryo derived meristematic buds in MS medium.

Plant formation and survival in soil

The shoots (3-5 cm) differentiated from the buds elongated and rooted in medium containing MS and 0.54 µM NAA within 4 weeks (Fig.3.1.13). In this medium 90 % shoots rooted. From a single somatic embryo 10-18 plantlets were developed. Those plants were transferred for hardening and 70 % plants survived (Fig.3.1.14) in the glass house.

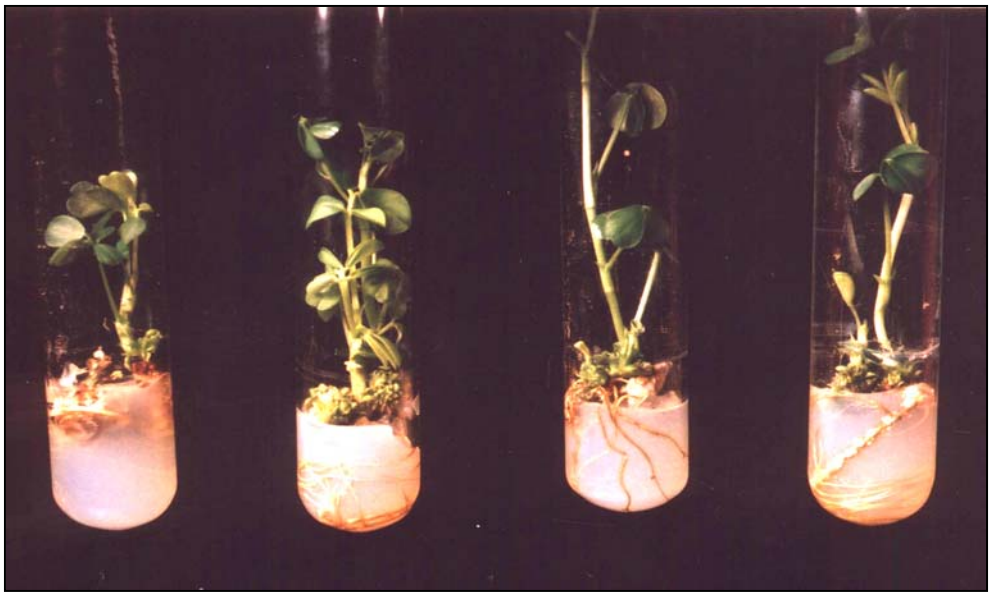


Fig. 3.1.13. TDZ induced somatic embryo derived shoots rooted in medium containing NAA (5.54 μM).

Fig. 3.1.14. Somatic embryo derived hardened plants in glasshouse.

NAA was used in our experiment to achieve optimum rooting frequency. This growth regulator is known for its influence in inducing rooting in Peanut shoots (Mckently *et al.* 1990). Earlier study

from this lab demonstrated a concentration of 0.1 mg/l (= 0.54 μM) NAA was optimum for induction of roots in 85% shoots (Chengalrayan *et al.* 1995).

In this experiment we developed a new approach for obtaining plants from somatic embryos of peanut. In this study we avoid the step of embryo conversion making the protocol more efficient for regeneration of plant from somatic embryos. The TDZ induced regeneration system appears to be repetitive, as several cycles of shoots were produced from the initial embryos and give a high number of regenerated plantlets. Such a regeneration system is ideally suited for genetic transformation because of ease in imposing antibiotic selection of transformed tissues, and *de novo* induction of shoot buds from primary somatic embryos. The site-specific activity of TDZ in somatic embryo is intriguing and very important for basic research.

Table 3.1.1 : Effect of TDZ with 2,4-D (3) on MZEDL derived embryogenic mass of peanut

medium MS + TDZ (μM)	**Response ± SD of embryogenesis with structure (%)**	**multilobule ±SD formation (%)**	**Site specific Response (%)**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
TDZ (2.27)	72.76 ± 4.27 [b]	68.86 ± 12.38	90.00 ± 7.58 [a]
TDZ (4.54)	74.54 ± 4.54 [b]	74.95 ± 13.68	69.39 ± 6.31 [b]
TDZ (9.08)	87.65 ± 3.85 [a]	78.33 ± 15.75	68.28 ± 18.11 [b]
TDZ (13.62)	45.83 ± 5.22 [c d]	79.29 ± 13.08	61.33 ± 6.88 [b]
TDZ (22.71)	55.84 ± 11.99 [c]	78.95 ± 14.82	29.00 ± 5.05 [c]
TDZ (45.41)	37.60 ± 6.07 [d]	60.86 ± 11.32	14.00 ± 19.22 [c]
ANOVA	S 1 %	NS	S 1%

***** Similar letters indicate data are at par; S 1%: Significant at 1%; NS: Non- Significant

3.2. Responses of Peanut Somatic Embryos to Thidiazuron

In peanut (*Arachis hypogaea* L), several protocols for somatic embryogenesis have been developed, but plant recovery has been limited (Ozias-Akins 1989, Mckently 1991, Baker and Wetzstein 1992, Durham and parrot 1992, Ozias-Akins *et al.* 1992b, Wetzstein and Baker 1993, Eapen *et al.* 1993, Reddy and Reddy 1993, Chengalrayan *et al.* 1994, Mckently 1995, Sabitha Rani and Reddy 1996). To increase the frequency of plant recovery attempts have been made to obtain normal somatic embryos by manipulating the culture medium prior to germination (Ozias-Akins *et al.* 1992b) by modifying the germinating medium (Trigiano *et al.* 1988), or by exposing the embryos to the germination medium and subsequently by triggering shoot formation from the plumule of rooted embryos in presence of kinetin (KIN) and benzylaminopurine (BAP) (Chengalrayan *et al.* 1995). Somatic embryo conversion was also achieved in presence of BAP and naphthalene acetic acid (NAA) (Venkatachalam *et al.* 1997, Venkatachalam *et al.* 1999b). In 2,4-dichlorophenoxy acetic acid (2,4-D) or NAA thidiazuron (TDZ), was effective in inducing differentiation in the plumule of the somatic embryos. Multiple shoot emergences were observed in 92 % of the rooted somatic embryos in eight weeks (Chengalrayan *et al.* 1997). However, the pathway of morphogenesis of multiple shoots in presence of TDZ remained unclear.

In previous experiments (Chengalrayan *et al.* 1997) attempt to trigger plumule differentiation in abnormal but rooted, somatic embryos of peanut, multiple shoots were induced in presence of 22.7 μM TDZ. Present experiment was designed to define whether the multiple shoot formation was due to (1) differentiation of the meristems of the fasciad embryos or (2) induction and proliferation of the shoot meristem, or (3) induction and subsequent differentiation of secondary embryos.

Materials and methods

The rooted somatic embryos obtained from mature zygotic embryo derived leaflets were subjected to 22.7 μM TDZ for shoot development following the method described (Chengalrayan *et al.* 1997). The early events at the plumular end of somatic embryos were studied microscopically. The differentiated structures developed from the plumule end of the rooted somatic embryos were exposed to three media combinations optimized earlier to achieve specific differentiation (Chengalrayan *et al.* 1995). The response of these structures in these media was studied closely.

Explants and culture conditions

Mature pods of a high yielding cultivar of peanut (*Arachis hypogaea* L.cv. JL-24) were collected from the Agriculture College, Pune, India. Embryo axes were surface sterilized as described in Materials and Method (section 2.6). Immature leaflets were dissected from swollen embryo axes and were used as explants. Somatic embryos were obtained from these leaflets using the method described by Chengalrayan *et al.* (1994). In brief, the leaflets were cultured in Murashige and Skoog's (MS) basal medium (Murashige and Skoog 1962) supplemented with 90.5 μM 2,4-D and 0.18 M sucrose for induction of embryogenic masses. Somatic embryos were developed from these masses in MS medium with 13.6 μM 2,4-D and 0.18 M sucrose. These were rooted in MS basal medium containing 0.06 M sucrose. The rooted embryos were transferred to MS medium supplemented with 22.7 μM TDZ and 0.06 M sucrose for conversion to plantlets (Chengalrayan *et al.* 1997). After incubation for 7 d the cultures were studied under the microscope.

After incubation for 10-12 d in same medium the rooted embryos were randomly divided into four groups. One group was retained in the conversion medium (MS+ 22.7 μM TDZ and 2 % sucrose) for further incubation of four weeks. The upper 4 – 6 mm part of embryos (i.e. plumule) in other three groups was isolated and cultured in three different media: (1) MS + 0.06 M sucrose; (2) MS + BAP 2.22 μ M + KIN 2.32 μ M + 0.06 M sucrose; (3) MS + BAP 13.32 μM + 0.06 M sucrose. The pH of all the media was adjusted to 5.8 and media were solidified with 0.6 % agar. The cultures were incubated at 25 ± 2°C in irradiance of 32 μmol $m^{-2} \cdot s^{-1,}$ 16 h photoperiod for 4 weeks. The developmental changes at the plumule were noted in all the groups. The experiment was repeated three times with 20 - 30 explants in each medium.

Histology

The morphology of the structures in the plumule of the rooted somatic embryos was studied at various stages of development. The plumule end (3 – 5 mm) were isolated after 7 d, 21d and 28d in TDZ medium and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol: 5: 5: 90, v/v) for 48 h at room temperature. The tissues were dehydrated using graded concentrations of Tertiary Butyl Alcohol and embedded in paraffin using the procedure described (Sharma and Sharma 1980). Serial sections of 10 μm, were cut using a *Reichert - Jung 2050* (Wien Austria) rotary microtome. Sections were double stained with haematoxylin - eosin and mounted with DPX (Loba chemie, Mumbai, India).

Results and Discussion

The objective of the present investigation was to identify the structures, which appear in the plumule end of the rooted somatic embryos of peanut on exposure to TDZ containing medium. Microscopic examination after 7 days incubation revealed that often the cotyledons were fused and expanded to form a cup shaped structure. Small nodule like projections appeared from the central depression of cup shaped structure indicating that the structures develop from the plumule of the somatic embryos. Due to their position inside the depression, the origin of these was not clearly visible under stereo microscope. From histological studies (Fig. 3.2.1) it is apparent that instead of a single meristematic dome, there are several caulogenic buds. TDZ induces proliferation of the meristematic cells in the plumule of the somatic embryos. This results in formation of a meristematic zone (mz) spread over between the cotyledons. Several meristematic buds (mb) appear from this zone. Proliferation of meristem in response to TDZ was noted in *Mateuccia struthioopteris* L. Masses resembling miniature nodules were identified as meristematic nodule (Thakur *et al.* 1998). In the present experiment extended incubation of the cultures resulted in further expansion of the cotyledons. The meristematic buds grow and elongate to form structures, which resemble somatic embryos (Fig. 3.2.2 A, B).

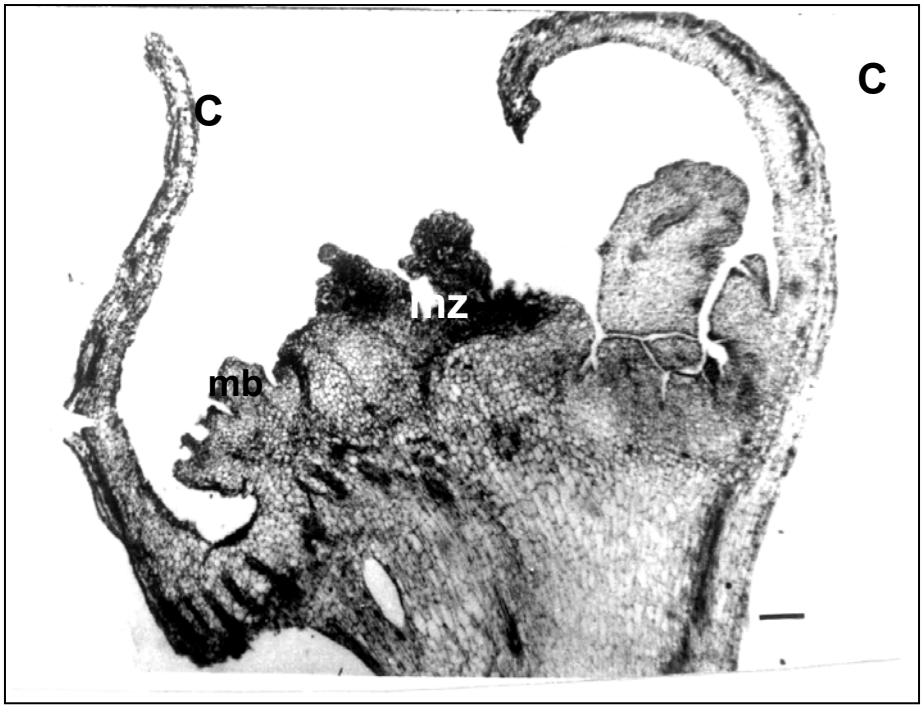
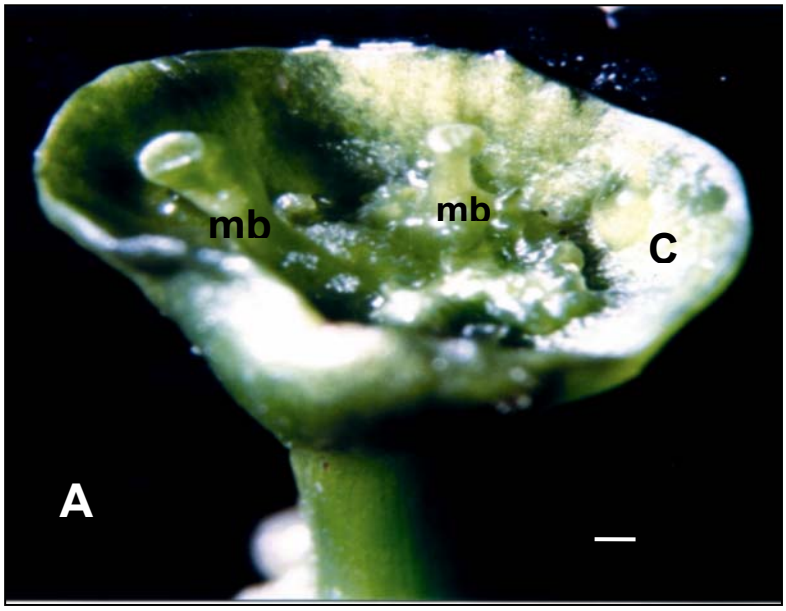


Fig. 3.2.1. Longitudinal section of somatic embryo after 7 days in TDZ (bar = 200 μm). A meristematic zone (mz) is formed due to proliferation of the cells from the plumule Surrounded by the cotyledons (c). Meristematic buds (mb) developed from the meristematic zone.



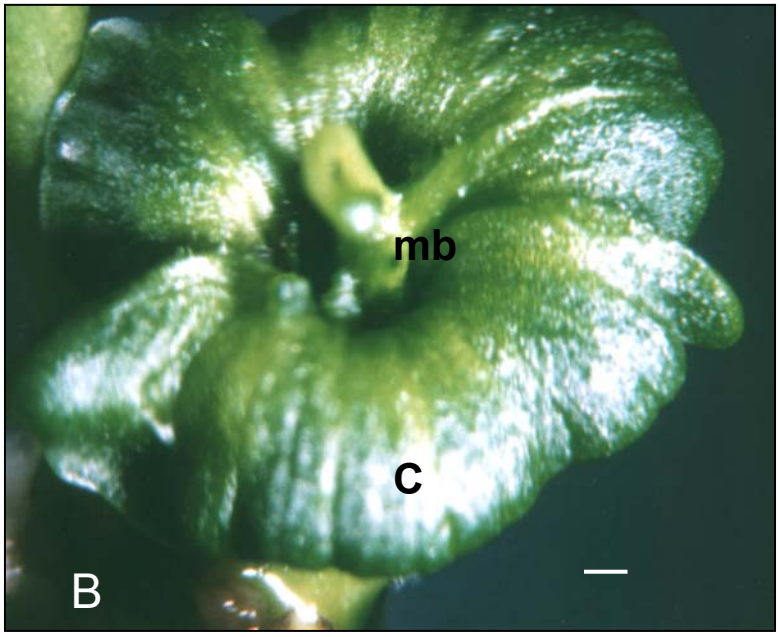


Fig. 3.2.2. "Somatic embryo" like meristematic buds (mb), appeared from central depression. A: cup shaped fused cotyledons (c) (bar = 500 μm), B: Bell shaped fused cotyledons (c) (bar = 500 μm).

Following further incubation for two weeks (total 21 days), more structures appear and elongate whereas the cotyledons expand and become fan shaped (Fig. 3.2.3). On histological examination (Fig. 3.2.4) these projections appear as meristematic buds (mb). Presumably due to rapid and uneven cell proliferation under the influence of TDZ, the meristematic center in some of the structures is shifted giving the appearance of cellular projections with meristematic patches. However

none of the projections had the characteristic morphology of the early somatic embryo of peanut (Hazra *et.al.* 1989, Chengalrayan *et al.* 2001). The morphology of somatic embryo is defined at a very early stage (Raghavan 1976, Raghavan 1986, Maheswaran and Williams 1985). Occasionally these

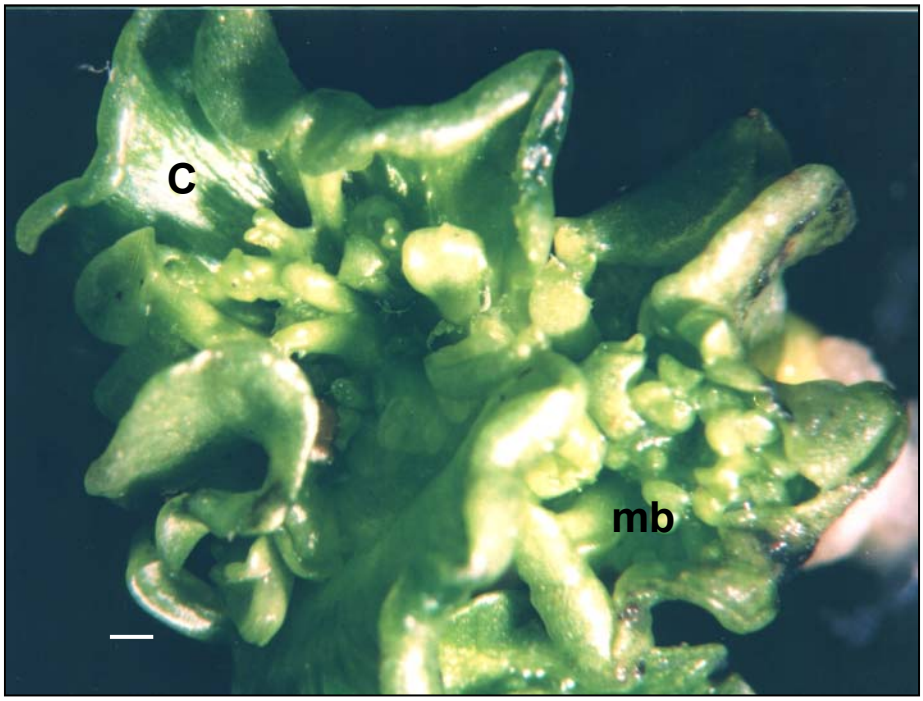


Fig. 3.2.3. Large number of meristematic buds (mb) and projections between the fan shaped cotyledons (c) after 21 days (bar = 500 μm).

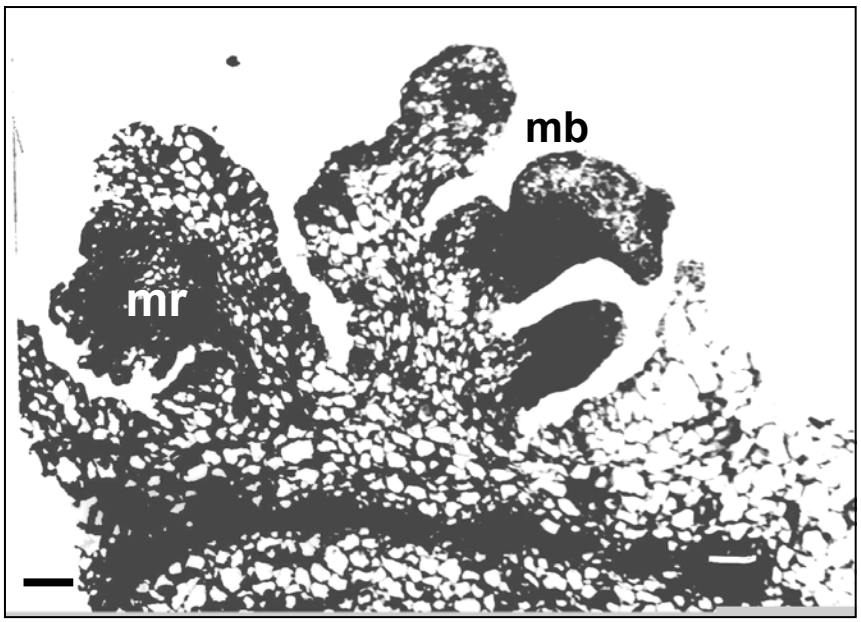


Fig. 3.2.4. Section of cluster of meristematic buds (mb). Bud with the meristematic region (mr) in one side (bar = 100 μm)

projections (Fig. 3.2.5) were similar to the structures, induced by TDZ at the cotyledonary node, petiole base, and on the rachis of the peanut leaf, and identified as “emergences” (Kanyand *et al.* 1997). Like the “emergences” these structures differentiated into shoots. In contrast to the characteristic white

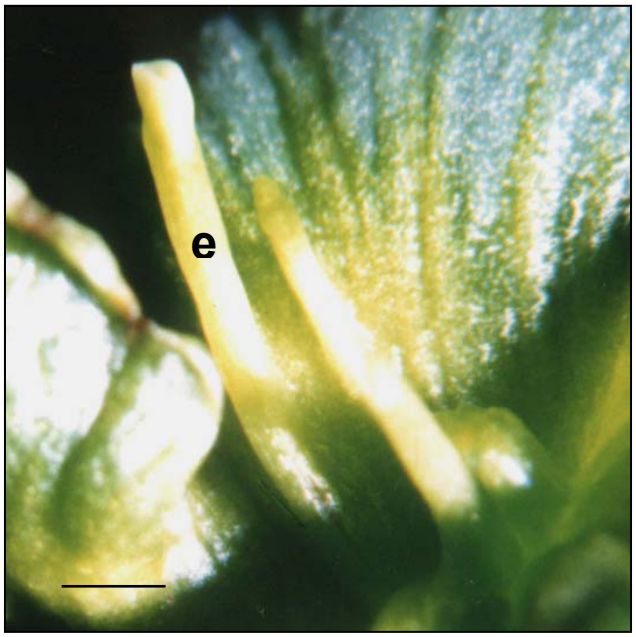


Fig. 3.2.5. Elongated meristematic buds appear as ‘emergences’ (e)

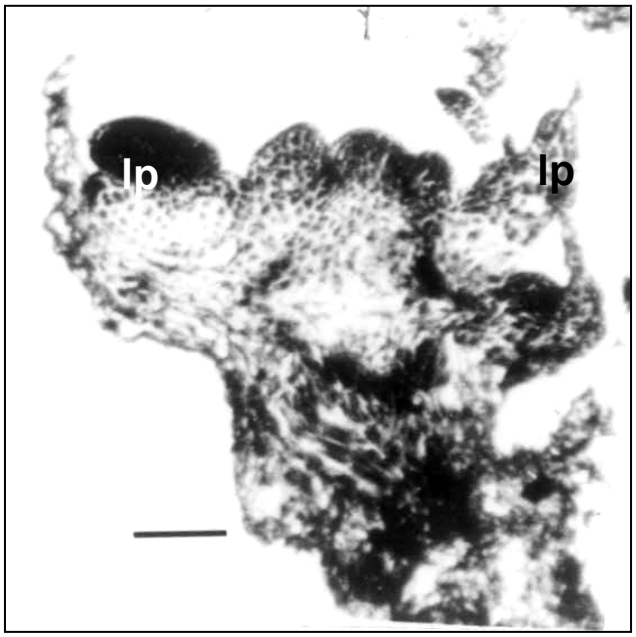


Fig. 3.2.6. Stunted shoot with multiple leaf primordia (lp) (bar = 100 μm). Differentiation of the internodal region between the leaves, restricted.

somatic embryos of peanut, all the structures developed in presence of TDZ in the present study were bright green from the tip to base. Unlike somatic embryos these were inseparable either individually or in cluster from each other or from the explant without injuring the base. All these evidences indicate that the structures developed from the plumule are not somatic embryos but projections with meristematic activity, which eventually differentiate to form shoots.

Similar to our earlier observation (Chengalrayan *et al.* 1997) the projections with meristematic activity (mb) differentiated to form cluster of small shoots on incubation of the rooted somatic embryos for an extended period of four weeks in the same medium containing TDZ. The root differentiation of the embryo was restricted. The shoot buds elongated to a limited extent and the leaves opened. Histological studies conducted after 28 d of culture shows development of stunted shoot (Fig. 3.2.6) due to restricted differentiation of the internodal region. Several such shoots appeared from the plumule of each somatic embryo. Thus the plantlets (Fig. 3.2.7A) with stunted shoots and roots (Chengalrayan *et al.* 1997) are formed.

The projections (Fig. 3.2.2 and 3.2.3), originated at the plumule of the rooted somatic embryos resembled the structures described earlier as somatic embryos (Gill and Saxena 1992, Victor *et al.* 1999 b). The responses of the "somatic embryo like" structures in the three media (Table 3.2.1), indicated that these are organogenic buds and not somatic embryos. In the PGR free medium (i) in which, somatic embryos of peanut cultivar JL-24 germinate to form root (Chengalrayan *et al.* 1994), the TDZ induced structures failed to develop roots. On the contrary in 41% of the explants the structures differentiated into leaves (Fig. 3.2.7 B). In medium (ii) containing MS + BAP 2.22 μ M + KIN 2.32 μ M + 0.06 M sucrose, 54% of the "somatic embryo like" caulogenic buds differentiated into shoots of varying height (Fig. 3.2.7 C). This is in concurrence with our earlier report describing shoot elongation in the rooted embryos following transfer to this medium (Chengalrayan *et al.* 1994). In medium (iii) containing BAP 13.32 μM the plumule with the structures converted into a dense mass (Fig. 3.2.7 D) of caulogenic buds. In our earlier study we demonstrated the efficiency of this medium in proliferation of caulogenic buds to form large number of buds (Chengalrayan *et al.* 1995).

Table 3.2.1 : Morphogenic responses in the TDZ induced somatic embryo like structures in the plumule of peanut somatic embryos in various optimized media

Media	Optimized for	No. of explants Tested	Response observed	Frequency of response in percent ± SD
DZ (22.7µm)	• Shoot induction in plumule of somatic embryos	83	Multiple shoots	79.21 ± 9.65
MS	** Rooting of somatic embryos/	59	No rooting, leaves	40.66 ± 3.78
BAP (2.22µm)+ KIN(2.32µm)	** Differentiation and elongation of shoots	61	Differentiation and elongation of shoots	54.29 ± 11.09
BAP (13.32µm)	** Caulogenic bud proliferation	58	Caulogenic bud proliferation	89.63 ± 0.64

* Chengalrayan *et al.* (1997); ** Chengalrayan *et al.* (1995)

It is apparent from the above evidences that TDZ triggers meristematic activity at the plumule of peanut somatic embryos. This result in formation of caulogenic buds, which resemble the structures, identified as somatic embryos (Gill and Saxena 1992, Victor *et al.* 1999 b). These buds differentiate to form shoots. The shoots are stunted due to restricted elongation of the internodal region. Therefore high frequency conversion of abnormal peanut somatic embryos (Chengalrayan *et al.* 1997) is due to TDZ aided caulogenic activity at the plumule.

In peanut tissue we used both adenine type cytokinins (KIN and BAP) and TDZ to induce caulogenic buds. Unlike the organogenic buds induced in presence of cytokinins, the bud induced by TDZ resembles the somatic embryos. Similar observation is reported by other researcher (Akasaka *et al.* 2000). Induction of direct somatic embryogenesis from the hypocotyl of intact seedlings of peanut in presence of 10 µM TDZ has been reported (Murthy *et al.* 1995, Gill and Saxena 1992). This is

contradictory to the report describing induction of caulogenesis in the hypocotyl tissue of seedling (Li *et al.* 1994) in presence of similar concentration TDZ and following the same method.

Keeping in view the earlier reports and on the basis of our observations we propose that TDZ induce organogenesis in peanut tissues and not somatic embryogenesis.

Fig. 3.2.7. Response of the meristematic buds in the plumule of the rooted somatic embryos in various culture conditions optimized for specific response:
A) Plantlet with stunted multiple shoots (ms) developed in TDZ 22.7 μM medium
B) Shoot induction and elongation in the MS medium containing BAP 2.22 μM and kinetin 2.32 μM;
C) Leaves differentiated from the meristematic buds cultured in medium devoid of growth regulator;
D) Proliferation of caulogenic buds in medium with BAP 13.32 μM.

Conclusion

The major snag in peanut somatic embryogenesis is its very low conversion frequency of somatic embryos to a normal plant. Though several attempts have been made to obtain normal somatic embryos and improve the frequency of plant recovery in peanut, the information is still limited

and obscure. This study has demonstrated two pathways of plant regeneration from somatic embryos using TDZ. Incorporation of TDZ in the early stage of embryo development induced caulogenic buds on the surface of the developing somatic embryos. Experimental results narrated efficacy of TDZ in the induction of multiple buds in a specific site of somatic embryos at lower concentration (2.27 μM). Multiple globular structures appeared when the explants were exposed to 9.08-22.71 μM of TDZ. Shoot buds appeared on these multiple globular structures. Shoots developed from these buds in MS basal medium were rooted in 0.54 μM NAA to obtain plantlets. Ten to 18 plants were obtained from a single embryo. The site-specific action of lower concentration (2.27 μM) of TDZ envisages interesting basic studies to understand the mechanism of action of TDZ. Higher number of plantlet recovery through this protocol dictates its utility in genetic transformation studies. The unusual projections morphologically simulating somatic embryos gave rise to shoots.

Influence of TDZ is at the plumule when the somatic embryos were exposed to this growth regulator at a very late developmental stage i.e. after rooting. On exposure to TDZ (22.7 μM), projections resembling somatic embryos appeared from the plumule. Histological examination revealed that these are caulogenic buds and not somatic embryos. These buds differentiated to form shoots. Plantlets could be recovered from these shoots by inducing roots.

The target of TDZ activity varied with the developmental stage of peanut somatic embryos. It induced morphogenic buds on the surface of the embryos when incorporated at the initial stage of embryogenesis whereas it affected the plumule when fully developed somatic embryos were exposed to this growth regulator. However in both the cases caulogenic buds were induced.

This part of work has been reported in the following publication:

Responses of Peanut Somatic Embryos to Thidiazuron.

Joshi MV, Sahasrabudhe NA, and Hazra S. Biologia Plantarum, Vol. 46, (in press).

CHAPTER 4:

INDUCTION, MAINTENANCE AND REGENERATION OF EMBRYOGENIC CALLUS

Introduction

Peanut (*Arachis hypogaea* L), a rich source of oil and protein, is commercially grown throughout the world for human consumption. However culturing peanut tissue *in vitro*, remained a difficult task for peanut workers. Availability of a reliable and reproducible protocol for induction, proliferation and regeneration of transformable tissue is a standard pre-requisite for peanut transformation. In spite of several documented reports on in vitro regeneration of peanut via embryogenesis (Hazra *et al.* 1989; Ozias-Akins, 1989) and organogenesis (Li *et al.* 1994; Kanyand et al, 1994), studies on callus culture of peanut are scanty and had been least attempted (Ozias- Akins *et al.* 1993; Venkatachalam et al 1996; Little *et al.* 2000) . An efficient callus protocol, if established, will not only serve the needs of genetic transformation studies, but also prove critical for study of somaclonal variations and protoplast fusion. Callus can be regenerated via both embryogenesis and organogenesis pathway, but development of transgenic plants through somatic embryos are preferred due to their single cell origin and ability to give rise to genetically stable, nonchimeric plants (Gill and Ozias-Akins, 1999).

There are several reports on peanut somatic embryogenesis using different explants, eg. Immature zygotic embryo (Hazra *et al.* 1989; McKently 1995; Ozias -Akins *et al.* 1992b), immature cotyledon (Durham and Parrott 1992; Ozias-Akins 1989; Ozias -Akins *et al.* 1992a), leaflet from germinating seedling (Baker and Wetztein 1992), and mature zygotic embryo axes (Baker *et al.* 1995; George and Eapen 1993). However, reports on repetitive embryogenesis are rather scanty. There are several advantages in using secondary embryos as source explant in transformation. It ensures availability of regenerating tissues all the year round, and also recovery of somatic embryo from a single transgenic cell via repetitive embryogenesis. It also offers the possibility to obtain a fully transgenic embryo from a transgenic sector (Merkle et al, 1990) and reduction of chimeric events by continued repetitive embryo production under selection (Parrott *et al.* 1991).

Callus growth and organogenesis in peanut tissue culture was reported as early on 1978 by Guy *et al.* They evaluated the relative ability of three different peanut cultivars for callus induction and regeneration. The growth regulators 2,4-D, NAA and Kinetin were employed to induce callus growth from the cotyledon and amino acid analysis were carried out for callus and cotyledon tissue.

Vajranabhaiah *et al.* (1993) reported the regeneration potential of callus derived from hypocotyl tissue of groundnut cv. TMV-2. They have shown the induction of callus on medium

supplemented with NAA (2m/l) and Kinetin (0.25 mg/l) and a compact mass of green tissue was obtained when transferred to MS medium containing IAA (0.25 mg/l) and Kinetin (2.5 mg/l). Embryoids and plantlets appeared within 20-25 days.

Ilahi I (1993) reported callus initiation from shoot apices of groundnut variety NC7 on medium containing BA (1mg/l) and NAA (0.01mg/l). They achieved regeneration of callus on reduced concentrations of BA (0.5mg/l) and IAA (0.1mg/l), and 10 plants/callus were recovered.

A thorough study for callus induction and morphogenesis of different groundnut explants were reported by Venkatachalam *et al.* (1996). Immature leaf was identified as the most efficient explant in producing callus in medium having NAA (3mg/l) and Kin (0.5mg/l). They reported that the calli derived from cotyledonary node was superior for shoot bud differentiation and multiple shoot formation in medium supplemented with Kin or BAP 2mg/l and 5 mg/l respectively. Rooting was achieved in 65 % of the shoots in NAA (4 mg/l).

Gill and Ozias-Akins (1999) reported morphogenic callus induction from immature cotyledons and embryo axes of peanut when cultured in 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. Shoots were obtained from these calli on medium containing 1.44 μM GA_3 and 2.32 μM KIN. Roots were induced (75%) in these shoots in medium containing 4.92 μM IBA.

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 on MS liquid medium containing 40 mg/l 2,4-D. Approximately 4% embryo conversion frequency was obtained in MS basal medium.

Somatic embryogenesis and embryogenic callus formation was demonstrated by Ozias-Akins *et al.* (1992a) from immature cotyledon and embryo axis of seven peanut cultivars. The percentage of cultures forming shoots was highest (71 %) for cultivar Tifrun in MS basal medium containing 25 mg/l BAP followed by incubation in plain MS salt.

These researchers reported (Ozias-Akins *et al.* 1993) embryogenesis and repetitive embryogenesis in peanut using immature embryos as explant and 0.5 mg/l picloram with 3% sucrose as initiation medium. Embryogenic callus was maintained in MS medium supplemented with 3 mg/l

picloram with 3% sucrose and 1 g/l filter-sterilized glutamine. A three step regeneration protocol was followed to obtain plantlets from embryogenic cultures. Somatic embryos were transferred onto MS medium containing either 1 mg/l NAA or 0.5 mg/l BAP + 0.5 mg/l Kinetin + 0.5 mg/l zeatin + 0.1 mg/l NAA + 500 mg/l casein hydrolysate and incubated for 4 weeks. In second step the embryos were transferred to B5 medium supplemented with 0.1 mg/l BAP, 0.1 mg/l NAA and 25 μM $AgNO_3$. Shoot elongation was obtained in next 4 weeks on MS + 2% sucrose + 3 mg/l BAP + 1 mg/l gibberellic acid (GA_3).

