STUDIES ON *IN VITRO* PLANT REGENERATION AND ITS APPLICATIONS IN PIGEONPEA [*Cajanus cajan* (L.) Millsp.]

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR

THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN **BIOTECHNOLOGY**

BY

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JUNE 2001

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Acknowledgements

I wish to express my deep sense of gratitude to my research guide Dr. K. V. Krishnamurthy for his guidance, encouragement and fruitful discussions, which helped me in the successful culmination of the present endeavor.

I am highly grateful to Dr. S.S. Khuspe for the financial assistance provided and Dr. S.K. Rawal for giving valuable suggestions during the course of this work. I am highly indebted to Dr. (Mrs.) Hazra for support and discussions and lifting my spirits during difficult times.

I am thankful to Dr. M. M. Jana for his kind help in providing facilities for multiplication of seed material and maintenance of plants in the greenhouse. Thanks are also due to Dr. S.H. Patel and Dr. C.N. Patel of GAU, Dr. R. B. Deshmukh of MPKV and Dr. R. P. S. Pundir of ICRISAT for the supply of seeds. The help rendered by Mr. Parag Akkadkar in photography is gratefully acknowledged. I am thankful to Aparna and Bhushan for their help during Southern hybridization work. I am thankful to Professor Baldev, New Delhi for his valuable suggestions.

It was pleasure working in the environment constituting of senior scientists like, Mr. D. D. Kulkarni, Mr. R. B. Naidu and Mr. D. A. Godbole and colleagues Swati, Abhay, Suhasini, Anjali Kulkarni. Thanks to them for maintaining a cheerful atmosphere in the lab. My sincere thanks are due to Anjali Parasnis who helped me during the course of my research work. Further the help rendered by all other members of the Plant Tissue Culture Division - mostly in the form of fruitful discussions and cooperation, which helped me during the course of my work, is also acknowledged. It's pleasure to acknowledge the support and help provided by Jawahar, Tomal, Vijay, Vaidya, Jayashree, Seema, GT, Anjan, Satish, Jayeeta, Neelima, RC, Lata, Prasad, Madhumita, Shilpa, Gaurav, Manisha, Renu and Dinesh Chowk.

I am greatly indebted to Chengal, Vandana, Charu and Mohini for the timely help.

The joyful company of Subray, Vishnumurthy, Upadhya, Godwin, Dinesh, Keshavaraja, Prasanna, Nishikant, Badri, Nandu, members of New Hostel Mess and Research students is worth being cherished and remembered for words would not substitute of what has been accomplished. It is difficult to thank each one separately, who helped me directly and indirectly. I am thankful to all those who have contributed to the completion of my thesis.

Last, but not the least, it is difficult to word my gratitude towards my parents, wife, Prajwal, brother, sister, uncles, aunts, cousins, in-laws for their love, support, patience and motivation.

Finally, I am grateful to the University Grants Commission for the award of the Junior and Senior Research fellowship. I am grateful to Dr. R.A. Mashelkar then Director of NCL and Director General CSIR for permitting me to work in NCL and Dr. Paul Ratnasamy, Director, NCL for permission to submit this work in the form of a thesis.

(Mohan, M.L.)

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"STUDIES ON *IN VITRO* PLANT REGENERATION AND ITS APPLICATIONS IN PIGEONPEA [*CAJANUS CAJAN* (L.) MILLSP.]"** submitted by Mr. Mohan, M.L. was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. K. V. Krishnamurthy)
Research Guide

Pune

Date:

KEY TO ABBREVIATIONS

ABA	Abscisic acid
AdS	Adenine sulfate
ANOVA	Analysis of variance
B5	Gamborg's medium (1968)
BAP	6-Benzylaminopurine.
° C	Degrees celsius.
CaMV	Cauliflower mosaic virus
CDNA	Complimentary DNA
$CHCl_3$	Chloroform
CTAB	Cetyltrimethylammonium bromide
dATP	Deoxy adenosine triphosphate
Dicamba	3,6 Dichloro-2-methoxybenzoic acid
DNA	Deoxyribonucleic acid
DPX	DPX-4 1889-(2-chloro-N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl amino carbonyl) benzene sulfonamide
EC_6	Maheswaran and Williams medium (1984)
EDTA	Ethylenediaminetetraacetic acid.
FAO	Food and Agriculture Organization
GA_3	Gibberellic acid.
GFP	Green fluorescent protein
GUS	β-D-glucuronidase
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin or (6-furfuryl amino purine)
KOH	Potassium hydroxide
Kr	Kilorad
LD_{50}	Lethal dose 50
LS	Linsmaier and Skoog's medium (1965)

MB5	Modified B5 medium
mg/l	Milligrams per liter
µE	Microeinstein
µg	Microgram
µM	Micromolar
MS	Murashige and Skoog's medium (1962)
NAA	α-Napthalene acetic acid.
NaCl	Sodium chloride
$NaPO_4$	Sodium phosphate buffer
nm	Nanometer
*npt*II	Neomycin phospho transferase gene
OD	Optical density
PEG	Polyethylene glycol
Picloram	4 Amino-3,4,6-trichloro picolinic acid
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
rpm	Revolustions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SSPE	Saline sodium phosphate EDTA
TAE	Tris acetic acid EDTA buffer
T-DNA	Transfer-DNA
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thidiazol-5yl) urea
UV	Ultraviolet (light)
v/v	Volume/Volume (concentration)
w/v	Weight/Volume (Concentration)
YEB	Yeast extract broth
Zeatin	6-(4-hydroxy-3-methyl-2-butenylamino) purine
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid.

SYNOPSIS

Legumes are a group of plants which are of utmost importance, useful as a source of proteins in food for humans and animals. They are useful as rotation crops to improve the soil fertility. Until recently, legumes have been mainly used for human consumption as a very cheap source of protein. Recently alternate uses of legumes mainly in the canning and freezing industries have been recognized.

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a major grain legume of the semi-arid tropics. Development of pigeonpea lines, resistant to diseases (mainly wilt caused by *Fusarium udum* Butl.) and pests (principally caterpillars of *Helicoverpa armigera* Hubn.) is of considerable importance in view of the great losses the crop suffers and the slow progress of plant breeding to evolve such lines.

Innovative biotechnological approaches are suggested as an alternative to combat against major biotic and abiotic stresses in pigeonpea. This could be achieved by transfer of desirable traits to high yielding and adaptive cultivars. Integrated pest management and disease control and increase in nutritional quality and yield of grains are the other characteristics which need to be improved. For this, the basic pre-requisites such as high frequency efficient plant regeneration system either through organogenesis or somatic embryogenesis from various explants of pigeonpea and development of gene transfer methodologies are essential.

The present work entitled **"Studies on *In Vitro* Plant Regeneration and its Applications in Pigeonpea [*Cajanus cajan* (L.) Millsp.]"** is devoted to the fulfillment of the basic pre-requisites, in particular, development of *in vitro* regeneration of plantlets from various explants of pigeonpea.

The thesis has been divided into chapters, followed by a summary.

CHAPTER 1: GENERAL INTRODUCTION

This chapter gives a general information of legumes. A thorough literature survey of *in vitro* studies in legumes in general and pigeonpea in particular has been dealt with.

CHAPTER 2: MATERIAL AND METHODS

The source of glassware, plasticware and chemicals used in the present work has been elucidated in this chapter. This chapter also describes the procedures followed for cleaning of glassware and preparation of media. The composition of different media and the various techniques used during the course of this work have also been included.

CHAPTER 3: *IN VITRO* REGENERATION THROUGH ORGANOGENESIS I. FROM DISTAL COTYLEDONARY SEGMENTS

This chapter gives the details of plant regeneration via organogenetic pathway using explants of different cultivars of pigeonpea. Organogenesis was achieved with six different basal medium supplemented with 20 µM BAP, 2 µM Kinetin and 250 µM Adenine Sulfate. This chapter also deals with studies on indirect organogenesis via callus derived from various explants of pigeonpea cultivars.

CHAPTER 4: *IN VITRO* REGENERATION THROUGH ORGANOGENESIS II. FROM MATURE EMBRYO AXES AND SEEDLING DERIVED EXPLANTS

Results of experiments on organogenesis with various explants like epicotyl, leaf, DCMEA (DeCapitated Mature Embryo Axes) and ERMEA (Epicotyl Region of Mature Embryo Axes) are dealt in detail in this chapter. Shoot buds were obtained from epicotyl and leaf explants on EC_6 basal medium supplemented with 20 µM BAP, 2 µM Kinetin and 250 µM Adenine sulfate. DCMEA and ERMEA produced shoots when cultured on EC_6 basal medium supplemented with various concentrations and combinations of BAP and IAA.

CHAPTER 5: SOMATIC EMBRYOGENESIS AND PLANT REGENERATION

Various explants of different cultivars of pigeonpea were evaluated for induction of somatic embryogenesis. The effect of different parameters on induction of somatic embryos has been described in detail in this chapter. Somatic embryos were induced on distal halves of mature cotyledons on EC_6 basal medium supplemented with various concentrations of BAP and TDZ in 2 genotypes.

CHAPTER 6: GENETIC TRANSFORMATION STUDIES

Agrobacterium-mediated genetic transformation of pigeonpea was attempted using decapitated mature embryo axes, epicotyl and leaf explants. Transformation studies were carried out with GUS and GFP reporter genes. Transformed callus expressing GUS reporter gene and transformed plants expressing Green Fluorescent Protein gene have been reported.

SUMMARY

This part of the thesis summarizes the findings of the present investigation and its future implications.

RESEARCH WORK PUBLISHED

(1) **Mohan ML** and Krishnamurthy KV **(1998)** Plant regeneration in pigeonpea [*Cajanus cajan* (L.) Millsp.] by organogenesis. *Plant Cell Reports* **17**:705-710.

(2) **Mohan ML**, Naidu RB, Kulkarni DD and Krishnamurthy KV **(1997)** Regeneration of Plantlets in Pigeonpea [*Cajanus cajan* (L.) Millsp.] by Organogenesis" In: Recent Advances in Biotechnological Applications of Plant Tissue and Cell Culture Eds:GA Ravishankar and LV Venkataraman, Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, pp. 151-155.

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(1) **Mohan ML**, Naidu RB, Kulkarni DD and Krishnamurthy KV **(1995)** Regeneration of Plantlets in Pigeonpea [*Cajanus cajan* (L.) Millsp.] by Organogenesis. In:"All India symposium on recent advances in biotechnological applications of plant tissue and cell culture & XVIII Meeting Plant Tissue Culture Association of India", CFTRI, Mysore, 22-24 June, 1995.

(2) **ML Mohan** and KV Krishnamurthy (1999) Organogenesis and genetic transformation in pigeonpea [*Cajanus cajan* (L.) Millsp.]. In: Emerging Frontiers in Plant Biotechnology, NCL Golden Jubilee National Seminar, NCL, Pune October 28-29, 1999.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Grain Legumes

Legumes are a group of plants, which grow under diverse climatic conditions such as tropical jungles to temperate areas as annuals, perennials, bushes and trees and belong to one of the three largest families of flowering plants-the Leguminosae. The family Leguminosae encompasses 690 genera and 18,000 species, which are characterized by keel-shaped flowers and pod-shaped fruits (Hulse 1989). The legumes are economically important as they form the third largest food crop (190 m. tonnes), following cereals (2054 m. tonnes) and root and tuber crops (625 m. tonnes) (Anonymous 1999). The importance of legumes range from food to fodder, wood to spices and ornamentals. They also play a useful role in biological nitrogen fixation (Duke 1981; Parrott *et al.* 1992).

Grain legumes (pulses) are an important source of dietary proteins, fibre and calories (Bliss 1990; Singh and Singh 1992; Muehlbauer 1993). The protein concentration in grain legume seeds generally varies from 18 to 40% depending on the species and among cultivars within a species (Bliss 1990). Due to its high protein content, grain legumes are important in the production of livestock and fish (Davey *et al*. 1994). They contain approximately 70% of globulins forming the major storage proteins while the rest is made up by glutelins, albumins and free amino acids (Norton *et al*. 1985). Proteins which are abundant in grain legumes are dietically inferior because of low content of sulfur amino acids mainly methionine and cystine (Singh and Singh 1992). Poor digestibility and presence of antinutritional factors also make them inferior (Bliss 1990). However, the high level of lysine in grain legumes makes them a good supplement for cereals, which are deficient in this amino acid (Davey *et al*. 1994). Limited success in improving the nutritional quality of seed proteins has been achieved by conventional breeding methods primarily because genes encoding seed storage proteins with high levels of essential amino acids do not normally exist in any given species (Kriz and Larkins 1991). Modifications of genes coding for seed proteins thereby could be achieved using the tool of genetic engineering. The various approaches that could be advocated are protein sequence modification, development of synthetic genes, overexpression of homologous genes and transfer and expression of heterologous genes (Sun and Larkins 1993). A methionine-rich 2S albumin gene has been isolated from brazil nut and transferred to *Vicia narbonensis* (Saalbach *et al.* 1994).

Some of the grain legumes such as groundnut (*Arachis hypogaea* L.) and soybean (*Glycine max* L.) contain large amounts of edible oils (Christou *et al*. 1993). Grain

legumes are also an excellent source of vitamins (thiamine and niacin), minerals (calcium and iron) and also contain about 60% carbohydrates, mainly as starch (Davey *et al.* 1994).

Grain legumes have been the main crop of Indian agriculture. These crops are generally included in rotation with other crops in most of the areas to keep the soil alive and productive by symbiotic nitrogen fixation with species specific Rhizobia (Buttery *et al.* 1992). However, most of the grain legumes do not fix adequate amount of nitrogen to support luxurious growth and development of plants to yield large quantities of protein-rich seeds.

The yield of grain legumes has become static (**Fig 1.1**) (Muehlbauer 1993). Reduced yields are due to a range of factors, primarily abiotic (such as inclement soil and climatic conditions), biotic (such as pests and diseases) and drought. In addition, there are several constraints such as management, lack of improvement methods and inputs (Nene *et al*. 1989). Grain legumes are susceptible to various fungal, bacterial and viral diseases and to a host of insects and other pests.

Wild species may provide genetic diversity not present in cultivated species as they possess the traits for stress and disease resistance. However, these traits are often associated with undesirable characters such as seed shattering, hard seededness, indeterminate growth habit, which are difficult to overcome in breeding programs (Muehlbauer 1993). Hence development of a molecular marker based system for transfer of specific segment of a genome is required (Muehlbauer 1993).

The conventional breeding methods are time consuming and laborious and plant breeders take time to release new genotypes due to time consuming crossing, back-crossing and progeny selection (Filippone 1993). This has led the plant breeders to explore the feasibility of using alternative biotechnological approaches for the improvement of grain legumes, which include tissue culture techniques of plant differentiation, protoplast regeneration, somaclonal variation, somatic embryogenesis, somatic hybridization, embryo rescue and gene transfer with the help of *Agrobacterium*, biolistic gun and/or electroporation. However, for exploitation of the aforementioned non-conventional methods of crop improvement, the following requirements have to be fulfilled:

(1) an efficient *in vitro* explant to plant regeneration system

(2) a method to deliver foreign DNA to plant tissues

(3) regeneration of plants from stably transformed tissues

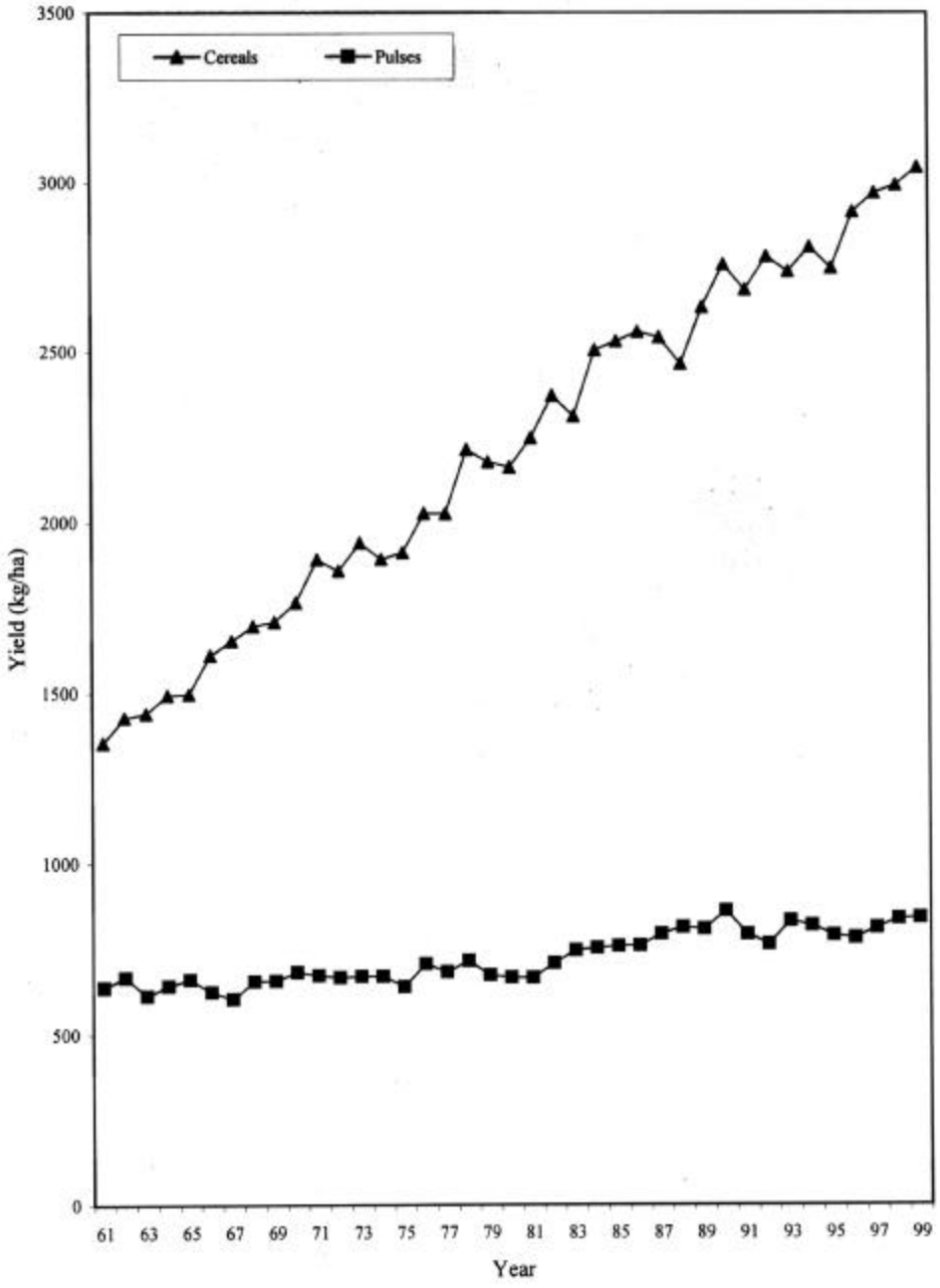


Fig 1.1 Comparison of increase in yield of cereals and pulses in the world

Biotechnology is now the cutting edge of plant science - offering new techniques, applications, and opportunities for crop improvement. Biotechnologists use a variety of techniques to identify genes that determine specific traits (for example, drought tolerance or disease resistance), make crosses between species previously believed to be incompatible, and produce improved genotypes much faster than was possible using conventional plant breeding. Application of biotechnological tools in crop improvement programs can be effective in three different, complementary ways:

→ speeding up the process of conventional breeding

→ creating genetic variability through tissue culture and

→ evolving novel genotypes through recombinant-DNA (r-DNA) technology (Chopra and Sharma 1991).

Using various techniques of genetic engineering and tissue culture, it is now possible to introduce isolated genes derived from different organisms from bacteria to mammals, into plants without causing any additional change in the cultivar. These genetically modified plants can subsequently be incorporated into the conventional plant breeding programs (Puonti-Kaerlas 1993a). Several methods have been developed for transfer of foreign DNA into plant cells.

1.1.1 Nonconventional methods of crop improvement in grain legumes

The *in vitro* culture methods, exploiting the regeneration capacity of plant cells, provided the opportunity to micropropagate elite plant clones by organogenesis or somatic embryogenesis. By manipulation of culture conditions, it is now possible to regenerate plants from intact organs, explants, callus and protoplasts (Davey *et al*. 1994). Plant gene technology has catalyzed progress in plant breeding, but has not yet been applied to food legume improvement on a large scale (Kahl *et al*. 1994). The advances made in the culture of grain legumes has been discussed in the following sections.

1.1.1.1 *In vitro* studies in grain legumes

Legumes exhibit a diversity of responses when cultured *in vitro* (Parrott *et al*. 1992). Until recently, grain legumes have found to be less amenable (recalcitrant) to regeneration *in vitro* (Hammatt *et al*. 1986b; Puonti-Kaerlas 1993a). Plant regeneration from cultured explants occurs via somatic embryogenesis and/or organogenesis, either directly or indirectly via an intervening callus phase (Parrott *et al.* 1992). Considerable progress has been made in the development of efficient plant regeneration systems for grain legumes.

1.1.1.1.1 Organogenesis

Organogenesis, the process by which a cell or a group of cells differentiates to form organs, may occur directly from the explanted tissue or from callus (Parrott *et al.* 1992). Proliferation from pre-existing meristems could be exploited for microprojectile-mediated transformation, as it has several advantages over *de novo* organogenesis since it is less subject to somaclonal variation (Parrott *et al.* 1992). The current status of regeneration by organogenesis in grain legumes is given in **Table 1.1**.

Table 1.1: *In vitro* studies in grain legumes-ORGANOGENESIS: Current status

No.	Species	Reference
1.	*Arachis hypogaea* L.	Illingworth 1968; Martin & Rabechault 1976; Mroginski & Fernandez 1980; Guy *et al.* 1980; Shyluk *et al.* 1981; Kartha *et al.* 1981; Bajaj *et al.* 1981a; 1981b; Mroginski *et al.* 1981; Sastri *et al.* 1982; Pittman *et al.* 1983; Oelck & Schieder 1983; Narasimhulu & Reddy 1983; Narasimhulu & Reddy 1984; Atreya *et al.* 1984; Mhatre *et al.* 1985; Bhatia *et al.* 1985; Seitz *et al.* 1987; Nataraja & Patil 1987; Banerjee *et al.* 1988; McKently *et al.* 1990; McKently *et al.* 1991; Daimon & Mii 1991; Dunbar & Pittman 1992; Cheng *et al.* 1992; Kachonpadungkitti *et al.* 1992; Eapen & George 1993b; Li *et al.* 1994; Feng *et al.* 1994; Kanyand *et al.* 1994; Chengalrayan *et al.* 1995; Ponsamuel *et al.* 1995; Sabita Rani & Reddy 1995; D'Silva & Podder 1995; Hopkins & Pinnow 1995; Ilahi *et al.* 1995; Heatly & Smith 1996; Feng *et al.* 1996; Venkatachalam *et al.* 1996; Cheng & Yeh 1997; Hu 1997; Venkatachalam & Jayabalan 1997; Kanyand *et al.* 1997; Morris *et al.* 1997; Venkatachalam *et al.* 1998a; Ponsamuel *et al.* 1998; Zharare *et al.* 1998; Venkatachalam *et al.* 1999b.

Table 1.1 Continued…

Table 1.1 (Contd)

No.	Species	Reference
1.	*Arachis hypogaea* L	Radhakrishnan *et al*. 1999; Pestana *et al*. 1999; Victor *et al.* 1999a; Gill & Ozias-Akins 1999.
2.	*Cajanus cajan* (L.) Millsp.	Shama Rao & Narayanaswamy 1975; Mehta & Mohan Ram 1980; Kumar *et al*. 1983; Kumar *et al*. 1984; Cheema & Bawa 1991; Sarangi & Gleba 1991; Eapen & George 1993b; George & Eapen 1994; Shiva prakash *et al*. 1994; Naidu *et al*. 1995; Sreenivasan *et al.* 1995; Eapen *et al.* 1998; Franklin *et al*. 1998; Geetha *et al*. 1998; Franklin *et al*. 2000.
3.	*Cicer arietinum* L.	Sharma *et al*. 1979; Bajaj 1979; Bajaj & Dhanju 1979; Kartha *et al*. 1981; Singh *et al*. 1982; Bajaj 1983; Khan & Ghosh 1983; Khan & Ghosh 1984; Altaf & Ahmad 1985; 1986; Neelam *et al* 1986a; 1986b; 1986c; Rao & Chopra 1987a; 1987b; Rao & Chopra 1989b; Sangvan *et al*. 1989; Sheila *et al*. 1991; Surya-Prakash *et al*. 1992; Malik & Saxena 1992a; Chandra *et al*. 1993; Brandt & Hess 1994; Barna & Wakhlu 1994; ; Murthy *et al*. 1996; Vani & Reddy 1996; Polisetty *et al*. 1997; Kanyand *et al*. 1997; Chandra *et al*. 1998; Nalini Mallikarjuna 1999.
4.	*Glycine canescens*	Widholm & Rick 1983; Grant 1984.
5.	*Glycine clandestina*	Hammatt *et al.* 1986a; Hymowitz *et al.* 1986.
6.	*Glycine max* L.	Oswald *et al*. 1977; Cheng *et al*. 1980; Kartha *et al*. 1981; Kameya & Widholm 1981; Wright *et al*. 1986; Barwale *et al*. 1986a; 1986b; Barwale & Widholm 1987; Wright *et al*. 1987; Freytag *et al*. 1989; Coble & Schapaugh Jr. 1990.

Table 1.1 (Contd…)

Table 1.1 (Contd)

No.	Species	Reference
6.	*Glycine max* L	Yang *et al*. 1990; Thome *et al*. 1995; Kaneda *et al*. 1997; Dan and Reichert 1998.
7.	*Glycine soja*	Barwale *et al.* 1986b.
8.	*Glycine* spp.	Hammatt *et al.* 1987a; 1987b.
9.	*Lathyrus* spp.	Malik *et al.* 1992; Malik *et al.* 1993.
10.	*Lens culinaris* (L.) Medic	Polanco *et al.* 1988; Malik & Saxena 1992a; Ahmad *et al*. 1996; Polanco and Ruiz 1997.
11.	*Lupinus* spp.	Sroga 1987; Mulin & BellioSpataru 2000.
12.	*Phaseolus acutifolius* L.	Dillen *et al.* 1996; Zambre *et al*. 1998
13.	*Phaseolus aureus* L.	Ghosh *et al.* 1979; Bajaj & Singh 1980.
14.	*Phaseolus coccineus* L.	Abou-Mandour & Hartung 1980; Angelini & Allavena 1989; Santalla *et al*. 1998
15.	*Phaseolus vulgaris* L.	Haddon & Northcote 1976; McClean & Grafton 1989; Franklin *et al*. 1991; Malik & Saxena 1991; Mohamed *et al*. 1992a; 1992b; Malik & Saxena 1992c; FernandezCaso *et al*. 1996; Benedicic *et al*. 1997; Santalla *et al.* 1998; Zambre *et al.* 1998
16.	*Pisum sativum* L.	Hildebrandt *et al*. 1963; Malmberg 1979; Mroginski & Kartha 1981; Hussey & Gunn 1984; Rubluo *et al.* 1984; Jackson & Hobbs 1990; Nauerby *et al.* 1991; Malik & Saxena 1992a; Nadolska-Orczyk *et al* 1994; B hmer *et al*. 1995; Sanago *et al*. 1996; Kosturkova *et al*. 1997; Popiers *et al.* 1997; Madsen *et al.* 1998
17.	*Vicia faba* L.	Thynn & Werner 1987; Khallafalla & Hattori 1999.
18.	*Vicia narbonensis* L.	Tegeder *et al.* 1996.
19.	*Vigna aconitifolia* (Jacq) Marechal	Bhargava & Chandra 1983; Godbole *et al.* 1984; Gill *et al.* 1986; Krishnamurthy *et al.* 1986.

Table 1.1 Continued…

Table 1.1 (Contd)

No.	Species	Reference
19.	*Vigna aconitifolia* (Jacq) Marechal	Jain & Chopra 1988; Sangeeta *et al*. 1988; Bhargava & Chandra 1989; Gehlot *et al*. 1989; Sankhla *et al*. 1990; Sankhla *et al.* 1991.
20.	*Vigna mungo*	Gosal & Bajaj 1983; Geetha *et al*. 1997a; 1997b; Sen and Guha Mukherjee 1998; Das *et al*. 1998; Ignacimuthu & Franklin 1998.
21.	*Vigna radiata* L.	Mendoza & Futsuhara 1990; Gulati & Jaiwal 1990; Gulati & Jaiwal 1994; Chandra & Pal 1995; Narciso *et al*. 1996; Narciso *et al*. 1997; Sen & Guha Mukherjee 1998; Betal & SenRaychaudhari 1999.
22.	*Vigna sinensis*	Pandey & Bansal 1989.
23.	*Vigna unguiculata* (L.) Walp	Pellegrineschi 1997; Brar *et al*. 1997; Soh *et al.* 1998; Brar *et al.* 1999a; 1999b.

1.1.1.1.2 Somatic Embryogenesis

Somatic embryogenesis - a process whereby a cell or a group of cells from somatic tissues form an embryo (Parrott *et al.* 1992) may be indirectly with an intervening callus phase or directly from the explants. **Table 1.2** represents the studies on somatic embryogensis in grain legumes.

Table 1.2: *In vitro* studies in grain legumes-SOMATIC EMBRYOGENESIS: Current status

No.	Species	Reference
1.	*Arachis duranensis*	Sabita Rani & Reddy 1996.
2.	*Arachis hypogaea* L.	Pittman *et al*. 1983; Banerjee *et al.* 1988; Ozias-Akins 1989; Hazra *et al*. 1989; Sellars *et al*. 1990; McKently 1991; Ozias-Akins *et al*. 1992a; Ozias-Akins *et al*. 1992b; Durham & Parrott 1992; Gill & Saxena 1992; Baker & Wetzstein 1992; Rau *et al*. 1992; Saxena *et al.* 1992; Wetzstein & Baker 1993.

Table 1.2 Continued...

Table 1.2 (Contd)

No.	Species	Reference
2.	*Arachis hypogaea* L.	George & Eapen 1993; Eapen & George 1993a; Eapen *et al*. 1993; Reddy & Reddy 1993; Mhaske & Hazra 1994; Baker & Wetzstein 1994; Baker *et al*. 1994; Chengalrayan *et al*. 1994; Feng *et al*. 1994; Baker & Wetzstein 1995; Baker *et al*. 1995; Chengalrayan *et al*. 1995; McKently 1995; Murthy *et al*. 1995; Sabita Rani & Reddy 1996; Venkatachalam & Jayabalan 1996; Venkatachalam *et al*. 1997; Chengalrayan *et al*. 1997; Chengalrayan *et al*. 1998; Baker & Wetzstein 1998; Venkatachalam *et al*. 1998a; Venkatachalam *et al*. 1999a; 1999c; Victor *et al*. 1999a; 1999b; Murch *et al*. 1999.
3.	*Cajanus cajan* (L.) Millsp.	Bajaj *et al*. 1980; Ramana Rao *et al*. 1992; George & Eapen 1994; Patel *et al*. 1994; Nalini Mallikarjuna *et al*. 1996; Sreenivasu *et al*. 1998; Anbazhagan & Ganapathi 1999.
4.	*Cicer arietinum* L.	Rao & Chopra 1989a; Shri & Davis 1992; Sagare *et al*. 1993; Barna & Wakhlu 1993; Dineshkumar *et al*. 1994; Islam 1994; Suhasini *et al*. 1994; Eapen & George 1994a; Kumar *et al*. 1995; Sagare *et al*. 1995a; 1995b; Dineshkumar *et al*. 1995; Barna & Wakhlu 1995; Adkins *et al*. 1995; Suhasini *et al*. 1996; Vani & Reddy 1996; Ramana *et al*. 1996; Murthy *et al*. 1996; Hita *et al*. 1997; Suhasini *et al*. 1997; Guru *et al*. 1999.
5.	*Glycine max* L.	Christianson *et al*. 1983; Gamborg *et al*. 1983; Lippmann & Lippmann 1984; Lazzeri *et al*. 1985; Li *et al*. 1985; Ranch *et al*. 1985.

Table 1.2 continued…

Table 1.2 (Contd)

No.	Species	Reference
5.	*Glycine max* L	Barwale *et al*. 1986a; Kerns *et al*. 1986; Ghazi *et al*. 1986; Lazzeri *et al.* 1987a; Buchheim *et al*. 1989; Christou & Yang 1989; Kiss *et al*. 1991a; Kiss *et al*. 1991b; Lazzeri *et al*. 1987b; Komatsuda & Ohyama 1988; Finer 1988; Finer & Nagasawa 1988; Hartweck *et al.* 1988; Hepher *et al*. 1988; Wright *et al*. 1991; Komatsuda *et al*. 1992; Gill & Saxena 1992; Liu *et al*. 1992; Ranch 1993; Lippmann & Lippmann 1993; Gyulai *et al*. 1993; Bailey *et al.* 1993a; 1993b; Ma *et al.* 1994; Nadolska-Orczyk & Orczyk 1994; Stejskal & Griga 1995; Li & Grabau 1996; Santos *et al*. 1997; Rajasekaran & Pellow 1997; Santarem *et al*. 1997; Samoylov *et al* 1998
6.	*Phaseolus acutifolius*	Kumar *et al*. 1988b; Malik & Saxena 1992b.
7.	*Phaseolus aureus* L.	Bajaj & Singh 1980; Malik & Saxena 1992b.
8.	*Phaseolus coccineus* L.	Rota *et al*. 1990; Genga & Allavena 1991; Malik & Saxena 1992b.
9.	*Phaseolus vulgaris* L.	Martin & Sondahl 1984.
10.	*Pisum sativum* L.	Jacobsen & Kysely 1984; Kysely *et al*. 1987; Lehminger-Mertens & Jacobsen 1989; Kysely & Jacobsen 1990; Tetu *et al*. 1990; Stejskal & Griga 1992; Nadolska-Orczyk *et al*. 1994; Loiseau *et al*. 1995; Bencheikh & Gallais 1996a; Bencheikh & Gallais 1996b; Loiseau *et al*. 1996; Loiseau *et al*. 1998
11.	*Psophocarpus tetragonolobus* (L.) DC.	Ahmed *et al.* 1996; Gupta *et al.* 1997.

Table 1.2 Continued…

Abbreviations used in the Table 1.3:

A.t - *Agrobacterium tumefaciens*; **A.r** - *Agrobacterium rhizogenes*; **I.P.I** – *In planta injection* **M.B** - Microprojectile bombardment; **Elect** - Electroporation; **PEG** - Polyethylene Glycol; **C** - Callus; **T** - Tumor; **R** - Rhizogenesis; **S.E** - Somatic embryos; **T.P** - Transgenic plant; **T.G** - Transient gene expression; **S** – Shoots; **P.P** - Protoplasts

Table 1.2 (Contd)

No.	Species	Reference
12.	*Vicia faba* L	Tegeder *et al.* 1995.
13.	*Vicia narbonensis* L.	Pickardt *et al*. 1989; Albrecht & Kohlenbach 1989; Tegeder *et al.* 1996.
14.	*Vigna aconitifolia* (Jacq) Marechal	Kumar *et al.* 1988a; Eapen & George 1990.
15.	*Vigna mungo* L. (Hepper)	Eapen & George 1990.
16.	*Vigna radiata* (L.) Wilczek	Eapen & George 1990.
17.	*Vigna sinensis*	Li *et al*. 1995a
18.	*Vigna unguiculata* (L) Walp	Kulothungan *et al.* 1995.

1.1.1.1.3 Genetic transformation

A range of somatic cell and molecular techniques are now available to supplement conventional plant breeding. Gene transfer (or DNA uptake) refers to a process, which moves a specific piece of DNA (usually a foreign gene ligated to a bacterial plasmid) into protoplasts or cells (Jenes *et al.* 1993). The introduction and expression of foreign DNA has been used to introduce commercially important characteristics such as herbicide and insect resistance, changes in oil and protein contents and virus tolerance (Walden 1989). Several techniques for DNA delivery into plant cells are available, ranging from *Agrobacterium*-mediated gene transfer, direct gene transfer through electroporation into protoplasts/intact tissues/either by PEG method or by uptake of DNA into naked protoplasts to injection and the use of microprojectile bombardment-mediated transformation to introduce DNA into intact tissues (**Table 1.3**).

Table 1.3: *In vitro* studies in grain legumes-GENETIC TRANSFORMATION: Current status

No.	Species	Method of Gene transfer	Remarks	Reference
1.	*Arachis hypogaea* L.	A.t (wild)	T	Lacorte *et al.* 1991.
		A.t	T	Mansur *et al.* 1993.
		M.B	S.E	Ozias-Akins *et al.* 1993.
		M.B	T.P	Schnall & Weissinger 1993.

Table 1.3 continued…

Table 1.3 (Contd)

No.	Species	Method of Gene transfer	Remarks	Reference
1.	*Arachis hypogaea* L.	A.t M.B	T.P T.P	Eapen & George 1994b. Brar *et al.* 1994.
		A.t	T.P	McKently *et al.* 1995.
				Schnall & Weissinger 1995
		A.t	T.P	Cheng *et al.* 1996.
		A.t	T.P	Cheng *et al.* 1997.
		A.t		De Freitas *et al.* 1997.
		M.B	T.G	Lacorte *et al.* 1997.
		A.t	S	Sarkar *et al.* 1997.
		A.r M.B	R T.P	Akasaka *et al.* 1998. Wang *et al.* 1998.
		A.t	T.P.	Egnin *et al.* 1998
		M.B	T.P	Yang *et al.* 1998.
		A.t.	T.P	Venkatachalam *et al.* 1998b
		M.B		Livingstone & Birch 1999
		M.B	T.P	Kim *et al.* 1999
		A.t	T.P	Rohini & Rao 2000
2.	*Cajanus cajan* L.	A.t.	**T.P**	Geetha *et al.* 1999
3.	*Cicer arietinum* L.	A.t (wild)	T	Mridula *et al.* 1988.
		A.t		Srinivasan *et al.* 1988.
		A.t		Srinivasan *et al.* 1991.
		A.t	T	Mohapatra & Sharma 1991.
		A.t	T.P	Fontana *et al.* 1993.
		A.t (wild)	T	Islam *et al.* 1994.
		A.r	T	Siefkes-Boer *et al.* 1995.
		A.t		Ramana *et al.* 1996.
		A.t	T.P	Kar *et al.* 1996.
		A.t	T.P	Krishnamurthy *et al.* 2000
4.	*Cyamopsis tetragonoloba* L.	A.t	T.P	Joersbo *et al.* 1999
5.	*Glycine max* L.	A.t/A.r	T/R	Owens & Cress. 1985.
		A.t	C	Baldes *et al.* 1987.
		Elect.	C/R	Christou *et al.* 1987.
		M.B	C	Christou *et al.* 1988.

Table 1.3 Continued…

Table 1.3 (Contd)

No.	Species	Method of Gene transfer	Remarks	Reference
5.	*Glycine max* L.	A.t	T.P	Hinchee *et al.* 1988.
		A.t	T	Owens & Smigocki 1988.
		A.t	T.P	Parrott *et al.* 1989.
		M.B	T.P	Christou 1990.
		Elect.	C	Christou & Swain 1990.
		Elect. M.B	C/S T.P	Dhir *et al.* 1991a; 1991b. Finer & McMullen 1991.
		A.t	T/T.P	McKenzie & Cress 1992.
		M.B	T.P	Sato *et al.* 1993a.
		A.t	T	Austin & Cress 1994.
		A.t (wild)	T	Bailey *et al.* 1994.
		A.t	T.P	Falco *et al.* 1995.
		A.t	T	Bond *et al.* 1996.
		M.B	C	Hadi *et al.* 1996.
		M.B A.t	T.P T.P	Stewart *et al.* 1996. Di *et al.* 1996.
		M.B	T.P	Liu *et al.* 1996.
		A.t	S	Santarem *et al.* 1998
		M.B	T.P	Maughan *et al.* 1999
		M.B	S.E	Santarem & Finer 1999
		I.P.I	T.P	Hu & Wang 1999
		A.t	T.P	Zhang *et al.* 1999
				Ponappa *et al.* 1999
6.	*Lathyrus sativus* L.	A.t/M.B	T.P	Barna & Mehta 1995.
7.	*Lens culinaris* (L.) Medic.	A.t	T	Warkentin & McHughen 1991.
		A.t	S	Warkentin & McHughen 1992.
8.	*Lupinus augustifolius* L.	A.t	T.P	Pigearie *et al.* 1997.
9.	*Phaseolus vulgaris* L.	A.t	T.P	Mariotti *et al.* 1989.
		M.B	T.G	Genga *et al.* 1991.
		M.B	T.G	Aragao *et al.* 1993.
		A.t	C	Franklin *et al.* 1993.
		A.t	T	Lewis & Bliss 1994.
		Elect.	T.G	Dillen *et al.* 1995.
		M.B	T.P	Kim & Minamikawa 1996.
		M.B	T.P	Aragao *et al.* 1996.

Table 1.3 Continued

Table 1.3 (Contd)

No.	Species	Method of Gene transfer	Remarks	Reference
9.	*Phaseolus vulgaris* L.	M.B/A.t	T.G	Brasileiro *et al.* 1996.
		M.B	T.P	Aragao & Rech 1997.
		A.t	S	Zhang *et al*. 1997.
		A.t, A.r	S	Barros *et al.* 1997.
		Elect.	P.P	Giovinazzo *et al.* 1997
		M.B Elect	T.P S	Aragao *et al.* 1998 Saker and Kuhne 1998
10.	*Pisum sativum* L.	A.t (wild) A.t	T T.P	Hobbs *et al.* 1989. Puonti-Kaerlas *et al.* 1989.
		A.t A.t	T.P T.P	De Kathen & Jacobsen 1990. Puonti-Kaerlas *et al.* 1990.
		A.t/A.r	T,S,R	Schaerer & Pilet 1991.
		A.t	T.P	Davies *et al.* 1993.
		A.t	T.P	Jordan & Hobbs 1993.
		PEG	T.G	Nicolaisen & Poulsen 1993.
		A.t	T.P	Schroeder *et al.* 1993.
		A.t	C	De Kathen & Jacobsen 1995.
		A.t	T.P	Grant *et al.* 1995.
		A.r	R	Nicoll *et al.* 1995.
		A.t	T.P	Bean *et al.* 1997.
		A.t A.t	T.P T.P	Grant *et al.* 1998 Simonenko *et al.* 1999
11.	*Vicia narbonensis* L.	A.t	T.P	Pickardt *et al.* 1991.
		A.t	T.P	Pickardt *et al.* 1995
				Saalbach *et al* 1995
12.	*Vigna aconitifolia* (Jacq) Marechal	A.t	C	Eapen *et al.* 1987.
		PEG	T.P	Köhler *et al.* 1987a.
		Elect.	T.P	Köhler *et al.* 1987b.
13.	*Vigna angularis* L.	A.t	T.P	Ishimoto *et al.* 1996.
14.	*Vigna mungo* (L.) Hepper	A.t	S	Pal *et al.* 1991.
		A.t	C	Karthikeyan *et al.* 1996.

Table 1.3 Continued…

Table 1.3 (Contd)

No.	Species	Method of Gene transfer	Remarks	Reference
15.	*Vigna unguiculata* (L.) Walp	A.t	C	Garcia *et al.* 1987.
		A.t	T.P	Penza *et al.* 1991.
		Elect.	C	Akella & Lurquin 1993.
		A.t	T.P	Muthukumar *et al.* 1996.

The ability to regenerate plants from cultured explants, tissues and protoplasts affords the opportunity to genetically manipulate legumes through somatic cell techniques (Davey *et al.* 1994). Though regeneration protocols exist for many legumes, genetic transformation reports are available only for a few species. The major reason for this is the fact that legume species have proven to be less amenable to transformation/regeneration procedures than most of the other dicotyledonous crop species (Kahl *et al.* 1994). Despite these difficulties, transgenic plants have been obtained from several leguminous crop species i.e., *Glycine max* (Hinchee *et al.* 1988; McCabe *et al.* 1988; Chee *et al.* 1989); *Vigna aconitifolia* (Köhler *et al.* 1987a & b); *Pisum sativum* (Puonti-Kaerlas *et al.* 1990) and *Cicer arietinum* (Fontana *et al.* 1993; Kar *et al.* 1996; Krishnamurthy *et al.* 2000).

1.1.1.1.4 Somaclonal variation

Plant breeders are continually searching for new genetic variability that is potentially useful in cultivar improvement (Parrott *et al.* 1992). Genetic variation that results as a consequence of frequent culturing of plant cells (somaclonal variation), is the simplest form of genetic manipulation (Larkin and Scowcroft 1981). It is rarely associated with gross cytological changes and is probably related to modifications in the nucleic acid composition. Although, many somaclonal variants exhibit characteristics which are detrimental compared to those of parental plants, individuals have been produced which express superior traits (Davey *et al.* 1994).

Somaclonal variations have been exploited recently to recover useful variants in *Glycine max* (Barwale and Widholm 1990). Among the variants observed, were chlorophyll deficiency, partial or complete sterility, numerous abnormalities in leaf morphology and number, twin seeds and multiple shoots. Changes in maturity date and

increases in oil content among regenerants have also been reported (Barwale and Widholm 1990). However, this variation was not inherited.

1.1.1.1.5 Somatic hybridization

Somatic hybridization has the potential for improving grain legumes, but the absence of regeneration from protoplast to plant systems in many a legumes has deterred the extension of the technique to grain legumes. In *Glycine max*, heterokaryons between *G. max* cv. HP-20-20 and perennial wild *Glycine* G1171 produced shoots, but these shoots failed to develop into plants (Hammat *et al.* 1992).

1.1.1.1.6 Embryo rescue

Wild relatives of crop plants comprise an important germplasm resource for plant improvement (Davey *et al.* 1994). Crosses between distantly related plants are generally unfruitful because of the abortion of embryos on the mother plant. These embryos can be precociously excised and cultured *in vitro* (Monnier 1990).

By using this technique, a large number of hybrid plants have been obtained and several genetic characteristics have been transferred in grain legumes. Important among those are hybrids produced in *Arachis* (Bajaj 1990b), *Glycine* (Grant 1990) and *Vigna* (Bhadra *et al.* 1989). Recently, this technique has been successfully exploited to create a viable hybrid in *Glycine max* (Hu and Zanettini 1995).

1.2 Pigeonpea [*Cajanus cajan* (L.) Millsp.]

1.2.1 The crop

Pigeonpea (Red gram), belonging to the family Papilionaceae and subfamily Papilionoideae (Purseglove 1988), is one of the major grain legumes grown in the world (**Table 1.4**) and in India, it has a large area under cultivation (**Table 1.5**). India is the largest producer of pigeonpea in the world. It is the second most important grain legume of India after chickpea. Nearly 85 % of the world's pigeonpea crop is grown in India (**Table 1.6**). It is grown in the kharif season in the Indian subcontinent under semi-arid conditions. It is a cheap source of protein in the human diet and its production has become stagnant (**Fig 1.2**). Roots of pigeonpea shed piscidic acid, which can dissolve iron phosphates in the soil, making it possible for the plants to absorb these nutrients through their roots. The pigeonpea plant can also improve the structure of the soil, as its deep roots bore channels deep into the ground, increasing the infiltration of water for subsequent crops (Madeley 1995).

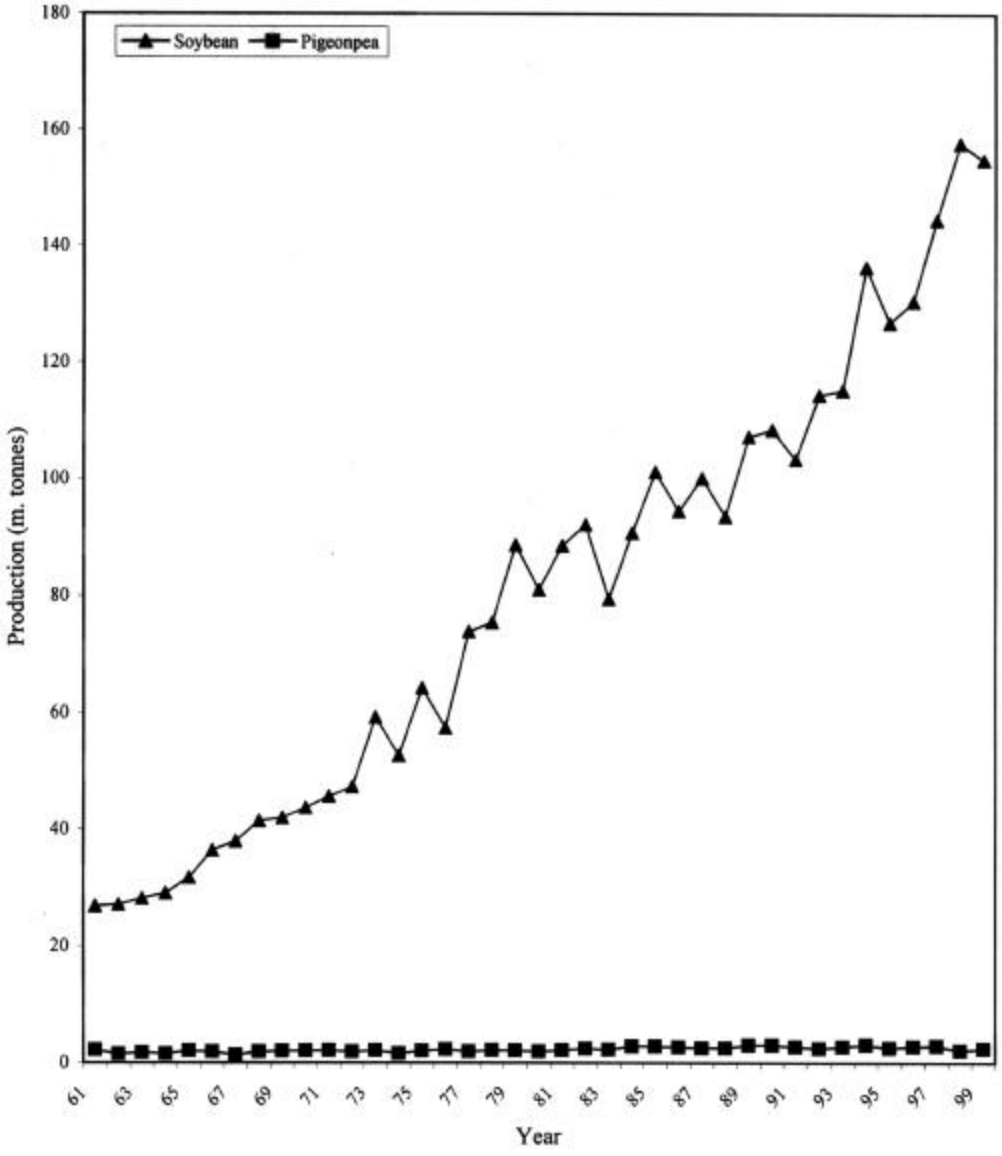


Fig 1.2 Comparison of increase in production of soybean and pigeonpea (world)

Table 1.4: World production, area and average yield of the major grain legumes in 1998

No.	Grain legume	Area m.ha	Production m. tonnes	Average yield kg/ha
1.	*Arachis hypogaea* L.	23.80	30.97	1,301
2	***Cajanus cajan* (L.) Millsp.**	**4.28**	**2.87**	**672**
3.	*Cicer arietinum* L.	11.20	8.59	767
4.	*Glycine max* (L.) Merr.	70.69	158.33	2,240
5.	*Lens culinaris* (L.) Medic.	3.40	2.99	8,776
6.	*Phaseolus vulgaris* L.	25.69	17.62	686
7.	*Pisum sativum* L.	6.90	13.19	1,912
8.	*Lupinus* spp	1.36	1.52	1,119
9.	*Vicia faba* L.	2.22	3.40	1,532
10.	*Vigna unguiculata* (L) Walp	6.66	2.44	366

Source : FAO 1999.