Baker and Wetzstein (1995) reported primary as well as repetitive embryogenesis from immature cotyledon of peanut in 30 or 40 mg/l 2,4-D medium. They obtained 90 % primary and 41-46 % repetitive embryogenesis and maintained the viability of secondary embryogenesis for over one year in the medium containing 20 mg/l 2,4-D. Conversion of those secondary embryos were not described.

Livingstone and Birch (1999) reported induction of embryogenic callus from mature embryo axes in medium containing 3 μg/ml picloram. Number of somatic embryos formed per zygotic embryo axis derived callus was 6.3 for cv. Gajah and 14.3 for cv. NC-7. They demonstrated a 22% regeneration rate for Gajah and 30% for NC-7 in medium supplemented with 10 μg/ml BAP. Four hours osmotic treatment on medium containing 0.2 M d-sorbitol and 0.2 M d-mannitol was found effective in improving the regeneration frequency upto 60 % for genotype NC-7. NAA 1 μg/ml was used for rooting.

A comprehensive study on effect of different auxins on peanut embryogenesis, secondary embryos, morphology of embryos and their conversion rate, was reported by Little *et al.* (2000). Picloram (83.0 μM) and Centrophenoxine (124.4 μM) were optimum for induction and conversion of repetitive embryos from epicotyl of mature embryo axes. Among the 4 genotypes tested (AT120, 59-4144, GK7 and VC1), the combination of genotype VC1 and picloram 83.0 μM, give rise to highest average number of mature embryos / mg of tissue. The conversion frequency of these embryos was 59.5 %.

Experiments conducted with an Indian cultivar JL-24 of peanut to formulate appropriate culture condition for induction, maintenance and regeneration of embryogenic callus are described in this chapter of the thesis. The conversion rate of the secondary embryo is also evaluated. This protocol, offers the possibility of using callus as well as somatic embryos as initial explant throughout the year for application of biotechnological approaches in improvement of peanut.

Materials and Methods

Collection of plant materials and surface sterilization procedure are described in Chapter II, 2.6 (Materials and Method).

Induction of Callus

Effect of picloram concentrations and Culture conditions:

Mature zygotic embryo derived leaflets were cultured for induction of morphogenic callus in MS basal medium supplemented with varied combinations of picloram including 2.07, 4.14, 12.42, 20.71 and 41.41 µM with 0.09 M sucrose. Medium pH was adjusted to 5.8 before autoclaving and the media were solidified with 0.6% agar (Qaligens fine chemicals, Mumbai).

20-25 leaflets were cultured in each petri dish (85 X 15 mm) containing 20 ml of medium. This PGR has been used earlier for induction of embryogenic callus from immature zygotic embryos of peanut (Ozias-Akins *et al.* 1993). For each treatment cultures were incubated in two incubation conditions. Five petri dishes containing 100-120 explants were cultured in dark and 5 petri dishes in light, in each replication. There were three replications in each experiment.

All cultures were incubated at 25 ± 2 ºC temperature. For light incubation, cultures were incubated under white fluorescent light 32 µMol. m^{-2}. s^{-1} with 16 h photoperiod.

Data were noted after 4 weeks. Number of explants showing induction of callus was counted. Experiment was repeated three times. Percent of response in each experiment was determined. Mean and standard deviation was calculated. The data was subjected to statistical analysis.

Effect of picloram and sucrose concentrations*:*

In the subsequent experiments three concentrations of picloram used were 2.07, 4.14, 12.42 µM. Sucrose 0.06. 0.09, 0.18 and 0.29 M was supplemented in medium containing picloram at each of the three concentrations. In 85 mm petri dish containing 20 ml of medium 20-25 explants were cultured. No. of leaflets showing appearance of embryogenic callus was scored after 4 weeks. Data were scored from 4-5 petri dishes in each replication and there were three replicates for each experiment. Every experiment was repeated two times.

The callus was maintained by subculturing at interval of 4 weeks in MS basal medium with picloram 4.14 µM and sucrose 0.06 M. All media were solidified with 0.6% agar (Qualigens fine chemicals, Mumbai) and the medium pH was adjusted to 5.8 before autoclaving. Cultures were incubated at 25 ± 2 ºC temperature and in dark.

Maintenance of embryogenic culture and secondary embryogenesis

Effect of picloram:

At the time of subculture every 4 weeks, clusters of globular embryos and mass of embryogenic callus were isolated selectively from the loose watery callus and transferred to fresh medium for further proliferation. This was continued for 3-4 passages of 4 weeks each till sufficient embryonic culture was available for experiments towards further refinement of the protocol for improved repetitive embryogenesis. The culture with predominantly somatic embryos is identified as embryonic culture. Different picloram concentrations (4.41, 12.42, 20.71 and 41.41 µM) in MS and 0.06 M sucrose were tested. In each petri dish (85 mm) containing 20 ml medium, 100 ± 5 mg embryonic culture was transferred as inoculum. Three petridishes were used for each treatment in each replication. The experiment was repeated three times.

The cultures of the present experiment and subsequent experiments were incubated in dark at 25 ± 2 ºC temperature unless otherwise mentioned. Similarly all the media were solidified with 0.6% agar. Media pH was adjusted to 5.8 before autoclaving.

The clusters of embryonic culture were transferred to sterile filter paper prior to weighing for absorption of adhering moisture. Approximately 100 ± 5 mg tissue was used as inoculam. Fresh weights were determined aseptically after incubation of 4 weeks in each passage. This was continued for three subcultures. Increase in fresh weight was determined from the difference between initial and final weight. Percent increase in fresh weight was calculated and was subjected to statistical analysis.

Effect of carbohydrates:

Carbohydrates like sucrose, fructose, glucose and maltose were tested at 0.06 M in MS supplemented with 12.42 µM picloram. In petri dish (85 mm) containing 20 ml medium, 100 ± 5 mg, embryonic tissue was cultured. For each combination three petri dishes were used in each replication. Every experiment was repeated three times with three replications.

Fresh weights of tissue were determined every 4 weeks prior to subculturing at 4, 8 and 12 weeks. Transferring the tissue briefly on sterilized filter paper prior to weighing it aseptically absorbed adhering moisture. Increase in fresh weight was estimated and expressed in percentage. The data was subjected to statistical analysis.

Effect of Glutamine:

For maintenance of repetitive embryogenesis 6.85 mM filter sterilized glutamine was supplemented in the medium as reported by Ozias-Akins et al (1993). The embryonic Callus (100 ± 5 mg) was cultured in petri dishes (85mm) containing 20 ml of MS basal medium supplemented with 12.42 µM picloram and 0.06 M sucrose. Medium without glutamine was used as control. Six plates were cultured for each medium in each replication. Experiment was repeated twice with two replications.

Callus fresh weight was determined aseptically on 4^{th} and 8^{th} week prior to subculturing. Difference in fresh weight was calculated as percent increase and subjected to statistical analysis.

Conversion of repetitive embryos

Morphologically well differentiated bipolar embryos with cotyledons from the solid cultures were selected for germination. Keeping in view the influence of NAA (1mg/l = 5.37 µM) for rooting of the peanut somatic embryos (Ozias-Akins *et al.* 1993; Livingstone & Birch, 1999) clusters (10-12 No.) having mainly the elongated embryos were cultured on 20 ml of MS basal salts supplemented with NAA (5.37 µM) and 0.06 M sucrose in 85 mm Petri dishes. For the first 3 weeks the embryos were incubated in dark followed by 1 week in light. The pH of the medium was adjusted to 5.8 and agar 0.6% was used as gelling agent.

The rooted embryos were separated from cluster and transferred on MS medium supplemented with 0.06 M sucrose and various concentration of BAP (4.44, 8.88 and 13.32 µM) or TDZ (4.54, 9.08 and 13.62 µM) singly. Single or two embryos were placed in each test tube containing 20 ml of medium and 10 test tubes were kept for each treatment in each replication. Cultures were incubated at 25 ± 2 ºC under white fluorescent light 32 µMol. m^{-2}. s^{-1} with 16 h photoperiod. Each embryo was counted as one explant.

Numbers of rooted embryos with elongated shoots were scored after 4 weeks and means were calculated from three replications. Experiments were repeated two times. Frequency of conversion was

calculated from the number of plantlets obtained to the number of embryos cultured and subjected to statistical analysis. The morphology of the plantlets was noted.

Regenerated plants showing stunted growth were transferred to MS basal medium with 0.06 M sucrose in test tube and cultured for further 3-4 weeks.

The statistical analysis for all the experiments were done as described in Chapter II, 2.8 (Materials and Method).

Results and Discussion

Induction of morphogenic callus

Effect of picloram concentrations with culture condition:

Ozias-Akins *et al.* (1993) demonstrated efficacy of picloram in induction of embryogenic callus from immature embryos of peanut cultivars Toalson and Florunner. In the present investigation mature zygotic embryo derived leaflets of an Indian cultivar JL-24 were used as explant for induction of embryogenic callus. In concurrence with the observation noted in immature embryos of peanut by Ozias-Akins *et al.* (1993), picloram was effective in inducing callus in the mature zygotic embryo derived leaflets too. Frequency of callusing response varied significantly with concentration of picloram. In all the concentrations, callusing frequency was higher in cultures incubated in dark compared to their counterpart incubated in light. The response was optimum in medium with 12.42 µM picloram (Table 4.1) in dark incubation. At higher concentration 20.71 µM the response was reduced. At 41.41 µM the response was least and the callus was hard and dry. The dissection of MZEDL was important for induction of callus. The cut edge in leaf base was essential for callus induction. Importance of cut in the leaf for somatic embryogenesis in seedling derived leaflets of peanut was also emphasized by Baker and Wetzstein (1992).

Callus obtained from medium containing 12.42µM picloram was compact, translucent and cream in color (Fig.4.1). Glossy embryogenic masses and globular embryos appeared from this calli after 1st subculture (Fig.4.2). Embryonic calli were surrounded with loose watery non-embryogenic callus. After 3/4 subcultures the culture was predominantly embryogenic with very less or no non-embryogenic callus. The result of Little *et al.* (2000) suggested picloram as an effective auxin for induction of embryogenic callus in mature embryo axis of peanut. Higher concentration (83.0 µM) of picloram was found suitable for induction and maintenance of embryogenic callus in their studies.

Table 4.1: Effect of picloram on mature zygotic embryo derived leaflets for induction of Embryogenic Callus under Light and Dark Culture Condition

Picloram (µM)	callus response (%)	
	light	dark
	mean ± SD	mean ± SD
2.07	40.5 ± 1.7 [b]	63.5 ± 1.8 [b]
4.14	51.1 ± 1.1 [a]	71.6 ± 2.1 [a]
12.42	55.0 ± 2.9 [a]	76.0 ± 4.3 [a]
20.71	45.2 ± 5.9 [b]	58.4 ± 7.1 [b]
41.41	33.2 ± 4.2 [c]	36.8 ± 3.3 [c]

Means within a column having the same letter are not statistically significant (P=0.01) according to New Duncan's Multiple range test. SD: Standard Deviation,

Analysis of variance for the interaction of picloram concentrations and culture incubation conditions

Source	d.f	SS	MSS	F	F-Tab5%	F-Tab 1%
Total	29	5990.09	206.55	0.33		
Treat	9	5684.54	631.62	41.34*	2.40	3.46
culture condition	1	3318.10	3318.10	217.19*	4.36	8.10
pic conc.	4	1981.58	495.40	32.43*	2.87	4.44
Pic Conc. x Cul.Cond	4	384.86	96.21	6.30*	2.87	4.44
Error	20	305.55	15.28			

d.f : degrees of freedom; S.S : Sum of squire; MSS : Mean sum of square,

* : Significant at 1.0% level

Highest callus induction frequency of 76 %, observed at picloram concentration of 12.42 µM was followed by 71.6% in 4.14 µM and 63.5% in 2.07µM. Although the analysis of variance is

significant at 1% level but the differences are not statistically significant. The interaction of picloram concentrations and culture incubation conditions was found significant for frequency of callus induction.

Previous work from this lab demonstrated that mature zygotic embryo derived leaflets (MZEDL) have an excellent capacity to give embryogenesis as well as organogenesis with the change in plant growth regulator and their concentration (Chengalrayan *et al.*1995). Using immature zygotic embryo derived explants Ozias-Akins *et al.* (1993) demonstrated that picloram is an effective auxin for peanut somatic embryogenesis via a short phase of callus. With both observations in view we cultured MZEDL in medium with various concentrations of picloram to get embryogenic callus. Mature embryo axes derived explants has certain advantages. It is readily available throughout the year for initiation of highly regenerable embryogenic callus. The disadvantages of using immature seed explant include seasonal availability and the difficulty of determining an exact developmental stage from external pod morphology. Moreover contamination problem during initiation of culture is more in immature embryos.

Role of culture environment in growth and differentiation of tissues in culture is emphasized by several researchers. Incubation of cultures in dark enhanced the callusing response in mature embryo derived leaflets of peanut from 55.0 % ± 2.9 in light to 76.0 % ± 4.3

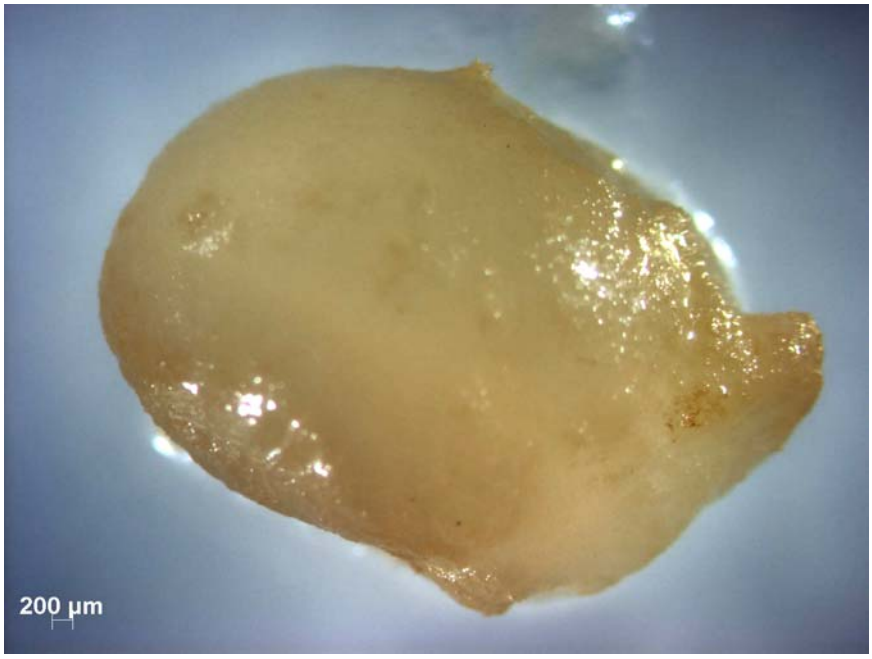


Fig. 4.1. MZEDL derived compact morphogenic callus in picloram (12.42 µM) and 0.06 M sucrose.

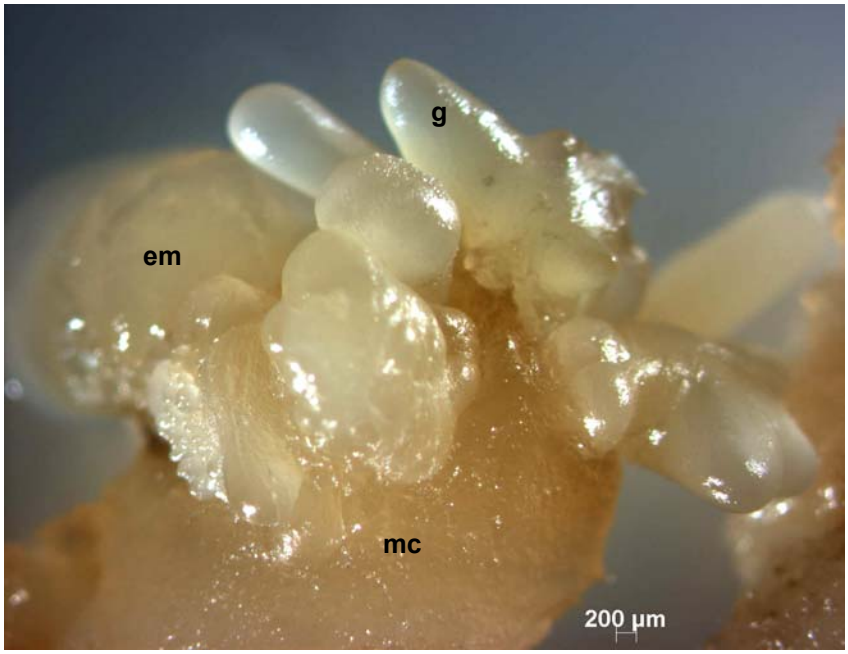


Fig. 4.2. Initiation of embryogenic mass (em) and globular embryos (g) from morphogenic callus (mc).

in dark the concentration of auxin being same. Physical and environmental conditions are critical to the process of embryogenesis (Tulecke, 1987), light being one of them. It is reported that initial divisions of explant and growth of callus tissue are sometimes prevented by light (George and

Sherrington, 1984). In concurrence with the observations reported by Ozias-Akins *et al.* (1993) using immature embryos, in our experiment dark incubation was more effective for induction of embryogenic callus in mature zygotic embryo derived leaflets. Similar result was reported by Pelkonen *et al.* (1999) in Lily where embryogenesis and differentiation was promoted when callus was incubated in dark during propagation and transferred to light prior to regeneration.

Effect of sucrose concentrations:

From the previous experiment (Table 4.1) it was observed that 2.07, 4.14 and 12.42M picloram were more effective in inducing callus in the MZEDL. Therefore picloram at these three concentrations was used to optimize the concentration of sucrose.

Frequency of embryogenic callus induction in MZEDL of peanut varied with varying concentrations of sucrose tested with three concentrations of picloram (Table 4.2). Optimum response 81.6% was observed for picloram concentration 4.14µM with 0.06M sucrose. Comparing the 76.0 ± 4.3% (Table 4.1) and 74.7 ± % (Table 4.2) response in 12.42µM picloram with 0.09M sucrose in two

different experiments, with 81.6 ± 3.6 % response in 4.14µM picloram with 0.06M sucrose (Table 4.2) it appears that the effect of picloram is influenced significantly by the sucrose concentrations. ANOVA for interaction of picloram and sucrose concentrations confirms the integrated role of picloram and sucrose on induction of embryogenic callus in peanut. In addition to its role as a carbon source, sucrose is the precursor of various compounds synthesized within the cell. Moreover it alters the micro environment of the cells in culture due to its role as penetrating osmoticum. Higher concentrations of sucrose were detrimental for callus induction. In the medium with 0.09 M, 0.18 M and 0.29 M sucrose with 4.14 µM picloram the response of callus induction frequency were 69.3, 47.0 and 8.0 % respectively indicating inhibitory role of sucrose at higher concentrations.

Table 4.2: Combined effect of sucrose and picloram on frequency of embryogenic callus induction

Sucrose	0.06 M Mean ± SD	0.09 M Mean ± SD	0.18 M Mean ± SD	0.29 M Mean ± SD
Pic 2.07 µM	74.5 ± 10.1	68.0 ± 10.6	56.0 ± 22.8	18.7 ± 11.8
Pic 4.14 µM	81.6 ± 3.6	69.3 ± 22.0	47.0 ± 13.2	8.0 ± 0.0
Pic 12.42µM	64.0 ± 17.9	74.7 ± 2.3	44.0 ± 8.6	8.0 ± 5.7

Analysis of variance for the interaction of picloram and sucrose concentrations

Source	d.f	SS	MSS	F	F-Tab5%	F-Tab 1%
Total	35	27829.22	795.12	0.23		
Treat	7	24569.06	3509.87	30.15*	2.4	3.4
sucrose conc.	3	17790.86	5930.29	50.93*	2.9	4.6
pic con.	2	1934.42	967.21	8.31*	3.3	5.5
sucrose x pic	6	4843.77	807.30	6.93*	2.5	3.5
Error	28	3260.16	116.43			

d.f : degrees of freedom; S.S : Sum of squire; MSS : Mean sum of square,

* : Significant at both 0.5 and 1.0% level

Sucrose has been identified as one of the factors, influencing induction of embryogenesis (Thorpe, 1988). Use of sucrose in increasing osmolarity and consequently suppression of

embryogenesis at higher concentrations was reported by Pence, 1981. In peanut, inhibition of embryogenesis in sucrose concentration above 7% was reported earlier (Eapen and George, 1993). Sucrose was identified as the superior carbon source tested for peanut and 6% sucrose gave the highest frequency of somatic embryos per explant. It has been demonstrated that merely by altering the sucrose concentration, the morphogenic pathway can be changed from organogenesis to embryogenesis and vice versa (Jeannin *et al.* 1995). In peanut 6 % sucrose was optimal for embryogenesis and 2 % sucrose was optimal for organogenesis (Chengalrayan *et al.* 1995). Tomar and Gupta (1988) observed the opposite phenomenon in *Albizzia richardiana,* i.e. higher concentration (4%) was responsible for organogenesis and 2% was for embryogenesis. In peanut 3% (0.09 M) sucrose were used for induction of embryogenic callus by other researchers (Livingstone and Birch, 1999; Little *et al.* 2000).

Induction and proliferation of secondary embryogenesis

Effect of picloram concentrations:

Repetitive growth of embryogenic callus was stabilized in 3 to 4 passages in culture by elimination of the non-morphogenic calli carefully during subculture. This somatic embryo rich culture, mentioned as embryonic culture was used in following experiments for further optimizations of conditions for optimum growth. For rapid proliferation and induction of secondary embryos, different concentrations of picloram were tested. An increase in tissue fresh weight of 5 fold in 4 weeks, 12 fold in 8 weeks and 34 fold in 12 weeks were obtained in 12.42 µM picloram (Table 4.3). The percent increase in callus growth reduced with every passage in other concentrations.

Table 4.3: Effect of picloram concentrations on proliferation of embryonic culture

Picloram		Callus fresh wt (mg).					
(µM)	Initial FW	1st SC	Percent increase	2nd SC	Percent increase	3rd SC	Percent increase
4.14	101	376 ± 32.5	372.28	657 ± 63.1	174.73	876 ± 65.2	133.33
12.42	104	523 ± 56.1	502.88	1245± 85.4	238.05	3421 ± 23.4	274.78
20.71	97	435 ± 43.3	448.45	983 ± 51.3	225.98	1654 ± 12.1	168.26
41.41	102	233 ± 31.4	228.43	365 ± 29.1	156.65	443 ± 26.6	121.37

SC: Subculture; FW: Fresh Weight,

MS medium supplemented with 12.42 µM picloram were found superior not only for giving optimum growth rate of embryonic culture (Table 4.3) but also a uniform embryo developmental stages (Fig 4.3, 4.4). Similar concentration was found suitable in earlier study by Ozias-Akins *et al.* (1993). Little *et al.* (2000) noted that higher concentration of picloram i.e. 83.0 µM is effective for maintaining the globular stage of repetitive embryos.

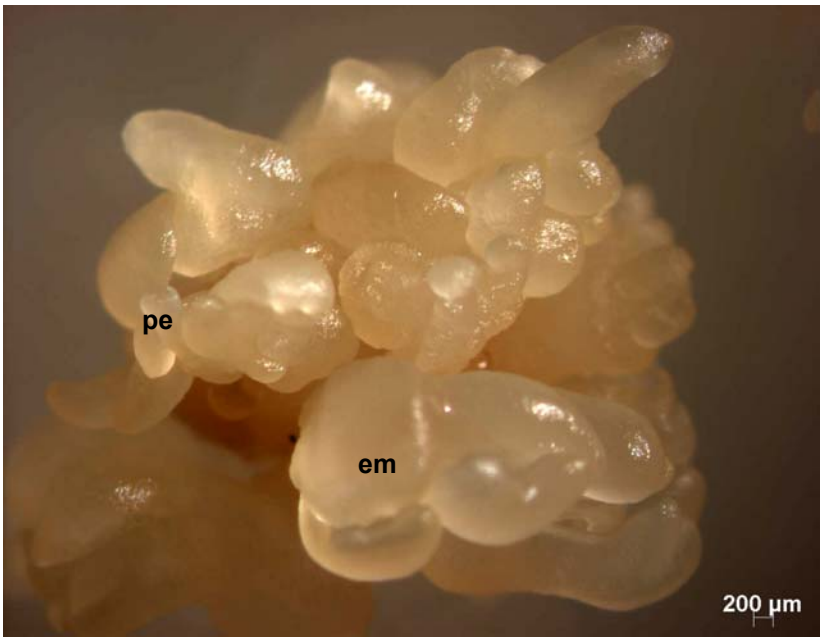


Fig. 4.3. Embryonic culture. Embryogenic masses (em) and primary embryos (pe) are predominant

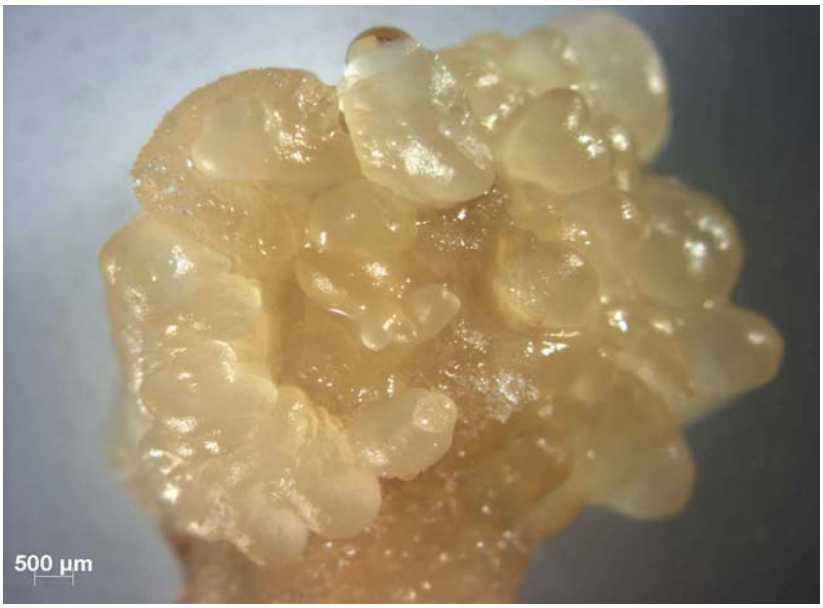


Fig. 4.4. Proliferating embryogenic masses and developing primary embryos growing in 12.42 µM picloram

Effect of different carbohydrates:

In previous experiments 0.06-0.09 M sucrose was used with various concentrations of picloram for obtaining embryogenic callus. To identify the best carbohydrate source for maintenance and proliferation of embryonic tissue, sucrose was substituted with similar concentration (0.06 M) of fructose, glucose and maltose in repetitive embryogenesis medium composed of MS basal medium and 12.42µM picloram. Comparing the growth in fresh weight every 4 weeks for a period of 12 weeks in media with four different carbohydrates, it was observed that sucrose was the most effective carbohydrate source followed by maltose (Fig 4.5). Fructose and glucose were less effective in proliferation of embryonic tissue.

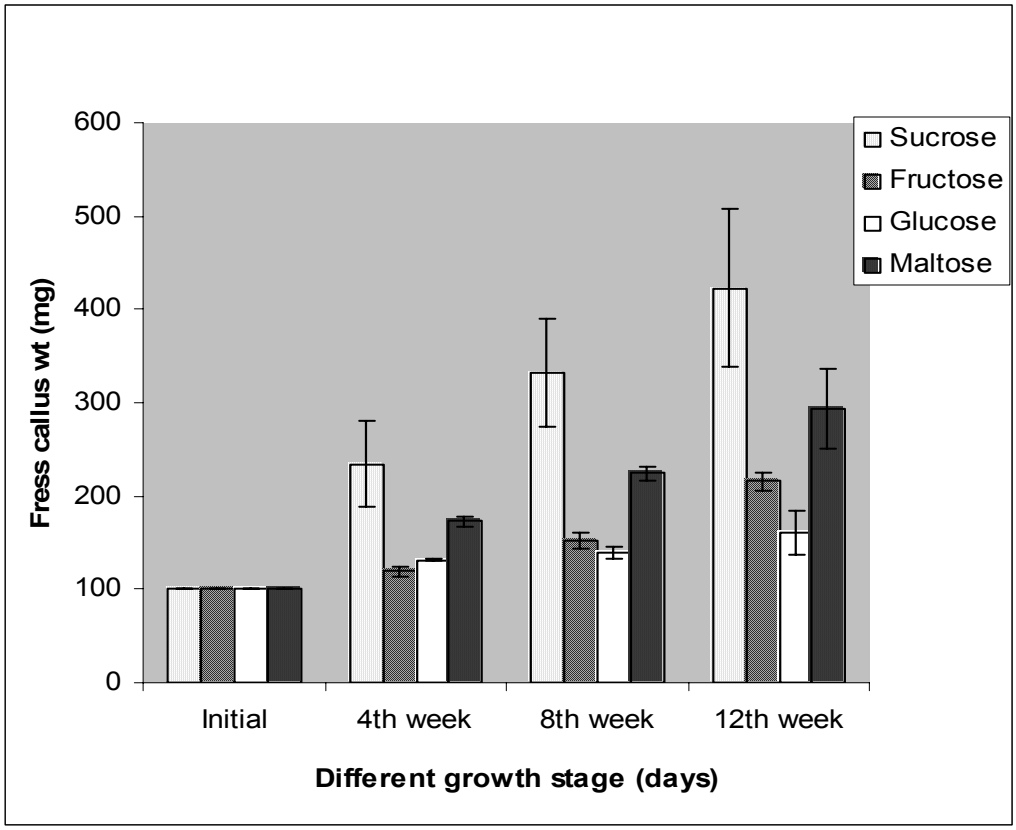


Fig. 4.5. Influence of various carbohydrate supplements on fresh weight of embryonic tissue of peanut.

Sucrose is assumed to be the best carbon source in cell and tissue culture media, because it is the main sugar translocated in the phloem of many plants (Giaquinta, 1980; Strickland et al, 1987). However, in several plant species including *Hevea brasiliensis*, *Miscanthus x ogiformis* Honda*, Spinacia oleracea, Hordeum vulgare,* significance of different carbohydrates is proved for induction of

embryogenic callus, embryo development and regeneration (Blanc *et al.*1999; Peterson *et al.* 1999; Komai *et al.* 1996; Scott and Lyne 1994). The change of carbohydrate has also proven beneficial in organogenesis in Cherry (Borkowska and Szczerba 1991), Rice (Jain *et al.* 1997) and many more. In earlier study (Chengalrayan *et al.* 1997), sucrose, maltose and glucose were found effective in inducing direct embryogenesis in the mature zygotic embryo derived leaflets of peanut. To identify the best carbohydrate in repetitive embryogenesis we used four different carbohydrates at the same molarity. Sucrose was found superior in terms of increase in fresh weight of the tissue, followed by maltose. Consequently, the media containing fructose or glucose contained half as many hexose equivalents as the media containing a disaccharide. In an attempt to study the effect of various carbohydrates on production of embryogenic calli Blanc *et al.*1999 proposed that the reduce growth of callus on media containing hexose could be due to an initial amount of carbon that was insufficient for stronger growth.

With sucrose and maltose treatments, equivalent quantities of soluble sugars were taken up by the calli. However, what happens to the absorbed carbon depends on the type of disaccharide in the medium (Scott et al, 1995). Blanc *et al.* (1999) reported that in *Hevea brasiliensis*, calli in the maltose treatment produced half as much calli dry matter while consuming equal amount of soluble sugar. They suggested that, although maltose was absorbed, the effect of the compound could be due to nutrient stress linked to a low ability of the callus to absorb maltose during the first days of culture. They reported that sucrose and maltose these disaccharides when used as a carbon source requires prior hydrolysis and a qualitative analysis of the carbohydrates in the medium revealed high extracellular hydrolysis of sucrose. Whereas, maltose shows slow hydrolysis. The maltose effect could be due to a nutrient deficiency linked to low absorption abilities and/or due to reduced maltose hydrolysis. Similarly in cucumber, 3% sucrose was found optimal for embryo development than maltose, glucose and fructose (Ladyman and Girard 1992).

Effect of glutamine:

The concept of incorporating glutamine in the medium as a source of organic nitrogen is not new. The potential of glutamine in enhancing embryogenic callus growth of peanut is demonstrated earlier (Ozias-Akins et all, 1993). With this report in view, in the present investigation glutamine 6.85mM was incorporated in the peanut embryonic callus culture medium to obtain optimum growth. The change is fresh was compared with the fresh weight of embryonic tissue cultured in medium without glutamine after 4 weeks and 8weeks. Comparing the increase in fresh weights in media with and without glutamine it appears that addition of 6.85 mM glutamine did not influence the growth rate

of secondary embryos raised from the callus initiated from mature explants significantly (Fig 4.6). Statistically they are at par.

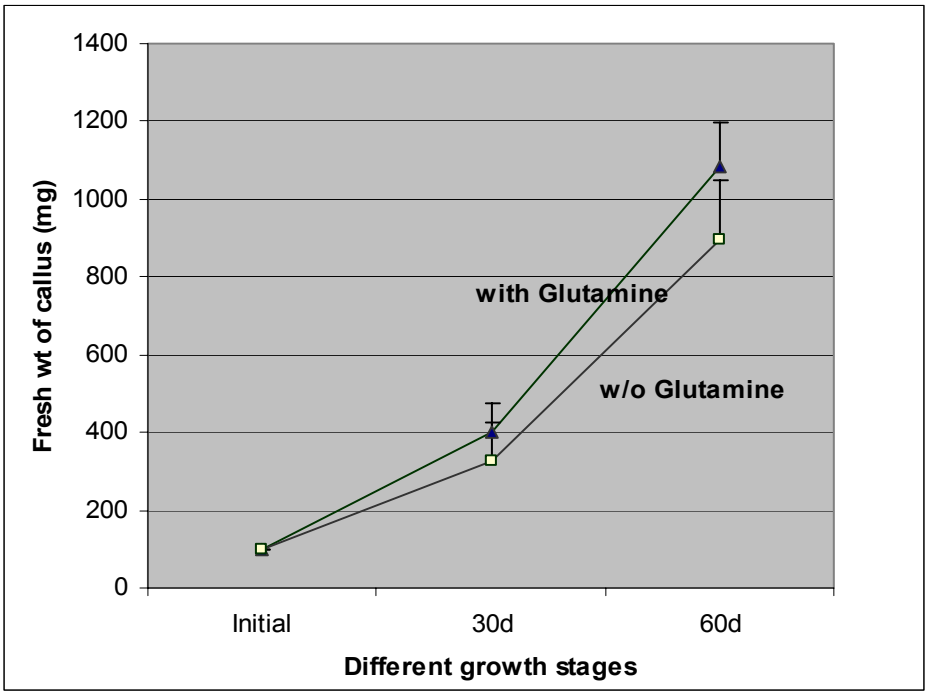


Fig.4.6: Effect of Glutamine (6.85 mM) in Secondary Embryogenesis

Morcillo *et al.* (1999) suggested that low vigor of plantlets resulting from somatic embryos may be due to insufficient levels of deposited storage proteins. In order to improve embryogenesis, embryo maturation and the vigor of regenerated plantlets, glutamine has been used in cultures of several species (Rajasegar *et al.* 1996; Witjaksono *et al.* 1998). In the present investigation addition of glutamine has not enhanced the callus growth significantly, but improved the quality of embryos in terms of size and synchronous development (Fig 4.7). Morphologically, embryos from glutamine containing medium appeared longer and less clumped. Supposedly, glutamine does not enhance callus growth significantly but does help in differentiation of the embryos. Similar observation was reported by Murch *et al.* (1999) in peanut embryogenesis where they have used glutamine (5 mM) along with other amino acids. By continuous subculture in picloram 12.42 µM with glutamine (6.85 mM) and sucrose 0.06 M we succeeded in getting profuse repetitive embryogenesis from mature zygotic embryo derived leaflets (Fig 4.8). Same amount of glutamine was used by Livingstone and Birch (1999) for maintenance of embryogenic callus in peanut.

Fig. 4.7. Uniform size of repetitive embryos indicate synchronous growth in presence of glutamine (6.85 mM).

Fig. 4.8. Profused repetitive embryogenesis in picloram (12.42 µM) with glutamine (6.85 mM) and sucrose (0.06 M)

Conversion of embryos

More than 80% embryos rooted after 4 weeks incubation in 5.37 µM NAA medium and plumule end of embryos turned green in light (Fig.4.9). In some of the embryos the leaves opened, but did not differentiate to shoot. On transfer of these rooted embryos to medium with BAP 4.44, 8.88 and 13.32 µM, the shoot formation in the plumule was achieved in 18, 23 and 42% respectively (Table 4.4). Whereas in medium with TDZ 4.54, 9.08 and 13.62 µM, the shoot induction were in 43, 65 and 71% of the embryos. In presence of either of these cytokinins, the root growth was stunted. The conversion frequency in TDZ 13.62 µM is significantly higher compared to any of the concentrations of BAP or low (4.54 µM) concentration of TDZ. Though optimum conversion frequency was achieved in the medium containing TDZ 13.62 µM, but the plants were morphologically stunted, bushy (Fig. 4.10) and with stunted and swollen, roots. All the plantlets raised from various concentrations of either cytokinin had multiple shoots. Transfer of these plantlets from cytokinin containing media to MS basal medium without growth regulators and growing for 2-3 weeks, was found effective for recovery of normal plants with elongated shoots and root (Fig.4.11).

Table 4.4: Conversion of repetitive embryos in BAP and TDZ

BAP		TDZ	
µM	% conversion mean ± SD	µM	% conversion mean ± SD
4.44	18 ± 2.1[c]	4.54	43 ±3.3[b]
8.88	23 ± 3.2[c]	9.08	65 ± 5.3[a]
13.32	41 ± 4.3[b]	13.62	71 ± 5.6[a]

SD: Standard Deviation; Same letter indicated that mean are not significant.

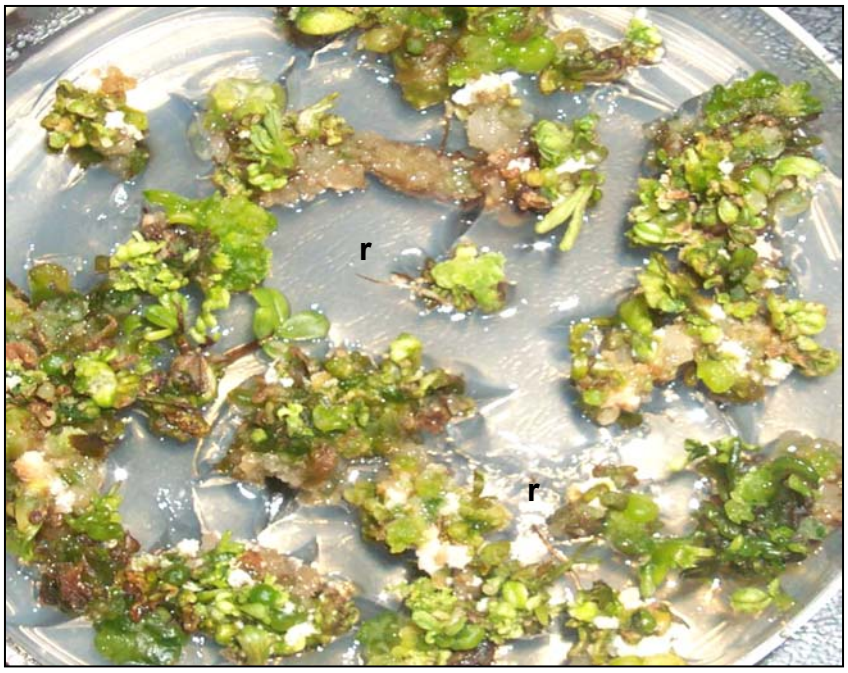


Fig. 4.9. Rooting (r) of somatic embryos in NAA (5.37 µM). Plumule turned green and started opening in light.

Fig. 4.10. Morphologically abnormal plantlets with stunted shoot and swollen roots developed in 13.62 µM TDZ.

Failure of primary somatic embryos of peanut to convert into plantlet was noted earlier (Chengalrayan et.al.1997). This limitation was overcome by incorporation of cytokinins in the germination medium. Similar limitation was encountered in germination and conversion of repetitive

somatic embryos in hormone-free medium (Durham and Parrott, 1992). Desiccation of somatic embryos or inclusion of BAP in germination medium found effective to enhance the conversion frequency of secondary embryos derived from both immature and mature embryos (Ozias-Akins et a. 1993; Livingstone and Birch, 1999; Little *et al.* 2000). Earlier study suggested that both BAP and TDZ are effective in triggering morphogenetic activity in the plumule of primary somatic embryos (Chengalrayan *et al.* 1997). Livingstone and Birch, (1999) achieved 60% conversion of peanut somatic embryos in 10 mg/l BAP. A pre selection and separation of individual or cluster of well-differentiated elongated embryos found effective in increasing the conversion rate.

In the present study, 65.4% conversion of the repetitive embryos was obtained with 9.08 µM TDZ in the medium. Withdrawl of TDZ by removing the plantlets from TDZ medium after 3-4 weeks and growing them in MS medium without growth regulators for further 2-3 weeks stimulated elongation of the somatic embryo derived plantlets.

The protocol standardised for in vitro regeneration of peanut via callus from immature embryo derived explant (Ozias-Akins *et al.* 1993) was extended to mature embryo axis derived immature leaflet explant. This protocol has certain advantages over and above the protocol described earlier for regeneration of callus via somatic embryogenesis.

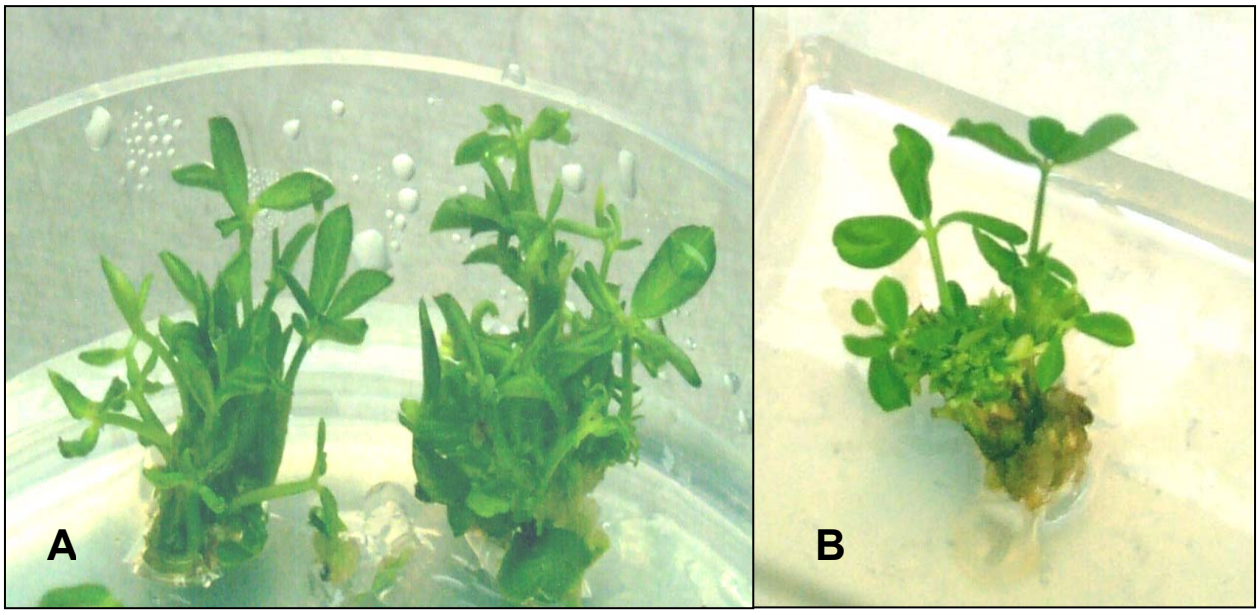


Fig. 4.11. Elongated plantlets with multiple shoots obtained from (A) TDZ (9.0 8 µM), (B) BAP (13.32 µM)

Conclusion

In this study a protocol for peanut callus induction and proliferation via primary and secondary embryogenesis is optimized using mature embryo axis derived explant. Role of picloram and its effectiveness in secondary embryogenesis of peanut is confirmed. The protocol describes a method for obtaining somatic embryos from somatic embryos via repetitive embryogenesis. The globular stages of repetitive embryos are very useful for transformation study. In concurrence with the earlier report TDZ was found effective in conversion of peanut embryos. To the best of our knowledge this is the first report where TDZ has been used for conversion of repetitive embryos of peanut successfully. In this study we found lower level of picloram (12.42 μM) is optimum for production of somatic embryos repetitively.

CHAPTER 5:

GENETIC TRANSFORMATION

5.1. Genetic Transformation via Particle Bombardment

5.1.1. Optimization of bombardment parameters

Introduction

Conventional breeding has led to the improvement of some peanut traits such as seed yield and drought tolerance. However, conventional peanut improvement has limited applicability to serve for many specific needs. These include resistance to insect pests as well as diseases caused by bacteria, nematodes, viruses such as *Tomato spotted wilt* and peanut stripe viruses, leaf spot fungi and to fungi such as *Aspergillus flavus* and *A. parasiticus* which produce carcinogenic aflatoxin. Other beneficial traits would include tolerance to water stress, uniform fruit maturity, and enhanced nutritional quality by modification of the amino acids and lipid compositions.

It is believed that a single hybridization event between *Arachis duranensis* and *A. ipaensis*, both diploid species, gave rise to what we know as peanut today. Thus peanut has a very narrow genetic base (Kochert. G,1996). Furthermore, introgression of desirable traits, if available, from wild *Arachis* relatives is difficult because of cross-incompatibility, especially when peanut is a self-pollinated species. Few lines derived from interspecific crosses have been made available to peanut growers. Lines showing resistance to *Cercospora arachidicola* and *Meloidogyne arenaria* have been released. However, these lines are low yielding with poor agronomic quality (Kokalis-Burelle *et al*. 1997). Simpson and Starr (2001) registered a groundnut cultivar COAN having root knot nematode resistance transferred from wild species of *Arachis*. Thus, extensive peanut breeding programs are needed in addition to the interspecific crossing to impart this resistance to commercially viable lines.

Introduction of novel traits into peanut using genetic engineering techniques will assist in the development of improved peanuts by allowing the introduction of genes from sources other than *Arachis* germplasm directly into commercially important cultivars without altering other characters (Higgins and Dietzgen, 2000).

Biolistic transformation

In light of the technical accuracy, automation and efficiency, transformation via particle bombardment is often considered as the most popular and widely used transformation system in number of crop species. In fact, in the opinion of Sanford J.C (2000), most of the presently grown transgenic crop acreage in the world has been achieved through use of the biolistic transformation. The biolistic process, deployed for the first time by Sanford, *et al.* (1987), employs high-velocity microprojectiles to deliver genetic materials into living cells and tissues. The system was variedly used

thereafter and recognized by synonymous terms like microprojectile bombardment method, the gene gun method, the particle acceleration method etc. Since then diverse applications of the biolistic process in biological sciences have been rapidly explored for both basic research and genetic engineering. Transformation of biological materials using the biolistic method has been shown to be a valuable technique for delivering DNA into the cells of plant (Klein *et al.* 1988 a, b), animal (Williams, *et al.*, 1991), and microbial species (Shark, *et al.*, 1991), as well as into subcellular organelles (Johnston *et al.* 1988; Daniell, *et al.*, 1990). Achieving higher rates of DNA expression in each of the systems under study often requires critical time that needs to be spent in optimizing the range of parameters involved in the transformation process. The system also needs to be optimized for the specific tissue to be transformed for subsequent stability and reproducibility of the results.

Biolistic transformation: Potential vis-à-vis conventional transformation:

Among the various available plant transformation techniques Particle Gun has an upper hand. The technique excels other procedures in its use for the production of transgenic plants, due to various aspects besides speed and instrumentational accuracy. Some of the most significant advantages of biolistic transformation method have been enumerated here.

- A direct gene transfer method and not restricted to any host range, unlike the narrow host range of *Agrobacterium*
- Plasmid construction simplified, compared to requirement for conserved border sequences from Ti plasmid for replication and transfer in *Agrobacterium* transformation
- False positive results from growth of *Agrobacterium* in host tissue are eliminated.
- Transformation protocols are simplified, since complex bacterial / plant interrelationship, which vary with each system, are eliminated.
- Biolistic transformation enjoys technical simplicity, speed and needs manipulations of physical parameters like velocity of microprojectile, distance of tissue from target etc, rather than otherwise complex interaction parameters like proximity of bacterial cells to wounded plant tissues, chemical responses, bacterial attachment to the plant cell surface, transfer of T-strands from bacteria to plant cells across the plant cell wall and membrane, etc., in other conventional transformation protocols.
- Possibility of routine cotransformation (introduction of multiple plasmids) with particle bombardment, rather than that of mobilizing a single plasmid containing multiple DNA sequences (Gray & Finer 1993).
- Comparatively tissue and genotype independent transformation technique.

Applications of biolistic transformation

The biolistic process was originally developed as a means to deliver foreign genes into the nuclear genome of higher plants. Extensive efforts were taken by many workers, resulting in successful biolistic transformation of a wide range of tissues in a wide range of plant species. Variety of plant tissues include cell suspensions, calli, immature embryos, mature embryo parts, meristems, leaf pieces, microspores and pollen are successfully used for transformation via particle bombardment (Sanford *et al.* 1993). The biolistic method has made it possible to transform such species that were otherwise impossible or very difficult to transform (McCabe, 1988; Fromm *et al.* 1990; Gordon-Kamm *et al*, 1990).

The biolistic process has proven to be effective even in very small cell types, and has therefore been useful in transforming diverse microbial species (Sanford *et al.* 1993).These include microbial eukaryotes such as yeast and filamentous fungi and algae; prokaryotes such as *Bacillus megaterium, Pseudomonas syringae, Agrobacterium tumefaciens, Erwinia amylovora, Erwinia stewartii, and Escherichia coli ;* and obligate fungal pathogens such as *Uncinula necator.*

The biolistic process first made possible the transformation of organelle genomes. Chloroplasts of *Chlamydomonas* (Boynton *et al.* 1988; Blowers *et al.* 1988), yeast and *Chlamydomonas* mitochondria (Johnston *et al.* 1988; Fox *et al.* 1988) have been successfully transformed by biolistics. Higher plant chloroplasts also have been either transiently (Daniell *et al.* 1990; Ye *et al.* 1990) or stably (Svab *et al.* 1990) transformed using the biolistic process.

Most dramatically, the biolistic process has proven useful in transforming animal cell lines (Zelenin *et al* 1989), primary animal cells (Williams *et al* 1991), and intact animals (Yang *et al* 1990; Williams *et al* 1991).

Functional maneuver of Particle Gun

The biolistic PDS-1000/He apparatus propels a macrocarrier loaded with millions of microscopic gold particles, microcarriers, which have been coated with biological material toward the target cells at high velocity. The macrocarrier is halted after a short distance by a stopping screen and the microcarriers continue traveling toward the target. The velocity of the microcarriers is sufficient to penetrate the cells.

High pressure helium, released by a rupture disk, is used to propel the macrocarrier. The launch velocity achieved is dependent upon helium pressure, the distance from the rupture disk to the macrocarrier, and the macrocarrier travel distance. The velocity of microcarrier at the point of impact

with cells is also dependent upon the amount of air in the sample chamber and the distance traveled. The chamber is partially evacuated to minimize the velocity loss due to air drag.

The helium pressure used is empirically determined and the rupture disk required is determined by the selected pressure. The rupture disk is inserted in the retaining cap and mounted on the gas acceleration tube (Fig. 5.1.1.1.). The stopping screen is placed in the lower portion of the microcarrier launch assembly. The macrocarrier holder, loaded with a macrocarrier coated with the gold microcarriers and the biological material of interest, is placed in the upper position of the launch assembly with the microcarrier facing the screen. The launch assembly is inserted in the chamber and the sample dish is placed below that. The chamber is then evacuated. An interlock switch prevents the gas acceleration tube from being filled with helium until a vacuum of at least 5 inches of mercury is reached. The tube is then filled with helium by pressing the fire switch until the rupture disk bursts. The rapid release of helium propels the microcarriers as described above. The vacuum is then released and the sample removed from the chamber.

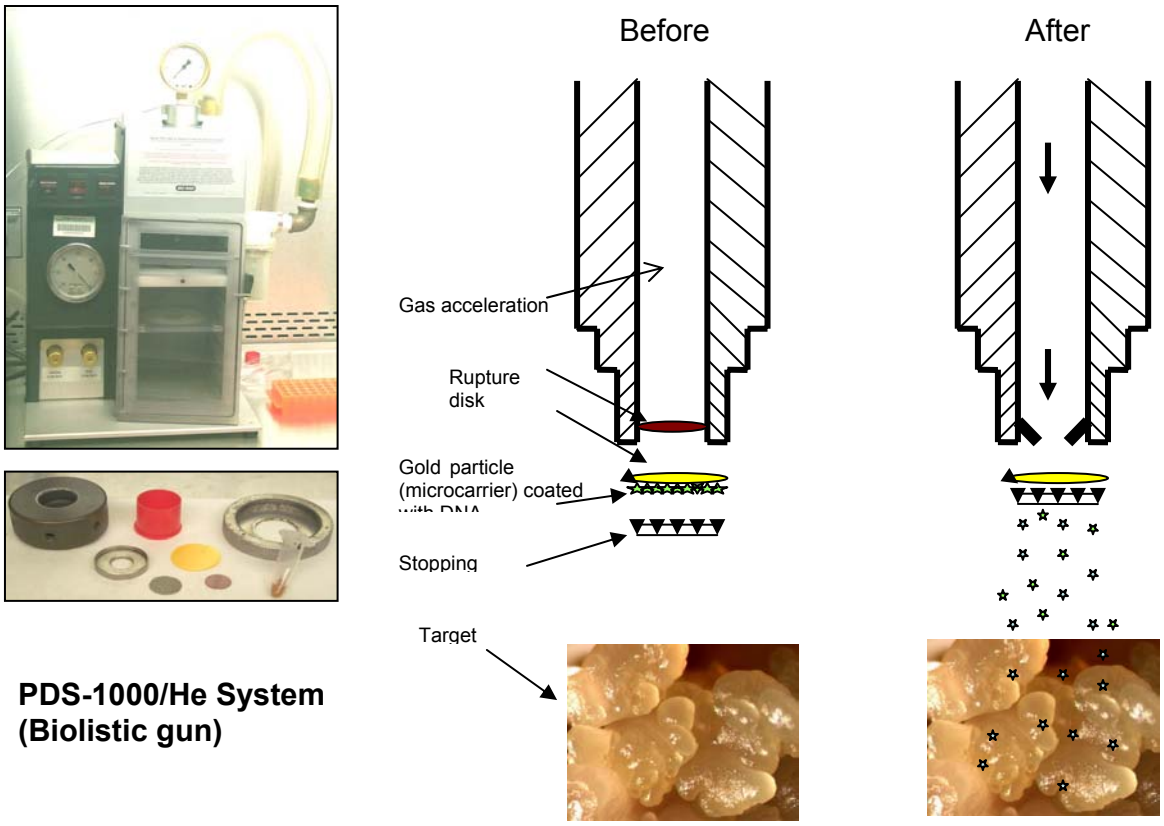


Fig 5.1.1.1. Functional maneuver of Particle Gun

The design produces an even distribution of the coated microcarriers which penetrate the sample. Up to ten cell layers of intact tissues can be penetrated by varying the macrocarrier placement and microcarrier travel. Improved microcarrier distribution provides delivery to a large sample size and results in greater cell survival.

Optimization of Bombardment Parameters:

Optimization of particle bombardment technique for any plant species necessitates manipulation of three broad categories of parameters like…….

1) Particle acceleration parameters – These will essentially involve helium pressure, macro- and micrcarrier travel distance, vacuum pressure inside the chamber, vacuum or residual gas, velocity of microprojectiles etc.

2) Microprojectile parameters- It will comprise choice of a suitable microprojectile (tungsten or gold), particle coating method composition and size of microcarrier etc.

3) Biological parameters – Living cells need more attention and hence consider Vector constructs, type of vector, concentration of DNA,cell age/ physiology, cell size, cell density, osmoticum, cell handling, transfers, selection method, etc.

Besides the parameters listed other parameters like temperature, humidity, photoperiod and biological factors including the nature of explants, pre and post bombardment culture conditions etc, may affect the outcome of transformation process.

There are reports on optimization of bombardment parameters in different plant species such as sorghum (Jason *et al.*2001), maize (Kemper *et al.* 1996), wheat (Rasco-Gaunt *et al.* 1999), Cassava (Schopke *et al.* 1997). But there very few reports on optimization of bombardment parameters in peanut (Livingstone and Birch, 1995; Wang *et al.*1998).

Transformation of peanut via particle bombardment

Clemente *et al.* (1992) were the first to report biolistic DNA transfer of GUS and NPTII gene in peanut leaflets. Though he observed the transient expression of GUS and the calli derived from the leaflets were growing on kanamycin selection, there was no stable transformation observed in regenerated plantlets.

Regeneration of stably transformed peanut plants was first time reported by Ozias-Akins *et al.* (1993). Embryonic tissue of *Arachis hypogaea* L. (cv. Toalson and Florunner) derived from immature zygotic embryos were used as recipient tissues for the introduction of a gene encoding hygromycin phosphotransferase (*hph*), conferring resistance to the antibiotic hygromycin, under the control of the

CaMV 35S promoter. The presence and integration of *hph* gene was confirmed by polymerase chain reaction (PCR) amplification and southern blot analysis of DNA from regenerated plants.

Schnall and Weissinger (1993) demonstrated a rapid method of biolistic transformation by the use of zygotic embryo axes as an explant. They have shown that by this method apical meristem housing the germ line cells was easily exposed for bombardment without compromising the viability of the plant.

Brar *et al.* (1994) reported the recovery of transgenic peanut plants using proprietary ACCELL technology, a microprojectile bombardment procedure. Shoot meristems of embryonic axes from mature seeds of peanut cv. 'Florunner' and 'Florigiant' were bombarded with plasmids encoding GUS, phosphinothricin acetyl transferase (*bar*) and tomato spotted wilt virus nucleocapsid protein (tswv-np) genes. The stable integration of the foreign genes in primary transgenics, and transmission of the transgenes to their progeny were demonstrated by molecular analysis. Integration of *bar* gene conferred resistance to BASTA^{TM}, a wide-spectrum herbicide, applied at 500 ppm of active ingredient at field level.

Livingstone and Birch (1995) optimized some microprojectile bombardment parameters for high transient expression rates of *uidA* or GUS reporter gene in peanut embryos and excised embryonic leaflets. They used the firefly luciferase reporter gene (*luc*) for the non-toxic assays, and showed the transient as well as stable expression of introduced gene in callus grown without selection for 8 weeks after bombardment of embryonic leaflets.

Singsit *et al.* (1997) introduced a gene encoding the *Bacillus thuringiensis cryIA(c)* protein and *hph* into peanut by microprojectile bombardment of somatic embryos. They have proven the presence and integration of *hph* and *Bt* genes by PCR and/or Southern blot analyses using the DNA from hygromycin-resistant embryogenic cell lines, regenerated plants, and their progeny. 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.

The effect of parameters involved in the transformation efficiency of peanut seed tissues by particle bombardment was evaluated by Lacorte *et al.* (1997), using the reporter gene ß-glucuronidase (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.

Direct DNA delivery via microprojectile bombardment was demonstrated by Wang *et al.* (1998), and to optimize their transformation protocol 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, and they found that GUS

expression driven by the *vspB* promoter was modulated by chemical and positional information and regulated in a tissue/organ specific manner.

The nucleocapsid protein (N) gene of the lettuce isolate of tomato spotted wilt virus (TSWV) was inserted into peanut (*Arachis hypogaea* L.) via microprojectile bombardment by Yang *et al* (1998). Constructs containing the *hph* gene for resistance to the antibiotic hygromycin and the TSWV N gene were used for bombardment of peanut somatic embryos and the integration of N gene into the peanut genome was confirmed by Southern blot analysis. They observed gene silencing in primary transgenic lines containing multiple copy insertions of the N transgene by northern blot, RT-PCR and ELISA analyses. Transgenic plants 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.

Livingstone and Birch (1999) described a system for the efficient production of fertile transgenic plants of diverse peanut cultivars, by particle bombardment of embryogenic callus derived from mature dry seed, followed by selection for resistance to hygromycin. B-glucuronidase (GUS) expression driven with different promoter constructs was quantitatively and histologically compared in peanut leaf tissue following microprojectile bombardment by Kim *et al.* (1999).

Magbanua *et al.* (2000) generated peanut lines transgenic for the antisense nucleocapsid (N) gene of Tomato spotted wilt virus (TSWV) by microprojectile mediated transformation of repetitive somatic embryos of cultivars VC1 and AT120. They have shown the resistance in transgenic progeny lines at field testing and northern blot analysis detected the transgene RNA and its transcript level after viral exposure.

Deng *et al.* (2001) demonstrated the use of cotyledons of immature peanut zygotic embryos as an explant for particle bombardment with a plasmid containing intron-GUS and *hph* gene conferring resistance to hygromycin. The presence and integration of foreign DNA was confirmed by PCR amplification and Southern analysis.

Materials and Method

DNA constructs

Plasmid p524EGFP.1 containing the cauliflower mosaic virus double 35S promoter sequence (Fleming *et al.* 2000) followed by alfalfa virus enhancer sequence (Datla *et al.* 1993) controlling expression of enhanced green fluorescent protein were used to study the bombardment parameters (Fig.5.1.1.2.).

For the study of osmoticum treatment two other constructs of EGFP and EYFP (Fig.5.1.1.3.) containing the CaMV 35 S promoter were used. The fluorescent protein gene variants were the product of Clontech and constructs containing these genes were developed and provided by Dr. Joe Nairn (School of Forest Resources, UGA).

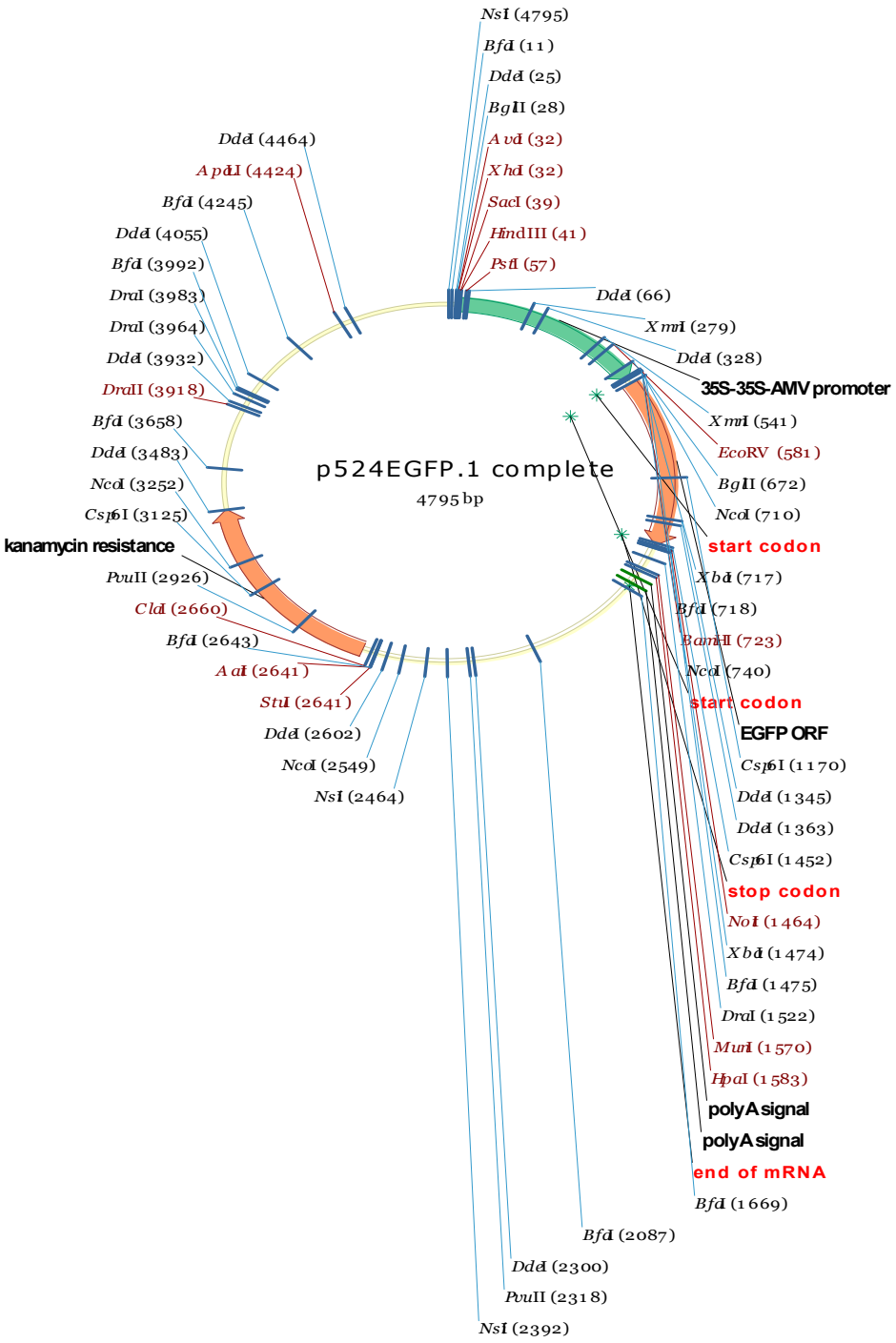


Fig.5.1.1.2. Map of plasmid p524EGFP.1 containing GFP gene driven by 35S-35S AMV promoter.

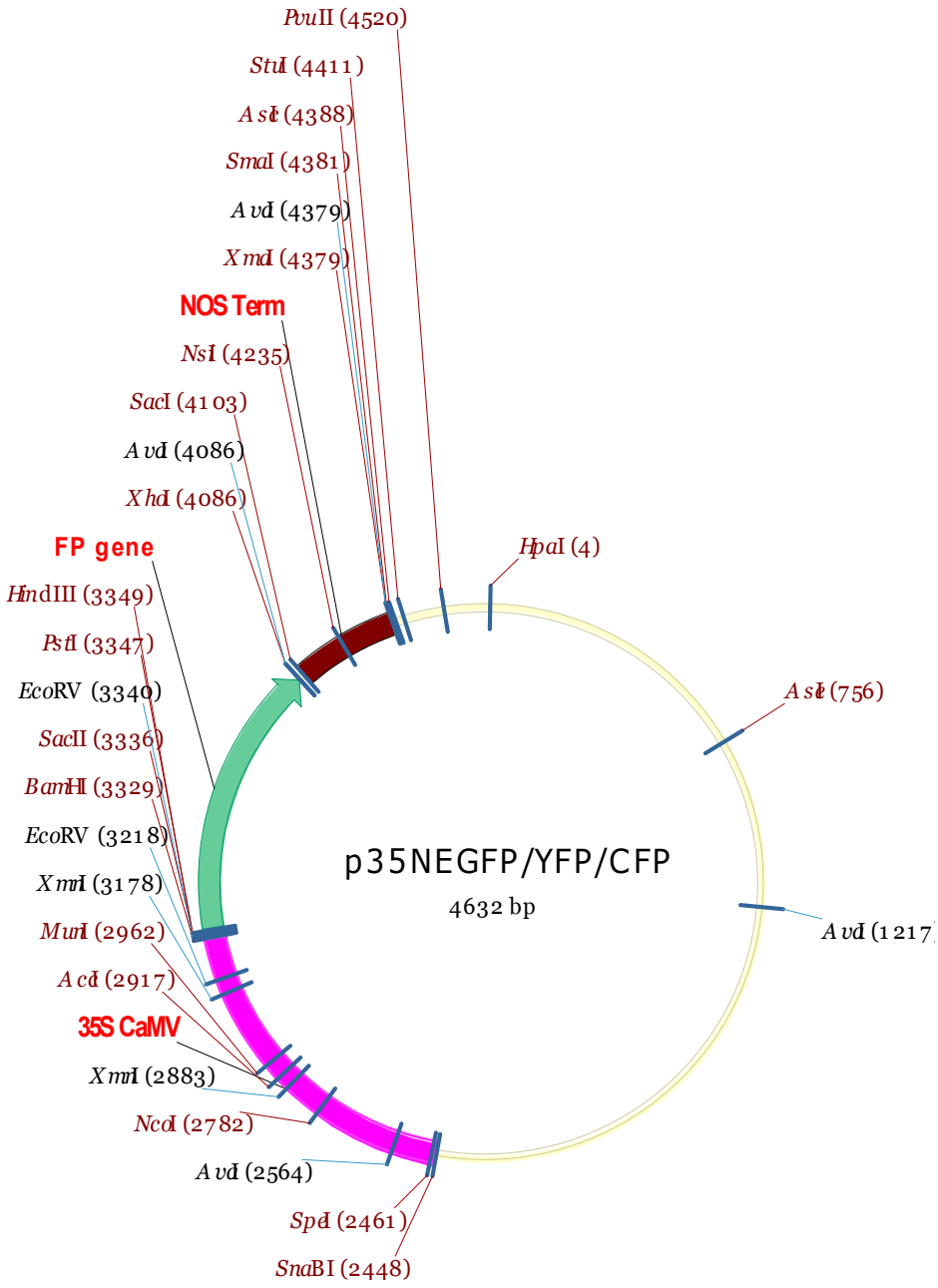


Fig.5.1.1.3. Map of plasmid P35 NEGFP/YFP/CFP

Plant materials and explant preparation for bombardment

Embryonic cultures were initiated from the mature zygotic embryos of peanut (cv. Georgia Green) by standardized methods (Ozias-Akins *et al.* 1993) and were routinely subcultured in MS

medium supplemented with 12.42 µM picloram, 6.85 mM filter sterilized glutamine and 0.6M sucrose. Cultures were maintained in the dark at 25 ± 2ºC and bombarded approximately two weeks after subculture.

In all bombardment experiments except for the osmoticum treatment the explants were arranged in the centre of Petri plates (1.2 cm radius) which were kept open in a hood for 2-3 hours to air dry.

Plasmid DNA isolation

Plasmid DNA from the E.coli cell line harboring p524EGFP.1.1 was isolated using QIAGEN-tip 500 columns (QIAGEN Inc., Chatsworth, CA) as per the manufacturer's specifications.

Bacterial culture was initiated and grown overnight in LB medium containing 50mg/l kanamycin. The bacterial cells were harvested by centrifugation at 4ºC for 15 min at 6000 rpm. All traces of supernatant were removed by inverting the open centrifuge tube until all medium has been drained. Pellets were frozen at -20ºC for storage or processed directly with the Qiagen protocol. The amount of the initial culture to be grown will depend on the plasmid type, i.e., high vs low copy number. To determine the yield, DNA concentration was estimated by both UV spectro-photometry and quantitative analysis on an agarose gel.