Table 1.5: Area, production and productivity of major grain legumes in India

No.	Grain legume	Area (m ha)	Production (mt)	Productivity (kg/ha)
1.	*Arachis hypogaea* L.	8.10	8.30	1025
2.	***Cajanus cajan* (L.) Millsp.**	**3.67**	**2.45**	**668**
3.	*Cicer arietinum* L.	7.22	6.01	832
4.	*Glycine max* (L.) Merr.	6.35	6.10	960
5.	*Lathyrus sativus* L.	0.85	0.49	576
6.	*Lens culinaris* (L.) Medic.	1.34	0.88	660
7.	*Phaseolus aureus* Roxb.	3.08	1.31	425
8.	*Phaseolus mungo* L.	3.15	1.49	473
9.	*Phaseolus vulgaris* L.	9.50	3.60	379
10.	*Pisum sativum* L.	0.88	0.74	841

Source : FAO 1999; The Hindu Survey of Indian Agriculture 1999

Table 1.6: World pigeonpea production

Location	Area (ha)	Production (MT)
World	4,276,789	2,873,916
Asia	3,952,700	2,634,472
Africa	281,300	204,300
North Central America	36,970	31,813
South America	5,819	3,331
Bahamas	195	140
Bangladesh	6,000	3,000
Burundi	2,300	2,300
Dominican Rep.	20,800	20,409
Grenada	500	580
Haiti	7,500	3,000
Myanmar	251,700	162,500
India	**3,670,000**	**2,450,000**
Jamaica	1,630	1,950
Malawi	143,000	99,000
Nepal	25,000	18,972
Panama	4,500	2,100
Puerto Rico	745	454
Tanzania	65,000	45,000
Trinidad & Tobago	1,100	3,180
Uganda	71,000	58,000
Venezuela	5,819	3,331

Source : FAO 1999.

1.2.2 Origin and distribution

It is grown mainly in Central and West Asia, South Europe, Ethiopia and North Africa (Yadav 1992). It is often stated that the genus *Cajanus* is monotypic, but in addition to cultigen *C. cajan* there are wild species. The chromosome number is $2n = 2x = 22$. Africa is the probable center of origin of cultivated pigeonpea, where it is found in wild form. However, according to Vavilov (Yadav 1992), India is the place of origin of pigeonpea.

Pigeonpea is divided into two botanical varieties "var. *flavus*" and "var. *bicolor*". The cultivars of var. *flavus* are earlier maturing, shorter plants with yellow standards, and

green glabrous pods, which are light colored when ripe, and are usually 3-seeded. These are the *tur* cultivars of India, where they are extensively cultivated in the Peninsula. The cultivars of var. *bicolor* are perennial, late maturing, large, bushy plants, with dorsal side of standard red or purple or streaked with these colors, and hairy pods blotched with maroon or dark colored, with 4-5 seeds, which are darker colored or speckled when ripe. These are the *arhar* cultivars of India, which are more extensively cultivated in the north of the country (Purseglove 1988).

1.2.3 Plant habit

It is a woody, short-lived, 1-4 m tall and often-cross pollinated perennial shrub grown as annual. Leaves are spirally arranged with a phyllotaxis of 2/5, trifoliate, ovate, hairy. It has a pronounced deep tap-root system with numerous laterals (Purseglove 1988). The pods are somewhat flattened, dehiscent and contain 2 to 8 seeds (Kay 1979).

1.2.4 Importance

Pigeonpea is the second most important pulse crop after chickpea in India. The young green seeds are eaten as a vegetable in many countries and are canned in Puerto Rico and Trinidad. The ripe seeds are boiled and eaten as a pulse. In India these are split and made into dhal. The green pods are sometimes used as a vegetable. The tops of the plants with fruits provide excellent fodder and are also made into hay and silage. Pigeonpea contains approximately 19.2% protein and 57.3% carbohydrates in dried seed. The protein and carbohydrate composition of Indian split dhal is 22.3% and 57.2% respectively (Purseglove 1988). It is also a good source of fibre, vitamins and minerals. Pigeonpeas are excellent source of vitamin B, average figures are: Thiamine 500 mg; riboflavin 150 mg; nicotinic acid 2.3 mg per 100 g of edible portion. The average mineral content is calcium 154-194 mg/100 g and phosphorus 238-372 mg/100 g. They are low in the essential amino acids methionine and cystine (Kay 1979).

1.2.5 Biotic stresses that affect pigeonpea

Pigeonpea suffers heavy losses in yield due to fungal, bacterial and viral diseases and insect pests because of its low and highly variable grain yield in the rainfed and low management input conditions under which it is usually grown. The low and unstable yields can be ascribed to the narrow genetic base of cultivars, which could be broadened by incorporating agronomically important traits from related wild species (Pundir and Mengesha 1995).

1.2.5.1 Fungal diseases

The most important and widespread fungal disease of pigeonpea is wilt, [*Fusarium oxysporum* f. sp. *udum*], which is soil-borne and affects the plant at all stages of its development. In India, where it is particularly serious, crop losses of 5-10 % are fairly commonplace, and in severe cases can amount to 50 % or more. Other fungal diseases of this crop are leaf spot (*Cercospora indica*), stem blight (*Phytophthora cajani*) and stem canker (*Diplodia cajani*) (Kay 1979).

1.2.5.2 Bacterial diseases

The major bacterial disease that plague pigeonpea is the bacterial leaf spot and stem canker caused by *Xanthomonas cajani* (Kay 1979).

1.2.5.3 Viral diseases

Several virus diseases affect pigeonpeas. A sterility disease (pigeonpea sterility mosaic), which is transmitted by mite or nematode, can cause almost complete crop failure. Other virus diseases are witches broom (transmitting vector is leafhopper) and cowpea mosaic (Kay 1979).

1.2.5.4 Insect pests

Substantial losses in yield also occur due to infestation by insects. The major pests are gram caterpillar (***Helicoverpa armigera***) (**Fig 1.3**), red gram plume moth (*Exelastis atomosa*), gram pod fly (*Melanagromyza obtusa*) and thrips (*Franliniella sulpurea* and *Taeniothrips nigriconis*). At the post-harvest stage, pulse beetles, *Callosobruchus chinensis*, are a very serious pest, where infestation can begin in the field. The female beetle lays eggs on the grain and emerging larvae feeds on the testa, subsequently boring into the pulse and pupating within the damaged portion of grain. The adult emerges out of the pupa and begins to feed on the grain (Kay 1979).

1.2.6 Abiotic stresses that affect pigeonpea

Drought, cold, heat and salinity are the abiotic stresses, which adversely affect the Pigeonpea crop. Pigeonpea is grown throughout India, but predominantly in the states of Uttar Pradesh, Gujarat and Maharashtra, which together contribute about 85% of the total growing area and production of India (Muller *et al*. 1990). More than 51% of the saline soils in India are located in these states (Agarwal *et al*. 1976). India's more than 7 million hectares of saline soils (Abrol 1991) coincides with agroclimatic zones otherwise favorable for pigeonpea cultivation. Among cultivated legumes, pigeonpea is classified as moderately sensitive to salinity (Keating and Fisher 1985).

Damage caused to the pigeonpea pods by the insect *Helicoverpa armigera* Hübner

1.2.7 Other factors that need improvement

1.2.7.1 Nutritional quality

In recent years plant breeders have been working to select varieties of grain legumes that are not only more productive but are also of an improved nutritional quality. In general, legume grains comprise an important part of the human diet in developing countries located in tropical and subtropical areas. The nutritional contribution is of paramount importance here, as these populations have limited access to foods of animal origin (Eggum and Beames 1983). Inadequate proportion of sulfur containing amino acids such methionine and cystine are the major limitations in pigeonpea followed by insufficient percentage of tryptophan. It appears that the protein quality of pulses in general is low, primarily due to the low content of sulphur-containing amino acids when compared to S-containing aminoacid's content and composition of cereals (Burr 1975).

Most nutritional and biochemical studies carried out so far with legume grains have dealt mainly with two factors that are important in determining their protein quality. One consists of the antiphysiological substances present in legume grains, of which the trypsin inhibitors, amylase inhibitor, and haemagglutinins are the most important. The second is the well-documented deficiency of sulfur-containing amino acids (Bressani and Elias 1977).

The full meaning of productivity is not complete if it refers merely to weight of grains per unit area. Rather, productivity must be viewed as the efficiency with which the total nutrient production meets the needs of the population, with a minimum of waste. It is recommended that the basis for selection of food crops must be based on production per hectare as the first component of productivity. The second component of productivity has to be nutritional quality, which refers mainly to protein. The pulses remain under-exploited source of edible protein. Greater attention needs to be given to their genetic diversity in order to improve amino acids profiles, in particular to improve the level of sulfur-containing amino acids and to eliminate anti-nutritional factors (Eggum and Beams 1983).

1.2.8 *In vitro* approaches of crop improvement

In recent years, rapid technological advances have resulted in the establishment of highly specialized techniques of tissue culture with potential applications for crop improvement (Mroginski & Kartha 1985). Despite the widely reported *in vitro* recalcitrance of legumes, 75 species from 25 genera have undergone *de novo* regeneration and limited contributions to crop improvement have been realized (Parrott *et al.* 1992). In the recent years, attention

has been focused on the development of regeneration systems amenable to gene transfer technology (Parrott *et al*. 1992). Asymmetric hybridization and genetic transformation methodologies involving protoplasts of cultivated and wild species of plants have been advocated to overcome impediments in the conventional breeding strategies.

Pigeonpea has wild relatives that are of potential value, through hybridization, in genetic improvement of the cultivated varieties. A remarkable trait of the wild species of pigeonpea is that most of them have high seed protein (**Table 1.7**). The maximum recorded is 33.4 % for *Cajanus mollis*, while the mean protein in cultivated pigeonpea is 22.1 %. Protein content of the pigeonpea could not be improved by conventional breeding due to incompatibility existing between the wild and cultivated pigeonpea (Remanandan *et al*. 1988). There are very few sources of resistance to blight and sterility mosaic. Sources of resistance to *Fusarium* wilt are not known among the wild relatives of pigeonpea. There are no known source of resistance to lepidopteran borers but high trypsin and chymotrypsin inhibitors are reported (**Table 1.7**). By conventional methods the progress has not yielded much results because of sexual incompatibility with its wild relatives. Therefore, biotechnological methods of improvement has been suggested to overcome the impediments in the conventional plant breeding. Pigeonpea is considered as one of the recalcitrant crops and not amenable to tissue culture.

Table 1.7 Seed protein contents and other characters of wild species of pigeonpea

No.	Wild Species	Seed Protein (%)	Remarks
1.	*Cajanus acutifolia* F.V. Muell	28.7	Sterility mosaic resistant, blight and podfly susceptible, high seed protein content, high trypsin and chymotrypsin inhibition.
2.	*C. albicans* (W.&A.) Benth.	28.7	Sterility mosaic resistant, blight and podfly susceptible, high seed protein content, high trypsin and chymotrypsin inhibition.
3.	*C. cajanifolia* Haines.	29.2	Highly susceptible to sterility mosaic, blight susceptible, susceptible to lepidopteran borers (Most probable progenitor of cultivated pigeonpea).
4.	*C. lineata* W.&A.	32.6	Sterility mosaic resistant, blight susceptible, high seed protein content.
5.	*C. mollis* Benth.	33.4	High seed protein content.

Table 1.7 Continued…

Table 1.7 (Contd)

No.	Wild Species	Seed Protein (%)	Remarks
6.	*C. platycarpa* Benth.	29.3	Blight resistant, sterility mosaic susceptible, high seed protein content.
7.	*C. reticulata* (Dryander) Benth.	24.1	-
8.	*C. scarabaeoides* (L.) Benth.	28.5	Susceptible to sterility mosaic and blight, *Hymnoptera* susceptible, antibiosis to *Heliothis armigera*, high seed protein content.
9.	*C. sericea* Benth.	28.6	Blight and sterility mosaic resistant, susceptible to lepidopteran borers, high seed proein content.
10.	*C. volublis* (Branco) Gamble	29.1	Sterility mosaic resistant, blight susceptible, high seed protein content, high trypsin and chymotrypsin inhibition.
11.	*Rhynchosia rothii* Benth. Ex. Benth.	28.7	High seed protein conent, extreme trypsin and chymotrypsin inhibition.
12.	*R. bracteata* Benth. Ex. Bak.	28.6	-
13.	*R. cana* DC	30.7	-
14.	*R. densiflora* DC	26.4	-
15.	*R. minima* DC	26.0	-
16.	*R. viscida* DC	28.4	-

Development of pest and disease resistance are hindered either due to cross incompatibility between resistant wild species and the cultivated varieties or due to unavailability of sources of resistance among the cross compatible species. To overcome these problems, a radical technique such as fusion of protoplasts of wild and cultivated *Cajanus* species could be an attractive proposition to transfer agronomically useful traits such as pest and disease resistance to the cultivated varieties. The use of molecular markers and the generation of a genetic map would also be desirable for marker assisted selection and the positional cloning of resistance genes in pigeonpea (Ramana Rao *et al*. 1992).

Abbreviations used in Tables 1.8-1.10:

AB - Axillary bud, **AM** - Apical meristem, **ANT** - Anther, **CA** - Callus, **CN** - Cotyledonary node, **CO** - Cotyledons, **EP** - Epicotyl, **HY** - Hypocotyl, **ICS** - Immature cotyledonary segments, **IEA** - Immature embryo axes, **L** - Leaf, **MCS** – Mature cotyledonary segments, **MEA** - Mature embryo axes, **MS** - Multiple Shoots, **NDS** – Nodular structures, **PL** - Plant, **PRO** - Protoplast, **R** - Root, **S** - Shoot, **SB** - Shoot bud, **SC** – Suspension culture, **SD** – Seed, **SE** - Somatic embryos.

The control of insect pest - *Helicoverpa armigera* (Hübner) could be achieved by transfer of insect resistant traits, but the lack of an efficient high frequency plant regeneration system has deterred the production of transgenic plants in pigeonpea.

1.2.8.1 Organogenesis in pigeonpea

There were many reports of regeneration of pigeonpea by organogenesis (**Table 1.8**). Multiple shoots were obtained from cotyledon explants (Mehta and Mohan Ram 1980; Kumar *et al*. 1984; Sarangi and Gleba 1991; Naidu *et al*. 1995); Geetha *et al*. 1998), apical meristem (Kumar *et al*. 1984; Cheema and Bawa 1991; Naidu *et al*. 1995; Franklin *et al*. 1998) mature embryo axes (Sarangi and Gleba 1991; Naidu *et al*. 1995; Franklin *et al*. 2000), cotyledonary node (Shiva Prakash *et al*. 1994; Geetha *et al*. 1998) and axillary bud (Franklin *et al*. 1998). All these regeneration systems were organogenesis from pre-existing meristems. Moreover, efficient transfer of plants to field was not achieved and even if transferred the information on number of plants transferred to field is not available. There are reports of *de novo* organogenesis from leaf (Eapen and George 1993b; George and Eapen 1994; Geetha *et al*. 1998; Eapen *et al*. 1998), epicotyl (Kumar *et al*. 1984; Naidu *et al*. 1995; Geetha *et al*. 1998) and hypocotyl (Cheema and Bawa 1991; Geetha *et al*. 1998). However, no histological evidence was provided for *de novo* origin of shoot buds. The transfer of plants obtained by *de novo* organogenesis to field was not achieved successfully in any of the earlier reports. No reports of organogenesis from distal cotyledonary segments are available. High frequency genotype independent protocol for organogenesis is still lacking in pigeonpea.

Table 1.8: *In vitro* studies in pigeonpea (*Cajanus cajan* (L.) Millsp.] : ORGANOGENESIS

No.	Cultivars	Explant	Mode of Regeneration	Reference
1.	Prabhat	CO	CO→MS→PL	Mehta & Mohan Ram 1980.
2.	ICP 6917, ICP 6974, ICP 7119, ICP 7263, Wild	CO AM EP	CO→MS→PL AM→PL EP→SB→PL	Kumar *et al.* 1984.
3.	AL-15	AM HY	AM→PL HY→SB→PL	Cheema & Bawa 1991.

Table 1.8 Continued…

Table 1.8 (Contd)

No.	Cultivars	Explant	Mode of Regeneration	Reference
4.	-	CO MEA	CO→MS→PL MEA→MS→PL	Sarangi & Gleba 1991.
5.	ICPL 161	L	L→SB→PL	Eapen & George 1993b.
6.	ICPL 161	IEA ICS EP SD R CO L	IEA→NDS ICS→NDS EP→CA SD→SB R→SB→PL CO→SB→PL,R L→SB→PL	George & Eapen 1994
7.	Gaut-89-8, Gaut-88-29, BP-86-34, SPMA-4	CN	CN→MS→PL	Shiva Prakash *et al.* 1994.
8.	T-21, PT-22, T-Vishaka-1, ICPL 87, N-290-21	MEA CO EP	MEA→MS→PL CO→MS→PL EP→SB→PL	Naidu *et al.* 1995.
9.	BDN-2	S	S→R	Sreenivasan *et al.* 1995.
10.	ICPL 161, ICPL 88039, UPAS 120	L	L→SB→PL	Eapen *et al.* 1998.
11.	-	AM AB	AM→PL AB→PL	Franklin *et al.* 1998.
12.	Hyderabad C	CN EP HY CO L	CN→MS→PL EP→MS→PL HY→MS→PL CO→MS→PL L→MS→PL	Geetha *et al.* 1998
13.	VBN1, VBN2, SA1, CO5	MEA	MEA→MS→PL	Franklin *et al.* 2000

1.2.8.2 Callus culture

Very few reports of differentiation of callus into plants are available in the literature. Basal part of the embryo and the part of the cotyledon adjacent to the embryo gave multiple shoots via callusing in MS medium containing 0.5 mgL^{-1} BAP. Distinct variation has been observed in the regenerated plants (Sarangi and Gleba 1991). Kumar *et al.* (1983) obtained regeneration from cotyledonary callus and leaf callus cultures when

incubated on Blaydes' medium fortified with BAP, NAA and Gibberellic acid. Shama Rao and Narayanaswamy (1975) reported that hypocotyl segments obtained only from γ-irradiated (5 kr) seeds produced abundant calli and shoot buds in 50% of the cultures but no plants were obtained from these shoot buds. However, no histological evidence of formation of shoot buds via callus has been demonstrated and the plants could not be transferred to field successfully in any of the reports on callus differentiation.

1.2.8.3 Somatic embryogenesis

There are very few reports of somatic embryogenesis in pigeonpea (**Table 1.9**). George and Eapen (1994) obtained somatic embryos from immature embryonal axes and immature cotyledons. Very few somatic embryos were obtained but plantlets capable of transfer to the field could not be obtained from the somatic embryos. Patel *et al*. (1994) reported somatic embryogenesis from distal halves of cotyledons. Transfer of plantlets to field and histological observations to demonstrate the initiation and development of somatic embryos were not reported. Somatic embryogenesis has also been reported from cotyledons. Inclusion of BAP in the medium resulted in the formation of well-developed embryo-like structures (Nalini Mallikarjuna *et al*. 1996). Transfer of plantlets to the field could not be obtained. Sreenivasu *et al*. (1998) obtained indirect somatic embryogenesis via callus from cotyledon and leaf explants. Evidence for the presence of various developmental stages of somatic embryogenesis and histological observations to show different stages of somatic embryo development were not presented. Somatic embryos were obtained from suspension cultures derived from leaf callus (Anbazhagan and Ganapathi 1999). No histological observations were made to demonstrate the various developmental stages of somatic embryos and the transfer of plantlets to field was not achieved. The successful transfer of complete plants to pots has been achieved in a very few systems.

Table 1.9: *In vitro* studies in pigeonpea [*Cajanus cajan* (L.) Millsp.] : SOMATIC EMBRYOGENESIS

No.	Cultivars	Explant	Mode of Regeneration	Reference
1.	ICPL 161	ICS IEA	ICS→SE IEA→SE	George & Eapen 1994.

Table 1.9 Continued…

Table 1.9 (Contd)

No.	Cultivars	Explant	Mode of Regeneration	Reference
2.	T-15-15, Gaut-82-90, Bandapalera, NP(WR)15	MCS	MCS→SE→PL	Patel *et al.* 1994.
3.	ICPL 87	L R	L→CA→SE→PL R→CA→SE→PL	Nalini Mallikarjuna *et al.* 1996.
4.	Pusa-606, Pusa-609, Pusa-852, Pusa-855, Pusa-856, H-86-5	L CO	L→CA→SE→PL CO→CA→SE→PL	Sreenivasu *et al.* 1998.
5.	Vamban-1	L	L→CA→SC→SE→PL	Anbazhagan & Ganapathi 1999

1.2.8.4 Protoplast culture

Protoplast technology is well established now for many plant species and is being routinely used for somatic hybridization and direct gene transfer (Puite 1992). Limited reports of protoplast isolation and culture are available. The regeneration of pigeonpea plants from protoplast derived callus has not been achieved so far (**Table 1.10**). Pigeonpea protoplasts were first isolated from leaves (Zhihong *et al*. 1985). High yields of protoplasts and formation of calli were also observed in pigeonpea and its wild relatives (Kulkarni and Krishnamurthy 1989; Ramana Rao *et al*. 1992; Sarangi *et al*. 1992).

Table 1.10: *In vitro* studies in pigeonpea [*Cajanus cajan* (L.) Millsp.] : PROTOPLAST REGENERATION

No.	Cultivars	Explant	Mode of Regeneration	Reference
1.	-	L	L→PRO→CA	Zhihong *et al.* 1985.
2.	T-21, T-148	L	L→PRO→CA	Kulkarni & Krishnamurthy 1989.
3.	ICPL 87, Rayagada Local, 82208 Kandula	L	L→PRO→CA	Sarangi *et al.* 1992.

1.2.8.5 Anther culture

Pollen embryogenesis was induced in the *in vitro* cultured anthers of pigeonpea. A suspension of pollen from anthers incubated in drop cultures on agar-agar medium

developed to form embryoids and colonies of callus (Bajaj *et al*. 1980). In anther culture, pollen grains underwent division and multicellular structures were found by Mohan Ram *et al*. (1982). No plantlet regeneration has been achieved so far using anther culture method.

1.2.8.6 Somaclonal variation

Studies on somaclonal variation for the varietal improvement of pigeonpea were carried out by Chintappalli *et al*. (1997). Molecular characterization of somaclonal variants was also done recently (Prasannalatha *et al*. 1999).

1.2.8.7 Embryo rescue

Hybrid vigor is shown in crosses between pure lines, but this is difficult to utilize because of technical difficulties and the production of hybrid seed would be costly (Purseglove 1988). The embryos from wide crosses could be rescued at early stages of development and cultured to get a viable hybrid.

Patel *et al.* (1992) standardized technique for embryo rescue of intervarietal and intergeneric hybrids by culturing on modified MS medium supplemented with IAA, kinetin and coconut water. The success in intervarietal crosses was 80-90 % but the cross of cultivars with wild *Atylosia lineata* was unsuccessful.

Nalini Mallikarjuna and Moss (1995) developed a interspecific hybrid between *Cajanus platycarpus* and *Cajanus cajan* using an efficient embryo rescue technique to overcome the barrier, which is post-zygotic in nature in hybridization experiments. All earlier efforts to hybridize *C. platycarpus* with *C. cajan* were unsuccessful (Ariyanayagam and Spence 1978; Kumar 1985; Dundas 1985; Pundir and Singh 1987). The *C platycarpus*, a wild species of pigeonpea has many desirable characters important for the improvement of cultivated pigeonpea but is incompatible with the cultigen. The F1 hybrids were found completely pollen sterile.

Dundas (1985) recommended that the transfer of desirable genes from *C platycarpus* may be possible by finding bridge-cross combinations between *C platycarpus* and other compatible wild *Cajanus* species.

1.2.8.8 Genetic transformation

Only a single report of genetic transformation in pigeonpea is available. Geetha *et al*. (1999) obtained transgenic plants of pigeonpea using *Agrobacterium*-mediated transformation with GUS reporter gene and *npt*II gene as selectable marker.

1.3 Aims of the thesis

At the time of initiation of this work there were no reports of somatic embryogenesis and genetic transformation in pigeonpea. The very few reports of regeneration in pigeonpea on organogenesis were restricted mainly to pre-existing meristems.

The main objectives of the present work with pigeonpea were therefore :

(1) to develop an *in vitro* regeneration system via organogenesis using different explants;

(2) to develop an *in vitro* regeneration system via somatic embryogenesis using different explants;

(3) to standardize conditions for *Agrobacterium* mediated genetic transformation.

CHAPTER 2

MATERIALS AND METHODS **(GENERAL)**

This chapter describes the techniques routinely followed in plant tissue culture work. The materials and methods which were specific to the particular experiments have been dealt in detail in the respective chapters. Techniques of genetic transformation by *Agrobacterium* method used in the present study has been described in the chapter 6 of the thesis.

2.1 Glassware

Glassware used in all the experiments was procured from "Borosil" India. Test tubes (25x150 mm), Petri dishes (85 mm x 15 mm), Conical flasks (100 ml, 250 ml, 500 ml and 1000 ml capacity) and Pipettes (1, 2, 5 and 10 ml capacity) were used for conducting the experiments.

2.1.1 Preparation of glassware

Glassware was cleaned by an initial boiling in a saturated solution of sodium bicarbonate for 1 h and subsequent washing in tap water repeatedly. It was then immersed in 30% nitric acid solution for 30 min followed by a thorough wash with tap water. The glassware, thus washed, was then rinsed twice with glass distilled water and allowed to dry in an oven at 200^{o}C for two hours.

Test tubes and flasks were plugged with absorbent cotton (Veer Industrial Corp., Meerut, India) and petri dishes were wrapped in brown paper prior to sterilization. Graduated pipettes and Pasteur pipettes were packed in aluminium pipette canisters. Autoclaving was carried out at 1.4 $kg.cm^{-2}$ for 1 h.

2.2 Plasticware

Sterile disposable Petri dishes (35 mm, 55 mm and 85 mm diameter) and filter sterilization units were obtained from "Laxbro" (India). Microfuge tubes (1.5 ml capacity) and Microtips (0-200 µl and 200-1000 µl capacity) were obtained either from "Biorad" (USA), or "Laxbro", India or from "Tarsons", India.

2.3 Chemicals

The chemicals used in the course of the study were of analytical grade and were obtained from "Qualigens", "S.D's fine chemicals", E-Merck and "Hi-Media", India. All vitamins, phytagel, antibiotics (except cefotaxime), growth regulators, chemicals used in the molecular biological study were obtained from "Sigma Chemical Co.", USA. Cefotaxime was procured from Russel India Ltd., Mumbai, India. Agar-agar and sucrose were obtained from "Qualigens" and "S.D's fine chemicals" and "Hi-Meda", India.

2.4 Preparation of culture media

Analytical grade chemicals and glass distilled water was used for the preparation of culture media. After addition of all constituents of media, the pH was adjusted to 5.8 using 0.1 N KOH or HCl. Gelling agent (agar-agar) was added as per the requirement and the medium was steamed to melt the agar for 1/2-1 h depending on the volume of the media and the concentration of the agar. It was then dispensed into test tubes or flasks and was autoclaved at 1.4 $kg.cm^{-2}$ for 20 min. Heat labile constituents like antibiotics, growth regulators, vitamins etc., were filter sterilized by passing through a millipore membrane (0.22 µm pore size) and added aseptically to the autoclaved medium just before gelling. The composition of various basal media used for culturing of explants has been given in **Table 2.1**.

Table 2.1: Composition of inorganic and organic components (mg/l) of seven plant tissue culture basal media

Major salts	**Whites**[a]	**MS**[b]	**1/2MS**	**LS**[c]	**B5**[d]	**MB5**[e]	**EC_6**[f]
NH_4NO_3	-	1650	825	1650	-	-	600
KNO_3	80	1900	950	1900	3000	2500	950
$MgSO_4.7H_2O$	360	370	185	370	500	250	185
$(NH_4)_2SO_4$	-	-	-	-	134	150	-
KCl	65	-	-	-	-	-	-
KH_2PO_4	-	170	85	170	-	-	170
NaH_2PO_4	16.5	-	-	-	150	150	-
Na_2SO_4	200	-	-	-	-	-	-
$CaCl_2.2H_2O$	-	440	220	440	150	250	166
$Ca(NO_3)_2.4H_2O$	200	-	-	-	-	-	-

Table 2.1 Continued…

Table 2.1 (Contd)

Minor salts	**Whites**[a]	**MS**[b]	**1/2MS**	**LS**[c]	**B5**[d]	**MB5**[e]	**EC_6**[f]
Na_2-EDTA.$2H_2O$	-	37.3	37.3	37.3	37.3	37.3	37.25
$FeSO_4.7H_2O$	-	27.8	27.8	27.8	27.8	27.8	27.85
H_3BO_3	1.5	6.2	6.2	6.2	3.0	3.0	0.62
$CoCl_2.6H_2O$	-	0.025	0.025	0.025	0.25	0.25	0.0025
$CuSO_4.5H_2O$	0.01	0.025	0.025	0.025	0.25	-	0.0025
$MnSO_4.H_2O$	5.04	-	-	-	10.0	-	-
$Na_2MoO_4.2H_2O$	-	0.25	0.25	0.25	0.25	0.25	0.025
KI	0.75	0.83	0.83	0.83	0.75	0.75	0.083
$ZnSO_4.7H_2O$	2.67	8.6	-	-	3.0	3.0	0.86
$MnSO_4.4H_2O$	-	22.3	22.3	22.3		10.0	2.23
MoO_3	0.001	-	-	-	-	-	-
$Fe(So_4)_3$	2.5	-	-	-	-	-	-
Organics							
Myo-inositol	-	100	100	100	100	100	100
Nicotinic acid	-	0.5	0.5	-	1.0	1.0	1.0
Pyridoxin.HCl	-	0.5	0.5	-	1.0	1.0	1.0
Thiamine.HCl	-	0.1	0.1	0.4	10.0	10.0	10.0
Glycine	-	2.0	2.0	-	-	-	-

[a] White (1963); [b] Murashige & Skoog (1962); [c] Linsmaier & Skoog (1965); [d] Gamborg *et al.* (1968); [e] Mante & Boll (1975); [f] Maheswaran & Williams (1984)

2.5 Collection of plant material

Seeds of cultivars of pigeonpea Gaut-82-90, Gaut-82-99, T-15-15, N-290-21, T-21, TV-1, PT-22, ICPL-87, ICPL-87119, ICP-6917, ICP-7128, ICP-7182, BDN-1 and BDN-2 were used for the present study. These cultivars were collected from Mahatma Phule Agricultural University, Rahuri, Gujarat Agricultural University, Baroda, and International Crops Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad.

2.6 Preparation of plant material

2.6.1 Mature explants

Sixty seeds were taken in 250 ml Erlenmeyer flask and washed with 1% (v/v) detergent Labolene® for 5 min. Seeds were then washed 5-6 times with distilled water and treated with 70% ethanol for 1 min followed by 5-6 rinses with distilled water. All further operations were carried out under sterile conditions in laminar air flow cabinet. Seeds were then transferred to a sterile 250 ml Erlenmeyer flask containing 80 ml 0.1% (w/v) mercuric chloride solution and agitated for 5 min followed by 5-6 washes with sterile distilled water and left in about 250 ml sterile distilled water for soaking for 18 h at 25 ± 2°C in the dark as stationary or kept on a gyratory shaker at 200 rpm. Presoaked seeds were used for preparation of mature embryo axes, mature cotyledons and distal cotyledonary segments or various mature embryo axes derived explants like DCMEA and ERMEA. Details on preparation of explants are provided in the respective chapters.

2.6.2 Seedling explants

The surface sterilized (as described in section **2.6.1**) seeds were inoculated immediately after surface sterilization in 250 ml flasks (7 seeds/flask) containing 80 ml of MS basal medium. The 10 day old seedlings were used for the preparation of various explants like leaf, epicotyl, cotyledon, cotyledonary node and root. Details on preparation of explants are provided in the respective chapters.

2.7 Handling of explants

All inoculations were carried out in a laminar air flow cabinet. All dissections were done on a sterile filter paper or in a glass Petri dish. Sterile surgical blades No. 11 and No. 23 (Kehr Surgical and Allied Products Pvt. Ltd., Kanpur, India) were used for cutting the explants. Forceps, blade holders and other instruments used were dipped in 95% alcohol and flamed prior to the inoculations.

2.8 Statistical analysis

The data were analyzed using ANOVA technique and treatment means were compared (Panse and Sukhatme 1967).

2.9 Culture conditions

The cultures were incubated in the culture room at 25 ± 2°C in dark, diffuse light (4.6 $\mu Ein.m^{-2}.s^{-1}$), medium light (38 $\mu Ein.m^{-2}.s^{-1}$) and high light (140 $\mu Ein.m^{-2}.s^{-1}$) intensity. The details of culture conditions have been mentioned in the description of each set of experiments separately.

2.10 Histology

For histological confirmation of the initiation and delvelopment of structures, the explants at different stages of development were fixed in FAA (Formalin:Acetic acid:ethanol) (5:5:90 v/v) for 72 h at RT. Tissues were dehydrated by passing stepwise through t-butanol series, followed by paraffin wax (58-60 °C mp) embedding as described by Sharma and Sharma (1980). Embedded tissues were cut into 10 µM thick sections, using rotary microtome (Reichert-Jung 2050 supercut, Germany). The sections were dewaxed by treating with xylene for 5-10 min. After removing paraffin wax, sections were passed through the following solutions in coplin jars, 5 min each (except hematoxylin and eosin solutions): Xylene → Xylene:Absolute ethanol (1:1) → Xylene:Absolute ethanol (1:1) → Absolute ethanol → 70 % ethanol → 40 % ethanol → 20 % ethanol → water → 4 % iron alum (Aqueous – w/v) → water → 1 % hematoxylin (Aqueous – w/v) → water → 20 % ethanol → 40 % ethanol → 70 % ethanol → Absolute ethanol → Absolute ethanol → 1 % eosin (w/v in absolute ethanol → Absolute ethanol → Xylene:Absolute ethanol (1:1) → Xylene:Absolute ethanol (1:1) → Xylene → Xylene (Kept for 10-15 min.). The slides were removed from xylene and the sections were mounted in DPX-4 1889-(2-chloro-N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl amino carbonyl) benzene sulfonamide (DPX) (BDH) mountant and observed microscopically, photographed using a camera attached to a microscope (Docuval, Carl Zeiss, Germany), on a 100 ASA black and white film.

2.11 Hardening of the plantlets

In vitro rooted shoots were taken out from the test tubes and gently washed under tap water to remove the agar and medium sticking to it. The shoots were dipped in 0.5 % of a systemic fungicide Bavistin® (BASF, India) for 10-15 min and then washed with tap water. The treated shoots were transferred in 8 cm earthen pots containing a mixture of autoclaved vermiculite and soil (1:1). The pots were covered with polypropylene bags and kept in the hardening room under diffused light conditions. The plants were watered once in a week. The top corners of polypropylene bags were cut after 2 weeks to gradually expose the plants to the outside environment. After 3-4 weeks, the polypropylene bags were completely removed.

2.12 Gene tic transformation

Details of materials and methods used for *Agrobacterium tumefaciens* mediated transformation have been described in the chapter 6.

CHAPTER 3

IN VITRO REGENERATION THROUGH ORGANOGENESIS I. FROM DISTAL COTYLEDONARY SEGMENTS

3.1 Introduction

Origin and changes in the specific form (shape, structure, organization) during the development of an organism and all such changes on and in the organism are called morphogenesis (Thorpe 1983). The recent adaptation of descriptive terms from animal development has stimulated a better understanding of morphogenesis from cells and tissue cultures. Cells and tissue cultures are viewed as first acquiring competence, which is associated with altered differential gene regulation and expression (Thorpe 1983).

The commitment of competent cells for morphogenesis is affected by many factors including complex interaction between genotypes, the explant (and its stage of development), medium, etc. Morphogenesis is triggered usually after competent cells are subcultured into a less complex medium allowing the expression of new developmental potential. This trigger is referred to as permissive induction (Thorpe 1983).

Morphogenesis *in vitro* occurs in two different patterns i.e. embryogenesis and organogenesis. Organogenesis is the process by which cells and tissues are manipulated to undergo changes, which lead to the production of unipolar structures namely a shoot or root primordium, whose vascular system is often connected to the parent tissues. In contrast, somatic embryogenesis leads to the production of bipolar structures containing a root/shoot axis with a closed independent vascular system. Both of these can occur directly on explants or indirectly via callus (Thorpe 1994).

Research on morphogenesis *in vitro* began in the early 1900s when Haberlandt theorized that the entire plant could be produced from a single living cell (Haberlandt 1902). Early studies on tobacco callus culture by Miller and Skoog (1953) indicated that auxins and cytokinins could be used to manipulate morphogenesis *in vitro*. In subsequent studies, critical factors determined were the types, concentrations and ratios of various plant growth regulators used (Sutter 1988). Pioneering work by Steward (1958) and Reinert (1958) on induction of embryogenesis in carrot suspension cultures introduced more questions than answers concerning the role of plant growth regulators and other controlling factors in morphogenesis (Sutter 1988).

With the advent of micropropagation and genetic engineering, plants from hundreds of species have been grown *in vitro* (Bajaj 1986). Still many species remain recalcitrant in culture conditions. It is important that regeneration of whole plants from single cells or simple tissues of recalcitrant species be accomplished if the possibilities of genetic engineering are to be fully implemented. Until recently, standardization of successful methods for regeneration have proceeded empirically. The literature abounds

with methods, which have taken years to develop because a basic understanding of the regulatory processes of morphogenesis were not known. Furthermore, knowledge gained from successful manipulation of one species or cultivar is often not applicable to other species or cultivars even if the plants are closely related genetically. Development of successful methods for regeneration of recalcitrant plant species *in vitro* would benefit from improved understanding of fundamental regulatory mechanisms of morphogenesis.

Since the discovery of phytohormones and the hypothesis of regulation by auxin/cytokinin ratio (Skoog and Miller 1957), significant progress has been made leading to important applications in agronomy and industry and to the promising technology of haploidization and of protoplast fusion leading to genetic engineering.

However, changes in the macro-micronutrient ratio and the addition of various substances such as charcoal or organic compounds (vitamins, amino acids, polyamines, polypeptides, steroids or diverse plant extracts) and carbohydrates, a great number of combinations and variations of light (quality and quantity), pH, water potential, temperature, gaseous atmosphere, container shape etc., can affect morphogenesis (Tran Thanh Van 1981). It is believed that neither the outburst of miscellaneous factors nor the unique hypothesis of auxin-cytokinin ratio can bring one closer to a basic understanding of the whole process of morphogenetic differentiation as long as the target cells are scattered among a heterogeneous mass of cells. The need for having a common cell origin for all morphogenetic patterns emerges from these considerations in order to localize more closely the target cells and the role of morphogenetic signals (Tranh Thanh Van 1981). This has resulted in voluminous literature describing various factors that influence morphogenetic response in plant tissues, but the regulatory processes of morphogenesis still remain unknown.

Plant tissues cultured *in vitro* are able to differentiate and form organs *de novo*. Such organs include roots, shoots and flowers (Thorpe 1980). The earliest report on controlled shoot formation *in vitro* was by White (1939). He observed that shoots were formed on callus of *Nicotiana glauca* X *Nicotiana langsdorffii* hybrid when it was submerged in a liquid medium, but not when cultured on the surface of nutrient agar. In the same year the first observation of root formation from callus was reported by Nobecourt (1939) using carrot callus. White's observation was confirmed and extended by Skoog (1944) who showed that auxins could stimulate root formation and inhibit shoot formation. A similar conclusion on the role of auxin in rooting was made by Gautheret (1945). In addition, Skoog (1944) found that the inhibitory effect of auxin on shoot

formation could be partially overcome by increasing the concentration of sucrose and organic phosphate in the medium.

For manipulating organogenesis *in vitro*, many growth promoters, phytohormones as well as other types of compounds have been included in the medium. Although, a large number of plant species respond to suitable auxin/cytokinin balance by forming shoots and roots, the permissive phytohormone balance leads to the induction of organogenetic tissue only in a number of cases (Hicks 1994). This will then develop into organs in a medium with an altered phytohormonal balance. Steward *et al*. (1964) pointed out that various growth regulating stimuli may need to be applied to cells, not only in right amounts, but also in the right sequences and under the right cultural conditions. Despite the vast amount of information on hormonal control, largely through trial and error, knowledge of the fundamental biology underlying *in vitro* induction of organogenesis remains scanty (Hicks 1994). For example, the identity of specific root or shoot forming genes remains unknown. Gene expression associated with organ specific inductive events is poorly characterized. The mechanism of action of auxins and cytokinins in organogenesis is still a mystery.

The development of thin layer culture techniques has allowed the study of direct organogenesis *in vitro* (Tran Thanh Van 1973). Higher cytokinin levels (10 µM) resulted in flower inhibition or morphological abnormalities (Van den Ende *et al*. 1984) or stimulated vegetative bud formation in *Begonia rese* (Chyłah and Tran Thanh Van 1975), *Brassica napus* (Klimaszewska and Keller 1985), *Beta vulgaris* (Detrez *et al*. 1988) and *Petunia hybrida* (Mulin and Tran Thanh Van 1989).

Regeneration of pigeonpea plants via callus cultures (Kumar *et al*. 1983) and direct differentiation from leaf (Eapen and George 1993b; Eapen *et al*. 1998) have been reported. George and Eapen (1994) have reported organogenesis from diverse explants of pigeonpea. Multiple shoot production was achieved from cotyledonary node explants (Mehta and Mohan Ram 1980; Kumar *et al*. 1984; Shiva Prakash *et al*. 1994; Naidu *et al*. 1995) and from epicotyl explants (Kumar *et al*. 1984; Naidu *et al*. 1995). According to Shama Rao and Narayanaswamy (1975) hypocotyl segments, obtained from gamma irradiated (5 Kr) seeds, produced abundant calli and shoot buds in 50% of the cultures. George and Eapen (1994) observed shoot regeneration from the distal end of cotyledons when whole cotyledons were cultured. Geetha *et al*. (1998) obtained formation of multiple shoots in different seedling explants such as leaf, hypocotyl, epicotyl, cotyledon

and cotyledonary node explants. Franklin *et al.* (2000) obtained regeneration of viable plants from embryonal axes.

In most of the above reports the regeneration was achieved with pre-existing meristems and the systems need germination of seeds. George and Eapen (1994) reported formation of shoot buds on distal ends of whole cotyledons on culture. However, successful transfer of well-developed shoots to field has not been achieved. Organogenesis from distal halves of cotyledons cultured alone has not been reported so far. Therefore the distal cotyledonary segments separated from the rest of the whole cotyledons were evaluated in the present study for their regeneration potentiality. Results obtained from these experiments are presented in this chapter. This chapter also describes the results on differentiation of callus obtained from distal cotyledonary segments.

3.2 Materials and Methods

3.2.1 Plant Material

Seeds of pigeonpea genotypes Gaut-82-90, T-15-15, Gaut-82-99, BDN-1, BDN-2, ICPL-87, ICPL-87119, PT-22, TV-1, T-21, N-290-21, ICP-7182, ICP-7128 and ICP-6917 were surface sterilized as described in chapter 2, section 2.6.2. The surface sterilized seeds were then soaked in sterile distilled water for 18 h in darkness at 28 ± 2° C. A total of 1890 seeds of each genotype were used for preliminary experiments described in the section 3.2.3.1. For the experiments with various basal media (section 3.2.3.2), 180 seeds each of the genotypes Gaut-82-90 and T-15-15 were used. For the experiments described in the section 3.2.3.3, a total of 390 seeds of each of the genotypes Gaut-82-90 and T-15-15 were used.

3.2.2 Preparation of explant

Cotyledons were split open from the pre-soaked seeds and the proximal meristematic ends were removed. Only the distal halves (3.5-4.0 mm^2) (**Fig 3.1A**) without any pre-existing axillary buds were used as explants. Two explants were inoculated per test tube with adaxial surface touching the medium. There were 20 explants per treatment and the experiment was repeated 3 times.

3.2.3 Induction of shoot buds

The influence of various cytokinins on shoot bud induction from explants was tested. MS basal medium was used in the preliminary experiments. All media were supplemented with 3 % sucrose and 0.8 % agar-agar and the pH of the media was adjusted to 5.8 before

sterilization by autoclaving at 1.4 $kg.cm^{-2}$ for 20 min. All the cultures were incubated at 25±2 °C under cool white fluorescent light (38 $\mu E.m^{-2}.s^{-1}$) under continuous light.

3.2.3.1 Effect of different growth regulators on shoot bud induction

In a preliminary experiment, MS basal medium supplemented with cytokinins BAP, kinetin and AdS in various concentrations and combinations were tested to evaluate the shoot bud induction capacity of the explants. Distal cotyledonary segments were cultured in test tubes containing MS basal medium (20 ml) supplemented with BAP (5, 10 and 20 µM), kinetin (1, 2 and 20 µM), AdS (200, 250 and 300 µM) and various combinations of BAP, kinetin and AdS. The cultures were incubated for 4 weeks under conditions mentioned as above. After 4 weeks, the explants response was scored for the shoot bud induction.

3.2.3.2 Effect of basal media on induction of shoot buds

The optimized concentrations of BAP, kinetin and AdS in MS basal medium were further standardized by varying the basal media keeping the concentration of cytokinins and AdS constant in this set of experiments. Six different basal media viz. MS medium (Murashige and Skoog 1962), EC_6 medium (Maheswaran and Williams 1984), LS medium (Linsmaier and Skoog 1965), White's medium (White 1963), B5 medium (Gamborg *et al*. 1968) and modified B5 medium (Mante and Boll 1975) were used in the experiments. All the media were supplemented with BAP (20 µM), kinetin (2 µM), AdS (250 µM), 3 % sucrose and 0.8 % agar-agar and the pH of the media was adjusted to 5.8 before autoclaving. All the cultures were incubated under the conditions mentioned as above for 4 weeks.

3.2.3.3 Effect of EC_6 basal medium supplemented with various combination of BAP, kinetin and AdS on shoot bud induction

The standardized shoot bud induction medium of EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS was modified by (a) excluding each growth regulator in turn and (b) by varying the concentrations of each of the growth regulators, one at a time keeping the other two constant, in an attempt to achieve more shoot bud formation per explant. All the media were supplemented with 3 % sucrose, 0.8 % agar-agar and the pH of the media was adjusted to 5.8 before autoclaving. All the cultures were incubated under the conditions mentioned as above for 4 weeks.

3.2.4 Elongation of shoot buds obtained from distal cotyledonary segments

The shoot buds along with explants obtained on EC_6 basal medium supplemented with BAP (20 µM), kinetin (2 µM) and AdS (250 µM) were transferred to harmone-free half-strength MS basal medium containing 0.8 % agar-agar and 3 % sucrose or to various half-strength MS basal media in test tubes supplemented with 2 µM BAP+0.2 µM kin+10 µM AdS, 0.5-2.0 µM NAA, 0.5-2.0 µM IAA, 0.5-2.0 µM IBA, 0.5-2.0 µM BAP, 0.5-2.0 µM BAP plus 0.5 µM NAA, 0.5-2.0 µM BAP plus 0.5 µM IAA, 0.5-2.0 µM BAP plus 0.5 µM IBA, 1-4 µM GA_3 for elongation of shoot buds into shoots. In addition the shoot buds were also cultured on half-strength MS basal medium supplemented with 0.25 % and 0.5 % activated charcoal. All the cultures were incubated at 25±2 °C under cool white fluorescent lights (38 $\mu E.m^{-2}.s^{-1}$) with 16/8 h photoperiod for 4 weeks. The cultures were transferred to freshly prepared media after 4 weeks twice or thrice at 4 weeks interval.