Particle prep

The biolistic particle delivery instrument PDS-1000/He (Bio-Rad, Hercules, CA, USA) was used as a particle delivery system for transformation experiments. The stock solutions were prepared and stored as per requirement.

1) Preparation of microcarrier

Gold particles of 0.6 µm size (except for size comparison) were used as a micrrocarrier in this study. 60 mg of gold particles were weighed into a microcentrifuge tube and suspended in 1 ml 100% ethanol. The suspension was vortexed on high speed for 1-2 min and repeated twice (total vortex time for 3-6 min). Particles were pelleted at 10,000 g for 1 min. Supernatant was removed and replaced with 1 ml sterile distilled water. The resuspended gold particles were again pelleted and rinsed once more with another 1 ml of sterile water. The microcarriers were finally resuspended in 1 ml sterile water and stored at -20ºC for 3-4 weeks.

2) Preparation of 0.1 M Spermidine stock

To give a 0.1M solution, 254.6 mg of spermidine (Sigma, USA) were dissolved in 10 ml of sterile distilled water. Aliquots of 40 µl spermidine solution in 1.5 ml microcentrifuge tubes were stored at -20ºC.

3) Preparation of 2.5 M $CaCl_2$ stock

Calcium chloride (1.837 g of $CaCl_2.2H_2O$) was dissolved in 5 ml of deionized water to give a 2.5 M solution. The solution was filter sterilized and dispensed in 100 µl aliquots in 1.5 ml microcentrifuge tubes and stored at -20ºC.

4) Absorption of DNA onto microcarriers

All the microcentrifuge tubes of gold microcarrier, plasmid DNA, $CaCl_2$, and spermidine were gathered under hood the with sterile pipette tips, p20, p100, p1000 pipettes, a few sterile microcentrifuge tubes and a vortex mixer. For each prep (good for 6 bombardments) the following were added to a sterile microcentrifuge tube in order, while vortexing: 50 µl of gold microcarrier suspension in water (vortexed before extracting), 5 µg (calculated volume should not be more than 20 µl) of plasmid DNA, 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. The tube was vortexed for 3 minutes and centrifuged briefly at 10,000 g. The supernatant was discarded and 250 µl of high grade 100% ethanol was added to each tube. Each tube was vortexed briefly, spin, and the supernatant was discarded. Finally, 70 µl of 100% ethanol were added to each tube and the particles were resuspended by vortexing.

5) Particle bombardment Protocol:

Hood and Equipment Set-up

Initially, the hood was switched on and then the hood surface and outside of the machine was swabbed down cleanly. Two stages and bursting plate holder were removed from the machine and thoroughly rinsed with 95% alcohol. The machine chamber was swabbed with 70 % alcohol from inside. Macrocarriers (large, yellow discs) were placed in a sterile petri plate with 70% alcohol as per the requirement of the experiments planned. Corresponding number of rupture discs (small brown discs -we have used 1800 psi discs for all purpose except for the helium pressure comparison study) were subsequently added and kept soaking not more than 5 minutes. Rupture discs were then removed first (removed as much of alcohol as possible) and put onto a sterile petri plate to dry. Macrocarriers were

lifted using forceps and allowed to dry on an autoclaved paper towel. The stopping screens (1 for every three shots), macrocarrier holders (silver discs) and macrocarrier punch (red plastic) were placed in a separate petri plate with 70 % alcohol After soaking for at least 5 minutes they were removed and placed on sterile paper towel to dry.

Preparation of Macrocarrier

The macrocarrier was placed onto holder with sterile forceps. The macrocarrier punch was used to seat the macrocarrier in place. A pipette was kept ready with a sterile pipette tip (usually for 10 µl). DNA prep tube with DNA was then held on the tip of the sonicator briefly to suspend the gold particles. 10 µl aliquots of gold were then taken out and dispersed into the center area of macroprojectile. The preps were then dried for approximately 10 minutes.

Shots

Using sterilized forceps, sterile dry rupture disc was placed into rupture disc holder and then screwed the holder in machine. A screen was placed into slot of particle stage with forceps. The macrocarrier assembly (from above) was placed into appropriate slot over the screen and screwed on holding plate. The particle stage (second slot from top) and prepared plate placed on target stage (third level from top) were inserted in the machine. Machine was switched on followed by turning the valve on the tank for helium gas. Vacuum pump was turned on and the pressure was allowed to reach 27 to 28 in Hg, when it was ready to take a shot. Shot switch was pressed continually till the shot was fired. The vacuum pump was switched off between two shots and pressed again to de-pressurize. Tissue sample plate was then removed out, rewrapped, and labeled accordingly.

Cleanup

The spent bursting plate, macrocarrier preps and screens were removed out after last shot. Bursting plate holder, particle stage, target stage, chamber of machine were cleaned with 95% ethanol. Gas tank was turned off. Vacuum pump and vacuum switch on machine was turned on and pressed the shot button continually until all gas was vented from system (observe gas gauge). Vacuum pump, hood and machine were turn off after cleaning.

Parameters studied for bombardment

To optimize the bombardment parameters the below variables were tried.

i) Different sizes of microcarrier- 0.6 µm, 7.5 µm and 1.0 µm gold particles were used.

ii) Helium pressure – rupture disc attended by using of 1350, 1550 and 1800 psi.

iii) Chamber vacuum pressure – two vacuum pressures of 27 and 28 in Hg were tested.

iv) Effects of osmoticum – explants were arranged 2-3 hours before bombardment in the above mentioned medium supplemented with 0.4M mannitol as an osmoticum. In this case there was no air drying and explants were kept in the same medium for 18-24 hours after bombardment.

Culture maintenance and transient expression

After bombardment, explants were kept on the same medium (except for mannitol study) in the dark. In the case of osmoticum treatment, cultures were shifted after 24 hours to without osmoticum regular medium. Transient expression of GFP can be observed in UV light after 24 hours of bombardment by using a Zeiss SV11 epifluorescence stereomicroscope equipped with a 100 W mercury bulb light source, a 480 ± 30 nm excitation filter and a 515 nm long-pass emission filter (Chroma Technology Crop., Brattleboro, VT, USA) . For the quantitative data the tissues were photographed using a Zeiss Axiocam digital camera attached with the microscope with a specific magnification (magnification 4.0) for all variables, after 48 hours of bombardment. Spots indicating transient GFP expression were counted from a 1000 μm^2 area from three different pieces of tissue per shot. A minimum of 9-10 counts were taken for each variable and each experiment was repeated three times. Proc GLM from SAS was used to detect significance differences in transient expression.

Cultures were visually monitored every 2-3 days and subcultured after 3-4 weeks in the same medium.

Results and Discussion:

Explant, GFP construct and Transient gene expression

The plasmid DNA of p524EGFP.1 (Fig.5.1.1.2.) isolated from E.coli (DH5-α) having a high concentration (approx. 2µg/ml) and quality was stored at -20ºC and; then used for bombardment as and when required (mentioned previously in material and methods). Embryonic callus of peanut was used as the target tissue. Those embryonic cultures, sub-cultured approximately two weeks (growth between lag and stationary phases) before the bombardment were found more responsive. Green Fluorescent Protein, a widely used reporter gene, owing to its features like non-destructive and early localization has been used in several species for the rapid establishment and optimization of plant transformation protocols (Elliott *et al.* 1999; Jordan M.C. 2000). We have thus used GFP constructs with varying efficiencies (details in Materials & Method, Fig. No.5.1.1.2 &3.) to optimize gun parameters for peanut transformation. Transient expression of GFP was observed 24 hours after bombardment. GFP spots

were counted in 1000 sq µm area (Fig.5.1.1.4.) after 48 hr. of bombardment. By using the transient expression as a criterion, different bombardment parameters were studied for optimizing the micro projectile bombardment protocol for peanut.

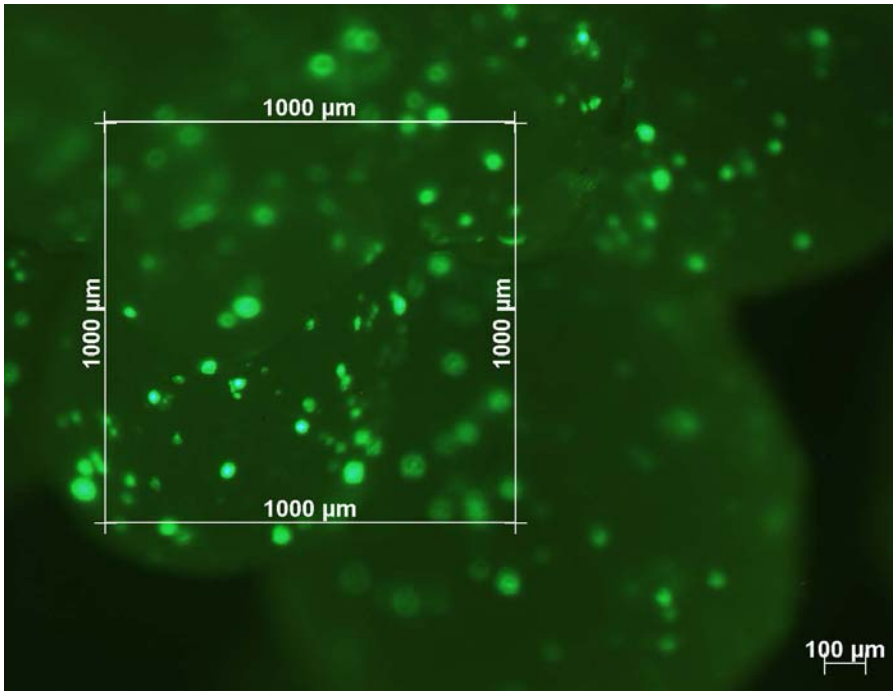


Fig.5.1.14. Transient expression of p524EGFP.1, 48 h post- bombardment, spots were counted from 1000 sq µm sector of embryogenic callus.

Optimization of System Parameters for Particle Gun

Earlier from this lab Dr. Ozias-Akins has reported optimization of various factors comprising distance of target tissue, DNA quantity per bombardment, radius from the center of the target platform etc relevant to bombardment of peanut tissue (Wang *et al.* 1998). Enhancement of the efficiency and specificity of bombardment would be accomplished by manipulating and evaluating accessory parameters of the gun. To achieve reproducibility and consistency in the peanut transformation experiments, we continued the efforts to define bombardment parameters more stringently. We focused our efforts on the parameters like size of the microprojectile, helium and vacuum pressure, and osmoticum to maximize transient expression in peanut tissue bombardment. Though gold particles are considered a best carrier to deliver the genetic material to the penetrated cells, the surface area and diameter of those particles plays a crucial role in their effectiveness. The particle size not only affects the acceleration speed but also determines available surface area for the DNA to bind to. The size of the particle also may be proportional to the penetration depth inside the tissue. Delivery of particle is

equally dependent on the barrel helium and vacuum pressure. The path of a micro projectile in a vacuum and the speed of its acceleration are the physical parameters that account for successful delivery of the carrier. In light of the pronounced involvement of these parameters in bombardment, an experiment was designed to evaluate the effect of variation in specific parameters on transient expression of the marker gene GFP.

Choice and Size of the Microprojectile

Among the two available types of microprojectiles, gold is often preferred over tungsten. Currently gold particles are available in a very limited range of sizes (Bio-Rad, USA), and are much rounder and more uniform in size compared to those of heterogeneous tungsten particles. Besides, tungsten is considered as potentially toxic to certain cell types and is subjected to surface oxidation that can critically alter DNA binding and cause degradation, over time. Though considered more expensive, a practical advantage of gold particle is not just their uniformity but their biological inertness and non-toxicity towards any cell. It is safe for any biological material and has been approved by FDA as a human therapeutic agent. Unlike tungsten, gold does not catalytically attack DNA bound to it (Sanford *et al.* 1993). Unfortunately gold microprojectiles are not very stable in sterile aqueous suspensions, and often agglomerate irreversibly over a period of time (Sanford *et al.* 1993). We preferred to use gold microprojectiles and always prepared it freshly before bombardment.

Size of the microcarrier is considered as a crucial factor in the delivery of the genetic material in bombardment experiments (Rasco-Gaunt *et al.* 1999). As described previously, we evaluated three different sizes of gold particles via transient expression of GFP (p524EGFP.1) number of spots observed per μm^2 of bombarded peanut tissue. The data were collected for transient expression 48 hours after bombardment. Among the three different sizes of gold tested, results in terms of number of spots per μm^2 were more and fairly consistent for the small sized particles (Fig. 5.1.1.5.). Gold microprojectiles, 0.6 μm in diameter resulted in a finer expression with a significantly higher number of spots (78.11 spots/ μm).

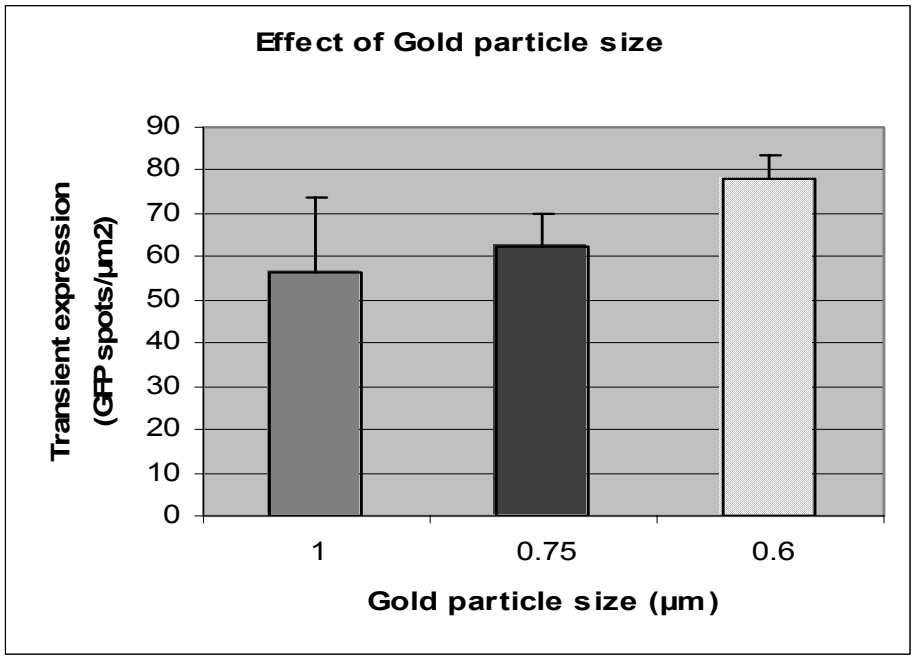


Fig. 5.1.1.5. Effect of gold particle size on transient expression of p524EGFP.1

Earlier Schopke *et al.* (1997) reported that the transient expression varies with particle size and further concluded that the smallest particle size, i.e. 1.0 µm was best. Randolph-Anderson *et al.* (1995) measured a 5 to 7 fold increase in anthocynin-expressing stable sectors of maize Type I callus bombarded with 0.6µm gold particles rather than 0.1µm gold particles. Further they suggested that the increase in stable sector recovery was due to the increased survival rate of cells bombarded with the smaller gold particles. But later Rasco-Gaunt *et al.* (1999) demonstrated that particle aggregation during precipitation is less pronounced with 0.6 µm particles than with larger (1.0- 1.2 µm) particles. Moreover, the use of smaller particles is expected to minimize tissue damage. The size of the particle chosen for biolistic transformation usually should be based upon the size of the target cells. As a rule of thumb, particles should be roughly one-tenth of the diameter of the cell (Sanford *et al.* 1993). The peanut tissue we used for bombardment experiments averaged between 0.5 to 1.0 mm.

Effect of helium pressure (psi) and Chamber vacuum pressure on transient expression

Vacuum/Residual gas

Helium has remained a choice gas in most of the guns used for bombardment of biological samples. Because of the lightness and low molecular weight, helium reduces the possible drag observed due to deacceleration of microprojectiles as well as transmission of potentially damaging shock waves,

which otherwise affects the efficiency of biolistic transformation. Most commonly, as much of the overlying air is removed with a vacuum pump as is practical, to reach a vacuum pressure of 28-29 in Hg (about 710-740 mm Hg). Higher vacuum pressures are not generally practical because of residual water vapor pressure from the biological sample itself (Sanford *et al.* 1993). We tried two possible vacuum pressures, i.e. 27 and 28 inches Hg, which can be used for DNA delivery with the PDS-1000/He gun. We found that 28 in. Hg is more efficient for peanut transformation (Fig. 5.1.1.6.). While working with wheat, Rasco-Gaunt *et al.* (1999) also reported similar observations. In the present experiment, somatic embryos were bombarded with three different rupture disc pressures. Based on the highest level of transient expression, we concluded that 1800 psi gas pressure was most appropriate for peanut calluses when used for transformation experiments (Fig. 5.1.1.7.). Earlier while working with peanut, Livingstone and Birch (1995) have also demonstrated similar observations. Confirmatory results were also produced later in other species like cassava (Schopke *et al.* 1997) and maize (Kemper *et al.* 1996). Contradictory result showing low acceleration pressures resulted in larger areas being covered by particles than higher acceleration pressures were reported by Rasco-Gaunt *et al.* (1999) in wheat tissue.

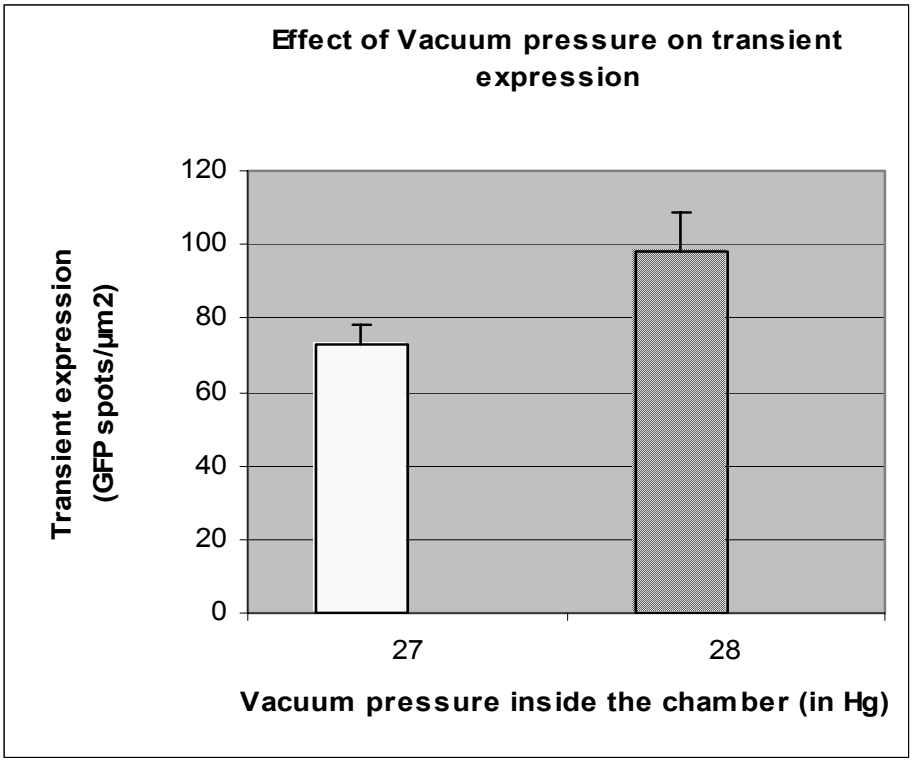


Fig. 5.1.1.6. Effect of vacuum pressure on transient expression of p524EGFP.1

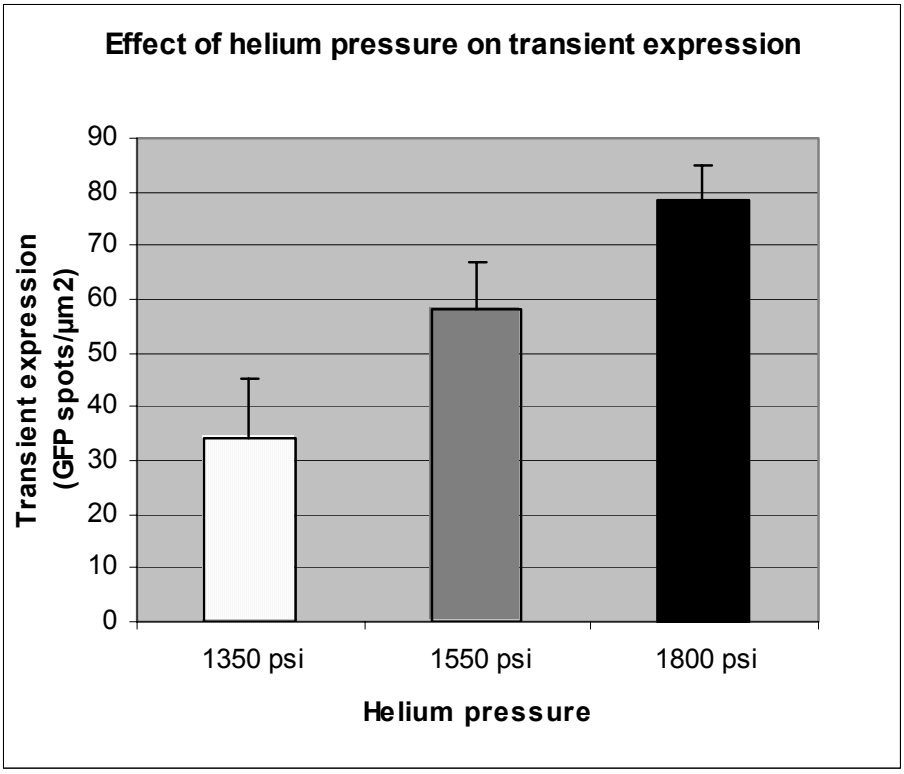


Fig. 5.1.1.7. Effect of helium pressure on transient expression of p524EGFP.1

Effect of osmoticum on transient expression by using different constructs

The effect of a short-term osmotic pre-conditioning (plasmolysis) of target cells or tissues on transient and stable transformation has been reported in several studies (Iglesias *et al.* 1994; Perl *et al.* 1992; Altpeter *et al.* 1996). Non-metabolisable osmotica such as mannitol, sorbitol and PEG or combinations of these are commonly used at osmotic levels ranging from 0.25M to 0.5M (Perl *et al.* 1992; Zhou *et al.* 1995; Altpeter *et al.* 1996; Ortiz *et al.* 1996).Short-term high osmotic treatments, typically for a few hours before or after bombardment, are thought to reduce turgor pressure, preventing the cells from leakage and bursting (Sanford *et al.* 1993). In addition, part of the increase in transient transformation rates could also be due to the penetration increment by itself, reaching more viable target cells (Kemper *et al.* 1996). We evaluated the effect of air-drying and exposure to mannitol (0.4M) on transient expression for three different GFP constructs viz. p524EGFP.1, EGFP and EYFP. The three different constructs used were expressed under two different promoters. Pre incubation of somatic embryos on osmoticum (0.4 M mannitol) resulted in an increase of transient expression over that of air-drying for all three constructs (Fig.5.1.1.8.).

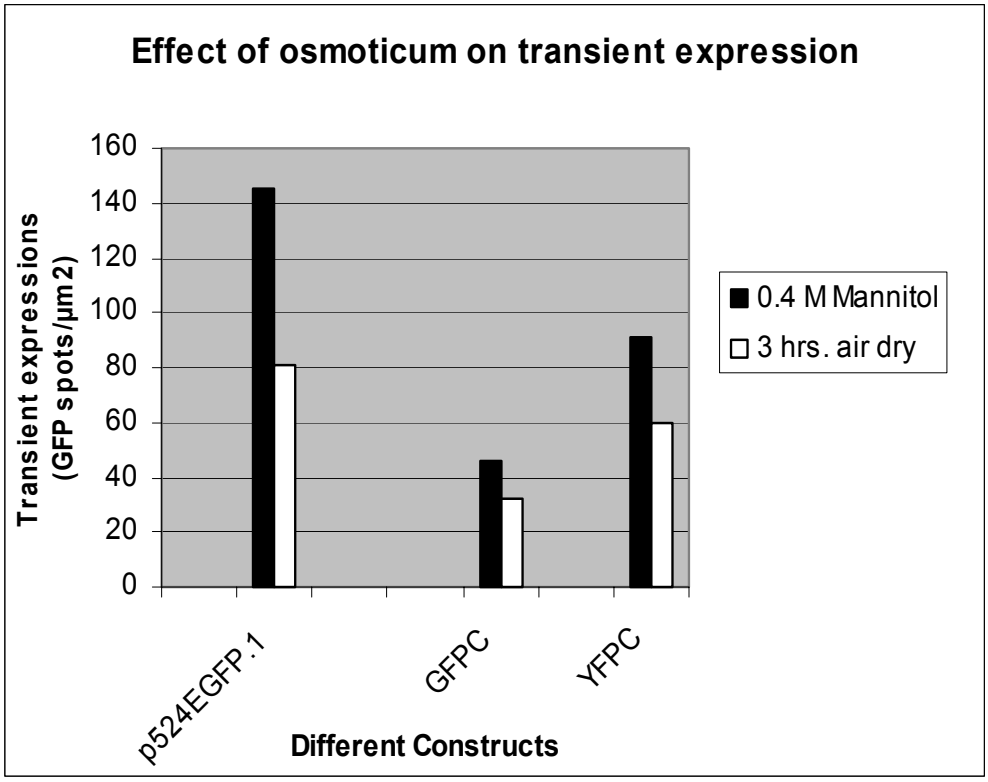


Fig.5.1.1.8. Effect of osmoticum on transient expression of three fluorescent proteins constructs.

The effect of osmoticum in improving particle penetration could be associated with the reduction in cell turgor pressure by mannitol, allowing particles to penetrate deeper in the cell layers (Kemper *et al.* 1996). These observations are in accordance with those reported by Livingstone and Birch (1995), who used sorbitol (0.2M) and mannitol (0.2M) as an osmoticum in peanut. It has been well demonstrated earlier that pre-treatment with osmoticum, added to the bombardment medium can enhance the rate of the transient transformation (Sanford *et al.* 1993; Vain *et al.* 1993; Schopke *et al.* 1997; Nandadeva *et al.*, 1999). In our experiments, mannitol treated cultures were more stable even up to 10 days as evidenced by the presence of almost 10-20% of the prominent transient spots. Transient expression from air dried tissues was reduced to 2-5% after 10 days with subsequently diminishing color intensities.

5.1.2. Stable transformation of green fluorescent protein in peanut (*Arachis hypogaea* L.)

Introduction

An optional selectable marker gene for plant transformation would be one that is visually detectable at any time and stage of plant cell growth, without cell disruption, and without the addition of substrates, cofactors or selective agents. In addition the gene product should not adversely affect cell growth, regeneration, or fertility, or impart any selective advantage if transferred to weedy relatives of a transformed crop (Kaemppler *et al.* 2000).

Green Fluorescent Protein (GFP): A perfect reporter gene for transformation studies.

Availability of a suitable reporter gene is a pre-requisite for monitoring transformation events. Reporter genes are nucleic acid sequences encoding easily assayed proteins. They are used to replace other coding regions whose protein products are difficult to assay. Reporter genes can be attached to other sequences so that only the reporter protein is made or so that the reporter protein is fused to another protein (fusion protein). Among the various available and more commonly used reporter genes are those for the following proteins:

Protein	**Activity & Measurement**
CAT(chloramphenicol acetyltransferase)	Transfers radioactive acetyl groups to chloramphenicol; detection by thin layer chromatography and autoradiography
GAL (□galactosidase)	Hydrolyzes colorless galactosides to yield colored products.
GUS (□glucuronidase)	Hydrolyzes colorless glucuronides to yield colored products.
LUC (luciferase)	Oxidizes luciferin, emitting photons.
GFP(green-fluorescent protein)	Fluoresces on irradiation with UV.

Transgenes encoding luciferase, □-glucuronidase (GUS), and anthocyanins have been the most widely utilized visual reporters in genetic transformation systems (McElroy and Brettell 1994; Wilmink and Dons 1993). However, both luciferase and GUS assays are destructive and require addition of a substrate at optimized levels at particular times to detect the enzymes visually. Also, GUS expression

assays are usually toxic to plant cells, and some cereal monocot cultures express an endogenous GUS-like activity, interfering with detection of transgene encoded GUS (Hansch *et al.* 1995; Hodal *et al.* 1992).

Green-fluorescent protein (GFP), one of the hottest biological tools, is responsible for the stunning bioluminescence of the Pacific Northwest Jellyfish*, Aequorea victoria*. GFP emits a green colored light when it accepts blue light from a calcium activated photoprotein in its native host. Since the cloning of the GFP gene in 1992 by Douglas Prasher (of USDA/APHIS) it has been demonstrated that the GFP gene can be expressed in non-homologous species, from yeast and plant cells to Drosophila and vertebrates, including humans (Prakash, 1997). GFP is likely to dominate as the best available reporter system owing to its features like requirement of no substrates (other than oxygen) to fluoresce, and has no physiological effect on cell metabolism. GFP is considered to be the most powerful tool for many of its applications in biological research and commercial biotechnology including: measuring gene expression in vitro, selecting transgenic cells, studying fusion proteins, studying intracellular protein traffic (and thus identifying signal sequences), determining cell lineage, assessing promoter activity, developing cell- and tissue-specific markers, investigating the pathogen movement and disease development, biomonitoring of organisms released into the environment, developing bioindicators for detecting environmental pollutants, ensuring the containment of genetically modified organisms and in evolutionary and ecological studies of transgenic organisms (Prasher, 1995)

Wild type GFP is a 27- kDa monomer consisting of 238 amino acids that has the unique characteristic of emitting green light when excited with UV (360-400 nm) or blue (440-480 nm) light. GFP is the only well characterized example of protein that displays strong, visible fluorescence without any additional substrates or co-factors (Heim and Tsien, 1996). The most attractive character of Green fluorescent protein is, it remains fluorescent when fused to another protein both on the N- and C-terminal ends, which makes it a fluorescent tag to monitor sub cellular activities. At the cellular level GFP is being used as an in vivo reporter gene to assess the frequency of transient and stable transformation (Leffel *et al.* 1997).

GFP, a perfect reporter gene, enjoys many advantages over other candidate genes and can be briefly summarized as:

- A visual selectable marker
- It is a non-destructive scorable marker gene
- No additional proteins, substrates or cofactors required to emit light

- Stable and species independent
- Can partially replace antibiotic selection
- Can identify early transformation events
- Helpful to standardize any new protocol
- Useful for understanding intracellular events in plant cells

Green fluorescent protein: Trafficking earlier cellular events

The utility of GFP in plant transformation and its expression was first demonstrated by Niedz *et al.* (1995) in sweet orange protoplasts using the wild- type *Aequorea* GFP. Observations of Hu and Cheng (1995) in *Arabidopsis* and tobacco and Sheen *et al* (1995) in corn and *Arabidopsis* demonstrated that successful GFP detection is highly dependent on the strength and source of the excitation sources, which encouraged researchers to modify and synthesize it as more suitable for plants (Stewart, 2001). Haseloff *et al.* (1997) reported that a cryptic intron existed in the wild-type *Aequorea* GFP and caused aberrant splicing in plant cells. They ….to alter cryptic intron sites with silent mutations and produced a variant called mGFP4. This mGFP4 was successfully expressed in soybean suspension cultured cells (Plautz *et al.* 1996), *Arabidopsis* (Haseloff *et al.* 1997), tobacco (Stewart 1996) and in many other plants. A synthetic human codon-optimized-GFP with a wild-type chromophore was created by Haas *et al.* (1996) eliminating the cryptic intron. Since human and corn have very similar codon usage, the gene proved to be well expressed in plants and yielded 20 times more fluorescence than the wild-type gene (Chiu *et al.* 1996).

Several other important modifications have been made by different scientists to improve GFP expression, fluorescence, solubility etc. and developed different GFP variants superior for plant transformations (Pang *et al.* 1996; Yang *et al* 1996; Crameri *et al.* 1996). These GFP variants have been extensively used to optimize transformation protocols in different species like wheat (McCormac *et al.* 1998), apple (Maximova *et al* 1998), soybean (Ponappa *et al.* 2000), fescue (Cho *et al* 2000), Canola (Halfhill *et al.* 2001), etc. Elliot *et al.* (1999) proposed use of GFP for the replacement of antibiotic selection in tobacco, corn and lettuce transformation. GFP has been successfully used as a visual selectable marker (Kaeppler *et al.* 2000), for in vivo promoter analysis (Nehlin *et al.* 2000), to optimize the Sonication Assisted *Agrobacterium*-mediated Transformation procedure (Finer and Finer 2000), for plasmid transformation (Sidorov *et al.* 1999; Jang *et al.* 1999), to study protein-protein interaction (Garamszegi *et al.* 1997), to monitor the expression of linked transgenes even at the field level (Harper *et al.*1999; Steward *et al.*, unpublished data), to study gene flow and pollination behavior (Hudson *et al.* 2001), and many more. There are several reports in peanut transformation with reporter

(Ozias-Akins *et al.* 1993; Lacorte *et al.* 1997; Livingstone and Birch, 1995, 1999) as well as economically important genes (Yang *et al* 1998; Magbanua *et al.* 2000; Rohini and Rao, 2001). To the best of our knowledge, there are no reports on GFP as an integrated transgene in the peanut genome. There are reports for use of GFP as a selectable marker in transformation, but reports are very scanty for its utility in stable tissue transformation and subsequent regeneration solely as a visual selection marker (Kaeppler *et al.* 2000).

In our present study we have evaluated three fluorescent protein mutants for their transient expression efficiencies after particle bombardment of embryogenic cultures of the peanut. The fluorescent protein variants, namely, Enhanced Green Fluorescent Protein (EGFP), Enhanced Yellow Fluorescent Protein (EYFP) and Enhanced Cyan Fluorescent Protein (ECFP) differing in their emission and excitation peaks, were used. Several GFP chromophore variants have been developed at Clontech and by other collaborators to improve the utility of GFP as a reporter for gene expression and protein localization. These variants are ideal for fluorescence microscopy and flow cytometry studies. Red-shifted variants (EGFP, EYFP, ECFP) contain mutations in the chromophore that shift the maximal excitation peak to approximately 490 nm. EGFP contains the double-amino acid substitutions Phe-64 to Leu and Ser-65 to Thr (GFPmut1; Cormack *et al.* 1996). Due to an increase in its excitation coefficient (E_m) and a higher efficiency of chromophore formation, EGFP fluoresces 35-fold more intensely than wt GFP when excited at 488 nm.

The EYFP variant contains four different amino acid substitutions that shift fluorescence from green (509 nm) to yellow-green (527 nm). Although EYFP's fluorescence excitation maximum is 513 nm, it can be efficiently excited at 488 nm giving a signal even brighter than that of EGFP. The ECFP variant contains six different amino acid substitutions. One of these mutations, Tyr-66 to Trp, shifts the chromophore's excitation maxima to 433 nm (major peak) and 453 nm (minor peak), and the emission maxima to 475 nm with a smaller shoulder at 501 nm. The other five substitutions enhance the brightness and solubility of the protein in a manner similar to the other EGFP variants.

In order to maximize the net fluorescence and localization, we have used all the three variants of GFP expressed under the general constitutive CaMV35S promoter. In a fourth construct, EGFP was expressed under the control of a double 35S promoter with an AMV enhancer sequence for enhanced expression effects.

Materials and Methods

Plasmid constructs

For the evaluation of transient effects of Green fluorescence protein (GFP) in peanut somatic embryos; three mutants i.e. EGFP, EYFP and ECFP (Fig.5.1.1.3) driven by the CaMV 35S promoter (kindly provided by Dr. Joe Nairn (UGA) and p524EGFP.1 (Fig. No. 5.1.1.2., described in 5.1.1) were used for bombardment. For the stable transformation studies plasmid p524EGFP.1 alone was used when visual selection for transgenic tissues was carried out. For indirect GFP selection plasmid p524EGFP.1 was co-bombarded with a second plasmid (pRT66 cpo-p) carrying a nonheme chloroperoxidase gene and a hygromycin phosphotransferase gene (hph) each driven by the CaMV 35S promoter. The CPO-P gene has been predicted to increase pathogen defense in plants. Dr. Niu Chen and Dr. Ozias Akins' group have used the CPO-p gene in peanut transformation studies in order to isolate potential resistant peanut lines.