3.2.5 Rooting of elongated shoots

The elongated shoots derived from half-strength MS medium supplemented with 3 µM GA_3 were excised and transferred to half-strength MS medium containing 0.8 % agar-agar, 3 % sucrose with 0.5 µM IBA for rooting. The cultures were incubated under conditions as described in section 3.2.5 for 3 weeks.

3.2.6 Hardening of plantlets

The rooted plantlets were transferred to pots as described in chapter 2, section 2.11 and kept for hardening in pots with a soil:vermiculite (1:1) mixture in the hardening room at 25±2 °C under diffuse light (16/8 h photoperiod) conditions for 3-4 weeks.

3.2.7 Statistical analysis

The data was analyzed using Analysis of Variance technique for completely randomized design and the treatment means were compared.

3.2.8 Histology

For histological observations of shoot bud origin and development the distal halves of cotyledons were fixed in formalin:glacial acetic acid : ethanol (5:5:90 v/v) for 72 h Histology was carried out as described in the Chapter 2, Section 2.10.

3.2.9 Initiation of callus from distal cotyledonary segments

Distal cotyledonary segments were cultured in test tubes containing EC_6 basal medium (20 ml) with 3 % sucrose gelled with 0.8 % agar-agar and was supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS for initiation of callus. The pH of the medium was adjusted to 5.8 before sterilization by autoclaving at 121 °C at 1.4 $kg.cm^{-2}$ pressure for 20 min. All the cultures were incubated at 25±2 °C under cool white fluorescent lights (38 $\mu E.m^{-2}.s^{-1}$) with 16/8 h photoperiod for 4 weeks.

3.2.10 Effect of various phytohormones on differentiation of callus

The nodular calli obtained from distal cotyledonary segments on EC_6 basal medium supplemented with BAP (20 µM), 2 µM kinetin and 250 µM AdS were separated from the explants and transferred to half-strength MS medium supplemented with various levels of BAP (5, 10 and 20 µM), zeatin (1, 2, and 3 µM), IAA (0.5 and 1.0 µM) and BAP (5, 10 and 20 µM), zeatin (1, 2 and 3 µM) in combination with IAA (0.5 and 1.0 µM) or GA_3 (1, 2, 3 µM) to evaluate the differentiation of the callus into shoots/shoot buds. All the cultures were incubated under the conditions as mentioned above for 4 weeks. The calli were transferred to freshly prepared medium 4 times at an interval of 4 weeks each.

3.3 Results and Discussion

3.3.1 Effect of growth regulators on induction of shoot buds

In an initial experiment, distal cotyledonary segments cultured on MS basal medium supplemented with various concentrations of BAP (5, 10 and 20 µM), kinetin (1, 2 and 3 µM) and AdS (200, 250 and 300 µM) alone did not induce shoot buds. A combination of BAP (5, 10 and 20 µM) and kinetin (1, 2 and 3 µM), BAP (5, 10 and 20 µM) and AdS (200, 250 and 300 µM), Kinetin (1, 2 and 3 µM) and AdS (200, 250 and 300 µM) did not support any shoot bud formation (**Table 3.1**).

Table 3.1 Effect of MS basal medium supplemented with different concentrations BAP, Kin and AdS and their combination on induction of shoot buds from distal cotyledonary segments

Growth Regulator (mM)	Gaut 82-90	T-15-15	Gaut 82-99	N-290-21	T-21	TV-1	PT-22
BAP (5)	NR	NR	NR	NR	NR	NR	NR
BAP (10)	NR	NR	NR	NR	NR	NR	NR
BAP (20)	NR	NR	NR	NR	NR	NR	NR
Kin (1)	NR	NR	NR	NR	NR	NR	NR
Kin (2)	NR	NR	NR	NR	NR	NR	NR
Kin (3)	NR	NR	NR	NR	NR	NR	NR
AdS (200)	NR	NR	NR	NR	NR	NR	NR
AdS (250)	NR	NR	NR	NR	NR	NR	NR
AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (1)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (2)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (3)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
Kin (1) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
Kin (1) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
Kin (1) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR

NR – No response (No shoot bud formation, explants remained as it is or turned necrotic)

Table 3.1 Continued…

Table 3.1 (Contd)

Growth Regulator (■M)	ICP 6917	ICP 7128	ICP 7182	ICPL-87	ICPL 87119	BDN-1	BDN-2
BAP (5)	NR	NR	NR	NR	NR	NR	NR
BAP (10)	NR	NR	NR	NR	NR	NR	NR
BAP (20)	NR	NR	NR	NR	NR	NR	NR
Kin (1)	NR	NR	NR	NR	NR	NR	NR
Kin (2)	NR	NR	NR	NR	NR	NR	NR
Kin (3)	NR	NR	NR	NR	NR	NR	NR
AdS (200)	NR	NR	NR	NR	NR	NR	NR
AdS (250)	NR	NR	NR	NR	NR	NR	NR
AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (1)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (2)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (3)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
Kin (1) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
Kin (1) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
Kin (1) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR

NR – No response (No shoot bud formation, explants remained as it is or turned necrotic)

The formation of shoot buds was, however, obtained in genotypes T-15-15 and Gaut-82-90 on media with combinations of BAP (20 µM), kinetin (2 and 3 µM) and AdS (200, 250 and 300 µM) (**Table 3.2**). The cotyledonary segments swell and turn green after 3 weeks in culture, producing small, green, dome-like structures (**Fig 3.1B**) all over the surface of the cotyledonary segment. After 4 weeks of culture these structures developed into shoot buds (**Fig 3.1C & Fig 3.2A**), without an intervening callus phase. All other combinations with three growth regulators (BAP, kinetin and AdS) did not induce shoot buds on cotyledonary segments (**Table 3.2**).

These results indicate the requirement of three cytokinins for induction of shoot buds. This is in contrast to the use of BAP (Kumar *et al*. 1984; Naidu *et al*. 1995; Geetha *et al*. 1998), TDZ (Eapen *et al*. 1998) or kinetin (Geetha *et al*. 1998) alone or BAP in combination with IAA (Mehta and Mohan Ram 1980; Eapen and George 1993b; George and Eapen 1994; Naidu *et al*. 1995) or NAA (Geetha *et al*. 1998; Franklin *et al*. 2000), TDZ in combination with IAA (Eapen *et al*. 1998) for regeneration of plants from different explants of pigeonpea. While a combination of BAP and kinetin was used for regeneration of plants from mature embryos and intact seeds (Naidu *et al*. 1995), use of a combination of BAP, kinetin and AdS for organogenesis has not been reported earlier. According to Eapen and George (1993b) addition of only BAP to the medium failed to regenerate plants from pigeonpea leaf discs, and is similar to our observations with cotyledonary segments. Organogenesis from distal cotyledonary segments separated from the rest of the cotyledon and cultured alone has not been reported earlier in the literature. However, George and Eapen (1994) observed formation of shoot buds on the distal end of cotyledons as well when whole cotyledons were cultured.

Patel *et al.* (1994) reported induction of somatic embryogenesis from cotyledons of pigeonpea when cultured on MS and B5 media supplemented with BAP, Kin and AdS. On the contrary, only organogenesis was observed in our experiments. The regeneration of pigeonpea from cotyledons was achieved earlier (Mehta and Mohan Ram 1980; Kumar *et al*. 1983; Kumar *et al*. 1984; Shiva Prakash *et al*. 1994; Naidu *at al*. 1995; Geetha *et al*. 1998). It was observed that either the presence of the embryonal axis stimulated the formation of shoot buds on cotyledons or the axillary meristems simply proliferated, resulting in the production of multiple shoots. In the present investigation, the multiplication of pre-existing axillary buds and their possible influence on shoot bud formation was ruled out because of the elimination of the proximal end of the cotyledon

A. Distal cotyledonary segments at the time of inoculation on various growth regulator combinations

B. Small, green, dome like structures appearing on all over the surface of distal cotyledonary segments after 3 weeks of culture on media supplemented with BAP, kinetin and AdS

C. Distal cotyledonary segment showing shoot buds induced on media supplemented with BAP, kinetin and AdS

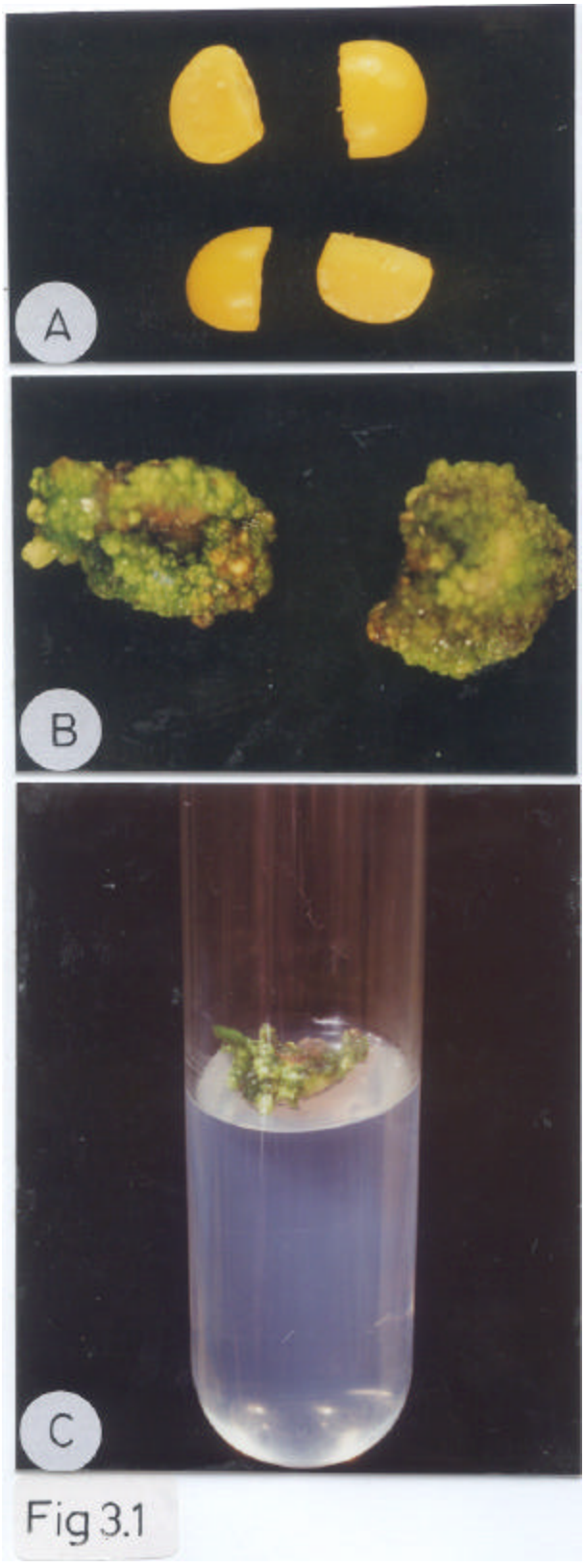


Fig 3.1

as well as the attached embryonal axis. Hence, there is enough evidence to say that the shoot bud induction was *de novo*. This was also confirmed by histological observations.

Out of the fourteen genotypes studied, only the cotyledonary segments of T-15-15 and Gaut-82-90 produced shoot buds and the cotyledonary segments turned necrotic in the genotypes BDN-1, BDN-2, ICPL-87, ICPL-87119, ICP-7182, ICP-7128, ICP-6917, PT-22, TV-1, T-21, N-290-21 and Gaut-82-99. The regeneration capacity of cotyledonary segments appears to be genotype-dependent, similar to the observation of Naidu *et al*. (1995) where regeneration of various explants of pigeonpea was genotype-dependent. The shoot bud formation was observed only in genotypes T-15-15 and Gaut-82-90, and hence these genotypes were selected for further experimentation.

Table 3.2 Effect of MS basal medium supplemented with various combination of BAP, Kin and AdS on induction of shoot buds from distal cotyledonary segments

Growth Regulator (µM)	Gaut 82-90	T-15-15	Gaut 82-99	N-290-21	T-21	TV-1	PT-22
BAP (5) + Kin (1) +AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (1) +AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (1) +AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1) +AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1) +AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1) +AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1) +AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1) +AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1) +AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (2) + AdS (200)	SB*	SB*	NR	NR	NR	NR	NR
BAP (20) + Kin (2) + AdS (250)	SB*	SB*	NR	NR	NR	NR	NR
BAP (20) + Kin (2) + AdS (300)	SB*	SB*	NR	NR	NR	NR	NR
BAP (20) + Kin (3) + AdS (200)	SB*	SB*	NR	NR	NR	NR	NR
BAP (20) + Kin (3) + AdS (250)	SB*	SB*	NR	NR	NR	NR	NR
BAP (20) + Kin (3) + AdS (300)	SB*	SB*	NR	NR	NR	NR	NR

NR – No response (No shoot bud formation, explants remained as it is or turned necrotic),
SB – Shoot buds, * Data is given in Table 3.3

Table 3.2 Continued…

Table 3.2 (Contd)

Growth Regulator (mM)	ICP 6917	ICP 7128	ICP 7182	ICPL-87	ICPL 87119	BDN-1	BDN-2
BAP (5) + Kin (1) +AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (1) +AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (1) +AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (5) + Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1) +AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1) +AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (1) +AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (10) + Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1) +AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1) +AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (1) +AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (2) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (2) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (2) + AdS (300)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (3) + AdS (200)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (3) + AdS (250)	NR	NR	NR	NR	NR	NR	NR
BAP (20) + Kin (3) + AdS (300)	NR	NR	NR	NR	NR	NR	NR

NR – No response (No shoot bud formation, explants remained as it is or turned necrotic)

The percent induction of shoot buds varied from 5 to 30 % in genotype Gaut-82-90 and 5 to 50 % in genotype T-15-15. The mean number of shoot buds per explant varied from 1.5 to 13.9 and 3 to 18.5 in genotypes Gaut-82-90 and T-15-15 respectively (**Table 3.3**). Even though shoot bud formation occurred on 6 combinations of BAP, kinetin and AdS, the percentage of shoot bud induction (50 % in T-15-15 and 30 % in Gaut-82-90) and the number of shoot bud formation per explant (18.5 in T-15-15 and 13.9 in Gaut-82-90) was significantly higher in the combination - 20 µM BAP, 2 µM kinetin and 250 µM AdS (**Table 3.3)**. Therefore, further experiments were carried out

using this combination of growth regulators to optimize the regeneration of plants from the explants of T-15-15 and Gaut-82-90 genotypes.

Table 3.3 Effect of MS basal medium supplemented with various combinations of BAP, Kin and AdS on shoot bud formation

	Gaut-82-90		T-15-15	
Growth Regulator (mM)	**Percentage response (mean ± se)**	**No. of shoot buds/explant (mean ± se)**	**Percentage response (mean ± se)**	**No. of shoot buds/explant (mean ± se)**
BAP (20) + Kin (2) + AdS (200)	20 ± 2^{c}	9.0 ± 0.7^{d}	30 ± 10^{c}	5.5 ± 0.2^{b}
BAP (20) + Kin (2) + AdS (250)	30 ± 8^{d}	13.9 ± 1.8^{e}	50 ± 13^{d}	18.5 ± 2.1^{c}
BAP (20) + Kin (2) + AdS (300)	15 ± 6^{bc}	6.0 ± 0.2^{c}	20 ± 4^{b}	4.5 ± 0.8^{ab}
BAP (20) + Kin (3) + AdS (200)	10 ± 3^{ab}	4.0 ± 1.3^{bc}	10 ± 5^{a}	3.0 ± 0.5^{a}
BAP (20) + Kin (3) + AdS (250)	10 ± 5^{ab}	1.5 ± 0.4^{a}	5 ± 3^{a}	4.0 ± 0.7^{ab}
BAP (20) + Kin (3) + AdS (300)	5 ± 2^{a}	2.0 ± 0.5^{ab}	5 ± 2^{a}	6.0 ± 0.7^{b}

Figures with different alphabets differ significantly at 0.05 probability.

3.3.2 Effect of various basal media on shoot bud induction

In the next set of experiments, BAP (20 µM), kinetin (2 µM) and adenine sulfate (250 µM) were used in combination to culture distal halves of cotyledons of the genotypes T-15-15 and Gaut-82-90 on various basal media. The response of shoot bud formation varied from 25-95 % in the genotype GAUT-82-90 and 55-95 % in the genotype T-15-15 (**Table 3.4**) depending on the basal medium. The average number of shoot buds per explant ranged from 12.5 to 32.3 and 17.5 to 40.5 in genotypes GAUT-82-90 and T-15-15, respectively (**Table 3.4**), with the maximum number of shoot buds on EC_6 basal medium in both the genotypes. The percentage induction of shoot buds in both the genotypes was significantly higher on EC_6 basal medium when compared to other basal media. The number of shoot buds per explant was significantly higher on EC_6 basal medium in both the genotypes Gaut-82-90 and T-15-15. Even though the percentage response was high on most of the basal media tried, the better response in terms of shoot bud formation on EC_6 basal medium prompted us to select only EC_6 basal medium for further studies (**Table 3.4**).

In contrary to our results, MS basal medium was used preferentially for regeneration of explants of pigeonpea (Eapen and George 1993b; Shiva Prakash *et al*. 1994; Naidu *et al*. 1995; George and Eapen 1994; Geetha *et al*. 1998; Franklin *et al* 2000). A few reports of use of B5 medium (Mehta and Mohan Ram 1980) and Blady's

medium (Kumar *et al*. 1984) however has been reported. Patel *et al*. (1994) have used six basal media for induction of somatic embryogenesis similar to our studies reported here. However, the effect of various basal media on induction of organogenesis from cotyledonary segments of pigeonpea has not been studied earlier.

Table 3.4 Response of cotyledonary segments on different basal media supplemented with 20 µM BAP, 2 µM Kin and 250 µM AdS

	Gaut-82-90		T-15-15	
Medium	**Percentage of Explants forming shoot buds (mean ± se)**	**No. of Shoot buds Per explant (mean ± se)**	**Percentage of explants forming shoot buds (mean ± se)**	**No. of shoot buds per explant (mean ± se)**
MS	25 ± 3^{a}	12.5 ± 1.3^{a}	55 ± 5^{a}	20.7 ± 2.0^{a}
B5	70 ± 7^{c}	26.5 ± 3.9^{c}	70 ± 8^{bc}	20.1 ± 2.8^{a}
Modified B5	50 ± 5^{b}	14.8 ± 1.4^{a}	80 ± 12^{c}	$18 .3 \pm 2.1^{a}$
EC_6	95 ± 10^{d}	32.3 ± 4.0^{d}	95 ± 15^{d}	40.5 ± 5.5^{b}
LS	75 ± 8^{c}	28.5 ± 3.2^{c}	65 ± 7^{ab}	17.5 ± 1.5^{a}
White's	80 ± 9^{c}	20.0 ± 2.5^{b}	75 ± 10^{bc}	18.2 ± 2.2^{a}

Figures with different alphabets differ significantly at 0.05 probability.

3.3.2.3 Effect of EC_6 basal medium supplemented with various levels of BAP, kinetin and AdS

The standardized shoot bud regeneration medium i.e. EC_6 basal medium supplmented with 20 µM BAP, 2 µM kinetin and 250 µM AdS was further modified by (a) excluding each growth regulator in turn and (b) by varying the concentrations of each of the growth regulators, one at a time keeping the other two constant, in an attempt to achieve more shoot bud formation per explant (**Table 3.5**). The percentage induction of shoot buds varied from 0 to 90 % in both the genotypes Gaut-82-90 and T-15-15. The number of shoot buds per explant varied from 1.5 to 32.5 in the genotype Gaut-82-90 and 4 to 42.5 in the genotype T-15-15. However, the optimum response and the mean number of buds produced per explant were found to be significantly high on the EC_6 basal medium supplemented with 20 µM BAP, 2 µM Kin and 250 µM AdS (**Table 3.5**). The EC_6 basal medium containing all the three cytokinins showed higher percentage of shoot bud formation (90 %) in both the Gaut-82-90 and T-15-15 genotypes and higher number of shoot buds per explant (32.5 and 42.5 in Gaut-82-90 and T-15-15 genotypes respectively). Even though the percentage response in EC_6 basal medium supplemented with BAP (20 µM), kinetin (2 µM) and AdS (250 µM) was not significantly different

from certain combinations (10 μM BAP + 2 μM kin + 250 μM AdS and 20 μM BAP + 2 μM kinetin) in the genotype T-15-15, the number of shoot buds formed per explant was significantly higher (**Table 3.5**).

Table 3.5 Effect of various growth regulator combinations (EC_6 Basal Medium) on induction of shoot buds on cotyledonary segments

Growth Regulator (mM)			Gaut-82-90		T-15-15	
BAP	Kin	AdS	Percentage of Explants forming Shoot Buds (mean ± se)	No. of Shoot buds Per explant (mean ± se)	Percentage of Explants forming Shoot Buds (mean ± se)	No. of Shoot buds Per explant (mean ± se)
0	2	250	0 ± 0^{a}	-	0 ± 0^{a}	-
5	2	250	10 ± 2^{b}	9.0 ± 0.7^{c}	40 ± 5^{cd}	5.5 ± 0.2^{a}
10	2	250	10 ± 3^{b}	1.5 ± 0.4^{a}	80 ± 16^{gh}	27.5 ± 0.6^{f}
20	**2**	**250**	90 ± 9^{c}	32.5 ± 1.3^{d}	90 ± 18^{h}	42.5 ± 0.6^{g}
40	2	250	10 ± 2^{b}	4.5 ± 0.4^{b}	60 ± 12^{ef}	17.5 ± 0.7^{d}
20	0	250	0 ± 0^{a}	-	70 ± 14^{fg}	12.5 ± 0.7^{c}
20	1	250	0 ± 0^{a}	-	70 ± 11^{fg}	27.5 ± 0.7^{f}
20	3	250	20 ± 5^{b}	11.0 ± 0.5^{c}	30 ± 6^{bc}	9.0 ± 0.4^{bc}
20	4	250	0 ± 0^{a}	-	50 ± 10^{de}	6.0 ± 0.2^{ab}
20	2	0	0 ± 0^{a}	-	80 ± 13^{gh}	23.3 ± 0.6^{e}
20	2	100	0 ± 0^{a}	-	60 ± 9^{ef}	22.7 ± 0.7^{e}
20	2	200	10 ± 2^{b}	9.0 ± 0.7^{c}	20 ± 4^{b}	4.0 ± 1.3^{a}
20	2	300	0 ± 0^{a}	-	0 ± 0^{a}	-

Figures with different alphabets differ significantly at 0.05 probability.

3.3.3 Elongation of shoot buds into shoots

The shoot buds obtained on shoot bud induction medium did not elongate on the same medium. Therefore the shoot buds obtained from cotyledonary segments on EC_6 basal medium supplemented with BAP (20 μM), kinetin (2 μM) and AdS (250 μM) along with explants were transferred as a mass to test tubes containing various media combinations (**Table 3.6**). The shoot buds did not elongate on hormone-free half-strength MS basal medium. The elongation of shoot buds was also not observed on half-strength MS basal medium supplemented with 2 μM BAP, 0.2 μM kinetin and 25 μM AdS (**Table 3.6**). The elongation of shoot buds could not be achieved when half-strength MS basal medium was supplemented with IAA (0.5-2.0 μM), IBA (0.5-2.0 μM) or charcoal (0.25 and 0.5 %) (**Table 3.6**). Half-strength MS basal medium supplemented with 0.5 μM IAA or 0.5 μM

IBA in combination with 0.5 to 2.0 µM BAP did not lead to elongation of shoot buds into shoots (**Table 3.6**). The shoot buds produced small shoots (**Fig 3.2B**) after 4 weeks of subculture on half-strength MS basal medium supplemented with NAA alone (0.5-2.0 µM), 0.5 µM NAA in combination with BAP (0.5-2.0 µM) or GA_3 (1-4 µM). On an average, 1.2 to 5.5 shoots were produced per explant (**Table 3.6**) depending on the growth regulator supplement and the genotype. Higher number of shoots were produced on the medium containing GA_3 at 3 µM concentration. The small shoots obtained on half-strength MS medium supplemented with NAA alone (0.5-2.0 µM), 0.5 µM NAA in combination with BAP (0.5-2.0 µM) did not elongate further on the same media combinations after 2-3 transfers at 4 weeks interval. The small shoots obtained on 3 µM GA_3 further elongated on 2-3 transfers at 4 weeks interval (**Fig 3.2C**). It was observed that half-strength MS basal medium containing 3 µM GA_3 enhanced the elongation of shoots (**Fig 3.3A**). Similarly, Mehta and Mohan Ram (1980) also observed that addition of GA_3 in the medium aided shoot elongation. However, GA_3 was found to support only sporadic elongation of shoot buds into shoots and simultaneous elongation of all shoot buds into shoots could not be achieved (**Fig 3.3B**). In fact, a large number of leafy shoots (leaves with petioles) were produced (**Fig 3.3C & Fig 3.4A**) when the explants were transferred to half-strength MS basal medium containing various growth regulators (**Table 3.6**). Similar observations were made in soybean cultures (Kiss *et al*. 1991b), where further development of meristems produced adventitious shoot tip primordia with only active lateral meristems. As a result, only leaf primordia started developing due to inhibition of shoot differentiation and elongation.

The topical supplementation of IAA as suggested by Shiva Prakash *et al*. (1994) was tried to elongate the shoot buds to get more shoots. This was not successful in our experiments, possibly because shoot bud formation on the distal end of the cotyledon is *de novo*, whereas Shiva Prakash *et al*. (1994) have reported the elongation of shoot buds produced on the cotyledonary node, which has pre-existing meristems and had partially differentiated cells. It was also observed that the growth of shoots was very slow as it took 2-3 subcultures for the shoot buds to form well-developed shoots. This is in accordance with the observations made by George and Eapen (1994). Even though, there were no significant differences with respect to formation of well-developed shoots between GA_3 and higher NAA/BAP treatments, a higher percentage of elongated shoots to leafy shoots were observed with GA_3 treatment (**Table 3.6**).

Table 3.6 Number of shoots recovered from cotyledonary segments after culturing on half-strength MS medium supplemented with various growth regulators

	Gsut-82-90			T-15-15		
Growth Regulator (µM)	**Leafy shoots[1] Per explant (mean ± se)**	**Shoots[2] per explant (mean ± se)**	**Percentage of shoots to Leafy shoots**	**Leafy shoots[1] Per explant (mean ± se)**	**No. of shoots[2] per explant (mean ± se)**	**Percentage of shoots to leafy shoots**
NIL	13.0 ± 0.7^{efghi}	0.0 ± 0.0^{a}	0^{a}	13.4 ± 0.9^{fgh}	0.0 ± 0.0^{a}	0^{a}
BAP (2) + Kin (0.2) + AdS (25)	10.7 ± 0.2^{abcd}	0.0 ± 0.0^{a}	0^{a}	15.3 ± 0.6^{hij}	0.0 ± 0.0^{a}	0^{a}
NAA (0.5)	11.8 ± 1.2^{cde}	2.4 ± 0.2^{bcd}	20^{e}	12.2 ± 1.6^{cdef}	1.4 ± 0.6^{b}	12^{b}
NAA (1.0)	9.7 ± 0.2^{ab}	1.2 ± 0.2^{b}	13^{bc}	10.4 ± 0.4^{abc}	1.2 ± 0.5^{b}	12^{b}
NAA (2.0)	15.0 ± 1.4^{ij}	1.4 ± 0.2^{bc}	9^{b}	14.4 ± 1.7^{ghij}	2.0 ± 0.3^{bc}	14^{b}
NAA (0.5) + BAP (0.5)	14.2 ± 1.5^{ghi}	2.2 ± 0.2^{bcd}	16^{cde}	16.0 ± 2.6^{jk}	1.8 ± 0.2^{b}	11^{b}
NAA (0.5) + BAP (1.0)	14.0 ± 1.8^{fghi}	2.6 ± 0.6^{bcd}	19^{de}	13.6 ± 1.9^{fghi}	2.4 ± 0.7^{bc}	18^{c}
NAA (0.5) + BAP (2.0)	20.2 ± 2.8^{k}	2.8 ± 0.5^{cde}	14^{bcd}	18.8 ± 2.2^{l}	3.4 ± 0.5^{cd}	18^{c}
IAA 0.5	8.9 ± 0.6^{a}	0.0 ± 0.0^{a}	0^{a}	13.1 ± 0.7^{fg}	0.0 ± 0.0^{a}	0^{a}
IAA 1.0	10.1 ± 0.6^{abc}	0.0 ± 0.0^{a}	0^{a}	12.6 ± 0.7^{defg}	0.0 ± 0.0^{a}	0^{a}
IAA 2.0	9.1 ± 0.9^{a}	0.0 ± 0.0^{a}	0^{a}	11.0 ± 2.7^{bcde}	0.0 ± 0.0^{a}	0^{a}
IAA (0.5) BAP (0.5)	10.7 ± 2.2^{abcd}	0.0 ± 0.0^{a}	0^{a}	9.0 ± 0.8^{ab}	0.0 ± 0.0^{a}	0^{a}
IAA (0.5) BAP (1.0)	8.7 ± 1.3^{a}	0.0 ± 0.0^{a}	0^{a}	23.5 ± 7.1^{m}	0.0 ± 0.0^{a}	0^{a}
IAA (0.5) BAP (2.0)	14.8 ± 1.5^{hij}	0.0 ± 0.0^{a}	0^{a}	17.9 ± 5.6^{kl}	0.0 ± 0.0^{a}	0^{a}
IBA (0.5)	11.9 ± 2.4^{cde}	0.0 ± 0.0^{a}	0^{a}	8.8 ± 4.4^{a}	0.0 ± 0.0^{a}	0^{a}
IBA (1.0)	14.3 ± 2.4^{ghij}	0.0 ± 0.0^{a}	0^{a}	13.8 ± 4.4^{fghi}	0.0 ± 0.0^{a}	0^{a}
IBA (2.0)	12.9 ± 1.2^{efgh}	0.0 ± 0.0^{a}	0^{a}	15.5 ± 6.3^{ij}	0.0 ± 0.0^{a}	0^{a}
IBA 0.5 BAP (0.5)	16.3 ± 2.5^{j}	0.0 ± 0.0^{a}	0^{a}	9.5 ± 1.7^{ab}	0.0 ± 0.0^{a}	0^{a}
IBA (0.5) BAP (1.0)	11.6 ± 0.8^{bcde}	0.0 ± 0.0^{a}	0^{a}	23.8 ± 6.8^{m}	0.0 ± 0.0^{a}	0^{a}
IBA (0.5) BAP (2.0)	13.1 ± 2.1^{efghi}	0.0 ± 0.0^{a}	0^{a}	18.8 ± 3.9^{l}	0.0 ± 0.0^{a}	0^{a}
GA_3 (1)	10.3 ± 1.3^{abc}	3.3 ± 0.3^{de}	32^{f}	12.9 ± 2.4^{efg}	3.7 ± 0.4^{d}	29^{d}
GA_3 (2)	11.7 ± 0.5^{bcde}	3.5 ± 0.3^{de}	30^{f}	14.3 ± 1.2^{ghij}	3.9 ± 0.5^{d}	27^{d}
GA_3 (3)	12.6 ± 1.8^{defg}	4.3 ± 0.3^{e}	34^{f}	18.5 ± 2.2^{l}	5.5 ± 1.2^{e}	30^{d}
GA_3 (4)	12.1 ± 0.9^{cdef}	3.5 ± 0.7^{de}	29^{f}	16.3 ± 3.6^{jk}	4.3 ± 0.3^{de}	26^{d}
0.25 %	9.7 ± 1.3^{ab}	0.0 ± 0.0^{a}	0^{a}	10.6 ± 2.4^{abcd}	0.0 ± 0.0^{a}	0^{a}
0.50 %	11.8 ± 1.5^{cde}	0.0 ± 0.0^{a}	0^{a}	12.4 ± 1.7^{cdefg}	0.0 ± 0.0^{a}	0^{a}

1.- Leaves with petiole, 2.- Well developed, elongated shoots. Figures with different alphabets differ significantly at 0.05 probability.

A. Magnified view of the distal cotyledonary segment showing shoot buds induced on media supplemented with BAP, kinetin and AdS (bar = 350 µm)

B. Shoot buds converting to small shoots on half-strength MS basal medium supplemented with various growth regulators

C. Multiple shoots obtained on half-strength MS basal medium supplemented with 3 µM GA_3

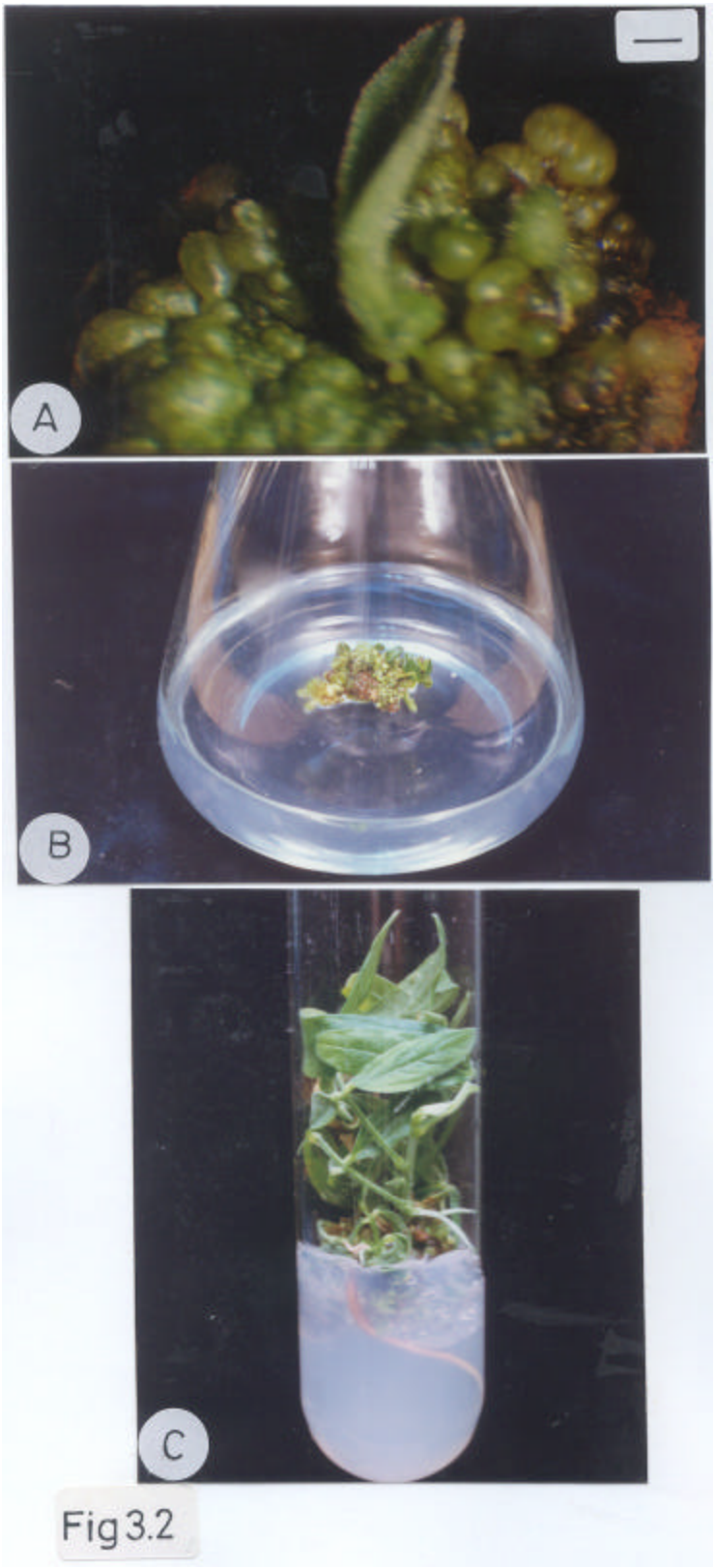


Fig 3.2

A. Elongated shoots obtained on half-strength MS basal medium supplemented with 3 μM GA_3

B. Sporadic elongation of shoot buds on half-strength MS basal medium supplemented with 3 μM GA_3

C. Formation of a large number of leafy shoots on half-strength MS basal medium supplemented with various growth regulators

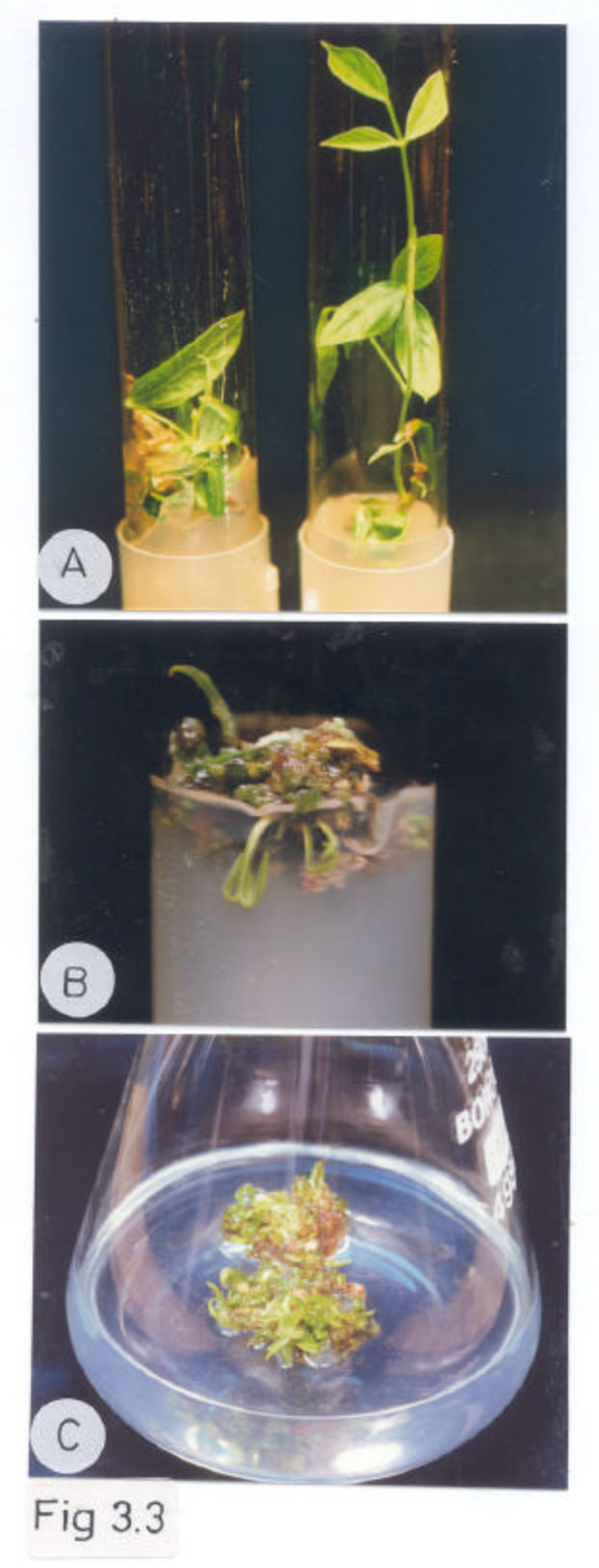


Fig 3.3

3.3.4 Rooting of the regenerated shoots

The shoots, which were elongated (3-4 cm length) on half-strength MS medium supplemented with 3 µM of GA_3 were rooted on half-strength MS medium with the growth regulator IBA (0.5 µM). Higher concentration of IBA (1 µM and 2 µM) resulted in abnormalities in shoot and root development (**Fig 3.4B & Fig 3.4C**) or formation of a lot of callus at the shoot tip (**Fig 3.5A**) or root-shoot junction (**Fig 3.5B**). On the other hand half-strength MS medium supplemented with 0.5 µM IBA produced profuse rooting with normal shoots (**Fig 3.5C and Fig 3.6A**). Eighty per cent of shoots produced well-developed roots in 15-20 days when half-strength MS medium was supplemented with 0.5 µM of IBA.

In pigeonpea, Kumar *et al*. (1983) had observed rooting with IAA or NAA and George and Eapen (1994) had observed 90 % rooting on NAA medium where as Geetha *et al*. (1998) and Shiva Prakash *et al*. (1994) found IBA as best auxin for rooting. IBA was most preferred auxin for rooting in pigeonpea (Shiva Prakash *et al*. 1994; Naidu *et al*. 1995; Geetha *et al*. 1998; Franklin *et al*. 2000), which is similar to our observations. Half-strength MS medium was used as basal medium for rooting in our studies. Similarly, Eapen and George (1993b), George and Eapen (1994), Naidu *et al*. (1995) and Franklin *et al*. (2000) also used half-strength MS medium for rooting. In contrast, MS basal medium (Shiva Prakash *et al*. 1994; Geetha *et al*. 1998; Eapen *et al*. 1998), B5 (Mehta and Mohan Ram 1980) and Blady's medium (Kumar *et al*. 1983; 1984) were also used for rooting of pigeonpea shoots.

3.3.5 Hardening of plantlets

The rooted plantlets were transferred to pots containing soil:vermiculite (1:1). The survival of plants in pots (**Fig 3.6B**) was 70 per cent after 3-4 weeks. When a total of 360 cotyledonary segments were cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS, 83 % formed shoot buds, of which 56 % formed small shoots. Only 18 % of these small shoots elongated on half-strength MS basal medium supplemented with 3 µM GA_3. The rooting percentage was 80 % on half-strength MS basal medium supplemented with 0.5 µM IBA and 70 % of the rooted plants survived hardening and 25 plants were transferred to soil.

This type of information on the number of cotyledons cultured and the number of plants transferred to soil were not reported earlier in the literature. No report of transfer of shoots originating from *de novo* organogenesis is available. To our knowledge this is the

A. Cotyledonary segments showing leafy shoots produced on half-strength MS basal medium supplemented with various growth regulators

B. Abnormal shoot and root development on half-strength MS medium supplemented with 1 and 2 µM IBA

C. Abnormal shoot and root development on half-strength MS medium supplemented with 1 and 2 µM IBA

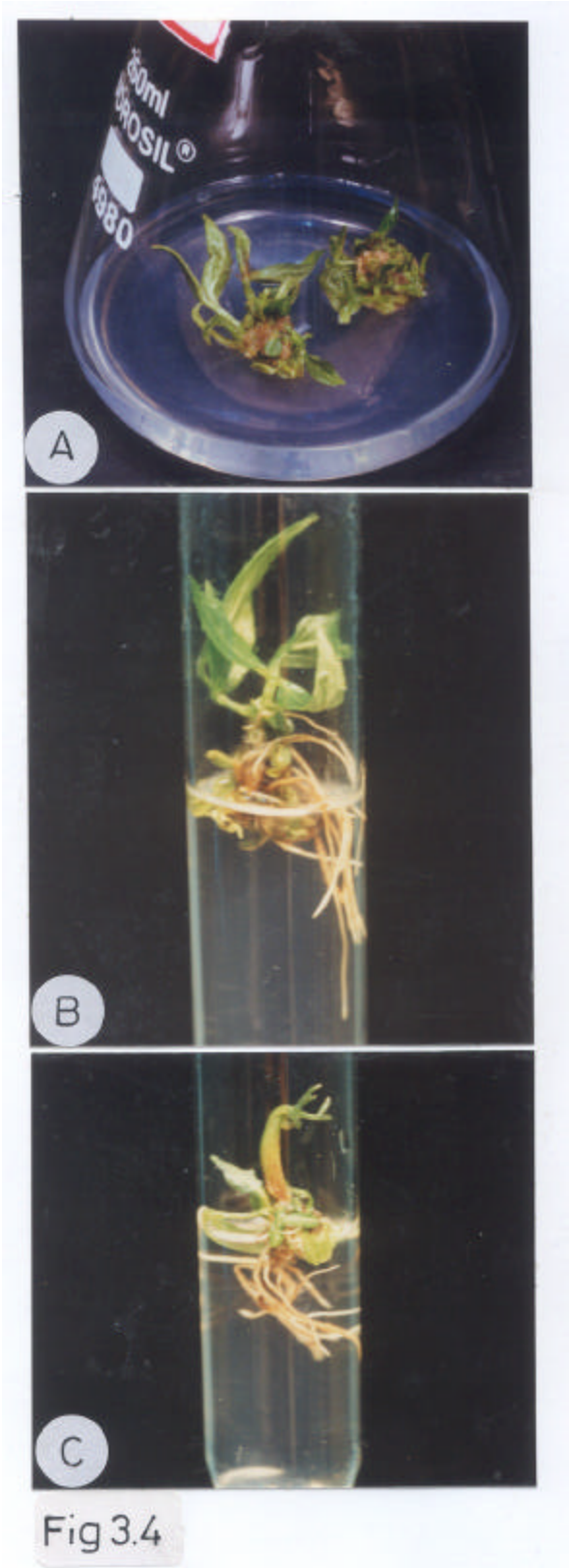


Fig 3.4

A. Callus formation at shoot tip on half-strength MS medium supplemented with 1 and 2 µM IBA

B. Callus formation at root-shoot junction on half-strength MS medium supplemented with 1 and 2 µM IBA

C. Initiation of root on half-strength MS medium supplemented with 0.5 µM IBA

Fig 3.5

A. Profuse rooting on half-strength MS medium supplemented with 0.5 µM IBA

B. Hardened plants obtained from distal cotyledonary segments surviving in pots

Fig 3.6

first report of organogenesis from distal cotyledonary segments as explants. Even though George and Eapen (1994) mentioned the formation of shoot buds on distal end of cotyledons when whole cotyledons were cultured, formation well-developed shoots and transfer of plantlets to soil has not been achieved. Histological evidence has not been provided for confirmation of *de novo* origin of shoot buds.

3.3.6 Histology

The origin of shoot buds from cotyledonary segments was examined by histological preparations. Formation of a layer of non-meristematic compact mass of cells (**Fig 3.7A**), which was due to the swelling and formation of small dome like structures on the surface of cotyledonary segments, not easily separable from the explant, was observed prior to the development of meristematic pockets of small cells (**Fig 3.7B**) with dense cytoplasm and darkly stained nuclei. The anatomy of differentiated shoot buds along with leaf primordium (**Fig 3.7C**) originating from the compact mass of cells confirms the organogenetic pathway of morphogenesis.

3.3.7 Effect of phytohormones on differentiation of callus

There was formation of green nodular calli (**Fig 3.8A & Fig3.8B**) also originating from the cut surface when distal cotyledonary segments were cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS. The differentiation of callus into shoot buds was not observed when the green nodular callus obtained on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS were subcultured on half-strength MS basal medium supplemented with various concentration of BAP (5, 10 and 20 µM), zeatin (1, 2 and 3 µM), IAA 0.5 and 1.0 µM), BAP (5, 10 and 20 µM) + IAA (0.5 µM and 1.0 µM) or zeatin (1, 2 and 3 µM) + IAA (0.5 and 1.0 µM). In all the above combinations the calli remained green for 2-3 subcultures and then turned necrotic and no regeneration of shoot buds could be observed (**Table 3.7**).

There was formation of shoot (**Fig 3.8C, Fig 3.9A & Fig 3.9B**), when callus derived from distal half of cotyledon cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS, was subcultured 2-3 time at 4 weeks interval each on half-strength MS basal medium containing GA_3 (3 µM) (**Table 3.7**). This shoot formation however, occurred very rarely and sporadically and was non-reproducible.

A. Cotyledonary segments (c) surrounded by dome-like (d) masses after 3 weeks of culture (bar = 330 µm)

B. Meristematic pocket (m) indicating initiation of differentiation (bar = 75 µm)

C. Differentiated shoot bud (s) with leaf primordium (l) (bar = 125 µm)

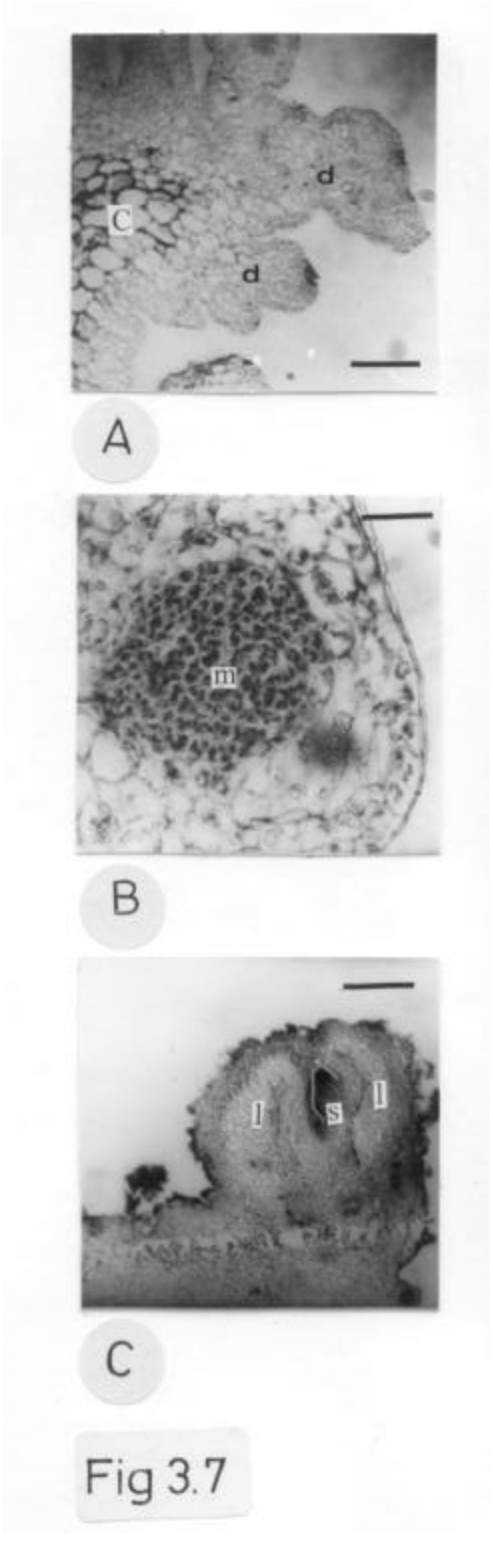


Fig 3.7

Table 3.7 Effect of half-strength MS basal medium supplemented with various growth regulator combinations on regeneration of callus originating from distal cotyledonary segments on EC_6 basal medium supplemented with BAP (5 µM) and IAA (1 µM)

GR (µM)	Gaut-82-90	T-15-15
Nil	No regeneration	No regeneration
BAP (5)	No regeneration	No regeneration
BAP (10)	No regeneration	No regeneration
BAP (20)	No regeneration	No regeneration
Zea (1)	No regeneration	No regeneration
Zea (2)	No regeneration	No regeneration
Zea (3)	No regeneration	No regeneration
BAP (5) + IAA (0.5)	No regeneration	No regeneration
BAP (10) + IAA (0.5)	No regeneration	No regeneration
BAP (20) + IAA (0.5)	No regeneration	No regeneration
BAP (5) + IAA (1.0)	No regeneration	No regeneration
BAP (10) + IAA (1.0)	No regeneration	No regeneration
BAP (20) + IAA (1.0)	No regeneration	No regeneration
ZEA (1) + IAA (0.5)	No regeneration	No regeneration
Zea (2) + IAA (0.5)	No regeneration	No regeneration
Zea (3) + IAA (0.5)	No regeneration	No regeneration
Zea (1) + IAA (1.0)	No regeneration	No regeneration
Zea (2) + IAA (1.0)	No regeneration	No regeneration
Zea (3) + IAA (1.0)	No regeneration	No regeneration
IAA (0.5)	No regeneration	No regeneration
IAA (1.0)	No regeneration	No regeneration
GA_3 (1)	No regeneration	No regeneration
GA_3 (2)	No regeneration	No regeneration
GA_3 (3)	Regeneration of shoot rarely. Not reproducible	Regeneration of shoot rarely. Not reproducible

There is no report of regeneration of callus derived from explants devoid of pre-existing meristems. In contrary to our observations of no differentiation of shoots from callus derived from distal cotyledonary segments, Kumar *et al.* (1983) reported plant regeneration from callus cultures derived from whole cotyledons. Similarly hypocotyl segments, obtained from γ-irradiated (5 Kr) seeds, produced abundant calli and shoot buds in 50 % of the cultures (Shama Rao and Narayanaswamy 1975) but no plants could be regenerated. Since our observation made involves the rare regeneration of shoots from the callus derived from the cut surface it can only be useful if it could be repeated frequently.

A. Hard callus appearing on cut surface of distal cotyledonary segment cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS (bar = 800 µm)

B. Nodular callus appearing on cut surface of distal cotyledonary segment cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS (bar = 600 µm)

C. Shoot emerging from callus obtained from distal cotyledonary segment on half-strength MS basal medium supplemented with 3 µM GA_3 (bar = 450 µm)

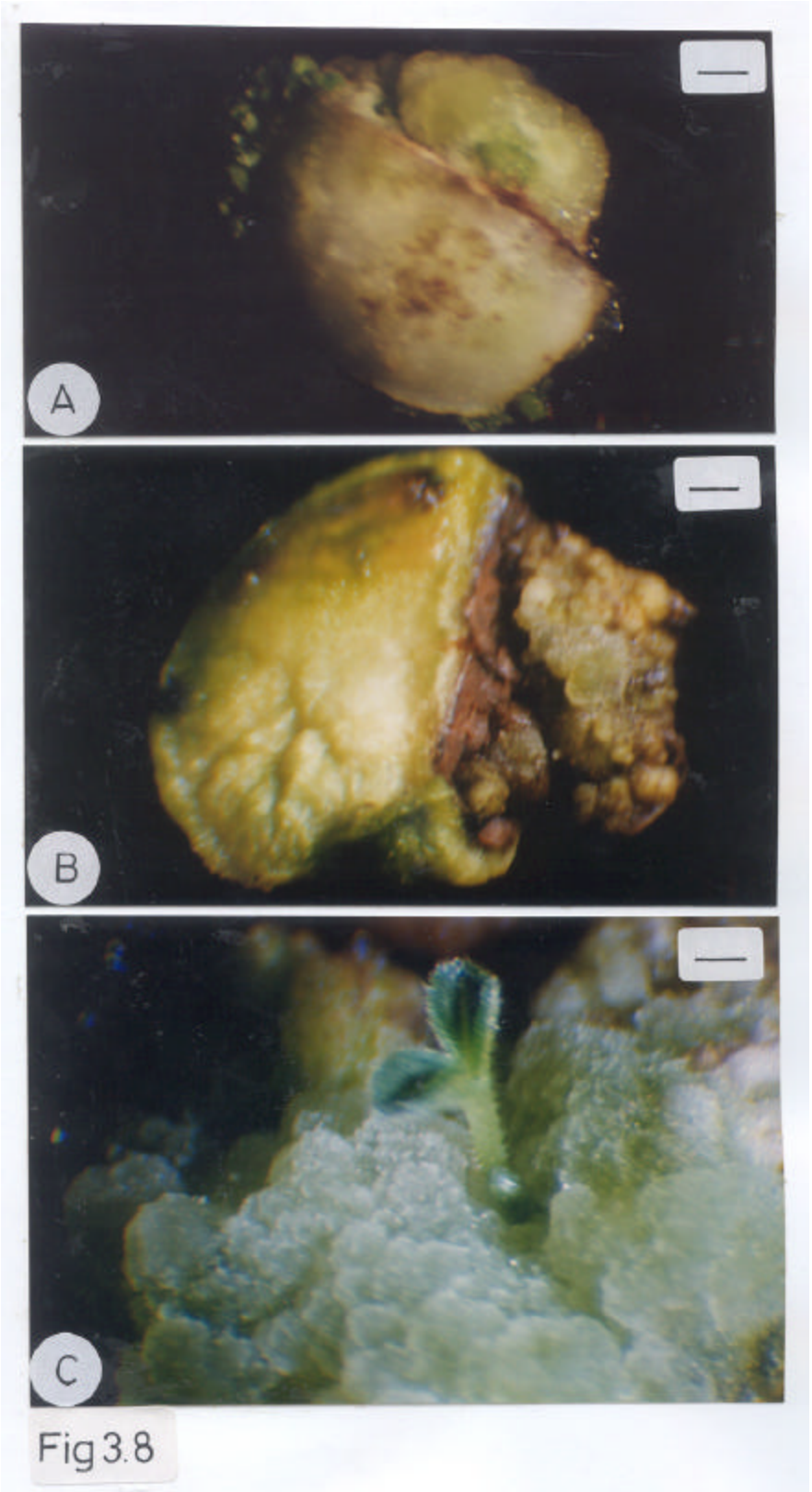
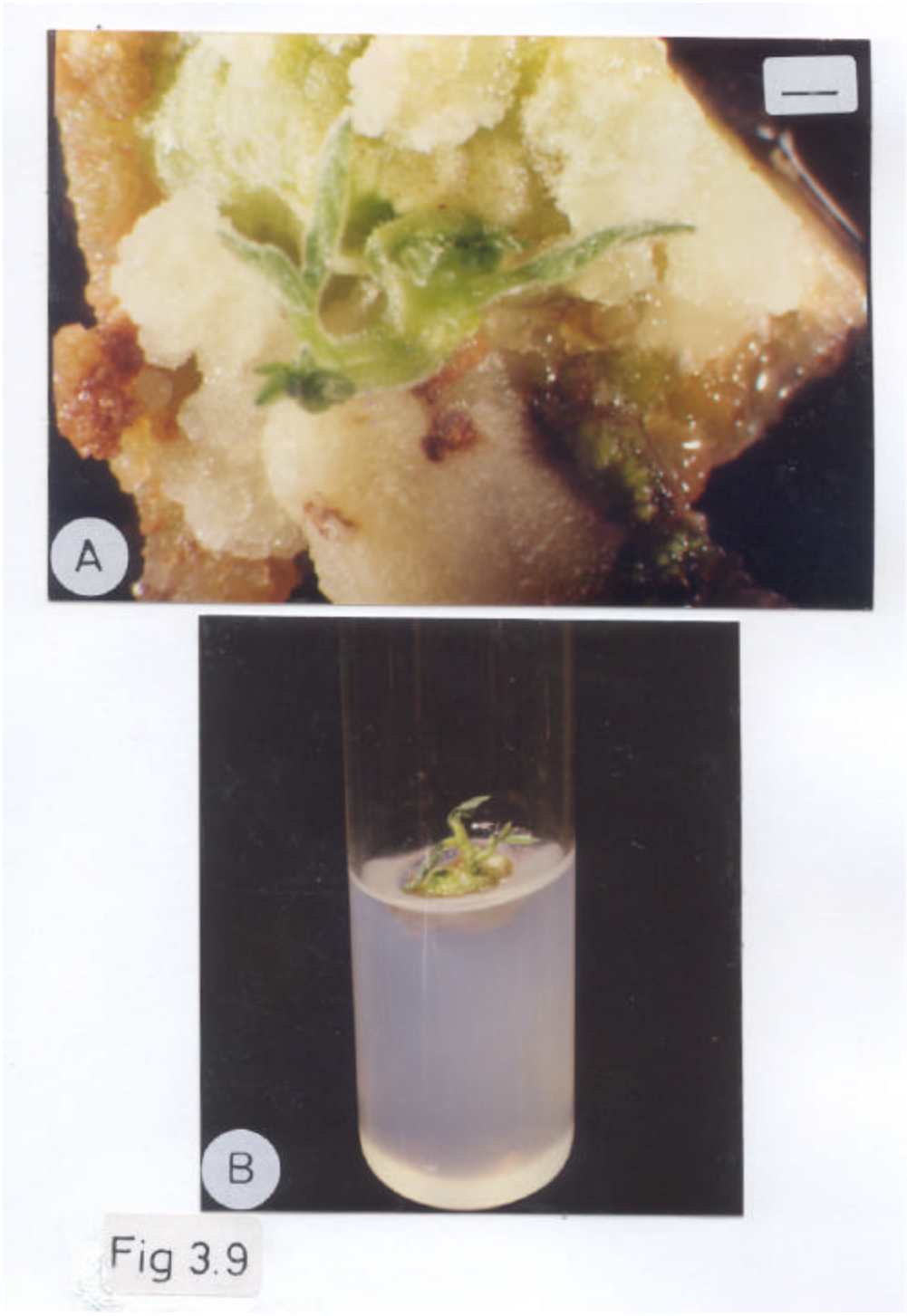


Fig 3.8



A. Shoot emerging from callus obtained from distal cotyledonary segment on half-strength MS basal medium supplemented with 3 µM GA_3 (bar = 450 µm)

B. Shoot emerging from callus obtained from distal cotyledonary segment on half-strength MS basal medium supplemented with 3 µM GA_3

Fig 3.9

3.4 Conclusions

In the present study, efforts were made to develop a regeneration system from distal cotyledonary segments of genotypes Gaut-82-90 and T-15-15. Induction of a large number of shoot buds could be achieved from distal cotyledonary segments devoid of proximal meristematic region . Maximum number of shoot buds were obtained on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS. The shoot buds formed shoots and elongated on half-strength MS medium supplemented with GA_3. The shoots were rooted on half-strength MS medium with 0.5 µM IBA and the plantlets were hardened with 70 % success. The regeneration of shoots from the callus derived from the cut surface of distal cotyledonary segments was rare. The regeneration system described above may be useful for introduction of new genes into pigeonpea genome by microprojectile-bombardment mediated as well as by *Agrobacterium*-mediated genetic transformation. Totipotent cells are apparently available and are distributed all over the surface of the explant, as shown by the production of buds all along the explant with a large number of buds clustered at the periphery. Availability of a large number of totipotent cells on the surface of a single cotyledonary segment (explant) enhances the possibility of genetic transformation by microprojectile-bombardment mediated transformation. Bud formation is also associated with a wounding site, which is a prerequisite for *Agrobacterium*-mediated transformation (Bolten *et al*. 1986; Sheikholeslam and Weeks 1987). *De novo* regeneration systems have been substantiated to be amenable to *Agrobacterium*-mediated transformation (Hinchee *et al*. 1988). Since the present protocol fulfills the requirement of genetic transformation, there is a possibility of transformation of the explants to achieve transgenic pigeonpea.

The regeneration achieved using distal cotyledonary segments has been published as a paper entitled "**Plant regeneration in pigeonpea [*Cajanus cajan* (L.) Millsp.] by organogenesis**" by Mohan ML and Krishnamurthy KV, (1998) *Plant Cell Reports*, **17**:705-710.

CHAPTER 4

IN VITRO REGENERATION THROUGH ORGANOGENESIS II. FROM MATURE EMBRYO AXES AND SEEDLING DERIVED EXPLANTS

4.1 Introduction

Genetic improvement of pigeonpea has not yet been achieved, even though various regeneration systems have been developed. Gosal and Bajaj (1979) obtained multiple shoots from *in vitro* cultured zygotic embryos. Mehta and Mohan Ram (1980) induced five to 35 shoots from cotyledons of young seedlings raised on B5 medium supplemented with BAP. Multiple shoot production was observed when cotyledons were cultured on B5 medium containing BAP at a concentration of 10^{-5} M. Phenolic compounds were absorbed with PVP resulting in better growth of plantlets (Mohan Ram *et al*. 1981). Shoot buds (2-3) were induced from cotyledon and hypocotyl explants when cultured on MS medium having BAP and NAA (Cheema and Bawa 1991). Eapen and George (1993b) observed *in vitro* shoot regeneration from leaf discs of pigeonpea on MS medium supplemented with BAP and IAA or IAA-amino acid conjugates. The frequency of shoot regeneration and the average number of shoot buds produced was dependent on the type of auxin present in the medium. Shiva prakash *et al*. (1994) obtained a mass of multiple shoot initials from the axillary bud region of the cotyledonary node explanted from the seedlings. Topical supplementation with IAA increased the formation of shoot initials. The plant regeneration was obtained from leaves, mature cotyledons and whole seeds of cultivar ICPL-161 when cultured on MS + BAP + IAA (George and Eapen 1994). Kumar *et al*. (1984) induced multiple shoots from epicotyl segments, excised cotyledons and shoot tips of pigeonpea and *Atylosia* on Blaydes' medium with BAP. Multiple shoots were obtained from mature embryo axes and mature cotyledons when cultured on MS medium supplemented with BAP alone or in combination with kinetin or IAA. Shoot buds were induced on epicotyl explant derived from 10-12 day old seedlings by culturing on MS medium containing BAP (1 mg/l) and IAA (0.1 mg/l) (Naidu *et al*. 1995). Eapen *et al*. (1998) obtained shoot regeneration from primary leaf segments when cultured on MS medium supplemented with TDZ alone or in combination with IAA. Geetha et al. (1998) obtained multiple shoots in different seedling explants such as leaf, hypocotyl, epicotyl, cotyledon and cotyledonary node explants. Franklin *et al*. (2000) obtained regeneration of viable plants from embryonal axes. However, reports on successful transfer of plants to field are a few. In addition, the data on the total number of plants transferred to field for evaluation are not available in any of the reports. Although, we obtained *de novo* plant regeneration from distal halves of mature cotyledon, very few shoots arise from the cut surfaces, which are essential for attachment of *Agrobacterium* cells to the explant. Moreover, from our preliminary experiments it was found that

cotyledons were not infected with *Agrobacterium* and the elimination of bacterium was found to be difficult due to over growth.

Therefore, with a view to find a suitable alternate explant for genetic transformation studies, several explants were tested for their regeneration potential *in vitro*. This chapter describes an efficient and rapid method of direct organogenesis from mature embryo axes (MEA) derived explants such as epicotyl region of mature embryo axes (ERMEA) and decapitated mature embryo axis (DCMEA) and the results obtained with various seedling explants like leaf, epicotyl, proximal cotyledonary segment, distal cotyledonary segment, cotyledonary node and root.

4.2 Materials and Methods

4.2.1 Plant Material

Seeds of pigeonpea genotypes T-15-15 and Gaut-82-90 were surface sterilized as described in chapter 2, section 2.6.1. The seeds were soaked in sterile distilled water and incubated on a gyratory shaker (200 rpm) for 18 h in darkness at 28 ± 2 °C.

4.2.2 Explant preparation

4.2.2.1 MEA derived explants

The pre-soaked seeds were washed twice with sterile distilled water and cotyledons were split open. The embryo axis was extracted (**Fig 4.1A**) and the shoot apex region and the root pole were removed as shown in **Fig 4.1B**. The segment of the embryo between shoot apex and cotyledonary node (referred to as epicotyl region of mature embryo axes: ERMEA) (**Fig 4.1C**) and the mature embryo axis in which both shoot and root pole were removed (referred to as decapitated mature embryo axis: DCMEA) (**Fig 4.2A**) were used as explants. There were 20 explants per treatment and the experiments were repeated thrice. The cultures were incubated at 25±2°C under cool white fluorescent lights (38 $\mu E.m^{-2}.s^{-1}$) under 16/8 h photoperiod for 4 weeks.

4.2.2.2 Seedling explants

The seeds immediately after surface sterilization were inoculated in 250 ml Erlenmeyer flasks (7 seeds/flask) containing 80 ml of hormone-free MS basal medium with 3 % sucrose and 0.8 % agar-agar. They were incubated under continuous illumination provided by cool white fluorescent light (140 $\mu E.m^{-2}.s^{-1}$) at 25 ± 2 °C for germination for 10 days. Various explants were prepared from 10 day old seedlings (**Fig 4.2B**).

4.2.2.2.1 Leaf

Leaf pieces (2-3 mm^2) were taken from primary leaves of 10 day old seedlings. The leaves were separated from the seedling and the explants were prepared. The petiole and

A. Embryo axis extracted from seeds pre-soaked for 18 h in dark at 25 ± 2 °C on a gyratory shaker at 200 rpm (rp - root pole, er - epicotyl region, sa - shoot apex (bar = 1000 µm)

B. The figure showing shoot apex (sa), epicotyl region (er) and DCMEA (dcmea) removed from the embryo axis (bar = 800 µm)

C. The epicotyl region of mature embryo axis (ERMEA) explant used for culturing on EC_6 basal medium supplemented with BAP and IAA (bar = 500 µm)

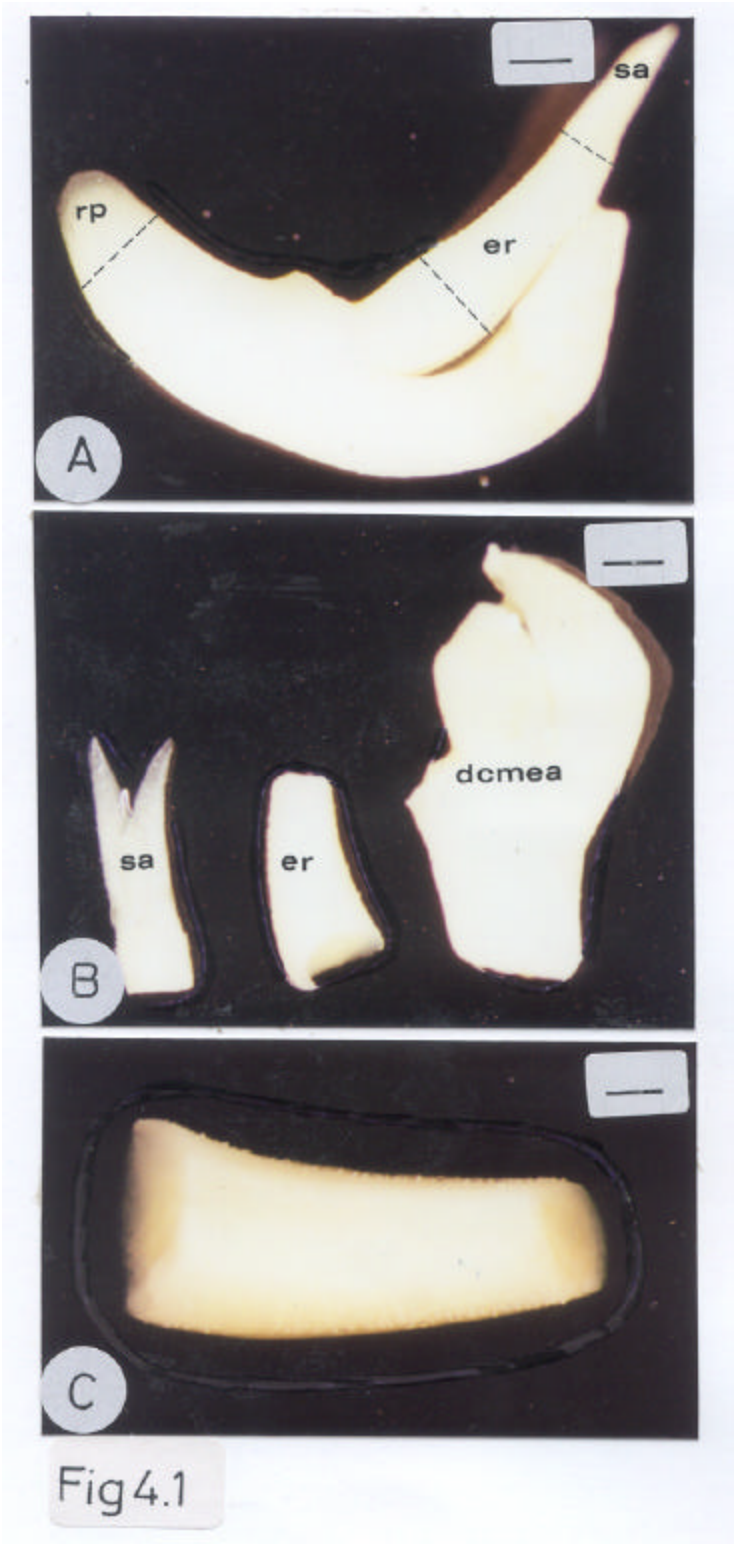


Fig 4.1

A. DCMEA explant used for culturing on EC_6 basal medium supplemented with BAP and IAA (bar = 600 μm)

B. The seedlings (10 day old) growing on hormone-free MS basal medium used for preparation of different explants

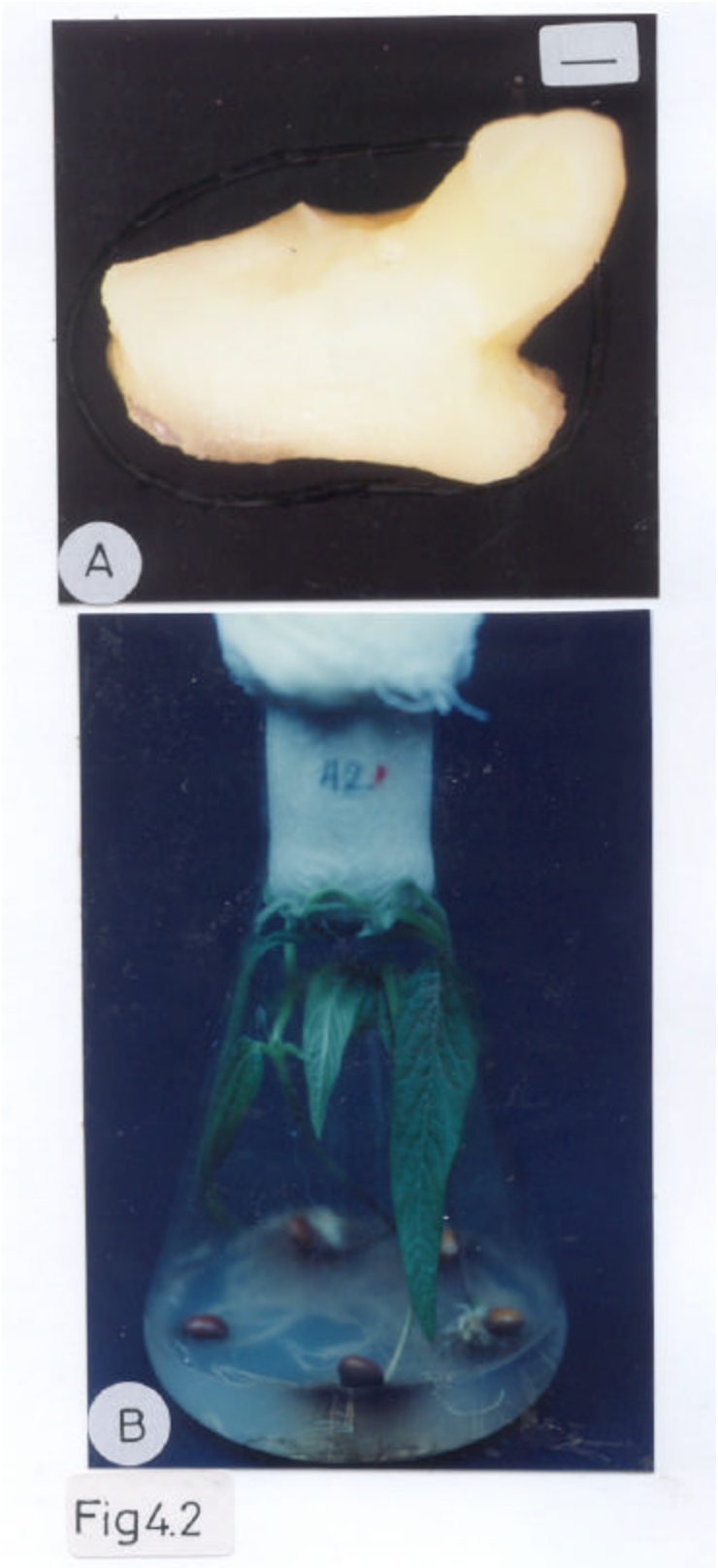


Fig4.2

leaf apex were removed. The leaf was given a cut along the midrib and the portion with midrib was made into pieces (2-3 mm^2) and cultured.

4.2.2.2.2 Epicotyl

The long epicotyl was separated by making cuts just below primary leaves and just above cotyledonary node. The epicotyl was cut into 0.5-1.0 cm long pieces and were used as explants.

4.2.2.2.3 Root

The tap root was cut into 0.5-1.0 cm long pieces and used as explants.

4.2.2.2.4 Cotyledonary segments

Cotyledons were separated from seedlings and were cut transversely at the middle to inoculate as proximal and distal cotyledonary segments separately.

4.2.2.2.5 Cotyledonary node

Cotyledons were separated from seedlings and cuts were given just above and below the position of attachment of cotyledon to seedlings. The resulting portion was used as cotyledonary node (CN) explants.

Schematic diagram of various seedling explants used in the experiment is represented in **Fig 4.3**. Two explants per tube containing 20 ml medium were cultured. Each set of experiment had 10 tubes and the experiments were repeated thrice. All the cultures were incubated at 25 ± 2 °C under cool white fluorescent lights (38 $\mu E.m^{-2}.s^{-1}$) under 16/8 h photoperiod for a period of 4 weeks.

4.2.3 Basal Medium

EC_6 basal medium was found to be best for induction of shoot buds from distal cotyledonary segments as described in chapter 3 hence, EC_6 basal medium supplemented with 3% sucrose and 0.8% agar-agar was used in all the experiments. All the media were sterilized by autoclaving at 1.4 $kg.cm^{-2}$ for 20 min.

4.2.3.1 Medium for MEA derived explants

The EC_6 basal medium supplemented with BAP (5 µM) and (10 µM) in combination with IAA (0.5, 1.0, 1.5, 2.0 and 3.0 µM) was used for culturing MEA derived explants, ERMEA and DCMEA. The BAP was added to the medium before adjusting the pH to 5.8 and sterilization. The medium was dispensed in 250 ml capacity Erlenmeyer flasks (150 ml/flask) and autoclaved at 1.4 $kg.cm^{-2}$ for 20 min. Filter sterilized IAA was added to the medium before dispensing from 250 ml flasks into 85X15 mm plastic petridishes.

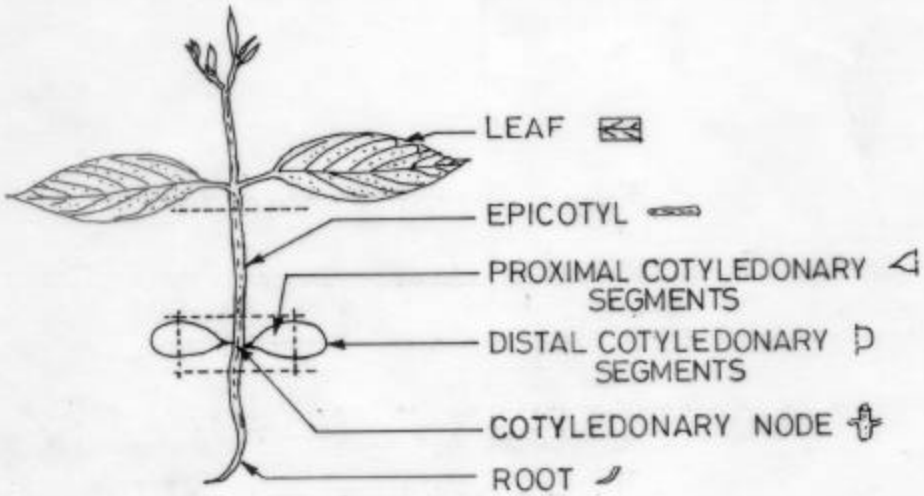


Fig 4.3 Schematic diagram of seedling and various explants used for experiments.

4.2.3.2 Medium for seedling explants

The combination of BAP (20 μM), Kin (2.0 μM) and AdS (250 μM), which was found optimum for induction of shoot buds from distal cotyledonary segments (Chapter 3) was used for evaluating the regeneration capacity of various seedling explants also. The pH of the medium was adjusted to 5.8 and dispensed into test tubes (20 ml/tube) before autoclaving at 1.4 $kg.cm^{-2}$ for 20 min.

4.2.4 Elongation of shoots

The shoots obtained from ERMEA and DCMEA explants on EC_6 basal medium supplemented with 5 μM BAP and 1 μM IAA were transferred along with explants for elongation of shoots on half-strength MS basal medium supplemented with 3 % sucrose, 0.8 % agar-agar and 3 μM of GA_3 (since elongation of shoot buds from distal cotyledonary segments was better on this medium as mentioned in chapter 3). The cultures were incubated under the conditions mentioned as above for 4 weeks.

The explants with shoot buds obtained on EC_6 basal medium suplemented with 20 μM BAP, 2 μM kinetin and 250 μM AdS from epicotyl, cotyledonary segments (proximal and distal), cotyledonary node and root explants were transferred to half-strength MS medium supplemented with 3 % sucrose, 0.8 % agar-agar and 3 μM GA_3 for elongation of shoot buds into shoots. The leaf pieces along with shoot buds were transferred to hormone-free half-strength MS basal medium containing 3 % sucrose and 0.8 % agar-agar in test tubes or to half-strength MS basal medium supplemented with BAP (2 μM) + kin (0.2 μM) + AdS (10 μM), 0.5-2.0 μM NAA, 0.5-2.0 μM BAP plus 0.5 μM NAA or 3 μM GA_3 for elongation of shoot buds into shoots. The cultures were incubated under the conditions mentioned as above for 4 weeks.

4.2.5 Rooting and Hardening

The shoots elongated on half-strength MS basal medium supplemented with 3 μM of GA_3 were excised and transferred to half-strength MS basal medium containing 0.5 μM IBA for rooting. The cultures were incubated under the conditions mentioned as above for 3 weeks. The rooted plantlets were hardened in pots with soil:vermiculite (1:1) mixture at 25±2°C under diffuse light (16/8 h photoperiod) conditions for 3-4 weeks.

4.2.6 Statistical analysis

The data were analyzed by ANOVA technique for a Completely Randomized Design and the treatment means were compared.

4.3 Results and Discussion

4.3.1 MEA derived explants

4.3.1.1 ERMEA

The explants swelled and turned green within 3 weeks of culture, producing small, green shoots directly from the cut surface of the ERMEA segments (**Fig 4.4A and 4.4B**). The percentage formation of shoots varied from 3-68 % in genotype T-15-15 and 28-43 % in genotype Gaut-82-90 depending on the concentration of BAP and IAA (**Table 4.1**). The mean number of shoots per explant was 0.4-10.5 (T-15-15) and 2.1-3.1 (Gaut-82-90) (**Table 4.1**). The highest percentage of shoot induction and number of shoots per explant was observed on EC_6 basal medium supplemented with 5 µM BAP and 1 µM IAA and the genotype T-15-15 was best in terms of regeneration from ERMEA explants. Very low percentage of ERMEA explants of genotype T-15-15 formed shoots when the BAP concentration was increased to 10 µM BAP or when 10 µM BAP was used in combination with IAA at 0.5, 1.0, 1.5, 2.0 and 2.5 µM (**Table 4.1**). Therefore, these combinations were not tried with ERMEA explants of the genotype Gaut-82-90. When 100 ERMEA explants of each genotypes were cultured on EC_6 basal medium supplemented with 5 µM BAP and 1 µM IAA, 68 explants of genotype T-15-15 and 37 explants of genotype Gaut-82-90 formed small shoots.

Table 4.1 Effect of various combinations of BAP and IAA on shoot regeneration on ERMEA explants of pigeonpea genotypes

		T-15-15		GAUT-82-90	
BAP (µM)	IAA (µM)	Percentage Induction (mean±se)	No. of Shoots/Explant (mean±se)	Percentage Induction (mean±se)	No. of Shoots/Explant (mean±se)
5	Nil	12 ± 4^{cd}	4.8 ± 0.3^{f}	30 ± 10^{a}	2.3 ± 0.5^{a}
5	0.5	52 ± 7^{e}	2.3 ± 0.4^{bcde}	43 ± 12^{c}	2.7 ± 0.8^{a}
5	1.0	68 ± 6^{f}	10.5 ± 2.6^{g}	37 ± 11^{bc}	3.1 ± 0.2^{a}
5	1.5	7 ± 2^{abc}	3.7 ± 0.7^{ef}	33 ± 8^{ab}	2.3 ± 0.6^{a}
5	2.0	5 ± 1^{ab}	2.0 ± 1.0^{bcd}	28 ± 2^{a}	2.1 ± 0.3^{a}
5	2.5	10 ± 5^{bcd}	2.0 ± 0.5^{bcd}	40 ± 7^{c}	2.3 ± 0.3^{a}
10	Nil	13 ± 3^{d}	3.5 ± 0.7^{def}	NT	NT
10	0.5	10 ± 3^{bc}	3.2 ± 0.8^{cde}	NT	NT
10	1.0	3 ± 2^{a}	1.7 ± 1.0^{abc}	NT	NT
10	1.5	6 ± 3^{ab}	1.5 ± 0.9^{ab}	NT	NT
10	2.0	10 ± 6^{bcd}	0.4 ± 0.2^{a}	NT	NT
10	2.5	13 ± 7^{d}	1.8 ± 1.0^{abc}	NT	NT

Figures with different alphabets in superscript differ significantly at 0.05 probability. NT – Not tried

4.3.1.2 DCMEA

The DCMEA explants turned green and produced small shoots at shoot apex region (**Fig 4.4C, 4.5A and 4.5B)** in 3 weeks of culture and many shoot buds at cotyledonary node region. The percentage response of shoot formation at the shoot apex region was 5-30 % and 21-31 % in the genotypes T-15-15 and Gaut-82-90 respectively (**Table 4.2**). The number of shoots per explant varied from 3.0-5.2 in the genotype T-15-15 and 2.0-2.8 in the genotype Gaut-82-90 (**Table 4.2**). The percentage induction of shoots and the number of shoots per explant were significantly higher in T-15-15 genotype when 5 µM BAP and 1 µM IAA were used. There was no significant difference between treatments in the genotype Gaut-82-90 in terms of percentage induction of shoots and the number of shoots per explant. Even though there was no significant difference between the genotypes T-15-15 and Gaut-82-90 in percentage induction of shoots, the number of shoots per explant was significantly higher in the genotype T-15-15. Out of 100 DCMEA explants of T-15-15 and Gaut-82-90 cultured on EC_6 basal medium supplemented with 5 µM BAP and 1 µM IAA, 30 and 31 explants of T-15-15 and Gaut-82-90 genotypes respectively formed small shoots at the shoot apex region.

A. Shoots arising from ERMEA explants cultured on EC_6 basal medium supplemented with BAP and IAA

B. Magnified view of shoots arising from ERMEA explant cultured on EC_6 basal medium supplemented with BAP and IAA (bar = 1200 μm)

C. Shoot emerging from shoot apex region of DCMEA explant cultured on EC_6 basal medium supplemented with BAP and IAA (bar = 1000 μm)

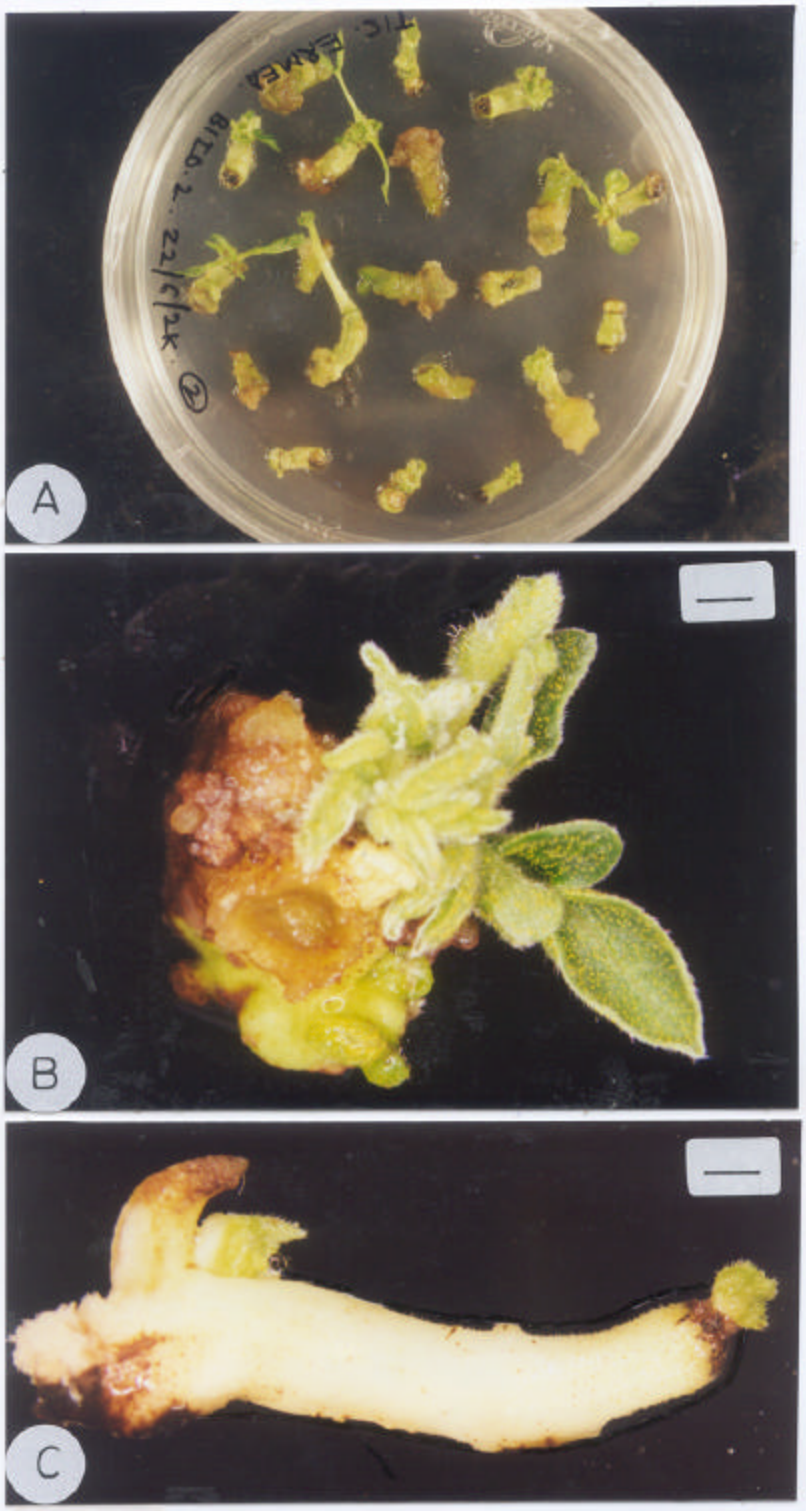


Fig 4.4

A. Magnified view of shoots appearing from shoot apex region of DCMEA explant cultured on EC_6 basal medium supplemented with BAP and IAA (bar = 750 µm)

B. Magnified view of shoots appearing from shoot apex region of DCMEA explant cultured on EC_6 basal medium supplemented with BAP and IAA (bar = 750 µm)

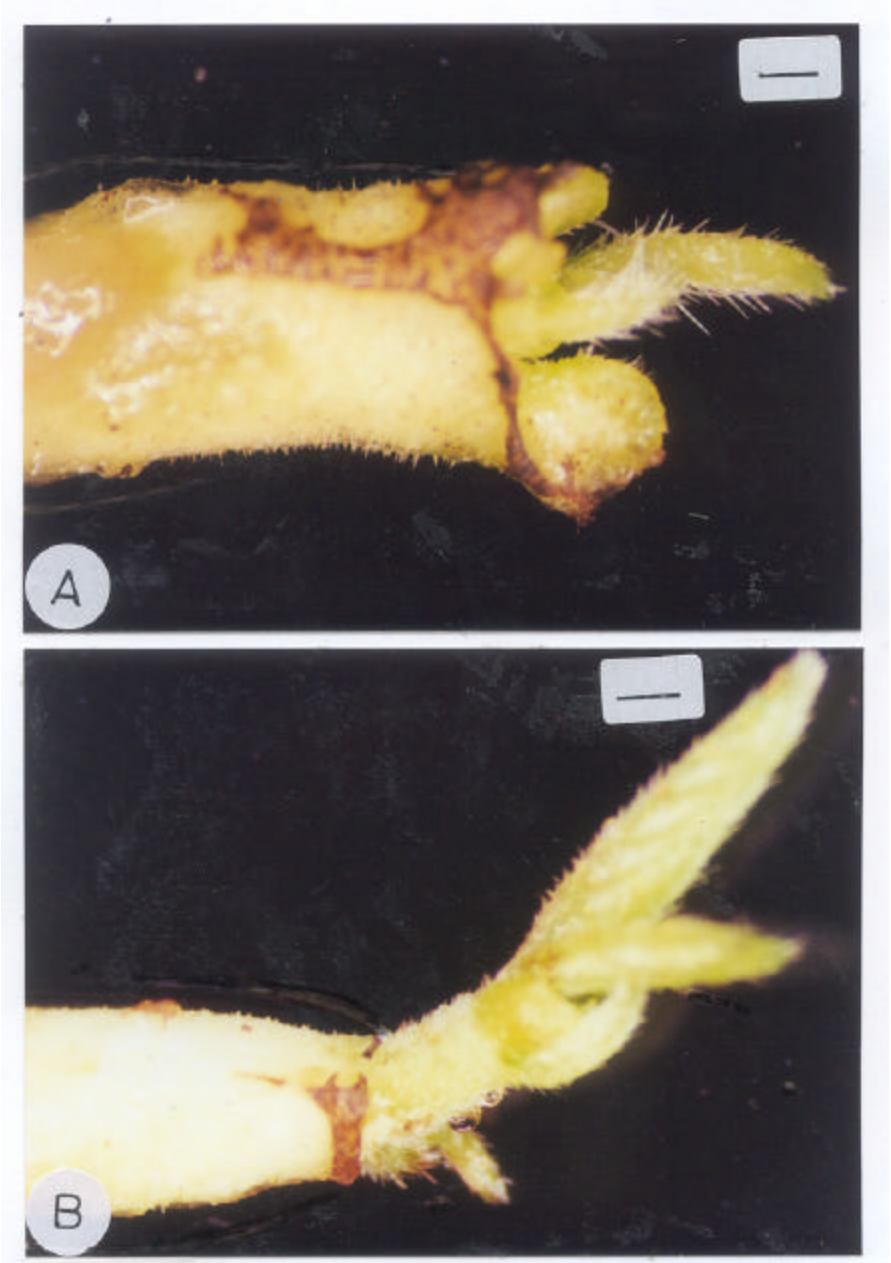


Fig 4.5

Table 4.2 Effect of various combination of BAP and IAA on shoot* regeneration on DCMEA explants of pigeonpea genotypes

	T-15-15		GAUT-82-90	
BAP(5 µM) + IAA (µM)	Percentage Induction (mean±se)	No. of Shoots/Explant (mean±se)	Percentage Induction (mean±se)	No. of Shoots/Explant (mean±se)
Nil	6 ± 1^{a}	4.5 ± 0.6^{cd}	23 ± 5^{a}	2.0 ± 1.1^{a}
0.5	5.0 ± 1^{a}	3.0 ± 0.6^{ab}	27 ± 9^{a}	$2.3 \pm .0.7^{a}$
1.0	30 ± 13^{d}	5.2 ± 0.8^{d}	31 ± 4^{a}	2.7 ± 0.4^{a}
1.5	13 ± 3^{b}	3.0 ± 0.9^{ab}	23 ± 6^{a}	2.1 ± 0.5^{a}
2.0	22 ± 6^{c}	3.9 ± 0.2^{bc}	21 ± 5^{a}	2.8 ± 1.4^{a}
3.0	13 ± 3^{b}	2.5 ± 1.0^{a}	25 ± 7^{a}	2.5 ± 0.8^{a}

Figures with different alphabets in superscript differ significantly at 0.05 probability.

*Only shoots arriving from shoot apex region were considered and the shoots arriving from cotyledonary node region were not counted.

4.3.1.3 Elongation of shoots derived from ERMEA and DCMEA explants

The explants along with shoots induced on EC_6 basal medium supplemented with 5 µM BAP and 1 µM IAA were transferred to elongation medium (half-strength MS + 3 µM of GA_3) after 4 weeks. Only 33 % of ERMEA and 25 % of DCMEA of these explants gave rise to elongated shoots **(Table 4.3)** (**Fig 4.6A**) in the genotype T-15-15. The small shoots produced in the genotype Gaut-82-90 did not elongate to form well-developed shoots. All the small shoots turned into leafy shoots. Elongation of shoots in the genotype T-15-15 occurred in only 22 of the ERMEA explants and 5 of the DCMEA explants transferred on half-strength MS basal medium supplemented with 3 µM GA_3 in 4 weeks time. The number of elongated shoots were only 1.4 and 1.3 per explant in ERMEA and DCMEA explants respectively (**Table 4.3**). A total of 30 elongated shoots from the ERMEA explants and 6 elongated shoots from the DCMEA explants could be obtained. While many small shoots did not elongate and turned into leafy shoots. Similarly, difficulty in elongation of shoot buds arising even from embryonal axes was observed by Franklin *et al.* (2000). The same was our experience with distal cotyledonary segments. The regeneration of shoots using ERMEA and DCMEA explants has not been reported so far in literature.

Table 4.3 Elongation of shoots derived from the MEA derived explants of the genotype T-15-15 on the EC_6 basal medium supplemented with 5 ■M BAP and 1 ■M IAA

Explant	Percentage elongation (mean±se)	No. of shoots/explant (mean±se)
ERMEA	33 ± 10	1.4 ± 0.3
DCMEA	25 ± 5	1.3 ± 0.3

4.3.1.4 Rooting and Hardening of shoots obtained from the ERMEA and the DCMEA explants

The 30 elongated shoots obtained from the ERMEA explants and 6 elongated shoots from the DCMEA explants of genotype T-15-15 on half-strength MS basal medium supplemented with 3 µM GA_3 were separated and transferred to test tubes containing half-strength MS basal medium supplemented with 0.5 µM IBA for rooting. The rooting (65 %) took place in 3 weeks time (**Fig 4.6B**). 20 rooted plantlets obtained from the ERMEA explants and 4 rooted plantlets from the DCMEA explants were transferred to pots filled with soil:vermiculite (1:1) mixture for hardening and kept at 25 ± 2 °C under diffuse light (16/8 h photoperiod) conditions for 3-4 weeks.

Geetha *et al* (1998) also observed greater multiple shoot formation with combinations of BAP and an auxin, similar to the observations made in the present study. Although, the regeneration of plants from epicotyl explants has been reported earlier (Naidu *et al* 1995; Kumar *et al* 1984; Geetha et al. 1998), the explants were prepared from 10-15 day old seedling. In our experiments, however, the epicotyl region of mature embryo axis was used as explant.

4.3.2 Seedling explants

4.3.2.1 Induction of shoot buds from leaf explants

The leaf segments enlarged and produced shoot buds after 4-5 weeks in culture (**Fig 4.7A**). The percentage of shoot bud formation was in 50-70 % cultures of the genotype T-15-15 and 10-62 % cultures of the genotype Gaut-82-90 (**Table 4.4**). The number of shoot buds per explant varied from 2.3-15.2 and 2.5-13.3 in the genotypes T-15-15 and Gaut-82-90 respectively. The combination of 20 µM BAP, 2 µM kin and 250 µM AdS was best both in percentage response of shoot bud formation and in the number of shoot buds produced per explant, even though shoot bud induction was observed on BAP alone or in combination with AdS. The genotype T-15-15 exhibited better response both in terms of percentage of shoot bud formation and in the number of shoot buds per explant (**Table 4.4**). Forty two leaf explants of the genotype T-15-15 and 37 leaf explants of

A. Elongated shoots obtained from ERMEA and DCMEA explants on half-strength MS basal medium supplemented with 3 µM GA_3

B. Rooted shoots obtained from ERMEA and DCMEA explants on half-strength MS basal medium supplemented with 0.5 µM IBA

Fig4.6

genotype Gaut-82-90 formed shoot buds on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS.

Studies on organogenesis from leaf explants reported earlier employed MS basal medium and a growth regulator combinations of BAP and IAA or IAA conjugates (Eapen and George 1993b; George and Eapen 1994), TDZ alone or in combination with IAA (Eapen *et al*. 1998) or BAP (Geetha *et al*. 1998). In contrast, we used EC_6 basal medium and cytokinins BAP, kin and AdS in various combinations. The genotypes used in the present study are different than the ones used in the earlier reports.