Bombardment procedure

Procedures for preparation of explants, method of bombardment, media composition and all other parameters for particle bombardment were followed as per the protocols described previously. Bombardment was done with gold microcarriers 0.6 µm in diameter, 1800-psi helium pressure, 28 in Hg vacuum pressure and 0.4 M mannitol as an osmotic treatment. Observations were taken 48 hours after bombardment and the number of fluorescent spots was counted from photographs taken at a magnification of 100 X. The same procedures were applied for experimental design and data analysis as mentioned earlier. For visual selection small pieces of embryonic callus that showed green fluorescence were separated and sub-cultured every 4 weeks. In the case of co-bombardment soon, hygromycin selection was began 3-4 days after bombardment, callus were put in liquid medium supplemented with 20 mg/l hygromycin. The hygromycin containing medium was changed every two weeks.

DNA Isolation

DNA was isolated from embryonic callus using small pestles to homogenize tissue in a 1.5 ml microcentrifuge tube. According to the brief protocol described here putative transformed tissue (50-100 mg) was homogenized in 700 µl 2X CTAB buffer [2% CTAB, 5% PVP (plyvinylpyrrolidon), 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl, pH 8.0] with 2% mercaptoethanol (freshly added), using the TRI-R STIR-R Model K-41(set on #2 speed). Microfuge tubes were incubated in a water bath for 15 min at 65ºC. Chloroform: Phenol: isoamyl alcohol (24:1:1) 800 µl was added and mixed well by

inversion. Tubes were centrifuged at high speed (10,000 g) for 5 min. The upper aqueous phase was transferred to a new tube and DNA was precipitated with 0.6 vol of iso-propanol by inverting the tubes several times. Tubes were then centrifuged at high speed (10,000 g) for 5 min. Pellets were washed with 70% ethanol and air dried to remove all the traces of ethanol. Pellets were dissolved in 50 µl of sterilized water and treated with 0.5 µl Rnase for one hour at room temperature before use. DNA was later quantified with a spectrophotometer.

Molecular confirmation of Putative Transgenics

Polymerase chain Reaction (PCR)

Primary transformants, obtained through visual as well as hygromycin selection were analyzed for the presence of the GFP gene by the Polymerase Chain Reaction (PCR). DNA was isolated from100 to 200 mg of tissue (as described earlier) and quantified by spectrophotometry**.** A 548-bp ORF of GFP was amplified using a 20-nucleotide sense primer EGFPS1: AAG GGC GAG GAG CTG TTC AC and an antisense primer EGFPAS1: TTC TGC TGG TAG TGG TCG GC

Amplification of GFP was carried out using a Perkin-Elmer Cetus DNA thermal cycler under the following conditions: 95º C for 60 sec (denaturation), 60ºC for 60 sec (annealing), 72ºC for 60 sec (extension), for 40 cycles followed by an extension of 10 min at 72ºC and 4ºC cycle until recovery. The amplified product was assayed by electrophoresis in 1 % agarose (Seakem HGT) gels in 1X TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.2).

Southern Blot Analysis

DNA (10 mg) was digested with *Hind* III, which cuts at only one site in the plasmid p524EGFP.1. Digested DNA was subjected to electrophoresis overnight at 25 V on a 0.8% agarose gel (Seakem HGT) in 1X TBE buffer. DNA was later transferred by the capillary method (Southern, 1975) to a nylon membrane (GreenScreen Plus). For capillary transfer of the DNA fragments to membrane, the gel was soaked in (enough to cover the gel) 0.25 HCl (20 ml of conc. HCl in 1L) for 20 min, and rinsed briefly in water. DNA was denatured by soaking the gel in 0.4 N NaOH for 30 min. Green Screen Plus membrane was allowed to float on the surface of water until it was wet completely from beneath and then was immersed in 0.4 N NaOH for 5 min. Denatured DNA was transferred from gel to the membrane in 0.4 N NaOH . After transfer, the membrane was washed in excess 2x SSC for 1-2 min, blotted with filter paper and baked at 80ºC for 1 hour. The blot was hybridized with a 548 bp PCR amplified, ^{32}P-labeled probe and exposed to phosphor screens overnight which were then read in a Phosphoimager (Packard, Meriden, USA.).

Fluorometric quantification of GFP

The visually selected and cobombarded lines were subjected to fluorometric quantification of GFP. Extraction of protein was carried out according to Remans *et al.* (1999) using a Packard Fluorocount Microplate Fluorometer. Callus of transformed lines (50 mg) was grounded in 1 ml protein extraction buffer (10 mM Tris-EDTA, pH 8.0; 0.02% sodium azide). Extraction buffer was used as control to zero the fluorometer at 485 nm excitation and 530 nm emission wavelengths. The linearity of fluorescence was tested using a dilution series (50-200 µl vol.) of the brightest fluorescent plant sample.100 µl of plant samples were measured for Relative Fluorescent Units (RFU).

Regeneration of transgenic lines

Embryonic calluses stably transformed with GFP were sub-cultured onto MS medium containing NAA (5.37 µM) and 2% sucrose and kept in the dark for the first 3 weeks followed by a week in light. Embryos having well developed shoot-root axes were then transferred to MS medium supplemented with TDZ (9.08 µM) and 2% sucrose under a photoperiod of 16 hr light and 8 hr dark.

RESULTS & DISCUSSION

Transient expression of fluorescent proteins in peanut

Among the four different constructs used, the p524EGFP.1 was found to exhibit the brightest transient expression followed by EYFP. The differences in the fluorescence could be observed through the microscope (Fig 5.1.2.1.). The quantitative data showed a higher number of transiently expressed EYFP spots (Table 5.1.2.1), although it did not differ significantly from the number observed with p524EGFP.1. Interestingly, for the p524EGFP.1 construct, more than 10% of the transiently expressed spots were found to persist even 10 d after bombardment. After evaluation of different promoters using different GFP constructs during sweet orange transformation studies, Fleming *et al.* (2000) also demonstrated the efficacy of this construct. Their results have also demonstrated the utility of the p524EGFP.1.1 construct containing a double CaMV 35S promoter along with AMV enhancer for stable transformation. Furthermore, they indicated that the GFP protein accumulation may not be sufficient for detection in multicellular structures when the same EGFP was driven by an unenhanced CaMV 35S or FMV promoter. For further stable transformation experiments by visual selection as well as cobombardment, only the p524EGFP.1 construct was used.

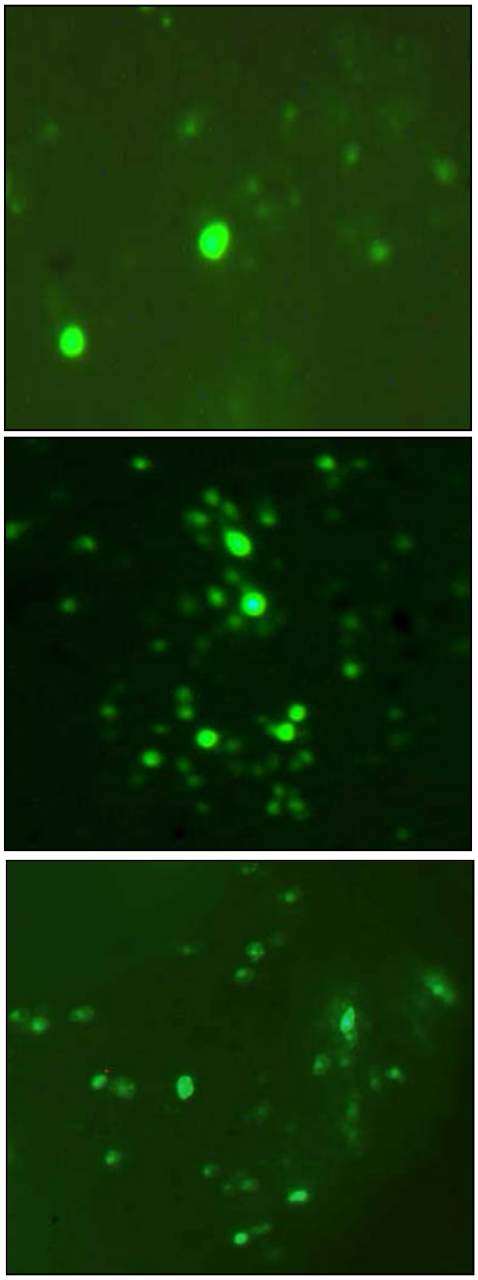


Fig. 5.1.2.1 Different GFP constructs varied in their brightness 48 h after bombardment

In our study, little transient expression was detected from ECFP, perhaps because the filter set was not optimal. Alternatively, EYFP was found to be the best for transient expression among the three

fluorescent constructs containing the CaMV 35S promoter. Both EYFP and p524EGFP.1 would be good candidates for fluorescent protein expression in peanut.

Table. 5.1.2.1. Different GFP transient expression after 48 hours and their stability After 10 d.

Different Constructs	Mean	Duncan grouping	expression remaining after 10 days
EYFP	61.63	A	5%
p524EGFP.1	57.71	AB	10%
EGFP	40.93	B	2%
ECFP	1	C	-

*Spots were counted on photo prints taken at a standard magnification after 48 hours of bombardment. Each experiment was repeated three times with a minimum of three replications.

Stable transformation of p524EGFP.1

Visual selection

Green fluorescent spots were visible in peanut somatic embryos as early as 24 hours after bombardment (Fig 5.1.2.2.). Transient GFP expression was apparent on the surface of embryonic tissues with maximum brightness at 2nd day and continued till 4th day of bombardment. More than 80% transient expression were completely extinguished after 10 days, leaving small, dimly fluorescent sectors of cells on few number of embryos. Only those very prominent spots observed visually continued to stay till even upto three weeks.

After transient expression data were recorded, cultures were monitored periodically under the fluorescence microscope for larger GFP-expressing sectors. Small fluorescent parts of embryos were found to carry stably transformed GFP within the first 6-7 weeks. Due to the relatively small size of these sectors, further separation and handling of transformed regions was almost impossible. Instead, tissues were allowed to grow without segregation for the next 2 months and were separated when they were big enough to work with (Fig 5.1.2.3.). Those embryos showing sectors of GFP expression, when allowed to grow, gradually resulted in completely transformed somatic embryos (Fig 5.1.2.4. A-D).

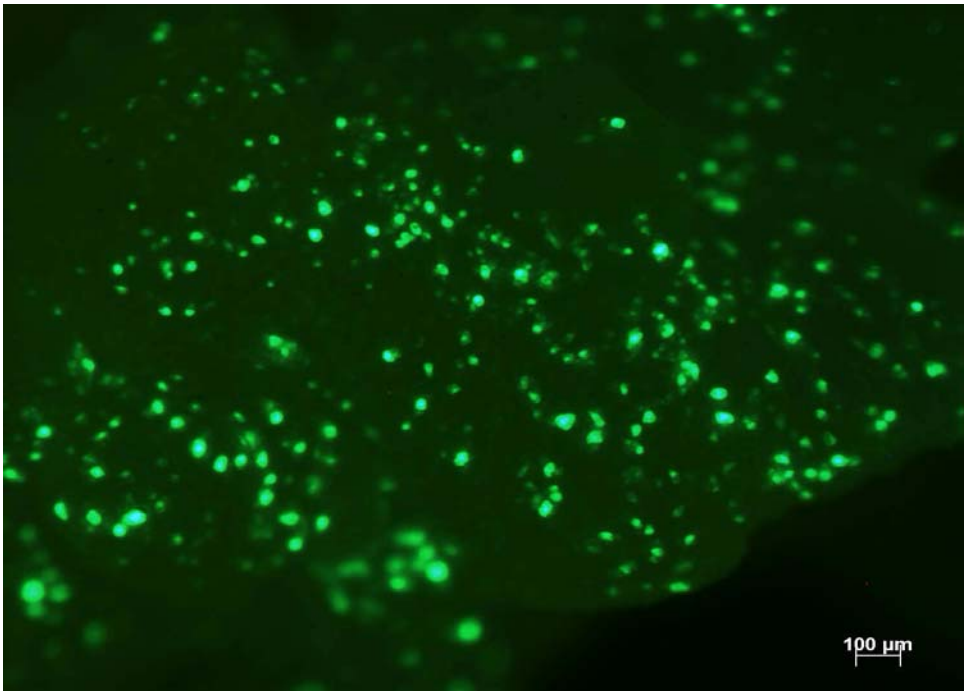


Fig.5.1.2.2. Transient expression of P524EGFP.1 48 h after bombardment

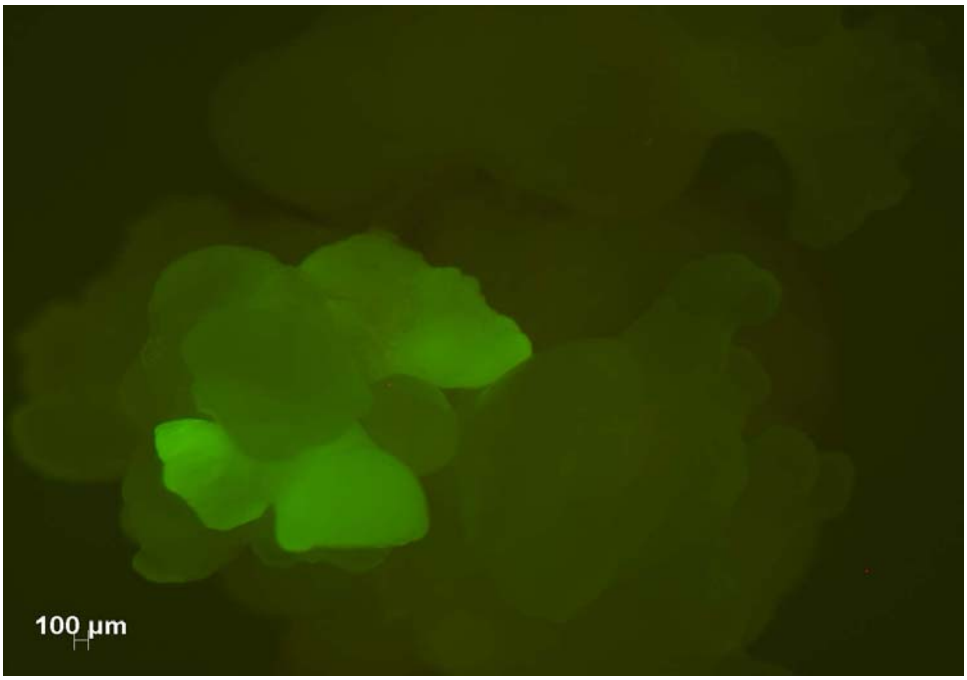


Fig.5.1.2.3. Initiation of stably transformed GFP embryos after 4 weeks of bombardment

Two lines of transformed peanut were visually selected from two separate bombardment experiments. Like that of the conventional somatic embryo development cycle, these embryos were monitored for

various embryogenic events. To the best of our knowledge, these observations are the first in peanut where transformed cell lines can be visually screened right from embryo development. Our observations showed all the developmental stages of embryogenesis in these stably transformed lines (Fig 5.1.2.5. A-D). Stability of GFP expression throughout the embryo development cycle makes GFP a perfect reporter gene to understand many early cellular events. Some of biochemical pathways, often associated with growth regulator signaling, may be specific for cellular differentiation processes during embryogenesis. Many of the genes are thought to get upregulated as well as some getting silenced during embryo development. GFP as a marker gene would be useful to study gene regulation in peanut. The genetic analysis of embryogenesis usually gets hampered by the shortage of morphological markers. This limitation can be overcome by using molecular markers, i.e. reporter genes like GFP expressed in specific regions of the developing embryo. Earlier Imlau *et al.* (1999) studied macromolecular trafficking within the sieve element–companion cell complex, phloem unloading, and post-phloem transport using green fluorescent protein (GFP).

During the process of regeneration, GFP expression was more pronounced in developing root cells (Fig 5.1.2.6 A, B.). Expression in shoot tissues was often confounded with fluorescence from other plant pigments like chlorophyll. In leaves, because of the chlorophyll development and its red fluorescence, GFP often showed a pink to pinkish-yellow color. The masking of GFP expression by other pigments made it difficult to screen at the plantlet stage for true transformants. If observed critically, GFP transformed plants can be distinctly identified from those of non-transformed by monitoring the dark red color of non transformed leaves under UV light. Such color variations in GFP transformed plants were also observed earlier in tobacco (Molinier *et al.* 2000). From visually selected lines, 20-25 plants were regenerated.

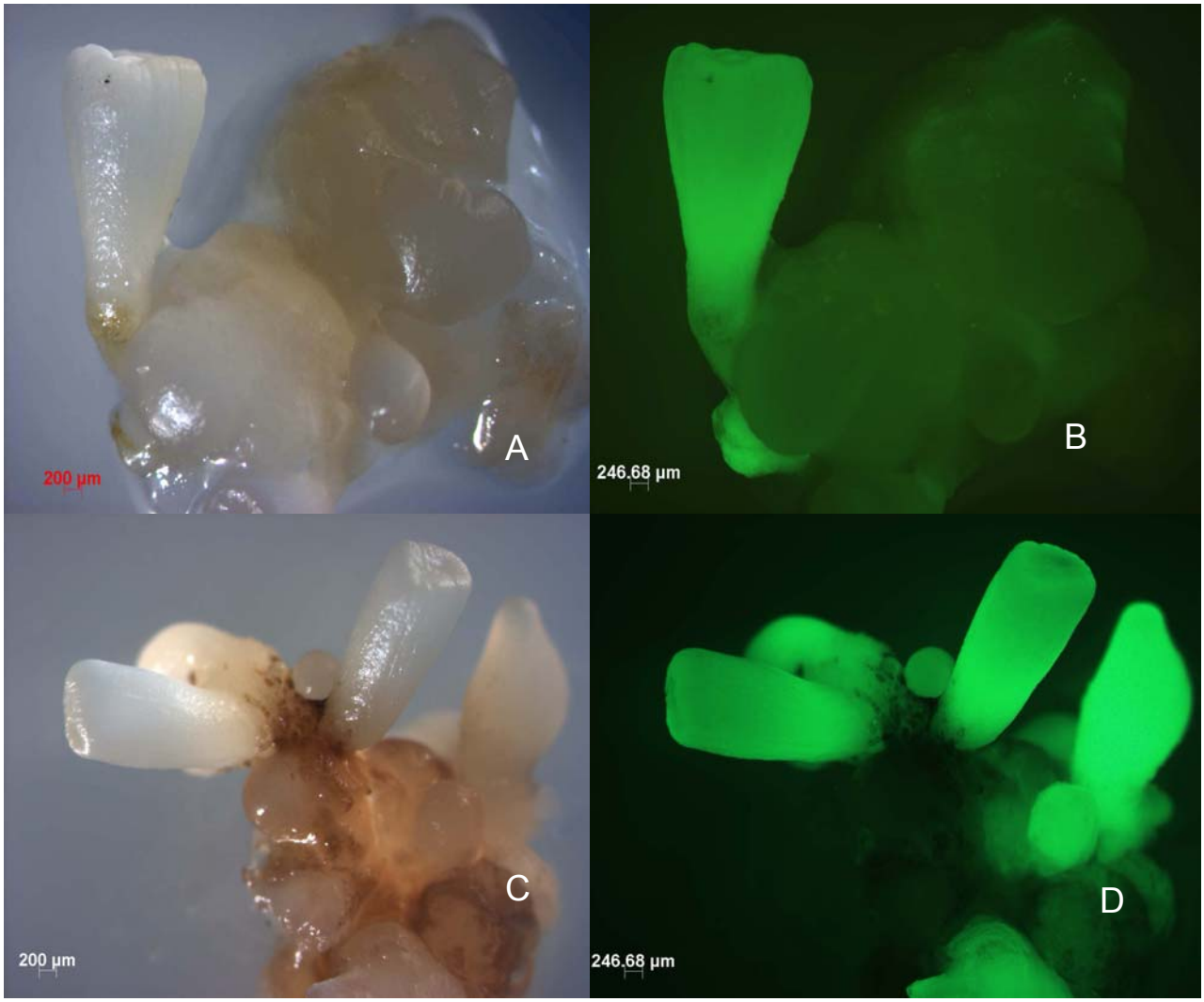


Fig.5.1.2.4. Peanut embryo transformed with GFP, showing GFP expression in A) Visual light, B) in UV light ; Multiple embryos C) in visual light, D) in UV light

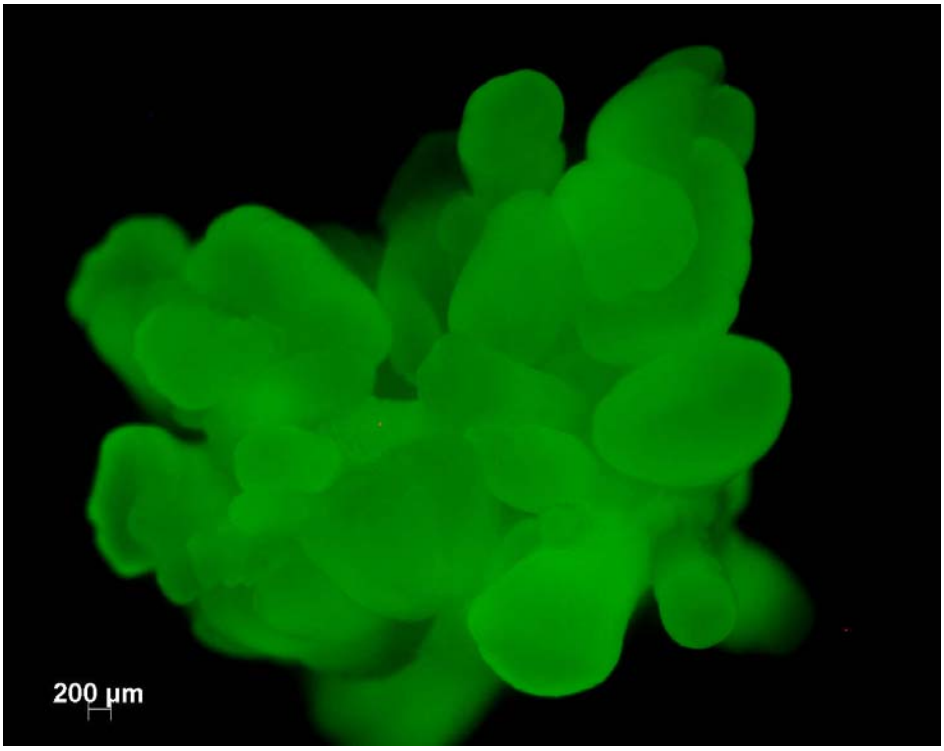


Fig5.1.2.5.A. Transformed embryos proliferation via repetitive embryogenesis

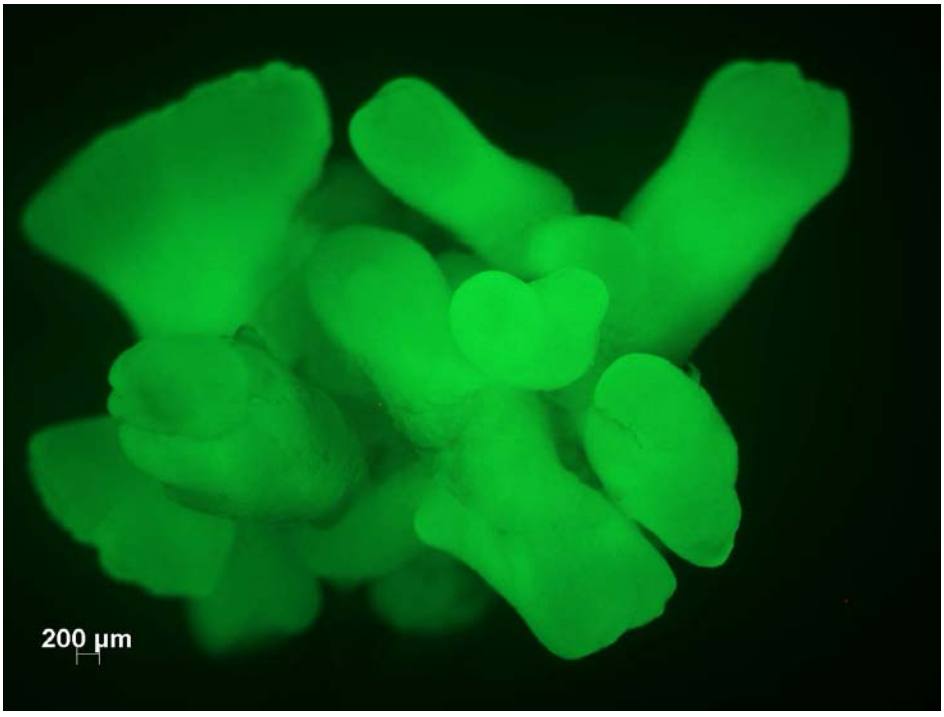


Fig.5.1.2.5.B. Various embryogenic stages of transformed peanut embryos expressing GFP in UV light

Fig.5.1.2.5.B. Developmental stages of transformed peanut embryos expressing GFP in UV light

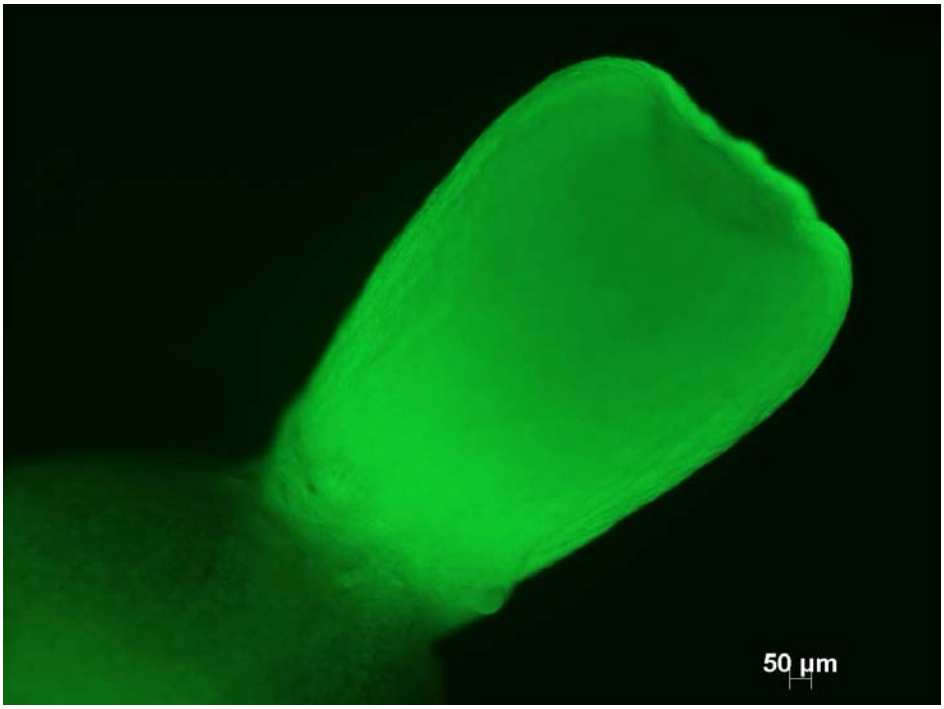


Fig.5.1.2.5.D. Single GFP transformed embryo for rooting

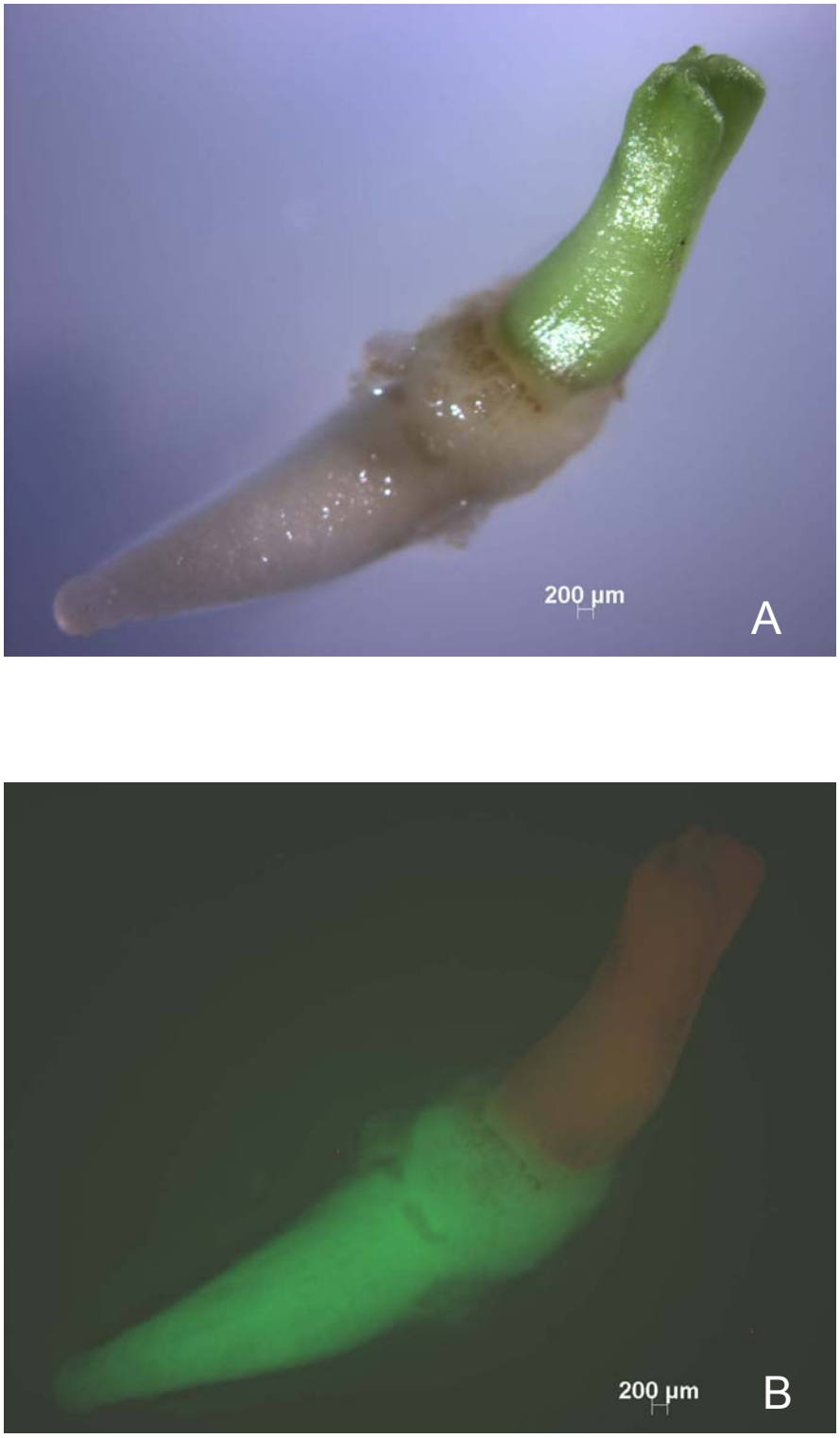


Fig.5.1.2.6. rooting of transformed embryo in NAA (5.37 µm) medium : plumule in green color in visual light and B) light pink color in UV light. Radicle is green expressing GFP in UV light.

The presence of sectors of transformed and non-transformed tissues during visual selection posed problems in transgenic screening. Due care was taken to avoid any mixing up of cell lines with non-transformed cell lines There is always a competition between growth of transformed and non-transformed tissues which was also observed by Elliott *et al.* (1999) in sugarcane. Chimeric calluses and embryos can occur which reduced the GFP brightness in our visually selected tissues. We have dissected the less bright GFP embryos and found layers of GFP transformed and non-transformed cells (Fig 5.1.2.7.). Where else, in case of bright GFP lines from hygromycin selection, all cells layers were found fully GFP expressed (Fig 5.1.2.8.). This made us to conclude that because of this competition of non-transformed tissue growth there is a chance to get less bright GFP lines and ultimately chimeric GFP callus.