Table 4.4 Effect of various growth regulator combinations (EC_6 Basal Medium) on induction of shoot buds on leaf pieces

	T-15-15		GAUT-82-90	
Growth Regulator (µM)	% of explants forming Shoot buds (mean ± se)	No. of Shoot buds per explant (mean ± se)	% of explant s forming Shoot buds (mean ± se)	No. of Shoot buds Per explant (mean ± se)
BAP(20)	0 ± 0^a	-	10 ± 2^b	2.5 ± 0.4^a
Kin(2)	0 ± 0^a	-	0 ± 0^a	-
AdS(250)	0 ± 0^a	-	0 ± 0^a	-
BAP(20) + Kin (2)	0 ± 0^a	-	0 ± 0^a	-
BAP(20) + AdS(250)	50 ± 10^b	2.3 ± 0.2^a	50 ± 8^c	3.9 ± 0.5^a
Kin(2) + AdS(250)	0 ± 0^a	-	0 ± 0^a	-
BAP(20) + Kin(2) + AdS(250)	70 ± 6^c	15.2 ± 0.5^b	62 ± 19^d	13.3 ± 1.1^b

Figures with different alphabets in superscript differ significantly at 0.05 probability.

4.3.2.2 Elongation of shoot buds obtained from leaf explants

When the shoot buds obtained from leaf explants on EC_6 basal medium supplemented with BAP (20 µM), kin (2 µM) and AdS (250 µM) were transferred to various media combinations, there was no elongation of shoot buds into shoots in any of the media except on half-strength MS basal medium supplemented with 3 µM GA_3 (**Table 4.5**). All other media produced only leafy shoots. Sporadic elongation of shoots (**Fig 4.7B & 4.7C**) was observed on half-strength MS basal medium supplemented with 3 µM GA_3. Only 5 % of the cultures showed further elongation on GA_3 medium into shoots producing 2.3 shoots per explant in the genotype T-15-15. In the genotype Gaut-82-90 the shoot buds did not convert into shoots in any of the explants transferred to half-strength MS basal medium supplemented with 3 µM GA_3. A total of 5 shoots were obtained from 60 leaf pieces of the genotype T-15-15. However, none of the shoots produced roots on the rooting medium containing half-strength MS basal medium supplemented with 0.5 µM

A. Shoot buds appeared on leaf explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

B. Shoot buds and shoots appeared on leaf explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

C. Elongated shoots obtained from leaf explants on half-strength MS basal medium supplemented with 3 µM GA_3

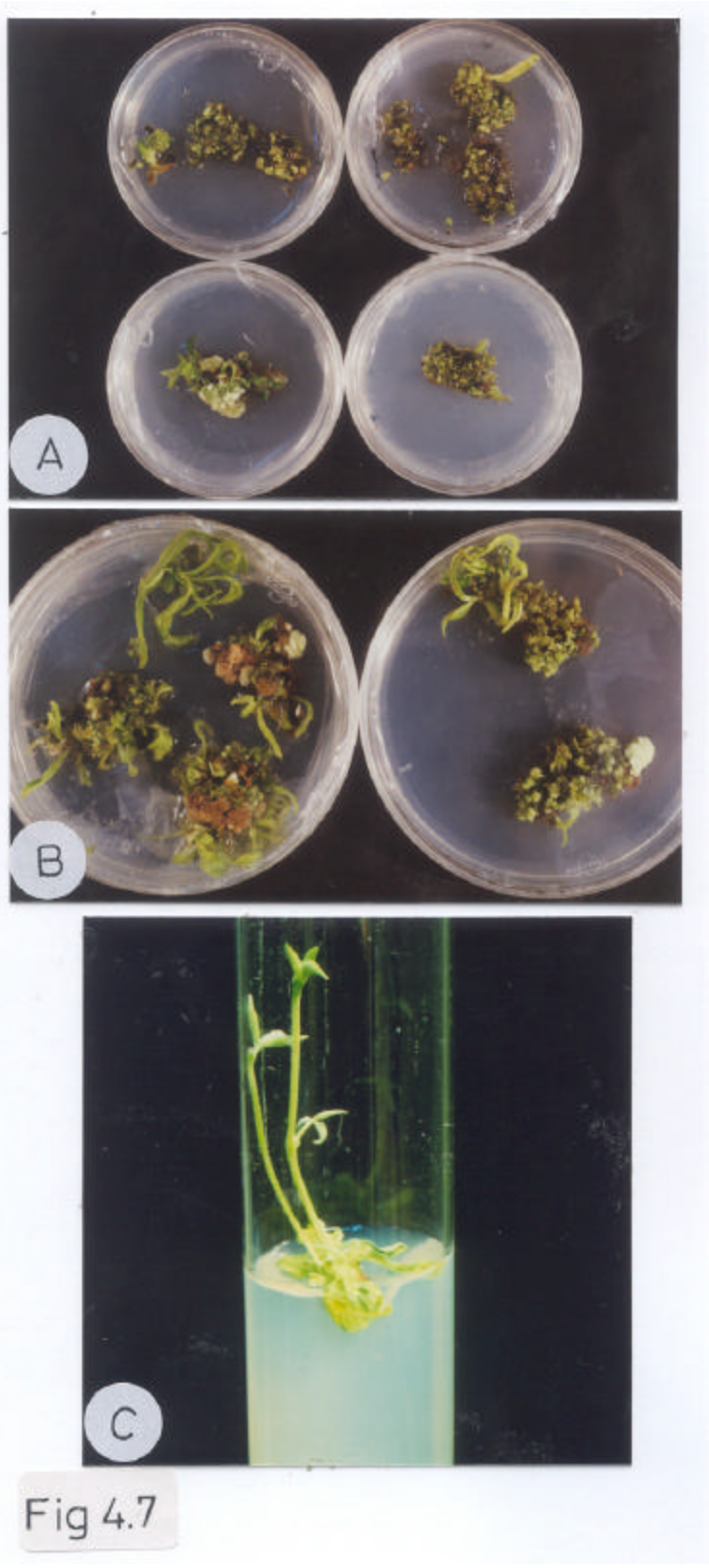


Fig 4.7

IBA and died after 4 weeks of culture and therefore could not be hardened. The regeneration frequency was very low. Similarly, low frequency of elongation of shoot buds obtained from leaves was also reported earlier by Eapen *et al.* (1998).

Table 4.5 Number of shoots recovered from leaf pieces after culturing on half-strength MS medium supplemented with various growth regulators for elongation

	T-15-15			Gaut-82-90		
Growth Regulator (µM)	Leafy shoots[1] Per explant (mean ± se)	Shoots[2] Per explant (mean ± se)	Percentage Of shoots to leafy shoots	Leafy shoots[1] Per explant (mean ± se)	Shoots[2] Per explant (mean ± se)	Percentage Of shoots to Leafy shoots
NIL	11.0 ± 0.9	0.0 ± 0.0	0	16.1 ± 3.1	0.0 ± 0.0	0
BAP (2) + Kin (0.2) + AdS (25)	7.4 ± 2.4	0.0 ± 0.0	0	4.7 ± 0.4	0.0 ± 0.0	0
NAA (0.5)	12.1 ± 2.9	0.0 ± 0.0	0	12.6 ± 1.9	0.0 ± 0.0	0
NAA (1.0)	14.5 ± 2.0	0.0 ± 0.0	0	8.8 ± 1.8	0.0 ± 0.0	0
NAA (2.0)	5.5 ± 1.2	0.0 ± 0.0	0	6.6 ± 1.1	0.0 ± 0.0	0
NAA (0.5) + BAP (0.5)	11.5 ± 1.1	0.0 ± 0.0	0	7.0 ± 0.6	0.0 ± 0.0	0
NAA (0.5) + BAP (1.0)	5.2 ± 1.5	0.0 ± 0.0	0	8.5 ± 0.9	0.0 ± 0.0	0
NAA (0.5) + BAP (2.0)	4.0 ± 0.9	0.0 ± 0.0	0	8.3 ± 0.8	0.0 ± 0.0	0
GA_3 (3)	10.2 ± 2.1	2.3 ± 0.5	23	7.5 ± 1.7	0.0 ± 0.0	0

[1]Leaves with petiole
[2]Well developed, elongated shoots

4.3.2.3 Induction of shoot buds from epicotyl explants

Seventy five per cent of the epicotyl segments of both T-15-15 and Gaut-82-90 genotypes produced shoot buds (**Fig 4.8A**) (**Table 4.6**). The formation of shoot buds was associated with abundant callus surrounding them (**Fig 4.8B**). The average number of shoot buds per explant was 6 for the genotype T-15-15 and 4 for the genotype Gaut-82-90 (**Table 4.6**). Out of 60 epicotyl explants of the genotypes T-15-15 and Gaut-82-90 cultured, 45 explants formed shoot buds.

Regeneration from the epicotyl explants were reported earlier by Geetha *et al.* (1998) on MS basal medium with BAP or Kinetin supplements, by Naidu *et al.* (1995) on MS medium supplemented BAP alone or in combination with IAA, and by Kumar *et al.* (1984) on Blady's medium with BAP or Kinetin in combination with IAA. However, George and Eapen (1994) observed only callus and no shoot buds on epicotyl explants when cultured on MS medium containing BAP and IAA. We obtained both callus and

shoot buds when epicotyl explants were cultured on EC_6 basal medium supplemented with BAP (20 µM), Kinetin (2 µM) and AdS (250 µM).

4.3.2.4 Elongation of shoot buds obtained from epicotyl explants

The epicotyl segments of the genotypes T-15-15 and Gaut-82-90 forming shoot buds were transferred on half-strength MS basal medium supplemented with 3 µM GA_3 for 4 weeks for elongation of shoot buds into shoots. Elongation of shoots (**Fig 4.8C**) occurred in only 10 % of the explants of the genotype T-15-15. The elongation of shoots was not observed in the genotype Gaut-82-90. On an average 1.5 elongated shoots were obtained per explant. A total of 7 shoots were obtained from 60 epicotyl segments of the genotype T-15-15.

4.3.2.5 Induction of shoot buds from root segments

Root segments generally did not respond to the treatment (**Table 4.6**). However, very rarely formation of nodular structures (**Fig 4.9A**) and small shoot (**Fig 4.9B**) were observed in the genotype T-15-15. George and Eapen (1994) observed plant regeneration from roots of seedlings, which were preconditioned on 15 mg/L of BAP for 4 weeks and cultured on MS basal medium with BAP and IAA. However, in our studies the preconditioning of seedlings was not done before culturing of the root segments.

4.3.2.6 Elongation of shoot buds from root segments

The nodular structures and the small shoots, which appeared rarely on root segments did not convert or elongate into shoots when they were cultured on half-strength MS basal medium supplemented with 3 µM GA_3.

4.3.2.7 Induction of shoot buds from proximal cotyledonary segments

Proximal cotyledonary segments produced shoot buds and shoots with a large number of leaves after 3-4 weeks of culture (**Fig 4.9C**). The percentage of shoot bud formation was 63 % in the T-15-15 and 53 % in the Gaut-82-90. The average number of shoot buds produced was 16.4 and 17.5 per explant in the genotypes T-15-15 and Gaut-82-90 respectively (**Table 4.6**). The genotype T-15-15 was better than Gaut-82-90 in percentage induction of shoot buds, however, there was no significant difference between two genotypes in terms of number of shoot buds produced per explant. The formation of shoot buds from proximal cotyledonary segments is observed frequently and normally as they contain pre-existing meristems. Out of 60 proximal cotyledonary segments of each genotype cultured, 38 segments of T-15-15 and 32 segments of Gaut-82-90 formed shoot buds.

A. Shoot buds appeared on epicotyl explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

B. Magnified view of shoot buds appeared on epicotyl explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS (bar = 600 µm)

C. Elongated shoots obtained from epicotyl explants on half-strength MS basal medium supplemented with 3 µM GA_3

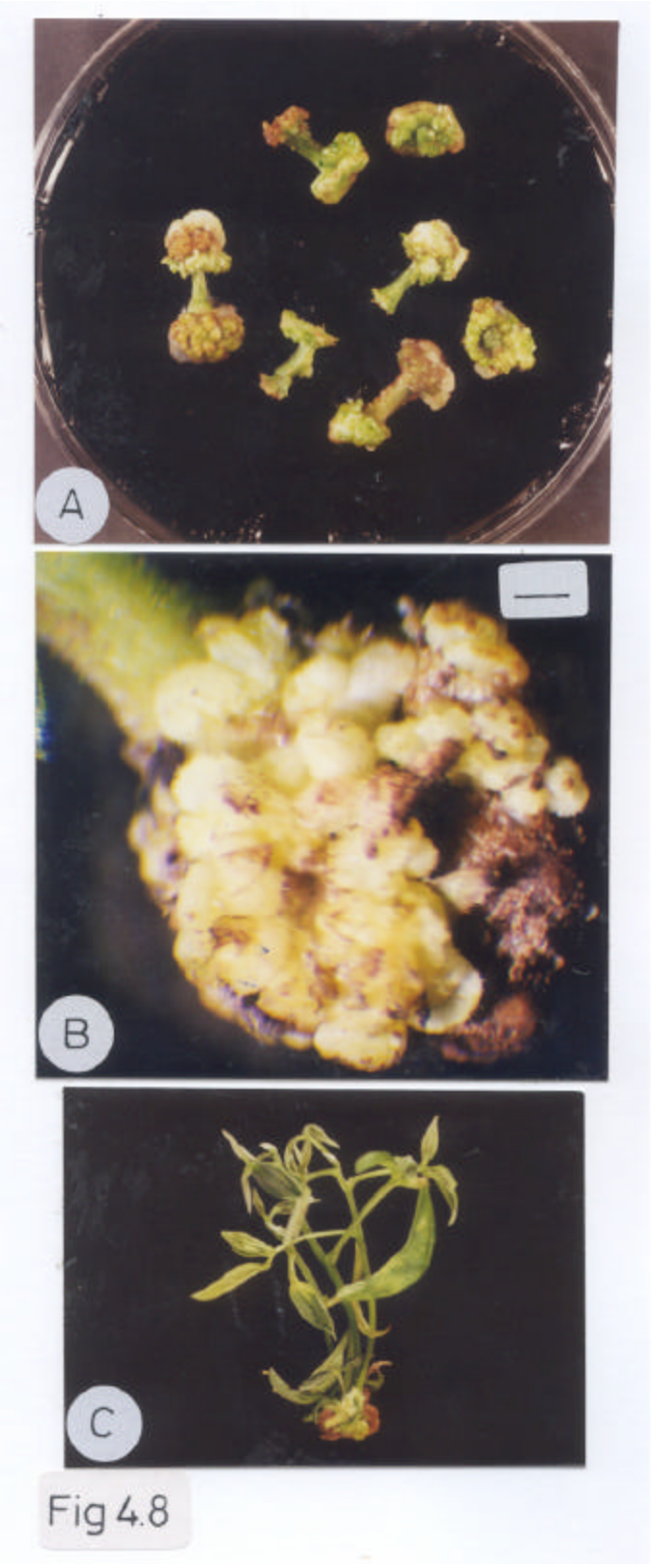


Fig 4.8

A. Nodular structures appeared on root explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

B. Small shoot appeared on root explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS (bar = 750 µm)

C. A large number of leaves appearing along with shoot buds on proximal end of cotyledonary segments cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

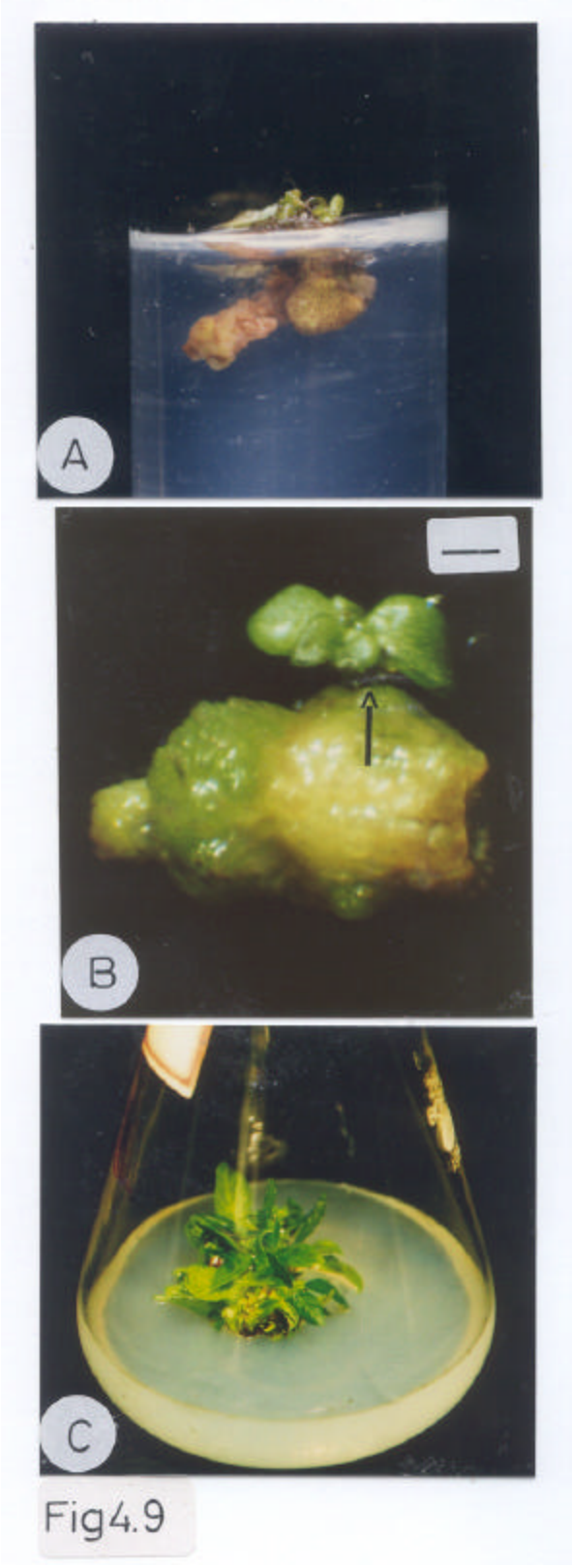


Fig4.9

Shoot regeneration was observed from cotyledons on MS basal medium with BAP or Kinetin (Geetha *et al* 1998), on MS medium with BAP alone or in combination with IAA or kinetin (Naidu *et al*. 1995), on B5 medium supplemented with BAP (Mehta and Mohan Ram 1980), on Blady's medium containing BAP (Kumar *et al*. 1984). Our studies differ in terms of basal medium and in the concentrations of BAP, kinetin and AdS used.

4.3.2.8 Elongation of shoot buds obtained from proximal cotyledonary segments

The shoot buds along with proximal cotyledonary segments were transferred on half-strength MS basal medium supplemented with 3 µM GA_3 for elongation for 4 weeks. Only 15 % (6 out of 38 explants transferred) of the explants produced elongated shoots (**Fig 4.10A**) in 4 weeks with an average of 1.7 shoots per explant in the genotype T-15-15. Most of the shoot buds turned into leafy shoots. In the genotype Gaut-82-90, all the shoot buds turned into leafy shoots and no well-developed shoot could be recovered. A total of 10 shoots were recovered from proximal cotyledonary segments of the genotype T-15-15.

4.3.2.9 Induction of shoot buds from distal cotyledonary segments

The percentage induction of shoot buds in distal cotyledonary segments was 87 % in the genotype T-15-15 and 67 % in the genotype Gaut-82-90. Each cotyledonary segment produced 14.3 and 10.1 shoot buds (**Fig 4.10B**) in the genotypes T-15-15 and Gaut-82-90 respectively (**Table 4.6**). The T-15-15 exhibited better response when compared to Gaut-82-90 both in terms of percentage induction of shoot buds and number of shoot buds per explant. When 60 distal cotyledonary segments were cultured, 52 cotyledonary segments of the genotype T-15-15 and 40 cotyledonary segments of the genotype Gaut-82-90 formed shoot buds. Similar to our studies, George and Eapen (1994) reported shoot bud formation on distal half of the cotyledon, however, they have used whole cotyledon from mature seeds for inoculation on MS medium supplemented with BAP and IAA, instead of distal cotyledonary segments separated from the whole cotyledon.

4.3.2.10 Elongation of shoot buds obtained from distal cotyledonary segments

The shoot buds elongated (**Fig 4.10C**) into shoots in only 18 % of the distal cotyledonary segments when they were transferred on half-strength MS basal medium containing 3 µM of GA_3 for 4 weeks in the genotype T-15-15. The elongation of shoot buds occurred in 9 cotyledonary segments producing 2 shoots per explant. A total of 18 shoots were obtained

Fig 4.10

A. Elongated shoot obtained from proximal end of cotyledonary segments on half-strength MS basal medium supplemented with 3 µM GA_3

B. Shoot buds appeared on distal end of cotyledonary segments cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

C. Elongated shoot obtained from distal end of cotyledonary segments on half-strength MS basal medium supplemented with 3 µM GA_3

from distal cotyledonary segments of the genotype T-15-15. The shoot buds did not convert to form shoots in distal cotyledonary segments of the genotype Gaut-82-90.

4.3.2.11 Induction of shoot buds from cotyledonary node explants

The cotyledonary node explants produced shoot buds (**Fig 4.11A**) in 65 % of T-15-15 cultures and in 60 % of Gaut-82-90 cultures (**Table 4.6**). The average number of shoot buds was 40.7 in the genotype T-15-15 and 32.5 in the genotype Gaut-82-90. The genotype T-15-15 was better in percentage induction of shoot buds as well as in the number of shoot buds produced per explant. Out of 60 cotyledonary node explants 39 explants of the genotype T-15-15 and 36 explants of the genotype Gaut-82-90 produced shoot buds on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS. Similar to our studies, Shiva prakash *et al*. (1994) obtained a large number of shoot buds from cotyledonary node explants by culturing on MS basal medium supplemented with BAP.

4.3.2.12 Elongation of shoot buds obtained from cotyledonary node explants

Elongation of shoot buds into shoots was observed only in the genotype T-15-15 and the shoot buds turned into leafy shoots in the genotype Gaut-82-90. Only 13 % of the 39 cotyledonary node explants of the genotype T-15-15 produced elongated shoots (average 2.3 per explant) (**Fig 4.11B**) when they were transferred to half-strength MS basal medium supplemented with GA_3 (3 µM) in 4 weeks. A total of 12 shoots have been obtained from 5 explants producing elongated shoots.

Table 4.6 Shoot bud induction in various seedling explants on EC_6 basal medium supplemented with 20 µM BAP, 2 µM Kin and 250 µM AdS

	T-15-15		Gaut-82-90	
Explant	% explants forming shoot buds (mean ± se)	No. of Shoot buds/ explant (mean ± se)	% explants forming shoot buds (mean ± se)	No. of Shoot buds/ Explant (mean ± se)
Epicotyl	75 ± 11	6.0 ± 0.7	75 ± 16	4.0 ± 0.3
Root	0 ± 0	-	0 ± 0	-
Cotyledon (Proximal end)	63 ± 5	16.4 ± 2.2	53 ± 3	17.5 ± 2.4
Cotyledon (Distal end)	87 ± 9	14.3 ± 1.3	67 ± 14	10.1 ± 1.6
Cotyledonary node	65 ± 7	40.7 ± 1.4	60 ± 6	32.5 ± 3.6

4.3.3 Rooting and Hardening

Rooting of shoots obtained from epicotyl (7), proximal cotyledonary segments (10), distal cotyledonary segments (18), cotyledonary node explants (12) were done on half-strength

A. Shoots appearing from cotyledonary node explants cultured on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS

B. Elongated shoots obtained from cotyledonary node explants on half-strength MS basal medium supplemented with 3 µM GA_3

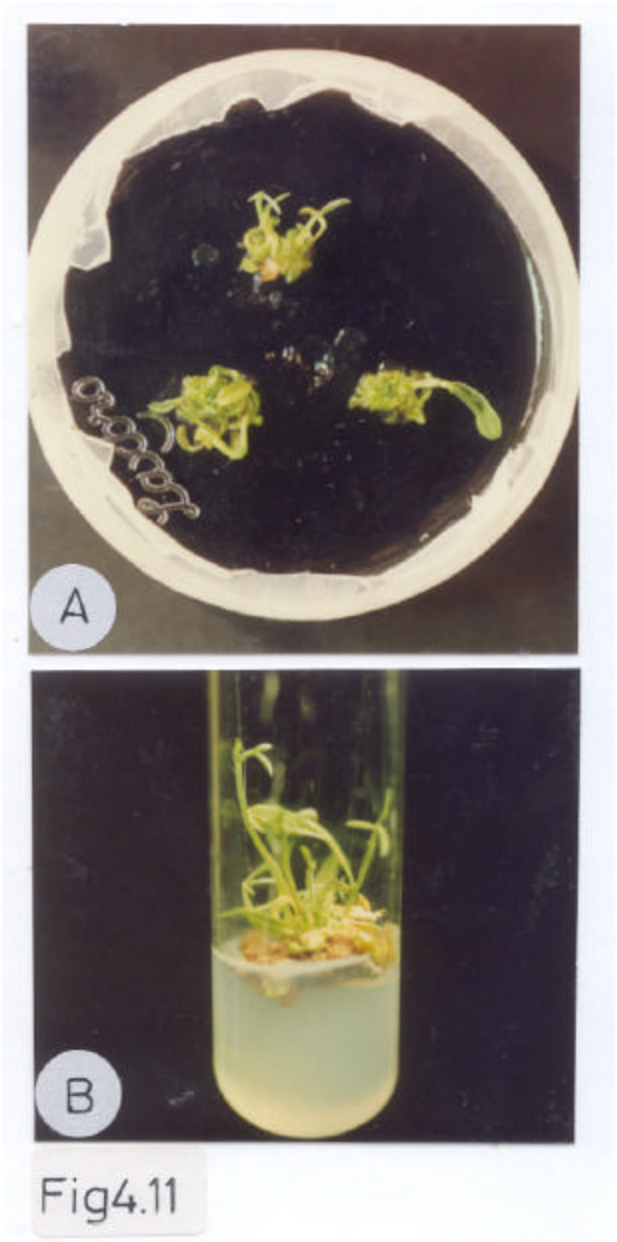


Fig4.11

MS basal medium supplemented with 0.5 µM IBA (**Fig 4.12A**). The frequency of rooting was 55 % and rooting took place in 15-20 days. Over all 26 rooted plantlets could be obtained from different seedling explants. Similar observations on rooting was observed by Naidu *et al*. (1995) and Shiva prakash (1994). Geetha *et al*. (1998) found IBA as best auxin for rooting. Other auxins used for rooting were NAA (Mehta and Mohan Ram 1980; Geetha *et al*. 1998; Eapen *et al*. 1998; Kumar *et al*. 1983; Eapen and George 1993b; George and Eapen 1994) and IAA (Kumar *et al*. 1983; Geetha *et al*. 1998).

The rooted plantlets were transferred to soil:vermiculite mixture (1:1) for hardening at 25±2 °C for 3-4 weeks in pots. The survival of plantlets in pots was 65 % (**Fig 4.12B**). A total of 32 plantlets from all the explants could be hardened in pots.

4.4 Conclusions

In the present studies, various explants of pigeonpea cultivars T-15-15 and Gaut-82-90 were evaluated for their organogenetic potentiality. Better shoot formation was observed from mature embryo axis derived explants like ERMEA and DCMEA on EC_6 basal medium supplemented with BAP (5 µM) and IAA (1 µM). The organogenesis was observed from various seedling derived explants such as leaf, epicotyl, proximal cotyledonary segments, distal cotyledonary segments and cotyledonary node. The shoot buds were induced on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS. The genotype T-15-15 was found to have better organogenetic potentiality when compared to the genotype Gaut-82-90. In all the explants used for study, the elongation of shoot buds into shoots was found to be difficult and elongation of shoots occurred in low percentages. Most of the shoot buds turned into leafy shoots and a very few shoots elongated sporadically. This has resulted in the problem of transferring a large number of plantlets from each explant and evaluating their performance in the field, even though a large number of shoot buds could be obtained on various explants.

Inherent in the development of a simple plant transformation method is the identification of a reliable and easy method for prolific plant regeneration. The method of organogenesis from DCMEA and ERMEA explants described avoids the germination of seeds for culturing of explants. When compared to other explants formation of shoots directly from DCMEA and ERMEA explants was observed and the shoot formation was associated with the cut surface of the explant. This makes the method highly useful for genetic manipulation of pigeonpea through *Agrobacterium*-mediated transformation procedure.

A. Rooting of shoots obtained from epicotyl, proximal cotyledonary segments, distal cotyledonary segments and cotyledonary node explants on half-strength MS basal medium supplemented with 0.5 µM IBA

B. Hardened plants in pots obtained from various mature embryos axis derived and seedling derived explants

Fig 4.12

CHAPTER 5

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION

5.1 Introduction

Somatic embryogenesis is the development of embryos from somatic cells, without the fusion of gametes, which develop into differentiated plants through characteristic developmental patterns not observed in zygotic embryogenesis (Tisserat *et al*. 1979; Williams and Maheswaran 1986; Rangaswamy 1986; Zimmerman 1993; Merkle *et al*. 1995). It is a pathway of *de novo* regeneration from *in vitro* cultured tissue mediated by callus (indirect) or from cells of an organized structure such as stem, leaf, hypocotyl or zygotic embryo (direct) (Williams and Maheswaran 1986). The somatic cells within a plant contain the genetic information necessary to form a complete and functional plant (Merkle *et al.* 1995).

The initiation of somatic embryogenesis occurs with the termination of the existing gene expression pattern in the explant tissue, and its replacement with an embryogenic gene expression program in those cells of the explant, which give rise to somatic embryos (Merkle *et al.* 1995). Embryogenic cells, which form after many divisions in culture and require growth regulators not only for re-entry into mitosis but also for determination of the embryogenic state are termed as IEDC's (induced embryogenic determined cells). Formation of IEDCs happens in case of indirect embryogenesis (Sharp *et al*. 1980; Williams and Maheswaran 1986). By contrast, direct embryogenesis in culture proceeds from cells which are pre-determined for embryogenic development i.e. they are PEDC's (pre-embryogenic determined cells), which require an external stimulus either in the form of growth regulators or favourable conditions conducive for the induction and development of embryos (Sharp *et al*. 1982; Williams and Maheswaran 1986; Carman 1990). After the induction of embryogenic determined cells, there is no difference between indirect and direct somatic embryogenesis (Williams and Maheswaran 1986). The embryogenic cells closely resemble the rapidly dividing meristematic cells of apical meristems, i.e., they are small, isodiametric, rich in cytoplasm, thin walled, with large nuclei and prominent nucleoli and minimally vacuolated (Tisserat *et al.* 1979; Williams and Maheswaran 1986).

The question of single or multiple-cell origin of somatic embryos is directly related to the coordinated behavior of the neighboring cells as a morphogenic group (Williams and Maheswaran 1986). Determined cells may operate singly or in groups to form embryos. In the case of indirect somatic embryogenesis (via callus or suspension culture), the origin of embryos was found to be from a clump of embryogenic cells - the proembryonal mass (PEM), from which one to many embryoids develop (Williams and

Maheswaran 1986). Direct somatic embryogenesis may also be multicellular (Haccius 1978), or may arise from a single superficial cell (Konar and Nataraja 1965) or by both of these pathways (Maheswaran and Williams 1986).

The ability to induce somatic embryogenesis in plants appears to be a universal trait whose occurrence depends on the interaction of an appropriate tissue with an appropriate induction stimulus. Induction of somatic embryogenesis in plants, one of the significant achievements of experimental embryology, has become an experimental tool of biotechnology.

Remarkable progress has been made in the commercialization of somatic embryogenesis since first described by Steward *et al*. (1958) in carrot. The potentials of somatic embryogenesis derive from a number of factors that involve high regenerative capacity, the ability to regenerate from single cells in both gametophyte and sporophyte tissues, the bipolarity of embryos and compactness and broad metabolic potential of embryogenic tissue (Janick 1993).

5.1.1 Uses of somatic embryogenesis

The major uses of somatic embryogenesis may be summarized as follows:

5.1.1.1 Clonal propagation

Somatic embryos offer some potential advantages over conventional micropropagation system like (i) high proliferation rate – as many as 1.35 million embryos per liter suspension culture (Janick 1993) (ii) singulation – each embryo being separate package that can be handled without the physical separation required from organogenesis or axillary branching systems. (iii) bipolarity – the well developed embryos contains root and a shoot meristem, indicating that conversion to seedling can be obtained in single step and (iv) the promise of clonal propagation via synthetic seed technology (Janick 1993).

5.1.1.2 Crop improvement

Somatic embryogenesis can be utilized as a regeneration technique for cell selection of natural or induced mutations. Selection for salt tolerance and disease resistance has proven efficient in embryogenically competent callus tissues of citrus (Litz *et al.* 1985).

5.1.1.3 Embryo rescue

Embryo rescue is a widely used technique to rescue embryos that normally abort in wide crosses and is an established technique to obtain viable plants from incompatible interspecific crosses. Somatic embryogenesis has been exploited as a means of obtaining plants from intergeneric hybrid embryos (McGranahan *et al*. 1988; Ozias-Akins *et al*. 1992b).

5.1.1.4 Transformation

More number of regenerants can be obtained originating from few or single cells, which increases the likelihood of achieving transformed cells. Chimeric transformants cycled in repetitive systems can lead to obtaining wholly transformed individuals (Baker and Wetzstein 1992).

5.1.1.5 Metabolite production

Cell/organ cultures have been suggested as a means to synthesize desirable metabolites (Al-Abta *et al*. 1979). Lipid synthesis has been followed in embryo cultures in a number of species including cocoa, jojoba, borage, rape seed, carrot (Janick 1991; Weber *et al*. 1992) and peanut (Mhaske and Hazra 1994).

5.1.1.6 Disease elimination

Because of the absence of vascular connections between the nucellus and other maternal tissues, poly embryonic species are generally free of infections that might have affected the parent plant. Similarly, plants derived via somatic embryogenesis from nucellus or nucellar callus would also be free of pathogens including viruses (Janick 1993).

5.1.1.7 Germplasm preservation

In some species like cocoa, coconut, mango and rubber, seeds are traditionally used for preservation of germplasm. Seeds of these species are desiccation sensitive and thus cannot be stored by conventional techniques. This can be overcome by cryopreservation of mature or immature zygotic or somatic embryos (Janick 1993).

5.1.1.8 Basic studies

Somtic embryogenesis also provide an important tool for the analysis of molecular and biochemical events that occur during induction and maturation.

5.1.2 Somatic embryogenesis in pigeonpea: Current status

George and Eapen (1994) obtained somatic embryos from immature embryonal axes and immature cotyledons on L_6 medium fortified with different auxins. Very few somatic embryos were obtained but plantlets capable of transfer to the field could not be obtained from the somatic embryos.

Patel *et al.* (1994) reported somatic embryogenesis from distal halves of cotyledons on various basal media supplemented with 3 cytokinins BAP, kinetin and AdS. However, transfer of plantlets to soil and studies to demonstrate the initiation and development of somatic embryos were not reported.

Somatic embryogenesis has also been reported from cotyledons on MS basal medium containing NAA at high concentration (50 mg/l) and BAP. Inclusion of BAP in

the medium resulted in the formation of well-developed embryo-like structures (Nalini Mallikarjuna *et al.* 1996). Transfer of plantlets to the field could not be achieved.

Sreenivasu *et al*. (1998) obtained indirect somatic embryogenesis via callus using high concentration of TDZ from cotyledons and leaf explants on MS basal medium. The presence of various developmental stages and histological observations were not reported to show different stages of somatic embryo development. The embryogenesis was obtained via callus which might lead to formation of somaclonal variants and are not suitable for genetic transformation.

Somatic embryos were obtained from suspension cultures derived from leaf callus (Anbazhagan and Ganapathi 1999). No histological observations were made to demonstrate the various developmental stages of somatic embryos and transfer of plantlets to field was not achieved.

Even though there are many reports of somatic embryogenesis in pigeonpea, highly reproducible complete plant regeneration protocols are very few (Sreenivasu *et al*. 1998; Anbhazagan and Ganapathi 1999). However, the data on the number of plants transferred to pots or field is not available in any of the above reports. Therefore, it is difficult to assess the efficiency of these protocols for use in genetic transformation studies. Moreover, when the present work was initiated no reports of somatic embryogenesis in pigeonpea were available in the literature. It is for these reasons attempts were made to induce somatic embryogenesis from distal cotyledonary segments and to regenerate normal healthy plants of pigeonpea in this study. The present chapter describes the results of the studies undertaken with the genotypes T-15-15 and Gaut-82-90. These genotypes were chosen for studies on somatic embryogenesis as regeneration potentiality via organogenesis was observed only in these two genotypes out of 14 genotypes used in our study using distal cotyledonary segments (Chapter 3).

5.2 Materials and Methods

5.2.1 Plant material

Seeds of pigeonpea genotypes T-15-15 and Gaut-82-90 were surface sterilized as described in chapter 2, section 2.6.1. The surface sterilized seeds (60 seeds/flask) were soaked in sterile distilled water for 18 h in the dark at 28 ± 2 °C and kept on gyratory shaker at 200 rpm.

5.2.2 Explant preparation

Cotyledons were removed from the pre-soaked seeds, split into halves and the proximal meristematic ends were removed. Only the distal halves (3.5-4.0 mm^2) without any pre-existing axillary buds were used as explants.

5.2.3 Induction of somatic embryogenesis

The effect of various auxins and cytokinins on induction of somatic embryogenesis from distal cotyledonary segments was evaluated. EC_6 basal medium with 3 % Sucrose and jelled with 0.8% agar-agar was used for induction of somatic embryos. The pH of the medium was adjusted to 5.8 before autoclaving. All the cultures were incubated at 25±2 °C under cool white fluorescent lights under a 16 h photoperiod.

5.2.3.1 Effect of growth regulators on induction of somatic embryogenesis

In a preliminary experiment, different phytohormones were tested to assess the morphogenetic response of the explant. Distal cotyledonary segments were cultured in test tubes (2 explants/tube) containing 20 ml of EC_6 basal medium supplemented with 2,4-D (5 - 100 µM), 2,4,5-T (5 - 100 µM), NAA (5 - 100 µM), Picloram (5 - 100 µM), Dicamba (5 - 100 µM), BAP (1 - 50 µM), TDZ (1 - 5 µM) and combination of 2,4-D (5 - 20 µM) + 2,4,5-T (5 - 20 µM), 2,4-D (5 - 20 µM) + NAA (5 – 20 µM), 2,4,5-T (5 – 20 µM) + NAA (5 – 20 µM). In addition, combinations of BAP (1 and 5 µM) and 2,4-D (5 - 100 µM), 2,4,5-T (5 - 100 µM), NAA (5 - 100 µM) were also tested. A total number of 20 explants were used per treatment and the experiment was repeated thrice. The cultures were incubated for 6 weeks under the conditions mentioned as above. At 6 weeks, the nature of explant's response in terms of globular embryos formed was recorded.

5.2.4 Development of cotyledonary structures

The explants along with globular embryos obtained on 5 µM BAP were transferred to: (a) hormone-free EC_6 and MS basal medium (b) EC_6 basal medium supplemented with various growth regulators such as BAP (1 and 5 µM), ABA (0.5 – 2 µM), GA_3 (1 - 5 µM) (c) MS basal medium with various hormones like BAP (1 and 5 µM), ABA (0.5 – 2 µM), GA_3 (1 - 5 µM) for further development of globular embryos into cotyledonary stage embryos. The explants along with globular embryos obtained on 10 µM TDZ were transferred to: (a) hormone-free EC_6 and MS basal medium (b) EC_6 basal medium supplemented with various growth regulators such as TDZ (1 and 10 µM), ABA (0.5 – 2 µM), GA_3 (1 - 5 µM) (c) MS basal medium with various hormones like TDZ (1 and 5 µM), ABA (0.5 – 2 µM), GA_3 (1 - 5 µM) for further development of globular embryos

into cotyledonary stage embryos. The cultures were incubated under the conditions as described above for a period of 8 weeks. After 8 weeks, the formation of cotyledonary stage embryos were recorded.

5.2.5 Maturation of cotyledonary stage of embryos

The cotyledonary stage somatic embryos obtained on MS basal medium supplemented with 3 µM GA_3 were separated from the explant and placed on half-strength MS basal medium with 0.8 % agar-agar, 3 % sucrose and 0.5 µM ABA in test tubes (1 cotyledonary embryo/tube) for maturation. The cultures were incubated at 25 ± 2 °C under cool white fluorescent lights with 16 h photoperiod for a period of one week.

5.2.6 Germination and conversion of mature somatic embryos

The mature somatic embryos were shifted to phytohormone-free half-strength MS basal medium containing 0.2 % phytagel and 3 % sucrose or to half-strength MS basal medium in 250 ml flasks (80 ml medium per flask) containing 0.5 and 1 µM BAP for germination and conversion. The cultures were incubated under the conditions described as above for 15 days.

5.2.7 Development of plantlets

Fully converted embryos with well-defined root and shoot were transferred to hormone-free half-strength MS basal medium in 250 ml flasks (80 ml medium per flask) containing 3% sucrose and 0.2% phytagel for further elongation of roots and shoots. The cultures were incubated under the conditions described as above for 15 days.

5.2.8 Transfer of emblings to soil

Plantlets were hardened as described in chapter 2 section 2.11. The plants were hardened for 3-4 weeks in the hardening room under diffused light conditions at 25±2 °C.

5.2.9 Statistical analysis

The data was analyzed using Analysis of Variance technique for completely randomized design and the treatment means were compared.

5.2.10 Histology

For histological confirmation of the origin and structure of somatic embryos, the explants were fixed in FAA for 72 h at various stages of development of somatic embryos. Tissues were dehydrated through t-butanol series. Paraffin embedding of tissue samples was done and sections of 10 µM thickness were cut, stained with hematoxylin-eosin and mounted with DPX mountant and observed microscopically (detailed description in chapter 2, section 2.10).

5.2.11 Parameter studies

Various parameters in the genotype Gaut-82-90 were evaluated to enhance the frequency of induction of somatic embryos. The genotype Gaut-82-90 was used for parameter studies since this genotype was better than T-15-15 in percentage induction of somatic embryos and in the number of globular embryos formed per explant and in the further development of globular somatic embryos.

5.2.11.1 Effect of different basal media

Six basal media such as EC_6, MS, B5, Modified B5, LS and White's supplemented with 3 % sucrose, 0.8 % agar-agar and 5 µM BAP were used to test the induction of somatic embryogenesis from distal cotyledonary segments in genotype Gaut-82-90. The cultures were incubated for 6 weeks at 25 ± 2 °C under light intensity of 38 $\mu Eiin.m^{-2}.s^{-1}$ with a 16 h photoperiod provided by cool white fluorescent lights. Induction of somatic embryos was recorded after 6 weeks.

5.2.11.2 Effect of explant source

The somatic embryogenesis potentiality of the explants such as mature embryo axes, mature cotyledons, distal cotyledonary segments and leaf of genotype Gaut-82-90 was tested. Seeds of pigeonpea genotype Gaut-82-90 were surface sterilized as described in chapter 2, section 2.6.1. The surface sterilized seeds were soaked in sterile distilled water for 18 h in the dark at 28 ± 2 °C and kept on gyratory shaker at 200 rpm.

The seed coat from the pre-soaked seeds was removed, cotyledons were split open, the embryo axes were extracted and used as mature embryo axes explants. The resulting cotyledons were cultured as mature cotyledons. The distal cotyledonary segments were prepared as described in section 5.2.2. The leaf explants were obtained from 10 day old seedlings (procedure for germination of seeds is described in chapter 2, section 2.6.2). Leaf pieces (2-3 mm^2) were taken from primary leaves of seedlings. The leaves were separated from the seedling and the explants were prepared by discarding the petiole and the leaf apex. The leaf was cut along the midrib and the portion with midrib was made into pieces of 2-3 mm^2 and cultured.

The explants were cultured on EC_6 basal medium supplemented with 5 µM BAP. The cultures were incubated under the conditions mentioned as above for 6 weeks. Induction of somatic embryos was recorded after 6 weeks.

5.2.11.3 Effect of carbohydrate source

Five carbohydrate sources like sucrose, glucose, maltose, glycerol and fructose were used to evaluate the somatic embryogenesis in distal cotyledonary segments of genotype Gaut-82-90. The cultures were incubated under the conditions mentioned as above for 6 weeks. Induction of somatic embryos was recorded after 6 weeks.

5.2.11.4 Effect dark v/s light incubation

The distal cotyledonary segments of genotype Gaut-82-90 were inoculated on EC_6 basal medium supplemented with 5 µM BAP. The cultures were incubated under different culture environments like 16 h photoperiod light, 24 h phtoperiod light and under dark conditions for 6 weeks. Induction of somatic embryos was recorded after 6 weeks.

5.2.11.5 Effect of agitation in liquid induction medium

The distal cotyledonary segments of genotype Gaut-82-90 were inoculated in 250 ml flasks containing 40 ml liquid EC_6 basal medium supplemented with 5 µM BAP (liquid induction medium) and the flasks were shaken at 200 rpm for 1 week under dark conditions. The cotyledonary segments were then inoculated on hormone-free EC_6 basal medium with 3 % sucrose and 0.8 agar-agar or on EC_6 basal medium supplemented with various concentration of BAP (0.5 – 2.0 µM) in test tubes (with 20 ml medium). The distal cotyledonary segments which were not agitated in liquid induction medium and cultured on EC_6 basal medium with 3 % sucrose, 0.8 % agar-agar and 5 µM BAP served as control. The cultures were incubated for 6 weeks at 25 ± 2 °C under light intensity of 38 $\mu E.m^{-2}.s^{-1}$ with a 16 h photoperiod provided by cool white fluorescent lights. Induction of somatic embryos was recorded after 6 weeks.

5.3 Results and Discussion

5.3.1 Effect of various phytohormones on induction of somatic embryognesis from distal cotyledonary segments

Reports on plant regeneration in pigeonpea via somatic embryogenesis were not available when the present work was initiated. Since our observations (Chapter 3) revealed that EC_6 basal medium was useful in obtaining higher percentage of regeneration of shoot buds from distal cotyledonary segments, EC_6 basal medium was used initially for evaluation of embryogenic potential of distal cotyledonary segments of genotypes Gaut-82-90 and T-15-15. Out of all combinations tested (**Table 5.1**) for induction of somatic embryos, induction of globular embryos was observed after 6 weeks of culture on EC_6 basal medium supplemented with BAP (1, 5, 10 and 20) or TDZ (1, 2, 3, 4 and 5 µM).

All other concentrations and combinations of auxins and combinations of auxins and BAP resulted in the formation of loose friable callus (**Table 5.1**).

Somatic embryogenesis has been induced from leaf and cotyledon explants of pigeonpea using TDZ (Sreenivasu *et al*. 1998), which is similar to our studies. Auxins were used for induction of somatic embryogenesis from leaf suspension culture (Anbazhagan and Ganapathi (1999) and immature cotyledons and immature embryonal axes (George and Eapen 1994), In contrast, our experiments with different auxins, produced only loose friable callus. Nailini Mallikarjuna *et al*. (1994) reported somatic embryogenesis on a medium containing NAA and BAP. In our experiments, combinations of different auxins and BAP resulted in formation of only friable callus. Patel *et al*. (1994) suggested a combination of 3 cytokinins (BAP, kin and AdS) for induction of somatic embryogenesis in pigeonpea, however, we found addition of only BAP or TDZ is sufficient to induce somatic embryogenesis from distal cotyledonary segments.

Table 5.1 Effect of EC_6 basal medium supplemented with various growth regulator concentrations and combinations on induction of somatic embryogenesis from distal cotyledonary segments

Growth Regulators (■M)	T-15-15	Gaut-82-90
2,4-D (5)	C	C
2,4-D (10)	C	C
2,4-D (20)	C	C
2,4-D (50)	C	C
2,4-D (100)	C	C
2,4,5-T (5)	C	C
2,4,5-T (10)	C	C
2,4,5-T(20)	C	C
2,4,5-T(50)	C	C
2,4,5-T(100)	C	C
NAA (5)	C	C
NAA (10)	C	C
NAA (20)	C	C
NAA(50)	C	C
NAA (100)	C	C
Picloram (5)	C	C
Picloram (10)	C	C
Picloram (20)	C	C
Picloram (50)	C	C
Picloram (100)	C	C
Dicamba (5)	C	C
Dicamba (10)	C	C
Dicamba (20)	C	C
Dicamba (50)	C	C
Dicamba (100)	C	C
2,4-D (5) + 2,4,5 -T (5)	C	C
2,4-D (10) + 2,4,5-T (10)	C	C
2,4-D (20) + 2,4,5-T (20)	C	C
2,4-D (5) + NAA (5)	C	C
2,4-D (10) + NAA (10)	C	C
2,4-D (20) + NAA (20)	C	C
2,4,5-T (5) + NAA (5)	C	C
2,4,5-T (10) + NAA (10)	C	C
2,4,5-T (20) + NAA (20)	C	C

C – callus

Table 5.1 (Contd.)

Growth Regulators (mM)		T-15-15	Gaut-82-90
Auxin	**Cytokinin**		
2,4-D(5)	BAP(1)	C	C
2,4-D(10)	BAP(1)	C	C
2,4-D(20)	BAP(1)	C	C
2,4-D(50)	BAP(1)	C	C
2,4-D(100)	BAP(1)	C	C
2,4-D(5)	BAP(5)	C	C
2,4-D(10)	BAP(5)	C	C
2,4-D(20)	BAP(5)	C	C
2,4-D(50)	BAP(5)	C	C
2,4-D(100)	BAP(5)	C	C
2,4,5-T(5)	BAP(1)	C	C
2,4,5-T(10)	BAP(1)	C	C
2,4,5-T(20)	BAP(1)	C	C
2,4,5-T(50)	BAP(1)	C	C
2,4,5-T(100)	BAP(1)	C	C
2,4,5-T(5)	BAP(5)	C	C
2,4,5-T(10)	BAP(5)	C	C
2,4,5-T(20)	BAP(5)	C	C
2,4,5-T(50)	BAP(5)	C	C
2,4,5-T(100)	BAP(5)	C	C
NAA(5)	BAP(1)	C	C
NAA(10)	BAP(1)	C	C
NAA(20)	BAP(1)	C	C
NAA(50)	BAP(1)	C	C
NAA(100)	BAP(1)	C	C
NAA(5)	BAP(5)	C	C
NAA(10)	BAP(5)	C	C
NAA(20)	BAP(5)	C	C
NAA(50)	BAP(5)	C	C
NAA(100)	BAP(5)	C	C
-	BAP (1)	SE	SE
-	BAP (5)	SE	SE
-	BAP (10)	SE	SE
-	BAP (20)	SE	SE
-	BAP (50)	NR	NR
-	TDZ (1)	SE	SE
-	TDZ(2)	SE	SE
-	TDZ(3)	SE	SE
-	TDZ(4)	SE	SE
-	TDZ(5)	SE	SE

C – callus, NR – No response (Explants turned necrotic), SE – Somatic embryos

The distal cotyledonary segments of the genotypes T-15-15 and Gaut-82-90 cultured on EC_6 basal medium fortified with various concentrations of BAP and TDZ produced a large number of globular embryos (**Fig 5.1A, Fig 5.1B and Fig 5.1C**) arising directly on the surface of the cotyledons. BAP at all concentrations except at 50 µM supported formation of globular embryos and the explants turned necrotic after 6 weeks of culture at 50 µM concentration of BAP (**Table 5.1**). The percentage induction of somatic embryogenesis on various concentrations of BAP (**Table 5.2**) varied from

A. Cotyledonary segment showing the formation of globular embryos after 6 weeks of culture on EC_6 basal medium supplemented with BAP or TDZ (bar = 1000 μm)

B. Globular embryos appearing on cotyledonary segments cultured on EC_6 basal medium supplemented with BAP or TDZ for 6 weeks (bar = 750 μm)

C. A large number of globular embryos formed on cotyledonary segments cultured on EC_6 basal medium supplemented with BAP or TDZ for 6 weeks (bar = 550 μm)

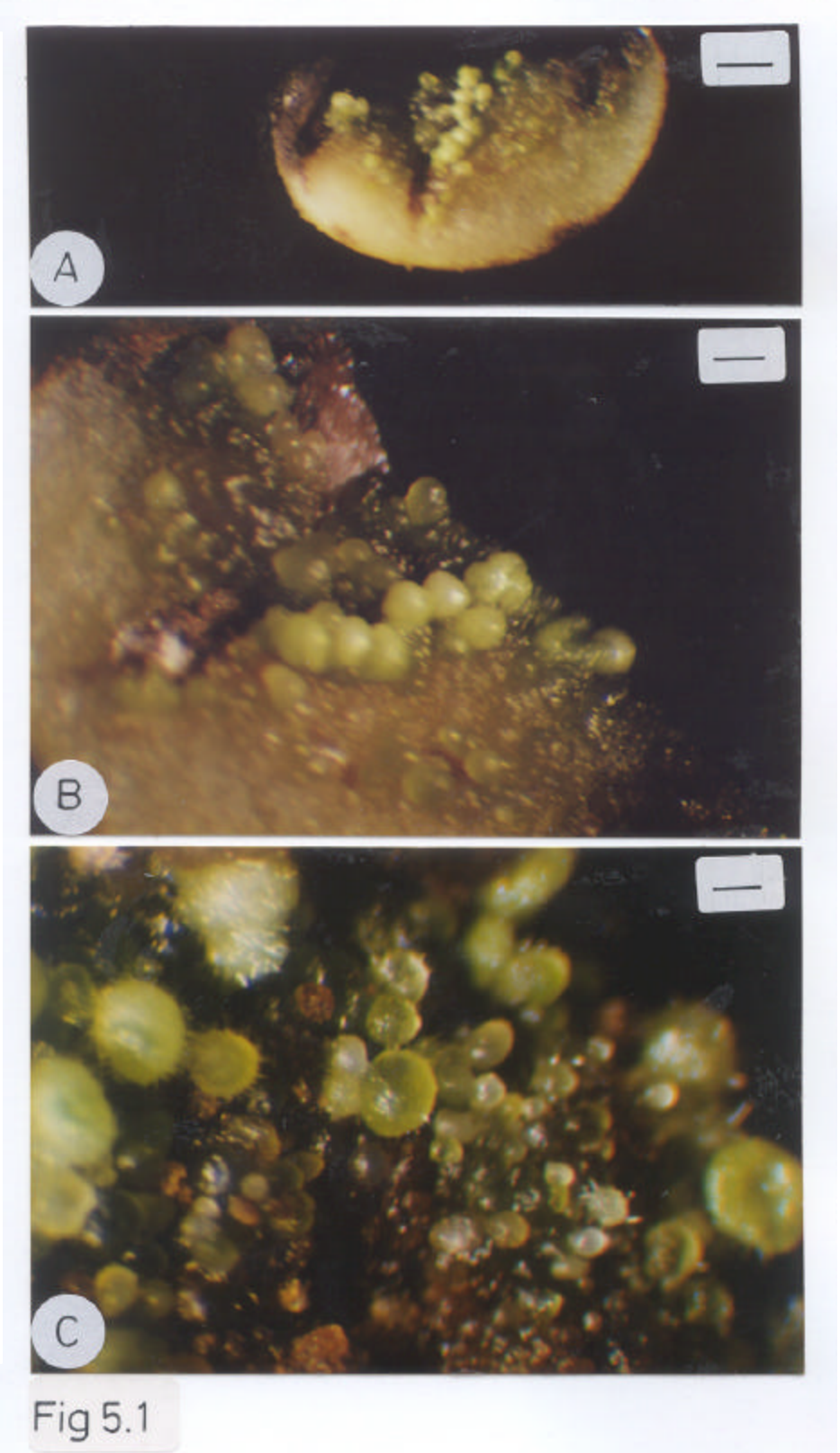


Fig 5.1

53-76 % in the genotype Gaut-82-90 and 62-80 % in the genotype T-15-15 with 5 µM BAP recording highest percentage of induction of globular embryos in both the genotypes. The average number of globular embryos per explant ranged from 13.9 to 27.6 and 10.8 to 16.7 in genotypes Gaut-82-90 and T-15-15 respectively with the cotyledonary segments producing maximum number of globular embryos on 5 µM BAP in both the genotypes. The induction percentage of somatic embryognesis was higher in the genotype T-15-15 whereas, the average number of globular embryos per explant was significantly higher in the genotype Gaut-82-90. (**Table 5.2**).

Table 5.2 Somatic embryo induction in cotyledonary segments on EC_6 basal medium supplemented with various levels of BAP

	GAUT-82-90		T-15-15	
BAP (µM)	**Percentage Induction (mean±se)**	**No. of globular Embryos per explant (mean±se)**	**Percentage Induction (mean±se)**	**No. of globular Embryos per explant (mean±se)**
1	53±3[a]	14.8±1.1[a]	62±5[a]	11.1±0.7[a]
5	76±2[b]	27.6±2.2[b]	80±1[b]	16.7±0.6[b]
10	70±3[b]	23.9±1.3[b]	64±2[a]	10.8±0.4[a]
20	57±2[a]	13.9±0.5[a]	69±2[a]	10.9±0.4[a]

Figures with different alphabets in superscript differ significantly at 0.05 probability.

The percentage induction of somatic embryogenesis from cotyledonary segments on various concentrations of TDZ ranged from 74 to 97 % in the genotype Gaut-82-90 and 75 to 91 % in the genotype T-15-15. The percentage response was highest at 10 µM TDZ in both the genotypes. The genotype Gaut-82-90 produced 19.2 to 38.4 globular embryos per explant while T-15-15 genotype recorded 13.0 to 20.9 globular embryos (**Table 5.3**). The number of globular embryos produced per explant was highest at 10 µM TDZ in the genotype Gaut-82-90 and at 5 µM TDZ in the genotype T-15-15. However, there was no significant difference between 5 µM and 10 µM TDZ concentrations in terms of number of globular embryos produced per explant in the genotype T-15-15. The genotype Gaut-82-90 was better when compared to genotype T-15-15 both in terms of percentage induction of somatic embryos and in the number of globular embryos produced per explant (**Table 5.3**).

In general, higher percentage of somatic embryogenesis and the number of globular embryos per explant were observed on 10 µM TDZ than 5 µM BAP in the genotype Gaut-82-90. In genotype T-15-15 higher percentage of somatic embryogenesis was on 10 µM TDZ when compared to 5 µM BAP. However, there was no significant

difference between TDZ (10 µM) and BAP (5 µM) in terms of the number of globular embryos formed per explant in genotype T-15-15 (**Table 5.2 v/s Table 5.3**).

From the above study it can be concluded that 5 µM concentration of BAP and 10 µM concentration of TDZ are the most potential growth regulators for induction of somatic embryogenesis from distal cotyledonary segments in genotypes Gaut-82-90 and T-15-15. Therefore, further experiments were carried out using only these concentrations of BAP (5 µM) and TDZ (10 µM).

Table 5.3 Somatic embryo induction in cotyledonary segments on EC_6 basal medium supplemented with various levels of TDZ

	GAUT-82-90		T-15-15	
TDZ (µM)	**Percentage Induction (mean±se)**	**No. of globular Embryos/explant (mean±se)**	**Percentage Induction (mean±se)**	**No. of globular Embryos/explant (mean±se)**
0.5	64±9^{a}	27.8±3.4^{ab}	84±4^{ab}	13.0±2.4^{ab}
1	88±3^{bc}	29.7±2.8^{bc}	75±5^{a}	10.4±1.3^{a}
5	74±2^{ab}	28.6±3.3^{ab}	80±4^{ab}	20.9±2.3^{c}
10	97±3^{c}	38.4±4.9^{c}	91±6^{b}	17.8±1.1^{bc}
20	75±5^{ab}	19.2±1.7^{a}	90±1^{b}	14.0±1.5^{a}

Figures with different alphabets in superscript differ significantly at 0.05 probability.

Cytokinin induced embryogenesis is rare, but has been achieved in *Trifolium* (Maheshwaran and Williams 1986), peanut (Gill and Saxena, 1992), *Phaseolus* sp. (Malik and Saxena 1992b), pigeonpea (Patel *et al*. 1994) and chickpea (Murthy *et al*. 1996). In those cases where both auxins and cytokinins were used together, the presence of cytokinin either had no effect or had a detrimental effect on the induction of embryogenesis (Parrott *et al*. 1992). However, Mallikarjuna *et al.* (1996) observed somatic embryogenesis only on addition of cytokinin to the auxin supplemented medium in pigeonpea.

Variation in response of induction of somatic embryos observed between different cultivars was similar to the earlier observations in soybean (Barwale *et al.* 1986a; Komatsuda and Ohyama 1988; Parrot *et al*. 1989; Bailey *et al*. 1993a) and peanut (Sellars *et al*. 1990; Ozias-Akins *et al*. 1992a; George and Eapen 1993; Baker *et al*. 1995; McKently 1995; Chengalrayan *et al.* 1998).

Patel *et al.* (1994) reported induction of somatic embryogenesis in cotyledons of pigeonpea when cultured on media supplemented with BAP (22.2 µM), Kin (2.3 µM) and

AdS (271.0 μM). In our experiments, however, induction of somatic embryogenesis was observed when the medium was supplemented with either BAP or TDZ alone.

The somatic embryo formation was observed on calli derived from cotyledon and leaf tissue of pigeonpea by Sreenivasu *et al*. (1998) or suspension cultures of leaf derived callus (Anbazhagan and Ganapathi 1999). However, in the present studies direct appearance of globular embryos on cotyledonary segments was observed without any callus formation.

George and Eapen (1994) could produce normal embryos from immature cotyledons and immature embryonal axes as explants on auxin supplemented medium, but failed to get plantlets. Nalini Mallikarjuna et al. (1996) also reported somatic embryogenesis on a medium containing NAA and BAP. In contrast, only BAP or TDZ supplementation was sufficient to induce somatic embryos in our experiments.

5.3.2 Development of embryos from globular stage to cotyledonary stage

The globular embryos did not develop further when cotyledonary segments with the globular embryos obtained on EC_6 basal medium supplemented with TDZ (10 μM) were transferred to hormone-free EC_6 or MS basal media. The further development of globular embryos also did not occur when the explants with globular embryos obtained on EC_6 basal medium supplemented with TDZ (10 μM) were transferred to (a) the same medium, (b) EC_6 basal medium supplemeted with reduced concentration of TDZ (1 μM) or ABA (0.5, 1 and 2 μM) or GA_3 (1, 2, 3, 4 and 5 μM) and (c) MS basal medium supplemented with reduced concentration of TDZ (1 μM) or ABA (0.5, 1 and 2 μM) or GA_3 (1, 2, 3, 4 and 5 μM). The observation that TDZ may not be ideal for further development of globular embryos was also reported earlier by Visser-Tenyenhuis *et al*. (1994) in geranium and TDZ is lethal to somatic embryo development. In contrast Sreenivasu *et al*. (1998) observed the development of pigeonpea somatic embryos induced on TDZ from leaf explants via callus.

The formation of heart and cotyledonary embryos was not observed when globular embryos induced from distal cotyledonary segments on EC_6 basal medium supplemented with BAP (5 μM) were transferred to hormone-free EC_6 or MS basal media. The further development of globular embryos also did not occur when the explants with globular embryos obtained on EC_6 basal medium supplemented with BAP (5 μM) were transferred to (a) the same medium, (b) EC_6 basal medium supplemented with reduced concentration of BAP (1 μM) or ABA (0.5, 1 and 2 μM) or GA_3 (1, 2, 3, 4 and 5

μM) and (c) MS basal medium supplemented with reduced concentration of BAP (1 μM) or ABA (0.5, 1 and 2 μM). However, the development of globular embryos into heart-shaped (**Fig 5.2A**) and cotyledonary embryos (**Fig 5.2B, Fig 5.2C and Fig 5.3A**) was observed when the explants with globular embryos obtained on EC_6 basal medium supplemented with 5 μM BAP were transferred to MS basal medium containing various levels of GA_3 (1, 2, 3, 4 and 5 μM).

The medium with 3 μM GA_3 produced the highest number of cotyledonary embryos when compared to other concentrations of GA_3 (**Table 5.4**). The formation of cotyledonary embryos was observed in 15 % cultures of genotype Gaut-82-90 and 10 % cultures of genotype T-15-15 when the cotyledons producing globular embryos were transferred to MS basal medium supplemented with 3 μM of GA_3. On an average 2.5 and 2.6 cotyledonary embryos per explant were observed in genotypes Gaut-82-90 and T-15-15 respectively. The effect of GA_3 on development of somatic embryos has been very well demonstrated in papaya (Chen *et al*. 1987), tepary bean (Kumar *et al*. 1988b), Black mustard (Vibha *et al.* 1990) and spinach (Komai *et al.* 1996).

Table 5.4 Cotyledonary embryo formation on MS basal medium supplemented with various levels of GA_3

	GAUT-82-90		T-15-15	
GA_3 (μM)	**Percentage Induction (mean±se)**	**No. of cotyledonary Embryos/explant (mean±se)**	**Percentage Induction (mean±se)**	**No. of cotyledonary Embryos/explant (mean±se)**
1	00±0[a]	0.0±0.0[a]	00±0[a]	0.0±0.0[a]
2	10±5[c]	1.0±0.4[b]	05±1[b]	1.00±0.6[b]
3	15±4[d]	2.5±0.6[c]	10±3[c]	2.6±0.5[c]
4	05±2[b]	1.0±0.3[b]	05±1[b]	1.7±0.3[b]
5	05±1[b]	1.3±0.3[b]	00±0[a]	0.0±0.0[a]

Figures with different alphabets in superscript differ significantly at 0.05 probability.

From the above pilot experiment, it could be concluded that the formation of cotyledonary embryos can be achieved when the explants with globular embryos derived from EC_6 basal medium supplemented with 5 μM BAP were transferred to MS basal medium supplemented GA_3 and 3 μM concentration of GA_3 is the optimum concentration for such a response. It is also observed that genotype Gaut-82-90 is a better genotype than T-15-15 in terms of the number of globular embryos formed per explant (**Table 5.2**) and in terms of percentage of cotyledonary embryos produced (**Table 5.4**). Therefore, further experiments for improving the percentage formation of globular embryos by various

A. Heart shaped somatic embryo formed on cotyledonary segment cultured on MS basal medium supplemented with GA_3 (bar = 500 µm)

B. Cotyledonary stage somatic embryos formed on cotyledonary segment cultured on MS basal medium supplemented with GA_3 (bar = 1000 µm)

C. A single isolated cotyledonary stage somatic embryo (bar = 425 µm)

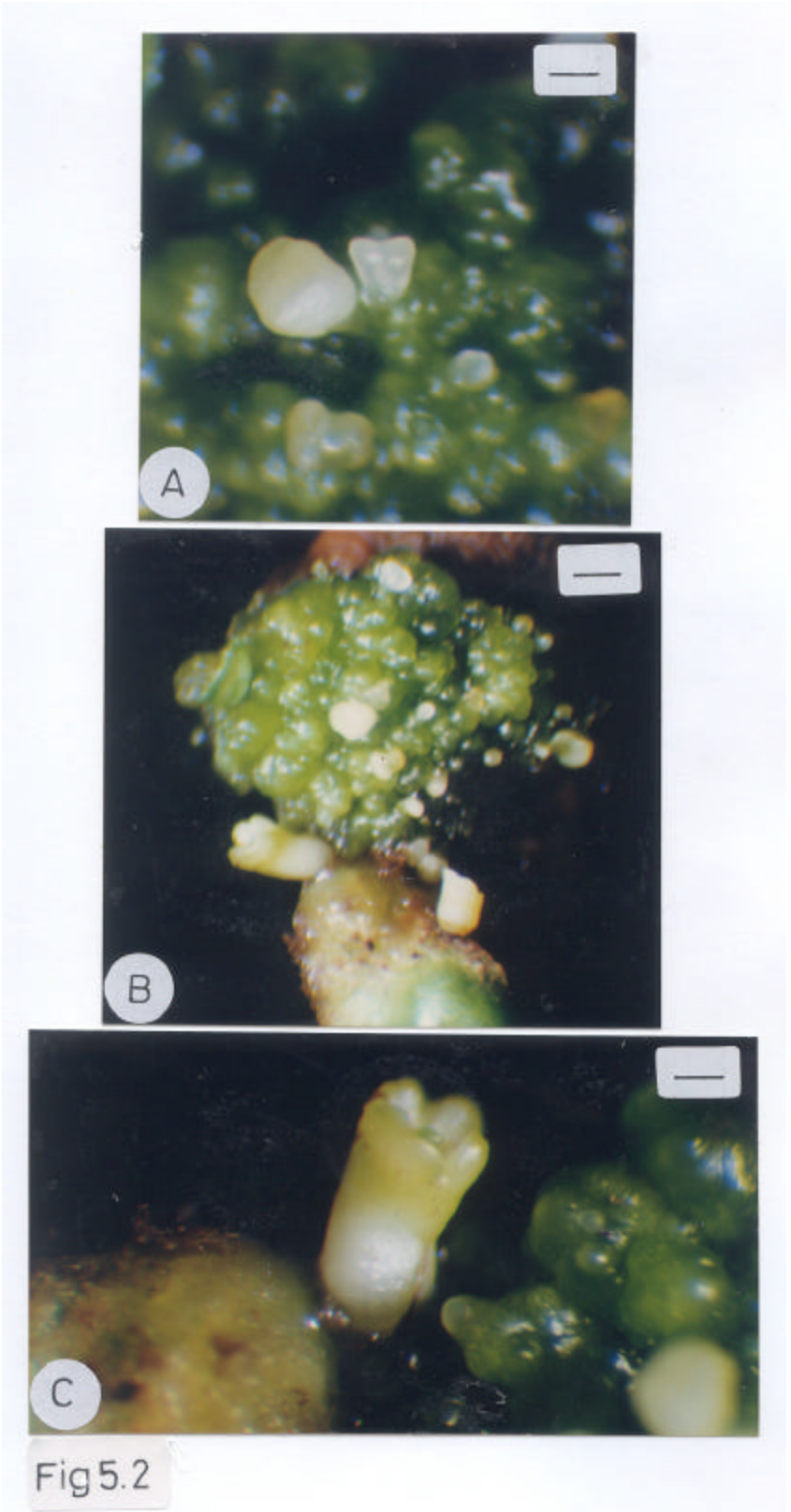


Fig 5.2

parameters (section 5.3.7) were carried out with Gaut-82-90 genotype only and EC_6 basal medium supplemented with BAP (5 µM) for induction of somatic embryos.

When a total of 560 cotyledonary segments were cultured on EC_6 basal medium supplemented with 5 µM BAP, 425 explants produced globular embryos. These globular embryos in 63 explants formed 158 cotyledonary stage embryos, when the explants with globular embryos were transferred to MS basal medium supplemented with 3 µM GA_3. A large number of cotyledonary embryos formed were morphologically abnormal (63 %) (out of 158 embryos obtained 100 embryos were found to be morphologically abnormal). Abnormal embryos were characterized by either absence of shoot or root primordia, uneven growth of cotyledons. These abnormal embryos failed to develop further. The normal cotyledonary stage embryos were selected based on the presence of shoot and root primordia and even growth of cotyledons. Fifty eight normal cotyledonary embryos were obtained. The morphological variations of cotyledonary stage embyos include horn shaped embryos (**Fig 5.3B**), bell shaped embryos (**Fig 5.3C**), embryos with single cotyledon (**Fig 5.4A**), dicotyledonary embryos (**Fig 5.4B**), tricotyledonary embryos (**Fig 5.4C**) and cone shaped embryos (**Fig 5.5A**). This observation was similar to the earlier reports in peanut (Hazra *et al*. 1989; Ozias-Akins 1989), soybean (Hartweck *et al*. 1988; Lazzeri *et al.* 1987a; Buchheim *et al.* 1989) and chickpea (Suhasini *et al.* 1996).

5.3.3 Maturation of somatic embryos

Maturation of somatic embryos is marked by expansion of cells and accumulation of storage reserves (Raghavan 1986). The term 'maturation' used here denotes the development of cotyledonary stage somatic embryos into distinct bipolar structures with well defined shoot and root pole and expanded cotyledons. The normal cotyledonary stage somatic embryos derived on MS basal medium supplemented with 3 µM GA_3 produced mature somatic embryos (**Fig 5.5B**) when placed on half-strength MS basal medium supplemented with ABA (0.5 µM) after one week. Mature somatic embryos had well-developed root and shoot pole. The maturation percentage of normal cotyledonary embryos was 30 %. Out of 58 cotyledonary embryos transferred, 31 embryos matured while the remaining embryos formed callus or turned necrotic. The effect of ABA on maturation of somatic embryos in pigeonpea is similar to earlier report in chickpea (Suhasini *et al.* 1994).

ABA prevents precocious germination of the cotyledonary stage somatic embryos. Precocious or premature development, particularly germination is a major problem

A. A single cotyledonary stage somatic embryo (bar = 535 µm)

B. Horn shaped cotyledonary stage somatic embryo (bar = 460 µm)

C. Bell shaped cotyledonary stage somatic embryo (bar = 550 µm)

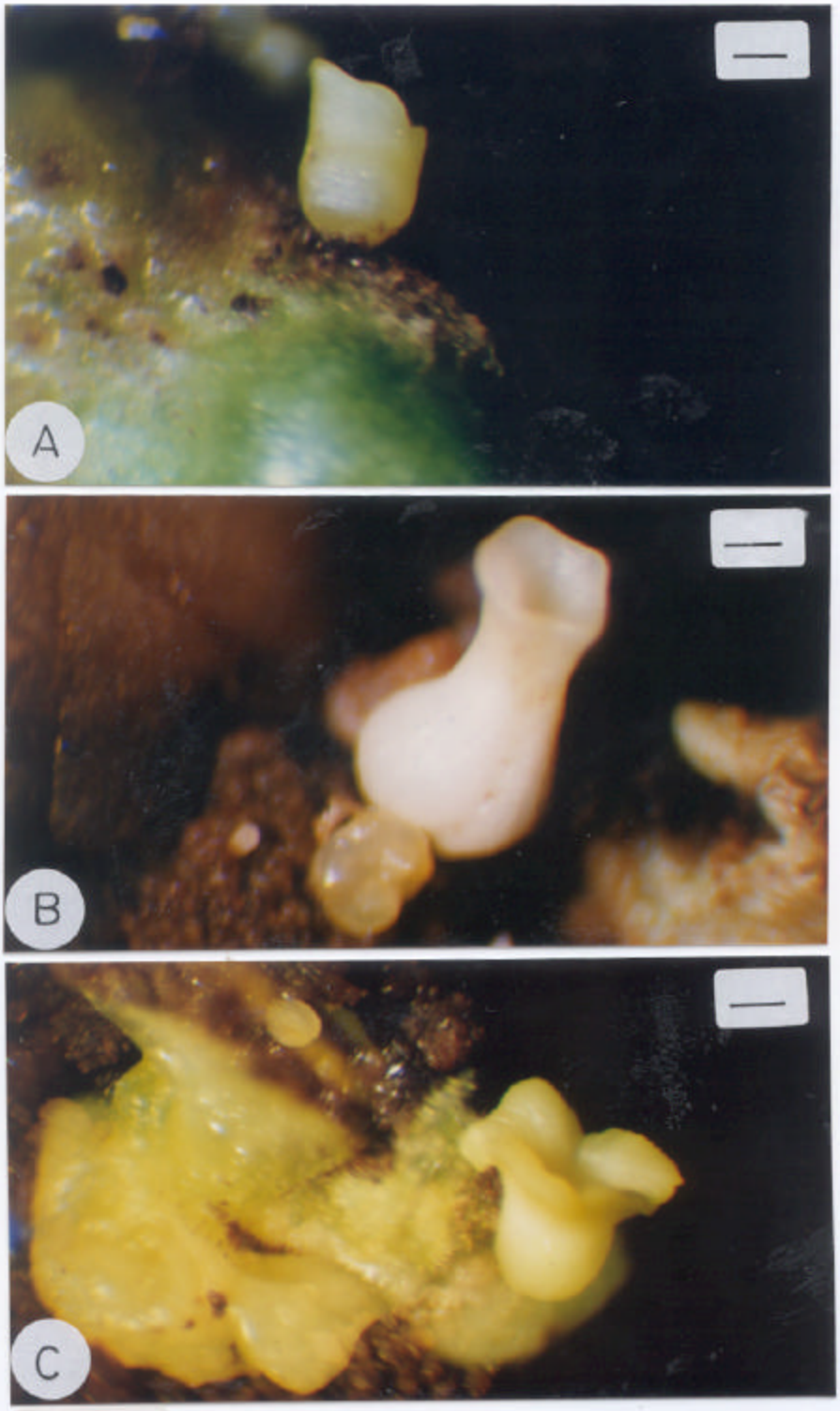


Fig 5.3

A. Cotyledonary stage somatic embryo with single cotyledon (bar = 575 µm)

B. Dicotyledonary somatic embryo (bar = 600 µm)

C. Tricotyledonary somatic embryo (bar = 540 µm)

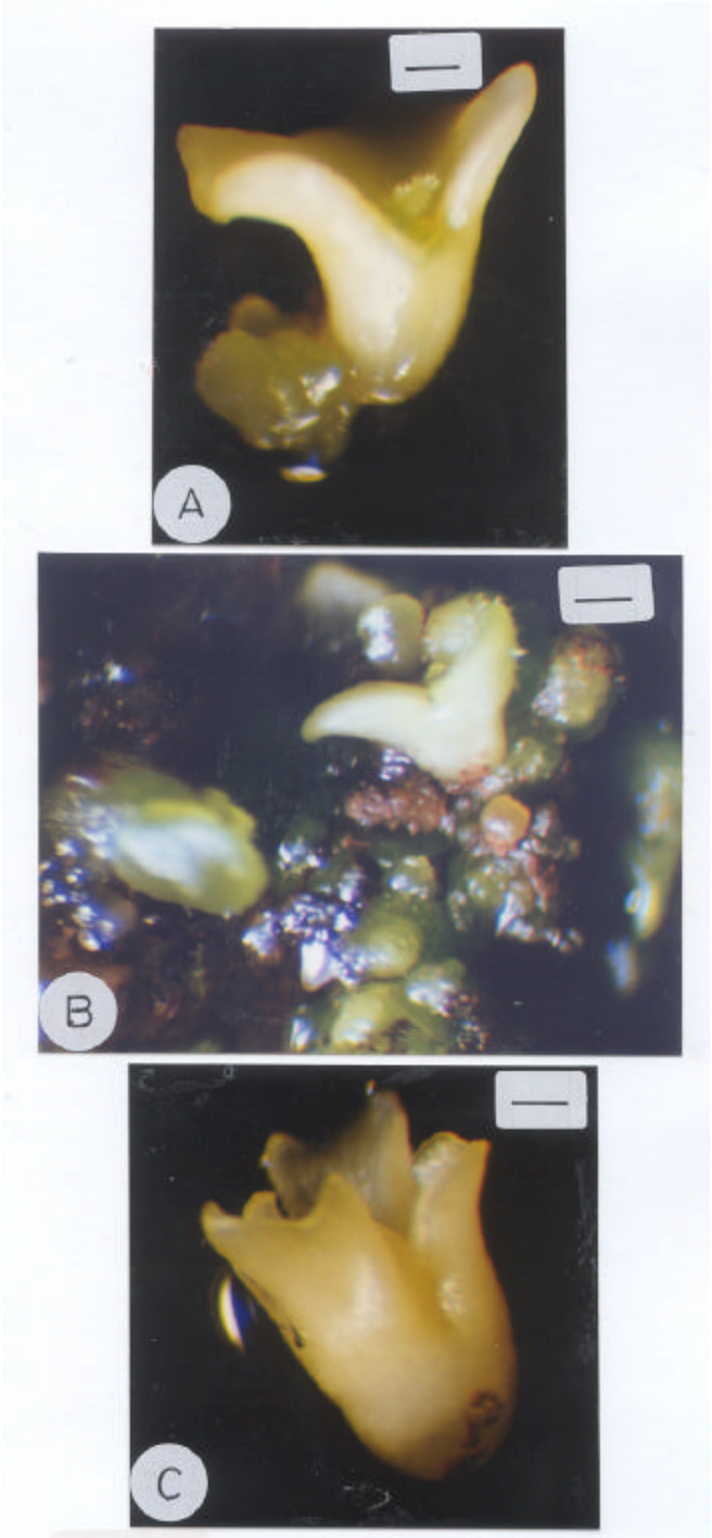


Fig 5.4

during somatic embryo development and this is controlled either by increasing the osmolarity of the maturation medium with additional sucrose (Carman 1989) or by incorporating ABA into the medium (Ammirato 1974). ABA may be necessary during embryogenesis to initiate the synthesis of storage proteins and proteins involved in desiccation tolerance (Galau *et al*. 1990). ABA was seen to influence development of carrot somatic embryos and in particular affected their capacity to develop functional shoot meristem (Nickle and Yeung 1994). A decrease in endogenous levels of ABA through fluridone (ABA synthesis inhibitor) application has been coupled with rapid vacuolation of cells in the apical bilayer. This vacuolation was concurrent with a decline in conversion of embryos (Nickle and Yeung 1994). It has been shown earlier that maturation of somatic embryos on ABA is necessary in *Phaseolus* (Malik and Saxena 1992a) and alfalfa (Redenbaugh *et al.* 1991).

5.3.4 Germination and conversion of somatic embryos

The term 'germination' denotes the elongation of the primary root and 'conversion' refers to the development of plantlets with a well-established root system and shoot with at least the first pair of leaves (Mathews *et al*. 1993). Germination (**Fig 5.5C**) and conversion of somatic embryos was observed when 31 mature somatic embryos obtained from half-strength MS basal medium supplemented with 0.5 µM ABA were placed on half-strength MS basal medium supplemented with 0.5 µM BAP. When embryos were incubated on half-strength MS medium without any growth regulator, only development of roots was observed (**Fig 5.6A**), while BAP (1 µM) supported shoot formation and swelling of cotyledons without root development (**Fig 5.6B**), 0.5 µM BAP supported both shoot and root formation (**Fig 5.6C**). 39 % of the embryos germinated and converted to plantlets. Out of 31 mature somatic embryos 12 germinated and formed plantlets (**Fig 5.7A**).

5.3.5 Transfer of plantlets to soil

On transfer of 12 plantlets to pots containing a soil : verimiculite mixture (1:1) for 15 days 42 % of plantlets survived (**Fig 5.7B**). Only 5 plants could thus be well established in the pots after hardening.

5.3.6 Histology

At the time of culture, the cotyledonary segments showed a single layered epidermis and the parenchyma was filled with food reserves (**Fig 5.8A**). A section passing through the cotyledonary segment revealed the development of globular embryos directly from

A. Cone shaped cotyledonary stage somatic embryo (bar = 575 µm)

B. Mature somatic embryo with well developed shoot and root pole obtained on half-strength MS basal medium supplemented with 0.5 µM ABA (bar = 225 µm)

C. Germinated somatic embryo obtained on half-strength MS basal medium supplemented with 0.5 µM BAP (bar = 1000 µm)

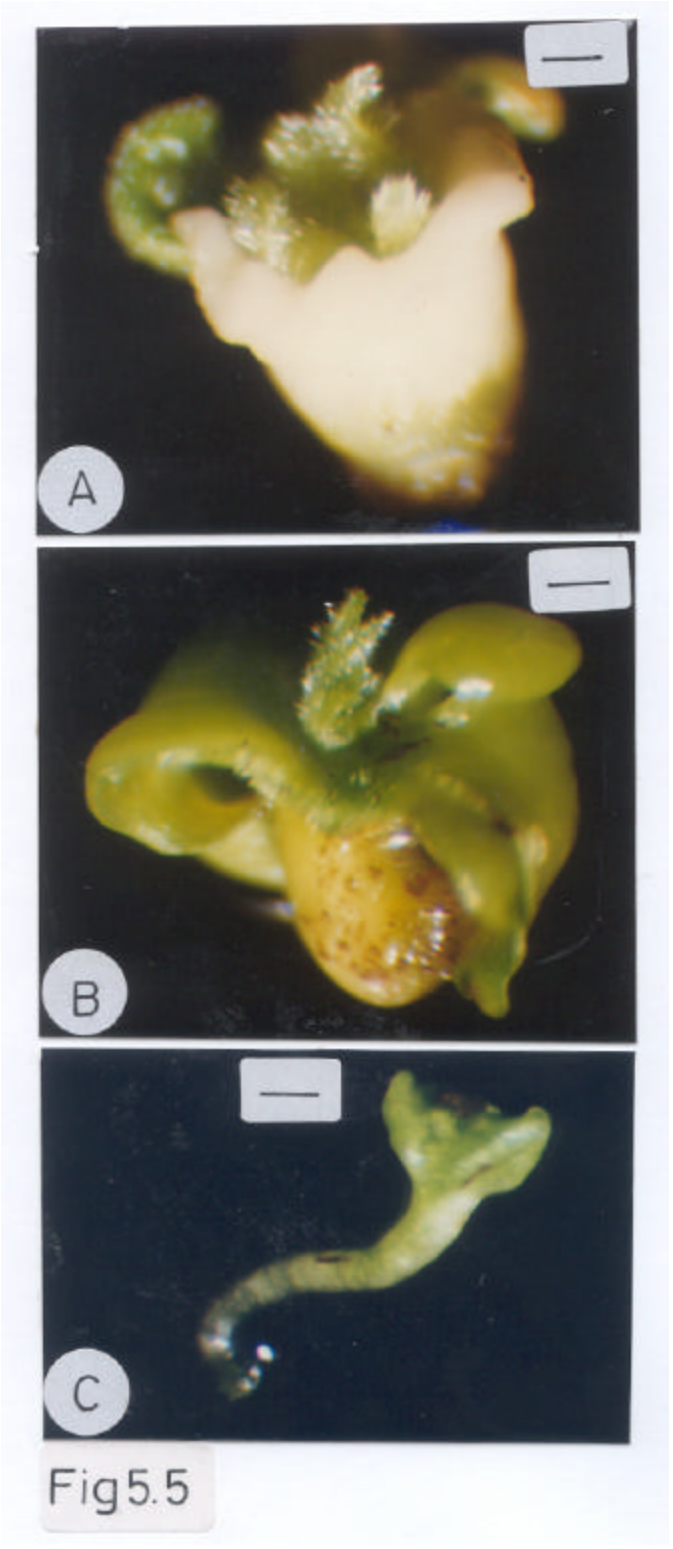


Fig 5.5

A. Somatic embryo producing only roots on hormone-free half-strength MS basal medium

B. Somatic embryo showing only shoot development and swelling of cotyledons on half-strength MS basal medium supplemented with 1 µM BAP

C. Somatic embryo showing normal shoot and root growth on half-strength MS basal medium supplemented with 0.5 µM BAP

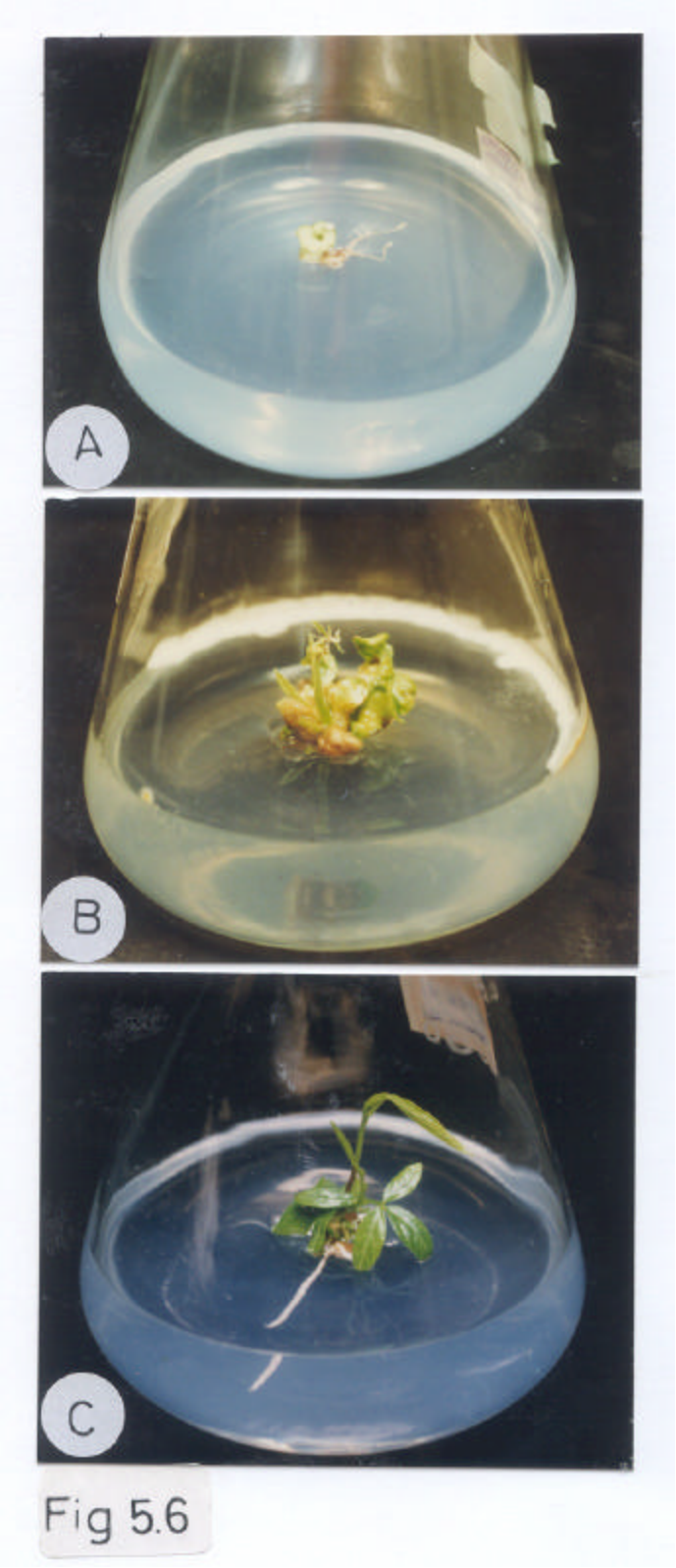


Fig 5.6

A. Converted plantlets with well-developed root system

B. Hardened plant surviving in pots

Fig 5.7

epidermal and subepidermal layers along the periphery of the explant (**Fig 5.8B**). Initiation of embryos from single cells was not observed.

Similarly in immature cotyledons of soybean, embryos developed from epidermal and subepidermal layers involving initiation of embryos both from single-cells and multiple-cells (Hartweck *et al*.1988) and from the superficial layer of cells (Barwale *et al*. 1986a; Hepher *et al*. 1988). In pea, histological studies of immature cotyledons revealed the division of cells in the superficial layers mainly in the epidermal and sub-epidermal layers (Tetu *et al*. 1990).

A section of early cotyledonary stage somatic embryo showed vascular initials, cotyledons and a developing shoot meristem region (**Fig 5.8C**). Mature somatic embryo has well developed cotyledons with pro-vascular strand, shoot pole and root pole. The section passing through the center of the mature somatic embryo showed the leaf primordia at the shoot pole and a prominent root pole (**Fig. 5.8D**).

The histological observations of different stages of somatic embryo development revealed the direct origin of globular embryos from epidermal and sub-epidermal layers of the cotyledonary segments involving multiple cells. The observations also confirmed the bipolar nature of the structures obtained, which is necessary for classifying the structures observed as indeed somatic embryos.

5.3.7 Parameter studies

Three factors such as explant, culture medium and culture environment play a major role in the production of somatic embryos *in vitro*. As seen in all cases of organized development *in vitro*, there is an inter-play between the explant, culture medium and culture conditions. To achieve optimum responses, the interactions of the above factors must be determined empirically (Thorpe 1988). It is for this reason, various basal media, explants, carbohydrates, culture environments and agitation in liquid induction medium have been tried to improve the percentage of induction of somatic embryogenesis obtained in the genotype Gaut-82-90 as well as to achieve higher percentage of further development of globular embryos found in Gaut-82-90 on EC_6 basal medium with 5 µM BAP. The various parameters were studied only with Gaut-82-90 as this genotype response is much better than T-15-15 in percentage induction of somatic embryos (see section 5.3.2 for details) and in the further development of globular somatic embryos on EC_6 basal medium supplemented with 5 µM BAP.

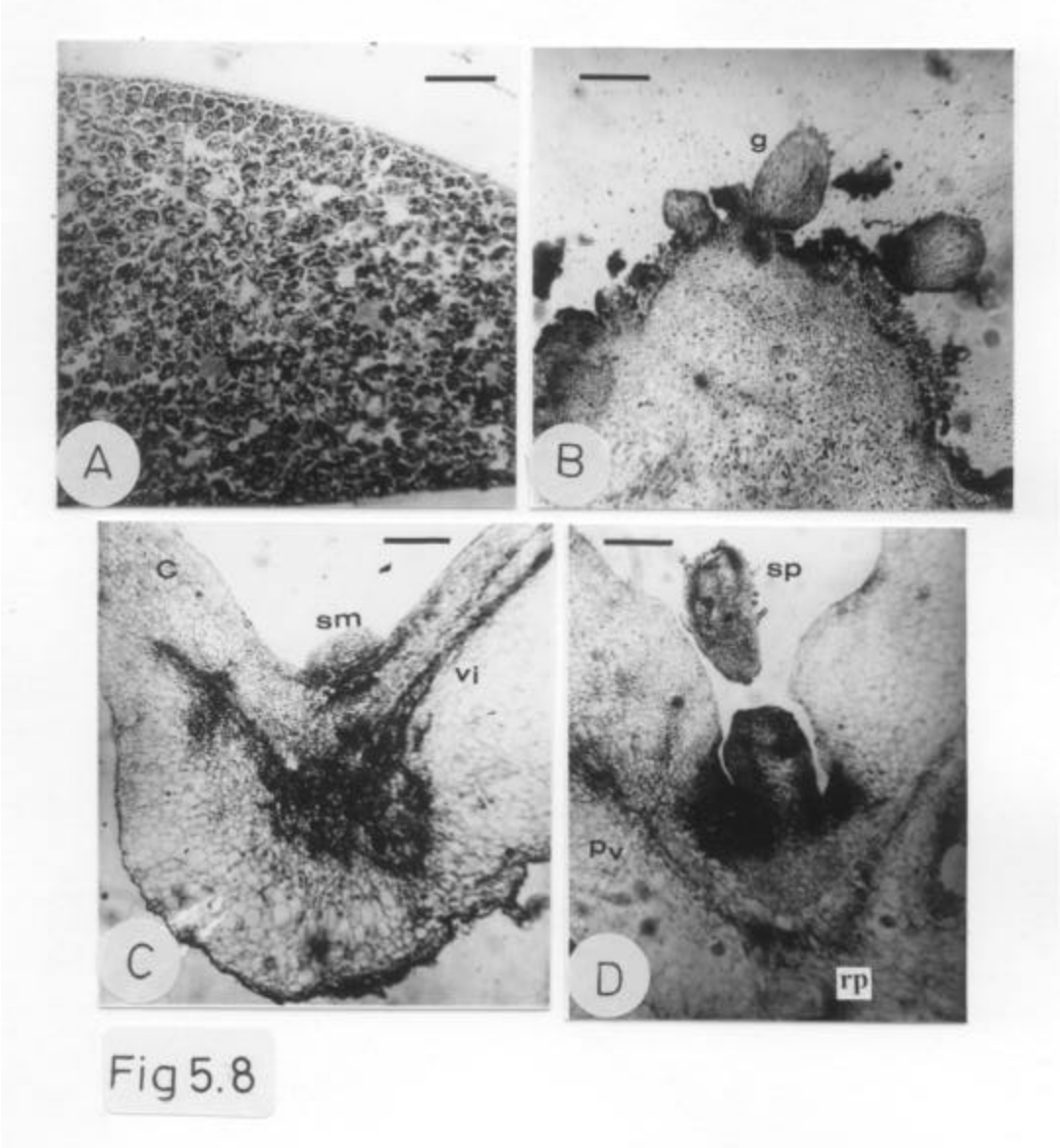


Fig 5.8

A. Section of explant at the time of culture, showing parenchymatous cells filled with food reserves (bar = 500 μm)

B. Section of cotyledonary segments showing formation of globular (g) embryos (bar = 500 μm)

C. Section of early cotyledonary stage somatic embryo with cotyledon (c) developing shoot meristem (sm) and vascular initials (vi) (bar = 825 μm)

D. Longitudinal section of mature cotyledonary stage somatic embryo showing root pole (rp), shoot pole (sp)and provascular strand (pv) (bar = 500 μm)

5.3.7.1 Effect of different basal media

The percentage induction of somatic embryos varied from 12 to 76 with MS basal medium recording the lowest and EC_6 basal medium recording the highest. LS medium produced the minimum number of globular embryos (1.57) per explant when compared to the maximum number of globular embryos (27.6) per explant on EC_6 basal medium (**Table 5.5**). It can be concluded from the above studies that EC_6 basal medium is best suited for induction of somatic embryogenesis from distal cotyledonary segments of pigeonpea. The optimized EC_6 basal medium supplemented with 5 µM BAP was therefore used for further optimization of parameters such as explant source, carbohydrate source and agitation in liquid induction medium only on EC_6 basal medium with 5 µM BAP.

In contrast to our observations, MS basal medium is the most commonly used medium for tissue culture of grain legumes. Induction of somatic embryogenesis using MS medium has been reported in peanut (Hazra *et al*. 1989; Ozias-Akins 1989; McKently 1991; Baker and Wetzstein 1992; Wetzstein and Baker 1993; Murthy and Saxena 1994; Chengalrayan *et al*. 1995), Pea (Tetu *et al*. 1990), Chickpea (Barna and Wakhlu 1993; Sagare *et al*. 1993 and Suhasini *et al*. 1996), pigeonpea (Nalini Mallikarjuna *et al*. 1996; Sreenivasu *et al*. 1998), winged bean (Ahmed *et al.* 1996) soybean (Christianson *et al.*1983; Ranch *et al*. 1985; Barwale *et al*. 1986a; Hepher *et al*. 1988) and *Vicia narbonensis* (Pickardt *et al*. 1989; Albrecht and Kohlenbach 1989). However, in our experiments EC_6 basal medium found to be the best for induction of somatic embryogenesis. Patel *et al*. (1994) also observed induction of somatic embryogenesis in pigeonpea using EC_6 basal medium, which is similar to our results.

Table 5.5 Effect of various basal media supplemented with 5 µM BAP on somatic embryogenesis from distal cotyledonary segments of genotype Gaut-82-90

Medium	Percentage induction (mean±se)	No. of globular embryos/explant (mean ± se)
EC_6	76 ± 3^{e}	27.6 ± 2.2^{c}
MS	12 ± 2^{a}	1.67 ± 0.8^{a}
B5	22 ± 4^{b}	3.60 ± 0.9^{b}
Modified B5	32 ± 5^{c}	2.40 ± 0.7^{b}
LS	36 ± 6^{c}	1.57 ± 0.4^{a}
White's	54 ± 18^{d}	5.54 ± 0.5^{b}

Figures with different alphabets in superscript differ significantly at 0.05 probability.