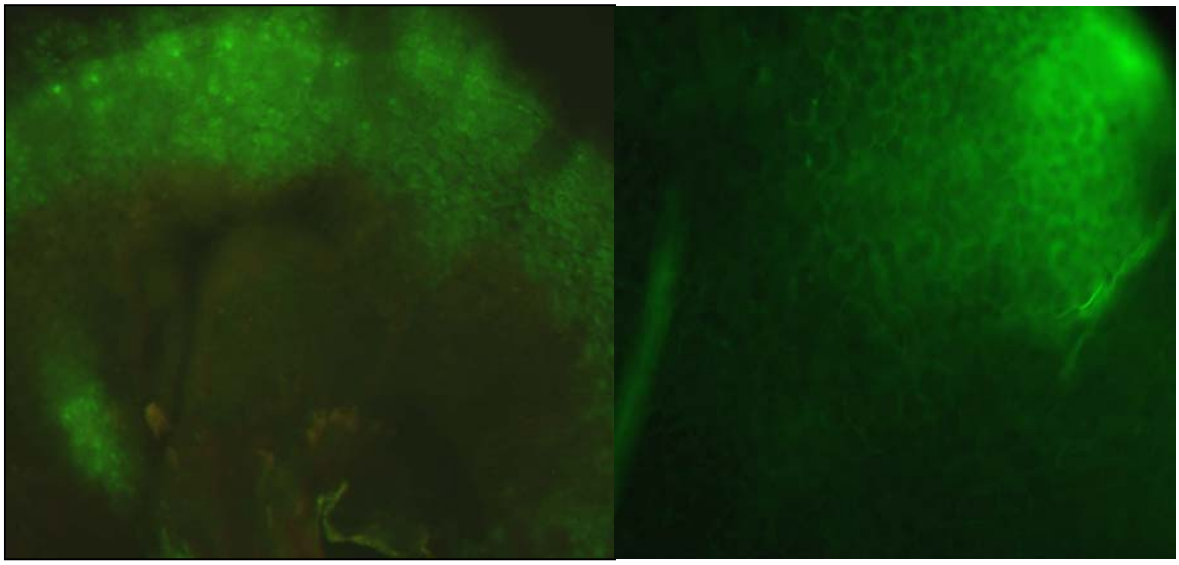


Fig 5.1.2.7. Layers of GFP transformed and non-transformed cells in visually selected transformed lines

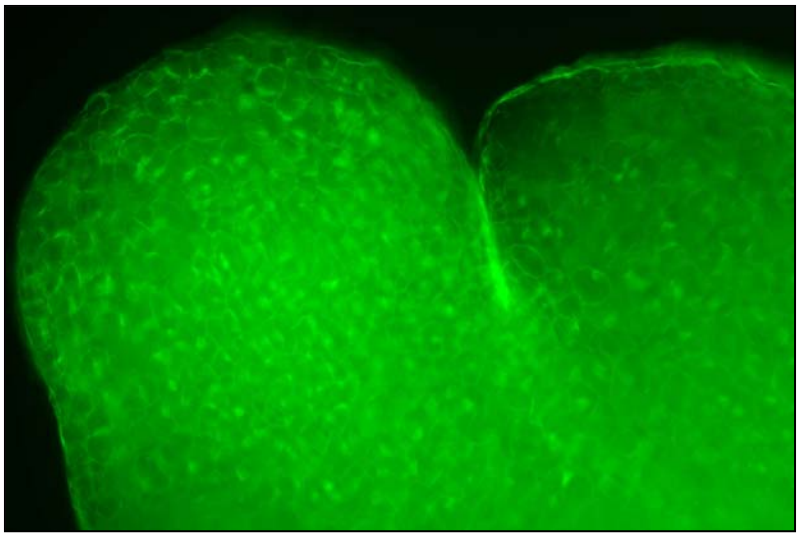


Fig 5.1.2.8. Fully GFP expressed layers of cells in hygromycin selected lines

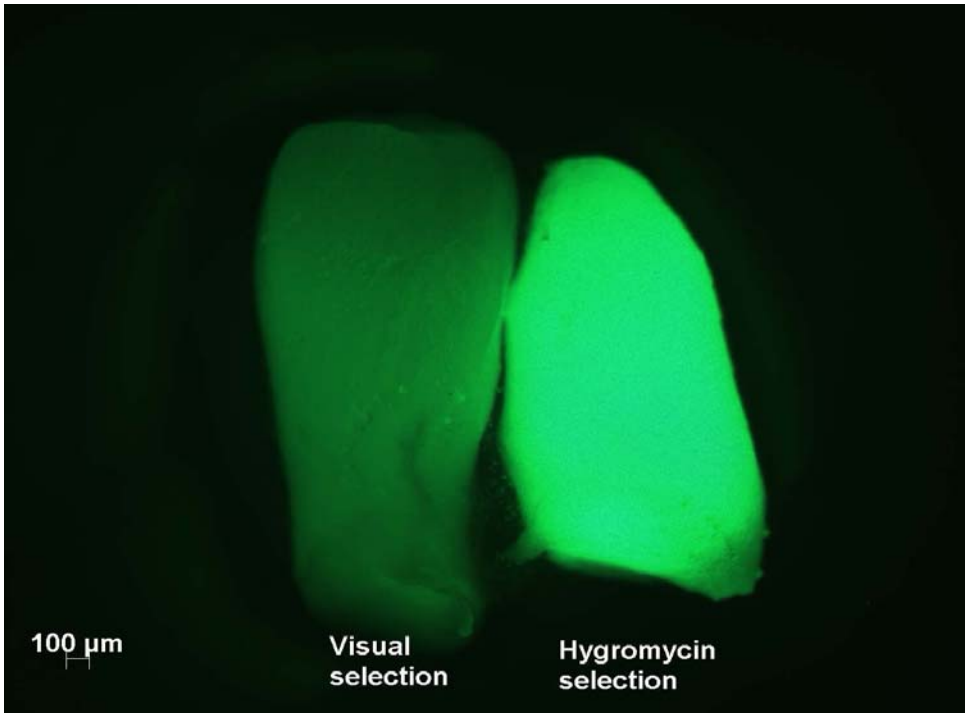


Fig 5.1.2.9. Difference in GFP brightness among visually selected and hygromycin selected embryogenic callus

Hygromycin selection

Under hygromycin selection (20mg/l), seven different cell lines resulting from co-bombardment experiments were isolated. Such a high frequency under antibiotic selection was not

unexpected. Jordan (2000), while working with wheat transformation using GFP as a visual marker, also observed such a high transformation frequency with antibiotic selection. He further concluded that it was very likely due to easier identification of later germinating transgenic embryos which otherwise would have become masked by the massive outgrowth of non-transformed tissues during visual selection. Similarly, Elliott *et al.* (1999) used GFP expression for the early identification of transformed sugarcane cell lines under antibiotic selection. They found that the antibiotic selection along with visual selection enabled the removal of untransformed tissue at an early age and facilitated the identification of transformed cell clusters. We found that the hygromycin-selected peanut lines were initially brighter than the visually selected lines (Fig 5.1.2.9.). The findings of Elliott *et al.* (1999) also suggest that for visually selected lines it was difficult to maintain the preferential growth of green-fluorescent cells in culture, even with regular selective subculturing to remove non-transformed cells.

GFP Quantification

Transformed lines with different GFP brightness recovered from both visual and hygromycin selection were subjected to fluorometric quantification to estimate the GFP protein expression. GFP protein extracted from the brightest lines was used to determine the appropriate dilution. A dilution series ranging from 50 to 200 µl of GFP protein in extraction buffer were scored using the Packard Flourometer and expressed as Relative Fluorescence Units (RFUs). From the RFU readings of different lines (Fig 5.1.2.10.) it was evident that control plants (non-transformed callus extract) had

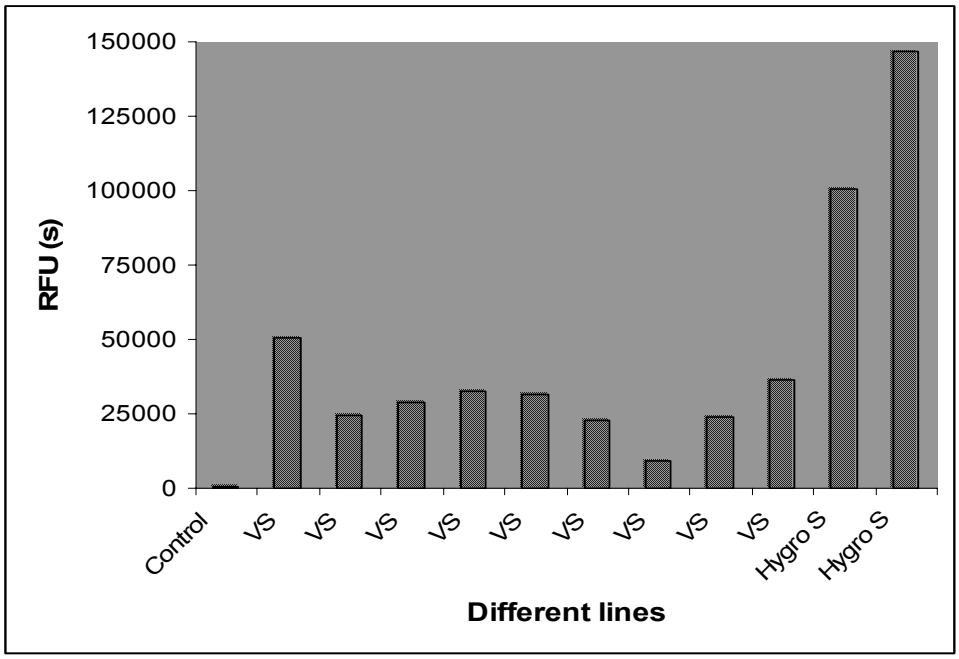


Fig 5.1.2.10. Expression of GFP as Relative Fluorescence Units (RFUs) from different lines (Control= non-transformed; VS=Visual selection; Hygro S= Hygromycin selection).

very low RFUs as compared to GFP transformed lines. Transformed lines with different brightness showed different RFUs which can be correlated with visual observations. The hygromycin-selected lines exhibited the highest RFUs compared to those of visually selected lines. These observations led us to conclude that the fluorometric data shows the same classes like that of visual selection and are strongly correlated with GFP expression level. Leffel *et al.* (1997) while working with tobacco plants also observed different classes of fluorescence in transformed plants and correlated the fluorescence level with the amount of GFP.

Integration of transgenes

The presence of a 548 bp GFP fragment in peanut genomic DNA was confirmed by PCR amplification. Total six lines of transgenic peanuts derived by both visual (VL) and hygromycin (HL) selection were found positive for GFP amplification. No amplification was observed from control, non-transformed tissue (NT) (Fig 5.1.2.11.).

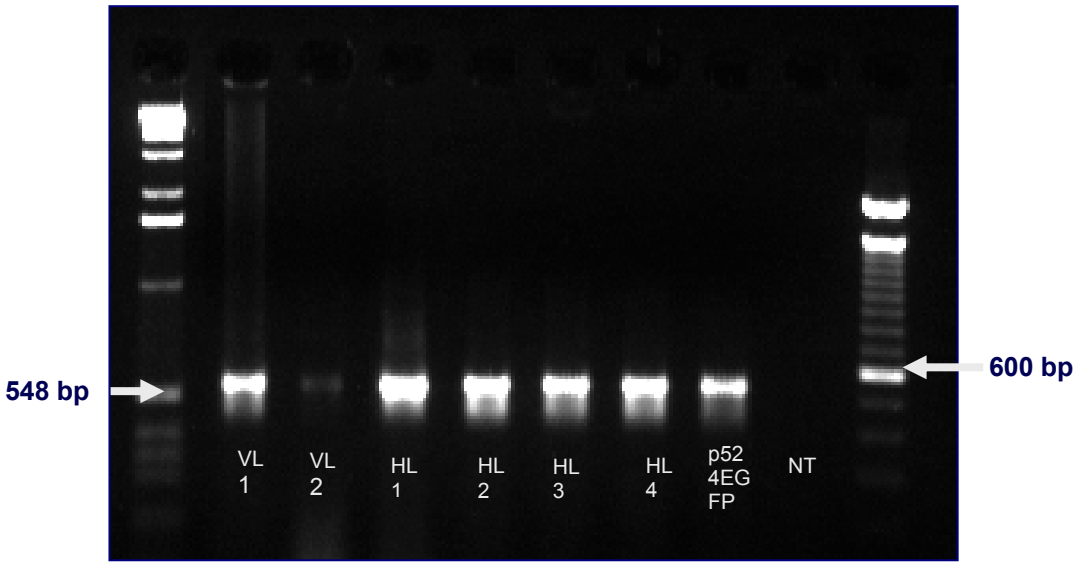


Fig 5.1.2.11. An ethidium bromide-stained gel showing amplification of the expected 548 bp fragment of the GFP gene from 6 transgenic lines. No amplification was observed from the controlled (NT: Non-transformed) callus DNA.

The GFP lines with different brightness isolated from visual selection and one from hygromycin selection were further screened by Southern blot to confirm integration and to estimate the

number of copies of GFP. When digested with *Hind*III restriction enzyme, multiple bands, both larger and smaller than the 4.7 kb GFP hybridizing fragment from plasmid, were observed (Fig 5.1.2.12.). These results indicate the presence of multiple copies at a single locus or a multiple loci. Deletions, duplications, and other physical alternations of the transforming DNA are common in biolistic approach and are likely the cause of the additional hybridization bands seen here. Visually selected lines showing the identical integration patterns necessarily indicate a single transformation event.

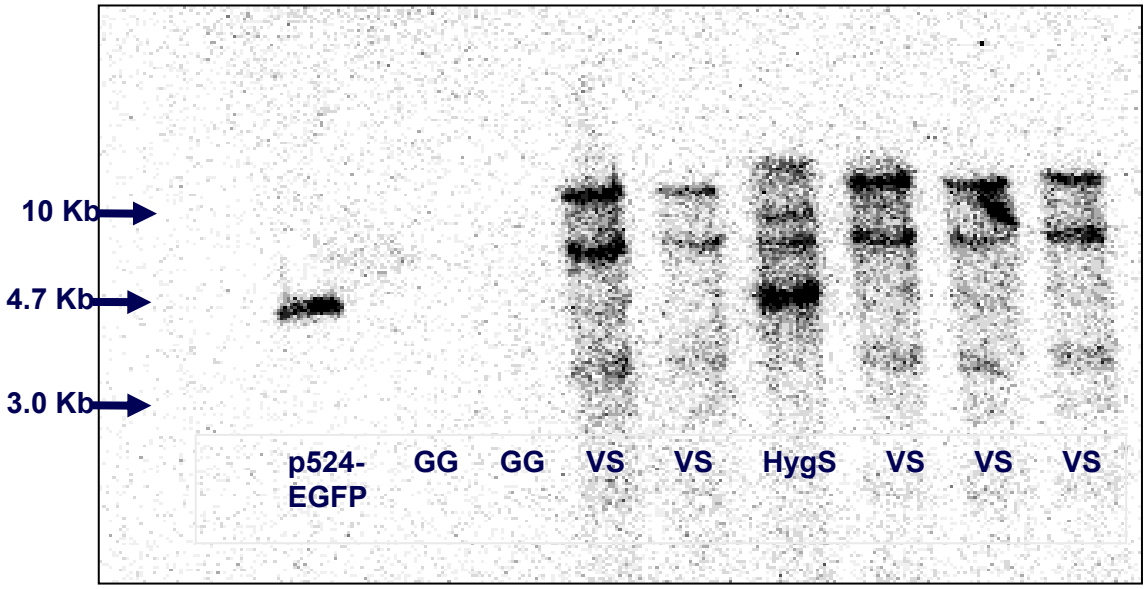


Fig 5.1.2.12. Southern blot analysis of *Hind* III digested genomic DNA from transgenic peanut plants using ^{32}P-labeled p524EGFP.1.1 as a probe. P524EGFP.1 is the 4.7 kb plasmid DNA, VS and HygS represents transgenic lines by visual selection and Hygromycin selection respectively; GG (Georgia Green) is the controlled non-transformed lines.

The co-bombarded GFP lines were found to be positive for *CPO-p* gene integration when screened by PCR, Southern and Northern blot analysis (data not shown). Molecular confirmation of CPO-p insert integration along with reporter gene, GFP was very important for estimating the frequency of cotransformation of linked and unlinked genes. In co-bombardment experiments we found a very high frequency of co-transformation and co-expression of both the genes. In microprojectile bombardment, where genes are frequently introduced via separate plasmids, application of GFP as a secondary marker has a great importance. Harper *et al.* (1999) showed the importance and application of GFP as a marker to monitor the expression of a Bt *cry1Ac* gene at field level.

Regeneration of transformed plants

Plants were regenerated from both visually and hygromicin-selected lines. Expression of GFP in regenerated lines was visually confirmed at the time of shoot initiation. (data not shown). Although GFP expression could be visually detected in all tissues, it was more pronounced in tissues like root tips containing very low amounts of chlorophyll.

GFP-transformed plants were regenerated on MS medium containing TDZ (2mg/l). Morphologically, GFP-expressing plantlets appeared the same as non-transformed plantlets (Fig 5.1.2.13.). To the best of our knowledge, this is the first report where GFP has been transformed into and expressed in peanut. The new selection system based solely on GFP expression can be utilized to efficiently produce transgenic peanut plants.

Fig 5.1.2.13. hardened peanut plantlets transformed with GFP.

Conclusion

In the history of transformation research several reporter genes have been used over a period of time. The ability to non-destructively visualize transient and stable gene expression has made Green Fluorescent Protein (GFP) a most efficient reporter gene for routine plant transformation studies. In our

study we found that GFP is a very useful visual marker to identify early transgenic events. By using this characteristic we have standardized different bombardment parameters like size of gold particle, effect of helium pressure, chamber pressure and effect of osmoticum.

However, the success of stable tissue transformation and subsequent regeneration of transgenic plants harboring GFP varies with different forms of GFP and the target plant species. In order to optimize a peanut transformation system using GFP as the selectable marker, we evaluated three fluorescent protein mutants for their transient expression efficiencies after particle bombardment of embryogenic cultures of the peanut cultivar, Georgia Green. The fluorescent protein variants used in the present study (Enhanced Green Fluorescent Protein (EGFP), Enhanced Yellow Fluorescent Protein (EYFP) and Enhanced Cyan Fluorescent Protein (ECFP)) differed in their emission and excitation peaks expressed under CaMV 35S promoter. A fourth construct expressing EGFP under double 35S promoter with an AMV enhancer sequence also was compared. The brightest fluorescent signal was observed from the construct containing EGFP driven by the enhanced double 35S promoter. Bombardments with this construct produced tissue sectors expressing GFP that could be visually selected under the fluorescence microscope over multiple subcultures. Embryogenic lines showing stable expression of GFP over an eight to twelve month period have been obtained. Over a long period lower GFP brightness has been observed in visually selected lines. This may be due to a mixture of transgenic and non-transgenic cells. Plants were regenerated from those transformed embryogenic lines. We concluded that stable transformation of GFP in peanut by visual selection was successful.

5.1.3. Transformation of peanut with the reporter gene ß-glucuronidase (*gus*) and Hygromycin resistance as a selectable marker

Introduction

Earlier results from this study have demonstrated that GFP can be effectively transferred to peanut tissues using the particle gun and can be stably expressed. GFP will serve as a perfect visual marker for many cellular and molecular studies in early embryonic events in peanut in-vitro culture. On the other hand, ß-glucuronidase (*gus)* also has been a widely used reported gene, particularly where equipment to visualize fluorescence is not affordable. It was found that the transformation frequency could vary with plasmid construct and promoters used in microprojectile bombardment (Wang *et al.* 1998; Kim *et al.* 1999). On many occasions, the expression of the foreign gene mobilized can be system specific and it is more important to monitor its specificity based on reporter gene used. Mantis and Tague (2000) showed that GUS and GFP governed by the same promoter could vary for their expression in same bombardment setup. GUS is the most exclusively used, reliable and favorite reporter for many of the molecular biologists. Though there are several reports of its stable transformation in peanut, like that of on any other species, standardization of any new transformation protocol needs a stable reporter like GUS (Wang *et al.* 1998; Deng *et al.* 2001). Using a standard pre-established biolistic protocol from our lab (Ozias-Akins *et al.* 1993), the present study was conducted with a plasmid carrying GUS as a reporter gene and a hygromycin phosphotransferase (*hph*) gene (resistance to hygromycin) as a selectable marker.

Materials and methods

Plant materials and Bombardment

Somatic embryos and embryogenic callus were used as explants for bombardment. Plant materials and all other bombardment parameters were kept same like that of the one described earlier for GFP transformation (section 5.1.).

Selection and regeneration of putative transformed cell lines

Transient expression of GUS was monitored 48 hr after bombardment using a histochemical GUS assay. Bombarded cultures were transferred to liquid selection medium (same as that described for embryo culture medium) supplemented with 20 mg/l hygromycin 3-4 days after bombardment. Medium was subsequently changed at least weekly, for the first three subcultures, and then later changed at an interval of two weeks. During liquid selection, the cultures were kept on a shaker at 110 rpm in dark. Consequently, hygromycin resistant embryogenic tissues appearing after 6-8 weeks were transferred to solid embryo culture medium with 20 mg/l hygromycin, followed by routine subculture onto fresh medium at an interval of 4 weeks. Later, a three-step regeneration protocol (Ozias-Akins *et al.* 1993) was followed to obtain plants from stably transformed somatic embryos.

Histochemical GUS assay

GUS expression was assayed by placing the tissue in a GUS staining solution (Jefferson, 1987) which consisted of 5 mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.5% (v/v) Triton X-100, 0.005% 5-bromo-4-chloro-3-indolyl ß-D-glucuronidase (X-gluc) in 100 mM sodium phosphate (pH 7.0), and incubating overnight with gentle agitation at 37ºC. The blue foci were observed under the microscope to confirm the transient expression of the GUS gene.

Plasmid construct

Plasmid pCAMBIA1301 (from CAMBIA, Australia) was used for the present study. The plasmid carries, a *gusA* gene with an intron and a hygromycin phosphotransferase gene (*hph*) that confers resistance to the antibiotic hygromycin. Both genes are driven by the CaMV 35S promoter. (Fig.5.1.3.1.).

Molecular confirmation of Insert Integration

Ten putative transgenic lines were selected on hygromycin-containing medium. Six of these were tested for the presence and integration of the hygromycin resistance gene by the Polymerase Chain Reaction (PCR) and Southern blot analysis. Protocols for isolation of DNA, PCR and Southern blotting were same as those described earlier in GFP transformation section (5.1.2.).

A 417-bp fragment from the *hph* ORF was amplified using a sense primer H706 (5'-GAA TTC CCC AAT GTC AAG CAC TTC CG-3') and an antisense primer H705 (5'-ATA TCT CCA CTG ACG TAA GGG ATG ACG-3'). The PCR amplification was carried out using a thermal cycler

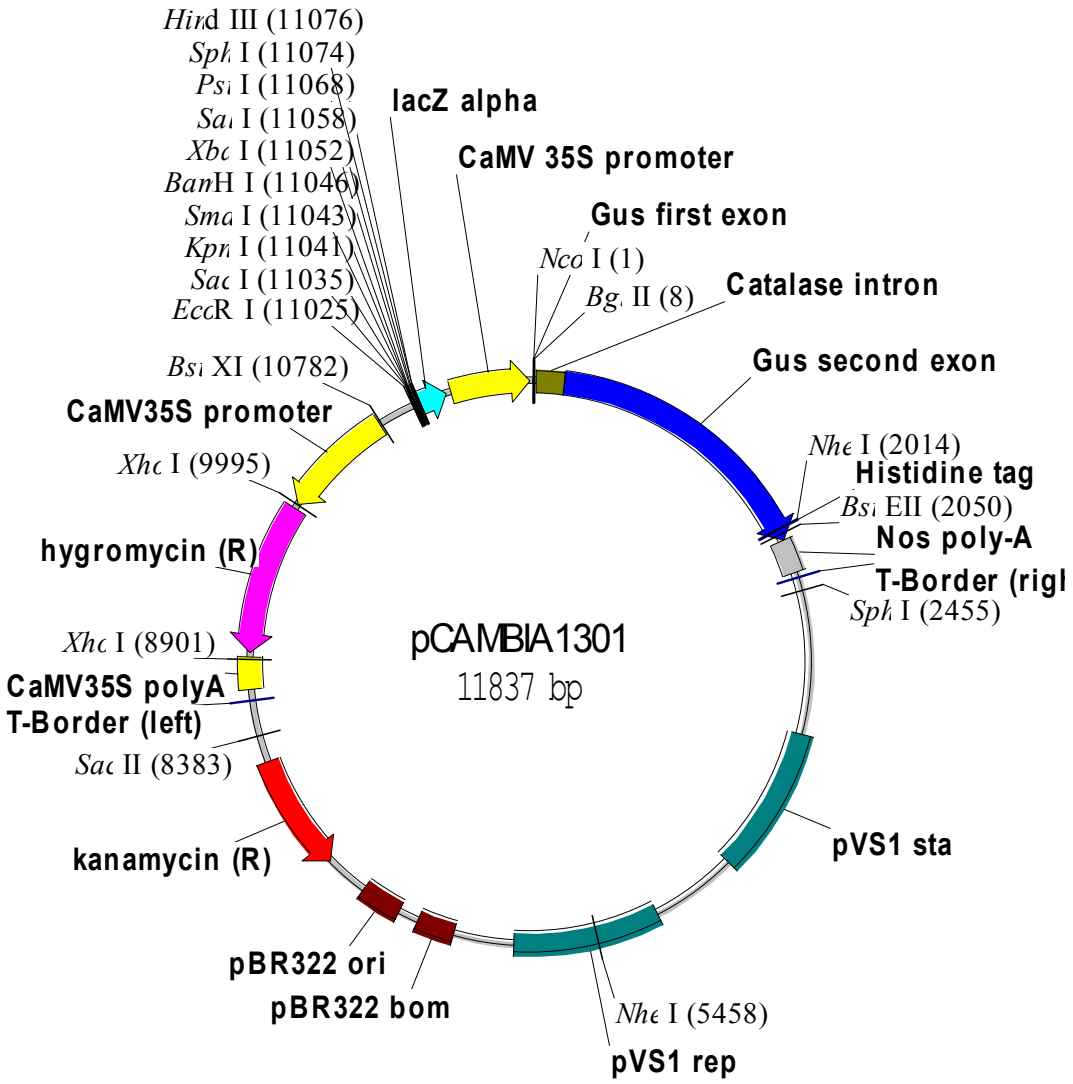


Fig.5.1.3.1. Map of plasmid pCAMBIA1301 containing the selectable marker gene for hygromycin resistance and the reporter gene, *GUS*, driven by the CaMV 35S promoter

(Perkin-Elmer Cetus, Foster City, Calif.) under the following conditions: initial denaturation at 94 ^{0}C for 5 min followed by 40 cycles of amplification at 94 ^{0}C for 1 min, 55 ^{0}C for 1 min, 72 ^{0}C for 1 min, and a final extension for 10 min at 72^{0}C. The amplified product was assayed by electrophoresis in 1% agarose (Seakem HGT) gel in 1X TBE. The same primers and PCR conditions were followed later to make probes for Southern and Northern hybridization.

For Southern blot analysis, peanut genomic DNA (10–20 µg) was digested with *Hind*III or *Sac*I and separated by electrophoresis on a 0.8% agarose gel. Both of the restriction enzymes cut the plasmid pCAMBIA1301 in a single site. DNA was transferred to Gene Screen Plus nylon membrane (NEN Research Products, Boston, Mass.) using 0.4 N NaOH. After hybridization with the 417 bp PCR-amplified, ^{32}P-labeled probe for *hph*, blots were exposed to phosphor screens overnight and images were generated with the Cyclone (Phosphoimager, Packard, Meriden, USA). Blots were stripped after hybridization with the *hph* probe and re-probed with a GUS probe made by using the same PCR program with two different primers (*uidA* : 5'-TGC CAG TTC AGT TCG TTG TTC-3' and CAMV35S : 5'-TCA TTG CGA TAA AGG AAA GGC C-3') amplifying a 875 bp fragment from the ORF.

Transgene expression by Northern analysis

For the confirmation of transgene expression by Northern blot analysis, total RNA was isolated from eight selected embryogenic callus lines using the Qiagen RNA kit. 50-100 mg plant tissue was first soaked in 5 to10 times RNA later (Ambion, Cat # 7020) for four hours and then RNA was isolated following the protocol of Qiagen, except that the tissues were homogenized in a microcentrifuge tube with a small pestle. Total RNA (10 µg) was electrophoretically fractionated on a 1.0% denaturing formaldehyde (2.2 M) gel using 1×MOPS running buffer as described (Sambrook *et al.* 1989). RNA was transferred to Gene Screen Plus using 7.5 mM NaOH as transfer solution and hybridized with the 417 bp ^{32}P-labelled, hygromycin gene, PCR product as a probe. Image was generated same as Southern blot analysis.

Results and Discussion

Transient expression of GUS was monitored under microscope 48h after bombardment (Fig.5.1.3.2.). Ten hygromycin-resistant cell lines were derived from two bombardment experiments. Out of these ten, six lines having enough tissue were selected for molecular analysis. The presence of

plasmid sequences was confirmed by the PCR analysis of all six lines using two primers specific to the hygromycin gene. All the lines were found to be PCR-positive for the hygromycin gene, and no amplification was observed from control (GG) or non-transformed tissue (NT) (Fig.5.1.3.3.).

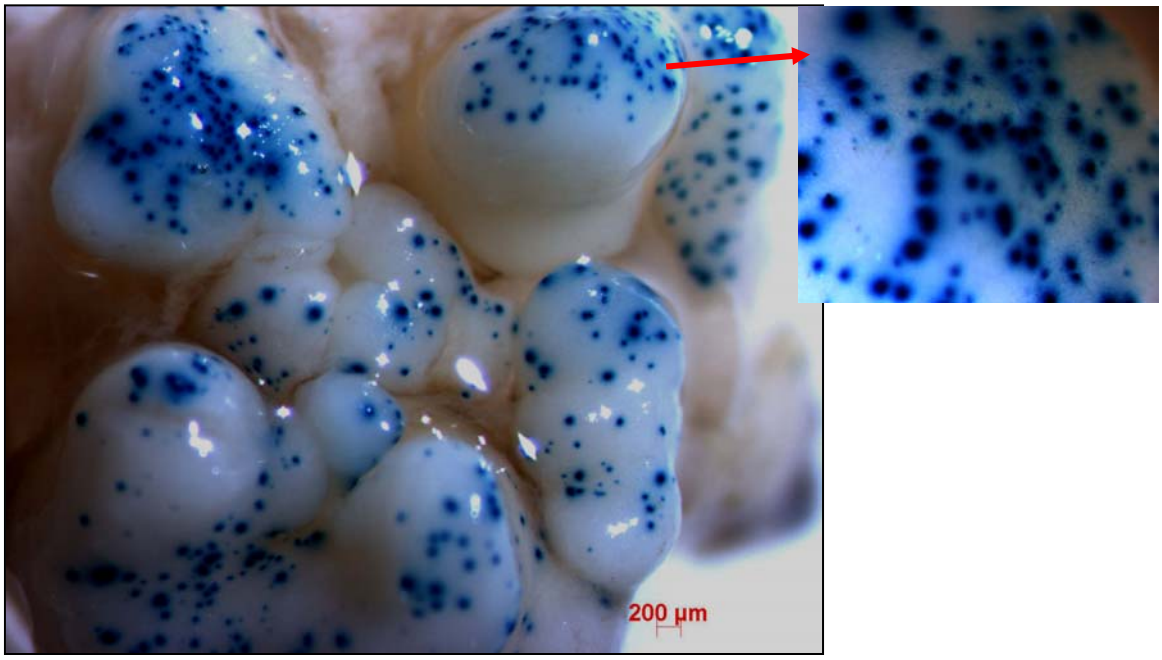


Fig.5.1.3.2. Transient expression of *GUS* in embryogenic callus, 48 h post-bombardment.

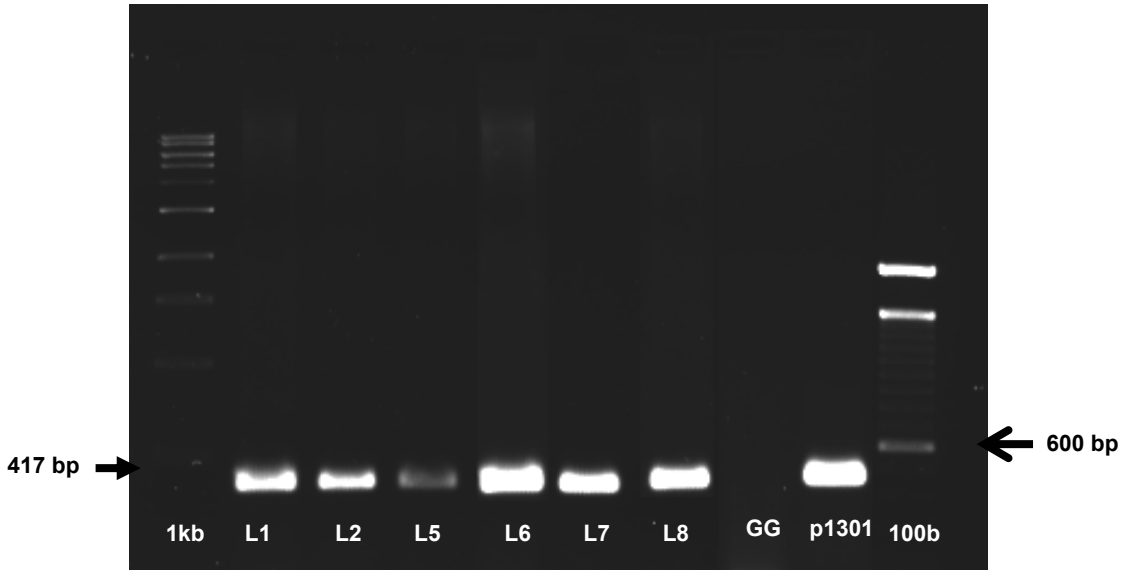


Fig.5.1.3.3. An ethidium bromide-stained gel showing amplification of the expected 417 bp fragment of the chimeric hygromycin resistance gene from 6 transgenic cell lines. No amplification was observed from the control callus DNA [Georgia Green (GG)]. Strong amplification was observed from the positive control (p1301 plasmid only) DNA.

Southern blot analysis confirmed the genomic integration of both hygromycin and GUS genes. After digestion with *Sac*I, (Fig.5.1.3.4. A,B) and *Hind*III, (Fig.5.1.3.5. A,B) multiple bands were found when hybridize with probes for the *hph* and *gus* genes. Most of these bands were smaller than the linearized plasmid. In the blot digested with *Sac*I, for lines L1, L2, L6 and L7, the fifth band was found markedly bigger in size for hygromycin (~ 3 kb) than that for GUS (~ 3.5 kb) probe. This difference is quite expected possibly due to rearrangement during integration. All of the transformed lines showed positive signals in northern analysis confirming expression of the hygromycin gene (Fig.5.1.3.6.).

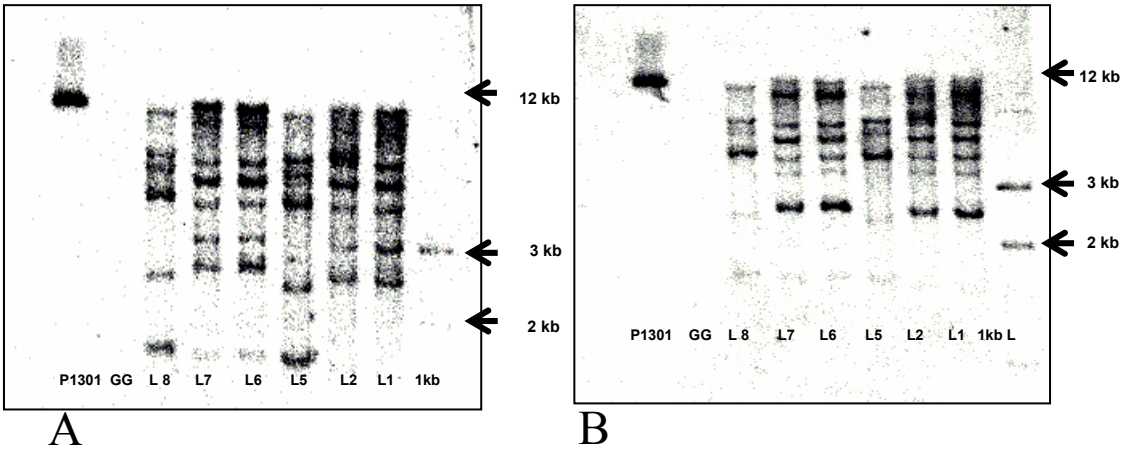


Fig.5.1.3.4. Southern blot of transgenic pCAMBIA1301 cell lines digested with *Sac*1: A) Probed with 417 bp hygromycin gene, B) Probed with 875 bp *GUS* gene

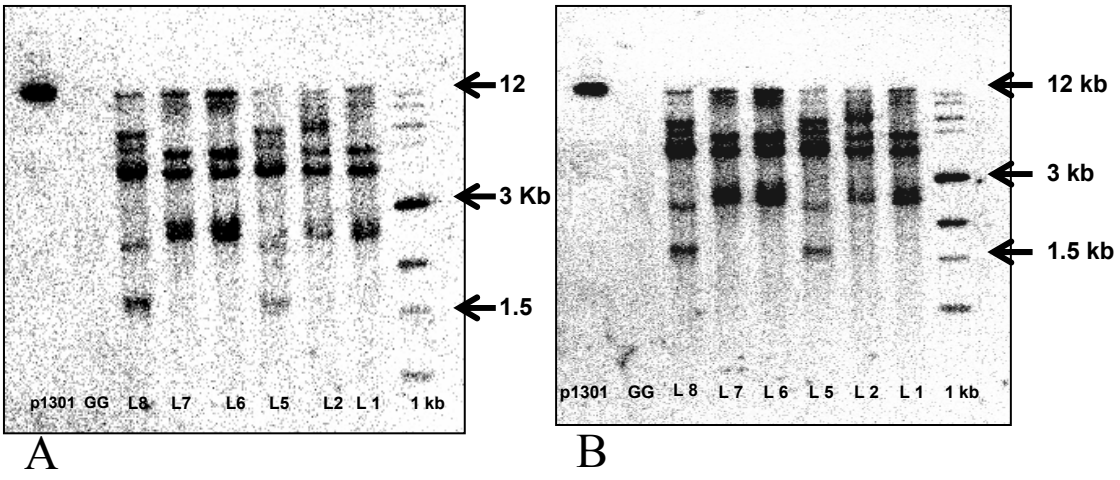


Fig.5.1.3.5. Southern blot of transgenic pCAMBIA1301 cell lines digested with *Hind*III : A) Probed with 417 bp hygromycin gene, B) Probed with 875 bp GUS

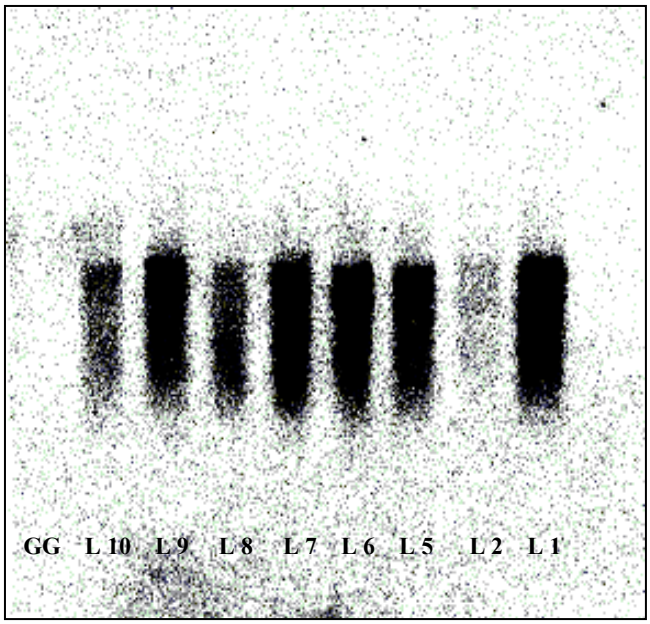


Fig.5.1.3.6. Northern blot of pCAMBIA1301 gene transcript in transgenic cell lines probed with *hph* gene. No expression in control (GG: Georgia Green).