5.3.7.2 Effect of explant source

Among explants like mature embryo axes, mature cotyledons (whole), distal cotyledonary segments and leaf, the induction of somatic embryogenesis was highest in terms of percentage induction as well as in the number of globular embryos formed per explant in distal cotyledonary segments (76 %) followed by mature cotyledons (whole) (50 %) and leaf (42 %). The number of globular embryos per explant was significantly less in leaf (4.7) and mature cotyledons (whole) (10.2) when compared to distal cotyledonary segments (27.6). There was no induction of somatic embryogenesis from mature embryo axes (**Table 5.6**).

Table 5.6 Effect of EC_6 basal supplemented with 5 mM BAP on somatic embryogenesis from different explants of genotype Gaut-82-90

Explant	Percentage induction (mean±se)	No. of globular embryos/explant (mean±se)
Mature Embryo axes	NR	-
Mature Cotyledons (Whole)	50 ± 6^{b}	10.2 ± 0.8^{b}
Distal cotyledonary segments	76 ± 3^{c}	27.6 ± 2.2^{c}
Leaf	42 ± 8^{a}	4.7 ± 1.4^{a}

NR - No Response, Figures with different alphabets in superscript differ significantly at 0.05 probability.

5.3.7.3 Effect of Carbohydrate source

The influence of carbon sources such as glucose, fructose, sucrose and maltose on embryogenic response of cultivar Gaut-82-90 was compared. The best embryogenic response was obtained with 3 % sucrose (76 %) followed by maltose (44 %) and Glucose (30 %). No embryogenesis was observed when glycerol and fructose were supplemented to the medium. The number of globular embryos per explant was also highest in sucrose supplemented medium (27.6) when compared to maltose (14.7) and glucose (10.4) (**Table 5.7**).

The plant tissues are incapable of autotrophic growth under *in vitro* conditions, so carbohydrates are added to provide energy source (Kozai 1991; Leifert *et al*. 1995). Carbohydrates are also necessary in tissue culture as osmotic agents (Thorpe 1982). The most commonly used carbohydrate for culturing of plant tissues *in vitro* is sucrose. In nature, carbohydrate is transported within plant tissues as sucrose and tissue may have an inherent capacity for uptake, transport and utilization of sucrose (Eapen and George 1993a). Despite the widespread use of sucrose supported by numerous successes, other sugars have also been reported as being suitable carbon sources for embryogenesis of different species (Genga and Allavena 1991). Glucose has been demonstrated to be

effective for *Phaseolus cocccineus* (Genga and Allavena 1991), while maltose proved to be an efficient carbon source for somatic embryo production in alfalfa (Strickland *et al*. 1987). Maltose has also been used instead of sucrose for maturity of somatic embryos in soybean (Finer and McMullen 1991) and alfalfa (Denchev *et al.* 1991).

Table 5.7 Effect of EC_6 basal medium supplemented with 5 mM BAP and various carbohydrates on somatic embryogenesis from distal cotyledonary segments of Gaut-82-90

Carbohydrate (3 %)	Percentage induction (mean±se)	No. of globular embryos/explant (mean±se)
Sucrose	76 ± 3^{c}	27.6 ± 2.2^{b}
Glucose	30 ± 9^{a}	10.4 ± 0.8^{a}
Maltose	44 ± 7^{b}	14.7 ± 3.4^{a}
Glycerol	NR	-
Fructose	NR	-

NR - No Response, Figures with different alphabets in superscript differ siginificantly at 0.05 probability.

5.3.7.4 Effect of dark v/s light incubation

The percentage induction of somatic embryogenesis was better when the cultures were incubated under 16 h photoperiod (76 %) when compared to 24 h light conditions (51 %) and dark incubation (48 %). But there was no significant difference between 24 h light incubation and dark incubation. However, the number of globular embryos produced in light at 16 h photoperiod was highest (27.6) followed by 24 h photoperiod (11.8) and dark incubation (6.5) (**Table 5.8**).

The culture environment also influences the process of somatic embryogenesis. Somatic embryogenesis can occur under a variety of light/dark regimes, but in general, darkness may be better (Thorpe 1988). However, in pigeonpea incubation under light at 16 h photoperiod was better when compared to dark incubation. According to Thorpe (1988) requirement with respect to illumination of cultures varies among plants.

Table 5.8 Effect of EC_6 basal medium supplemented with 5 µM BAP on somatic embryo induction from distal cotyledonary segments of genotype Gaut-82-90 incubated under different culture environment.

Culture environment	Percentage induction (mean ± se)	No. of globular embryos/explant (mean ± se)
Light (16 h photoperiod)	76 ± 3^b	27.6 ± 2.2^c
Light (24 h photoperiod)	51 ± 5^a	11.8 ± 0.8^b
Dark	48 ± 8^a	6.5 ± 0.3^a

Figures with different alphabets in superscript differ siginificantly at 0.05 probability.

5.3.7.5 Effect of agitation in liquid induction medium

The highest percentage of somatic embryo induction was achieved when the distal cotyledonary segments were agitated in liquid induction medium at 200 rpm for 1 week and then transferred to EC_6 basal medium supplemented with 0.5 µM BAP followed by EC_6 basal medium supplemented with 1 µM BAP (65 %), hormone free EC_6 basal medium (60 %) and EC_6 basal medium supplemented with 2 µM BAP (55 %). The treatment EC_6 basal medium supplemented with 0.5 µM BAP was found be the best (24.1) in producing greater number of globular embryos per explant when compared to EC_6 basal medium supplemented with 1 µM BAP (14.7), EC_6 basal medium supplemented with 2 µM BAP (14.2) and hormone-free EC_6 basal medium (12.6) (**Table 5.9)**. There was no significant difference in the controls where in the agitation in liquid induction medium was not carried out compared to the experimental treatment where in the cotyledonary segments were agitated in liquid induction medium and transferred to EC_6 basal medium supplemented with 0.5 µM BAP in terms of percentage induction of somatic embryos and in the number of globular embryos per explant (**Table 5.9)**. However, the induction of somatic embryogenesis on hormone-free EC_6 basal medium when the cotyledonary segments were agitated in liquid induction medium was found to be lower than the controls (**Table 5.9**).

Table 5.9 Effect of agitation in liquid induction medium on somatic embryo production from distal cotyledonary segments of genotype Gaut-82-90

BAP (μM)	Percentage induction (mean±se)	No. of globular embryos/explant (mean±se)
Control*	76 ± 3^{c}	27.6 ± 2.2^{b}
Nil	60 ± 6^{ab}	12.6 ± 2.6^{a}
BAP 0.5	75 ± 11^{c}	24.1 ± 3.1^{b}
BAP 1.0	65 ± 15^{b}	14.7 ± 1.4^{a}
BAP 2.0	55 ± 21^{a}	14.2 ± 1.6^{a}

Figures with different alphabets in superscript differ significantly at 0.05 probability.

*The distal cotyledonary segments were inoculated on EC_6 basal medium supplemented with 5 μM BAP without agitating at 200 rpm for 1 week.

5.4 Conclusions

In the present investigations, efforts were made to standardize the protocol for Somatic embryogenesis from distal halves of cotyledonary segments. The somatic embryos were induced and a large number of globular embryos were produced by culturing the cotyledonary segments on EC_6 basal medium supplemented with BAP or TDZ. However, further development of globular embryos and formation of cotyledonary stage embryos occurred only when the globular embryos obtained on EC_6 basal medium supplemented with BAP were transferred to MS basal medium supplemented with GA_3 (2 - 5 μM). Various parameter studies were carried out to improve the efficiency of somatic embryogenesis achieved on optimized parameters of culture conditions such as EC_6 basal medium supplemented with 5 μM BAP in genotype Gaut-82-90 with distal cotyledonary segments as explants. In spite of various efforts, the formation of cotyledonary embyros, germination and conversion of embryos into plantlets was very low. The protocol needs to be improved further for use in genetic transformation experiments. The system may be useful for basic studies of embryogenesis since various morphological variations were observed in the development of cotyledonary stage somatic embryos.

Part of this chapter has been published as a paper entitled "**Somatic embryogenesis and plant regeneration in pigeonpea [*Cajanus cajan* (L.) Millsp.]**" by Mohan ML and Krishnamurthy KV (2001) *Biologia Plantarum* (In Press)

CHAPTER 6

GENETIC TRANSFORMATION STUDIES

6.1 Introduction

One of the most important events in the human history perhaps occurred when man stopped merely gathering food and became a farmer. That was probably the beginning of genetic engineering (Kemp 1983). Through classical breeding, countless plants were domesticated by man either by creating genetic diversity by crossing individuals or by selecting offspring that showed some useful trait/s (Kemp 1983).

Classical plant breeding, however, has its limitations as it depends on sexual compatibility and often takes 10-15 years to release a new variety (Pauls 1995). Due to the unsuccessful crosses and narrow gene pool available within a species, genetic engineering as an additional tool, is being used, to keep pace with the burgeoning population in the developing countries. Genetic engineering is defined as the manipulation of plant genomes via the introduction of a characterized DNA segment (Comai 1993).

Genetic engineering offers many opportunities for improving agriculture and public health. An elite variety could be modified for a single trait with the gene/s coding for insect, disease (viral and fungal) resistance or herbicide tolerance from entirely different organisms. Other quality traits such as protein and carbohydrate content, modified plant oil and fatty acid composition for health reasons, enhanced flavor and texture, longer shelf life could also be introduced (Smith 1994). Potential benefits include higher yields and enhanced nutritional value for crops and livestock, reduction in pesticide and fertilizer use, development of disease resistance and the use of plants to produce valuable heterologous molecules, which yield many new products (Flavell 1995). Transgenic plants have been engineered to produce a variety of products using their high capacity of self-assembling fermentors, which operate in non-sterile conditions at a cost of only several hundred dollars per ton of biomass (Raskin 1996).

With the worldwide market for herbicides at $ 6 billion, the first target for research was herbicide resistant transgenic plants. Herbicide tolerant plants have a number of commercially and environmentally desirable properties and would provide more cost effective and environmentally friendly weed control (Smith 1994).

Disease resistance has also been targeted as an advantageous trait in the major crops and a number of strategies have been employed to achieve this goal (Smith 1994). Virus resistance is important for good crop yields and also to reduce the amount of chemicals required to control the insect vectors that transmit the virus.

Introduction of bacterial insecticide genes from *Bacillus thuringiensis* into plants to confer pest resistance by inhibition of insect vectors has also been achieved (Fujimoto *et al*. 1993; Koziel *et al*. 1993). Cotton, which is transgenically insect resistant will probably be the first insect resistant plant to be commercialized (Smith 1994). Shade *et al*. (1994) described the use of α-amylase inhibitor to provide resistance against bruchid beetles in legume seeds. In transgenic peas, gene expression was observed in the seed and the protein was accumulated at sufficient levels to prevent infestation with the bruchid beetle weevils.

The 2S storage albumin of Brazil nut (*Bertholeita excelsa*) has been a popular target for increasing the level of sulfur-amino acids in legumes, because it contains multiple methionine and cystine residues (Habben and Larkins 1995). Saalbach *et al*. (1994) have constructed a chimeric 2S albumin cDNA that is constitutively expressed throughout the seeds and reveals that the Brazil nut protein is synthesized in this organ, albeit in low concentrations. Storage lipid modification via engineering of the fatty acid biosynthesis enzymes has also been achieved (Murphy 1992), which will contribute to the production of healthier foods as well as production of chemical feed stocks.

Crop losses can occur due to cold and freeze damage. Cold tolerance could be induced in cold-susceptible plants by a process called "acclimation" and one of the changes that occur is the production of organic compounds and antifreeze proteins (Hew and Yang 1992), which lower the freezing temperature of tissue liquids. Georges *et al*. (1990) expressed a fusion between chloramphenicol acetyl transferase and a synthetic gene encoding the founder antifreeze protein in maize tissues. They observed that tissue extracts, which showed the presence of the protein, displayed a reduction in ice crystal formation.

The ability to create transgenic organisms offers the opportunity of programming plants to accumulate foreign proteins in seeds, roots or leaves – to enhance their quality, value or even provide a new source of valuable pharmaceuticals, including antibiotics. The FLAVR SAVR^{TM} tomato is the first genetically engineered plant product to be released, and has a synthetic gene that inhibits the expression of polygalacturonase (Fray and Grierson 1993), which normally accelerates fruit softening and contributes to the over ripening of tomatoes. Transgenic plants with improved seed protein and oil content in corn (Berquist *et al*.

1992), cotton plants resistant to boll worm (Perlak *et al*. 1990), plants with ability to synthesize antibodies and vaccines (Mason *et al*. 1992), plants which synthesize biodegradable plastics (Nawrath *et al*. 1994; Porrier *et al*. 1992; Porrier *et al*. 1995) are some of the important examples of genetically engineered plants.

6.1.1 History of genetic transformation

In 1983, the era of plant transformation was initiated when *Agrobacterium*-mediated gene delivery was used for producing transgenic plants (Fraley *et al*. 1983). Following years of unsuccessful experiments with variations in feeding isolated DNA to plant tissues and organs, gene transfer became a reality soon after it was discovered that the soil bacterium *Agrobacterium tumefaciens* and *A. rhizogenes* are considered as natural genetic engineers due to their ability to transfer and intergrate DNA into plant genomes through a unique integrative gene transfer mechanism (Jouanin *et al*. 1993).

The first transgenic plants were developed in the early 1980's using a disarmed version of the Ti plasmid of *Agrobacterium tumefaciens*, a pathogenic bacterium, which can transfer part of its "T-DNA" into the plant genome (Smith 1994). The genes causing crown gall disease were removed from the Ti plasmid, while leaving the DNA transfer mechanism intact. Replacing the tumour causing genes with foreign genes and the subsequent conversion of plant cells to kanamycin antibiotic resistance by the transfer of a bacterial selectable marker gene (Neomycin phosphotransferase) into the plant cells, the expression of foreign genes into plants could be achieved (Smith 1994).

The production of transgenic plants depends on the stable introduction of foreign DNA into the plant genome, followed by regeneration of host cells into intact plants, and the subsequent expression of the introduced gene(s) (Walden and Wingender 1995). Initial successes in transformation studies were limited to Solanaceae, tobacco (*Nicotiana tabacum* L.) in particular. This has dramatically changed throughout the 1980's and into the 1990's where now it is possible to transform a wide range of plants, many of which are of agronomic importance (Songstad *et al*. 1995) using genetically engineered avirulent strains of *Agrobacterium* as vectors (Herrera-Estrella *et al*. 1983).

Host range limitations of *Agrobacterium*-mediated gene transfer prompted the search for alternate gene transfer systems, leading soon to the development of "direct gene transfer to protoplasts" (Potrykus 1995). Further limitations in these gene transfer systems led to the exploration of various other approaches such as pollen

transformation, pollen tube pathway, electrophoresis, microlaser, liposome-fusion and injection, macroinjection, direct DNA application etc. (Potrykus 1995). However, none of these approaches have been developed into a reproducible gene transfer technique (Potrykus 1995). Transformation can also be achieved by methods which include the direct insertion of DNA into protoplasts by microinjection (Crossway *et al.* 1986) or electroporation (Horn *et al.* 1988).

The biolistic transformation (particle bombardment) system – coating DNA on tiny metal particles and shooting these into plant tissues was the next breakthrough (Klein *et al.* 1987; Sanford 1988), which not only led to the efficient production of transgenic "model plants", but also opened a route for genetic engineering of major crop plants (Potrykus 1995). This method depends far less on sophisticated tissue culture procedures and is independent of the limitations of tissue culture. This method has proved to be particularly successful with plants that are less amenable to tissue culture, such as cereals and legumes (Walden and Wingender 1995). The transformation of cereals and legumes has received much attention, because of the agronomic importance of these crops. The details of different methods adopted to engineer plants are outlined in the following sections.

6.1.2 Methods of gene transfer

6.1.2.1 *Agrobacterium* mediated gene transfer

Agrobacterium tumefaciens is a gram-negative soil bacterium causing crown gall tumors at wound sites of infected dicotyledonous plants (Gelvin 1993). It attaches to plant cells and then transfers part of its tumor-inducing (Ti) plasmid called T (transferred) DNA, to some of these plant cells. The T-DNA becomes integrated into one of the chromosomes of the plant cell and expression of the gene located on the T-DNA leads to the formation of proteins involved in the production of an auxin and a cytokinin, which causes the tumorous phenotype (Hooykaas and Beijersbergen 1994). Gene transfer systems based on *Agrobacterium* exploit the natural DNA transfer ability of these plant pathogens. The T-DNA genes can be replaced by any other gene of interest, which can be transferred to the plant genome. For plant species that are readily amenable to tissue culture, *Agrobacterium*-mediated gene transfer, the first widely adopted means of creating transgenic plants, remains the most popular technique (Walden and Wingender 1995). Probably the greatest advantage of the system is that it offers the potential to generate transgenic cells at relatively high frequency without significant reduction in plant regeneration rates (Walden and

Wingender 1995). Plants are usually transformed with relatively simple constructs, in which the gene of interest is coupled to promoter of plant, viral or bacterial origin, some promoters confer constitutive expression, while others may be selected to permit tissue-specific expression, or environmentally inducible expression. The cauliflower mosaic virus (CaMV) 35S RNA promoter is often used because it directs high levels of expression in most tissues (Walden and Wingender 1995). Genetic transformation that is effected by *Agrobacterium* is dependent on the use of disarmed Ti plasmid. The *Agrobacterium* genes that are responsible for tumor formation can be replaced with foreign genes that are expressed following infection of plant cells (Litz and Gray 1995). The inserted genes could be :

1) a selectable marker, such as the gene for neomycin phosphotransferase (NPT II), which confers resistance to antibiotic kanamycin;
2) a gene that encodes a scorable marker, such as β-glucuronidase (GUS) which undergoes a useful histochemical color reaction (Jefferson *et al.* 1987);
3) a sequence that encodes a promoter, such as 35S from caulflower mosaic virus, for expressing the different genes that have been introduced into the plasmid;
4) and genes that have agricultural interest (Litz and Gray 1995).

Agrobacterium-mediated transformation involves incubation of cells or tissues with the bacterium, followed by regeneration of plants from the transformed cells directly and/or via callus stage. Transformation mediated by *Agrobacterium* has provided a reliable means of creating transgenics in a wide variety of species that are amenable to tissue culture and regeneration (Walden and Wingender 1995). Earlier reports of genetic transformation by *Agrobacterium* generally involved tissues that regenerate through organogenesis such as leaves (Horsch *et al*. 1985). However, for species that are not easily regenerated by organogenesis, such as walnut (McGranahan *et al*., 1988) and mango (Mathews *et al*., 1992), embryogenesis has increasingly been preferred.

Agrobacterium-mediated gene trasnfer remains the most common method of transforming dicotyledonous plants, including legumes (Kumar and Davey 1991). Legumes are natural hosts to *Agrobacterium* and many grain legumes produce stably transformed callus (Puonti-Kaerlas 1993b) and plants.

6.1.2.2 Direct gene transfer

DNA can be introduced directly into plant protoplasts by techniques that are similar to those used for animal and yeast cells. The freely accessible plasma membrane allows the easy entry of the DNA (Jenes *et al*. 1993). The advantage of this procedure is that it can be used on any plant from which protoplasts can be obtained (Draper *et al*. 1982). It was first reported by Paszkowski *et al*. (1984) in tobacco. The simple application of naked DNA under defined conditions to plant protoplasts has been shown to result in the uptake and integration of DNA into the plant (Draper *et al*. 1982). It was developed as an alternative to *Agrobacterium*-mediated gene transfer because of host range limitations.

Direct uptake of DNA into protoplasts can be promoted by chemical and physical treatment, e.g., with polyethylene glycol (PEG), and application of electric pulses (electroporation) respectively or a combination of both.

The basic procedure involves the preparation of protoplasts from plant tissue by enzymatic digestion, the addition of DNA to the protoplast suspension, the uptake of DNA stimulated by various methods, the selection for expression of a transformed gene usually applied at some point in the regeneration process from the treated protoplast to plants.

6.1.2.2.1 Electroporation mediated gene transfer

A widely used physical treatment for enhancing the frequency the of transformation of protoplasts is to subject them to electric pulses. This treatment is believed to create transient pores in the cell membranes through which the DNA present in the external solution gains entry under appropriate conditions (pH, concentration). This leads to either transient expression of introduced DNA, or stable integration of the DNA into a small proportion of the protoplasts, and the transgenic tissues and plants can be regenerated (Jones 1995). Conditions should be chosen such that the pore formation is reversible and the protoplasts recover from the treatment (Jones 1995).

6.1.2.2.1.1 Electroporation of protoplasts

Electroporation causes the uptake of DNA into protoplasts by temporary permeabilization of the plasma membrane to macromolecules (Hinchee *et al*. 1994). This is achieved by application of a high intensity electric field to protoplasts contained in a buffer between two electrodes. DNA diffusion occurs immediately after the electric field is applied and until the pores in the membrane reseal (Fromm *et al*. 1987). The efficiency of gene transfer and viability of protoplasts is influenced by

the amplitude and duration of the electric pulse and composition of the electroporation medium (Jenes *et al.* 1993). Electroporation has been used successfully for transient (Songstad *et al*. 1995) and stable transformation (Negrutiu *et al*. 1987; Shillito *et al*. 1985) of protoplasts from a wide range of species and tissue sources. Stable transformation of maize protoplasts (Fromm *et al.* 1986) and carrot protoplasts (Langridge *et al.* 1985) has been reported.

6.1.2.2.1.2 Electroporation of entire tissue s

Electroporation of intact leaf tissue of rice (Dekeyser *et al*. 1990) has been achieved. The applicability of this technique to other monocots including wheat, maize and barley has also been reported (Jenes *et al.* 1993).

6.1.2.2.2 PEG (polyethylene glycol) method.

PEG is the most widely used chemical treatment for facilitating DNA uptake into plant protoplasts. PEG acts to increase the permeability of cell membranes and has been used as an efficient protoplast fusion agent. PEG mediated transformation involves mixing freshly isolated protoplasts with DNA and immediately adding a given concentration of PEG dissolved in a buffer. PEG concentration can affect protoplast viability and gene transfer efficiency (Jenes *et al*. 1993). Production of transgenic plants is dependent on the regeneration competency of the resultant transformed callus (Hinchee *et al.* 1994).

6.1.2.2.3 Microprojectile bombardment-mediated transformation

This method is capable of circumventing the host-range restrictions of *Agrobacterium tumefaciens*, and the regeneration problems of protoplast transformation (Klein *et al*. 1987). Microprojectile-mediated transformation is a mechanical method of introducing DNA into almost any plant species and genotype (Hinchee *et al*. 1994; Walden and Wingender 1995). This mechanical method may be highly advantageous when major biological barriers exist to either *Agrobacterium* or protoplast mediated transformation (Hinchee *et al*. 1994). Heavy microprojectiles (tungsten/gold) coated with DNA are accelerated into cells and tissues. The cells can survive the intrusion of particles thus this technique facilitates the transport of genes into any type of intact cells or tissues (Jenes *et al*. 1993) and was demonstrated for the first time in maize cells (Klein *et al*. 1987). Particle-mediated plant transformation technology has been used to transform several plant species, including relatively recalcitrant species such as maize (Fromm *et al*. 1990), soybean (McCabe *et al*. 1988) and wheat (Vasil *et al*.

1992). Stable transformation using this method has been achieved in tobacco (Klein *et al.* 1988; Tomes *et al.* 1990).

6.1.2.2.4 Other methods

Due to host range limitations of *Agrobacterium* and the absence of regeneration protocols from protoplasts for the direct gene transfer, alternate approaches of gene transfer were explored.

Pollen tube pathway (Luo and Wu 1988), electrophoresis (Ahokas 1989), microlaser (Guo *et al*. 1995), sonication (Joersbo and Brunstedt 1990), incubation of dry embryos in DNA (Toepfer *et al*. 1989), silicon fiber mediated gene transfer (Kaeppler *et al.* 1990), microinjection (Neuhaus and Spangenberg 1990; Potrykus 1990) etc. are the methods reported. These methods can be extended for the genetic transformation of legume species especially, pigeonpea [*Cajanus cajan* (L.) Millsp.].

6.1.3 Transformation studies in grain legumes

The major problem in the development of commercially viable *Agrobacterium*-mediated transformation methods is the lack of efficient plant regeneration systems in which a large proportion of cells in the explant are capable of regeneration (Jordan and Hobbs 1994). The earlier reports on legume transformation employed wild type oncogenic strains of *Agrobacterium* leading to the production of crown gall tumors or hairy roots (Kumar and Davey 1991). However, these tumors failed to regenerate shoots in most of the cases studied. With the establishment of efficient plant regeneration systems in legumes, desirable genes have been introduced into the plant genome through binary vectors in many grain legumes **(Table 1.3)**.

Progress with legumes is limited compared to plants of other families such as solanaceae, however, since legumes are gradually becoming amenable to tissue culture, somatic cell techniques will be increasingly targeted to these species (Kumar and Davey 1991). Transgenic grain legumes have been produced using a combination of particle bombardment technology and *de novo* explant to plant regeneration systems. Suspension cultures have also been utilized, although, success has been very limited (Christou 1995). Among legumes, only soybeans and peanuts have received much attention as far as stable transformation is concerned (Christou *et al*. 1993). For important species such as soybean, peanut and *Phaseolus*, elite varieties can be engineered relatively easily (Christou 1995). Genetically engineered soybean was recovered using embryonic axes from mature and immature soybean seeds (McCabe *et al*. 1988) and cotyledon explants (Hinchee *et al*. 1988). Transgenic pea plants were

also recovered from cotyledonary node explants (Jordan and Hobbs 1993). However, the low transformation frequency was attributed to the limited number of competent cells in the explants (Jordan and Hobbs 1994). Transgenic plants in legumes have also been recovered from soybean (McCabe *et al*. 1988; Hinchee *et al*. 1988), pea (Puonti-Kaerlas *et al*. 1989; Schroeder *et al*. 1993; Grant *et al*. 1995), peanut (Schnall and Weissinger 1993; Eapen and George 1994b; Brar *et al*. 1994; McKently *et al*. 1995), *Phaseolus* (Mariotti *et al*. 1989, cowpea (Penza *et al*. 1991) and chickpea (Fontana *et al*. 1993; Kar *et al*. 1996; Krishnamurthy *et al*. 2000).

Despite much progress, *A. tumefaciens*-mediated transformation of legumes is still not sufficiently effective in many species for commercial use (Jordan and Hobbs 1994). Wild type *A. tumefaciens* does not attack all plants, and it is important to establish that *Agrobacterium* strain to be used and the species and genotypes to be transformed are compatible. Wild type strains have been found to be virulent on soybean (Hood *et al*. 1987; Owens and Cress 1985; Hood *et al*. 1986; Pedersen *et al*. 1983; Byrne *et al*. 1987), pea (Puonti-Kaerlas *et al*. 1989; Hawes *et al*. 1989; Hobbs *et al*. 1989), dry bean (El Khalifa and Lippincott 1968; McClean *et al*. 1991), lentil (Warkentin and McHughen 1991) and moth bean (Gill *et al*. 1988). However, there is considerable variation among the legume species, as well as in the ability of the different strains to infect legumes (Byrne *et al*. 1987; Puonti-Kaerlas *et al*. 1989; Hobbs *et al*. 1989; McClean *et al*. 1991; Warkentin and McHughen 1991) and in the response of the different genotypes within a species to that infection (Owens and Cress 1985; Byrne *et al*. 1987; Puonti-Kaerlas *et al*. 1989; Hawes *et al*. 1989; Hobbs *et al*. 1989; McClean *et al*. 1991; Armstead and Webb 1987). Such interactions can be very important as, depending on the strain-genotype combinations used, soybean (Owens and Cress 1985) and alfalfa (Mariotti *et al*. 1984) ranged in response from nonsusceptible to very highly susceptible. Therefore, other successful means of legume transformation (e.g. biolistics) are essential for potential improvement of legumes (Jordan and Hobbs 1994).

6.1.4 Transformation studies in pigeonpea : Current status

To date, only a single report of genetic transformation in pigeonpea is available. Geetha *et al*. (1999) obtained transgenic plants of pigeonpea using *Agrobacterium*-mediated transformation with GUS reporter gene and *npt*II gene as selectable marker. The data on the number of transgenic plants transferred to field and their performance is not available in the above report. No report on pigeonpea transformation is also

available using GFP gene as reporter gene. Moreover, there were no reports of genetic transformation in pigeonpea when this work was initiated.

Therefore studies have been carried out to standardize an *Agrobacterium*-mediated transformation protocol for pigeonpea. The Standardized protocol may be useful to achieve the aforementioned goals – production of disease/pest resistant high yielding cultivars of pigeonpea.

6.2 Materials and methods

6.2.1 Plant material

The genotype T-15-15 was found to be better in terms of the number of shoots produced from DCMEA explants as described in chapter 4. Therefore, the genotype T-15-15 was used for transformation experiments. Seeds of the genotype T-15-15 were surface sterilized as described in section 2.6.1. The surface sterilized seeds were soaked in sterile distilled water for 18 h in the dark at 28 ± 2 °C and kept on gyratory shaker at 200 rpm. They were dissected under sterile conditions and the embryo axes were separated from the cotyledons. These embryo axes were injured by the removal of 1-2 mm of shoot and root apical meristems (referred to as decapitated mature embryo axes – DCMEA) and were used as explants for co-cultivation with *Agrobacterium* strains pGV2260-35S-GUSINT and pBIN 35S-mgfp5-ER containing marker and reporter genes. Around 180-240 DCMEA explants were used per experiment and the experiments were repeated thrice in case of treatment with *Agrobacterium* strain pGV2260-p35SGUSINT and 6 times in case of treatment with *Agrobacterium* strain pBIN 35S-mgfp5-ER. Forty DCMEA explants inoculated on the M1 medium, without treatment with *Agrobacterium*, served as control.

6.2.2 *Agrobacterium* strain

6.2.2.1 GUS reporter gene

The *Agrobacterium tumefaciens* strain LBA4404 containing the pGV2260-35S-GUSINT plasmid is pBIN19 derivative, which carries a chimeric NPTII gene and a GUS gene construct with a ST-LS1 gene derived intron (Vancanneyt *et al*. 1990) (**Fig 6.1A**) was kindly provided by Dr. Deepak Pental (University of Delhi, South Campus, New Delhi, India) (**Table 6.1**).

6.2.2.2 GFP reporter gene

The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.* 1983) harboring a binary plasmid pBIN 35S-mgfp5-ER (**Fig 6.1B**) was used as vector for

transformation. The construct was kindly provided by Dr. Jim P Haseloff from MRC Laboratory of Molecular Biology, Cambridge, England (**Table 6.1**). pBIN 35S-mgfp5-ER is a plant binary vector derived from pBI121 (Jefferson *et al*. 1987) in which the Bam HI - Sst I fragment containing the GUS reporter gene has been replaced with Bam HI – Sst I fragment containing mgfp5-ER gene. The nucleotide substitutions that alter codon usage (but not amino acid sequence) eliminate cryptic splicing in *Arabidopsis* by destroying the splice sites and branch point and lowering the A-U content around the cryptic intron (Haseloff *et al*. 1997). Fusion of the N-terminal signal sequence from *Arabidopsis thaliana* basic chitinase and C-terminal HDEL sequence allows compartmentalization of GFP away from the nucleoplasm and cytoplasm and into the lumen of the endoplasmic reticulum resulting in highly fluorescent transgenic plants and an apparent decrease in phytotoxicity (Haseloff *et al*. 1997). GFP5 has dual excitation peaks (395 nm and 473 nm) of approximately equal amplitude, which can be visualized well with either long wavelength UV (e.g. hand held lamp) or blue light (e.g. argon laser) (Siemering *et al.* 1996).

Table 6.1 *Agrobacterium* and plasmid strain used in the present investigation

***Agrobacterium*/Plasmid**	**Culture conditions**	**Reference**
pGV2260-p35SGUSINT	**YEB + 100 µg/ml Kanamycin + 50 µg/ml Streptomycin + 100 µg/ml Rifampicin**	**Vancanneyt *et al.* 1990**
pBIN 35S-mgfp5-ER	**YEB + 50 µg/ml Kanamycin + 250 µg/ml Rifampicin**	**Haseloff *et al.* 1997**

6.2.3 Mobilization of GFP gene into *Agrobacterium*

The plasmid containing pBIN 35S-mgfp5-ER was introduced into the *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method described by Dityatkin *et al*. (1972) with modifications. The *Agrobacterium* strain LBA4404 containing helper Ti plasmid was grown overnight at 28 °C in 5 ml of YEB medium containing 250 mg/l of rifampicin. Two millilitre of the overnight grown culture was added to 50 ml of YEB medium in a 250 ml Erlenmeyer flask and the culture was shaken at 250 rpm at 28 °C until the culture grows to an OD_{600} of 0.5. The culture was chilled on ice and the cell suspension was centrifuged at 3000 g for 5 min. at 4 °C. The supernatant was discarded and the cells were re-suspended in 1 ml of 20 mM ice cold $CaCl_2$ solution.

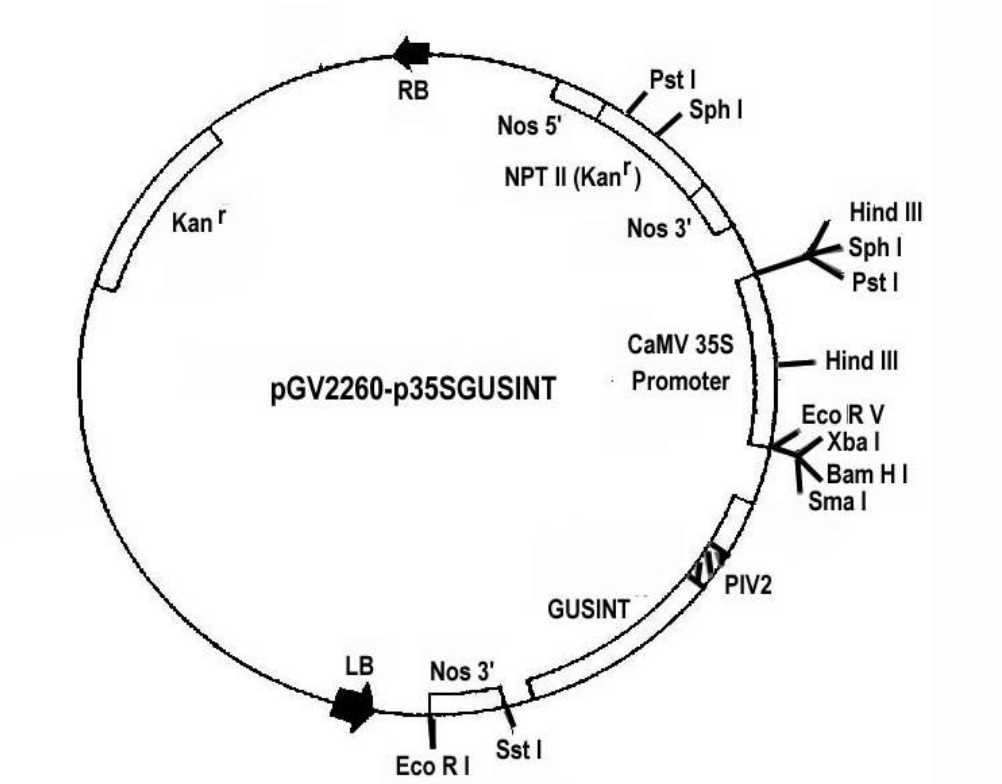


Fig 6.1A Plasmid map of the *Agrobacterium* strain pGV2260-p35SGUSINT used in the experiments

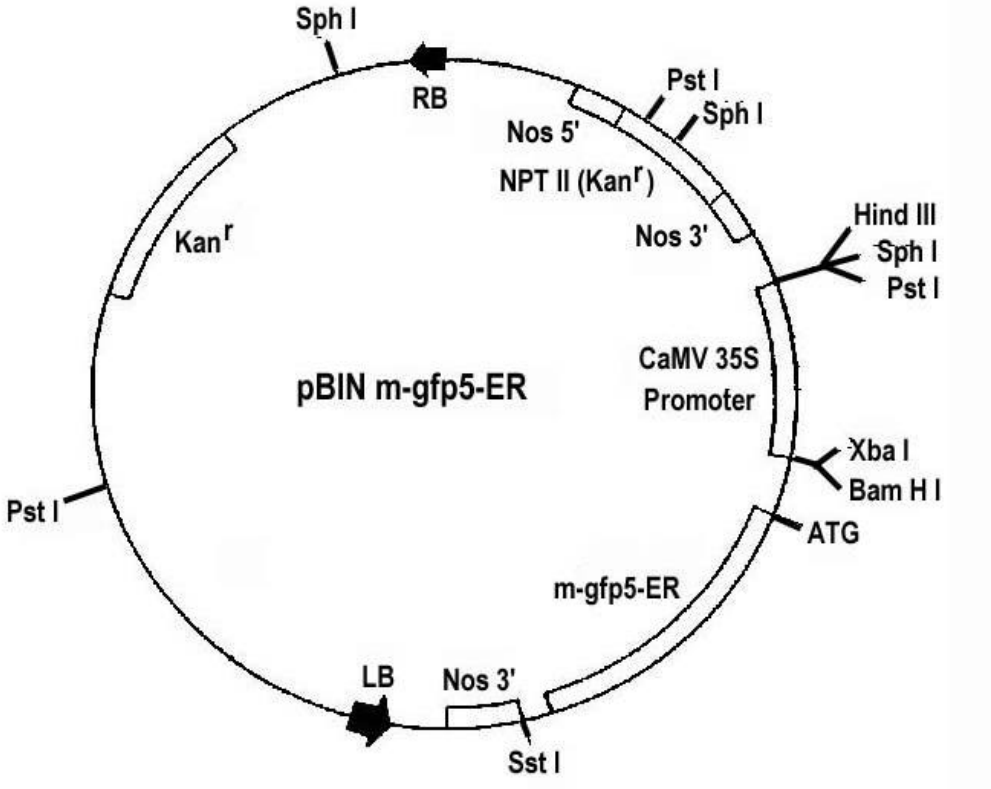


Fig 6.1B Plasmid map of the *Agrobacterium* strain pBIN 35S-mgfp5-ER used in the experiments

The cell suspension was dispensed into pre-chilled microfuge tubes as 0.1 ml aliquots. One microgram of pBIN 35 mgfp5-ER plasmid DNA was added to the cells. The cells were frozen in liquid nitrogen and thawed by incubating the microfuge tubes in a 37 °C water bath for 5 min. The volume of the culture was made up to 1 ml using YEB medium and incubated at 28 °C for 2-4 h with gentle shaking. The cells were spun down for 30 s and the supernatant was discarded. The cells were re-suspended in 0.1 ml YEB medium and were subsequently spread on YEB agar plate containing 250 mg/l rifampicin and 50 mg/l kanamycin. The plates were incubated at 28 °C for 2-3 days. The transformed colonies were picked and grown. The bacterial colonies were maintained on YEB agar plates containing 50 mg/l kanamycin and 250 mg/l rifampicin at 4 °C or as glycrol stocks at –70 °C.

6.2.4 Bacteriological medium

Agrobacterium strains (pGV226-p35SGUSINT and pBIN 35S-mgfp5-ER) were grown in YEB (1 g/l of Bacto-yeast extract; 5 g/l Beef extract; 5 g/l Bacto peptone; 5 g/l Sucrose; 0.5 g/l $MgSO_4$) medium (Shaw 1988). The pH of the medium was adjusted to 7.2 before sterilization by autoclaving.

6.2.5 Growth media and conditions for *Agrobacterium*

A single bacterial colony was inoculated in 5 ml of liquid YEB medium with antibiotics (concentrations as mentioned in **Table 6.1**) and grown at 28 °C on a gyratory shaker at 200 rpm for 48 h. One hundred and fifty micro liter of this culture was suspended in 15 ml of YEB medium with antibiotics and incubated at 28 °C on a shaker at 200 rpm for 24 h. In order to find out the growth pattern of *Agrobacterium* strains used, the cell density of bacterium was measured at an optical density at 600 nm at 4 h interval for a period of 24 h and plotted against time (**Fig. 6.2**). Based on this graph the 18 h grown culture (late log phase) was used for treating the explants.

6.2.6 Regeneration media

DCMEA explants in all the experiments were cultured on EC_6 basal medium supplemented with 3 % sucrose, 0.8 % agar-agar, BAP (5 µM) and IAA (1 µM) for shoot regeneration (M1 medium) (The medium on which the shoot regeneration was achieved from DCMEA explants as described in chapter 4). The shoots were cultured on half-strength MS basal medium supplemented with 3 µM GA_3 for elongation (M2 medium).

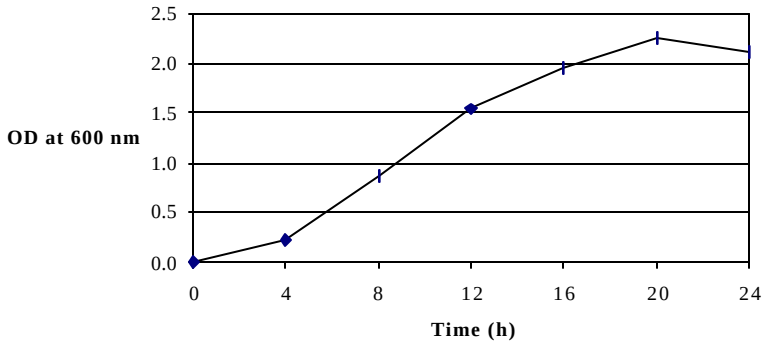


Fig 6.2 Characteristic *Agrobacterium* growth curve plotted OD_{600} against time

Half-strength MS basal medium supplemented with 0.5 µM IBA (M3 medium) was used for rooting. DCMEA explants were cultured on EC_6 basal medium supplemented with 3 % sucrose, 0.8 % agar-agar, BAP (0.5 µM) and 2,4-D (5 µM) for callus formation and maintenance (M4 medium).

6.2.6.1 Determination of lethal dose of kanamycin

The LD_{50} of kanamycin used, as selective pressure was determined by culturing 30 DCMEA explants (6 explants per petri dish and 5 petri dishes per treatment) in M1 medium and supplemented with various concentrations of kanamycin (25, 50, 75, 100, 200, 300 and 400 mg/l). The cultures were incubated for 4 weeks at 25±2 °C under 16 h photoperiod at light intensity of 38 $\mu E.m^{-2}.s^{-1}$ provided by cool white fluorescent lights.

6.2.6.2 Co-cultivation of explants

DCMEA explants were treated with *Agrobacterium* strain pGV2260-p35SGUSINT or pBIN 35S-mgfp5-ER. *Agrobacterium* culture grown in 5 ml YEB medium for 18 h with antibiotics as mentioned in **Table 6.1** was centrifuged at 5000 rpm at RT for 10 min. The pellet was resuspended in 2.5 ml of liquid M1 medium. This suspension was then used for co-cultivation experiments. Treatment of explants was carried out by dipping DCMEA explants in a batch of 20 in *Agrobacterium* suspension for 20 min.

All the explants treated by dipping were blotted dry on a sterile filter paper and co-cultivated on M1 medium for 72 h. These were then washed with sterile distilled water, blotted dry on sterile filter paper and incubated on M1 medium containing 500 mg/l cefotaxime for one week. After one week of culture the embryo axes were transferred to M1 medium containing 250 mg/l cefotaxime (Claforan – Russel India Ltd.) and 50 mg/l kanamycin. Cultures were incubated under the conditions mentioned as above for 4 weeks.

6.2.6.3 Regeneration of shoots

The shoots regenerated on M1 medium containing 50 mg/l kanamycin and 250 mg/l claforan after 4 weeks of incubation were transferred to a freshly prepared M1 medium containing 125 mg/l of claforan and 50 mg/l kanamycin and were incubated for another 4 weeks. The green shoots growing in the presence of 50 mg/l kanamycin were then transferred to M2 medium supplemented with 50 mg/l kanamycin and incubated for 4 weeks. The shoots surviving on M1 medium supplemented with 50 mg/l kanamycin for 8 weeks were scored for GUS staining or green fluorescence.

6.2.6.4 Rooting and hardening

The shoots obtained from DCMEA explants treated with *Agrobacterium* strain pBIN 35S-mgfp5-ER surviving on M2 medium supplemented with 50 mg/l kanamycin (13 weeks after co-cultivation) were transferred to M3 medium for rooting and incubated for 3 weeks. The rooted plantlets were hardened as described in chapter 2, section 2.11 and maintained in the controlled environment at 25±2 °C under 16 h photoperiod of a light intensity of 38 $\mu E.m^{-2}.s^{-1}$ conditions for 2 weeks.

6.2.6.5 Callus development from DCMEA

In a separate experiment, 20 DCMEA explants treated with *Agrobacterium* by dipping were blotted dry on a sterile filter paper and co-cultivated in M4 medium for 72 h. These were then washed with sterile distilled water, blotted dry on sterile filter paper and incubated on M4 medium containing 500 mg/l cefotaxime for one week. After one week of culture the DCMEA explants were transferred to M4 medium again containing 250 mg/l cefotaxime (Claforan – Russel India Ltd.) and 50 mg/l kanamycin. Cultures were incubated under the conditions mentioned as above. Calli developed after 4 weeks of culture, were transferred to fresh M4 medium having kanamycin (50 mg/l) and claforan (125 mg/l) and incubated for another 4 weeks. After 4 weeks of incubation the calli were again transferred to fresh M4 medium containing 50 mg/l of kanamycin for another 4 weeks. The calli obtained on M4 medium without kanamycin or claforan from DCMEA explants not treated with *Agrobacterium* served as controls. Calli were tested for GUS activity and green fluorescence after 4 and 8 weeks of their culture in M4 medium with kanamycin and claforan.

6.2.7 Tissue staining for GUS activity

Histochemical analysis was carried out to determine the β-glucuronidase activity in callus growing on M4 medium after 4 and 8 weeks. The leaves of the shoots surviving on M1 medium supplemented with kanamycin (50 mg/l) was tested after 8 weeks. The tissues were cut into small bits (0.5 cm) and immersed in 1 mM X-Gluc solution (**Table 6.2**) in microtiter multiwell plates (Sigma Chemical Co., USA) and incubated overnight at 37 °C (Stomp 1992). The tissues were bleached in 100 % ethanol before observation.

Table 6.2 Reagent mix for GUS assay

Stock Solution	Final concentration	Reagent mix (ml/ml)
1.0 M $NaPO_4$ buffer, pH 7.0	0.1 M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M K-ferricyanide, pH 7.0	0.5 mM	100
0.005 M K-ferrocyanide pH 7.0	0.5 mM	100
0.002 M X-glucuronide	1.0 mM	50
10 % Triton X-100 (Optional)	0.1 %	10
Distilled water	-	600

6.2.8 Fluorescence microscopy

Visualization of GFP fluorescence in plant tissues was achieved using a Leica Wild MPS 32 stereo microscope (Leitz Wetzlar, Germany) fitted with G filter. Photographs were taken using Leica MPS 32 photoautomat camera set to take automatic exposure (under dark field) on Fujichrome 400 ASA film.

6.2.9 DNA isolation

6.2.9.1 Plant DNA isolation

Genomic DNA was extracted from putatively transformed plants, calli and untransformed pigeonepea plants and callus using Doyle and Doyle (1987) method with modifications. Two putatively transformed plants obtained (0.5 g tissue) 16 and 18 weeks after co-cultivation with *Agrobacterium* strain pBIN 35S-mgfp5-ER were used. The callus (1 g) developed and maintained for 12 weeks on M4 medium supplemented with 50 mg/l kanamycin after treating DCMEA explants with *Agrobacterium* strain pBIN 35S-mgfp5-ER was used as transformed callus. The leaf tissue (1 g) of seedlings grown on hormone-free MS basal medium for 4 weeks was used as control. The callus (1 g) produced and maintained on M4 medium from DCMEA explants, which were not treated with *Agrobacterium* was used as control.

The tissues were crushed in liquid nitrogen in a pre-cooled mortar and pestle to a fine homogeneous powder. The powder was quickly transferred to a SS34 tube containing 7.5 ml of ice cold Extraction Buffer (0.35 M Sorbitol; 0.1 M Tris.HCl pH 7.5; 5 mM EDTA, pH 7.5). The tube was gently shaken and 7.5 ml of Nuclei Lysis Buffer (2 M NaCl; 0.2 M Tris, pH 7.5; 50 mM EDTA, pH 7.5; 2 % CTAB) was quickly added. To this 3 ml of 5 % Sarkosyl solution was added. The tube was incubated at 65 °C for 20 min. The contents were cooled down and 18 ml of $CHCl_3$:Isoamylalcohol (24:1) mixture was added. The tube was centrifuged at 4000

rpm for 15 min at 4 °C. The aqueous phase (supernatant) was taken into a new SS34 tube and was extracted with 15 ml of $CHCl_3$: Isoamylalcohol (24:1). 1.5 ml 3 M sodium acetate was added and mixed. 16.5 ml of isopropyl alcohol was added and incubated at RT for 10 min. The contents were centrifuged at 8000 rpm for 10 min at 4 °C. The pellet was washed with 0.5 ml of chilled 70 % ethanol by suspending the pellet and centrifuging at 10000 rpm for 10 min at 4 °C. The supernatant was removed completely and the pellet was dried by inverting the tube on paper towel. The pellet was dissolved in 50 µl of sterile milli Q water. RNAse at a concentration of 10 µg/ml was added and incubated at 37 °C for 1 h. The DNA was re-precipitated by phenol:chloroform extraction and the pellet was dissolved in sterile milli Q water.

6.2.9.2 Plasmid DNA isolation and preparation of probe

The hybridization probe consisted of a 880 bp mgfp5-ER sequence excised from the pBIN 35S-mgfp5-ER plasmid. Plasmid DNA was isolated from overnight (18 h) grown *Agrobacterium* culture using the modified Birnboim and Doly (1979) protocol. Isolated plasmid DNA was treated with RNAse and purified using phenol:chloroform method. The plasmid DNA was cut using the restriction enzymes *Xba* I (Amersham, USA) and *Sac* I (to isolate the GFP fragment, which was purified by agarose gel electrophoresis and oligolabelled with ^{32}P (Feinberg and Vogelstein 1984) to produce a highly radioactive probe. This probe was then denatured by boiling to give a single stranded probe suitable for addition to the hybridization solutions (Draper *et al.* 1988).