Interestingly, none of these stably transformed lines showed GUS expression during histochemical assay even when kept for 24 h in GUS reaction buffer. In a prolonged incubation (3-4 days) of embryonic cell lines in GUS solution, only two lines (L5 and L7) showed 3-4 very faint blue patches in 2-3 embryo clusters. Nevertheless, integration of the GUS gene was confirmed by Southern blot analysis. None or very little *gus* transcript was detected by Northern blot analysis of these lines confirming the less expression or silencing of the GUS gene. Such gene silencing is very common for GUS and has been observed in many earlier studies (Wang and Waterhouse, 2000; Buck *et al.* 2001; Deng *et al.* 2001). The cause of such frequent gus gene silencing is unknown. Buck *et al.* (2001) suggested that convergent transcription of transgenes in an inverted repeat is an important parameter to trigger their silencing, and the spacer region in between the inverted genes plays a role in the efficiency of initiating and maintaining silencing. Silencing induced by transgene integration into the plant genome in inverted- repeat configurations is supported by many researchers in different species (Wang and Waterhouse, 2000; Morino *et al.* 1999). Kohli *et al.* (1999) concluded that the integrity of integrated transgenes was a major factor in the onset of silencing. In transgenic rice they observed that the presence of truncated sequences of transgenes capable of generating incomplete transcripts, resulting in aberrant RNA species, may be responsible for silencing.

Beaujean *et al.* (1998) suggested that the CaMv 35S promoter is frequently associated with transgene silencing (Matzke and Matzke, 1995) and they showed that an initial high level of GUS expression in transgenic tobacco plants obtained using the 35S promoter subsequently induced a post-transcriptional silencing phenomenon. In peanut, Deng *et al.* (2001) also reported such gene silencing using the same plasmid, pCAMBIA130, for cotyledonary tissues of peanut

Regeneration of transformed plants

Plants were regenerated from all selected lines using the protocol mentioned in earlier section (5.1.2.). Our bombardment protocol was found suitable for other plasmids and reporter genes like GUS for transient expression. Stable transformed lines were recovered and there were no escapes observed for hygromycin selection.

Conclusion

Using the newly standardized bombardment parameters we have successfully transformed the ß- glucuronidase (*GUS*) and hygromycin phosphotransferase (*hph*) gene in peanut somatic embryos. Integration of *GUS* and hygomycin genes was confirmed by southern hybridization for the former and expression of hygromycin gene by northern for the later. Though there was no escape observed on hygromycin selection, but evidence to report GUS gene silencing was obtained.

5.1.4. Use of Phosphinothricin (*Bar)* as a selectable marker gene in peanut transformation

Introduction

The success of genetic transformation with genes for novel traits has relied largely on the use of selectable marker genes conferring antibiotic or herbicide resistance. Selectable marker genes are still necessary for efficient plant transformation. The *bar* gene, isolated from *Streptomyces hygroscopicus*, codes for the enzyme phosphinothricin acetyl transferase (PAT) and is often used as a selectable marker gene in transformation (Block *et al.* 1995; Vasil *et al.* 1992; Kuai *et al.* 2001; Lohar *et al.* 2001). L-Phosphinothricin (PPT), or the ammonia salt, glufosinate, is the active compound of the non-selective herbicide Basta. L-Phosphinothricin, an analog of L-glutamic acid, is an inhibitor of glutamine synthetase (GS), a key enzyme in ammonia metabolism (Leason *et al.* 1982; Manderscheid and Wild 1986). Glutamine synthetase catalyses the ATP-dependent incorporation of ammonium into the amide position of glutamate, resulting in the formation of glutamine. This is the major route for capturing the toxic ammonium produced during photorespiration (Martin *et al.* 1983; Wild *et al.* 1987; Sechley *et al.* 1992; Last 1993). High concentrations of ammonium interfere with the electron-transport systems of both chloroplasts and mitochondria, resulting in the production of free radicles (Krogmann *et al.* 1959; Puritch and Barker 1967) and ultimately leading to the death of the plant. The enzyme PAT, encoded by the *bar* gene, inactivates PPT by acetylation (Bottermen *et al.* 1991). Transgenic plants expressing the *bar* gene are tolerant to or completely resistant to the herbicide Basta. The mode of action of PPT in vitro, especially at the callus level, is less clear. Although the use of PPT also results in an accumulation of ammonium in vitro, it is doubtful that ammonium accumulation is the main cause of cell death (Krieg *et al.* 1990; Palmer and Oelck 1992).

In peanut transformation, the most used selectable marker gene is hygromycin phosphotransferase (*hph*), a gene for resistance to the antibiotic hygromycin (Singsit *et al.* 1997; Yang *et al.* 1998; Wang *et al.* 1998; Livingstone and Birch, 1999; Magbanua *et al.* 2000). There is only one report of incorporation of *bar* gene for resistance to a non-selective herbicide in peanut (Brar *et al.* 1994) by using ACCELL method. They have demonstrated the effectiveness of the *bar* gene at the field level showing resistance to the herbicide Basta (500 ppm). So the efficiency of the *bar* gene as a selectable marker in peanut has not been studied yet. In our present study we tried to insert a plasmid

carrying the phosphinothricin acetyl transferase (*bar*) gene and optimize the use of PPT as a selectable marker in peanut transformation.

Materials and Method

Plasmid construct

Plasmid pCAMBIA3301 (from CAMBIA, Australia) was used for the present study. Plasmid pCAMBIA3301 contained the selectable *bar* gene, encoding the enzyme phosphinothricin acetyl transferase (PAT) that confers resistance to the herbicide phosphinithricin. The plasmid also contained a GUS reporter gene with an intron. Both *uidA* and *bar* were driven by the CaMV 35S promoter. (Fig.5.1.4.1).

To evaluate the expression of the *bar* gene under a different promoter, we have used the plasmid pAHC25 (Christensen *et al.* 1996) (Fig. 5.1.4.2) containing *bar* and *uidA* under the control of a maize ubiquitin promoter (*Ubi1*).

Explant preparation, bombardment and selection of putative transformed lines

Explants were prepared and bombardments carried out as described previously for GFP transformation (5.1.1.). Two days after bombardment, tissue pieces were selected from the centre and two random locations of the bombardment circles for GUS histochemical assay to evaluate the effect of promoter or GUS transient expression. GUS assay was carried out as previously described in section no. 5.1.3, and the tissue was photographed using a Zeiss Axiocam digital camera.

After 5-7 days, bombarded tissues were transferred to 25 ml liquid embryo culture medium (MS+ 12.42 µM Picloram + 0.06 M sucrose) supplemented with 15 mg/l phosphinothricin (PPT) [diluted from 60% glufosinate ammonium, a gift from AgrEvo USA company, Pikeville, NC] for selection of herbicide resistant tissue. The cultures were incubated in dark at 26ºC and 110 rpm shaking. Well developed somatic embryos were transferred to the solid medium of same compositions after 4-6 weeks of selection. Presence and integration of the *bar* gene were checked by PCR analysis of the selected lines.

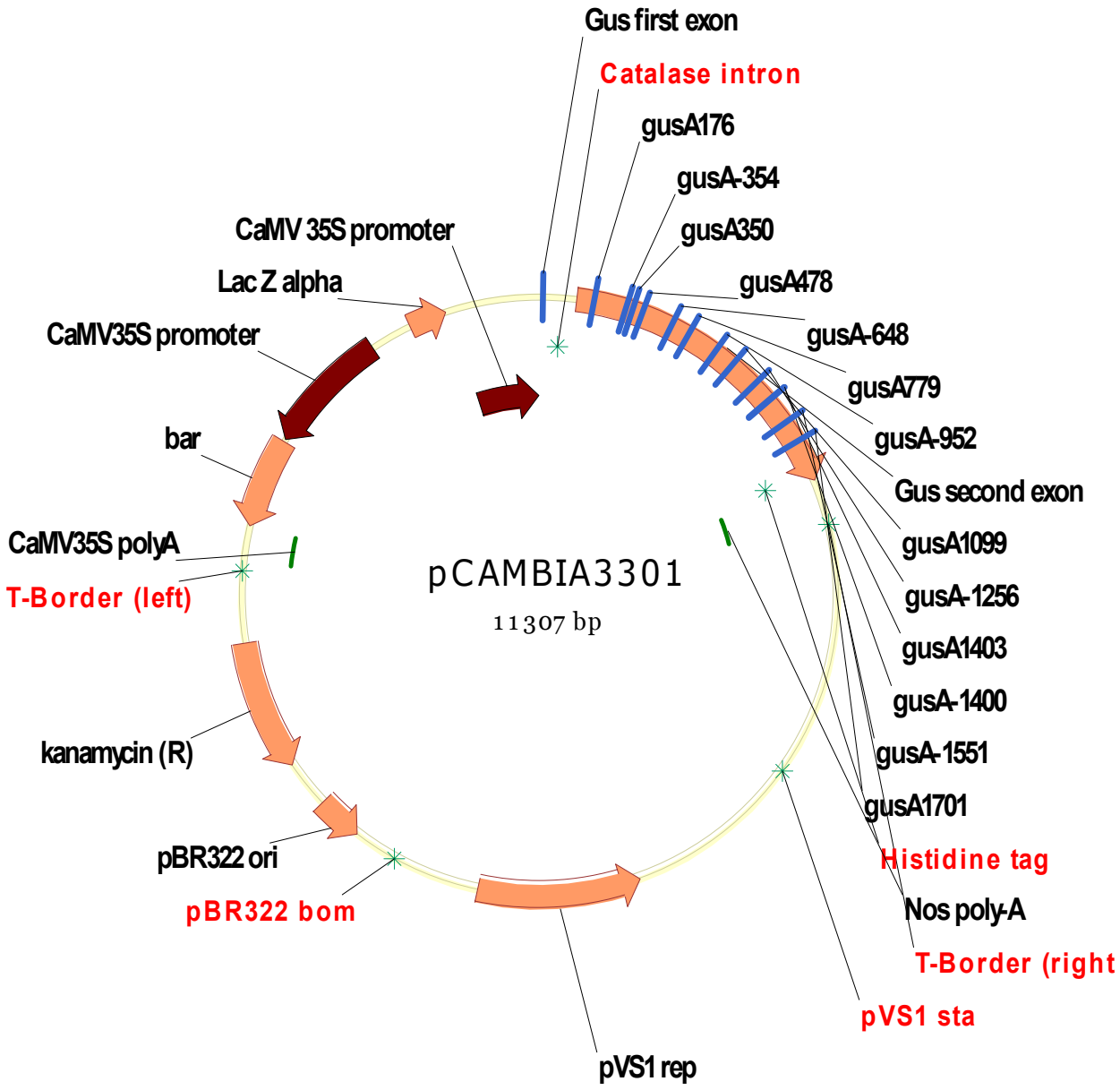


Fig.5.1.4.1. Map of the plasmid pCAMBIA3301 containing the selectable marker gene for *PPT* resistance and the reporter gene, *GUS,* driven by the CaMV 35S promoter

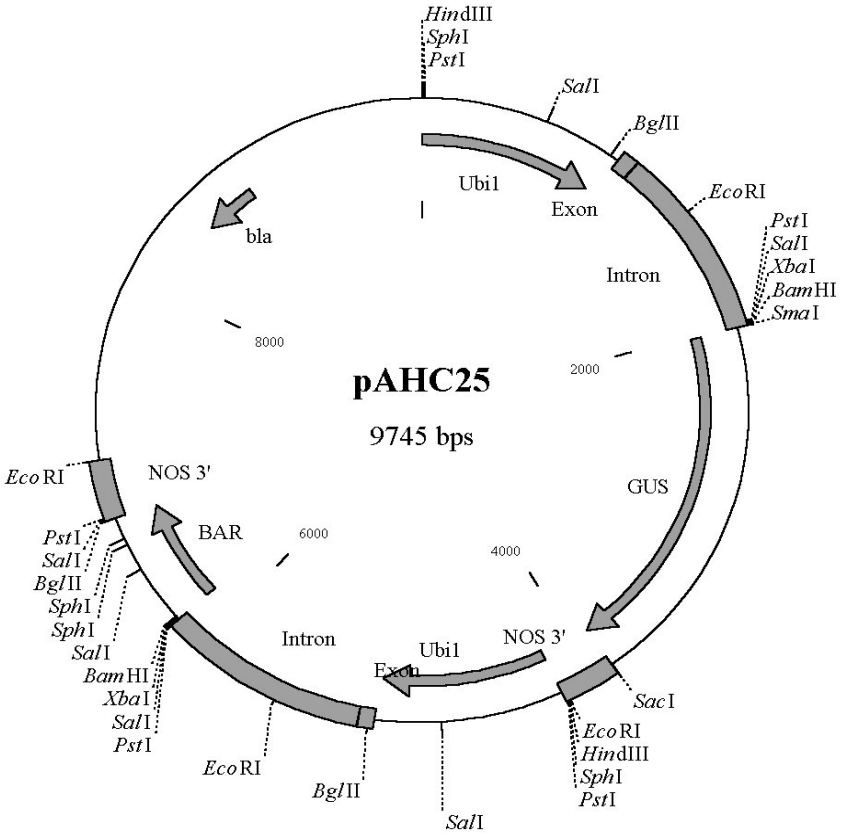


Fig.5.1.4.2. Map of the plasmid pAHC25 containing the selectable marker gene for *PPT* resistance and the reporter gene, *GUS,* driven by the *Ubi1* promoter

Kill curve

The growth of embryonic tissues of peanut in different concentrations (0 - 100 mg/l) of PPT was tested. Embryogenic callus were weighed and 100 ± 5 mg of callus were cultured in liquid embryo culture medium supplemented with varied concentration of PPT. Fresh weights of callus were taken at 10, 20, 30, and 45 days after treatment, while subculturing. The experiment was repeated three times with three replicate for each treatment including no PPT as a control. Mean data were used for growth curve.

Molecular confirmation of Insert Integration

Two transgenic lines selected on phosphinothricin were tested for the presence of the *bar* gene by PCR using the same protocols for isolation of DNA and PCR described earlier (page no…). A 419-bp fragment of the phosphinothricin ORF was amplified using a 26 nucleotide sense primer *BAR*R25 (5'-GCC AGT TTC CCG TGC TTG AAG CCG GC-3') and an antisense primer *BAR*F25 (5'-GGC GGT CTG CAC CAT CGT CAA CCA C-3'). The PCR amplification was carried out in a thermal cycler (Perkin-Elmer Cetus, Foster City, Calif.) under the following conditions: initial denaturation at 94 ^{0}C for 5 min followed by 40 cycles of amplification at 94 ^{0}C for 1 min, 66.5 ^{0}C for 1 min, 72 ^{0}C for 1 min. After the final cycle, a 10 min extension at 72 ^{0}C was added.

Results and Discussion

As there is no report on the PPT selection of peanut somatic embryos, it was essential to check the growth of embryonic callus of peanut in media containing PPT. It was found that the increasing concentration of PPT in the culture medium led to a significant inhibition of growth and proliferation of somatic embryos (Table 5.1.4.1). All PPT treatments showed a reduction in growth rate compared with the control (Fig.5.1.4.3). The weight losses of tissues on medium with 10-15 mg/l PPT after 20 days indicate the cell death or no growth. After 30 days at 15 mg/l PPT most of the tissue had become brown, leaving very few new embryos to emerge. Selection for PPT resistance subsequently was carried out at 15 mg/l PPT, because this level of phosphinothricin was sufficient to stop the growth of non-transformed tissues but often did not kill them immediately.

Although the experiment determined an apparent lethal dose of PPT it was found that the growth pattern was not always consistent for a specific concentration and was dependent upon the size of embryo clusters and stage of embryo development. This inconsistency in PPT response was also observed by other researchers and suggested that ammonium accumulation is not the only cause of cell death, but that it may be influenced by the metabolic status of the tissue (Palmer and Oelck, 1992; Block et al, 1995).

Table 5.1.4.1. Growth response of the peanut somatic embryos on Phosphothricin (PPT) kill Curve

PPT	callus weight (mg)			
mg/l	10days	20days	30days	45days
PPT-0	221.2954	492.1447	531.8308	588.019
PPT-2	127.6336	163.7552	187.23	209.345
PPT-5	100.7465	133.4992	145.353	153.43
PPT-10	114.3387	120.81	102.9135	102.3389
PPT-15	105.9629	110.273	78.39417	79.91562
PPT-25	97.08425	95.17702	83.97707	87.74225
PPT-50	99.53598	97.88181	92.81323	92.32263
PPT-100	98.98578	94.09295	83.63012	83.1738

Initial callus weight was 100 ± 5 mg.

Selection and recovery of transformed lines

For transient expression from plasmid pAHC25, where *bar* and uidA genes are under the control of the maize *Ubi1* promoter, the number of GUS spots was very few (Fig 5.1.4.4. A, B). Only 2-3 spots in 4-5 embryos / plate were observed. For plasmid pCAMBIA3301 more than 90% of the embryos had GUS spots (Fig 5.1.4.5.). More than 75% of callus became brown after 4-6 weeks of selection in liquid medium with 15 mg/l PPT. Small white embryonic callus began to appear from the brown callus after 3-4 weeks (Fig 5.1.4.6.). As the growth of peanut somatic embryos is slow compared to other plants it takes 3-4 more weeks to get enough tissue of putative cell lines to separate them by subculture. Two lines selected as putative transformed lines were numbered separately at the time of DNA isolation. For confirmation of the presence of the *bar* gene oligonucleotide primers specific to sequences for the *bar* region amplified the expected 419 bp nucleotide fragment from both of the PPT-resistant cell lines. No amplification was observed from control non-transformed tissue (Fig 5.1.4.7). Transformation and expression of the *bar* gene governed by the maize ubiquitin promoter was reported in rice (Toki *et al.* 1992), sugarcane (Gallo-Meagher and Irvine, 1993) and Norway spruce (Elfstrand, 2001); but it was not found to be suitable for peanut transformation. On other hand expression and integration of *bar* gene

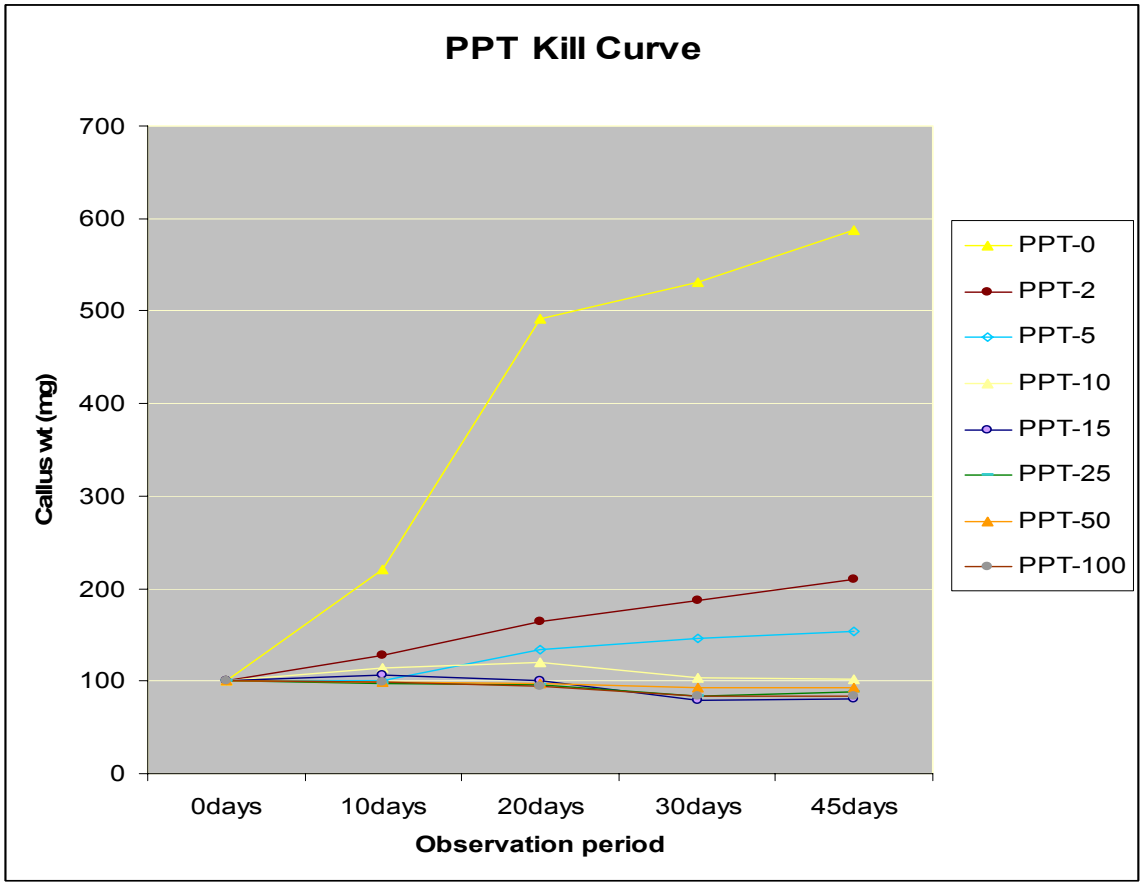


Fig 5.1.4.3. Growth of peanut somatic embryos on media containing Phosphothricin (PPT)

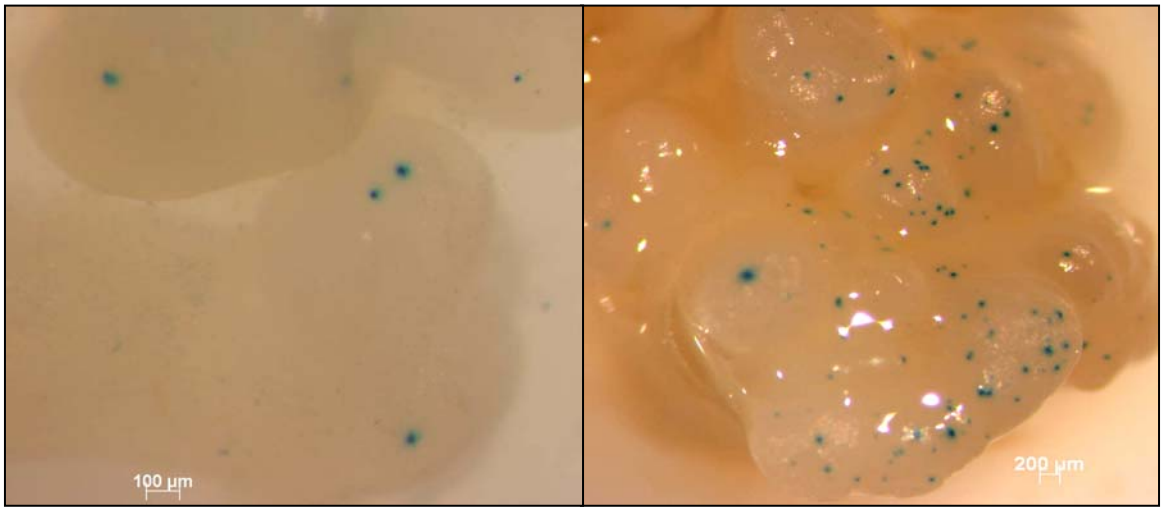


Fig 5.1.4.4. Transient GUS expression in embryogenic callus 48 h post-bombardment
A) GUS expression of plasmid pAHC25 with *Ubi1* promoter, B) GUS expression of plasmid pCAMBIA3301 with CaMV 35S promoter

controlled by 35S CaMV promoter was successful. There was no stable transformed line recovered from the plasmid having the *bar* gene control by the *Ubi1* promoter. Christensen *et al.* (1996) suggested that the maize *Ubi1* promoter may be monocot specific and highly expressed only in monocots.

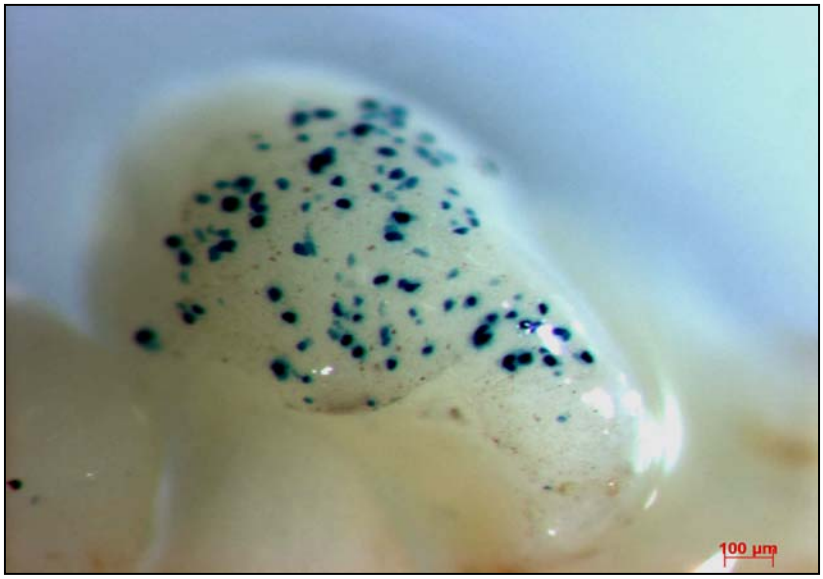


Fig 5.1.4.5. Transient expression of GUS (pCAMBIA 3301)

Fig 5.1.4.6. White embryonic callus appeared after 4 weeks from the brown callus in the media containing 15 mg/l PPT as initiation of stable transformation.

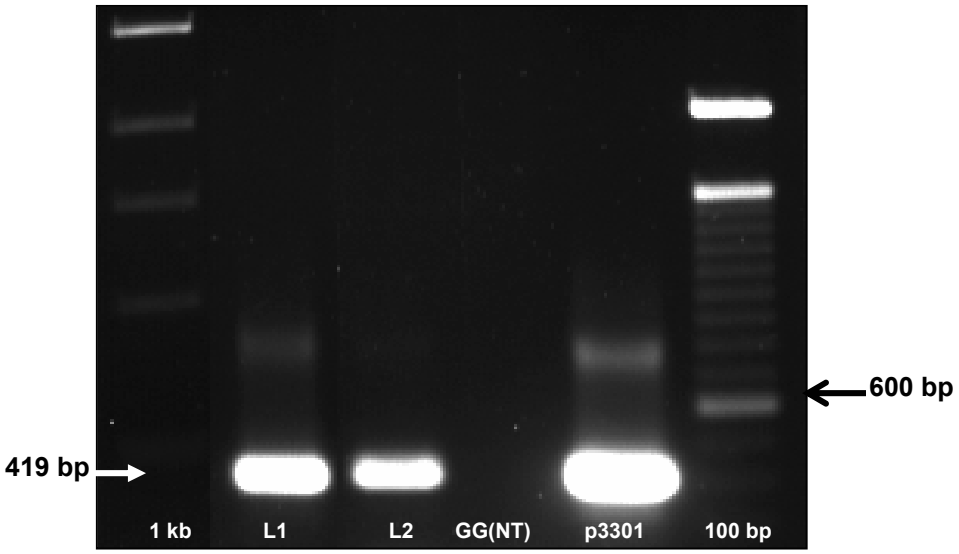


Fig 5.1.4.7. An agarose gel showing amplification of the expected 419 bp fragment of phosphinothricin gene from 2 transgenic cell lines. No amplification was observed from the control callus DNA [Georgia Green (GG)]. Strong amplification was observed from the positive control (p3301 plasmid only) DNA.

In this study we have standardized a PPT selection protocol for peanut somatic embryos. Herbicide selection was not always obvious even with a concentration of 15 mg/l PPT in the dark. It was reported that more chimeric plants or escapes were possible with PPT selection than hygromycin, and the selection pressure with hygromycin is much stronger (Cho *et al.* 2000; Lambe *et al.* 2000). Here we have standardized the selection pressure in liquid medium which is more efficient and faster for eliminating non-transformed tissues (Ozias-Akins *et al.* 1993). In peanut this is the first report for the use of PPT as a selectable marker gene.

Conclusion

Study of genetic transformation is often relied on the use of selectable marker genes like those conferring herbicide resistance. For present studies we have used phosphinothricin (*Bar*) gene as a selectable marker gene in peanut transformation. The tissue growth response over varied concentrations of phophinothricin was evaluated. Transformed lines were isolated after a long selection in liquid medium with phosphinothricin. The presence of *Bar* gene in transformed lines was demonstrated by PCR analysis. This is the first report to use of phosphinothricin (*Bar*) gene as a selectable marker.

5.2. *Agrobacterium tumefaciens* Mediated Genetic Transformation in Peanut (*Arachis hypogaea* L.)

Introduction

Agrobacterium tumefaciens, a gram-negative, soil-borne plant pathogen has the ability to insert foreign DNA sequences into the plant genome. The major advantage of *Agrobacterium*-mediated gene transfer over biolistics is its ability to integrate fewer copies of foreign inserts into the plant genome, thereby reducing the risk of transgene rearrangements and gene silencing. It is also considered to be comparatively inexpensive method.

Plant genetic transformation using *Agrobacterium* is an integrated process which relies on a protocol that is dependent considerably upon plant species, type of explant, bacterial strain, plasmid construct, culture conditions etc. An improved gene transfer procedure via *Agrobacterium* has been optimized in several plant species (Chyi *et al.* 1987; Chabaud *et al.* 1988; Dong and McHughen 1991; Sangwan *et al.* 1991, 1992; Pawlicki *et al.* 1992; De Bondt *et al.* 1994).

Though transformation of peanut via *Agrobacterium* has been reported earlier by many workers (Dong *et al.* 1990; Mansure *et al.* 1993; Egnin *et al.* 1998; Sharma and Anjaiah, 2000; Rohini and Rao, 2000, 2001), low efficiency of transformation (Cheng *et al.* 1994; 1996) has always remained a cause of concern. The genotype specificity of the transformation-regeneration system (Cheng *et al.* 1994; McKently *et al.* 1995) and high incidence of sterility in regenerated peanut plants (Cheng *et al.* 1994; Eapen and George, 1994) has been observed. An understanding of factors influencing the efficiency of T-DNA transfer and associated gene expression in peanut may enable the rapid development of transgenic peanut plants (Egnin *et al.* 1998).

Transformation of peanut by Agrobacterium tumefaciens

Susceptibility of peanut to *Agrobacterium tumefaciens* has been demonstrated by Dong *et al.* (1990), Lacorte *et al.* (1991) and Mansur *et al.* (1993). Lacorte *et al.* (1991) were able to induce crown gall formation in a range of Brazilian cultivars using the best wild type strain A281.

Mansur *et al.* (1993) suggested that the susceptibility of peanut (cv Tatu) explants to *A. tumefaciens* was strongly influenced by explant type and incubation condition.

The first report of peanut transformation using the disarmed *Agrobacterium* strain was documented by Eapen and George (1994). They reported regeneration of primary transgenic plants from leaf discs of peanut cv. TAG-24, co-cultivated with the *A. tumefaciens* strain LBA 4404 harboring the binary plasmid pBI 121, carrying GUS and NPT II genes. Unfortunately transgenic plants obtained from these experiments were sterile.

McKently *et al.* (1995) used the zygotic embryo axes from mature seed as explants and co-cultivated with *Agrobacterium* strain EHA105 harboring the plasmid pMON9793 containing GUS and NPT II genes. Integration of T-DNA into the host genome was confirmed by the polymerase chain reaction.

Cheng *et al.* (1996) reported the recovery of fertile primary transformants produced via *Agrobacterium* and their R1 progeny. Bacterial strain EHA105 harboring the binary vector pBI121 containing GUS and NPT II was found effective for the peanut CV. New Mexico Valencia A, when leaf sections were used as explants.

Cheng *et al.* (1997a) evaluated the stability of GUS expression in the next generation of transformed plants. They demonstrated the recovery of both homozygous and heterozygous T1 plants and Mendelian inheritance of the GUS gene in T2 plants.

Li *et al.* (1997) reported the successful regeneration of transgenic peanut plants of cultivar New Mexico Valencia A, containing the nucleocapsid gene of TSWV. They demonstrated that protection against tomato spotted wilt virus can be achieved in transgenic peanut plants by expression of the sense DNA of the tomato spotted wilt virus nucleocapsid gene.

Sarker *et al.* (1997) used the organogenesis pathway for transformation and regeneration with leaflets as explants for four peanut cultivars viz, Dhaka-1, DM-1, DG-2 and ACC No.12. They also reported transient expression of GUS by histochemical assay.

Egnin *et al.* (1998) optimized a protocol for *Agrobacterium* transformation in peanut cv. New Mexico (Valencia 'Select' market type) using the binary plasmid pIG121 harboring *uidA* (*gusA*) gene, driven by the CaMV 35S promoter. The disarmed *Agrobacterium* tumefaciens strain EHA101 was found to be superior to the disarmed strain C58, and a combination of high auxin and low cytokinin in

the co-cultivation medium was better than hormone free medium. Further they reported the effect of explant type on transient expression frequency.

Venkatachalam *et al.* (1998) demonstrated an efficient transformation protocol where cotyledons were co-cultured with *Agrobacterium* strain LBA 4404 harboring the binary vector pBI121 containing the *uidA* (GUS) and *NPTII* genes. Embryos were induced on an embryo induction medium containing 0.5 mg/l NAA and 5.0 mg/l BAP supplemented with 75 µg/ml kanamycin and 300 µg/ml cefotaxime, and prolific shoots developed from those embryos on a MS medium containing 0.5 mg/l BAP and 50 µg/ml kanamycin with a 47% transformation efficiency. Transformation was confirmed by PCR analysis and southern hybridization.

A high frequency of *Agrobacterium*-mediated transformation in peanut was also demonstrated by Sharma and Anjaiah (2000). They showed successful transformation of cotyledon explants by using *Agrobacterium* strain C58 carrying plasmid pBI121 containing NPT II and GUS genes and the coat protein gene of IPCVcp and NPT II in a separate plasmid named pROKII: IPCVcp. More than 75% of transgenic progeny were with single copy integration of the IPCVcp gene which was expected to be characteristic of a single locus trait.

Transgenic peanut *(Arachis hypogaea* L. cv. TMV-2) plants have been produced by a tissue culture-independent *Agrobacterium tumefacien*s-mediated transformation procedure (Rohini and Rao, 2000). They have demonstrated a procedure where embryo axes of mature seeds were co-cultivated and directly germinated in vivo. Molecular characterization of primary transformants as well as the T1 and T2 generation plants provided evidence of insertion, expression and inheritance of foreign genes in peanut.