6.2.10 Southern hybridization

The presence and integration of the GFP gene in the kanamycin resistant plants and callus was analyzed by Southern blots (Southern 1975). For this 15 µg of DNA isolated from putatively transformed plants and calli was digested with restriction endonuclease *Eco* RI (10 units/µg of DNA), separated through a 1 % agarose gel prepared in 1x TAE (**Fig 6.3A**) and transferred (Sambrook *et al*. 1989) and blotted on to Hybond N^+ Nylon membrane (Amersham) by vacuum transfer. Negative controls consisted of DNA isolated from untreated pigeonpea plants and callus. The 880 bp fragment of mgfp5-ER was labelled with ^{32}[α-dATP] and used for hybridization performed according to Sambrook *et al*. (1989) in 6x SSPE, 5x Denhardt's solution, 0.4 % SDS, 0.1 mg/l denatured Solmon Sperm for 24 h at 55 ° C before washing membranes with 2x SSPE, 0.5 % SDS solution at 55 ° C. Hybridizing bands were detected by 5 days exposure to Kodak X-OMAT AR autoradiography film at – 70 ° C.

6.3 Results and discussion

The worldwide efforts to transform an ever increasing number of plant species with high efficiency has resulted in a number of promising gene transfer systems that continue to become more refined (Fisk and Dandekar 1993). Although, *Agrobacterium*-mediated transformation remains the method of choice, alternative methods such as electroporation and particle bombardment have been developed in order to circumvent the poorly understood biological barriers, which prevent its application to certain plants. (Fisk and Dandekar 1993).

The fact that leguminous plants are difficult to regenerate *in vitro* prevented their transformation and the production of transgenic plants for a long time. However, significant progress has been made recently and reports of transformation are now available for many food legumes viz., cowpea (Penza *et al.* 1991), pea (Davies *et al*. 1993; Jordan and Hobbs 1993); Schroeder *et al*. 1993), peanut (Ozias-Akins *et al*. 1993; Eapen and George 1994b; McKently *et al.* 1995), lentil (Warkentin and McHughen 1992), *Vicia narbonensis* (Pickardt *et al*. 1991), chickpea (Fontana *et al*. 1993; Kar *et al.* 1996; Krishnamurthy *et al*. 2000) and soybean (Hichee *et al*. 1988; Parrot *et al.* 1989; Falco *et al.* 1995).

Pigeonpea, an important grain legume suffers heavy losses due to fungal diseases and insect pests, mainly pigeonpea pod borer. Although, wild species of *Cajanus* have numerous traits, the cross incompatibility between the wild and cultivated varieties has deterred the improvement of the crop by conventional plant breeding techniques (van Rheenen *et al*. 1993). Insect resistant plants could be developed by the transformation of plants with synthetic genes that encode for insect resistance (Raffa 1989). Pod borer larvae *Helicoverpa armigera* (Huebner) are susceptible to *Bacillus thuringiensis* (Bt) endotoxins when expressed in plant cells (Mohapatra and Sharma 1991). The pre-requisite for the transfer of genes of desirable traits are the establishment of an efficient plant regeneration system *in vitro* from an explant amenable for transformation and standardization of an efficient genetic transformation protocol. Only one report of pigeonpea transformaton is available where transgenic plants were obtained using GUS reporter gene (Geetha *et al.* 1999).

The results obtained from *Agrobacterium*-mediated transformation in DCMEA explants are presented here.

6.3.1 Determination of lethal dose of kanamycin

DCMEA explants (**Fig 6.3B**) formed shoots on M1 medium supplemented without kanamycin (control) after 4 weeks of incubation. The shoot formation was also observed on M1 medium supplemented with kanamycin at 25 and 50 mg/l concentrations. No shoot formation was observed on M1 medium with 75, 100, 200, 300 and 400 mg/l of kanamycin. Inhibition of proliferation shoots in more than 50 % of DCMEA explants was observed when cultured on M1 medium containing 50 mg/l of kanamycin (**Fig 6.3C**) for 4 weeks. Survival of few shoots was observed on 25 mg/l kanamycin after another passage of 4 weeks. However, the shoots formed on 50 mg/l kanamycin bleached completely in another 4 weeks when transferred to M1 medium containing 50 mg/l kanamycin. Therefore 50 mg/l kanamycin was used as the optimal selective pressure for transformation experiments. In a previous report by Geetha *et al.* (1999) also, 50 mg/l kanamycin was used as the selection pressure.

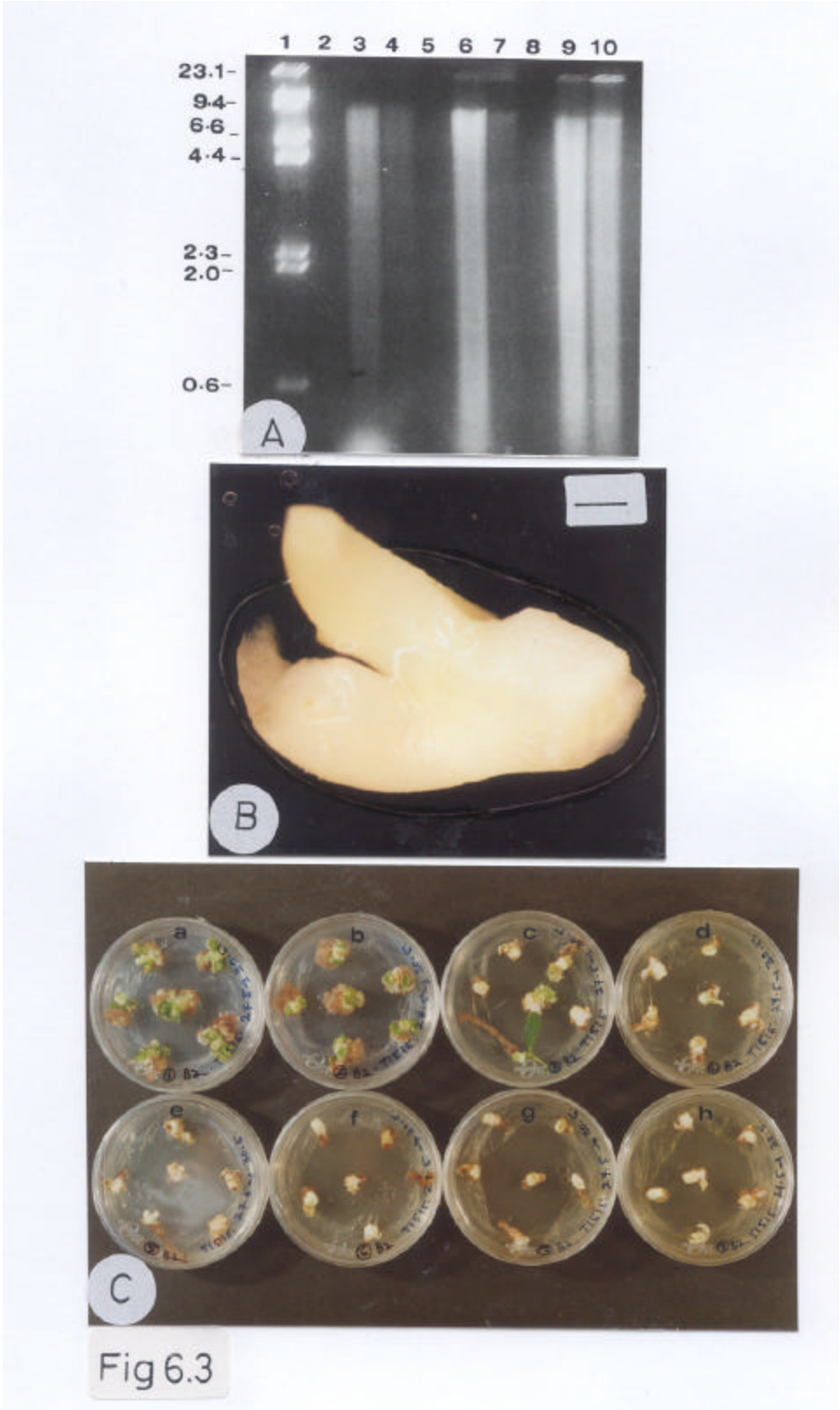
6.3.2 Co-cultivation

Dipping of explants in the *Agrobacterium* culture for 15-20 min was done as longer periods of incubation posed problems in the elimination of bacteria and contamination in subsequent cultures of DCMEA explants *in vitro*. After dipping of explants for 15-20 min the explants were transferred to M1 medium and incubated for co-cultivation for 72 h. At the end of the co-cultivation period of 72 h, the explants were transferred to M1 medium containing an antibiotic (cefotaxime) that will specifically inhibit bacterial growth. After 1 week, the explants were transferred on a selective agent (kanamycin) that will select those cells receiving and expressing the gene transfer casette. The method is similar to the transformation system used in soybean (Hinchee *et al.* 1994).

6.3.3 Transformation studies with *Agrobacterium* strain pGV2260-p35SGUSINT

6.3.3.1 Development of shoots

A few explants (3 out of 616 explants treated with *Agrobacterium* strain with pGV2260-p35SGUSINT) survived and formed shoots (**Fig 6.4A**) on M1 medium containing kanamycin (50 mg/l) after 4 weeks of culture. The shoots did not grow and elongate further after 8 weeks of culture on M1 medium supplemented with 50 mg/l kanamycin. The untreated explants cultured on M1 medium containing 50 mg/l kanamycin did not survive.



A. Agarose gel electrophoresis of *Eco*RI digested plant genomic DNA from control plant (lane 3), control callus (lane 4), transformed callus (lane 6 & 7), putatively transformed plants (lane 9 & 10). Lane 1 is λ-DNA *Hind*III digest molecular weight marker.

B. DCMEA explants explant used for treating with *Agrobacterium*

C. Determination of lethal dose (LD_{50}) of kanamycin in pigeonpea DCMEA explants: a=control, b=25 mg/l, c=50 mg/l, d= 75 mg/l, e=100 mg/l, f=200 mg/l, g=300 mg/l, h=400 mg/l.

Fig 6.3

Prior to our study, DCMEA explants have not been used for transformation of pigeonpea. Geetha *et al*. (1999) have recently reported the use of shoot apex and cotyledonary nodes from 5 day old germinated seeds and development of transgenic plants in 12 weeks of incubation.

Embryo axes has become the explant of choice for development of transgenic plants via *Agrobacterium* co-cultivation in chickpea (Kar *et al*. 1996; Krishnamurthy *et al*. 2000), peanut (McKently *et al*. 1995), peas (Davies *et al*. 1993). Use of embryo axis as an explant has several advantages: (1) due to its smaller size, it is amicable to both *Agrobacterium* as well as particle bombardment mediated transformation techniques, (2) the explants take the least time to develop into single shoot (10-15 days) compared to several months in case of plant regeneration via callus phase, (3) somaclonal variations can be avaoided if callus phase can be bypassed, (4) regeneration through embryo axis is genotype independent.

6.3.3.2 Induction of callus

DCMEA explants treated with *Agrobacterium* strain pGV2260-p35SGUSINT and cultured on M4 medium containing kanamycin (50 mg/l) and cefotaxime (250 mg/l) induced callus after 2 weeks of incubation at the cut ends. Proliferation of callus was achieved on transfer to fresh M4 medium containing 50 mg/kanamycin and 125 mg/l cefotaxime after 4 weeks. The calli were taken at 4 and 8 weeks for GUS histochemical assay.

6.3.3.3 GUS activity in shoots and callus

The control explants, which were not co-cultivated with *Agrobacterium* strain pGV2260-p35SGUSINT did not show GUS activity. There was no GUS activity in the shoots (putative transformants) obtained on the medium containing 50 mg/l kanamycin after 8 weeks of culture. When compared to untransformed callus, transformed calli obtained from DCMEA explants showed intense blue color after 4 weeks (**Fig 6.4B**) and 8 weeks of culture (**Fig 6.4C**). Absence of GUS activity in shoots may be due to premature termination of the introduced T-DNA during the transfer process (Cousins *et al.* 1991).

Bacterial β-glucuronidase (*uidA* and *gusA*), commonly referred to as GUS gene, has become the major reporter gene used as a tool for the analysis of plant gene expression (Walden and Schell 1990). The assay is extremely sensitive and uses X-gluc (5-bromo, 4-chloro, 3-indolyl, β-D-glucuronide), which can qualitatively show

C. Shoots surviving on M1 medium supplemented with 50 mg/l kanamycin after treating with *Agrobacterium* strain pGV2260-p35SGUSINT

D. GUS expression in callus derived from transformed DCMEA explants after 4 weeks of culture (bar = 1000 µm)

E. GUS expression in callus derived from transformed DCMEA explants after 8 weeks of culture (bar = 1000 µm)

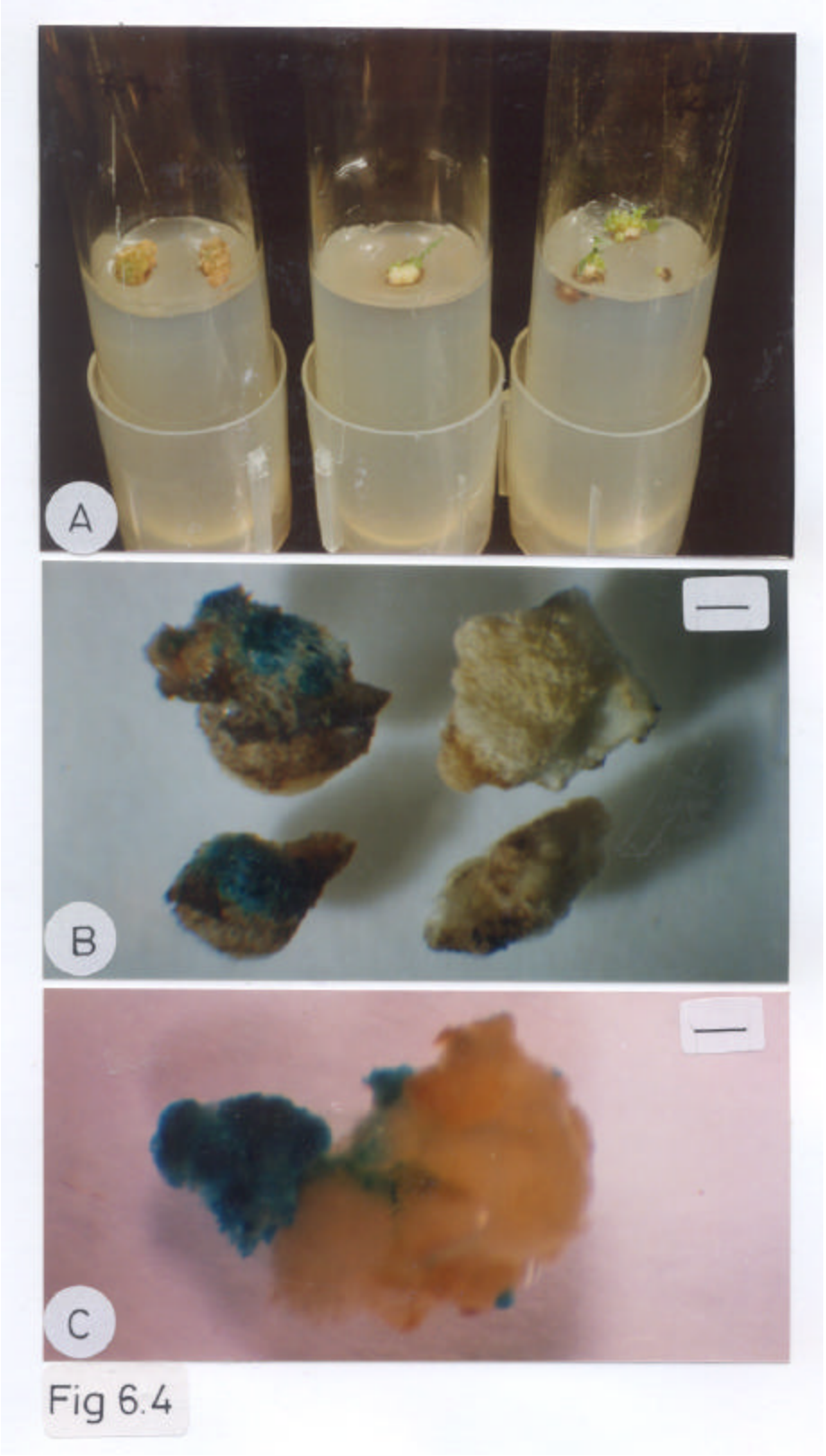


Fig 6.4

cell and tissue specificity (Jefferson 1989) Cleavage of X-gluc by the enzyme β-glucuronidase produces the final insoluble blue color precipitate dichloro dibromo indigo (ClBr-indigo) (**Fig 6.5**). It is readily detectable at low concentrations, final cleavage product is insoluble in water, reaction proceeds to an insoluble intermediate, which on oxidative dimerization gives intense blue color, and this product allows precise cellular localization and little loss of enzyme product on tissue processing.

In the present study, use of *Agrobacterium* strain containing a portable intron in the β-glucuronidase (GUS) gene ruled out the possibility of false GUS activity (blue color) in histochemical test of the explants due to bacterial contamination. The introduction of the portable intron into the GUS gene leads to nearly complete repression of its expression in *Agrobacterium* because of the absence of the eukaryotic splicing apparatus in prokaryotes. Use of such construct avoids the confusion of GUS gene expression in inoculated explants (Vancanneyt *et al.* 1990).

Since shoots obtained from DCMEA explants after co-cultivation with *Agrobacterium* strain pGV2260-p35SGUSINT strain did not survive after 8 weeks of selection pressure on M1 medium supplemented with 50 mg/l kanamycin, southern hybridization could not be performed.

The GUS gene has been used extensively as a reporter for gene expression in plants (Jefferson *et al*. 1987). Transformed tissues or patterns of gene expression can be identified histochemically, but this is generally destructive test and is not suitable for assaying primary transfomants, for following the time course of gene expression in living plants, nor as a means of rapidly screening segregating populations of seedlings. The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* can be directly visualized, and therefore, shares none of these problems.

6.3.4 Transformation studies with *Agrobacterium* strain pBIN 35S-mgfp5-ER

6.3.4.1 Development of shoots

DCMEA explants of genotype T-15-15 were treated with a plant binary vector pBIN 35S-mgfp5-ER using *Agrobacterium* co-cultivation method. They are an attractive alternative to co-cultivation of leaf bits or for direct gene transfer in the case of leguminous plants (Penza *et al*. 1991). One of the early steps in transformation by *A. tumefaciens* is the attachment of the bacteria to the plant host cell and the bacterium appears to be the active partner in attachment (Matthyse 1994).

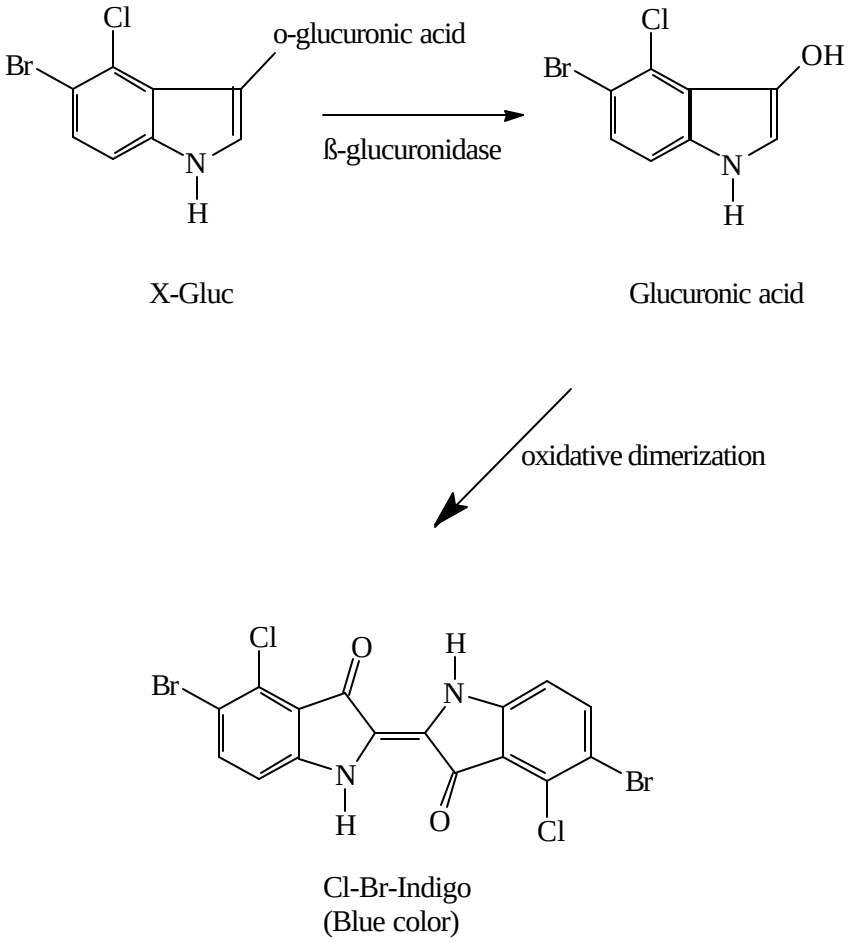


Fig 6.5 Cleavage of 5-bromo, 3-indolyl, β-D-glucuronide by the enzyme β-glucuronidase into dichloro-dibromo-indigo (Cl-Br-indigo)

The DCMEA explants after treatment with *A. tumefaciens* were cultured on M1 medium containing 500 mg/l cefotaxime and selection pressure of 50 mg/l kanamycin was applied after one week instead of immediate application of selection pressure as reported by Geetha *et al*. (1999). Kanamycin selection was beneficial in producing transgenic calli and shoots as the selection pressure enriched the growth of transformed tissue and suppressed the growth of un-transformed tissue similar to observation in an earlier report of soybean (Hinchee *et al*. 1988). By contrast, no selection pressure was applied at all in a report of cowpea by Penza *et al*. (1991).

The putative transformants were identified by the virtue of their survival on M1 medium containing 50 mg/l kanamycin in all the experiments in which kanamycin selection was applied after 1 week following *Agrobacterium* co-culture similar to an earlier report in chickpea (Fontana *et al*. 1993), where the selective pressure was applied 3 weeks after co-cultivation and as against an earlier report of soybean (Hinchee *et al*. 1988), where in kanamycin selection was utilized immediately. Even though varied number of explants survived and developed shoots on medium supplemented with 50 mg/l of kanamycin depending on the batch, their number decreased in subsequent transfer to fresh medium. The initial survival of explants and formation of shoots varied from 8.11 to 26.79 % after 4 weeks of incubation, but only 1.7 to 6.7 % of the explants with shoots survived on kanamycin after 8 weeks of culture. Untreated explants cultured on medium containing kanamycin (50 mg/l) formed shoots in 28 % of the cultures after 4 weeks, however, these shoots bleached and died after 8 weeks of culture. The results obtained with transformation with GFP gene is presented in **Table 6.3**.

Table 6.3 Shoots recovered in various experiments after treatment with *Agrobacterium* strain pBIN 35S-mgfp5-ER and selection on 50 mg/l kanamycin

No.	Explants Treated	Explants forming Shoots (after 4 weeks of selection)	% shoot formation	Survival (after 8 weeks of selection)	% survival	No of shoots showing green fluorescence
C-1	100	52	52.0	52	52.0	-
C-2	100	28	28.0	0	0.0	-
1	186	38	20.4	11	5.9	4
2	222	18	8.1	7	3.2	0
3	203	26	12.8	6	3.0	0
4	209	56	26.8	14	6.7	5
5	190	25	13.2	7	3.7	1
6	233	21	9.0	4	1.7	0

C1 - Control without selection pressure, C2 - Control with selection pressure of 50 mg/l kanamycin, 1-6 – different batches of co-cultivation experiments

After 4 weeks of incubation on M1 medium supplemented with 50 mg/l kanamycin, the shoots arising from shoot apex region of DCMEA explants were cut at the base of the shoots and were subcultured in test tubes (**Fig 6.6A**) on M1 medium supplemented with 50 mg/l of kanamycin for another 4 weeks. The green shoots continued growing on this medium containing 50 mg/l kanamycin for 4 weeks (**Fig 6.6B**). Healthy and phenotypically normal kanamycin resistant shoots were obtained when shoots produced on M1 medium were transferred on M2 medium containing 50 mg/l of kanamycin (**Fig 6.6C**) for 4 weeks. Most of the shoots appeared bleached and some shoots, which were green initially bleached out gradually, leaving only a few green shoots. The shoots growing on selection pressure (M1 medium supplemented with 50 mg/l kanamycin) for 8 weeks were selected for analysis of green fluorescence. The control leaves for analysis of green fluorescence were taken from the shoots obtained from DCMEA explants, not treated with *Agrobacterium*, growing for 8 weeks on M1 medium without kanamycin.

Genetic transformation using GFP reporter gene has not been reported so far in pigeonpea. However, Geetha *et al*. (1999) have recently reported *Agrobacterium*-mediated transformation in pigeonpea using GUS (*uidA*) reporter gene. GFP is increasingly being used in plant biology from the cellular level to whole plant level. GFP is the first truly *in vivo* reporter system useful in whole plants (Leffel *et al*. 1997).

A. Shoot obtained on M1 medium supplemented with kanamycin (50 mg/l) after 4 weeks of selection

B. Shoots surviving on M1 medium supplemented with 50 mg/l kanamycin after 8 weeks of selection

C. Elongated shoot surviving on M2 medium supplemented with 50 mg/l kanamycin

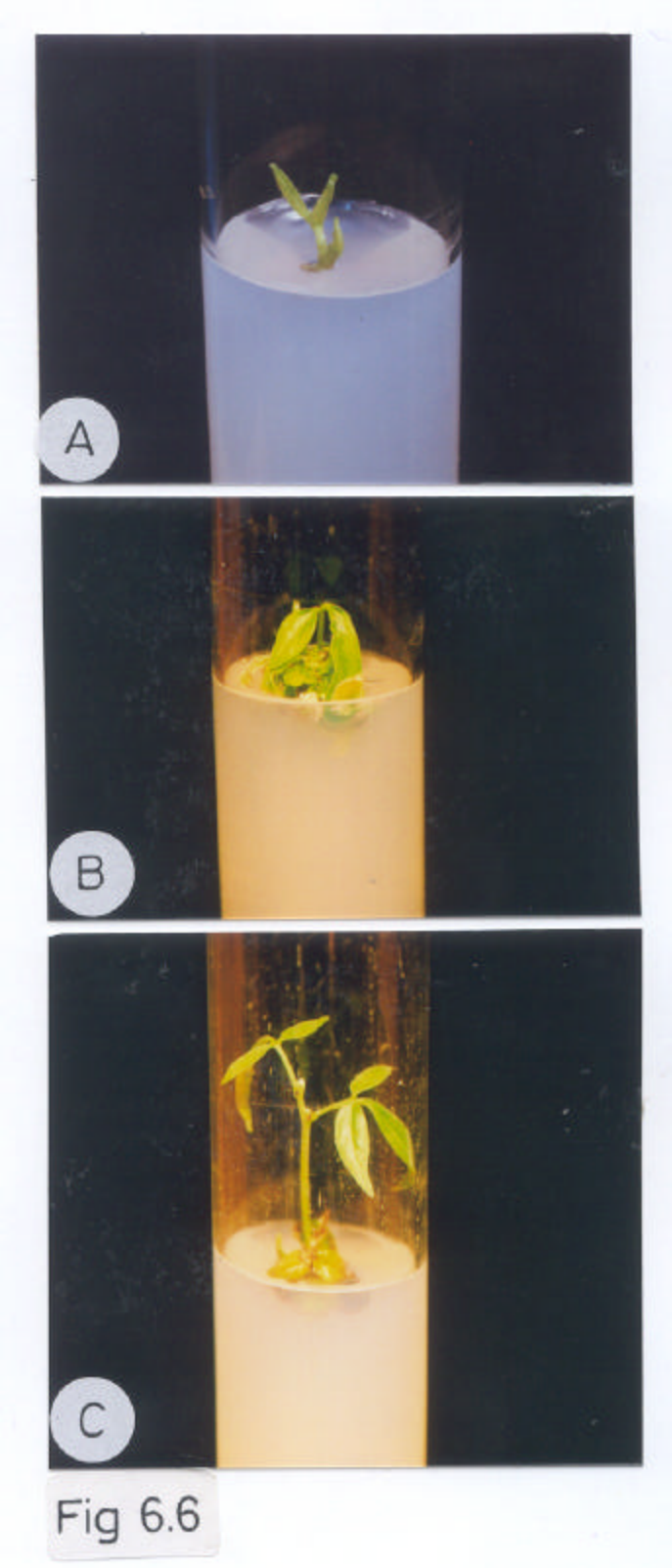


Fig 6.6

6.3.4.2 Callus induction

DCMEA explants treated with *Agrobacterium* strain pBIN 35S-mgfp5-ER and cultured on M4 medium containing kanamycin (50 mg/l) and cefotaxime (250 mg/l) induced callus after 2 weeks of incubation at the cut ends. Proliferation of callus was achieved on transfer to fresh medium containing 50 mg/l kanamycin and 125 mg/l cefotaxime. Eight weeks old callus was used for visualizing green fluorescence and 12 weeks old callus was used for southern hybridization.

6.3.4.3 Visualization of green fluorescence

The leaf bits analyzed for GFP presence by fluorescence microscopy showed green fluorescence (**Fig 6.7A**) as compared to red auto-fluorescence by the control leaves (**Fig 6.7B**). The calli growing on kanamycin containing medium was also analyzed for green fluorescence. The transformed callus also showed green fluorescence (**Fig 6.7C**). Out of all the experiments 10 shoots showed green fluorescence. Most of the shoots showing green fluorescence eventually died under selection pressure in 1-2 transfers on M2 medium supplemented with 50 mg/l kanamycin at 4 weeks interval and only 2 shoots from all the experiments could be recovered.

6.3.4.4 Rooting and hardening

The putatively transformed shoots, which survived selection pressure on M2 medium supplemented with 50 mg/l kanamycin produced roots on M3 medium in 3 weeks (**Fig 6.8A)**. One shoot started wilting during rooting stage and the other rooted plant was hardened under controlled conditions (**Fig 6.8B**). However, hardened plant (**Fig 6.8C**) could not survive long and started wilting after 2 weeks. Both these plants were used for southern analysis.

Fusion of the N-terminal signal sequence from *Arabidopsis thaliana* basic chitinase and C-terminal HDEL sequence in the construct mgfp5-ER allows compartmentalization of GFP away from the nucleoplasm and cytoplasm and into the lumen of the endoplasmic reticulum resulting in highly fluorescent transgenic plants and an apparent decrease in phytotoxicity. This results in better regeneration of transgenic plants expressing GFP gene. Similarly, when GFP was targeted to the endoplasmic reticulum, transformed cells regenerated routinely to give highly fluorescent *Arabidopsis* plants (Haseloff *et al.* 1997).

A. Section of leaf from putative transformants showing green fluorescence (bar = 600 µm)

B. Section of control leaf showing red autofluorescence (bar = 900 µm)

C. Transformed callus showing green fluorescence (bar = 600 µm)

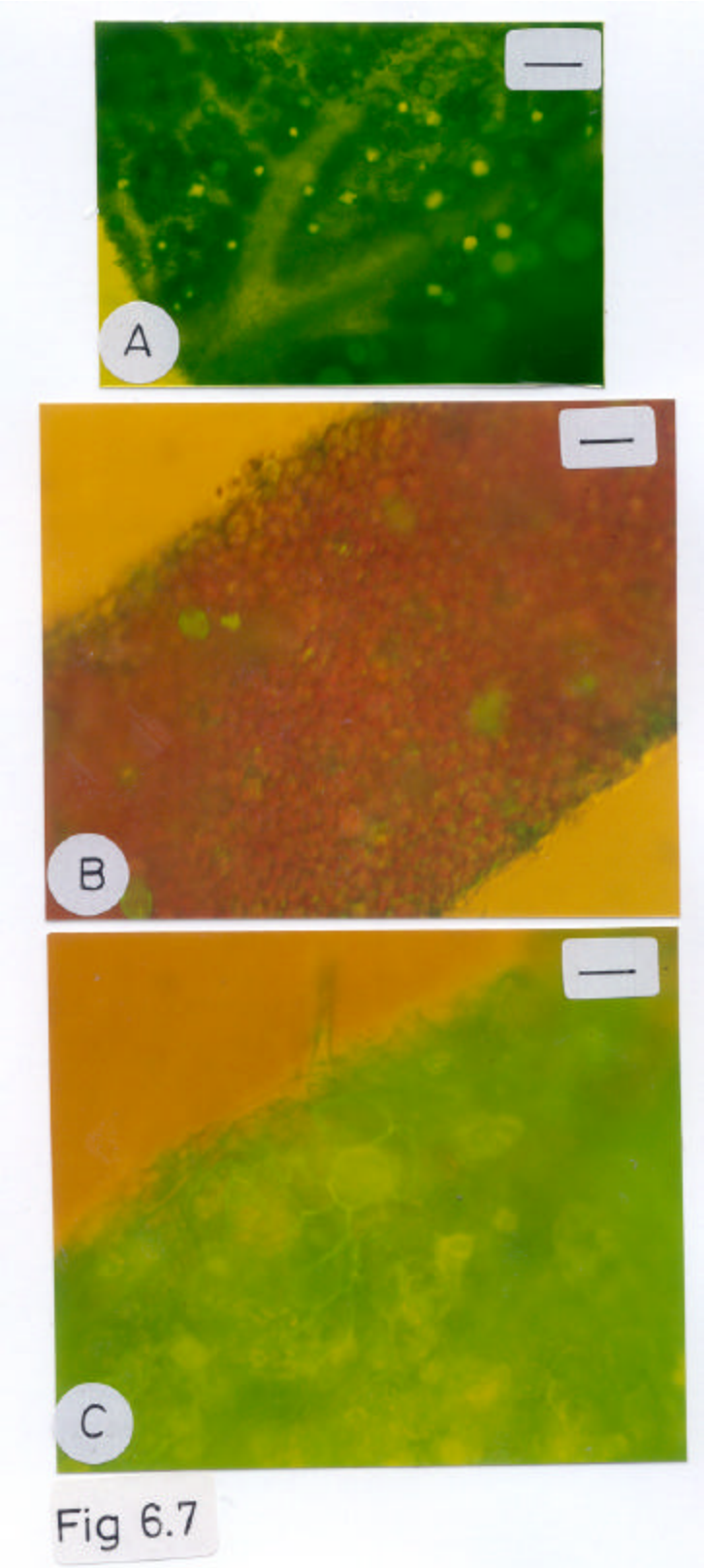


Fig 6.7

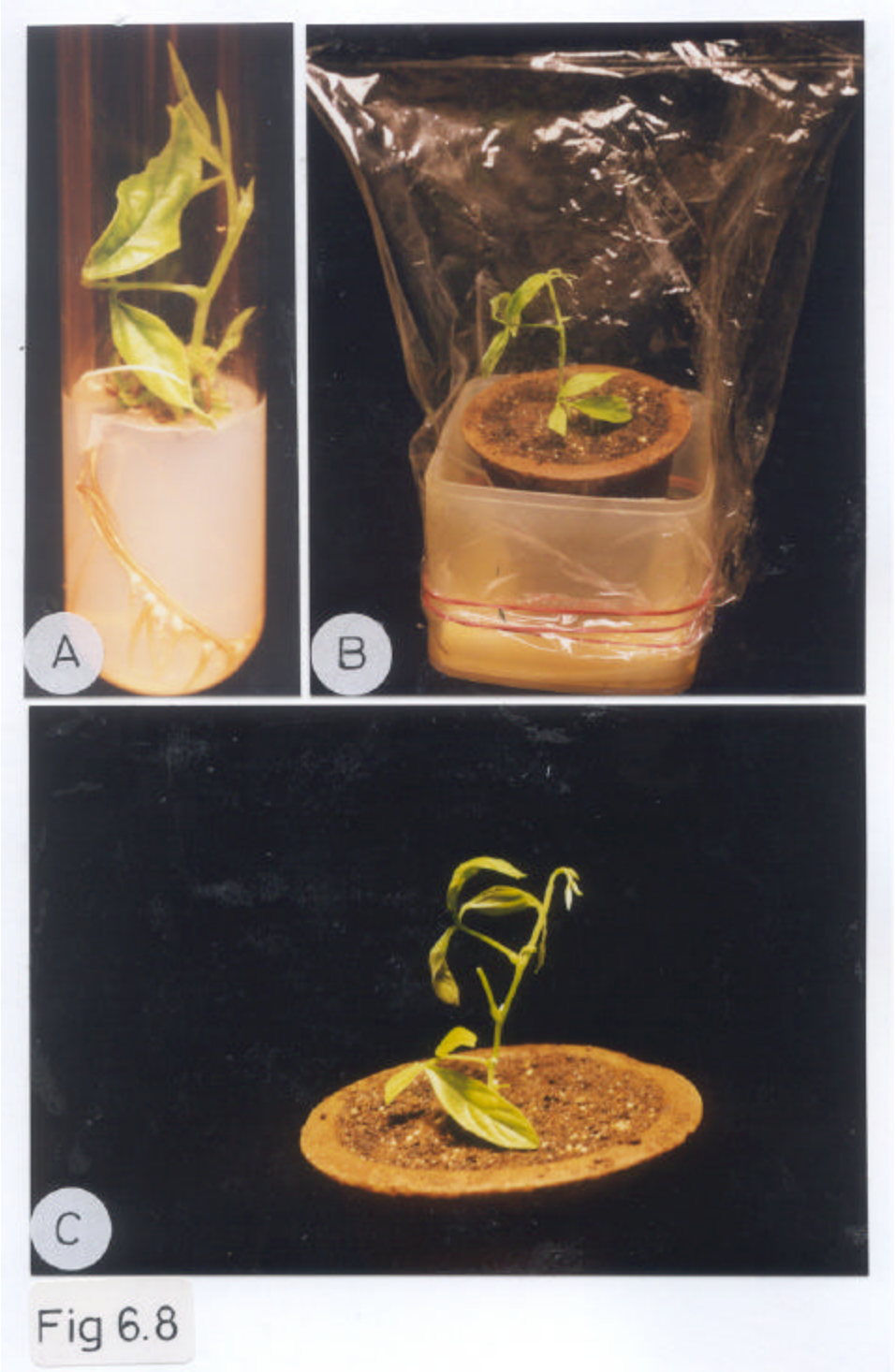


Fig 6.8

A. Rooted putatively transformed shoot of the genotype T-15-15
B. Hardening of putatively transformed plantlet of the genotype T-15-15
C. Hardened putatively transformed plant growing in pot

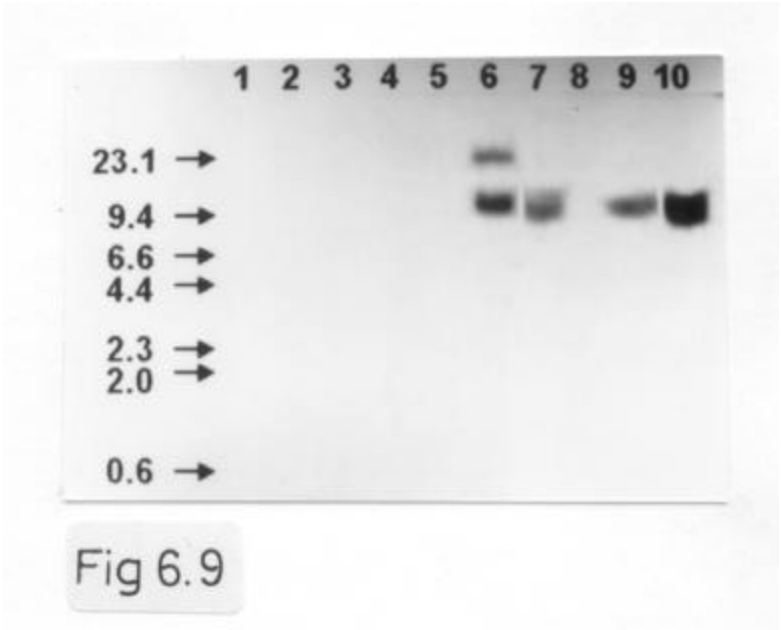
6.3.5 Southern analysis

Southern hybridization of plants confirmed the integration of the GFP gene confirming that pigeonpea is susceptible/amenable to transformation by *A. tumefaciens*. Integration of the GFP DNA was in unique sites in each transgenic plant. Southern hybridization also confirmed the presence of the gene in transgenic calli. No hybridization could be detected for un-transformed plants and callus (**Fig. 6.9**).

6.4 Conclusions

In the present study, an attempt was made to standardize protocol for *Agrobacterium*-mediated genetic transformation system by co-cultivation method using *Agrobacterium* strains harboring plasmid p35SGUSINT and pBIN 35S-mgfp5-ER. The integration of GUS gene in callus has been confirmed by histochemical analysis. The integration of GFP gene in callus and putatively transformed plants has been confirmed by both fluorescence microscopy and southern analysis.

In legumes, stable transgenics have been obtained. Even though the present protocol does not give a fool-proof method for production of transgenic plants of pigeonpea with desirable traits, nevertheless it is a step towards obtaining transgenic pigeonpeas. Since transformation was confirmed by GUS and green fluorescence in callus cultures, production of transgenic plants through regeneration of callus cultures into plants is an exciting probability. Although, we have shown that DCMEA explant of pigeonpea is susceptible to *A. tumefaciens*, further optimization of the protocol is necessary to obtain high frequency transformation. *Agrobacterium*-mediated transformation has been the primary tool for the transformation of many dicotyledonous crops. This is because of the relative ease and precision of gene transfer to intact, regenerable explants (Hinchee *et al*. 1994) and the other methods of gene transfer via protoplasts being limited by the technical difficulties in reproducibly achieving regeneration from protoplasts (Hinchee *et al*. 1994).



Southern hybridization of *Eco*RI digested DNA from control plant (lane 3), control callus (lane 4), transformed callus (lane 6 & 7), putatively transformed plants (lane 9 & 10)

SUMMARY

Biotechnology is now the cutting edge of plant science offering new techniques, applications, and opportunities for crop improvement. Application of biotechnological tools in crop improvement programs can be effective in three different, complementary ways: (a) speeding up the process of conventional breeding (b) creating genetic variability through tissue culture and (c) evolving novel genotypes through recombinant-DNA technology. It can provide solutions to problems like pests, biotic and abiotic stresses and can be useful in increasing the productivity of the specific crop.

Grain legumes are an important source of dietary proteins, fibre and calories. The legumes are economically important as they form the third largest food crop (190 m. tonnes), following cereals (2054 m. tonnes) and root and tuber crops (625 m. tonnes). The importance of legumes range from food to fodder, wood to spices and ornamentals. They also play a useful role in biological nitrogen fixation.

Pigeonpea belongs to the family Papilionaceae and subfamily Papilionoideae and is one of the major grain legumes grown in the world and in India, it has a large area under cultivation (3.67 million hectares). India is the largest producer of pigeonpea in the world (2.45 million tonnes). Nearly 85 % of the world's pigeonpea crop is grown in India. It is the second most important grain legume of India after chickpea. The crop is plagued by many biotic and abiotic stresses, the major one being the pod borer insect *Helicoverpa armigera* Hubn and the conventional breeding methods yielded not much results in improving the crop resistance against major pests and diseases. India is the major country among the pigeonpea producing countries, hence, serious efforts are being made to improve the crop using biotechnological methods.

An efficient plant regeneration system is a major pre-requisite for development of transgenic plants. At the time of initiation of this work, there were no reports of somatic embryogenesis and genetic transformation in pigeonpea. The very few reports of regeneration in pigeonpea on organogenesis were restricted mainly to pre-existing meristems. Therefore, this study was carried out with an objective of developing a highly efficient and reproducible *in vitro* plant regeneration method via organogenesis or somatic embryogenesis using different explants. The study was also aimed to standardize conditions for *Agrobacterium* mediated genetic transformation and their molecular characterization so that pigeonpea cultivars with agronomically desirable traits could be evolved through biotechnological methods.

The main findings of the entire work have been summarized as follows:

A. *In vitro* regeneration through organogenesis from distal cotyledonary segments

1. A protocol of plant regeneration from distal cotyledonary segments of genotypes T-15-15 and Gaut-82-90 has been developed.
2. Induction of shoot buds was achieved from distal cotyledonary segments devoid of proximal meristematic ends of cotyledons of mature seeds.
3. Maximum number of shoot buds were induced from distal cotyledonary segments on MS basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS.
4. The highest number of shoot buds were induced on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS, and was more than MS, B5, Modified B5, LS or White's basal media supplemented with same growth regulators.
5. The genotype T-15-15 (32.3 shoot buds/explants) was better in forming shoot buds than the genotype Gaut-82-90.
6. Elongation of shoot buds was obtained on half-strength MS basal medium supplemented with 3 µM GA_3.
7. Rooting (80 %) of elongated shoots was achieved on half-strength MS basal medium supplemented with 0.5 µM IBA.
8. Rooted plants survived (70 %) after hardening in pots.
9. Regeneration of shoots from callus was observed rarely when callus derived from distal cotyledonary segments on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS was subcultured on half-strength MS basal medium supplemented with 3 µM GA_3.
10. The origin of shoot buds from cotyledonary segments was examined by histological preparations. The anatomy of differentiated shoot bud along with the leaf primordium originating from the compact mass of cells confirms the organogenetic pathway of morphogenesis.

B. *In vitro* regeneration through organogenesis from mature embryo axes and seedling derived explants

1. Multiple shoots were obtained from various mature embryo axes derived explants such as ERMEA and DCMEA explants of genotypes T-15-15 and Gaut-82-90.
2. Induction of maximum number of shoots was achieved from ERMEA and DCMEA explants on EC_6 basal medium supplemented with 5 µM BAP and 1 µM IAA.

3. The genotype T-15-15 was found to be better in organogenetic potentiality of ERMEA and DCMEA explants than Gaut-82-90.
4. Elongation of shoots was achieved on half-strength MS basal medium supplemented with 3 µM GA_3.
5. Rooting (65 %) of elongated shoots was done on half-strength MS basal medium supplemented with 0.5 µM IBA.
6. Shoot buds were induced from various seedling derived explants such as leaf, epicotyl, root, proximal cotyledonary segments, distal cotyledonary segments and cotyledonary node explants of genotypes T-15-15 and Gaut-82-90.
7. The shoot buds were induced on EC_6 basal medium supplemented with 20 µM BAP, 2 µM kinetin and 250 µM AdS.
8. The genotype T-15-15 was found to be better in organogenetic potentiality of various seedling explants than Gaut-82-90.
9. Elongation of shoots was achieved on half-strength MS basal medium supplemented with 3 µM GA_3.
10. Rooting (65 %) of elongated shoots of epicotyl, proximal cotyledonary segments, distal cotyledonary segments and cotyledonary node explants was done on half-strength MS basal medium supplemented with 0.5 µM IBA.
11. The rooted plantlets were hardened with 65 % success.

C. Somatic embryogenesis and plant regeneration

1. A plant regeneration system via somatic embryogenesis was developed using distal cotyledonary segments of genotypes Gaut-82-90 and T-15-15.
2. The somatic embryogenesis was induced from distal cotyledonary segments on EC_6 basal medium supplemented with BAP or TDZ.
3. The formation of cotyledonary embryos was observed from globular embryos obtained on EC_6 basal medium supplemented with 5 µM BAP.
4. The genotype Gaut-82-90 was better in producing somatic embryos when compared to the genotype T-15-15.
5. Further development of globular embryos was achieved on MS basal medium supplemented with 3 µM GA_3.
6. Maturation of somatic embryos was done on half-strength MS basal medium supplemented with 0.5 µM ABA.

7. Germination and conversion of somatic embryos occurred on half-strength MS basal medium supplemented with 0.5 µM BAP.
8. On transfer of 12 plantlets transferred to pots, 42 % of plantlets (5) survived.
9. The histological observations of different stages of somatic embryo development revealed the direct origin of globular embryos from epidermal and sub-epidermal layers of the cotyledonary segments involving multiple cells. The observations also confirmed the bipolar nature of the structures obtained which is necessary for classifying the structures observed as indeed somatic embryos.
10. The effect of various parameters like basal media, explant source, carbohydrate source, cultural environment and agitation in liquid induction medium on induction of somatic embryo were studied. The EC_6 basal medium, distal cotyledonary segments, sucrose at 3 % level, incubation at 16/8 h photoperiod in light were optimum for somatic embryo induction in genotype Gaut-82-90.
11. The highest percentage of somatic embryo induction was achieved when the distal cotyledonary segments were agitated in liquid induction medium at 200 rpm for 1 week and then transferred to EC_6 basal medium supplemented with 0.5 µM BAP

D. Genetic transformation studies

1. The DCMEA explants of T-15-15 genotype were used for *Agrobacterium* mediated transformation.
2. *Agrobacterium tumefaciens* strain LBA4404 containing the plasmid pGV2260-35S-GUSINT or pBIN 35S-mgfp5-ER was used as a vector for transformation.
3. The plasmid containing pBIN 35S-mgfp5-ER was introduced into the *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method.
4. LD_{50} for selection of transformed shoots was found to be 50 mg/l kanamycin.
5. No plants showing GUS activity were obtained when the DCMEA explants were treated with *Agrobacterium* strain pGV2260-p35SGUSINT. Callus obtained from DCMEA explants were treated with *Agrobacterium* strain pGV2260-p35SGUSINT showed GUS activity in histochemical assay.
6. Green fluorescent and Southern positive plants and callus were obtained when the DCMEA explants were treated with *Agrobacterium* strain pBIN 35S-mgfp5-ER.

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