By using this non tissue culture approach the fertile transgenic plants of peanut *(Arachis hypogaea* L.) cv. TMV-2 expressing tobacco chitinase and neomycin phosphotransferase *(npt* II) genes were generated using an *Agrobacterium tumefaciens* (LBA4404:pBI121-pBTex) -mediated transformation system (Rohini and Rao, 2001). Their studies demonstrated that the expression of the heterologous chitinase gene driven by the CaMV 35S promoter led to a high level of activity in some of the transgenic plants which showed resistance to the fungal pathogen *Cercospora arachidicola* to different degrees.

Most of the peanut transformation studies with *Agrobacterium* have used leaf sections as explants. In the present study, we have attempted to optimize an *Agrobacterium*-mediated transformation system for peanut using embryonic callus and somatic embryos as explants. We tested

different parameters to evaluate their effects on transient expression. The experiment was set up for two different approaches of *Agrobacterium* transformation, viz.

A) Sonication Assisted *Agrobacterium*-mediated Transformation (SAAT)

B) Agrobacterium cells coated onto microprojectiles

5.2.1. Sonication Assisted *Agrobacterium*-mediated Transformation (SAAT)

Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT) (Trick and Finer, 1997) is an easy, low cost method of substantially enhancing the efficiency of *Agrobacterium*-mediated transformation of low or non-susceptible plant species. SAAT consists of subjecting the target tissue to ultra sound while immersed in *Agrobacterium* suspension. The enhanced transformation rates using SAAT probably result from micro wounding, where the energy released by cavitations (Frizzel L.A, 1988) causes small wounds both on the surface of and deep within the target tissue. These wounding patterns permit *Agrobacterium* to travel deeper and more completely throughout the tissue than conventional microscopic wounding, increasing the probability of infecting plant cells. Sonication enhances the delivery of naked DNA into tobacco protoplasts (Joersbo and Brunstedt, 1990) and seedlings (Zhang *et al.* 1991), but has recently been shown to enhance *Agrobacterium*-mediated transformation of plant tissue (Trick and Finer, 1997).

In our study we have tried to optimize the *Agrobacterium*-mediated transient transformation of somatic embryos of peanut using SAAT.

Materials and Methods

Plasmid Construct and Transformation of Agrobacterium Strains

Plasmid pCAMBIA1301 (from CAMBIA, Australia) has the *gusA* gene present as a reporter and the hygromycin gene as a selectable marker, both under control of the CaMV 35S promoter (Fig 5.1.3.1). The multiple cloning site (MCS) is between *gusA* (proximal to the right border of T-DNA transfer) and hygromycin (proximal to the left border). Additionally, there is an intron present in the *gusA* coding sequence, which ensures that the gene is not expressed in bacteria, but only upon transfer to the plant. A hexahistidine 'tail' has been engineered at the carboxyl end of GUS, allowing for protein purification by immobilized metal ion affinity chromatography (IMAC).

The plasmid was transformed into *A. tumefaciens* strains LBA4404, EHA105 and AGL1 using the freeze-thaw method (Holsters *et al.*, 1978). The colonies of *A. tumefaciens* carrying plasmid pCAMBIA1301 were selected based on resistance to rifampicin (10mg/l) and kanamycin (50 mg/l) for LBA4404 and only kanamycin (50mg/l) for the other two strains. Three independent transformed colonies of each strain were randomly selected and checked for the insertion by PCR. Transformation and expression studies were initially done with tobacco as a control.

Preparation of explant and A. tumefaciens for co-cultivation

Somatic embryos and embryogenic cultures of peanut cultivar Georgia Green were initiated and cultured as described earlier in particle bombardment section (5.1.1). The well-grown embryonic clusters were used for co-cultivation with *A. tumefaciens.*

Agrobacterium cultures were grown for 16-18 hours in liquid LB (containing 10g/l Tripton, 5 g/l Yeast Extract, and 10 g/l NaCl, pH 7-7.2) medium containing 50 mg/l kanamycin and an addition of 10 mg/l rifampicin for LBA4404, with agitation at 28ºC. Cultures were centrifuged at low speed (4000 x g) for 5 min and washed with sterile water. Finally the bacterial pellets were resuspended in double (original culture vol.) volume of liquid peanut embryo culture medium (MS+3mg/l picloram). The culture adjusted to an OD_{600} of 0.1 – 0.3 using a spectrophotometer.

Sonication and Co-cultivation

Ten clusters of somatic embryos were placed in a 1.5 ml microcentrifuge tube containing 0.5 ml *Agrobacterium* suspension. Explants were gently resuspended and placed in a float at the center of the bath sonicator (Solid state/ Ultrasonic FS-28, Fisher Scientific, USA). An electronic timer monitored the sonication time. After sonication, explants were vacuum infiltrated for 60 min at 20 in. Hg and transferred from the tubes on to sterile filter paper to blot off excess bacteria before transferring to the co-cultivation medium.

Explants were spread on solid co-cultivation medium containing MS plus 12.42 µM Picloram and 6.85 M Glutamine and 100 µM Acetosyringone (Aldrich) in 85 mm petri dishes (as embryo culture medium stated before in 5.1.). As a control for the effect of acetosyringone, one experiment was designed using co-cultivation medium without acetosyringone. Cultures were co-cultivated for 2 days at 26ºC in the dark.

After co-cultivation, embryogenic clusters were collected in 100 ml sterile beaker and thoroughly washed with autoclaved water containing 400 mg/l cefotaxime (Agri-bio, USA). Excess water from explant was blotted in sterile filter paper and subcultured on peanut embryo culture medium supplemented with 400 mg/l cefotaxime to prevent *Agrobacterium* growth. After two days in the dark, explants were checked for GUS expression by histochemical assay. On the third day explants were placed in selection medium containing MS supplemented with 12.42 µM picloram, 0.06 M sucrose and 20 mg/l hygromycin (filter sterilized).

To evaluate the effects of sonication duration, explants were sonicated for 0, 2, 6, 10, 30, 60, 120, 180, 240 seconds using three *Agrobacterium* strains LBA4404, EHA105 and AGL1.

Based on survival and tissue tolerance, as evident from fresh weight of calluses, a kill curve was set to determine the optimum sonication time. Sonicating the tissue for 0, 10, 60, 180 and 300 seconds assessed the effect of sonication treatment on tissue growth and recovery. Initially 100 ± 5 mg embryogenic callus were weighed for each sonication treatment and 3 petri dishes containing 100 ± 5 mg/dish were cultured for each treatment in each replication. Experiment was carried out two times with three replications. Fresh weights of embryonic callus were taken after 2, 4, and 6 weeks. Mean and Standard deviation was calculated using Microsoft excel.

Other parameters like surfactant, Silwet L-77 (500 ppm), or, an antioxidant, L-cysteine (400 mg/l) were tested in co-cultivation medium to increase the transformation frequency. These compounds were incorporated in the medium aseptically. GUS expression was assayed for each parameter 2 days after co-cultivation.

Histochemical GUS assay

GUS expression was assayed by placing the 5-7 embryogenic cluster in a 1.5 ml microcentrifuge tube containing 0.5 ml of GUS staining solution (Jefferson, 1987) which consisted of 5 mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.5% (v/v) Triton X-100, 0.005% 5-bromo-4-chloro-3-indolyl ß-D-glucuronidase (X-gluc) in 100 mM sodium phosphate (pH 7.0), and incubating overnight with gentle agitation at 37ºC. The numbers of blue foci were counted under the microscope to score the cells and tissues expressing the GUS gene.

Results and Discussion

It is evident from most of the earlier reports that transformation of peanut through *Agrobacterium* has been achieved with varied success (Eapen and George, 1994; McKently *et al.*, 1995; Cheng *et al.*, 1996; Sarker *et al.*, 1997; Egnin *et al.*, 1998 and Rohini and Rao, 2000, 2001). These studies reflect variable responses of explants on transformation efficiency. Explants right from zygotic embryo axes from mature seeds, leaflets, leaf sections and axis to cotyledons were attempted. Taking into account the well established peanut regeneration system from somatic embryos (Ozias-Akins *et al.* 1993), experiment were carried out to use somatic embryos and embryonic callus for *Agrobacterium* transformation.

After mobilizing the plasmid DNA to *Agrobacterium* by the freeze-thaw method, transformation of the bacterial strains was confirmed by PCR [using a portion of chimeric hygromycin phosphotransferase (hph) gene (Fig.5.2.1.1.)] and transient expression of the GUS gene in co-cultivated tobacco leaves (Fig. 5.2.1. 2.).

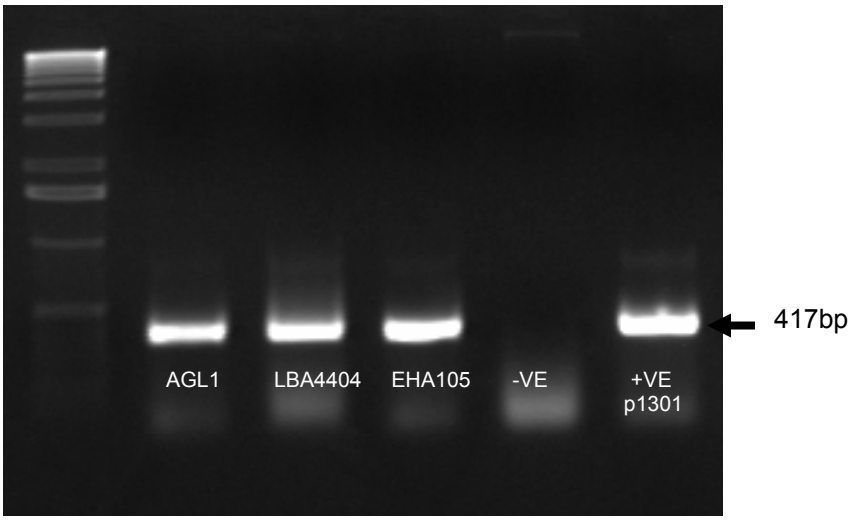


Fig. 5.2.1.1. PCR analysis of three *Agrobacterium* strains transformed with plasmid pCAMBIA1301 showing amplification of the expected 417 bp fragment of the chimeric hygromycin resistance gene. No amplification was observed from the control non-transformed *Agrobacterium* strain (LBA4404). Strong amplification was observed from the positive control (plasmid only) DNA.

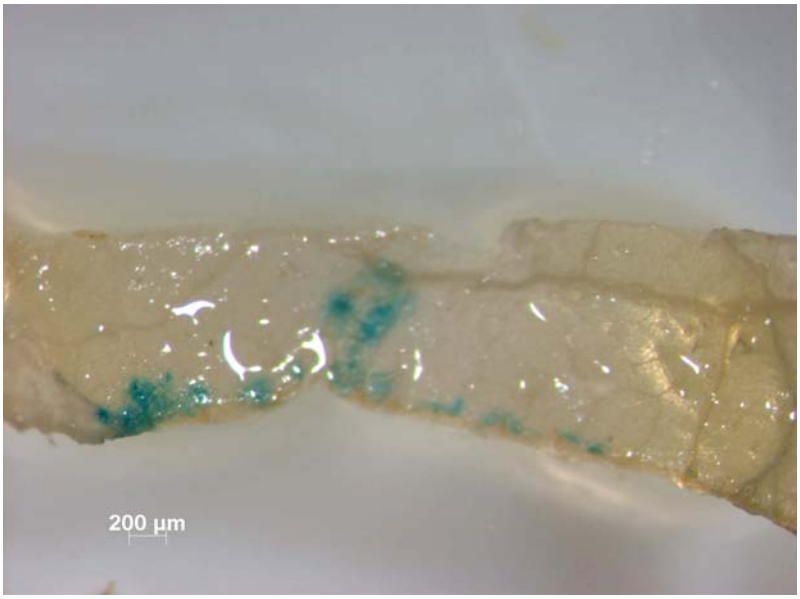


Fig. 5.2.1.2. Transient *GUS* expression in tobacco leaves transformed with pCAMBIA1301, 48 h after co-cultivation.

Evaluation of Accessory Parameters for Agrobacterium Transformation

Acetosyringone, a synthetic inducer of T-DNA transfer (Stachel *et al.* 1985), enhances the transformation process due to activation of *vir* genes and is now routinely used in transformation studies. The present study acetosyringone was used to enhancement of transformation efficiency of somatic embryos. Acetosyringone was used at a concentration of 100 µM in both conventional (without Sonication) and Sonication-assisted transformation. Results were promising than that of the respective untreated controls. In earlier studies it was found that the inclusion of acetosyringone in the co-cultivation medium either had no effect (Mansur *et al.* 1993) or doubled the frequency of transient GUS expression (Newton, 1997). Higgins and Dietzgen (2000) suggested that this effect appears to be dependent on the peanut cultivar, *Agrobacterium* strain and plasmid used. It is well known that the transfer of T-DNA is mediated by the expression of virulence genes and that transcription of the *vir* region is induced by various phenolic compounds. Supplementary acetosyringone could overcome the problem of comparatively less synthesized phenolics or exudates from the wounded embryos than that of the leaf tissues or embryo axes.

Besides acetosyringone, vacuum infiltration has also been found effective for *Agrobacterium* transformation (Amoah *et al.* 2001). Results demonstrated that 60 min vacuum infiltration at 20 in. Hg pressure enhanced transformation efficiency beyond the control (without vacuum). Similarly, vacuum

infiltration also was found to be effective for transformation of several plant species including *Arabidopsis* (Clough and Bent, 1998) and wheat (Cheng *et al.*, 1997b).

Other parameters also were evaluated for their influence on transformation efficiency of somatic embryos and embryonic calluses of peanut. Cysteine has a potential to increase the capacity of *Agrobacterium* to infect plant tissue and stably transfer its T-DNA by reducing wound and pathogen defense responses in plants through the action of its thiol group. Use of L-Cysteine (400 mg/l) was found to be very effective in transformation of soybean (Olhoft and Somers, 2001). During the present investigation cysteine was used exclusively in the co-cultivation medium. No apparent effect was observed either for embryos or embryonic calluses with or without addition of cysteine.

Use of the surfactant Silwet L-77 has been shown to increase transformation efficiencies in *Agrobacterium*-mediated transformation of *Arabidopsis (*Clough and Bent, 1998). They found that Silwet L-77 reduces surface tension at doses with low phytotoxicity and ultimately enhances the entry of bacteria into relatively inaccessible plant tissues. However, our observations suggested that there was no positive effect of such a surfactant on peanut transformation.

None of the parameters studied were found to affect the transformation efficiency of peanut embryonic tissues.

Why did we studied so many parameters? We speculated that there are some inherent factors in somatic embryos and embryonic callus of peanut which inhibit attachment or transfer of the T-DNA. Unlike many other crops, where manipulation of such parameters gave marked increases in transformation efficiencies, peanut somatic embryos are least responsive to such external stimuli.

Sonication Assisted Agrobacterium Transformation

Effect of sonication duration on tissue viability was evaluated by measuring the fresh weight of the sonicated tissue. A reduction in fresh weight of sonicated tissue compared with the control allowed a kill curve for Sonication treatment to be generated. Fresh weights of sonicated embryonic tissue that were not treated with *Agrobacterium* revealed that sonication caused a noticeable drop in the growth rate in the first 2 weeks after treatment (Table 5.2.1.1.). After 2 weeks, all tissues showed a similar growth rate except for the longest. This pattern of growth suggests that there is some tissue damage due

to sonication, but peanut embryogenic tissues were hardy enough to overcome the damage caused by up to 180 sec sonication and could regain their regular growth rate. For the 300 sec treatment, a very slow growth rate was observed until the sixth week (Fig.5.2.1.3), indicating extensive cell damage caused by excessive Sonication. A similar result was reported earlier by Trick and Finer (1998) while working with in soybean sonication and transformation.

Table 5.2.1.1: Effect of sonication on growth of peanut somatic embryos

Sonication duration (Sec)	Sample fresh weight (mg ± SE) at each time point (weeks) 0	2	4	6
0	100±5	180.33 ±5.85	365.05 ±62.99	1226.66 ± 16.50
10	100±3	151.66 ±8.08	302.90 ±63.26	1037.00 ±72.54
60	100±3	126.33 ±8.38	278.00 ±60.09	956.00 ±52.88
180	100±2	121.00 ±2	253.13 ±35.92	802.66 ±8.14
300	100±4	112.00 ±3.60	184.00 ±34.87	307.00 ±32.12

Different strains and sonication treatments

In order to evaluate strain specificity, three different strains of bacteria were studied for the range of sonication duration (Fig 5.2.1.4). No reproducibility in number of GUS spots or percent of

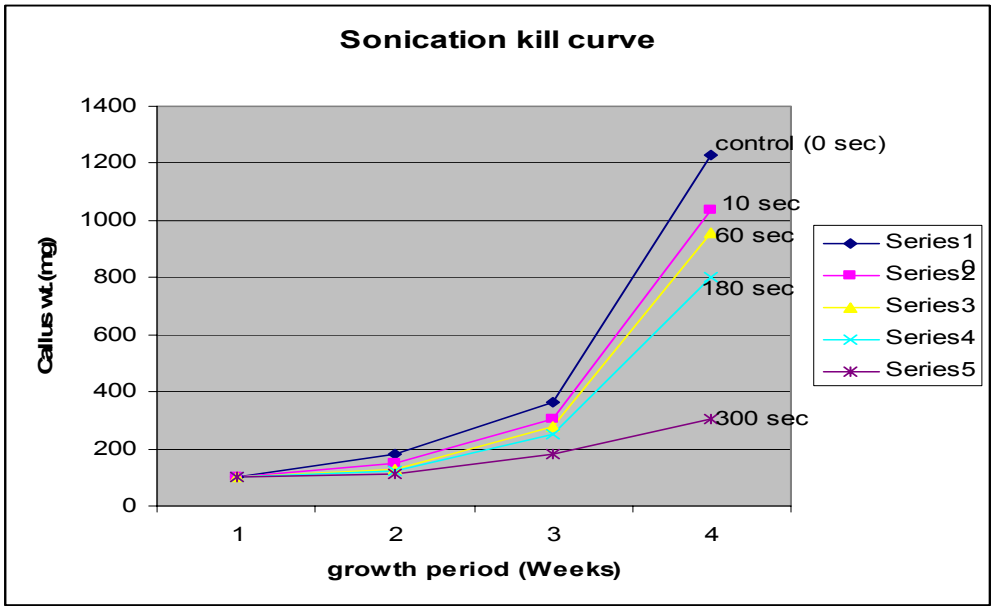


Fig.5.2.1.3. Sonication kill curve.

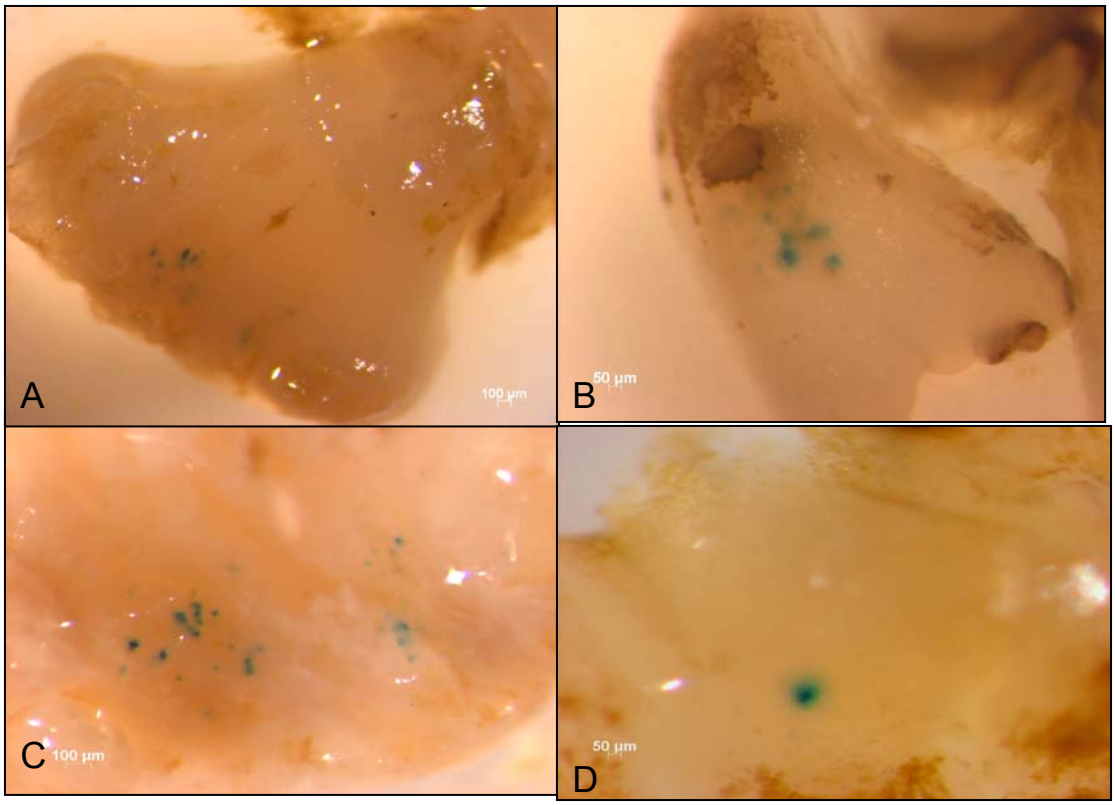


Fig 5.2.A.4. Transient expression of GUS 3 d after co-cultivation with *Agrobacterium*, (A) co-cultivation with *Agrobacterium* strain AGL1; (B) co-cultivation with EHA105; (C) co-cultivation with LBA4404; (D) Single prominent spot by LBA4404

embryos showing GUS expression were observed in repeated experiments. Observations from independent experiments suggested that transformation efficiency, based upon the percent of embryos showing GUS expression, was inconsistent for different sonication timings for LBA 4404 (Table 5.2.1.2.). While for AGL1 the results were more distinct only at higher sonication timings. Among the three stains, EHA105 was more consistent in its transient expression in every experiment with an average of 15-20 % of the embryos showing GUS expression.

If we generalize, the sonication timing ranging from 30 sec to 2 minutes were having more pronounced effects. In case of LBA4404 though the percent of embryos showing GUS expression was found less (~10%), the response was comparatively reproducible even at a low sonication duration like 30 sec. Visual observations suggested that AGL1 was showing transformation in loose cells more often than those of compact embryonic callus.

No results were observed in other sonication treatments (Table 5.2.1.2).

Table 5.2.1.2. Sonication assisted *Agrobacterium*-mediated transformation of peanut somatic embryos using plasmid pCAMBIA 1301

Strains	Sonication time	No. of spots	% embryos showing GUS expression
LBA4404	30s	12-15	8.3 (1 emb /out of 12 emb.)
	30 s	5-6	10 (1 emb /out of 10 emb.)
	30 s	14-15	11.11 (1emb/out of 9 emb.)
	1 m	2	10 (2 emb/out of 20 emb.)
	2 m, 3 m, 4m	-	-
AGL1	30 s	-	-
	1 m	2	6.6 (2 emb/out of 30 emb.)
	2m	10-12	10 (1 emb/out of 10 emb.)
	3 m, 4m	-	-
EHA	30s	5 to 7	13.33 (2 emb./out of 15 emb.)
	1 m	5 to 10	21.4 (3 emb./out of 14 emb.)
	2 m	5 to 10	20 (3 emb./out of 15 emb.)
	3 m, 4m	-	-

5.2.2. *Agrobacterium* cells used as microprojectile coating

Plant wound response is a key factor in the interaction between *Agrobacterium* and the host plant thereby affecting the efficiency of *Agrobacterium*-mediated gene transfer (Potrykus, 1991). Among the different strategies used to increase the *Agrobacterium* transformation frequency, several workers have found that wounding plant tissue before *Agrobacterium* infection, by means of microprojectile bombardment with uncoated particles, improves the transformation efficiency. Particle bombardment methods using *Agrobacterium* were previously used to transform tobacco leaves and sunflower meristems (Bidney *et al.*, 1992), banana meristems (May *et al.*, 1995) and common and tepary bean meristems (Brasileiro *et al.*, 1996). Bidney *et al.* (1992) and Sanford *et al.* (1993) have

suggested the use of *Agrobacterium* cells as microprojectiles. Rasmussen *et al.* (1994) reported biolistic transformation of plant cells using intact bacteria as microprojectiles for the first time, although the number of stably transformated colonies was low. Integrated biolistic and *Agrobacterium* transformation was used later as an alternate method of transformation in several species (Kikkert *et al.* 1999; Droste *et al.* 2000). Cordero de Meso *et al.* (2000) demonstrated enhanced stable transformation of strawberry by using *Agrobacterium* cells as a microprojectile coating.

In order to enhance the transformation frequency of peanut we have tried two different techniques: 1) *Agrobacterium* cells as a microprojectile coating and 2) wounding the explants by bombardment with uncoated microprojectiles followed by inoculation with *Agrobacterium* cells. The latter method combines the advantages of *Agrobacterium* transformation with the ability of particle bombardment to generate micro-wounds, thus enhancing the attachment of bacteria and subsequent gene transfer.

Materials and Methods

Agrobacterium cells as a microprojectile coating

Bacterial microprojectile preparation was done as per the method described by Rasmussen *et al.* (1994) with some modifications. All the three stains of *Agrobacterium* viz. LBA4404, EHA 101 and AGL 1 were tested. An overnight culture of *Agrobacterium* growing in LB medium at pH 5.4 supplemented with 50 mg/l kanamycin was centrifuged at 2500 x g and diluted with sterile water to an OD of 600nm=10. Aliquots of 100 µl of this culture were placed in microcentrifuge tubes, centrifuged for 1 min at 2300 x g and washed with 1 ml of TE buffer (10mM Tris, pH 8, 1mM EDTA). Cells were centrifuged and resuspended in 50 µl of TE buffer by vortexing. An aliquot of 30 µl of gold (0.6 µm) particles at a concentration of 60 mg/ml was spun for 1 min at 15000 x g, resuspended in 40 µl of bacterial suspension and vortexed for 15 sec. Explants were bombarded using 10 µl of this mixture spread on to a microcarrier. Explants were placed on the co-cultivation medium for 2 days after bombardment and then washed in sterile water containing 400 mg/l cefotaxime, blotted dry on filter paper and maintained like that of other transformation studies.

Wounding with uncoated microprojectiles followed by inoculation with Agrobacterium

For wounding of the explants with uncoated gold, the microprojectiles were prepared according to the procedure described earlier in particle bombardment section (5.1.1.). $CaCl_2$ (2.5 M) was intentionally reduced to 25 µl/l as suggested by Droste *et al.* (2000). Immediately after the bombardment, embryonic calluses were incubated with diluted *Agrobacterium* cultures for 30 min prior to co-cultivation.

All the experiments were repeated three times with 3-4 replications.

Results and Discussions

Microwounding explants by means of microprojectile bombardment enhance the frequency of *Agrobacterium* transformation in several species (Bidney *et al.* 1992; Knittel *et al.* 1994; Brasileiro *et al.* 1996).

In the present work, we attempted to increase transformation efficiency of somatic embryos of peanut, combining the previously developed protocols for bombardment transformation.

In the experiment where *Agrobacterium* was coated on to gold particles, we found a much lower frequency of transformation, compared to that of conventional sonication (SAAT). For *Agrobacterium* strains LBA 4404 and EHA 105, 2 out of 20 embryos (10%) showed GUS positive foci (2-3 spots in each embryo). No GUS expression was detected for AGL1.

Where explants were wounded by bombardment and then inoculated with *Agrobacterium* was, no GUS positive results were observed for any of the strains.

Rasmussen *et al.* (1994) while working with tobacco suspension cells, reported transient GUS expression using *E. coli* cells as a microprojectile coating but eventually failed to produce the results with *Agrobacterium*. Kikkert *et al.* (1999) demonstrated the use of phage, yeast and bacteria as microprojectiles to bombard suspension cells from tobacco, maize, petunia and rice, and also petunia leaves, with limited success.

Results indicated that the integrated bombardment and *Agrobacterium* system would be less

promising for peanut transformation using proliferative embryogenic tissue than Sonication-assisted transformation. Bidney *et al.* (1992) showed that wounding of tobacco leaves and sunflower meristems by bombardment prior to *Agrobacterium* treatment increases the transformation frequencies. Later May *et al.* (1995) obtained transgenic banana plants by *Agrobacterium* inoculation of previously bombarded meristems. The results of these transformation protocols are difficult to compare due to differences in plant species, physiological status of the source tissue, type of explant and culture system (Droste *et al.* 2000). Though it is obvious that the microwounds caused by particle bombardment can enhance transformation frequency in other plant species and target tissues, our observations based on the GUS assay suggest that the method holds little promise to enhance *Agrobacterium* infection to obtain stable transformation of peanut.

Conclusion

A well standardize regeneration protocol via somatic embryogenesis has been developed in this laboratory (Ozias –Akins *et al.* 1993). By using the pre-established regeneration protocol and somatic embryos as explant we have tried to standardize an Agrobacterium–mediated transformation protocol in peanut. Three different bacteria strains viz. LBA4404, EHA-105 and AGL 1 were used. For a high frequency transformation different parameters and various methods to induce tissue damage; principally involving Sonication Assisted Agrobacterium mediated transformation (SAAT), and Agrobacterium cells as microcarrier coating were studied.

In transient expression study, results with SAAT were more promising and consistent. Exposure to 30 seconds to 2 min sonication treatment was optimum for peanut embryogenic tissue infected with all three bacteria stains. Use of Agrobacterium cells as microcarrier, and use of particle bombarded for inducing damage to the tissues followed by co-cultivation with bacteria was not found much effective in peanut somatic embryos.

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 biotic factors including various diseases caused by fungal pathogens, viruses, bacteria and nematodes. Biotechnology offers an impressive option to supplement the ongoing efforts on developing genetically modified germplasm to prevent the loss in crop yield. In addition to classical and molecular breeding approaches, genetic transformation to introduce novel genes into plants for quality production offers an attractive option. With this in view attempts were made to standardize protocols for in vitro regeneration and genetic transformation of peanut tissues.

Present study was formulated to address some basic questions associated with peanut embryogenesis, callus induction and transformation of peanut. The work was carried out with three major objectives

1. Studies on somatic embryogenesis for improved conversion to plantlet.
2. Optimization of condition for embryogenic callus induction and regeneration via somatic embryogenesis.
3. Genetic transformation of peanut using Particle gun and *Agrobacterium tumefaciens.*

The major snag in peanut somatic embryogenesis is its very low conversion frequency of somatic embryos to a normal plant. In this study we incorporated Thidiazuron (TDZ) in early embryo development medium with a view to get normal somatic embryos having high germination frequency. TDZ was ineffective in producing somatic embryos with normal plumule but exhibited intriguing response in the developing somatic embryos. Morphogenic buds, appeared particularly in the equatorial region of the embryos at lower concentration (2.27 μM) of TDZ, suggesting presence of potent cells around that region. At higher concentrations (9.08-22.7 μM) morphogenic projections appeared in the plumular part of the somatic embryos which subsequently differentiated into shoot buds. Appearance of these projections was restricted to the plumular half of the somatic embryos. The bud elongated and leaves rolled out on MS basal medium. Roots emerged in these shoots on transferring to MS basal medium supplemented with 0.54 μM NAA. Ten to 18 shoots were obtained from a single somatic embryo. Higher number of plantlet recovery through this protocol dictates its utility in genetic transformation studies. In contrast to the response in the plumule the radicle did not show any

morphological changes indicating lack of sensitivity towards TDZ. The site-specific action of TDZ envisions studies for basic research.

Role of TDZ in conversion of rooted somatic embryo into plantlet was demonstrated earlier. However the pathway of shoot regeneration in the abnormal somatic embryos remains unexplained. TDZ triggered multiple shoot differentiation in the plumule of the peanut somatic embryos. Keeping in view the ability of TDZ to induce both organogenesis and embryogenesis in peanut tissues, experiments were conducted to determine the pathway of morphogenesis in the plumule of the somatic embryos. On exposure to TDZ (22.7 μM), projections resembling somatic embryos appeared from the plumule. Histological examination revealed that these are caulogenic buds and not somatic embryos. These buds differentiated to form shoots. In concurrence with the earlier reports on TDZ induced organogenesis it can be concluded that this growth regulator induces organogenic response in peanut tissues.

A protocol for induction of embryogenic callus and its proliferation and regeneration was optimized after testing various combinations of growth hormone and carbohydrates. The media formulation containing MS basal salts supplemented with 4. 14 μM picloram, and 0.06 M sucrose was effective in inducing embryogenic callus from mature zygotic embryo derived leaflets. A medium composed of MS with 12.42 μM picloram, 0.06 M sucrose and 6.85 mM glutamine was optimized for maintenance of embryogenic callus via primary and secondary embryogenesis. Plantlets were recovered from these embryos in medium containing MS basal salts, 9.08 μM TDZ and 0.06 M sucrose. Thus a protocol for callus induction of morphogenic from mature embryo axis derived explant and proliferation via primary and secondary embryogenesis is optimized. To the best of our knowledge this is the first report describing use of TDZ to convert the repetitive embryos of peanut successfully. Picloram 12.42 μM was optimum for production of somatic embryos repetitively.

Experiments were conducted for optimization of parameters for particle bombardment using a Green Fluorescence Protein (GFP) gene. We obtained stably transformed lines with GFP gene by visual as well as antibiotic selection and successfully regenerated the transgenic plants. In order to optimize a peanut transformation system using GFP as the selectable marker, we evaluated three fluorescent protein mutants for their transient expression efficiencies after particle bombardment of embryogenic cultures of the peanut cultivar, Georgia Green. The fluorescent protein variants used in the present study (Enhanced Green Fluorescent Protein (EGFP), Enhanced Yellow Fluorescent Protein (EYFP) and Enhanced Cyan Fluorescent Protein (ECFP)) differed in their emission and excitation

peaks expressed under CaMV 35S promoter. A fourth construct expressing EGFP under double 35S promoter with an AMV enhancer sequence also was compared. The brightest fluorescent signal was observed from the construct containing EGFP driven by the enhanced double 35S promoter. Bombardments with this construct produced tissue sectors expressing GFP that could be visually selected under the fluorescence microscope over multiple subcultures. Embryogenic lines showing stable expression of GFP over an eight to twelve month period have been obtained. Over a long period GFP brightness has been observed in visually selected lines at reduced intensity. This may be due to a mixture of transgenic and non-transgenic cells. Plants were regenerated from those transformed embryogenic lines. This is the first report of stable transformation of GFP in peanut by visual selection. Using the newly standardized bombardment parameters we successfully transformed the ß-glucuronidase (*GUS*) and hygromycin phosphotransferase (*hph*) gene in peanut somatic embryos. The parameters standardized earlier (with GFP) for bombardment, were found suitable here for both the reporter genes. Integration and expression of foreign gene has been demonstrated by molecular analysis like PCR, Southern blot and Northern blot analysis. Though there was no escape observed on hygromycin selection, but evidence to report GUS gene silencing was obtained.

We have shown the use of herbicide resistance gene; Phosphinothricine acetyl transferase (*Bar*) as a selectable marker in peanut transformation. Transformed lines were isolated after a long selection in liquid medium with phosphinothricin. The presence of *Bar* gene in transformed lines was demonstrated by PCR analysis. This is the first report of using phosphinothricin (*Bar*) gene as a selectable marker.

By using somatic embryos as explant we tried to standardize an *Agrobacterium*-mediated transformation protocol in peanut. Three different bacteria strains viz. LBA4404, EHA-105 and AGL 1 were used. For a high frequency transformation different parameters were tested. Various methods to induce tissue damage, principally involving Sonication Assisted Agrobacterium mediated transformation (SAAT), and Agrobacterium cells as a microprojectile coating were studied.

In transient expression study, results with SAAT were more promising and consistent. Sonication treatment of 30 seconds to 2 min was optimum for peanut embryogenic tissue infected with all three bacterial stains. Use of *Agrobacterium* cells as microprojectile coating and induce damage to the tissues by bombardment, followed by co-cultivation with bacteria was not found much effective in transformation of peanut somatic embryos.

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AUTHOR'S PUBLICATION

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